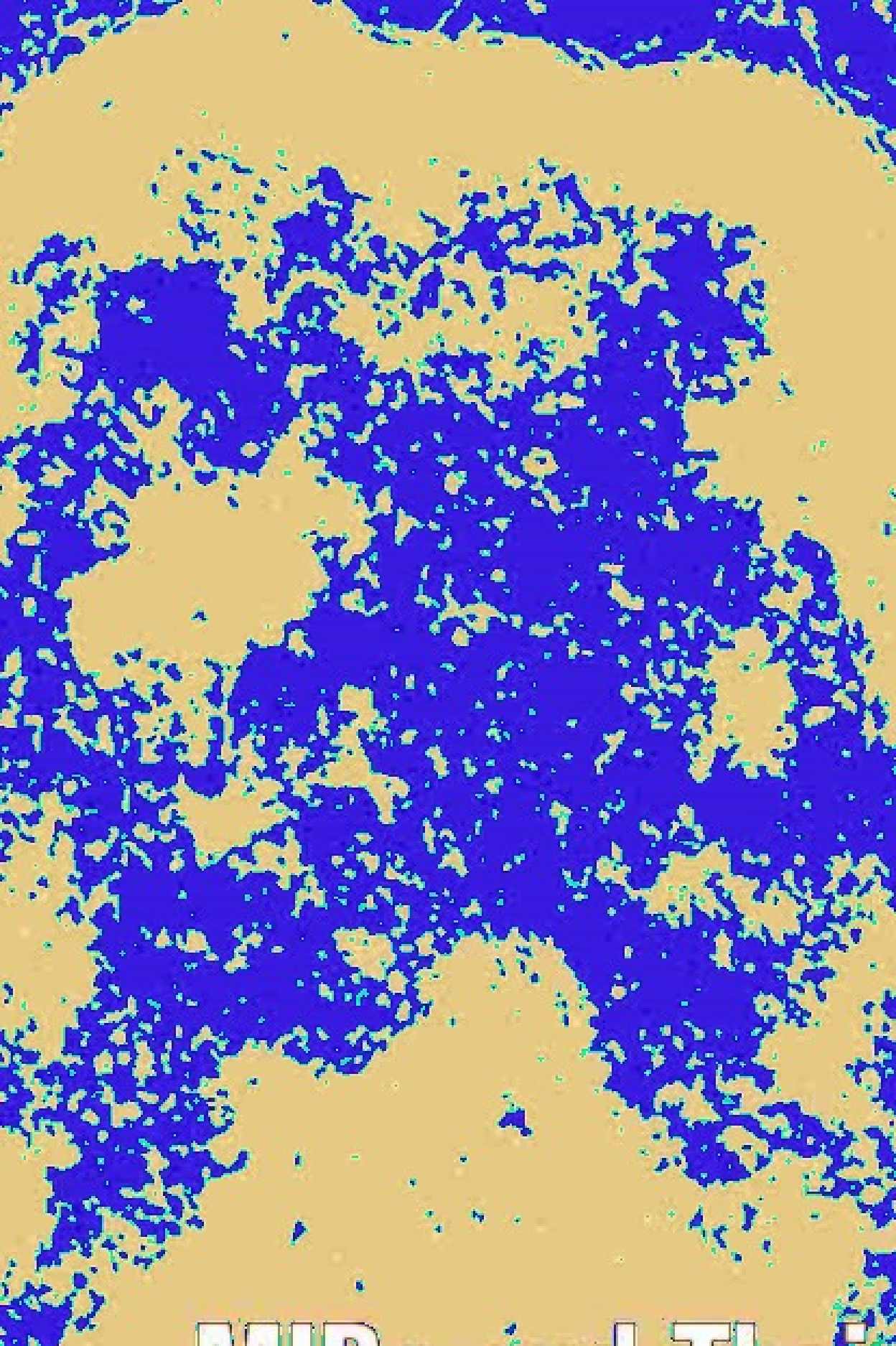


MIPs and Their Role in the Exchange of Metalloids

ADVANCES IN EXPERIMENTAL MEDICINE AND BIOLOGY

Recent Volumes in this Series

Volume 671
FRONTIERS IN BRAIN REPAIR
Edited by Rahul Jandial

Volume 672
BIOSURFACTANTS
Edited by Ramkrishna Sen

Volume 673
MODELLING PARASITE TRANSMISSION AND CONTROL
Edited by Edwin Michael and Robert C. Spear

Volume 674
INTEGRINS AND ION CHANNELS: MOLECULAR COMPLEXES AND SIGNALING
Edited by Andrea Becchetti and Annarosa Arcangeli

Volume 675
RECENT ADVANCES IN PHOTOTROPHIC PROKARYOTES
Edited by Patrick C. Hallenbeck

Volume 676
POLYPLOIDIZATION AND CANCER
Edited by Randy Y.C. Poon

Volume 677
PROTEINS: MEMBRANE BINDING AND PORE FORMATION
Edited by Gregor Anderluh and Jeremy Lakey

Volume 678
CHEMO FOG: CANCER CHEMOTHERAPY-RELATED COGNITIVE IMPAIRMENT
Edited by Robert B. Raffa and Ronald J. Tallarida

Volume 679
MIPS AND THEIR ROLE IN THE EXCHANGE OF METALLOIDS
Edited by Thomas P. Jahn and Gerd P. Bienert

MIPs and Their Role in the Exchange of Metalloids

Edited by

Thomas P. Jahn, PhD
Department of Agriculture and Ecology, Faculty of Life Sciences
University of Copenhagen, Frederiksberg, Denmark

Gerd P. Bienert, PhD
Unité de Biochimie Physiologique, Institut des Sciences de la Vie
Université catholique de Louvain, Louvain-la-Neuve, Belgium

Springer Science+Business Media, LLC

Landes Bioscience

Springer Science+Business Media, LLC
Landes Bioscience

Printed in the USA.

Springer Science+Business Media, LLC, 233 Spring Street, New York, New York 10013, USA
http://www.springer.com

Please address all inquiries to the publishers:
Landes Bioscience, 1002 West Avenue, Austin, Texas 78701, USA
Phone: 512/ 637 6050; FAX: 512/ 637 6079
http://www.landesbioscience.com

The chapters in this book are available in the Madame Curie Bioscience Database.
http://www.landesbioscience.com/curie

MIPs and Their Role in the Exchange of Metalloids, edited by Thomas P. Jahn and Gerd P. Bienert. Landes Bioscience / Springer Science+Business Media, LLC dual imprint / Springer series: Advances in Experimental Medicine and Biology.

ISBN: 978-1-4419-6314-7

Library of Congress Cataloging-in-Publication Data

MIPS and their role in the exchange of metalloids / edited by Thomas P. Jahn and Gerd P. Bienert.
p. ; cm. -- (Advances in experimental medicine and biology ; v. 679)
Includes bibliographical references and index.
ISBN 978-1-4419-6314-7
1. Aquaporins. 2. Semimetals--Physiological transport. I. Jahn, Thomas P., 1966- II. Bienert, Gerd P., 1978- III. Series: Advances in experimental medicine and biology ; v. 679.
[DNLM: 1. Aquaporins. 2. Arsenic--toxicity. 3. Boron--toxicity. 4. Plants--metabolism. 5. Protein Transport. 6. Silicon--toxicity. W1 AD559 v.679 2010 / QU 55.7 M669 2010]
QP552.A65M57 2010
572'.696--dc22
2010010017

PREFACE

Sixteen years have passed since human aquaporin-1 (AQP1) was discovered as the first water channel, facilitating trans-membrane water fluxes. Subsequent years of research showed that the water channel AQP1 was only the tip of an iceberg; the iceberg itself being the ubiquitous super family of membrane intrinsic proteins (MIPs) that facilitate trans-membrane transport of water and an increasing number of small, water-soluble and uncharged compounds. Here we introduce you to the superfamily of MIPs and provide a summary about our gradually refined understanding of the phylogenetic relationship of its members. This volume is dedicated to the metalloids, a recently discovered group of substrates for a number of specific MIPs in a diverse spectrum of organisms. Particular focus is given to the essential boron, the beneficial silicon and the highly toxic arsenic. The respective MIP isoforms that facilitate the transport of these metalloids include members from several clades of the phylogenetic tree, suggesting that metalloid transport is an ancient function within this family of channel proteins.

Among all the various substrates that have been shown to be transported by MIPs, metalloids take an outstanding position. While water transport seems to be a common function of many MIPs, single isoforms in plants have been identified as being crucially important for the uptake of boric acid as well as silicic acid. Here, the function seems not to be redundant, as mutations in those genes render plants deficient in boron and silicon, respectively. Various isoforms seem to play very specific role in either uptake or redistribution within the plant.

The discovery of metalloid transport through MIPs and in particular the transport of arsenic and antimony has been instrumental to further develop strategies in medical research and application.

With the present work we try to put into context the recent advances that have changed our view on how transport of metalloids through these MIPs is integrated into a network of molecular players including metabolic enzymes and other transporters that together aid in homeostatic processes at the cellular level.

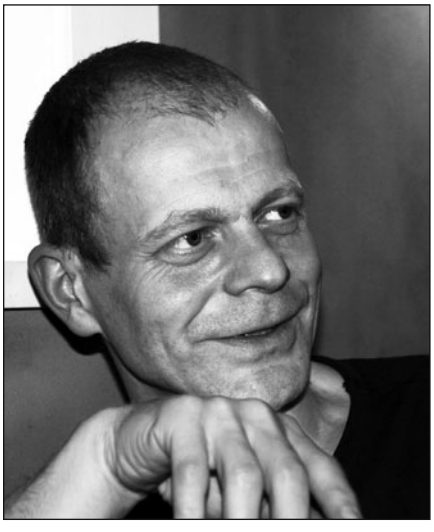
This book also provides aspects of the environmental chemistry of metalloids relevant for the reader to understand the role of MIPs in the exchange of metalloids between organisms and their environment. Finally, a chapter is included about

biomimetic membranes, artificial membrane systems that are currently being used in combination with MIPs for water purification providing an example for biotechnological interest in the large group of MIPs with various specificities.

It has been an exciting experience to be part of the discovery of metalloid transport through MIPs and it has been a pleasure to edit this book, a collaborative effort of experts, whose contribution is gratefully acknowledged. We would also like to express our special thanks to Elsbeth Bienert for providing the artistic illustrations at the beginning of each chapter.

Thomas P. Jahn, PhD
Gerd P. Bienert, PhD

ABOUT THE EDITORS...



THOMAS P. JAHN is an Associate Professor and group leader at the Department of Agriculture and Ecology, Faculty of Life Sciences, University of Copenhagen. He studied biology at the University of Bonn, Germany. From early on in his scientific career he was interested in transport processes in plants and their molecular mechanisms. More recently his group contributed to the field of aquaporin research culminating in the identification of several new substrates for members of this superfamily of channel proteins.

The overall scope of his current research focuses on the elucidation of networks comprising molecular components engaged in the responses to nutritional stresses, including elements of transport, assimilation, storage and stress signaling.

ABOUT THE EDITORS...

GERD P. BIENERT is currently a Marie Curie Fellow at the Institute of Life Science at the Université Catholique de Louvain in Louvain la Neuve, Belgium. His work focuses on the molecular characterisation of the intracellular trafficking and hetero-oligomerisation of aquaporins in plants.

In 2008, he received his PhD in Molecular Plant Nutrition from the University of Copenhagen, Denmark. During his PhD, Gerd Patrick Bienert made significant advances in the scientific understanding on the substrate selectivity of plant aquaporins for uncharged solutes. The work resulted in the molecular identification of the first arsenite, antimonite and hydrogen peroxide channels in plants.

Gerd P. Bienert studied biology at the Julius-Maximilians-University Würzburg and at the Technical University Darmstadt, Germany. During his education he emphasized molecular plant physiology and biophysics, genetics and biotechnology.

Gerd P. Bienert's main research interests focus on the molecular transmembrane transport processes involved in the uptake, translocation and extrusion of compounds that are relevant for plant physiology. In addition, intracellular regulation and trafficking of the transport proteins themselves are also contemplated.

Gerd P. Bienert was born in Rothenburg an der Tauber, Germany. In his home region, Tauber-Franken, he began to develop his enthusiastic curiosity for biology by exploring and studying nature. He became fascinated by insects, especially the members of the order of hymenoptera to which he still devotes his free-time. The existing overlap between entomology and botany has aroused his interest in understanding the physiology of plants.

PARTICIPANTS

Eric Beitz
Department of Pharmaceutical
and Medicinal Chemistry
University of Kiel
Kiel
Germany

Gerd P. Bienert
Unité de Biochimie Physiologique
Institut des Sciences de la Vie
Université catholique de Louvain
Louvain-la-Neuve
Belgium

François Chaumont
Institut des Sciences de la Vie
Université catholique de Louvain
Louvain-la-Neuve
Belgium

Jonas Å.H. Danielson
Department of Biochemistry
Molecular Protein Science Center
Center for Chemistry and Chemical
Engineering
Lund University
Lund
Sweden

Toru Fujiwara
Biotechnology Research Center
University of Tokyo
Tokyo
Japan

Charles Hachez
Institut des Sciences de la Vie
Université catholique de Louvain
Louvain-la-Neuve
Belgium

Peter E. Holm
Department of Basic Sciences
and Environment
Faculty of Life Sciences
University of Copenhagen
Frederiksberg
Denmark

Thomas P. Jahn
Department of Agriculture and Ecology
Faculty of Life Sciences
University of Copenhagen
Frederiksberg
Denmark

Urban Johanson
Department of Biochemistry
Molecular Protein Science Center
Center for Chemistry and Chemical
Engineering
Lund University
Lund
Sweden

Takehiro Kamiya
Biotechnology Research Center
University of Tokyo
Tokyo
Japan

Zijuan Liu
Department of Biological Sciences
Oakland University
Rochester, Michigan
USA

Enzo Lombi
Center for Environmental Risk Assessment and Remediation
University of South Australia
Mawson Lakes Campus
Mawson Lakes, South Australia
Australia
and
CRC CARE
Salisbury, South Australia
Australia

Jian Feng Ma
Research Institute for Bioresources
Okayama University
Kurashiki
Japan

Kyoko Miwa
Biotechnology Research Center
University of Tokyo
Tokyo
Japan

Rita Mukhopadhyay
Department of Molecular Microbiology and Infectious Diseases
Florida International University College of Medicine
Miami, Florida
USA

Claus Hélix Nielsen
Quantum Protein Center
Department of Physics
Technical University of Denmark
and
AQUAporin A/S
Lyngby
Denmark

Barry P. Rosen
Department of Biochemistry and Molecular Biology
Wayne State University School of Medicine
Detroit, Michigan
USA

Markus J. Tamás
Department of Cell and Molecular Biology/Microbiology
University of Gothenburg
Gothenburg
Sweden

Mayuki Tanaka
Biotechnology Research Center
University of Tokyo
Tokyo
Japan

CONTENTS

Elsbeth Bienert

Chapter 1

Aquaporins:
A Family of Highly Regulated Multifunctional Channels

Charles Hachez and François Chaumont*

Abstract

Aquaporins (AQPs) were discovered as channels facilitating water movement across cellular membranes. Whereas much of the research has focused on characterizing AQPs with respect to cell water homeostasis, recent discoveries in terms of the transport selectivity of AQP homologs has shed new light on their physiological roles. In fact, whereas some AQPs behave as "strict" water channels, others can conduct a wide range of nonpolar solutes, such as urea or glycerol and even more unconventional permeants, such as the nonpolar gases carbon dioxide and nitric oxide, the polar gas ammonia, the reactive oxygen species hydrogen peroxide and the metalloids antimonite, arsenite, boron and silicon. This suggests that AQPs are also key players in various physiological processes not related to water homeostasis. The function, regulation and biological importance of AQPs in the different kingdoms is reviewed in this chapter, with special emphasis on animal and plant AQPs.

Introduction—The Discovery of Aquaporins

Water is the most abundant substance in cells and organisms and is indispensable for life. Transport of water across phospholipid membranes is a universal requirement for any living organism, but, for decades, transmembrane water flow was explained only by the simple diffusion of water molecules through the phospholipid bilayer. However, this process is very slow and requires a high activation energy (E_a > 10 kcal/mol). The model of simple diffusion failed to explain why the membrane permeability of some cell types is so high that the bulk movement of water across the membrane occurs as fast as if no membrane was present and why the activation energy is comparable to that of water molecules diffusing freely in solution (E_a < 5 kcal/mol). Moreover, plasma membrane water permeability varies greatly for different cell types and, for a given cell type, transient variations in membrane water permeability are observed in response to the application of diuretic and anti-diuretic compounds or blocking agents, such as mercury chloride. These observations could only be explained if water channels existed in the membrane. The existence of such "hydrophilic pores" within biological membranes was first hypothesized in 1956 by Stein and Danielli[1] to account for the high membrane water permeability of red blood cells, which cannot be explained by a simple diffusion model.[2] In plants, the existence of such aqueous pores was first postulated by Ray.[3] However, the exact nature and role of these water channels remained obscure for years. The discovery, in the beginning of the nineties, of

*Corresponding Author: François Chaumont—Institut des Sciences de la Vie, Université catholique de Louvain, Croix du Sud 5-15, B-1348 Louvain-la-Neuve, Belgium. Email: francois.chaumont@uclouvain.be

MIPs and Their Role in the Exchange of Metalloids, edited by Thomas P. Jahn and Gerd P. Bienert.

proteinaceous channels that enhanced the water permeability of the membranes solved this mystery. These water channels, named aquaporins (AQPs), shed a new light on water movement regulation, but, as the number and range of known AQP-permeating compounds constantly increases, it appears that AQPs also play a central role in many other biological processes not related to cell water balance regulation. AQP research has recently demonstrated that AQPs act as multifunctional channels and have unexpected roles. In this chapter, we will describe the nature of AQPs, as well as their regulation and involvement in cell physiology.

The first report of protein-mediated water transport through membranes was by Gheorghe Benga in 1986.[4] In 1987, the archetypal animal water channel was accidentally copurified with the blood group antigen Rh from cell membranes of Rh (D)-positive erythrocytes.[5,6] This 28 kDa protein turned out to be extremely hydrophobic and, after isolation and characterization, was found to present some similarities with other channel-forming proteins[7] and was therefore designated CHIP28 for "channel-like integral protein of 28 kDa".[8] CHIP28 was found to be expressed in tissues with a high water permeability[5] and showed homology to other uncharacterized proteins: a 26 kD major intrinsic protein (MIP) from bovine lens cells, *Drosophila* big brain, soybean nodulin 26 and *Escherichia coli* glycerol facilitator (GLPF) protein. In 1992, evidence that CHIP28 displayed water channel activity when heterologously expressed in *Xenopus* oocytes or inserted in proteoliposomes constituted a major breakthrough in the field and paved the way to AQP research.[9] CHIP28 was renamed AQP1 and MIP, as the original prototype member, was renamed AQP0. In 1999, Agre[10] reported the first high-resolution images of the three-dimensional structure of AQP1 at 4.5 Å resolution. Further studies using computer simulations identified the path of water molecules as they move through the channel and demonstrated how a pore can allow water to pass without allowing the passage of small solutes.[11,12] Peter Agre was awarded the 2003 Nobel Prize for chemistry for his role in the discovery and characterization of AQP1.

AQPs belong to the ancient MIP family, the members of which are found in all kingdoms, with more than 450 AQPs having been identified in organisms ranging from vertebrates and insects to plants, fungi, bacteria[13,14] and even viruses.[15] MIPs form channels through membranes, facilitating the passive transport of water and other small neutral solutes. Although the term "aquaporin" was initially restricted to water-transporting MIPs, it is now used to describe all MIPs. While five to seven AQP homologues have been identified in different insect species[14] and 13 in mammals,[16] higher plants show a higher multiplicity of isoforms, with 35, 33, or 36 homologs in *Arabidopsis thaliana*, *Oryza sativa,* or *Zea mays,* respectively.[17-19] Plant AQPs are classically grouped into four subfamilies on the basis of sequence similarity, these being the plasma membrane intrinsic proteins (PIPs), the tonoplast intrinsic proteins (TIPs), the nodulin 26-like intrinsic proteins (NIPs) and the small basic intrinsic proteins (SIPs). Recently, three other subfamilies, the GlpF-like intrinsic proteins (GIP), the hybrid intrinsic proteins (HIPs) and the uncategorized X intrinsic proteins (XIPs), have been discovered in the moss *Physcomitrella patens*.[20,21]

Whereas much of the research effort has focused on characterizing AQPs with respect to cell water homeostasis, recent discoveries about the transport selectivity of MIP homologs suggest that some AQPs are more than just water channels and that AQPs are involved not only in cell water relations, but also in many other major physiological processes.[22,23] For example, in mammals, a role of AQPs in osmotically driven transepithelial fluid transport, as occurs during urine concentration and glandular fluid secretion, has been clearly demonstrated. In addition, involvement of AQPs in other physiological processes has been revealed, e.g., in cell migration, brain and corneal swelling, skin hydration, adipocyte fat accumulation, neural signal transduction and apoptosis.[24-27] In plants, AQPs are involved in many major processes not strictly related to osmoregulation, including nutrient acquisition, nitrogen and carbon fixation, cell signaling and stress responses.[28,29] This sheds new light on their physiological roles in living organisms that will be emphasized throughout this chapter.

Topology of Aquaporins

AQPs are transmembrane proteins composed of 243 to 302 amino acids and typically containing six membrane-spanning α-helices, with the N- and C-termini located on the cytoplasmic side of the membrane. The N- and C-terminal halves form two tandem repeats of three membrane-spanning α-helices (TM1-TM6) showing significant sequence similarity. Each half contains two small hydrophobic loops (loops B and E). This tandem repeat structure probably originates from an ancient intragenic duplication preceding the divergence between pro- and eukaryotes 2.5 billion years ago.[30] The cytosolic loop (loop B) between the second and third transmembrane domains and the extra-cytosolic loop (loop E) between the fifth and sixth transmembrane domains form short helices that are relatively hydrophobic. These two loops contain a asparagine-proline-alanine (NPA) motif, highly conserved in the MIP family, suggesting an essential role for protein function. The two halves of the protein show an obvious structural symmetry, with each of the NPA motif-containing loops dipping into the middle of the lipid bilayer to form two hemipores that, together, create a water-filled channel. The pore formed in AQP1 allows 3×10^9 water molecules per monomer per second to pass through the membrane.[11,31,32] The narrowest part of the pore only measures about 3 Å, which is slightly larger than the 2.8 Å diameter of the water molecule.[11] The highest degree of conservation is found in the transmembrane domains, particularly in the two regions containing the NPA motifs.

The three-dimensional structure of several AQPs has been determined at high spatial resolution (5 to 2.2 Å) by electron microscopy and X-ray crystallography.[11,33-45] The AQP1 channel consists of three topological elements, an extracellular vestibule and a cytoplasmic vestibule connected by an extended narrow pore acting as the selectivity filter. As a result of the pore topology and spatial arrangement of amino acids within the pore region, AQP selectivity results from a combination of mechanisms, including size exclusion at two highly conserved constriction regions (the NPA motif and the aromatic/arginine filter) and stereoscopic recognition of the substrate mediated by spatially defined H-bonding and hydrophobic interactions within the pore.[11,34,46] Analysis of the AQP pore indicates that proton transport through the channel is energetically highly unfavorable. Indeed, whereas water molecules can permeate the pore with a minimal energy barrier, transfer of protons is blocked by a combination of electrostatic repulsion, unfavorable dipole orientation and a H-bond isolation mechanism.[11,12,34,47-49]

AQPs usually form tetramers. This tetrameric assembly seems to be crucial for the proper folding, stability, targeting to the plasma membrane and even functionality of AQPs.[33,42,50,51] According to Murata et al,[11] each monomer interacts with two neighboring monomers through the membrane-spanning α-helices. Nevertheless, the monomer is the functional unit, transporting water independently of the other subunits in the complex.[34,52,53] However, a recent report has shown that, even though each monomer provides its own functional water pore, the properties of this pore are determined by a cooperative interaction of the entire tetramer, allowing stabilization of the water pore structure.[54]

Selectivity of Aquaporins

AQPs were originally characterized as water channels and have been shown to increase the membrane water permeability by up to 20-fold. Their classical role in facilitating transcellular water transport in many fluid-transporting tissues is now well characterized in mammals and plants, as well as in single cell organisms.[27,46] In mammals, involvement of AQP in the urine concentrating mechanism, gland fluid secretion and swelling of tissues under stress is currently being extensively studied using AQP-knockout mice and their unexpected role as water channels in processes such as cell migration has been recently demonstrated.[27,55] In plants, metabolic control of the membrane water permeability by regulation of AQP activity constitutes a way to deal with environmental cues and control tissue water balance.[28,56,57] Plant water balance is a broad concept which encompasses multiple aspects of plant-water relations, such as the regulation of long distance water transport (in the presence or absence of transpiration-driven fluxes), the appropriate water supply for maintenance of cell turgor and elongation and the regulation of cell homeostasis.

The time when AQPs were considered as simple water conduits is now past, as a constantly increasing number of studies demonstrate that AQPs are multifunctional channels. Although some members of each of the main AQP subfamilies have been shown to act as water channels, AQPs are being found to have an ever-increasing range of substrates.[22,23] Together with the fact that the different subfamilies of AQPs are distributed differently in tissues and cell compartments, the idea that some of these isoforms may not be restricted to water conductance, but are involved in solute transport, has recently found experimental support in the AQP community, with the identification of AQP substrates of physiological importance.[14,28,58,59] Whereas some AQPs, such as SoPIP2;1,[35] behave as "strict" water channels, others can conduct a wide range of nonpolar solutes, such as urea or glycerol and even more unconventional permeants, such as the nonpolar gases carbon dioxide and nitric oxide, the polar gas ammonia, the reactive oxygen species hydrogen peroxide and the metalloids antimonite, arsenite [As(III)], boron (B) and silicon (Si).[23,55,58] This indicates that AQPs are key players in various physiological processes not related to water homeostasis. For example, in mammals, there is now strong evidence for the involvement of aquaglyceroporin-facilitated glycerol transport in skin hydration and fat cell metabolism.[58] In plants, GIP1;1, NtAQP1 and Nt-TIPa have been shown to facilitate the passage of urea and/or glycerol.[20,60,61] Absorption of compounds, such as ammonia/ammonium or even formamide, has been shown to be facilitated by TaTIP2.[62] Recently, the diffusion of reduced and noncharged species of the metalloids Si, B, arsenic and antimony was shown to be facilitated by MIPs from different organisms (reviewed in refs. 22,55). For example, Si uptake as silicilic acid is facilitated in rice by NIP Lsi1 (see also chapters by Ma as well as Bienert and Jahn in this book). Facilitated transport of Si is important in higher plants, as Si can account for up to 10% of the shoot dry weight.[63] Boron uptake under conditions of B limitation is achieved through AtNIP5; 1 acting as a plasma membrane boric acid channel (see also the chapter by Miwa et al in this book).[64] AtNIP2;1 transports lactic acid, with a preference for the protonated acidic form and its expression is highly up-regulated in response to water logging and anoxia stress. This suggests that AtNIP2;1 might play a role in adaptation to lactic fermentation under anaerobic stress.[65] The finding that some plasma membrane AQPs facilitate CO_2 diffusion in plants has recently shed new light on their function in carbon bioassimilation in leaves.[29,66] Hydrogen peroxide is used in signal transduction pathways and acts as both an intra- and inter-cellular signaling molecule[55,67] and its diffusion has been shown to be facilitated by mammalian AQP8, but not by AQP1 and by two tonoplast AQPs from Arabidopsis (AtTIP1;1 and AtTIP1;2).[23] Interestingly, another nonwater permeant, ammonia, is conducted by AQP8, but not AQP1,[62] which strongly suggests that close AQP orthologs show different selectivity profiles in a given organism. This fact, together with their tissue localization, is currently believed to be one of the main reasons for the diversity of AQP homologs found in a given organism.

Measurement of Aquaporin Activity and Water Movement

Different methods exist to measure the contribution of AQPs to membrane water permeability, each with advantages and disadvantages. We have chosen to illustrate four of the most commonly used approaches. It is worth noting that these methods are not restricted to measuring the water channel activity of AQPs, but can also be used to measure the passage of other solutes or gases. The different methods do not provide the same kind of information and are often complementary.

Cell Swelling Assays

Cell swelling assays were among the first methods used to investigate the water channel activity of AQPs. Different methods exist, but the most common employs defoliculated oocytes of the African clawed frog *Xenopus*, which exhibit low permeability to water due to the fact that their cell membrane is essentially devoid of water channels. An expression vector containing the *AQP* cDNA of interest (or in vitro transcribed mRNA) is microinjected into defoliculated oocytes, then, after sufficient time to allow the heterologous expression of the AQP protein (usually 48 to 72 hours), the oocytes are examined under a microscope. Following hypoosmotic challenge, their volume change is recorded in real time. This method allows calculation of the water permeability

associated with the expression of a given AQP isoform in the oocyte plasma membrane and has been widely used in the AQP field.[68,69] The *Xenopus* oocyte swelling assay has been used to assess functional consequences of amino acid substitutions or deletion on AQP activity and to measure cooperative effect of AQP proteins in regulating cell water permeability.[68]

The main drawback of this method is that it is laboratory animal-based and so faces increasing legal restrictions aimed at guaranteeing animal welfare. Moreover, heterologous expression of AQPs does not always lead to the insertion of these proteins in the oocyte plasma membrane. Therefore a negative result in terms of swelling needs to be confirmed by localization of GFP fusion proteins or immunodetection. It is worth noting that oocyte swelling assays can be used to assess the selectivity of AQP isoforms by measuring the incorporation of radioactively labeled potential substrates in control and AQP-expressing oocytes.[20] AQP-mediated gas conductance (NH_3, CO_2) as well as AQP-mediated passage of metalloids (Si, As(III) and B) have also been studied using this expression.[63,70-72]

In the plant field, a similar approach using plant cell protoplasts has been widely used to measure the activity of endogenous AQPs[57,73-77] or of transiently or constitutively overexpressed isoforms.[51,78] Results using this expression system must be interpreted with caution, as results with protoplasts do not necessarily reflect the cell membrane water permeability measured *in planta*.[77] The advantage for plant biologists is that protoplasts offer an easy-to-handle homologous expression system, but this method has important limitations in terms of the interpretation of the results, as the assignment of the protoplast to a given cell type is difficult and as protoplasting probably modifies the expression of *AQP* genes.[77,79]

Stopped-Flow Spectrophotometry

Stopped-flow spectrophotometry, a technique widely used in AQP research, relies on the analysis of particle light scattering.[80-83] Each suspended particle passing through a laser beam scatters the light in some way. This combination of scattered light is picked up by detectors and, by analyzing fluctuations in brightness at each detector, it is possible to correlate this with variations in the volume of the particle. Cytoplasmic volume variations can be monitored either by fluorescence quenching or by 90° scattered light. Using this technique, the kinetics of plasmolysis or deplasmolysis of AQP-expressing bacterial or yeast cells and of AQP-enriched reconstituted proteoliposomes in response to osmotic challenges are usually recorded in real-time. One advantage of this technique is that, once it has been correctly set up, repeatable results for a large panel of specific AQP isoforms can be rapidly acquired and the process can even be automated. Another is that analyses are performed on easy-to-transform single cell organisms (yeast, bacteria) or even under cell-free conditions (reconstituted proteoliposomes), which can allow the determination of the intrinsic water permeability of a single AQP isoform. Using such expression systems, care should be paid to the correct folding and targeting to the biological membrane, of the heterologously expressed proteins, as mentioned in the case of Xenopus oocytes.

Cell Pressure Probe Measurements

Pressure probe measurements allow determination of the cell hydraulic conductivity, L_p ($m \cdot s^{-1} \cdot MPa^{-1}$), by modulation of the cell turgor. This is currently the technique of choice to probe AQP activity *in planta*,[77,84] but is unfortunately not applicable to cells lacking a rigid cell wall, such as animal cells. After puncturing of the cell using a microcapillary, it is possible to modulate its turgor pressure. The transmembrane water flow results from a hydraulic flow driven by the hydrostatic pressure gradient, ΔP, generated by the pressure probe which causes water to flow out of, or into, the cell. The parameter of interest is the half time ($T^{w}_{1/2}$) value, the time required for half of the turgor change from a given value to the equilibrium value. The elastic modulus (ε) is the other important physiological parameter that must be measured to correlate the volume and pressure of the cell and characterize the mechanical rigidity of the cell wall. High values of ε refer to a low extensibility or a rigid cell wall, i.e., big changes in pressure cause small changes in cell volume. Low values of ε, on the other hand, mean a highly extensible cell wall. The elastic modulus

has the dimensions of pressure and is usually given in MPa. These two parameters, $T^{w}_{1/2}$ and ε are used to calculate the cell hydraulic conductivity L_p according to Steudle et al.[85]

Proton NMR

Proton NMR technology uses the magnetic properties of hydrogen (^1H) nuclei, present mostly in water. This technique detects changes in the properties of water protons as they move from one physical state or chemical configuration to another and thus permits the non-invasive detection of water movement in different compartments within an organism.[86-88] The determination of the transport processes involved in water balance is a subject of considerable interest in many fields of research, e.g., in medicine or in plant and environmental sciences. The advantage of proton NMR over other analytical methods is its non-invasiveness and the possibility of simultaneously acquiring measurements at different organizational levels, from the whole organism to the tissue, or even the cellular, level. Magnetic resonance imaging allows visualization of both static and dynamic water phenomena. Imaging of water movement can be studied either by following the local intensity in time-controlled sequential images (e.g., for monitoring slow de- or rehydration processes) or by directly mapping proton displacement in a well defined time interval based on pulsed field gradient NMR or displacement imaging.[86,88,89]

Aquaporin Inhibition

Blocking of AQPs has been observed with compounds that can oxidize the Cys residues associated with the pore region of the protein or that can bind to protein sulfhydryl groups. A traditional method for determining the involvement of AQPs in physiological processes is the use of AQP inhibitors, such as mercury chloride ($HgCl_2$).[9,46,90] Treatment with a reducing agent, such as β-mercaptoethanol or dithiothreitol, partially reverses the inhibition. It should be noted that the use of this type of blocker can have many side effects, as they also perturb the ionic homeostasis of the cell and the integrity of its membrane. Phloretin, a flavonoid compound, is another transport blocker that has been shown to block AQPs in animals[91,92] and plants.[75,77,93,94] This compound is known to adsorb to lipid surfaces and to alter the dipole potential of lipid bilayers. Its mechanism of inhibition is still unclear, but it might interfere with the conformation of the AQP protein in the membrane.

Most, if not all, plant plasma membrane AQPs are specifically blocked by intracellular acidification due to the presence of charged His residues in the cytosolic loop D that control its conformation and the pore conductance.[35,95] This mechanism can be used to artificially inhibit AQP activity; cell (or tissue) acid loading with propionic acid (20 mM) artificially induces acidosis, which reversibly and specifically inhibits AQPs.[95] This reversible pH inhibition mechanism provides a novel and mild method for probing AQP activity in living organisms.

AQPs appear to play a central role in some human diseases, so it is possible that the manipulation of water channel proteins using compounds developed for this purpose might be exploited for clinical benefit.[27] These could be used to treat clinical disorders in which water depletion or water overload is an important factor, as in brain edema, glaucoma, epilepsy, obesity, or cancer. The search for AQP-targeting drugs using site modeling analysis and systematic screening is currently in progress.

Phenotype Analysis Reveals Involvement of Aquaporins in Key Physiological Processes

In some cases, AQP mutations in man have been used to study the biological significance of a given AQP isoform.[27,96-98] However, reverse genetics, when applicable, provides a more specific and elegant approach to probing AQP activity in vivo and is the strategy of choice for studying the physiological roles of AQPs in living organisms. This approach theoretically makes it possible to alter the expression of a single gene or a set of genes.[99] The role of AQPs in mammalian physiology has been mainly studied by phenotype analysis of transgenic mouse models with *AQP* gene deletions/mutations.

In both mice and humans, phenotype studies support the expected roles of AQPs as water channels mediating kidney fluid transport during urine concentration and fluid secretion from glandular epithelia. As thoroughly reviewed by Verkman et al,[27,100] the analysis of these mutants has revealed that AQPs are involved in many kinds of unexpected physiological phenomena related to their ability to transport water, such as brain and corneal swelling, neural signal transduction, regulation of intracranial and intraocular pressure, tumor angiogenesis and cell migration. The strong expression of some mammalian AQPs in tumor cells, particularly in aggressive tumor cell lines, has received increasing attention regarding their role in cancer development. The involvement of AQPs in cell migration and proliferation processes, as inferred from reverse genetic studies, suggests that they play key roles in cancer biology.[27,100] For example, while AQP1 is expressed ubiquitously in tumor vascular endothelium, AQP1-null mice show defective tumor angiogenesis due to impaired endothelial cell migration.[26] In contrast, enhanced migration of AQP-expressing cancer cells has been recorded in vitro and increased local tumor invasion, tumor cell extravasation and metastases have been found in vivo,[100] showing that AQPs are important in tumor growth and spread. As glycerol facilitators, aquaglyceroporins have been found to play unexpected roles in skin hydration, fat metabolism and tumorigenesis.[58,100] For example, AQP3-null mice have a dry skin, reduced skin elasticity and impaired epidermal biosynthesis.[58] In addition, a basal cell carcinoma was shown to have increased levels of AQP3, AQP3-null mice were found to be more resistant to skin tumorigenesis by a mechanism that might involve reduced tumor cell glycerol metabolism and ATP generation.[100]

In the plant field, given the high multiplicity of isoforms found in a single organism, modulation of AQP expression is considered the strategy of choice for elucidating the role of AQPs in plant physiology.[76,101-105] Silencing of plasma membrane AQPs usually results in a decrease in cell water permeability,[76,101-103] but, although PIP-silenced plants usually show lower cell water permeability than control plants, this does not necessarily result in lower tissue water conductivity, probably as a consequence of the different mechanisms used by plants to deal with lower membrane water permeability (for example, by increasing their root mass).[99] Recently, using antisense lines, tobacco NtAQP1 was shown to facilitate the passage of CO_2 through biological membranes.[66,106] NtAQP1-related CO_2 permeability seems to be of physiological importance under conditions in which the CO_2 gradient across a membrane is small, as is the case between the atmosphere and the plant cell cytosol.[66] A decrease in membrane resistance to CO_2 transport might increase CO_2 bioavailability and improve photosynthesis, ultimately affecting the degree of photorespiration and efficiency of nitrogen and water use. However, the biological significance of AQP-facilitated CO_2 diffusion is still controversial, as this process seems to be mainly governed and limited by the existence of unstirred layers.[107]

Although attractive, reverse or forward genetic approaches are generally complicated by many factors, such as the incomplete characterization of the transport selectivity of the targeted AQP, the fact that AQPs can act as multifunctional channels with multiple physiological roles, possible compensation/interaction mechanisms between close homologs and the number of posttranslational regulation mechanisms. It is also worth mentioning that these strategies can also represent a risk for false interpretation and that results should be supported by complementary approaches. The fact that RNAi-mediated loss of aquaporin AtTIP1;1 was first reported to result in early senescence and plant death[104] and that transposon insertion line, completely devoid of AtTIP1;1 protein did not result in any significant effect on plant metabolism[108] clearly illustrates this latter point.

Aquaporin Regulation: Gating and Localization

The presence of AQPs in cell membranes makes it possible to regulate water flux between and within cells. This fine regulation is assumed to be under metabolic control. Cells have the ability to alter their water conductivity within a few seconds or minutes in response to stimuli such as hormones (e.g., vasopressin in mammals and abscisic acid in plants) or diuretic or anti-diuretic compounds. Depending on the species and conditions, short-term changes of several orders of magnitude can be seen in membrane water conductivity. In mammals, AQPs are localized in

epithelia that require a high rate of water flux, such as the collecting duct of the kidney, the lung capillaries and the secretory cells of the salivary glands.[109,110] In plants, AQPs have been shown to be expressed in regions where a high cell-to-cell water permeability is required, such as in the vicinity of apoplastic barriers in roots[57] or in leaf vascular bundles[111,112] or reproductive organs.[113] In both mammals and plants, different AQPs in the same organism differ in their transcriptional or posttranslational regulation and subcellular distribution.[97,114] This complex and multilevel regulation allows a finely tuned and rapid control of membrane water permeability mediated by changes in the expression and activation of AQPs.[46,115,116]

The gating of AQPs makes it possible to control the water flow through the pore of the protein and is accomplished by a conformational change in the protein that blocks the channel and thus prevents the flow of water through the pore. In plants and mammals, different gating mechanisms have been observed; these involve the phosphorylation/dephosphorylation of certain serine or threonine residues, the protonation of specific histidine residues and the binding of divalent cations. The same AQP isoform can be simultaneously present in the same membrane in several forms with different isoelectric points.[117] This is proof of the importance of the co or posttranslational regulation of AQP activity. These posttranslational modifications affect the apparent size and isoelectric point of the protein and can be visualized using 2D gel electrophoresis.[117]

Regulation of subcellular localization through AQP trafficking may also represent a means of modulating the membrane water permeability. In fact, the subcellular localization of AQPs should not be seen as a static, but rather as a dynamic, phenomenon. This relocalization of AQP isoforms has been observed in mammalian and plant cells.[118,119] Although the details of the mechanism leading to AQP relocalization differ between these two organizational models, the principle remains the same: the density of functional AQPs in the cell membrane is controlled by the fusion (or endocytosis) of AQP-rich vesicles. As a result, the net density of active AQPs increases (or decreases), leading to enhanced (or decreased) cell membrane water permeability.

Phosphorylation

Phosphorylation of AQPs was first discovered as a posttranslational modification regulating the water channel activity of a TIP in plants[120] but was also shown to play a critical role in the regulation of AQP function in animals and humans.[27,121,122] Phosphorylation was shown to directly affect the water channel activity of the targeted protein, but its effect seems to vary greatly, ranging from stimulation of water transport activity (e.g., SoPIP2;1,[123] ZmPIP2)[69] to its inhibition (e.g., HsAQP4).[122] In addition phosphorylation of AQPs can affect their subcellular localization.[118]

In mammals, phosphorylation of AQPs is mediated by the activity of kinases belonging to the protein kinase A (PKA), protein kinase C (PKC) and protein kinase G (PKG) families.[118,121,124] PKA, also known as cAMP-dependent protein kinase, refers to a family of enzymes, the activity of which is dependent on the level of cyclic AMP (cAMP) in the cell. At low cAMP levels, the kinase is catalytically inactive and activation is triggered by an increase in the cAMP concentration (e.g., activation of adenylate cyclases by G protein-coupled receptors or inhibition of phosphodiesterases that degrade cAMP). The activated kinase then catalyzes the transfer of the terminal phosphate of ATP to serine or threonine residues in protein substrates. PKC is a family of protein kinases that require calmodulin, Ca^{2+}, diacylglycerol and phospholipids for activation. It is currently believed that a given AQP isoform may be the substrate for several kinases belonging to different families. For example, AQP2 is phosphorylated by PKA, but putative phosphorylation sites for PKG, PKC and casein kinase II are also present in the sequence. There is now compelling evidence that PKA-dependent phosphorylation of ser256 in the carboxy-terminal of AQP2 plays a critical role in its trafficking to the apical membrane of kidney duct epithelial cells, whereas activation of the PKC pathway leads to its endocytosis, which is independent of the phosphorylation state of ser256.[125]

In plants, CDPKs seem to be the predominant calcium-dependent kinases phosphorylating AQPs.[69,123,126] A typical CDPK is activated by an increase in free Ca^{2+} concentrations from nM

to μM levels which leads to the phosphorylation of its target. Several plant AQPs have been shown to be phosphorylated in vivo and in vitro at serine residues in the N- or C-terminal regions. For example, in vivo serine phosphorylation has been detected in a variety of plant isoforms, i.e., spinach SoPIP2;1, soybean NOD26, bean PvTIP3;1 and *Arabidopsis* and maize PIP1 and PIP2 isoforms.[69,117,123,127-132]

The effect of the phosphorylation of animal and plant AQPs has been demonstrated by the heterologous expression of mutated proteins in *Xenopus* oocytes, as kinase and phosphatase agonists or antagonists can be added to the medium and their effects measured. [69,120,123,128] For instance, the water channel activity of PvTIP3;1 in oocytes is increased by addition of cAMP agonists, which stimulate oocyte protein kinase A.[120] Similarly, the activity of SoPIP2;1 and GmNodulin26 is increased when oocytes are incubated in the presence of the phosphatase inhibitor, okadaic acid.[123,133] Mutation of Ser to Ala or dephosphorylation of highly conserved Ser residues in the C-terminus or in loops B and D leads to a decrease in water channel activity.[35,69,120,123,133]

Recently, two kinases acting on the phosphorylation sites ser115 and ser274 of SoPIP2;1 were characterized.[134] The kinase acting on ser274 is bound to the plasma membrane and belongs to the CDPK family. The kinase acting on ser115 is soluble and has still to be characterized. However, its recognition site is typical of that for PKA, CDPK, or PKC.[134]

pH and Divalent Cations

Regulation of AQP permeability by protons and/or Ca^{2+} has been reported for mammalian AQP0, AQP3 and AQP6[135-138] and for plant PIPs.[35,95,139]

The pH effects are different, depending on the isoform. For example, the water permeability of rat AQP3 is decreased by a shift to an acidic external pH (from pH 7.4 to 5.6), but no such effect is observed with rat AQP0, AQP2, AQP4, or AQP5.[140] On the other hand, the water permeability of bovine AQP0 is increased by a shift to an acidic pH.[137] Extracytosolic loops A and C, which contain histidine residues, contribute to this pH sensitivity and the differences were shown to originate from the position of these key His residues in the extracytosolic loops.[138] Mutation of each of these key His residues results in a shift in pH sensitivity. Interestingly, acid or alkaline sensitivity is induced in human AQP1 by adding His residues at positions 48 in loop A and 130 in loop C.[138] In plants, most, if not all, PIPs are blocked by intracellular acidification.[95] Mutagenesis experiments demonstrated the importance of his197 (located in cytosolic loop D) in this pH-dependent regulation mechanism: the H197A mutant shows less pronounced P_f inhibition, while H197D, in which a negatively charged amino acid residue is introduced, is pH-insensitive.[95] This AQP gating in response to intracellular pH acidification plays a role in the response to anoxia (oxygen deprivation) and other environmental stresses.[95] This pH-dependent inhibition of the membrane water permeability is also observed in *Arabidopsis* plasma membrane vesicles, in which acidification to pH 6.0 reduces water permeability to 20% of that seen at pH 8.3.[139] Similar observations were made at low pH on tonoplast vesicles isolated from the storage roots of *Beta vulgaris*.[141] As his197 is conserved in PIPs, this mechanism makes it possible to understand how plant roots decrease their water transport capacity in response to flooding, which leads to anoxia and cytosol acidification.[95] The inhibition mechanism has been modeled in detail by Tornröth-Horsefield et al.[35]

The divalent cation Ca^{2+} modulates the activity of both animal and plant AQPs.[35,83,138,139,142] Whereas some mammalian isoforms, such as bovine AQP0 or MIPfun (killifish AQP0), appear to be inhibited by Ca^{2+}, others, such as rat AQP4 and human AQP1, are not Ca^{2+}-responsive. When observed, this Ca^{2+} inhibition has been shown to be mediated by calmodulin. As the sites of Ca^{2+} inhibition and pH inhibition are distinct, the mechanisms of pH sensing and Ca^{2+} inhibition are completely separable.[138,143]

In plants, Ca^{2+} has also been shown to modulate the activity of AQPs, but the inhibition mechanism is somewhat different from that for mammalian isoforms, as no calmodulin is required for the binding of Ca^{2+} to the AQP. The hydraulic conductivity of *Arabidopsis* cells is reduced by up to 4-fold in the presence of Ca^{2+}.[83,139] Moreover, in the presence of Ca^{2+} chelators, a shift to

higher P_f values compared to control conditions is observed, suggesting downregulation of AQP activity through Ca^{2+} binding. Crystallographic studies and molecular dynamics simulations have demonstrated the importance of loop D in controlling whether the pore is open or closed.[35] Depending on its conformation, loop D acts as a flexible lid to the pore. By altering the conformation of this loop through a network of ionic interactions and hydrogen bonds between specific amino acids in the loop and the N-terminal part of the protein, divalent cations, such as Cd^{2+} or Ca^{2+}, stabilize the closed conformation of the pore.[35] Recently, Cd^{2+} and Mn^{2+} were shown to be potent inhibitors of AtPIP2;1,[83] probably acting via the same mechanism as Ca^{2+}, but the physiological significance of such inhibition needs further analysis.

Hetero-Oligomerization

Hetero-oligomerization is another way to regulate the activity/trafficking of oligomeric protein complexes and has been observed for many membrane proteins unrelated to AQPs.[144-146] For example, animal voltage-gated K^+ channels of the Shaker superfamily and their plant homologs result from the tetrameric assembly of subunits.[147] The data obtained with these animal and plant channels have demonstrated the existence of heteromerization between different types of subunits, generally, but not always, belonging to the same subfamily.[146,148,149] The case of the AKT2 subunit in plant K^+ channels is representative: hetero-oligomerization with the KAT2 subunit results in relocalization of the protein from the Golgi apparatus to the plasma membrane.[146]

A wide range of experimental data suggests that AQPs usually form homotetramers.[11,43,150] However, several plant isoforms might possibly form heterotetramers.[51,68,151] In *Lens culinaris* seeds, heteromerization of two TIPs was detected in crosslinking experiments, in which dimers, trimers and tetramers were irreversibly crosslinked using dithiobis-succinimidylpropionate.[151] In maize, a cooperative mechanism between ZmPIP1 and ZmPIP2 isoforms was recently demonstrated.[51,68] Whereas all PIP2s exhibit high water-channel activity in *Xenopus* oocytes, plant protoplasts, or yeast vesicles, PIP1s are often inactive or have low activity.[61,82,123,152-155] Interestingly, when ZmPIP2s are co-expressed with increasing amounts of ZmPIP1;2 in oocytes, a synergistic effect on AQP activity is observed, probably resulting from enhanced plasma-membrane targeting.[68] An increased P_f is seen compared to oocytes expressing ZmPIP2 alone. Co-expression of ZmPIP2s with GFP::ZmPIP1;2 improves the targeting of ZmPIP1;2 to the plasma membrane and/or its stability.

A similar effect has been observed in *Z. mays* cells.[51] When expressed alone in maize leaf protoplasts, ZmPIP1 fusion proteins are retained in the endoplasmic reticulum, whereas ZmPIP2s are found in the plasma membrane. Interestingly, when co-expressed with ZmPIP2s, ZmPIP1s are relocalized to the plasma membrane. This ZmPIP1 relocalization results from a physical interaction between ZmPIP1s and ZmPIP2s, as demonstrated using Förster resonance energy transfer/fluorescence lifetime imaging microscopy, as well as immunoprecipitation.[51] The way in which ZmPIP1s interact with ZmPIP2s is not known, but loop E of ZmPIP1s might play an essential role in their ability to interact with ZmPIP2s, as revealed by mutagenesis analysis of ZmPIP1;1 and ZmPIP1;2.[68] In addition to regulation of PIP1 trafficking, heteromerization might activate the channel activity of some monomers.[68] The question whether similar interactions exist between animal AQPs remains to be answered.

Modification of the Subcellular Localization

In mammals, the subcellular localization of AQP2 in the kidney collecting duct is controlled by the anti-diuretic hormone vasopressin, which induces a cascade of signaling events leading to AQP2 phosphorylation.[118,156-158] In the absence of vasopressin, the density of AQP2 in the apical plasma membrane of the collecting duct is quite low and, as a result, the apical membrane displays a low water permeability, preventing water from being reabsorbed from the urine. On the other hand, the basolateral membrane displays a constitutive high water permeability due to the presence of AQP3 and AQP4 in this membrane which facilitates a directional water flux. Binding of vasopressin to its receptor in the basolateral membrane activates adenylate cyclase, causing an increase in cAMP levels, which leads to protein kinase A-mediated phosphorylation of AQP2

and other targets. This phosphorylation event provokes the fusion of AQP2-containing subapical endosomes to the apical membrane, enhancing its water permeability and leading to water reabsorption from the urine. When vasopressin levels drop, AQP2 is endocytotically removed from the apical plasma membrane.[27,158,159] In plants, relocalization of AtPIP and AtTIP from the plasma membrane and tonoplast, respectively, to an endomembrane compartment was recently shown to occur in response to salt stress.[160] PIP2s are subjected to constitutive cycling, their endocytosis being clathrin-dependent.[161] Upon osmotic challenge, maize protoplasts are hypothesized to modulate their water channel activity through the fusion of AQP-rich endosomes with the plasma membrane.[75] Upon osmotic stress in *Mesembryanthemum crystallinum* roots, McTIP1;2 is relocalized from the tonoplast to the endosomes.[162] This process is dependent on AQP glycosylation and a cAMP-dependent pathway and, interestingly, can be inhibited by membrane trafficking inhibitors. As the redistribution of PIPs from the plasma membrane to internal compartments contributes to downregulation of root water uptake,[160] relocalization might act as a stress-response mechanism by rapidly and reversibly modulating the cell membrane water permeability. Alternatively, this relocalization process could lead to the degradation of the protein. Recently, phosphorylation/dephosphorylation of AtPIP2;1 at the C-terminus was shown to regulate its targeting to the plasma membrane or its internalization upon salt treatment.[132]

Conclusion

The roles of AQPs in cell physiology are currently increasing. As pointed out throughout this introduction, the discovery that some AQPs are more than simple water channels has shed new light on their physiological roles in living organisms.[22,23,27,29,55,63-66] Further characterization of the selectivity profile of AQPs and mutant phenotype analysis is being carried out and has already revealed unexpected cellular functions. Given the involvement of AQPs in many key cellular processes and their suggested role in facilitating tumor growth and spread, the quest for specific AQP inhibitors of possible clinical importance is receiving increasing attention. This is of particular interest in the cancer research field, as AQPs could turn out to be interesting drug targets for novel antitumor therapies.[100] Clearly, AQPs offer an exciting field for research and discovery.

Acknowledgements

This work was supported by grants from the Belgian National Fund for Scientific Research (FNRS), the Interuniversity Attraction Poles Programme–Belgian Science Policy and the "Communauté française de Belgique–Actions de Recherches Concertées".

References

1. Stein WD, Danielli JF. Structure and function in red cell permeability. Discussion Faraday Soc 1956; 21:238-251.
2. Benga G. Birth of water channel proteins—the aquaporins. Cell Biol Int 2003; 27:701-709.
3. Ray PM. On the theory of osmotic water movement. Plant Physiol 1960; 35:783-795.
4. Benga G, Popescu O, Pop VI et al. P-(chloromercuri)benzenesulfonate binding by membrane proteins and the inhibition of water transport in human erythrocytes. Biochemistry 1986; 25:1535-1538.
5. Denker B, Smith B, Kuhajda F et al. Identification, purification and partial characterization of a novel mr 28,000 integral membrane protein from erythrocytes and renal tubules. J Biol Chem 1988; 263:15634-15642.
6. Agre P, Saboori AM, Asimos A et al. Purification and partial characterization of the mr 30,000 integral membrane protein associated with the erythrocyte rh(d) antigen. J Biol Chem 1987; 262:17497-17503.
7. Preston GM, Agre P. Isolation of the cDNA for erythrocyte integral membrane protein of 28 kilodaltons: Member of an ancient channel family. Proc Natl Acad Sci USA 1991; 88:11110-11114.
8. Smith BL, Agre P. Erythrocyte mr 28,000 transmembrane protein exists as a multisubunit oligomer similar to channel proteins. J Biol Chem 1991; 266:6407-6415.
9. Preston GM, Carroll TP, Guggino WB et al. Appearance of water channels in xenopus oocytes expressing red cell CHIP28 protein. Science 1992; 256:385-387.
10. Mitsuoka K, Murata K, Walz T et al. The structure of aquaporin-1 at 4.5-a resolution reveals short alpha-helices in the center of the monomer. J Struct Biol 1999; 128:34-43.

11. Murata K, Mitsuoka K, Hirai T et al. Structural determinants of water permeation through aquaporin-1. Nature 2000; 407:599-605.
12. de Groot BL, Grubmüller H. The dynamics and energetics of water permeation and proton exclusion in aquaporins. Current Opinion in Structural Biology, Theory and simulation/Macromolecular assemblages 2005; 15:176-183.
13. Zardoya R. Phylogeny and evolution of the major intrinsic protein family. Biol Cell 2005; 97:397-414.
14. Campbell E, Ball A, Hoppler S et al. Invertebrate aquaporins: A review. J Comp Physiol B 2008; 178:935-955.
15. Gazzarrini S, Kang M, Epimashko S et al. Chlorella virus mt325 encodes water and potassium channels that interact synergistically. Proc Natl Acad Sci USA 2006; 103:5355-5360.
16. Ishibashi K. Aquaporin superfamily with unusual NPA boxes: S-aquaporins (superfamily, SIP-like and subcellular-aquaporins). Cell Mol Biol 2006; 52:20-27.
17. Johanson U, Karlsson M, Johansson I et al. The complete set of genes encoding major intrinsic proteins in arabidopsis provides a framework for a new nomenclature for major intrinsic proteins in plants. Plant Physiol 2001; 126:1358-1369.
18. Sakurai J, Ishikawa F, Yamaguchi T et al. Identification of 33 rice aquaporin genes and analysis of their expression and function. Plant Cell Physiol 2005; 46:1568-1577.
19. Chaumont F, Barrieu F, Wojcik E et al. Aquaporins constitute a large and highly divergent protein family in maize. Plant Physiol 2001; 125:1206-1215.
20. Gustavsson S, Lebrun AS, Norden K et al. A novel plant major intrinsic protein in Physcomitrella patens most similar to bacterial glycerol channels. Plant Physiol 2005; 139:287-295.
21. Danielson JJ, Johanson U. Unexpected complexity of the aquaporin gene family in the moss Physcomitrella patens. BMC Plant Biol 2008; 8.
22. Bienert GP, Schüssler MD, Jahn TP. Metalloids: Essential, beneficial or toxic? Major intrinsic proteins sort it out. Trends Biochem Sci 2008; 33:20-26.
23. Bienert GP, Moller ALB, Kristiansen KA et al. Specific aquaporins facilitate the diffusion of hydrogen peroxide across membranes. J Biol Chem 2007; 282:1183-1192.
24. Yool AJ. Aquaporins: Multiple roles in the central nervous system. Neuroscientist 2007; 13:470-485.
25. Lee W-K, Thevenod F. A role for mitochondrial aquaporins in cellular life-and-death decisions? Am J Physiol Cell Physiol 2006; 291:195-202.
26. Saadoun S, Papadopoulos MC, Watanabe H et al. Involvement of aquaporin-4 in astroglial cell migration and glial scar formation. J Cell Sci 2005; 118:5691-5698.
27. Verkman AS. More than just water channels: Unexpected cellular roles of aquaporins. J Cell Sci 2005; 118:3225-3232.
28. Maurel C. Plant aquaporins: Novel functions and regulation properties. FEBS Letters 2007; 581:2227-2236.
29. Uehlein N, Otto B, Hanson DT et al. Function of Nicotiana tabacum aquaporins as chloroplast gas pores challenges the concept of membrane CO_2 permeability. Plant Cell 2008; 20:648-657.
30. Reizer J, Reizer A, Saier MH. The MIP family of integral membrane channel proteins: Sequence comparisons, evolutionary relationships, reconstructed pathway of evolution and proposed functional differentiation of the two repeated halves of the proteins. Crit Rev Biochem Mol Biol 1993; 28:235-257.
31. Zeidel ML, Ambudkar SV, Smith BL et al. Reconstitution of functional water channels in liposomes containing purified red cell CHIP28 protein. Biochemistry 1992; 31:7436-7440.
32. Walz T, Smith BL, Zeidel ML et al. Biologically active two-dimensional crystals of aquaporin chip. J Biol Chem 1994; 269:1583-1586.
33. Daniels MJ, Chrispeels MJ, Yeager M. Projection structure of a plant vacuole membrane aquaporin by electron cryo-crystallography. J Mol Biol 1999; 294:1337-1349.
34. Sui H, Han BG, Lee JK et al. Structural basis of water-specific transport through the AQP1 water channel. Nature 2001; 414:872-878.
35. Tornroth-Horsefield S, Wang Y, Hedfalk K et al. Structural mechanism of plant aquaporin gating. Nature 2006; 439:688-694.
36. Heymann JB, Engel A. Structural clues in the sequences of the aquaporins. J Mol Biol 2000; 295:1039-1053.
37. Kukulski W, Schenk AD, Johanson U et al. The 5Å structure of heterologously expressed plant aquaporin sopip2;1. J Mol Biol 2005; 350:611-616.
38. Hiroaki Y, Tani K, Kamegawa A et al. Implications of the aquaporin-4 structure on array formation and cell adhesion. J Mol Biol 2006; 355:628-639.
39. Gonen T, Sliz P, Kistler J et al. Aquaporin-0 membrane junctions reveal the structure of a closed water pore. Nature 2004; 429:193-197.

40. Schenk AD, Werten PJ, Scheuring S et al. The 4.5 Å structure of human AQP2. J Mol Biol 2005; 350:278-289.
41. Savage DF, Egea PF, Robles-Colmenares Y et al. Architecture and selectivity in aquaporins: 2.5 Å x-ray structure of aquaporin z. PLoS Biol 2003; 1:334-340.
42. Ringler P, Borgnia MJ, Stahlberg H et al. Structure of the water channel AQPz from Escherichia coli revealed by electron crystallography. J Mol Biol 1999; 291:1181-1190.
43. Fu D, Libson A, Miercke LJ et al. Structure of a glycerol-conducting channel and the basis for its selectivity. Science 2000; 290:481-486.
44. Lee JK, Kozono D, Remis J et al. Structural basis for conductance by the archaeal aquaporin aqpm at 1.68 a. Proc Natl Acad Sci USA 2005; 102:18932-18937.
45. Horsefield R, Nordén K, Fellert M et al. High-resolution x-ray structure of human aquaporin 5. Proc Natl Acad Sci USA 2008; 105:13327-13332.
46. Maurel C, Verdoucq L, Luu D-T et al. Plant aquaporins: Membrane channels with multiple integrated functions. Ann Rev Plant Biol 2008; 59:595-624.
47. de Groot BL, Frigato T, Helms V et al. The mechanism of proton exclusion in the aquaporin-1 water channel. J Mol Biol 2003; 333:279-293.
48. Tajkhorshid E, Nollert P, Jensen MO et al. Control of the selectivity of the aquaporin water channel family by global orientational tuning. Science 2002; 296:525-530.
49. Chakrabarti N, Roux B, Pomes R. Structural determinants of proton blockage in aquaporins. J Mol Biol 2004; 343:493-510.
50. Borgnia M, Nielsen S, Engel A et al. Cellular and molecular biology of the aquaporin water channels. Annu Rev Biochem 1999; 68:425-458.
51. Zelazny E, Borst JW, Muylaert M et al. Fret imaging in living maize cells reveals that plasma membrane aquaporins interact to regulate their subcellular localization. Proc Natl Acad Sci USA 2007; 104:12359-12364.
52. Preston GM, Jung JS, Guggino WB et al. The mercury-sensitive residue at cysteine 189 in the CHIP28 water channel. J Biol Chem 1993; 268:17-20.
53. Verkman AS, Shi LB, Frigeri A et al. Structure and function of kidney water channels. Kidney Int 1995; 48:1069-1081.
54. Németh-Cahalan KL, Froger A, Hall JE. Zinc modulation of water permeability reveals that aquaporin 0 functions as a cooperative tetramer. J Gen Physiol 2007; 130:457-464.
55. Wu B, Beitz E. Aquaporins with selectivity for unconventional permeants. Cell Mol Life Sci 2007; 64:2413-2421.
56. Johansson I, Karlsson M, Johanson U et al. The role of aquaporins in cellular and whole plant water balance. Biochim Biophys Acta 2000; 1465:324-342.
57. Hachez C, Moshelion M, Zelazny E et al. Localization and quantification of plasma membrane aquaporin expression in maize primary root: A clue to understanding their role as cellular plumbers. Plant Mol Biol 2006; 62:305-323.
58. Hara-Chikuma M, Verkman A. Physiological roles of glycerol-transporting aquaporins: The aquaglyceroporins. Cell Mol Life Sci 2006; 63:1386-1392.
59. Liu LH, Ludewig U, Gassert B et al. Urea transport by nitrogen-regulated tonoplast intrinsic proteins in arabidopsis. Plant Physiol 2003; 133:1220-1228.
60. Gerbeau P, Guclu J, Ripoche P et al. Aquaporin nt-TIPα can account for the high permeability of tobacco cell vacuolar membrane to small neutral solutes. Plant J 1999; 18:577-587.
61. Biela A, Grote K, Otto B et al. The Nicotiana tabacum plasma membrane aquaporin NtAQP1 is mercury-insensitive and permeable for glycerol. Plant J 1999; 18:565-570.
62. Jahn TP, Moller ALB, Zeuthen T et al. Aquaporin homologues in plants and mammals transport ammonia. FEBS Letters 2004; 574:31-36.
63. Ma JF, Tamai K, Yamaji N et al. A silicon transporter in rice. Nature 2006; 440:688-691.
64. Takano J WM, Ludewig U, Schaaf G et al. The arabidopsis major intrinsic protein NIP5;1 is essential for efficient boron uptake and plant development under boron limitation. Plant Cell 2006; 18:1498-1509.
65. Choi WG, Roberts DM. Arabidopsis NIP2;1, a major intrinsic protein transporter of lactic acid induced by anoxic stress. J Biol Chem 2007; 282:24209-24218.
66. Flexas J, Ribas-Carbo M, Hanson DT et al. Tobacco aquaporin NtAQP1 is involved in mesophyll conductance to CO_2 in vivo. Plant J 2006; 48:427-439.
67. Bienert GP, Schjoerring JK, Jahn TP. Membrane transport of hydrogen peroxide. Biochim Biophys Acta 2006; 1758:994-1003.
68. Fetter K, Van Wilder V, Moshelion M et al. Interactions between plasma membrane aquaporins modulate their water channel activity. Plant Cell 2004; 16:215-228.

69. Van Wilder V, Miecielica U, Degand H et al. Maize plasma membrane aquaporins belonging to the PIP1 and PIP2 subgroups are in vivo phosphorylated. Plant Cell Physiol 2008; 49:1364-1377.
70. Nakhoul NL, Davis BA, Romero MF et al. Effect of expressing the water channel aquaporin-1 on the CO_2 permeability of xenopus oocytes. Ame J of Phys-Cell Physiol 1998; 43:543-548.
71. Ma JF, Yamaji N, Mitani N et al. Transporters of arsenite in rice and their role in arsenic accumulation in rice grain. Proc Natl Acad Sci USA 2008; 105:9931-9935.
72. Tanaka M, Wallace IS, Takano J et al. NIP6;1 is a boric acid channel for preferential transport of boron to growing shoot tissues in arabidopsis. Plant Cell 2008; 20:2860-2875.
73. Ramahaleo T, Morillon R, Alexandre J et al. Osmotic water permeability of isolated protoplasts. Modifications during development. Plant Physiol 1999; 119:885-896.
74. Morillon R, Chrispeels MJ. The role of aba and the transpiration stream in the regulation of the osmotic water permeability of leaf cells. Proc Natl Acad Sci USA 2001; 98:14138-14143.
75. Moshelion M, Moran N, Chaumont F. Dynamic changes in the osmotic water permeability of protoplast plasma membrane. Plant Physiol 2004; 135:2301-2317.
76. Kaldenhoff R, Grote K, Zhu JJ et al. Significance of plasmalemma aquaporins for water-transport in Arabidopsis thaliana. Plant J 1998; 14:121-128.
77. Volkov V, Hachez C, Moshelion M et al. Water permeability differs between growing and nongrowing barley leaf tissues. J Exp Bot 2007; 58:377-390.
78. Sommer A, Geist B, Da Ines O et al. Ectopic expression of Arabidopsis thaliana plasma membrane intrinsic protein 2 aquaporins in lily pollen increases the plasma membrane water permeability of grain but not of tube protoplasts. New Phytologist 2008; 180:787-797.
79. Zelazny EM, Miecilica U, Borst JW et al. An n-terminal diacidic motif is required for the trafficking of maize aquaporins ZmPIP2;4 and ZmPIP2;5 to the plasma membrane. Plant J 2008; in press.
80. Ohshima Y, Iwasaki I, Suga S et al. Low aquaporin content and low osmotic water permeability of the plasma and vacuolar membranes of a cam plant Graptopetalum paraguayense: Comparison with radish. Plant Cell Physiol 2001; 42:1119-1129.
81. Hubert J-Fo, Duchesne L, Delamarche C et al. Pore selectivity analysis of an aquaglyceroporin by stopped-flow spectrophotometry on bacterial cell suspensions. Biol Cell 2005; 97:675-686.
82. Suga S, Maeshima M. Water channel activity of radish plasma membrane aquaporins heterologously expressed in yeast and their modification by site-directed mutagenesis. Plant Cell Physiol 2004; 45:823-830.
83. Verdoucq L GA, Maurel C. Structure-function analysis of plant aquaporin AtPIP2;1 gating by divalent cations and protons. Biochem J 2008; 415:409-416.
84. Steudle E. Pressure probe techniques: Basic principles and application to studies of water and solute relations at the cell tissue and organ level. In: Smith JAC, Griffiths H, eds. Water Deficits: Plant Responses from Cell to Community. Oxford: Bios Scientific Publisher Ltd, 1993:5-36.
85. Steudle E, Murrmann M, Peterson CA. Transport of water and solutes across maize roots modified by puncturing the endodermis—further evidence for the composite transport model of the root. Plant Physiol 1993; 103:335-349.
86. Van der Toorn A, Zemah H, Van As H et al. Developmental changes and water status in tulip bulbs during storage: Visualization by NMR imaging. J Exp Bot 2000; 51:1277-1287.
87. Scheenen TWJ, van Dusschoten D, de Jager PA et al. Quantification of water transport in plants with NMR imaging. J Exp Bot 2000; 51:1751-1759.
88. Köckenberger WDP, De Panfilis C, Santoro D et al. High resolution NMR microscopy of plants and fungi. J Microsc 2004; 214:182-189.
89. Van As H, Lens P. Use of 1h NMR to study transport processes in porous biosystems. J Ind Microbiol Biotechnol 2001; 26:43-52.
90. Javot H, Maurel C. The role of aquaporins in root water uptake. Ann Bot (Lond) 2002; 90:301-313.
91. Abrami L, Berthonaud V, Deen PM et al. Glycerol permeability of mutant aquaporin 1 and other AQP-MIP proteins: Inhibition studies. Pflugers Arch 1996; 431:408-414.
92. Echevarria M, Windhager EE, Frindt G. Selectivity of the renal collecting duct water channel aquaporin-3. J Biol Chem 1996; 271:25079-25082.
93. Dordas C, Chrispeels MJ, Brown PH. Permeability and channel-mediated transport of boric acid across membrane vesicles isolated from squash roots. Plant Physiol 2000; 124:1349-1362.
94. Moshelion M, Becker D, Biela A et al. Plasma membrane aquaporins in the motor cells of Samanea saman: Diurnal and circadian regulation. Plant Cell 2002; 14:727-739.
95. Tournaire-Roux C, Sutka M, Javot H et al. Cytosolic pH regulates root water transport during anoxic stress through gating of aquaporins. Nature 2003; 425:393-397.
96. Deen PM VM, Knoers NV, Wieringa B et al. Requirement of human renal water channel aquaporin-2 for vasopressin-dependent concentration of urine. Science 1994; 264:92-95.

97. King LS, Kozono D, Agre P. From structure to disease: The evolving tale of aquaporin biology. Nat Rev Mol Cell Biol 2004; 5:687-698.
98. King LS, Yasui M, Agre P. Aquaporins in health and disease. Mol Med Today 2000; 6:60-65.
99. Hachez C, Zelazny E, Chaumont F. Modulating the expression of aquaporin genes in planta: A key to understand their physiological functions? Biochim Biophys Acta 2006; 1758:1142-1156.
100. Verkman A, Hara-Chikuma M, Papadopoulos M. Aquaporins: New players in cancer biology. J Mol Med 2008; 86:523-529.
101. Martre P, Morillon R, Barrieu F et al. Plasma membrane aquaporins play a significant role during recovery from water deficit. Plant Physiol 2002; 130:2101-2110.
102. Javot H, Lauvergeat V, Santoni V et al. Role of a single aquaporin isoform in root water uptake. Plant Cell 2003; 15:509-522.
103. Siefritz F, Tyree MT, Lovisolo C et al. PIP1 plasma membrane aquaporins in tobacco: From cellular effects to function in plants. Plant Cell 2002; 14:869-876.
104. Ma S, Quist TM, Ulanov A et al. Loss of TIP1;1 aquaporin in arabidopsis leads to cell and plant death. Plant J 2004; 40:845-859.
105. Jang JY, Rhee JY, Kim DG et al. Ectopic expression of a foreign aquaporin disrupts the natural expression patterns of endogenous aquaporin genes and alters plant responses to different stress conditions. Plant Cell Physiol 2007; 48:1331-1339.
106. Uehlein N, Lovisolo C, Siefritz F et al. The tobacco aquaporin NtAQP1 is a membrane CO_2 pore with physiological functions. Nature 2003; 425:734-737.
107. Missner A, Kugler P, Saparov SM et al. Carbon dioxide transport through membranes. J Biol Chem 2008; 283:25340-25347.
108. Schüssler MD, Bienert GP, Kichey T et al. The effects of the loss of TIP1;1 and TIP1;2 aquaporins in Arabidopsis thaliana. The Plant Journal 2008; 56:756-767.
109. Verkman AS. Role of aquaporin water channels in kidney and lung. Am J Med Sci 1998; 316:310-320.
110. Verkman AS, Matthay MA, Song Y. Aquaporin water channels and lung physiology. Am J Physiol Lung Cell Mol Physiol 2000; 278: 867-879.
111. Fraysse LC, Wells B, McCann MC et al. Specific plasma membrane aquaporins of the PIP1 subfamily are expressed in sieve elements and guard cells. Biol Cell 2005; 97:519-534.
112. Hachez C, Heinen R, Draye X et al. The expression pattern of plasma membrane aquaporins in maize leaf highlights their role in hydraulic regulation. Plant Mol Biol 2008; 68:337-353.
113. Alexandersson E, Fraysse L, Sjovall-Larsen S et al. Whole gene family expression and drought stress regulation of aquaporins. Plant Mol Biol 2005; 59:469-484.
114. Chaumont F, Moshelion M, Daniels MJ. Regulation of plant aquaporin activity. Biol Cell 2005; 97:749-764.
115. Maurel C, Chrispeels MJ. Aquaporins. A molecular entry into plant water relations. Plant Physiol 2001; 125:135-138.
116. Cochard H, Venisse JS, Barigah TS et al. Putative role of aquaporins in variable hydraulic conductance of leaves in response to light. Plant Physiol 2007; 143:122-133.
117. Santoni V, Vinh J, Pflieger D et al. A proteomic study reveals novel insights into the diversity of aquaporin forms expressed in the plasma membrane of plant roots. Biochem J 2003; 373:289-296.
118. Takata KM, Tajika T, Ablimit Y et al. Localization and trafficking of aquaporin 2 in the kidney. Histochem Cell Biol 2008; 130:197-209.
119. Boursiac Y, Chen S, Luu DT et al. Early effects of salinity on water transport in arabidopsis roots. Molecular and cellular features of aquaporin expression. Plant Physiol 2005; 139:790-805.
120. Maurel C, Kado RT, Guern J et al. Phosphorylation regulates the water channel activity of the seed-specific aquaporin alpha-TIP. EMBO J 1995; 14:3028-3035.
121. Zhang W, Zitron E, Homme M et al. Aquaporin-1 channel function is positively regulated by protein kinase C. J Biol Chem 2007; 282:20933-20940.
122. Han Z, Wax MB, Patil RV. Regulation of aquaporin-4 water channels by phorbol ester-dependent protein phosphorylation. J Biol Chem 1998; 273:6001-6004.
123. Johansson I, Karlsson M, Shukla VK et al. Water transport activity of the plasma membrane aquaporin PM28a is regulated by phosphorylation. Plant Cell 1998; 10:451-459.
124. Procino G, Carmosino M, Marin O et al. Ser-256 phosphorylation dynamics of aquaporin 2 during maturation from the endoplasmic reticulum to the vesicular compartment in renal cells. FASEB J 2003; 17:1886-1888.
125. van Balkom BWM, Savelkoul PJM, Markovich D et al. The role of putative phosphorylation sites in the targeting and shuttling of the aquaporin-2 water channel. J Biol Chem 2002; 277:41473-41479.
126. Weaver CD, Roberts DM. Phosphorylation of nodulin-26 by a calcium-dependent protein-kinase. FASEB J 1991; 5:A426-A426.

127. Johnson KD, Chrispeels MJ. Tonoplast-bound protein-kinase phosphorylates tonoplast intrinsic protein. Plant Physiol 1992; 100:1787-1795.
128. Johansson I, Larsson C, Ek B et al. The major integral proteins of spinach leaf plasma membranes are putative aquaporins and are phosphorylated in response to Ca^{2+} and apoplastic water potential. Plant Cell 1996; 8:1181-1191.
129. Santoni V, Verdoucq L, Sommerer N et al. Methylation of aquaporins in plant plasma membrane. Biochem J 2006; 400:189-197.
130. Miao GH, Hong Z, Verma DP. Topology and phosphorylation of soybean nodulin-26, an intrinsic protein of the peribacteroid membrane. J Cell Biol 1992; 118:481-490.
131. Weaver CD, Roberts DM. Determination of the site of phosphorylation of nodulin 26 by the calcium-dependent protein kinase from soybean nodules. Biochemistry 1992; 31:8954-8959.
132. Prak S, Hem S, Boudet J et al. Multiple phosphorylations in the C-terminal tail of plant plasma membrane aquaporins. Role in sub-cellular trafficking of AtPIP2;1 in response to salt stress. Mol Cell Proteomics 2008; 7:1019-1030.
133. Guenther JF, Chanmanivone N, Galetovic MP et al. Phosphorylation of soybean nodulin 26 on serine 262 enhances water permeability and is regulated developmentally and by osmotic signals. Plant Cell 2003; 15:981-991.
134. Sjövall-Larsen S, Alexandersson E, Johansson I et al. Purification and characterization of two protein kinases acting on the aquaporin SoPIP2;1. Biochim Biophys Acta 2006; 1758:1157-1164.
135. Yasui M, Hazama A, Kwon TH et al. Rapid gating and anion permeability of an intracellular aquaporin. Nature 1999; 402:184-187.
136. Zeuthen T, Klaerke DA. Transport of water and glycerol in aquaporin 3 is gated by H^+. J Biol Chem 1999. 274:21631-21636.
137. Nemeth-Cahalan KL, Hall JE, pH and calcium regulate the water permeability of aquaporin 0. J Biol Chem, 2000; 275:6777-6782.
138. Nemeth-Cahalan KL, Kalman K, Hall JE. Molecular basis of pH and Ca^{2+} regulation of aquaporin water permeability. J Gen Physiol 2004; 123:573-580.
139. Gerbeau P, Amodeo G, Henzler T et al. The water permeability of arabidopsis plasma membrane is regulated by divalent cations and pH. Plant J 2002; 30:71-81.
140. Zeuthen T, Klaerke DA Transport of water and glycerol in aquaporin 3 is gated by h(+). J Biol Chem 1999; 274:21631-21636.
141. Sutka M, Alleva K, Parisi M et al. Tonoplast vesicles of Beta vulgaris storage root show functional aquaporins regulated by protons. Biol Cell 2005; 97:837-846.
142. Hedfalk K, Tornröth-Horsefield S, Nyblom M et al. Aquaporin gating. Current Opinion in Structural Biology Membranes/Engineering and design 2006; 16:447-456.
143. Kalman K, Nemeth-Cahalan KL, Froger A et al. Phosphorylation determines the calmodulin-mediated Ca^{2+} response and water permeability of AQP0. J Biol Chem 2008; 283:21278-21283.
144. Torvinen M, Marcellino D, Canals M et al. Adenosine A2A receptor and dopamine D3 receptor interactions: Evidence of functional A2A/D3 heteromeric complexes. Mol Pharmacol 2005; 67:400-407.
145. Etxeberria A, Santana-Castro I, Regalado MP et al. Three mechanisms underlie KCNQ2/3 heteromeric potassium m-channel potentiation. J Neurosci 2004; 24:9146-9152.
146. Xicluna J, Lacombe B, Dreyer I et al. Increased functional diversity of plant K^+ channels by preferential heteromerization of the shaker-like subunits AKT2 and KAT2. J Biol Chem 2007; 282:486-494.
147. Daram P, Urbach S, Gaymard F et al. Tetramerization of the AKT1 plant potassium channel involves its C-terminal cytoplasmic domain. EMBO J 1997; 16:3455-3463.
148. Liu DT, Tibbs GR, Siegelbaum SA. Subunit stoichiometry of cyclic nucleotide-gated channels and effects of subunit order on channel function. Neuron 1996; 16:983-990.
149. Isacoff EY, Jan YN, Jan LY. Evidence for the formation of heteromultimeric potassium channels in xenopus oocytes. Nature 1990; 345:530-534.
150. Fotiadis D, Jeno P, Mini T et al. Structural characterization of two aquaporins isolated from native spinach leaf plasma membranes. J Biol Chem 2001; 276:1707-1714.
151. Harvengt P, Vlerick A, Fuks B et al. Lentil seed aquaporins form a hetero-oligomer which is phosphorylated by a Mg^{2+}-dependent and Ca^{2+}-regulated kinase. Biochem J 2000; 352:1183-1190.
152. Daniels MJ, Mirkov TE, Chrispeels MJ. The plasma membrane of Arabidopsis thaliana contains a mercury- insensitive aquaporin that is a homolog of the tonoplast water channel protein TIP. Plant Physiol 1994; 106:1325-1333.
153. Yamada S, Katsuhara M, Kelly WB et al. A family of transcripts encoding water channel proteins: Tissue- specific expression in the common ice plant. Plant Cell 1995; 7:1129-1142.
154. Chaumont F, Barrieu F, Jung R et al. Plasma membrane intrinsic proteins from maize cluster in two sequence subgroups with differential aquaporin activity. Plant Physiol 2000; 122:1025-1034.

155. Temmei Y, Uchida S, Hoshino D et al. Water channel activities of mimosa pudica plasma membrane intrinsic proteins are regulated by direct interaction and phosphorylation. FEBS Lett 2005; 579:4417-4422.
156. Christensen BM, Zelenina M, Aperia A et al. Localization and regulation of PKA-phosphorylated AQP2 in response to V(2)-receptor agonist/antagonist treatment. Am J Physiol Renal Physiol 2000; 278:29-42.
157. Nielsen S, Chou CL, Marples D et al. Vasopressin increases water permeability of kidney collecting duct by inducing translocation of aquaporin-cd water channels to plasma-membrane. Proc Natl Acad Sci USA 1995; 92:1013-1017.
158. Marples D, Frokiaer J, Nielsen S. Long-term regulation of aquaporins in the kidney. Am J Physiol Renal Physiol 1999; 276:331-339.
159. Verkman AS. Lessons on renal physiology from transgenic mice lacking aquaporin water channels. J Am Soc Nephrol 1999; 10:1126-1135.
160. Boursiac Y, Chen S, Luu D-T et al. Early effects of salinity on water transport in arabidopsis roots. Molecular and cellular features of aquaporin expression. Plant Physiol 2005; 139:790-805.
161. Dhonukshe P, Aniento F, Hwang I et al. Clathrin-mediated constitutive endocytosis of PIN auxin efflux carriers in arabidopsis. Current Biology 2007; 17:520-527.
162. Vera-Estrella R, Barkla BJ, Bohnert HJ et al. Novel regulation of aquaporins during osmotic stress. Plant Physiol 2004; 135:2318-2329.

Elsbeth Bienert

Chapter 2

Phylogeny of Major Intrinsic Proteins

Jonas Å.H. Danielson* and Urban Johanson*

Abstract

Major intrinsic proteins (MIPs) form a large superfamily of proteins that can be divided into different subfamilies and groups according to phylogenetic analyses. Plants encode more MIPs than other organisms and seven subfamilies have been defined, whereof the Nodulin26-like major intrinsic proteins (NIPs) have been shown to permeate metalloids. In this chapter we review the phylogeny of MIPs in general and especially of the plant MIPs. We also identify bacterial NIP-like MIPs and discuss the evolutionary implications of this finding regarding the origin and ancestral transport specificity of the NIPs.

Introduction

Major intrinsic proteins (MIPs) form channels in membranes that facilitate the permeation of water and other small uncharged polar molecules. The function of MIPs seems fundamental to life as we know it today since MIPs are found in virtually every organism. All studied MIPs share a common structure which consists of two transmembrane helices (TMHs), half a TMH followed by a third TMH. This topology is repeated once and due to the odd number of TMH in the first half, the second half is inserted in the opposite orientation in the membrane where the two half TMHs meet at a conserved NPA (Asn-Pro-Ala) motif situated at the N-terminus of each half TMH. A tetrad of amino acid residues situated in helix 2 and 5 and in loop E (H2, H5, LE1, LE2) referred to as the ar/R (aromatic/Arginine) selectivity region, appears to be the major determinant for substrate specificity. Water specific channels or orthodox AQPs (aquaporins) typically have the amino acid residues FHTR (one letter code) at these positions. Phylogenetic analyses of MIP sequences can shed light on how this super family has evolved and will potentially increase our understanding of the functions of the many different subfamilies that have been identified in these studies. In this chapter we review the phylogenetic analyses that have been reported regarding the whole super family irrespectively of species and in particular the studies of the abundant plant MIPs which are about three times as numerous as compared to MIPs in mammalian species. In addition we analyse the phylogeny of the plant nodulin26-like intrinsic protein (NIP) subfamily and discuss their evolutionary origin and ancestral selectivity region.

A Historical Account of the MIP Phylogeny

A prerequisite for phylogenetic analyses and recognition of protein families is of course that a sufficient number of homologous sequences are available. In hindsight it is surprising how quickly the MIPs were recognized as belonging to the same family, taking into account the few sequences available at the time and the differences in their proposed physiological roles. The nomenclature

*Corresponding Authors: Jonas Å.H. Danielson and Urban Johanson—Department of Biochemistry, Molecular Protein Science Centre, Centre for Chemistry and Chemical Engineering, Lund University, PO Box 124, S-221 00 Lund, Sweden. Emails: jonas.danielson@biochemistry.lu.se, urban.johanson@biochemistry.lu.se

MIPs and Their Role in the Exchange of Metalloids, edited by Thomas P. Jahn and Gerd P. Bienert.

for the different isoforms has changed several times over the years and can sometimes be confusing. In this chapter we are using the original protein name when first citing a publication but the accepted systematic names preceded by the initials of the species name are mentioned in brackets and subsequently used to allow the reader to easily classify the MIP and refer to the current literature and the phylogenetic trees presented in this chapter.

The first cDNA sequence of a MIP appeared in the 1984 publication on bovine MIP-26 (*Bos taurus*; BtAQP0).[1] BtAQP0 was believed to function as a gap junction protein in lens fiber tissue. Surprisingly though, comparisons of protein properties showed that the similarity to other known junctional proteins, such as connexins, was low. Further analyses indicated that BtAQP0 had six transmembrane helices similar to many of the transport proteins known at the time. In 1987, an integral membrane protein from soybean, NOD26 (*Glycine max* NOD26-like intrinsic protein; GmNIP1;1), was identified in a search for proteins associated with the peribacteroid membrane surrounding nitrogen fixating symbiotic bacteria in the root nodule and became the second MIP to have its cDNA sequenced.[2] In the following year two groups, independently of each other, noticed a surprisingly high degree of sequence similarity between the lens fiber protein BtAQP0 and the peribacteroidal membrane protein GmNIP1;1.[3,4] This was the very start of what would become the large and diverse MIP family of proteins. In 1989, sequencing of the *Escherichia coli glpF* gene encoding a glycerol transporter (EcGlpF) was finished[5] and in 1990 Baker et al noticed that EcGlpF, BtAQP0 and GmNIP1;1 were all similar and members of the same family of proteins sharing a common ancestor.[6] 1990 also saw three publications reporting five new MIP sequences, the *Drosophila melanogaster* (DmBIB),[7] two closely related bean tonoplast intrinsic proteins (*Phaseolus vulgaris*; PvTIP3s)[8] and two TIPs expressed in the root of *Arabidopsis thaliana* (AtTIP1;1) and tobacco (*Nicotiana tabacum*; NtTIP2) respectively.[9]

With a growing number of proteins recognized as MIPs, the first real phylogenetic analysis of the family was conducted in 1991.[10] Six MIPs were included in the analysis (EcGlpF, GmNIP1;1, BtAQP0, PvTIP3, DmBIB and a partial sequence of a bacterial GlpF from *Streptomyces coelicolor*) and although this was too few sequences to be able to draw any general conclusions, one test result still stands out as very interesting. By splitting each protein sequence into the two repeated halves, Pao et al actually got 11 sequences from the five full length MIPs and the partial MIP for their analysis. The result showed that all of the first halves form one cluster and all of the second halves form another. This is indicating that the corresponding halves of all MIPs are closer related to each other than to the other half of any of them. The authors thus concluded that the internal gene duplication responsible for the repeat must have happened before the split of eukaryotes and prokaryotes.

There was a rapid increase in the discovery rate of new MIPs following the publication of the first cDNA sequence of a human aquaporin CHIP28 (HsAQP1)[11] and in 1993 Reizer and colleagues made a new phylogenetic analysis of the 18 sequences known to be members of the MIP family at the time.[12] Even though this is also a very limited number of sequences, it is still possible to recognize five of the subfamilies as we group them today and a similar analysis of the sequence repeats as that conducted by Pao et al[10] confirmed the previous finding. By 1996 the number of known MIPs had increased to an amount that allowed a more comprehensive phylogenetic analysis. Of the 84 MIP sequences available at this time, Park and Saier analyzed the 52 most divergent and complete and found them to belong to 12 subfamilies (4 bacterial, 3 yeast, 3 plant and 2 animal subfamilies).[13] They concluded that all present MIPs originate from two divergent bacterial MIPs that gave rise to the aquaglyceroporin- (Glycerol facilitator-like protein; GLP) and the AQP-cluster respectively. The former cluster consists of 5 subfamilies as defined in this study but corresponds to one subfamily in phylogenetic analysis as of today where the different groups of GLPs in general reflect the phylogeny of the species. Animal water-specific AQPs as well as the plant specific subfamilies PIPs (plasma membrane intrinsic proteins), TIPs and NIPs are all very clearly resolved in the phylogenetic analysis. It is interesting to see that the water specific AQP from *E. coli* (EcAqpZ) clusters together with NIPs and that both these subfamilies are next to the GLPs in the phylogenetic tree.[13] Froger and colleagues used a set of 142 MIPs in a sequence

alignment to, among other things, identify positions potentially important for substrate specificity that could discriminate between GLPs and other MIPs.[14] The following year Heymann and Engel included a phylogenetic analysis in their review on aquaporins.[15] It was based on 46 MIPs, selected to reflect the diversity of the 160 MIP protein sequences known at the time and resulted in 16 subfamilies divided into the two clusters of AQPs and GLPs with a *Archaeoglobus fulgidus* aquaporin (the only archaeal MIP in the analysis) being the closest relative to the GLPs.

Between 2001 and 2005, Rafael Zardoya and colleagues published three articles forming the most comprehensive phylogenetic analysis of the MIP family of proteins available as of today. In the first of these papers all 153 full-length, nonredundant, MIP sequences available at the time were analyzed together.[16] Another very important improvement in the analysis was the use of bootstrapping to test the validity of the different nodes in the phylogenetic tree. Based on this analysis Zardoya and Villalba concluded that MIPs could reliably be grouped into six major paralogous groups (subfamilies) GLPs, animal AQPs, PIPs, TIPs, NODs (NIPs) and AQP8s. However, the exact phylogenetic relationship between these subfamilies was not resolved even though there was some support for PIPs, TIPs and animal AQPs being more closely related. Phylogenetic analysis and variability profiles also showed that the PIP group is highly conserved, likely due to stringent evolutionary constraints.[16,17] In an analysis published in 2002, Zardoya and coworkers saw a close phylogenetic relationship between NIPs and bacterial AQPs and concluded that plant NIPs likely arose by horizontal gene transfer 1190 million years ago.[18] This could however not be confirmed in a more thorough analysis published in 2005.[17] For the latter analysis, 463 nonredundant and complete or almost complete MIP sequences were aligned and subsets of these were analyzed to get an overall phylogeny of the complete MIP family as well as a more detailed analysis for each subfamily.

As the available MIP sequences have gone from scarce to plentiful, the problem has shifted from how to retrieve enough sequences to how to make an appropriate selection of sequences for your analysis. Since many of the early sequences of MIPs were picked up as cDNA sequences, there was a large bias toward highly expressed MIPs in the early sequence alignments, often resulting in having many sequences of close homologs in the same dataset. With more and more genomes being sequenced, it has become possible to instead retrieve all MIP sequences encoded in the genome of a species and in that way avoid the bias to highly expressed genes. This strategy has proven to be successful for identifying new subfamilies and also provides a way to find out whether MIPs from different species are separated by a gene duplication event (paralogs) or a speciation event (orthologs).[19,20] However, this approach has also introduced the problem of accidentally identifying pseudogenes as functional MIPs since it does not discriminate between expressed and non-expressed sequences.

The general phylogeny of the MIP superfamily as of today can be seen in Figure 1. The dataset used for this analysis was constructed to contain a few representative sequences from all of the major groups including the subfamilies identified by Zardoya.[17] In total 13 subfamilies are defined whereof five appear to be specific for plants. All of the central nodes, indicated by grey shading in Figure 1, have very low bootstrap values (≤52%) and hence the relationship between the subfamilies is not resolved. We would like to point out that what is often referred to as the AQP cluster or AQP group of MIPs is actually not a clear monophyletic clade, but rather constitutes a heterogeneous group, that can only be defined as all MIPs except the GLPs. The GLP cluster/group, on the other hand, is a monophyletic group with high bootstrap support and contains eukaryotic as well as bacterial sequences suggesting an ancient origin of the aquaglyceroporins. It is worth noting that higher plants are lacking GLPs whereas mosses contain a GLP (PpGIP1;1) suggesting that the GLPs were lost during the evolution of higher plants.[21]

Plant MIPs

Plant MIPs were early identified and subsequent studies have shown that plants have more isoforms than other organisms. In 1992, a total of six MIP sequences from plants were known in addition to GmNIP1;1 allowing a phylogenetic analysis.[22] Two paralogous TIP groups was

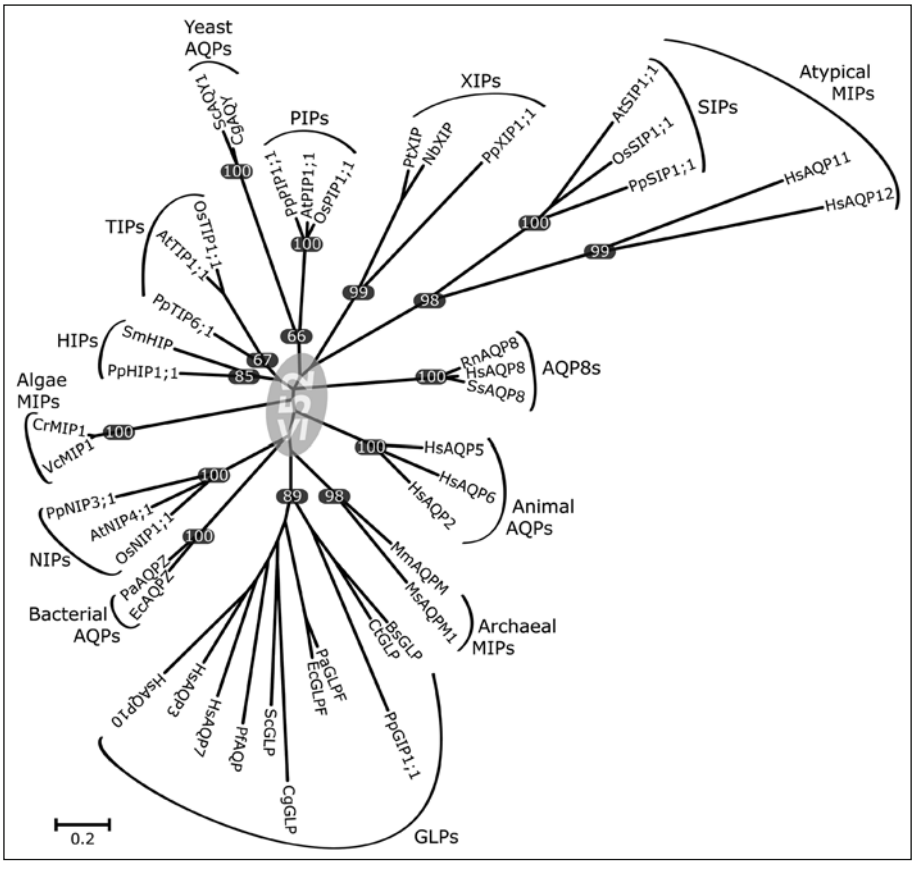


Figure 1. Phylogenetic analysis of MIPs. Thirteen different subfamilies are supported by high bootstrap values in a Neighbor-Joining analysis of 44 representative MIPs. Only integral regions were included in the analysis resulting in 182 positions in the final dataset. Bootstrap values of 1000 replicates are indicated for nodes of the different subfamilies. The shading in the middle of the tree marks the uncertainty of the positioning of the central nodes as inferred from bootstrap values ≤52%. Sequence identifiers are given in the Appendix at the end of this chapter.

discerned, the seed specific αTIPs (TIP3s) and the vegetatively expressed γTIPs (TIP1s). Although the two other MIPs included in the study were annotated as TIPs they were quite different and are now recognized as PIPs. It became evident from studies of the model plant *Arabidopsis thaliana* that plants express several isoforms of PIPs, classified as PIP1s or PIP2s based on their sequence.[23-25] By 1997 the rapidly growing sequence databases allowed the identification of MIP homologs in at least 15 different plant species. More importantly the large number of deposited EST sequences from *Arabidopsis thaliana* allowed a systematic search of expressed MIP genes in a single plant species.[26] In total 23 MIP-encoding genes were identified in Arabidopsis, representing an overwhelming multitude of genes compared to the five human AQP genes known at the time. The AtMIPs formed three distinct subfamilies, consisting of 1 NIP, 11 PIP and 11 TIP sequences. Although 11 of the sequences were only partial, 20 of these genes were later confirmed by comprehensive analyses of the MIPs encoded in the genomic sequence (see below). However, due to the many partial sequences the exact relationship especially within the TIP subfamily remained speculative. This was resolved in a phylogenetic analysis

of 38 TIPs from different plants.[27] In this study high bootstrap support was provided for three different groups that had emerged within the TIP subfamily, γTIPs (TIP1s), δTIPs (TIP2s) and the αTIPs (TIP3s). The analysis showed that at least the TIP1 and the TIP2 groups formed before the split of the monocots and the dicots. In addition, PIPs were clearly divided in PIP1s and PIP2s with a similar high support. Preliminary analyses of the nearly completed Arabidopsis genome identified about 30 MIP members and suggested that the TIP subfamily consisted of at least four groups. Most significant was the expansion of the NLM subfamily (NIPs) since six MIPs were classified as NIPs based on sequence comparisons.[28] At this time several groups of plant MIPs were well established phylogenetically and could be expected to be found in virtually every higher plant. However, there was not a common standard for how new MIPs should be named and some MIPs had several alternative names that could cause confusion. To facilitate identification of orthologs or co-orthologs, a uniform and systematic nomenclature based on phylogenetic analysis was informally agreed upon the third international aquaporin meeting, MIP2000, Gothenburg, Sweden, 2000.

In the following year three articles conforming to the new nomenclature were published. The first study was a systematic analysis and classification of ESTs from maize (*Zea mays*),[29] followed by two articles reporting the annotation and classification of AtMIP genes based on the recently published Arabidopsis genomic sequence.[19,30] In total 33 ZmMIP cDNAs and 35 full-length AtMIP genes were found. In addition three and seven pseudogenes encoding partial AtMIP sequences were identified by Quigley et al[30] and Johanson et al,[19] respectively, representing partial gene duplications and deletion events. Although bootstrap analysis was only employed by Johanson and colleagues,[19] the very similar results presented in all three articles demonstrated the high reliability of the phylogenetic classifications. Four different subfamilies in higher plants were now recognized, the PIPs, TIPs, NIPs and the small basic intrinsic proteins (SIPs). The latter subfamily is formed by rather divergent and atypical MIPs that are lacking a fully conserved first NPA motif (Fig. 1).[31] The gene structure in each subfamily was found to be conserved and furthermore the two PIP-, five TIP- and two SIP-groups were preserved in both dicots and monocots, suggesting that the last common ancestor had at least these groups of MIPs and the gene structure characteristic for each subfamily. Hence, these genes and gene structures could be expected in all monocot and dicot plants.[19] Regarding the NIP groups, the situation was more complicated. In Arabidopsis a fixed criterion of maximum distance of 30% within a group were applied in all subfamilies. The high diversity among AtNIPs resulted in seven NIP groups, whereas the phylogenetic classification of ZmNIPs and other plant NIPs without a specific criterion defined only three groups, where the NIP1 group included AtNIP1s to AtNIP4s. Interestingly, there were no orthologs in Arabidopsis to the ZmNIP2 group. The early diversification of plant MIPs was confirmed by an analysis of ESTs from the moss, *Physcomitrella patens*.[32] *P. patens* was found to have at least 12 different MIPs that could be classified into the four plant subfamilies. It was also concluded that the PIP1 and PIP2 groups formed before the divergence of mosses and the lineage leading to vascular plants. In contrast the five TIP groups found in higher plants were proposed to have evolved later in the lineage leading to vascular plants. It has been suggested that the NIPs evolved from a water-specific AQP to fill the functional role of GLPs, which are hitherto not found in higher plants.[18] In this context it was unexpected that further studies of *P. patens* ESTs identified a GLP homolog, PpGIP1;1 (GlpF-like intrinsic protein).[21] Interestingly, this MIP is closely related to the Type II GLPs generally found in Gram-positive bacteria and it was concluded that a GLP was most likely acquired from this group of bacteria by a horizontal gene transfer event about 1040 million years ago, i.e., 100 to 150 million years after the NIPs were suggested to have been acquired in plants.

Analysis of the rice genome provided the first comprehensive list of MIPs in a monocot. In an early study 33 full length MIP genes were identified and the encoded proteins were analyzed phylogenetically and classified according to the maize MIPs.[29,33] All the groups within the four subfamilies in maize were confirmed and in addition two MIPs, OsNIP4;1 and OsPIP2;8 were identified that could not be classified into any of the predefined groups. Although not supported by

the phylogenetic analysis, OsPIP2;8 was assigned to the PIP2 group based on sequence similarity. The other protein, OsNIP4;1, was considered as a founder of a new and fourth NIP group in rice. In a later study focusing on homology modeling of AtMIPs, ZmMIPs and OsMIPs the updated rice genome was reanalyzed regarding encoded MIPs.[34] The result of Sakurai et al[33] was confirmed and six additional MIP genes were included although two of these (Os*PIP1;4* and Os*PIP1;5*) originated from a different cultivar and might therefore represent allelic variation. All six new OsMIPs could be added to the existing groups as defined by Sakurai et al.[33]

Although comparisons of nucleotide sequences are potentially more informative than comparisons of protein sequences all phylogenetic analyses reviewed here except one are based on the protein sequences. Forrest and Bhave compared nucleotide sequences encoding PIPs and TIPs in wheat, rice and Arabidopsis.[35] Surprisingly, the *PIP1s* and *PIP2s* cluster together in an Arabidopsis clade and a monocot clade in this study, although it is well established that the PIP1 and PIP2 subfamilies separated long before the divergence of monocots and dicots. This result could be due to that much of the nucleotide variation is at synonymous sites since the amino acid sequence is highly conserved among the PIPs. Thus the two clades could reflect a difference in codon usage between the species and it might be more reliable to use protein sequences when PIPs are compared between monocots and dicots.

Analysis of MIPs in plant lineages that diverged from higher plants a long time ago can provide a better understanding of early evolution of the MIP family in terrestrial plants. We therefore identified and classified the MIPs encoded in the genome of *P. patens*.[20] The result shows that the bryophyte has an unexpected multitude and variation in the MIP family. In total 23 encoded MIPs were found that could be divided into seven subfamilies. One of the new subfamilies was the HIPs (hybrid intrinsic proteins). This class of protein was also found in spikemoss (*Selaginella moellendorffii*) and as indicated by the name has similarities to both TIPs and PIPs. The ar/R constriction was predicted to have a histidine both at H2 like the TIPs and at H5 like the PIPs. The other new subfamily was the XIPs (X intrinsic proteins) that are present also in many dicots. The physiological role of both HIPs and XIPs awaits further studies. The origin and early evolution of plant MIPs is still unresolved. None of the seven subfamilies are close homologs to the MIPs found in unicellular green algae, e.g., CrMIP1 from *Chlamydomonas reinhartii* (Fig. 1). Although the GIPs and the NIPs are suggested to have been acquired by horizontal gene transfer from bacteria, there is no evidence to suggest that any of the other five subfamilies originates from a similar event.

Phylogenetic Analysis of NIPs

As previously mentioned one of the first MIPs identified was a plant NIP from soybean, GmNIP1;1. This NIP is localized to the peribacteroid membrane of root nodules, but it was soon apparent that NIPs were not restricted to this highly specific membrane of leguminous plants. Today, NIPs are known to be one of the largest families of plant MIPs and also one of the most divergent, both in regard to substrate specificities as well as amino acid sequences. Phylogenetic analyses show that the NIP subfamily can be divided into several well defined subgroups which are remarkably well conserved across species (Fig. 2).[17,20,29] The NIP1, NIP2 and NIP3 subgroups are all present in higher plants, although NIP2s seem to be present only in some plants including—but not restricted to—monocots. Arabidopsis only possesses the NIP1 (the AtNIP1 to AtNIP4 groups) and NIP3 group (AtNIP5;1 and AtNIP6;1). The bryophyte *P. patens* has one NIP3 and three NIPs belonging to another distinct group, the NIP5s, so far only detected in primitive plants. There are also divergent NIPs that do not belong to any of these four groups, such as OsNIP4;1, PpNIP6;1 and AtNIP7;1. As seen in Figure 2 these diverse NIPs tend to group together due to the phenomenon known as long-branch attraction. The low levels of support in Figure 2 and previous analyses indicate that it is not clear how the different groups are related to each other.[20,36] It is interesting to note that at least the NIP3 group had already evolved in a common ancestor to bryophytes and higher plants. Thus it is possible that this conserved group of NIPs represents and has retained, the original function of NIPs in early terrestrial plants.

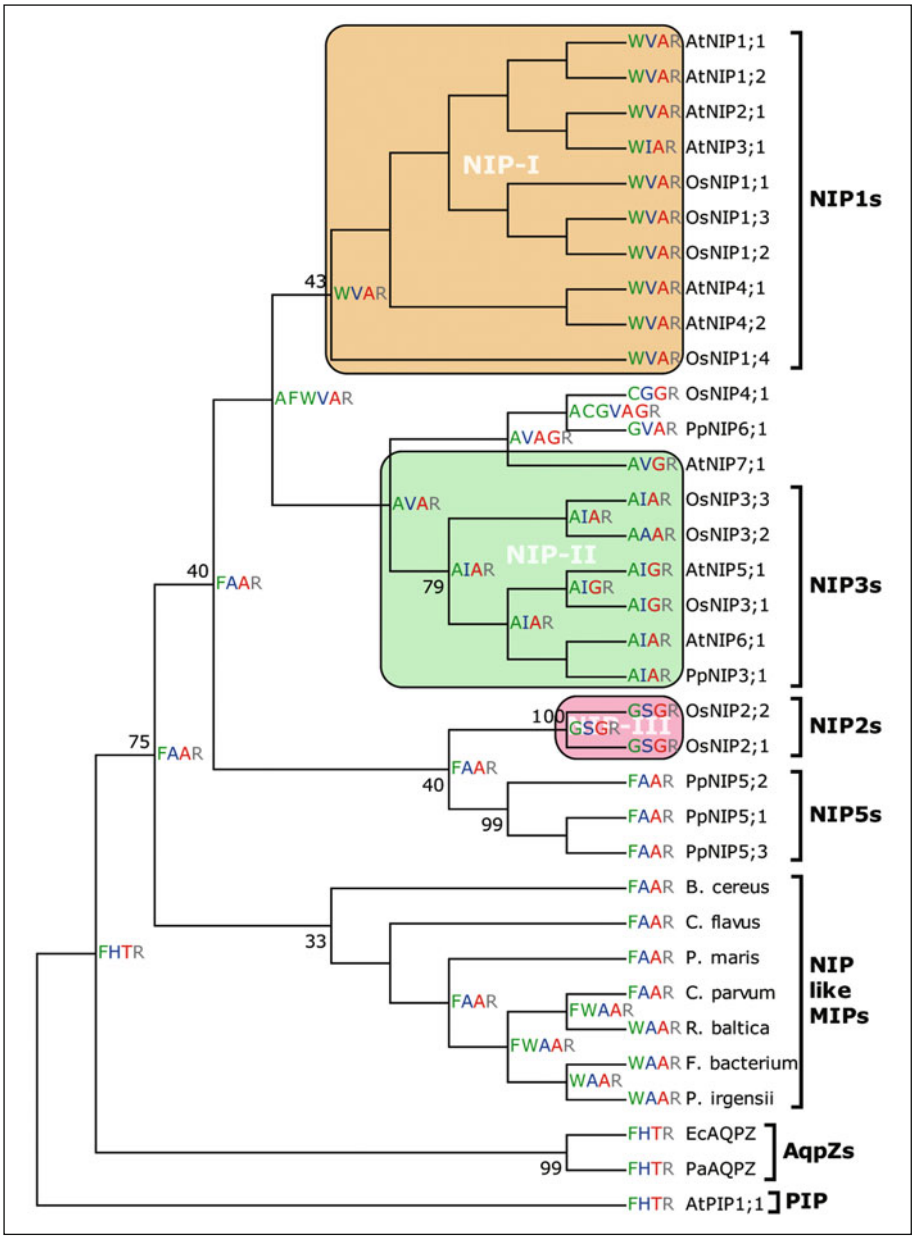


Figure 2. Phylogenetic analysis of NIPs and related MIPs. Parsimony analysis of NIPs from rice, Arabidopsis and *P. patens* and similar bacterial MIPs. The different phylogenetic NIP groups are marked by brackets and Arabic numbers whereas the three functional groups are indicated by shaded rectangles and roman numbers. The ar/R selectivity regions of all taxa are shown as well as the inferred ancestral states when nontrivial. Bootstrap values from 1000 replicates are shown for defined phylogenetic groups and basal nodes when >30%. AtPIP1;1 was used as outgroup to root the tree, and two sequences from the subfamily closest to NIPs in Figure 1 were included in the analysis. Sequence identifiers are given in the Appendix at the end of this chapter.

Solute Transport

The substrate specificity of MIPs is governed by the tetrad of amino acid residues at the most narrow region of the pore referred to as the ar/R region. In Figure 2 the tetrad of each isoform is shown as well as the ancestral state of each node according to parsimony analysis. Based on the structure of the ar/R constriction region, AtNIPs have been divided into two functional groups, NIP-I and NIP-II.[37] Whereas NIP-Is are able to transport both formamide, glycerol and to a moderate extend also water, NIP-IIs show very low water permeability but can instead transport urea.[38,39] This have been accredited to the very wide pore of NIP-IIs, as it has been shown that decreasing the aperture of the ar/R region, by replacing an alanine at the H2 position with a tryptophan, abolishes urea transport.[39] In 2006, Ma et al identified a rice NIP in a mutant screen for silicon (Si) transporters.[40] This NIP was found to be OsNIP2;1 and since then several other NIP2s have been shown to function as Si transporters.[41,42] Although, originally classified as NIP-II,[43] further analysis has revealed that these NIPs have an ar/R filter distinctly different from that of the NIP-I and NIP-II groups and they have therefore been suggested to form a separate functional group, NIP-III in Figure 2.[34,44-46] However, as seen in Figure 2, the functional grouping is at large covered by the phylogenetic groups and it would therefore be recommended that the phylogenetic grouping is used when referring to the NIP groups. This will avoid the confusion resulting from the discrepancy between the numbering of the functional and phylogenetic groups.

Apart from Si transport, NIPs have also been shown to be capable of transporting other metalloids (boron [B], arsenite [As(III)] and antimony) both in vivo[47-51] and in vitro.[44,36] It has been speculated whether metalloid transport is the original function of NIPs.[52] For example, B is essential in higher plants and needed in high amounts as it is used in the cross linking of the pectin Rhamnogalacturonan II in the cell walls. However, the low level of borate found in bryophytes[53] and the relatively complex NIP group found in the bryophyte *P. patens* suggest that at least B might not be the original physiological NIP substrate in mosses. This would favor the idea of another solute as the original substrate of NIPs, see further discussion below.

NIP-Like Bacterial MIPs and Ancestral State of ar/R Filter

The origin of NIPs is still unclear. In a very recent article the green algae *Ostreococcus lucimarinus* was claimed to encode a NIP, although no evidence for this statement was presented.[54] Furthermore, we were not able to find any support for this classification neither in phylogenetic analyses, in motif comparisons, nor in BLAST searches. In an attempt to resolve the ancestry of the NIPs we made TBLASTN searches for the closest prokaryotic homologs to PpNIP6;1, one of the basal NIPs according to our earlier analysis.[20] Some of the hits are included in the phylogenetic analysis and as seen in Figure 2 they neither appear to form a stable monophyletic group, nor do they correspond to a clear bacterial taxonomic subgroup. However, the relatively high bootstrap value for the common node with the NIPs shows that these bacterial sequences, representing a group of widely distributed but previously unrecognized NIP-like MIPs, are likely to be more closely related to NIPs than AqpZs are. A common origin of NIPs and the bacterial NIP-like MIPs (bNIPs) would exclude the suggested horizontal gene transfer of a bacterial AqpZ encoding gene as the origin of plant NIPs. However, a direct evolutionary link between AqpZs and an ancestral bNIP might still exist.

Interestingly, some bNIPs have an ar/R filter identical to that of PpNIP5 group (FAAR) and the parsimony analysis suggests that this was the ancestral state of NIPs and bNIPs (Fig. 2). Whether this ar/R tetrad would be specific for transport of metalloids can only be speculated since no MIP with this filter has yet been tested. The ar/R tetrad of HsAQP9, which transports the metalloid As(III), is similar (FACR) taking into account that it is mainly the backbone carbonyl oxygen of the Cys at loop E1 that is likely to interact with a substrate. However, this extrapolation might not be valid since HsAQP9 belongs to the GLP subfamily and therefore differs from NIPs at many other positions.

Conclusion

A coherent picture of the MIP superfamily divided into many different subfamilies has emerged. However, the relationship between the subfamilies and the earliest events that formed these subfamilies still remain unclear. Several of the different groups within the plant MIP subfamilies were already present in the last common ancestor of bryophytes and vascular plants suggesting an early evolution of e.g., the NIP3 group in terrestrial plants. However, none of the algal MIPs as identified today is closely related to MIPs in terrestrial plants. Here we identify bacterial NIP-like MIPs as close relatives of the plant NIPs, suggesting that NIP-like MIPs were already present in a common ancestor. Interestingly, the bryophyte NIP5 group has the same ar/R constriction region as these bacterial NIPs leading us to speculate that an original function is retained. Further studies are required to elucidate the substrate specificity of these MIPs and identification of MIPs in an algal sister clade of terrestrial plants is likely to contribute to our understanding of the early evolution of plant MIPs.

Acknowledgements

This work was supported by the Swedish Research Council (VR).

References

1. Gorin MB, Yancey SB, Cline J et al. The major intrinsic protein (MIP) of the bovine lens fiber membrane: Characterization and structure based on cDNA cloning. Cell 1984; 39:49-59.
2. Fortin MG, Morrison NA, Verma DP. Nodulin-26, a peribacteroid membrane nodulin is expressed independently of the development of the peribacteroid compartment. Nucleic Acids Res 1987; 15:813-824.
3. Shiels A, Kent NA, McHale M et al. Homology of MIP26 to NOD26. Nucleic Acids Res 1988; 16:9348.
4. Sandal NN, Marcker KA. Soybean nodulin 26 is homologous to the major intrinsic protein of the bovine lens fiber membrane. Nucleic Acids Res 1988; 16:9347.
5. Muramatsu S, Mizuno T. Nucleotide sequence of the region encompassing the GlpKF operon and its upstream region containing a bent DNA sequence of Escherichia coli. Nucleic Acids Res 1989; 17:4378.
6. Baker ME, Saier MH Jr. A common ancestor for bovine lens fiber major intrinsic protein, soybean nodulin-26 protein and E. coli glycerol facilitator. Cell 1990; 60:185-186.
7. Rao Y, Jan LY, Jan YN. Similarity of the product of the Drosophila neurogenic gene big brain to transmembrane channel proteins. Nature 1990; 345:163-167.
8. Johnson KD, Hofte H, Chrispeels MJ. An intrinsic tonoplast protein of protein storage vacuoles in seeds is structurally related to a bacterial solute transporter (GIpF). Plant Cell 1990; 2:525-532.
9. Yamamoto YT, Cheng CL, Conkling MA. Root-specific genes from tobacco and Arabidopsis homologous to an evolutionarily conserved gene family of membrane channel proteins. Nucleic Acids Res 1990; 18:7449.
10. Pao GM, Wu LF, Johnson KD et al. Evolution of the MIP family of integral membrane transport proteins. Mol Microbiol 1991; 5:33-37.
11. Preston GM, Agre P. Isolation of the cDNA for erythrocyte integral membrane protein of 28 kilodaltons: Member of an ancient channel family. Proc Natl Acad Sci USA 1991; 88:11110-11114.
12. Reizer J, Reizer A, Saier MH Jr. The MIP family of integral membrane channel proteins: Sequence comparisons, evolutionary relationships, reconstructed pathway of evolution and proposed functional differentiation of the two repeated halves of the proteins. Crit Rev Biochem Mol Biol 1993; 28:235-257.
13. Park JH, Saier MH Jr. Phylogenetic characterization of the MIP family of transmembrane channel proteins. J Membr Biol 1996; 153:171-180.
14. Froger A, Tallur B, Thomas D et al. Prediction of functional residues in water channels and related proteins. Protein Sci 1998; 7:1458-1468.
15. Heymann JB, Engel A. Aquaporins: Phylogeny, structure and physiology of water channels. News Physiol Sci 1999; 14:187-193.
16. Zardoya R, Villalba S. A phylogenetic framework for the aquaporin family in eukaryotes. J Mol Evol 2001; 52:391-404.
17. Zardoya R. Phylogeny and evolution of the major intrinsic protein family. Biol Cell 2005; 97:397-414.

18. Zardoya R, Ding X, Kitagawa Y et al. Origin of plant glycerol transporters by horizontal gene transfer and functional recruitment. Proc Natl Acad Sci USA 2002; 99:14893-14896.
19. Johanson U, Karlsson M, Johansson I et al. The complete set of genes encoding major intrinsic proteins in Arabidopsis provides a framework for a new nomenclature for major intrinsic proteins in plants. Plant Physiol 2001; 126:1358-1369.
20. Danielson JÅ, Johanson U. Unexpected complexity of the aquaporin gene family in the moss Physcomitrella patens. BMC Plant Biol 2008; 8:45.
21. Gustavsson S, Lebrun AS, Norden K et al. A novel plant major intrinsic protein in Physcomitrella patens most similar to bacterial glycerol channels. Plant Physiol 2005; 139:287-295.
22. Höfte H, Hubbard L, Reizer J et al. Vegetative and seed-specific forms of tonoplast intrinsic protein in the vacuolar membrane of Arabidopsis thaliana. Plant Physiol 1992; 99:561-570.
23. Kaldenhoff R, Kolling A, Richter G. A novel blue light- and abscisic acid-inducible gene of Arabidopsis thaliana encoding an intrinsic membrane protein. Plant Mol Biol 1993; 23:1187-1198.
24. Kammerloher W, Fischer U, Piechottka GP et al. Water channels in the plant plasma membrane cloned by immunoselection from a mammalian expression system. Plant J 1994; 6:187-199.
25. Yamaguchi-Shinozaki K, Koizumi M, Urao S et al. Molecular cloning and characterization of 9 cDNAs for genes that are responsive to desiccation in Arabidopsis thaliana: Sequence analysis of one cDNA clone that encodes a putative transmembrane channel protein. Plant Cell Physiol 1992; 33:217-224.
26. Weig A, Deswarte C, Chrispeels MJ. The major intrinsic protein family of Arabidopsis has 23 members that form three distinct groups with functional aquaporins in each group. Plant Physiol 1997; 114:1347-1357.
27. Karlsson M, Johansson I, Bush M et al. An abundant TIP expressed in mature highly vacuolated cells. Plant J 2000; 21:83-90.
28. Johansson I, Karlsson M, Johanson U et al. The role of aquaporins in cellular and whole plant water balance. Biochim Biophys Acta 2000; 1465:324-342.
29. Chaumont F, Barrieu F, Wojcik E et al. Aquaporins constitute a large and highly divergent protein family in maize. Plant Physiol 2001; 125:1206-1215.
30. Quigley F, Rosenberg JM, Shachar-Hill Y et al. From genome to function: The Arabidopsis aquaporins. Genome Biol 2001; 3:RESEARCH0001.
31. Johanson U, Gustavsson S. A new subfamily of major intrinsic proteins in plants. Mol Biol Evol 2002; 19:456-461.
32. Borstlap AC. Early diversification of plant aquaporins. Trends Plant Sci 2002; 7:529-530.
33. Sakurai J, Ishikawa F, Yamaguchi T et al. Identification of 33 rice aquaporin genes and analysis of their expression and function. Plant Cell Physiol 2005; 46:1568-1577.
34. Bansal A, Sankararamakrishnan R. Homology modeling of major intrinsic proteins in rice, maize and Arabidopsis: Comparative analysis of transmembrane helix association and aromatic/arginine selectivity filters. BMC Struct Biol 2007; 7:27.
35. Forrest KL, Bhave M. The PIP and TIP aquaporins in wheat form a large and diverse family with unique gene structures and functionally important features. Funct Integr Genomics 2008; 8:115-133.
36. Bienert GP, Thorsen M, Schüssler MD et al. A subgroup of plant aquaporins facilitate the bi-directional diffusion of As(OH)3 and Sb(OH)3 across membranes. BMC Biol 2008; 6:26.
37. Wallace IS, Roberts DM. Homology modeling of representative subfamilies of Arabidopsis major intrinsic proteins. Classification based on the aromatic/arginine selectivity filter. Plant Physiol 2004; 135:1059-1068.
38. Wallace IS, Wills DM, Guenther JF et al. Functional selectivity for glycerol of the nodulin 26 subfamily of plant membrane intrinsic proteins. FEBS Lett 2002; 523:109-112.
39. Wallace IS, Roberts DM. Distinct transport selectivity of two structural subclasses of the nodulin-like intrinsic protein family of plant aquaglyceroporin channels. Biochemistry 2005; 44:16826-16834.
40. Ma JF, Tamai K, Yamaji N et al. A silicon transporter in rice. Nature 2006; 440:688-691.
41. Mitani N, Yamaji N, Ma JF. Identification of maize silicon influx transporters. Plant Cell Physiol 2009; 50:5-12.
42. Chiba Y, Mitani N, Yamaji N et al. HvLsi1 is a silicon influx transporter in barley. Plant J 2009; 57:810-818.
43. Wallace IS, Choi WG, Roberts DM. The structure, function and regulation of the nodulin 26-like intrinsic protein family of plant aquaglyceroporins. Biochim Biophys Acta 2006; 1758:1165-1175.
44. Mitani N, Yamaji N, Ma JF. Characterization of substrate specificity of a rice silicon transporter, Lsi1. Pflugers Arch 2008; 456:679-686.
45. Rouge P, Barre A. A molecular modeling approach defines a new group of nodulin 26-like aquaporins in plants. Biochem Biophys Res Commun 2008; 367:60-66.
46. Ali W, Isayenkov SV, Zhao FJ et al. Arsenite transport in plants. Cell Mol Life Sci 2009; 66:2329-2339.

47. Kamiya T, Tanaka M, Mitani N et al. NIP1;1, an aquaporin homolog, determines the arsenite sensitivity of Arabidopsis thaliana. J Biol Chem 2009; 284:2114-2120.
48. Takano J, Wada M, Ludewig U et al. The Arabidopsis major intrinsic protein NIP5;1 is essential for efficient boron uptake and plant development under boron limitation. Plant Cell 2006; 18:1498-1509.
49. Isayenkov SV, Maathuis FJ. The Arabidopsis thaliana aquaglyceroporin AtNIP7;1 is a pathway for arsenite uptake. FEBS Lett 2008; 582:1625-1628.
50. Ma JF, Yamaji N, Mitani N et al. Transporters of arsenite in rice and their role in arsenic accumulation in rice grain. Proc Natl Acad Sci USA 2008; 105:9931-9935.
51. Tanaka M, Wallace IS, Takano J et al. NIP6;1 is a boric acid channel for preferential transport of boron to growing shoot tissues in Arabidopsis. Plant Cell 2008; 20:2860-2875.
52. Bienert GP, Schüssler MD, Jahn TP. Metalloids: Essential, beneficial or toxic? Major intrinsic proteins sort it out. Trends Biochem Sci 2008; 33:20-26.
53. Matsunaga T, Ishii T, Matsumoto S et al. Occurrence of the primary cell wall polysaccharide rhamnogalacturonan II in pteridophytes, lycophytes and bryophytes. Implications for the evolution of vascular plants. Plant Physiol 2004; 134:339-351.
54. Liu Q, Wang H, Zhang Z et al. Divergence in function and expression of the NOD26-like intrinsic proteins in plants. BMC Genomics 2009; 10:313.

Appendix. Identifiers for sequences used in the phylogenetic analyses

Taxa Name	Species	GI Number	CDS-Start	CDS-Stop	Locus Tag
PpPIP1;1	*Physcomitrella patens*	167997734			PHYPADRAFT_62169
PpTIP6;1	*Physcomitrella patens*	168016415			PHYPADRAFT_73809
PpNIP3;1	*Physcomitrella patens*	-[a]			
PpSIP1;1	*Physcomitrella patens*	167999246			PHYPADRAFT_112053
PpGIP1;1	*Physcomitrella patens*	168057250			PHYPADRAFT_171260
PpHIP1;1	*Physcomitrella patens*	-[a]			
PpXIP1;1	*Physcomitrella patens*	-[a]			
AtPIP1;1	*Arabidopsis thaliana*	145339736			AT3G61430
AtTIP1;1	*Arabidopsis thaliana*	145360688			AT2G36830
AtNIP4;1	*Arabidopsis thaliana*	18421689			AT5G37810
AtNIP1;1	*Arabidopsis thaliana*	145340407			AT4G19030
AtNIP1;2	*Arabidopsis thaliana*	42566939			AT4G18910
AtNIP2;1	*Arabidopsis thaliana*	145360604			AT2G34390
AtNIP3;1	*Arabidopsis thaliana*	186479109			AT1G31885
AtNIP4;1	*Arabidopsis thaliana*	18421689			AT5G37810
AtNIP4;2	*Arabidopsis thaliana*	18421690			AT5G37820
AtNIP5;1	*Arabidopsis thaliana*	145340067			AT4G10380
AtNIP6;1	*Arabidopsis thaliana*	42563382			AT1G80760
AtNIP7;1	*Arabidopsis thaliana*	145338170			AT3G06100
AtSIP1;1	*Arabidopsis thaliana*	186509744			AT3G04090
OsPIP1;1	*Oryza sativa*	115447784			Os02g0666200
OsNIP1;1	*Oryza sativa*	115445190			Os02g0232900
OsNIP1;2	*Oryza sativa*	13161410			Os01g0202800[b]
OsNIP1;3	*Oryza sativa*	54144479			
OsNIP1;4	*Oryza sativa*	58531193			
OsNIP2;1	*Oryza sativa*	115448656			Os02g0745100
OsNIP2;2	*Oryza sativa*	193811875			Os06g0228200
OsNIP3;1	*Oryza sativa*	20270142			Os10g0513200[b]
OsNIP3;2	*Oryza sativa*	58531195			
OsNIP3;3	*Oryza sativa*	37806235			
OsNIP4;1	*Oryza sativa*	115434109			Os01g0112400
OsTIP1;1	*Oryza sativa*	115450710			Os03g0146100
OsSIP1;1	*Oryza sativa*	115434915			Os01g0182200
HsAQP2	*Homo sapiens*	209180415			
HsAQP3	*Homo sapiens*	22165421			
HsAQP5	*Homo sapiens*	186910293			
HsAQP6	*Homo sapiens*	86792454			
HsAQP7	*Homo sapiens*	4502186			
HsAQP8	*Homo sapiens*	45446751			
HsAQP10	*Homo sapiens*	22538419			
HsAQP11	*Homo sapiens*	27370564			
HsAQP12	*Homo sapiens*	156447036			
CrMIP1	*Chlamydomonas reinhardtii*	159471951			
VcMIP1	*Volvox carteri*	167172578 + 167084018[c]			

continued on next page

Appendix. Continued

Taxa Name	Species	GI Number	CDS-Start	CDS-Stop	Locus Tag
PfAQP	*Plasmodium falciparum*	124804458			
MmAQPM	*Methano thermobacter marburgensis*	54040725[d]			
ScGLP	*Saccharomyces cerevisiae*	51012656			YFL054C
ScAQY1	*Saccharomyces cerevisiae*	45270021			YPR192W
CgAQY	*Candida glabrata*	50285982			CAGL0D00154g
CgGLP	*Candida glabrata*	50285778			CAGL0C03267g
MsAQPM1	*Methanosphaera stadtmanae*	84372150	1,146,881	1,147,615	Msp_0998
EcAQPZ	*Escherichia coli*	215485161	913,630	914,325	
EcGLPF	*Escherichia coli*	215485161	4,432,406	4,433,251	
PaAQPZ	*Pseudomonas aeruginosa*	115583796	1,010,865	1,011,554	aqpZ
PaGLPF	*Pseudomonas aeruginosa*	115583796	1,544,365	1,545,204	glpF
SsAQP8	*Sus scrofa*	159461726			LOC100127152
RnAQP8	*Rattus norvegicus*	2358276			
CtGLP	*Clostridium tetani*	28204652	2124330	2125034	CTC_01996
BsGLP	*Bacillus subtilis*	225184640	1002501	1003325	BSU09280
SmHIP	*Selaginella moellendorffii*	-[e]			
NbXIP	*Nicotiana benthamiana*	39858292 + 39862195[c]			
PtXIP	*Populus trichocarpa*	224103260			POPTRDRAFT_557139
C. parvum	*Chlorobaculum parvum*	193085153	474251	474964	Cpar_0442
R. baltica	*Rhodopirellula baltica*	32397972	72504	74108	RB4879
B. cereus	*Bacillus cereus*	218540236	250503	251225	BCAH820_B0297
F. bacterium	*Flavobacteriales bacterium*	163787877	253541	254209	FBALC1_07048
P. irgensii	*Polaribacter irgensii*	88803076	393143	393817	PI23P_12632
C. flavus	*Chthoniobacter flavus*	196229614	370933	371610	CfE428DRAFT_1912
P. maris	*Planctomyces maris*	149176345	17718	18404	PM8797T_07554

a) We have described in detail the annotation of this PpMIP in a previous study (Danielson JA, Johanson U. BMC Plant Biol 2008; 8:45); b) This locus only cover part of the MIP sequence used; c) These two EST sequences were combined to get the full length CDS used in the analysis; d) This GI number refers to the AA sequence, the NA sequence can be found in the original article, PMID: 16233136; e) This sequence was retrieved from the genomic sequence available from the *Selaginella moellendorffii* genome project at the Joint Genome Initiative (http://genome.jgi-psf.org/Selmo1/Selmo1.home.html) and partially corresponds to the gene model "estEXT fgenesh1 pm-C 30069".

Elsbeth Bienert

Chapter 3

Metalloids, Soil Chemistry and the Environment

Enzo Lombi* and Peter E. Holm

Abstract

This chapter reviews physical chemical properties, origin and use of metalloids and their relevance in the environment. The elements boron (B), silicon (Si), germanium (Ge), arsenic (As), antimony (Sb), tellurium (Te), polonium (Po) and astatine (At) are considered metalloids. Metalloids conduct heat and electricity intermediate between nonmetals and metals and they generally form oxides. The natural abundance of metalloids varies from Si being the second most common element in the Earth's crust to At as the rarest of natural elements on Earth. The metalloid elements Ge, Te, Po and At are normally present in trace or ultratrace levels in the environment and as such are not considered of relevance in terms of environmental health. The environmental geochemical processes, factors and parameters controlling the partitioning and the speciation of B, Si, As and Sb are reviewed in relation to the bioavailability of these metalloids. Approaches based on the hypothesis that metal toxicity is related to both the metal-ligand complexation processes and the metal interactions with competing cations at the cell surface (biotic ligand) have so far not been successful for assessing metalloid bioavailability. The chapter concludes that our understanding of metalloids toxicity will improve in the future if, in addition to the points discussed above, surface membrane potentials are considered. This should represent a robust approach to the prediction of metalloid toxicity.

Introduction

'Metalloids' is a term that is frequently used to group elements that possess physical and chemical characteristics that are intermediate between those of metals and nonmetals. For instance, they conduct heat and electricity better than nonmetals but not as well as metals (i.e., they are semiconductors). Also, they generally form amphoteric oxides, being an acid and a base. They are grouped along a diagonal line through the p block of the periodic table from boron to astatine (Fig. 1). Commonly the following elements are considered metalloids: boron (B), silicon (Si), germanium (Ge), arsenic (As), antimony (Sb), tellurium (Te), polonium (Po) and astatine (At).

Despite their similarities in chemical and physical characteristics, metalloids display a dramatic difference in their abundance in the environment. For instance, Si is the second most common element in the Earth's crust (the first being oxygen) making up over a quart of its mass. In contrast, the most long-lived natural isotope of astatine (^{210}At) has a half-life of only 8.3 hours. As a consequence, this element, which is produced by the decay of uranium and thorium,[1] is the rarest of the natural elements on Earth with only approximately 25 g present on the whole planet at any given time.[2]

*Corresponding Author: Enzo Lombi—Centre for Environmental Risk Assessment and Remediation, University of South Australia, Building X, Mawson Lakes Campus, Mawson Lakes, South Australia 5095, Australia. Email: enzo.lombi@unisa.edu.au; CRC CARE, PO Box 486, Salisbury, South Australia 5106, Australia.

MIPs and Their Role in the Exchange of Metalloids, edited by Thomas P. Jahn and Gerd P. Bienert.

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
H																	He
Li	Be											**B**	C	N	O	F	Ne
Na	Mg											Al	**Si**	P	S	Cl	Ar
K	Ca	Sc	Ti	V	Cr	Mn	Fe	Co	Ni	Cu	Zn	Ga	**Ge**	**As**	Se	Br	Kr
Rb	Sr	Y	Zr	Nb	Mo	Tc	Ru	Rh	Pd	Ag	Cd	In	Sn	**Sb**	**Te**	I	Xe
Cs	Ba	Lu	Hf	Ta	W	Re	Os	Ir	Pt	Au	Hg	Tl	Pb	Bi	**Po**	**At**	Rn
Fr	Ra	Lr	Rf	Db	Sg	Bh	Hs	Mt	Ds	Rg	Uub	Uut	Uuq	Uup	Uuh	Uus	Uuo

Figure 1. The periodic table of elements: metalloids are grouped along a diagonal line from boron to astatine.

The same variability among metalloids observed in terms of their natural abundance also extends to their interactions with living organisms. Only B is recognized as an essential element for plants, while Si is considered as 'agronomically essential' to specific crops such as rice.[3] In contrast, there is mounting evidence that B, Si and As may be essential for animals.[4]

This chapter will provide an historical perspective of our knowledge and use of metalloids and will review the relevance of these elements in the environment. Finally, the environmental chemistry of the most relevant metalloids (i.e., B, Si, As and Sb) will be summarized and discussed in relation to metalloids' bioavailability.

Historical Perspective

Among the metalloids, As is arguably the element that has been recognized and utilized by different cultures around the world for the longest time. While the term *arsenicum* was first mentioned by Dioscorides (circa 90-40 B.C.), As sulphide was already mentioned by Aristotle in the fourth century B.C.[5] Ancient Greeks employed As in medicines and its use in this context continued until recent times. For instance, the Fowler's solution (containing 1% arsenic trioxide) was one of the most frequently dispensed medicines in Western countries.[6] In more recent times As was used in insecticides, herbicides, animal feeds and as wood preservative but its direct application in the environment has significantly decreased as a consequence of its recognized toxicity and carcinogenicity.

The history of Sb and B is also intertwined with various civilisations since antiquity. Artifacts made of Sb and dating back to 3000 B.C. have been found in Egypt and present day Iraq.[7] For many centuries Sb was used to remove impurities from gold and, for the same reasons, was employed in early times to remove 'impurities' from the body. Medical applications of Sb and its compounds have continued over the centuries to the present day where Sb is used in tropical medicine notably in the treatment of leishmaniasis.[8] The first solid evidence for the use of borax (sodium borate) are related to the import of this mineral to Mecca and Medina in the eigth century; borax flux by European goldsmiths dates to about the 12th century.[9]

The use of silica for the production of glass also dates back to the early stages of human civilisation. In contrast, the discovery of the other metalloids is much more recent. Germanium was discovered by Clemens Alexander Winkler in the same year in which Coca Cola was formulated, 1886.[10] Polonium was named by Marie Curie[11] after her native land of Poland in 1898 while At was one of the two of the 'missing' elements, together with technetium, to be synthesized by man.[12]

Environmental Relevance

Elevated concentrations of metalloids in the environment may be the results of human activities or due to geogenic conditions. Generally, anthropogenic sources are limited to specific areas but agricultural and horticultural activities, such as the use of As-based pesticides or As-tainted irrigation water may result in widespread contamination, (e.g., see ref. 13). However, when considering the environmental relevance of metalloids a first consideration must be made between the occurrence of Si (and sometimes B) and that of other elements. As mentioned above Si represents approximately one quarter of the mass of the Earth crust. In contrast, all the other elements are generally present in trace concentrations. Consequently larger concentrations due to anthropogenic sources are regarded as instances of contamination or pollution.

Boron is widespread in the environment where it usually occurs as borax ($Na_2B_4O_7$ $10H_2O$) tourmaline (a borosilicate mineral) or in the form of Ca and Mg salts. Boron concentrations in soils vary between 10 and 300 mg kg^{-1} depending on various geological and environmental factors.[14] Generally the occurrence of B toxicity is less frequent than B deficiency.[15] However, B can naturally accumulate in some arid environments and where water rich in B or wastewater have been used for irrigation.[16]

Naturally elevated concentrations of As are often associated with the presence of sulphidic ore deposits which during weathering release As in associated waters and soils. Soils, sediments and water bodies displaying large concentration of As have been identified in many countries around the globe including China, Argentina, Chile, Mexico, Cambodia, Vietnam, Thailand, Nepal, Ghana, Taiwan, Finland and Hungary. Also there are increasing reports of chronic arsenism emerging from Europe and USA.[17] However, the biggest calamity associated with As contamination is related to the utilization of As-tainted water for human consumption and irrigation in various districts of West Bengal (India) and Bangladesh where tens of millions of people are at risk (e.g., see ref. 18). For many years the issue of As toxicity to humans in these areas has been mainly thought to be caused by to the use of As-contaminated drinking water. However, in recent years mounting evidence is accumulating that this problem may be compounded by the consumption of As-contaminated food and in particular rice (see also the chapter by Bienert and Jahn in this book). Meharg and Rahman[19] first brought to light in Bangladesh that rice grain As was becoming elevated through the extensive use of aquifer water for the irrigation of dry season paddy rice.

Mining and smelting operations are the greatest emission source of Sb into the environment.[20] Other sources of Sb in the environment are linked to its use in metal alloys, flame retardants and catalysts.[21] Also, on a more local scale, Sb can derive from the application of lead arsenate [As(V)] pesticides, since Sb is present as a contaminant in the Pb- and As-containing ores used to manufacture pesticides.[22] Modern applications of Sb have increased dramatically during the last decades and today Sb is used in a broad range of applications, such as batteries, cable sheating, sheets and pipes, plumbing solder and antifriction material, rubber, PVC products and other synthetic products, plastic bottles, ceramics, ammunition primers, medical drugs, bactericides and fungicides.[23] Of growing concern are also the findings of Sb contamination at shooting ranges (e.g., see ref. 24).

The remaining elements are normally present in trace or ultratrace levels in the environment and as such are not considered of relevance in terms of environmental health.

Finally, it should be noted that due to the widespread application of metalloids in semiconductor, other electronic applications and flame retardant materials their flow to the environment is likely to increase in the future as a result of electronic-waste disposal.[25] This is of particular concern in developing countries where end-of-life electronics are imported and recycled via a "backyard industry" using primitive processes.[26] For instance, a computer desktop and monitor may contain up to 17.5 g of Sb and 60 mg of As.[27]

Environmental Toxicity of Metalloids

As mentioned above some metalloids are (agronomically) essential while others are not. However, the majority of them are toxic when present in large concentrations (with the exception

of Si). Among the essential elements, the range between deficient and toxic concentration of B in the soil solution is smaller than for any other nutrient element.[28] The information regarding the environmental toxicity of ultratrace elements such as Po, Ge, Te and At is extremely scant and it is unlikely that any significant detrimental effect occurs (even though toxicity may not be excluded at mining or disposal sites).

In contrast, the ecotoxicology of As, B and Sb is well documented. The large information available in the literature for As and Sb has been recently screened and analysed by the U.S. Environmental Protection Agency in the frame of a process aiming at identifying Ecological Soil Screening Levels (EcoSSL). These levels are 'the concentrations of contaminants in soil that are protective of ecological receptors that commonly come into contact with and/or consume biota that live in or on soil' (i.e., plants, soil invertebrates, birds and mammals).[29] In the case of Sb, an EcoSSL value could not derive for plants but was set at 78 mg kg^{-1} for soil invertebrates.[30] Arsenic EcoSSL levels for plants were set at 18 mg kg^{-1} while no value could be reported for soil invertebrates due to insufficient data.[30] Even though some papers on B toxicity to plants and soil invertebrates have been published rigorous ecotoxicological information is still scant and a screening level for B is difficult to assess.

Factors Controlling Bioavailability

Biotic and abiotic factors control the bioavailability of metalloids in the environment. Biotic factors are mainly related to physiological and molecular processes responsible for the uptake, detoxification and efflux of these elements. These processes are discussed in details in several other Chapters in this book. However, these biotic processes do not operate in isolation but are closely linked to various abiotic processes controlling the partitioning and speciation of metalloids.

Solid: Solution Partitioning of Metalloids

Metalloids are generally present in the soil solution phase as either negatively charged ions or undissociated (uncharged) molecules. When present as negatively charged molecules their partitioning between the solution phase and the solid phase (soil or sediments) follows opposite trends to that of trace element cations. For instance, while high pHs move the partitioning of heavy metals toward the solid phase, the solubility of metalloids generally increases under alkaline conditions. This is due to a decrease in the number of positively charged sorption sites at high pH due to deprotonation of pH dependent sorption sites. Negatively charged metalloid ions, such as As(V) and antimonite [Sb(III)], display a strong affinity for Fe and Al oxy/hydroxides which often represent the primary sinks of these elements in the environment. The charge of these ions depend on pH (e.g., see refs. 31, 32). However, the effect of pH may be different in the case of some metalloids, such as B, which are uncharged in the neutral pH range. Applications of lime ($CaCO_3$) to soil increase B fixation as it raises the pH of the soil solution, causes B to become negatively charged and increases adsorption to soil surfaces.[28] However, metalloid movement in the soil pore water may increase at high pH due to an increase in colloidal generation at high pH. While numerous publications show that the generation of colloids is a major process controlling metal mobility in soils,[33-35] detailed studies for colloid-associated As and Sb are missing. In this case however, we should point out that metalloids' mobility would not be related to an increase in the partitioning of the elements in the soil solution but rather to colloidal-facilitated transport processes.

While organic matter present in soil and sediments is generally an effective sink for metal cations, the interactions between metalloids and organic matter are not well understood. Sadiq[36] suggested that a limited interaction between As oxyanions and organic matter is expected because of the similar type of electrical charge. Also, since simple organic acids and phenolics can be adsorbed by Fe oxides (e.g., see ref. 37), low-molecular-weight organics may compete with As oxyanions for adsorption sites on Fe oxides. However, more recent reports indicate that some metalloids, such as Sb, can be bound in considerable amounts to organic matter.[38,39] This association may be attributed to the formation of "bridging" products between Ca, organic matter and metalloid anions.

The partitioning of metalloids between the solid and solution phase is also controlled by the redox conditions of the system. The redox effect is due to both the importance of this parameter in controlling the stability of Fe and Mn oxides that can act as a sink for metalloids and the influence of redox on the metalloid speciation itself. The redox potential aspect is extremely relevant in the case of As because, as mentioned above, the accumulation of this element in rice is of increasing concern. Rice is predominantly cultivated under paddy conditions, which create a predominantly reduced environment in the soil (with the exception of the soil volume surrounding the roots). Even though Sb can undergo redox processes similar to those of As in soil (see below) no evidence for Sb accumulation in rice has been reported.

Speciation of Metalloids in the Environment

The solid phase speciation of metalloids in the environment is extremely complex due to the large heterogeneity that characterize soils and sediments. However, in general metalloids can be present in primary or secondary minerals in the environment or sorbed on a variety of solid phases ranging from clay minerals to oxides and organic matter. In this book chapter we will mainly focus on the speciation in the solution phase as this represent the dominant pathway of exposure for the majority of soil (and aquatic) organisms.

While Si is one of the most abundant elements in soil and sediments, most of the species present in the environment are not bioavailable. In fact, the majority of Si is in the form of Si dioxide or aluminosilicate minerals. The Si species that is bioavailable to organisms is orthosilicic acid (H_4SiO_4). Silicic acid in soil solution is mainly derived by weathering of minerals like sesquioxides, kaolinites and smectites instead of silica and quartz minerals that are rather insoluble.[40] Orthosilicic acid is under normal environmental condition present as an uncharged molecule since its first pKa is 9.84. Also low molecular weight polymeric species of silicic acid have been found in soil solutions but those are unstable in subalkaline conditions and only represent a small percentage of the total Si in solution.[41] Silicic acid polymerizes to form silica gel ($SiO_2\ nH_2O$) when its concentration exceeds 2 mM.[42] Very few reports relate to the volatilization of Si compounds and are mainly related to poly(dimethylsiloxane)s (PDMS). PMDS are widely used in various commercial applications and for this reason found their way in the environment and soil. In soil PMDS undergo soil-catalyzed hydrolysis to produce volatile dimethylsilanediol.[43]

Boron in soil is generally present in primary minerals, such as tourmaline, sorbed by clay minerals sesquioxides and organic matter or in soil solution.[16] In soil solution the undissociated H_3BO_3 species generally dominates and $B(OH)_4^-$ has negligible importance.[16] The undissociated boric acid is easily leached from the soil under wet conditions but tends to accumulate in arid environments. Boron does not undergo oxidation-reduction reactions and is not known to volatilize from soils.[28]

The pH and redox potential are key factors controlling As speciation and consequently its mobility in the environment. Because the surfaces of Mn, Al and Fe oxides/hydroxides and clay minerals are positively charged only below soil pH 3, 5, 8 and 4 respectively,[36] As adsorption on soil colloids is more pronounced at lower pHs. Under anaerobic conditions the reduced form of As, arsenite [As(III)], tends to accumulate while As(V) is the main species in aerobic environments. A diagram showing the various aqueous species that can be expected for As as a function of pH and redox potential is reported in Figure 2. Also, the charge of As oxyanions is pH dependent. In the normal range of soil pH (4-8) the dominant As species in solution is undissociated H_3AsO_3 in the case of As(III). In contrast, inorganic As(V) species are usually charged in the environment with uncharged H_3AsO_4 only occurring at very low pH. The dissociation constants of As(V) are similar to those of phosphate and under a circumneutral pH range, $H_2AsO_4^-$ and $HAsO_4^{2-}$ are expected to be the dominant species.[44] In addition to inorganic As species, methylated species are found in the environment as a result of anthropogenic activities and microbial mediated methylation. This latter process was first described by Gmelin in 1815 and provided an explanation for the occurrence of some instances of As poisoning. In fact, at that time, As was an essential constituent in many organic and inorganic pigments used in wallpaper and fungi living

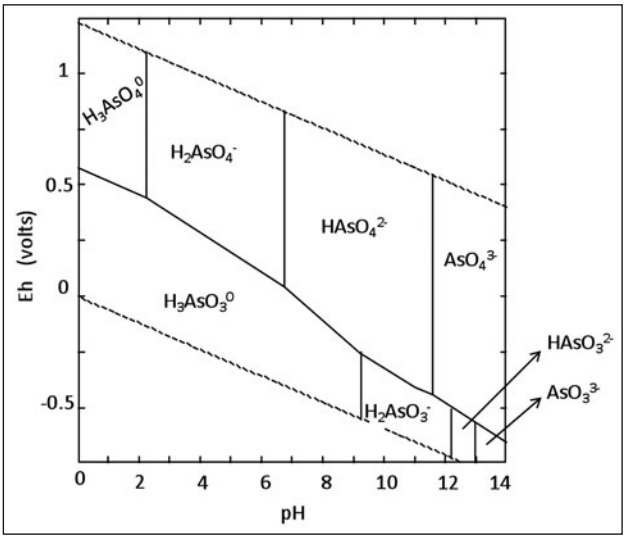


Figure 2. Eh-pH diagram for As. Modified with permission from Lombi et al. Journal of Plant Nutrition and Soil Science 1999:451-456; Copyright Wiley-VCH Verlag GmbH and Co. KGaA.

on wallpaper can converted inorganic As into methylated gaseous species (e.g., trimethylarsine). Microbial methylation of As in soil is common to both bacteria and fungi. This process seems to be favoured by anaerobic condition.[45] The microbial production of arsines from arsenicals in soil seems to contribute only marginally to the loss of As.[46] The most common methylated species in soil is generally dimethylarsinic acid (DMA). The Na salts of both monomethylarsonic acid (MMA) and DMA were also extensively used in agriculture. This brief discussion of the speciation of inorganic As species should demonstrate the importance of speciation both in terms of mobility of this element in the environment and in relation to uptake mechanisms. In fact, while As(V) is usually strongly retained in soils due to its negative charge, the uncharged As(III) has been found to be much more mobile in the environment.[32,47] Also, while As(V) may be taken up by phosphate transporters (e.g., see refs. 48,49) the uptake of As(III) occurs, as will be discussed in this book, through different mechanisms. Methylated As species have been found to be taken up by plants at a much smaller rate than inorganic species (e.g., see ref. 50).

The chemistry of Sb in aquatic systems has been thoroughly reviewed by Filella et al.[51] Sb environmental chemistry resembles that of As both in terms of factors controlling its availability and in terms of speciation. Common Sb minerals are stibinite (Sb_2S_3) and valentinite (Sb_2O_3).[39] Pentavalent Sb is the dominant species under aerobic soil conditions. The Sb(III) ion [$Sb(OH)_6^-$] is also the dominant Sb form in surface waters.[51] Under reduced condition the uncharged trivalent Sb(III) [$Sb(OH)_3^0$] is the dominant ion in the environmentally relevant pH range.[51] A Eh-pH diagram for Sb species is shown in Figure 3. In a comprehensive study of both the soil solid and solution phase Mitsunobu et al[52] compared the behavior of As and Sb under various soil redox conditions. They found that both metalloids were mainly associated with Fe oxides but also that their solution chemistry varied in response to changes in redox potential. With decreasing redox potential the total concentration of As in solution increased as a result of an increased proportion of As(V). In contrast, Sb was found to remain in solution as antimonate and the authors concluded than SbV is stable under reducing conditions. This is somehow in contrast with the Eh-pH diagrams reported in Figure 2 and 3. Similarly to As, Sb is also found in methylated species of biological origins. For instance, detected mono-, di- and tri-methyl and tri-ethyl Sb derivatives have been identified by Krupp et al.[53] However, the chemistry of these organic compounds seems to be different from that

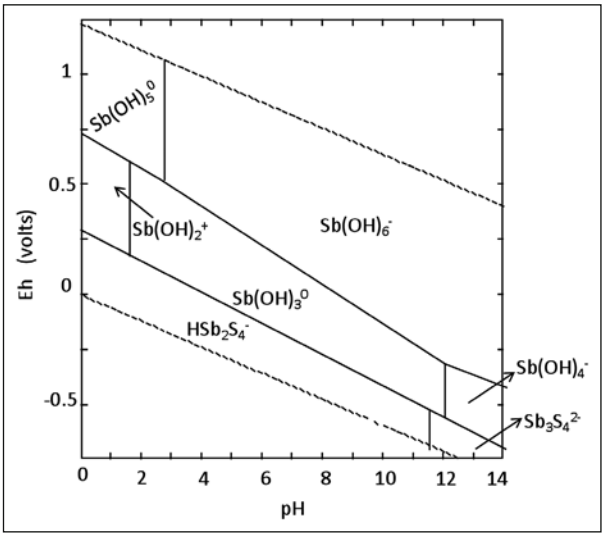


Figure 3. Eh-pH diagram for Sb. Modified from an original found in Krupka KM and RJ Serne. 2002. Geochemical Factors Affecting the Behavior of Antimony, Cobalt, Europium, Technetium and Uranium in Vadose Zone Sediments . PNNL-14126, Pacific Northwest National Laboratory, Richland, WA.

of their As counterparts. For example, while DMA is very soluble in water, dialkylstibinic acid is polymeric and showing poor water solubility.[54] Some of these methyl compounds are known to be volatile in a matter similar to Sb hydride derivatives. The soil processes that lead to the formation of these compounds are controlled by organisms and are particularly active under anaerobic conditions (e.g., see ref. 55). The processes and factors controlling speciation and bioavailability of As, B, Si and Sb are briefly summarized in Table 1.

Similar to Si, the most important form of germanium in aqueous solution is germanic acid ($H_4GeO_4^0$). Because of its similarities to Si, ^{68}Ge has been used as a tracer to investigate Si fluxes in freshwater sediments.[56] Polymeric forms of Ge have also been identified but, as pointed out by Wood and Samson,[57] these species are probably not environmentally relevant since average Ge concentrations would likely be too low to stabilize such species. The first and second dissociation

Table 1. Processes and factors controlling speciation and bioavailability of As, B, Si and Sb

	Processes		
Metalloid	**Redox**	**Sorption**	**Precipitation**
Arsenic, As	+[a]	+	-[b] (red[c]) + (ox[c]: with Ca, Mg, etc)
Boron, B	-	-[d]	As salt
Silicon, Si	-	-	+
Antimony, Sb	+	+	+

[a]+: to active and important process; [b]-: unlikely to be important process under these conditions; [c]red.: reducing conditions. ox: oxidizing conditions; [d]The dominant undissociated form H_3BO_3 adsorbs very little.

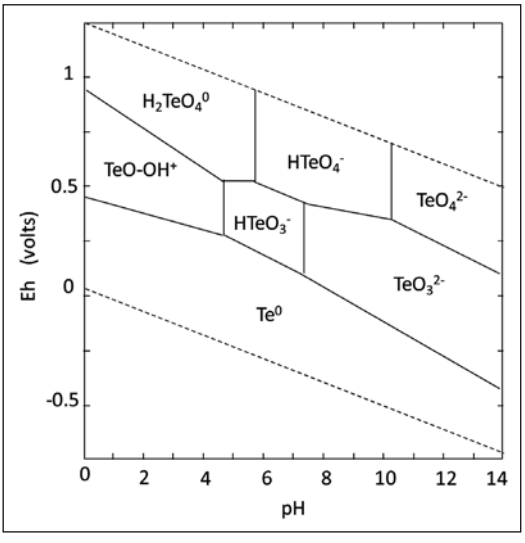


Figure 4. Eh-pH diagram for Te. Modified from Harada and Takahashi. Geochimica Et Cosmochimica Acta 2008; 72(5):1281-1294,[59] ©2008 with permission from Elsevier.

constants for germanic acid occurs at pH values of approximately 9 and 11.5 respectively and are therefore too high to be relevant under prevalent environmental conditions.[57] Methylated species of biological origins, such as monomethylgermanium (MMGe) and dimethylgermanium (DMGe), have also been reported in a variety of aquatic environments (e.g., see ref. 58).

Tellurium is chemically similar to Se and as such can be found in the environment in different oxidation states including Te0, TeIV and TeVI.[59] An Eh-pH diagram for Te is reported in Figure 4. Based on this diagram it appears that Te will be mainly present in the environment in the form of charged oxyanions or hydroxide ions. As it is the case for all the other redox active metalloids the toxicity and availability of Te depends on its speciation and oxidation state. The information regarding Te speciation in soils is rather scant. Harada and Takahshi[59] reported that TeVI was the predominant species in a soil water extracts under oxidizing conditions while TeIV predominated under anoxic conditions. They also observed that both oxidation states had a similar partitioning between the solution and solid phases. On the basis of X-ray absorption spectroscopy data, they suggested that this was due to the fact that both species can form inner-sphere complexes with Fe oxides. Various methylated and ethylated species of Te of biological origin have also been reported (e.g., see refs. 60,61). Several volatile species of Te including tellurium hydride, monomethyltellurium hydride, dimethyltellurium and some unidentified Te species have been reported by various authors (e.g.,see ref. 55).

The chemistry of At is not well understood even though a number of investigations have been conducted since this radioisotope is intensively investigated in radiotherapy. However, due to the vanishing small amounts present in the environment, its environmental chemistry has not been investigated in any detail.

Assessing Soil Bioavailability of Metalloids

The assessment of the bioavailability of inorganic contaminants has progressed significantly in the last decade. However, a large proportion of the resources have been targeted towards the development of assessment tools for metals rather than for metalloids. The current knowledge regarding bioavailability and toxicity of metals is based on the concept known as Free Ion Activity Model (FIAM).[62] According to this model, the free ions are able to equilibrate quickly

with the cell surface binding sites whereas metals bound to colloids or strong complexing agents cannot.[63] This concept has been used to develop the Biotic Ligand Model (BLM).[64,65] The BLM is based on the hypothesis that metal toxicity is related to both the metal-ligand complexation processes and the metal interactions with competing cations at the binding site (biotic ligand) at the cell surface. This model has obtained considerable acceptance as the U.S. Environmental Protection Agency (USEPA) has incorporated the BLM into its regulatory framework and other countries will probably consider this option.[66] However, while the knowledge and application of the BLM for aquatic systems has progressed substantially, the development of a terrestrial BLM[67] is at this stage still in progress.

The BLM has been successfully employed to model and predict the amelioration of toxicity caused by competing ions. For instance the effect of major cations such as H^+, Ca^{2+}, Mg^{2+}, Na^+ and K^+ on the decrease in toxicity of heavy metals has been interpreted as competition between cations at the BL.[64,65] However, as mentioned above the BLM has not been successfully developed or extended to the assessment of metalloids bioavailability.[68] In fact, the current BLM approach would fail to predict the enhanced toxicity of anions such as Se and As as a result of an increasing availability of usually ameliorating cations such as Ca^{2+}.[69] As the chemistry of many metalloids is dominated by anionic forms in solutions the discussed limitation in the BLM approach is particularly important for these elements. Kinraide and coworkers[68,70,71] have put forward that the limitation in the BLM approach is caused by this model neglecting the effect of the cell membrane surface potential (ψ_0). It is well known that cell membranes are negatively charged (e.g., see ref. 72). This charge plays an important role in relation to the activities of cations and anions at the cell surface. For instance, negative values of ψ_0 result in an increase in cation and in a decrease in anion concentrations at the surface of the membrane. Consequently, an increase in cations (including protons) in the medium surrounding a membrane reduces the negativity of ψ_0. This electrostatic effect causes a reduction in the surface activity of potentially toxic cations and an increase in the surface activity of anions, such as As(V). This mechanism explains why the extrinsic toxicity of Cu^{2+} is alleviated by ameliorating cations such as Ca^{2+}, whereas the extrinsic toxicity of anions is aggravated. Kinraide and Yermiyahu[72] has recently demonstrated that effects due to the plasma membrane surface potential may be more important in terms of bioavailability than site-specific competitions for cations. This approach has enabled us to effectively explain changes in As(V) toxicity as a result of changes in the concentration of cations in solution.[68] Therefore, the inclusion of ψ_0 effects is expected to increase our understanding of metalloids toxicity in the future and should represent a robust approach to the prediction of metalloid toxicity.

Despite this recent development, however, it should be kept in mind that several metalloid species are present in the environment as uncharged molecules. This is for instance the case of boric acid and As(III). Therefore, cationic competition and the consideration of surface potential will possibly be irrelevant for such species. Instead, new conceptual and numerical models will have to be developed to assess metalloid toxicity.

Conclusion

Based on a review of physical chemical properties, origin, use, partitioning, speciation and bioavailability of metalloids and their relevance in the environment it can be concluded that:

The elements boron (B), silicon (Si), germanium (Ge), arsenic (As), antimony (Sb), tellurium (Te), polonium (Po) and astatine (At) are all considered metalloids. Ge, Te, Po and At are typically present in very low levels in the environment and as such are not considered of relevance in terms of environmental health. The pH, redox potential and the reactive sorbents (clay minerals, Al, Fe and Mn oxides, organic matter) in soils and sediments are key factors in controlling the partitioning, speciation and bioavailability of the metalloid elements B, Si, As and Sb. Inclusion of the surface membrane potentials in availability models is expected to increase our understanding of metalloids toxicity in the future and should represent a robust approach to the prediction of metalloid toxicity.

References

1. Hyde EK, Ghiorso A. The alpha-branching of ack and the presence of astatine in nature. Phys Rev 1953; 90:267-270.
2. Anders E. Technetium and astatine chemistry. Annu Rev Nucl Sci 1959; 9:203-220.
3. Savant NK, Datnoff LE, Snyder GH. Depletion of plant-available silicon in soils: A possible cause of declining rice yields. Commun Soil Sci Plant Anal 1997; 28:1245-1252.
4. Nielsen FH. Importance of making dietary recommendations for elements designated as nutritionally beneficial, pharmacologically beneficial, or conditionally essential. Journal of Trace Elements in Experimental Medicine 2000; 13:113-129.
5. Azcue JM, Nriagu JO. Arsenic: historical perspectives. In: Nriagu JO, ed. Arsenic in the Environment. Part I: Cycling and Characterization. New York: Wiley, 1994:1-15.
6. Nriagu JO. Preface. In: Nriagu JO, ed. Arsenic in the Environment. Part II: Human Health and Ecosystem effects. New York: Wiley, 1999:ix.
7. Kroschwitz JI, Seidel A. Kirk-Othmer Encyclopedia of Chemical Technology, 5th ed. Hoboken: Wiley-Interscience, 2004.
8. McCallum RI. Antimony in Medical History: An account of medical uses and its compounds since early times to the present. Cambridge: Pentland Press, 1999.
9. Travis NJ, Cocks EJ. The Tincal Trail-A History of Borax. London: Harraps Ltd, 1984.
10. Haller EE. Germanium: From its discovery to SiGe devices. In: Symposium on Germanium-Based Semiconductors from Materials to Devices held at the 2006 EMRS Spring Meeting, Nice, France, 2006.
11. Curie P, Curie P, Bémont G. Sur une nouvelle substance fortement radio-active, contenue dans la pechblende. Comptes rendus de l'Académie des Sciences. 1898; 127:1215-1217.
12. Corson DR, MacKenzie KR, Segrè E. Artificially Radioactive Element 85. Phys Rev 1940; 58:672-678.
13. Jørgensen N, Laursen J, Viksna A et al. Multi-elemental EDXRF mapping of polluted soil from former horticultural land. Environ Int 2005; 31:43-52.
14. IPCS. Environmental Health Criteria 204: Boron. International Programme on Chemical Safety involving US EPA, UNEP, WHO and International Labour Organisation, CI, USA. 1998.
15. Gupta UC, Jame YW, Campbell CA et al. Boron toxicity and deficiency: A review. Canadian Journal of Soil Science 1985; 65:381-409.
16. Keren R, Bingham FT. Boron in water, soils and plants. In: Stewart BA. Advances in Soil Science. New York: Springer-Verlag, 1985;229-276.
17. Mead MN. Arsenic: in search of an antidote to a global poison. Environ Health Perspect 2005; 113:378.
18. Ng JC. Environmental contamination of arsenic and its toxicological impact on humans. Environ Chem 2005; 2:146-160.
19. Meharg AA, Rahman M. Arsenic contamination of Bangladesh paddy field soils: Implications for rice contribution to arsenic consumption. Environ Sci Technol 2003; 37:229-234.
20. Adriano DC. Trace Elements in the Terrestrial Environment. Heidelberg: Springer-Verlag, 1986. AEA Technology. WEEE and Hazardous Waste: A report produced for DEFRA, AEAT/ENV/R/1688, 2004.
21. Maeda S. Safety and environmental effects. In: Patai S, ed. The Chemistry of Organic Arsenic, Antimony and Bismuth Compounds. New York: John Wiley and Sons, 1994:725-759.
22. Wagner S, Peryea F, Filby R. Antimony impurity in lead arsenate insecticide enhances the antimony content of old orchard soils. J Environ Qual 2003; 32:736-738.
23. Shotyk W, Krachler M, Chen B. Anthropogenic impacts on the biogeochemistry and cycling of antimony. Met Ions Biol Syst 2005; 44:171-203.
24. Ackermann S, Giere R, Majzlan J. Antimony speciation in shooting ranges and its association with iron oxides. In: 17th Annual V M Goldschmidt Conference, Cologne, Germany. 2007.
25. Williams E, Kahhat R, Allenby B et al. Environmental, social and economic implications of global reuse and recycling of personal computers. Environ Sci Technol 2008; 42:6446-6454.
26. Basel Action Network (BAN) and Silicon Valley Toxics Coalition (SVTC). Exporting Harm: the High-tech Trashing of Asia, BAN, Seattle, WA and SVTC, San Jose, CA, 2002; http://www.ban.org/E-waste/technotrashfinalcomp.pdf
27. Malhammar G. Chemical Analysis of Printed Board Assemblies, High Density Packaging User Group (HDPUG) Report 2000.
28. Goldberg S. Reactions of boron with soils. Plant Soil 1997; 193:35-48.
29. US-EPA 2008 http://www.epa.gov/ecotox/ecossl/
30. US-EPA Ecological soil screening levels for arsenic. 2005; http://www.epa.gov/ecotox/ecossl/
31. Lombi E, Wenzel WW, Sletten RS. Arsenic adsorption on soil and iron oxides coated sand: changes over time. Zeit. Pflanzen. Bodenk 1999; 162:451-456.

32. Goldberg S. Competitive adsorption of arsenate and arsenite on oxides and clay minerals. Soil Sci Soc Am J 2002; 66:413-421.
33. Jensen DL, Ledin A, Christensen TH. Speciation of heavy metals in landfill-leachate polluted groundwater. Water Res 1999; 33:2642-2650.
34. Egli M, Fitze P, Oswald M. Changes in heavy metal contents in an acidic forest soil affected by depletion of soil organic matter within the time span 1969-93. Environ Pollut 1999; 105:367-379.
35. Denaix LR, Semlali M, Douay F. Dissolved and colloidal transport of Cd, Pb and Zn in a silt loam soil affected by atmospheric industrial deposition. Environ Pollut 2001; 113:29-38.
36. Sadiq M. Arsenic chemistry in soils: an overview of thermodynamic predictions and field observations. Water Air Soil Pollut 1997; 93:117-136.
37. McBride MB. Adsorption and oxidation of Phenolic compounds by iron and manganese oxides. Soil Sci Soc Am J 1987; 51:1466-1472.
38. Mergenthaler B, Richner T. Mobilität und geochemisches Verhalten von Antimon im Boden von Schießanlagen. 2002; http://ecollection.ethbib.ethz.ch/show?type=dipl&nr=47
39. Steely S, Amarasiriwardena D, Xing BS. An investigation of inorganic antimony species and antimony associated with soil humic acid molar mass fractions in contaminated soils. Environ Pollut 2007; 148:590-598.
40. Schachtschabel P, Blume HP, Brmmer G et al. eds.; Ferdinand Enke: Stuttgart 1998.
41. Wonisch H, Gerard F, Dietzel M et al. Occurrence of polymerized silicic acid and aluminum species in two forest soil solutions with different acidity. Geoderma 2008; 144:435-445.
42. Ma JF, Yamaji N. Silicon uptake and accumulation in higher plants. Trends Plant Sci 2006; 8:392-397.
43. Tuazon E, Aschmann S, Atkinson R. Atmospheric degradation of volatile methyl-silicon compounds. Environ Sci Technol 2000; 34:1970-1976.
44. O'Neill P. Arsenic. In: Alloway BJ, ed. Heavy metals in soils, 2nd ed. London: Blackie 1995;105-121.
45. McBride BC, Wolfe RS. Biosynthesis of dimethylarsine by Methanobacterium. Biochemistry 1971; 10:4312-4317.
46. Gao S, Burau RG. Environmental factors affecting rates of arsine evolution from and mineralization of arsenicals in soil. J Environ Qual 1997; 26:753-763.
47. Hamon RE, Lombi E, Fortunati P et al. Coupling speciation and isotope dilution techniques to study arsenic mobilization in the environment. Environ Sci Techno 2004; 38:1794-1798.
48. Asher CJ, ReayPF. Arsenic uptake by barley hordeum-vulgare cultivar zephyr seedlings. Aust J Plant Physiol 1979; 6:459-466.
49. Meharg AA, Naylor J, Macnair MR. Phosphorus nutrition of arsenate-tolerant and notolerant phenotypes of velvet grass. J Environ Qual 1994; 23:234-238.
50. Raab A, Williams PN, Meharg A et al. Uptake and translocation of inorganic and methylated arsenic species by plants. Eniron Chem 2007; 4:197-203.
51. Filella M, Belzile N, Chen YW. Antimony in the environment: a review focused on natural waters I. Occurrence. Earth Sci Rev 2002; 57:125-176.
52. Mitsunobu S, Harada T, Takahashi Y. Comparison of antimony behavior with that of arsenic under various soil redox conditions. Environ Sci Technol 2006; 40:7270-7276.
53. Krupp EM, Grumping R, Furchtbar URR et al. Speciation of metals and metalloids in sediments with LTGC/ICP-MS. Fresenius J Anal Chem 1996; 354:546-549.
54. Parris GE, Brinckman FE. Reactions which relate to environmental mobility of arsenic and antimony. II. Oxidation of trimethylarsine and trimethylstibine. Environ Sci Technol 1976; 10:1128-1134.
55. Meyer J, Schmidt A, Michalke K et al. Volatilisation of metals and metalloids by the microbial population of an alluvial soil. Syst Appl Microbiol 2007; 30:229-238
56. Tallberg P, Koski-Vahala J, Hartikainen H. Germanium-68 as a tracer for silicon fluxes in freshwater sediment. Water Res 2002; 36:956-962.
57. Wood SA, Samson IM. The aqueous geochemistry of gallium, germanium, indium and scandium. Ore Geology Reviews 2006; 28:57-102.
58. Lewis BL, Froelich PN, Andreae MO. Methylgermanium in natural waters. Nature 1985; 313:303-305.
59. Harada T, Takahashi Y. Origin of the difference in the distribution behavior of tellurium and selenium in a soil-water system. Geochimica Et Cosmochimica Acta 2008; 72:1281-1294.
60. Chasteen TG, Bentley R. Biomethylation of selenium and tellurium: microorganisms and plants. Chem Rev 2003; 103:1-25.
61. Pinel-Raffaitin R, Peckheyran C, Amouroux D. New volatile selenium and tellurium species in fermentation gases produced by composting duck manure. Atmospheric Environment 2008; 42:7786-7794.
62. Campbell PGC. Metal Speciation and Bioavailability in Aquatic Systems. New York: John Wiley and Sons, 1995:45-97.

63. Anderson MA, Morel FMM. The influence of aqueous iron chemistry on the uptake of iron by the coastal diatom Thalassiosira weisflogii. Limnol Oceanogr 1982; 27:789-813.
64. Di Toro DM, Allen HE, Bergman HL et al. Biotic ligand model of the acute toxicity of metals. 1. Technical basis. Environ Sci Technol 2001; 20:2383-2396.
65. De Schamphelaere KAC, Janssen CR. A biotic ligand model predicting acute copper toxicity for Daphnia magna: the effects of calcium, magnesium, sodium, potassium and pH. Environ Sci Technol 2002; 36:48-54.
66. Slaveykova VI, Wilkinson KJ. Predicting the bioavailability of metals and metal complexes: Critical review of the biotic ligand model. Eniron Chem 2005; 2:9-24.
67. Thakali S, Allen HE, Di Toro DM et al. A Terrestrial Biotic Ligand Model. 1. Development and application to Cu and Ni toxicities to barley root elongation in soils. Environ Sci Technol 2006; 40:7085-7093.
68. Wang P, Zhou DM, Kinraide TB et al. Cell Membrane Surface Potential (psi(0)) Plays a Dominant Role in the Phytotoxicity of Copper and Arsenate. Plant Physiol 2008; 148:2134-2143.
69. Yermiyahu U, Kinraide TB. Binding and electrostatic attraction of trace elements to plant root surfaces. In: Huang PM, Gobran GR, eds. Biogeochemistry of Trace Elements in the Rhizosphere. Amsterdam, The Netherlands: Elsevier, 2005:365-389.
70. Kinraide TB. Plasma membrane surface potential (ψ(PM)) as a determinant of ion bioavailability: A critical analysis of new and published toxicological studies and a simplified method for the computation of plant ψ(PM). Environ Toxicol Chem 2006; 25:3188-3198.
71. Kinraide TB. The controlling influence of cell-surface electrical potential on the uptake and toxicity of selenate (SeO42-). Physiologia Plantarum 2003; 117:64-71.
72. Kinraide TB, Yermiyahu U. A scale of metal ion binding strengths correlating with ionic charge, Pauling electronegativity, toxicity and other physiological effects. J Inorg Biochem 2007; 101:1201-1213.

Elsbeth Bienert

Chapter 4

Arsenic Transport in Prokaryotes and Eukaryotic Microbes

Barry P. Rosen and Markus J. Tamás*

Abstract

Aquaporins (AQPs) and aquaglyceroporins facilitate transport of a broad spectrum of substrates such as water, glycerol and other small uncharged solutes. More recently, AQPs have also been shown to facilitate diffusion of metalloids such as arsenic (As) and antimony (Sb). At neutral pH, the trivalent forms of these metalloids are structurally similar to glycerol and hence they can enter cells through AQPs. As- and Sb-containing compounds are toxic to cells, yet both metalloids are used as chemotherapeutic agents for treating acute promyelocytic leukemia and diseases caused by protozoan parasites. In this chapter, we will review the role of AQPs and other proteins in metalloid transport in prokaryotes and eukaryotic microbes.

Introduction

Arsenic (As) and antimony (Sb) are two toxic metalloids with similar chemical properties. Arsenic is classified as a group I human carcinogen and affects the health of millions of people through the drinking water and the food chain.[1,2] The toxic and carcinogenic effects of As may be caused by inactivation of specific enzymes of metabolism, induction of oxidative stress, inhibition of DNA repair mechanisms and deregulation of cell proliferation. Sb is less abundant in the environment than As and much less is known about its carcinogenicity and mechanisms of action.[3] Despite their toxicity, arsenicals and antimonials have a long history of usage as chemotherapeutic agents. Today, As- and Sb-containing drugs are used for treating acute promyelocytic leukemia and diseases caused by protozoan parasites. To act as a drug or poison, these metalloids need to enter cells, which makes it imperative to recognize their uptake pathways. Similarly, to avoid toxicity and acquire tolerance cells employ systems that remove these agents from the cytosol via efflux pathways. In this chapter, we will review the role of AQPs and other proteins in metalloid transport in prokaryotes and eukaryotic microbes.

Metalloid Transport in Prokaryotes

The two relevant oxidation states of As are pentavalent (arsenate [As(V)]) and trivalent (arsenite [As(III)]). In solution the oxyanion As(V) is an analogue of phosphate and competitively inhibits enzymes that use phosphate or have phosphorylated intermediates. In *Escherichia coli* the two phosphate transporters are Pit and Pst,[4] both of which catalyze As(V) uptake.[5,6] Given the structural similarities between As(V) and phosphate, it seems probable that As(V) is adventitiously taken up by phosphate transporters in most organisms, including humans.

*Corresponding Author: Markus J. Tamás—Department of Cell and Molecular Biology/Microbiology, University of Gothenburg, S-405 30 Gothenburg, Sweden. Email: markus.tamas@cmb.gu.se

MIPs and Their Role in the Exchange of Metalloids, edited by Thomas P. Jahn and Gerd P. Bienert.

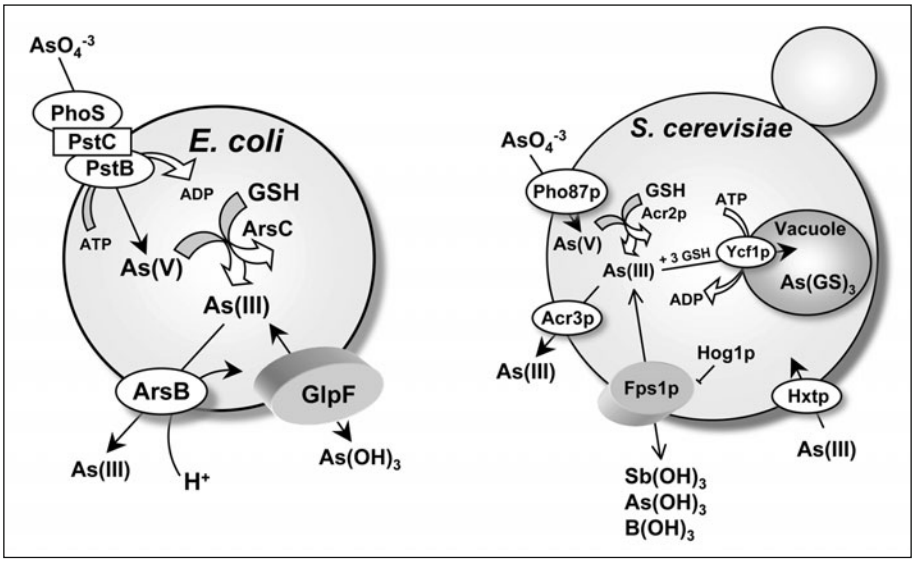


Figure 1. Pathways of arsenical uptake and detoxification in *E. coli* and *S. cerevisiae*. In both *E. coli* and yeast (and probably most cells) As(V) is taken up by phosphate transporters, while As(III) is taken up by aquaglyceroporins (GlpF in *E. coli* and Fps1p in yeast). Fps1p is negatively regulated by Hog1p. In yeast As(III) is also taken up by a number of the hexose transporters (Hxtp). In both *E. coli* and *S. cerevisiae*, As(V) is reduced to As(III) by an As(V) reductase, in *E. coli* this is ArsC and in yeast it is Acr2p. Although these two enzymes are unrelated evolutionarily, glutathione (GSH) and glutaredoxin serve as the source of reducing potential for both. As(III) detoxification results from removal of the metalloid from the cytosol. In *E. coli*, As(III) is extruded from the cells by ArsB alone or by the ArsAB ATPase. In yeast, Acr3p is a plasma membrane As(III) efflux protein, and Ycf1p, an ABC transporter in the MRP family, pumps $As(GS)_3$ into the vacuole.

In contrast, pathways of As(III) uptake were identified only recently.[7] The initial studies of As(III) transport in the Rosen lab began over 25 years ago, focusing initially on As(III) detoxification conferred by efflux pumps.[8] Many times over the years the question was asked how As(III), the most toxic inorganic form of As, enters cells. In 1973 Rosen identified mutants of *E. coli* that were resistant to antibiotics because they were unable to accumulate neomycin.[9] This led Rosen to consider a selection strategy for identification of mutants of *E. coli* that were resistant to the metalloids As(III) and antimonite [Sb(III)] because they could not take them up. Omar Sanders, an undergraduate and Chris Rensing, a postdoctoral associate in the Rosen lab, used random mutagenesis with the transposon Tn*phoA*.[10] An advantage of this selection is that insertions into the genes for membrane proteins can generate blue colonies when the gene for alkaline phosphatase (*phoA*) is exposed to the periplasmic space, enriching for insertions in the genes for transporters. They selected on media containing either As(III) or Sb(III) and isolated a single mutant, OSBR1, that is resistant to Sb(III)[11] and exhibited a 90% reduction in the rate of As(III) uptake.[12] The point of insertion was found to be within the *glpF* gene, which encodes the aquaglyceroporin, GlpF. These results clearly identify GlpF as the major route of As(III) entry in *E. coli* (Fig. 1). Although the mutant was resistant to Sb(III), it retained some sensitivity to As(III), indicating that there must be one or more additional uptake systems for As(III) that account for the residual 10% of uptake.

Uptake of glycerol in *E. coli* was first described by Edmund Chi Chien Lin (1928-2006) in 1968.[13] In 1972 he showed that the *glpF* gene encodes a transporter that catalyzes facilitated

diffusion of glycerol,[14] decades before identification of mammalian aquaporins; E.C.C. Lin should be given credit posthumously for the identification of the first member of the aquaporin (AQP) superfamily. In a series of papers he and his collaborator Thomas H. Wilson at Harvard Medical School characterized GlpF as a glycerol channel. In 1989 the sequence of the *glpF* gene was reported[15] and, in 1990, Lin cloned the *glpF* gene in collaboration with Winfried Boos.[16] Perhaps the most important event in the field was the identification of the human kidney aquaporin, AQP1, the long-sought after erythrocyte water channel by Peter Agre,[17] for which he was awarded the Nobel Prize in Chemistry in 2003. The aquaporin superfamily of small, hydrophobic, integral membrane proteins is expressed widely in animals, plants, insects, parasites, yeast and bacteria.[18] The AQPs can be functionally categorized into two subgroups, the orthodox AQPs, which are water specific channels and aquaglyceroporins, which allow the transport of water, glycerol and other small uncharged solutes (see also chapters by Hachez and Chaumont as well as Danielson and Johanson in this book).

This raised the question of how trivalent inorganic As, which was often considered to be the anion As(III) in solution, could be taken up by a channel for neutral species. However, it's pK_a is 9.2, so it would be expected to be protonated at physiological pH. To examine this question, Rosen and collaborators used X-ray absorption spectroscopy (XAS) to determine the nearest neighbor coordination environment of As(III) under a variety of solution conditions.[19] Extended X-ray Absorption Fine Structure (EXAFS) analysis demonstrated three oxygen ligands at 1.78 Å from the As atom, showing that the major species in solution is $As(OH)_3$, which is effectively an inorganic molecular mimic of glycerol.

Aquaglyceroporins are widespread in prokaryotes, which makes it likely that As(III) is taken into most bacteria adventitiously by these channels. One variation on the theme is AqpS of the legume symbiont *Sinorhizobium meliloti*, which participates in a novel mechanism of As detoxification, the only known example of an aquaglyceroporin with a physiological role in As resistance.[20] As(V) is taken up by *S. meliloti* via a phosphate transport system and is reduced to As(III) by ArsC, an As(V) reductase, generating As(III) in the cytosol. This internally generated As(III) is extruded from the bacterial cell by downhill movement through AqpS. This demonstrates that aquaglyceroporins are bi-directional channels that facilitate movement of As(III) both into and out of cells, depending on the orientation of the concentration gradient. Thus, in *S. meliloti* the combination of AqpS and ArsC effectively forms a novel pathway of As(V) detoxification.

The bacterial GlpF aquaglyceroporin has provided fundamental structural insights in the mechanism of aquaporin channels. To understand the way in which these channels conduct water and organic solutes, their structure was hotly sought-after. Cryo-electron microscopy of the mammalian AQP1 gave the first three-dimensional data at 6 Å resolution.[21] But it was the *E. coli* GlpF that provided the first atom-level details when Fu and Stroud solved its structure at 2.2 Å by X-ray crystallography.[22] This provided a wealth of information about the relationship of structural elements and conserved sequence regions to function. Since then a number of structures of homologues have been determined and they show that these highly conserved proteins possess an identical structural core with the same 'hour-glass' fold. When the structure was solved, it became apparent that GlpF and subsequently all AQPs, are homotetramers in which each monomer consists of six membrane spanning α-helices, with two membrane spanning half-helices interacting with each other from opposing sides through two highly conserved aspargine-proline-alanine (NPA) motifs that form a narrow pore that limits the size of the substrates. On the periplasmic side of the membrane, a constriction region about 8 Å from the NPA motifs, termed the aromatic/arginine (ar/R) region, forms a primary selection filter that controls selectivity for uncharged solutes. Since As(III) forms $As(OH)_3$ in solution, GlpF and a number of other aquaglyceroporins such as human AQP9 and plant NIPs are able to conduct this and other metalloids, which are most likely hydroxyacids in solution, across the membrane.[18] Of clinical relevance is the uptake of Trisenox in leukemia cells by AQP9.[23] Trisenox (arsenic trioxide), a major chemotherapeutic drug for the treatment of acute promyelocytic leukemia, is also $As(OH)_3$ in solution.

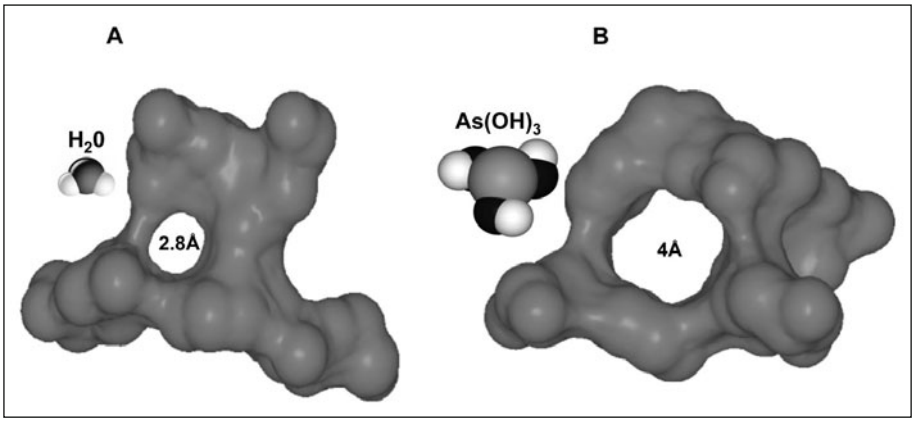


Figure 2. Cross-sectional views of (A) bovine AQP1 (Protein Data Base (PDB) IJ4N) and (B) *E. coli* GlpF glycerol channel (PDB 1LDI) showing the narrowest opening of the pore with a water or $As(OH)_3$ molecule, respectively.

Single crystal structures by themselves do not define mechanism. They are like single frames of the movie that plays out the mechanism. However, the structures are useful for conducting molecular dynamics simulations, which bridge the gap between experimental data and in silico analysis and are useful tools to test models that would be difficult to do experimentally and for predictions that can then be tested experimentally. Molecular dynamic simulations of GlpF showed that hydrogen bonding interactions between glycerol and residues within the channel could account for its stereoselectivity.[24] Although GlpF conducts glycerol equally well in both directions, the channel protein is structurally asymmetrical, with a periplasmic vestibule that, from mean force calculations, has an attractive force that increases the rate of glycerol conduction by 40-75%.[25] From direct comparison of the structures of GlpF, an aquaglyceroporin, with human AQP1 and with AqpZ, the *E. coli* aquaporin water channel, it appears that the diameter of the channel at its narrowest point is a major factor discrimination between water and glycerol because the diameter of the water channels are too small to allow for passage of glycerol[26] (Fig. 2). In GlpF the pore diameter is 4.0 Å, while in AqpZ, the side chains of residues leu21 and phe159 project inward, narrowing the pore to a diameter less than 3.0 Å, with a similar sized constriction of 2.8 Å in bovine AQP1.[27]

Molecular dynamic simulations that directly compared GlpF with AqpZ shed light on the mechanism of substrate selectivity in these channels, i.e., why GlpF is a glycerol channel.[28] In addition to the periplasmic vestibule present in GlpF and absent from AqpZ, there is a much larger barrier in AqpZ (22.8 kcal/mol) than in GlpF (7.3 kcal/mol) at the ar/R selectivity filter caused by steric restrictions in AqpZ. The latter difference can be attributed to the more pronounced structural asymmetry of GlpF, which may play a role in attracting glycerol.[25] Thus, molecular dynamic simulations are useful in exploring the mechanism of metalloid conduction by GlpF and in predicting which AQPs are metalloid channels.

Two different families of metalloid efflux proteins can be found in bacteria; the ArsB and the ACR3 family.[29] ArsB is a member of the Major Facilitator Superfamily of transporters and is the most widespread determinant for As resistance in bacteria and archea. ArsB from *E. coli* is the best characterized member of this family and it exhibits a dual mode of energy coupling depending on the subunit composition. In the presence of the ArsA, ArsB associates with ArsA to form the As(III)-transporting ArsAB ATPase. In contrast, in the absence of the ArsA, ArsB acts as an $As(III)/H^+$ antiporter.[12,30] Members of the ACR3 family are found in bacteria, archea and fungi. In general, less is known about the Acr3 proteins, although proteins from *Bacillus subtilis*[31,32] and yeast[33] have been functionally characterized (see below).

Metalloid Transport in Eukaryotic Microbes

Metalloid Uptake in Yeast

Three metalloid entry pathways have been characterized in *Saccharomyces cerevisiae* (budding yeast): the aquaglyceroporin Fps1p, hexose permeases and phosphate permeases (Fig. 1). The yeast aquaglyceroporin Fps1p was the first eukaryotic As(III) and Sb(III) uptake pathway to be described.[34,35] Besides As(III) and Sb(III), Fps1p may also mediate influx of the metalloid boron (B).[36] Fps1p is a plasma membrane glycerol channel with a central role in osmoregulation. It is inactive under hyperosmotic conditions, thus permitting glycerol accumulation and turgor recovery. In contrast, hypoosmotic shock triggers rapid activation of Fps1p and subsequent glycerol release, preventing cell bursting and death.[37] Regulation of Fps1p activity requires a short domain in the cytoplasmic N-terminal extension: without this domain, Fps1p cannot restrict transport and becomes hyperactive.[37,38]

Inactivation of Fps1p, either by increasing external osmolarity or by deleting the *FPS1* gene, results in enhanced As(III) and Sb(III) tolerance. Conversely, cells expressing a hyperactive Fps1p protein are highly As(III) and Sb(III) sensitive. Direct transport assays demonstrated that Fps1p mediates As(III) influx into cells: cells lacking *FPS1* accumulated little As(III), whereas cells harbouring hyperactive Fps1p accumulated large amounts of As(III).[34,35] Interestingly, this metalloid entry pathway is apparently regulated by As(III). First, expression of the *FPS1* gene is repressed when cells are exposed to As(III) or Sb(III), although the identity of the protein/signal transduction pathway involved is unknown.[34] Secondly, the mitogen-activated protein kinase (MAPK) Hog1p has a negative effect on Fps1p-mediated As(III) transport. From phenotypic analysis, Sb(III) influx into cells is mostly Hog1p regulated as well. Hog1p phosphorylates a threonine residue (thr-231) within the Fps1p N-terminus, resulting in reduced As(III) influx. Inactivation of Hog1p results in reduced Fps1p phosphorylation and increased As(III) influx through Fps1p. Hence, down-regulation of MAPK activity may be an effective way to sensitize cells and to reverse metalloid resistance by increasing influx.[39]

As mentioned above, As(III) also enters cells through hexose transporters. There are more than 20 hexose permeases in yeast, so, under laboratory conditions, about 80% of medium As(III) can be taken up by the aggregate of all of the glucose permeases. On the other hand, Fps1p has the highest rate of As(III) transport and is much more rapid than any individual glucose transporter. It is likely that during growth in the presence of glucose, where most glucose permeases are repressed, the majority of As(III) would enter via Fps1p. When glucose is absent from the growth medium, conditions that favor expression of many of the hexose permeases, most As(III) uptake would probably be via hexose permeases. Similarly, a strain that lacks all hexose carriers exhibits low level of As(III) uptake and improved tolerance. As(III) transport through individual hexose transporters is inhibited by glucose and vice versa, the presence of As(III) reduces sugar transport.[40] Polymerisation of three $As(OH)_3$ molecules has been predicted to form a six-membered ring structure that may be similar to the known structure of arsenious oxide (As_4O_6) and that of hexose sugars and it has been proposed that this putative structure could be recognized by the hexose carriers.[40] Although Fps1p and hexose transporters mediate the majority of As(III) influx into cells, genetic evidence[34] and transport data[40] suggest that additional uptake routes may exist. The molecular identity of the protein(s) catalyzing residual As(III) uptake remains to be unveiled.

The third pathway of metalloid uptake is as the pentavalent form, As(V), which, as in *E. coli*, occurs via phosphate transporters. In yeast, mutations in *PHO84*, which encodes a high-affinity phosphate transporter, or in the *PHO87* and *PHO88* genes encoding, respectively, a low affinity phosphate transporter and a putative phosphate transporter, confer As(V) tolerance,[41,42] indicating that at least three yeast phosphate transport systems catalyze As(V) uptake.

Metalloid Efflux in Yeast

Yeast also possesses systems for metalloid efflux and detoxification (Fig. 1). The *ACR3* gene was isolated in a screen for genes that confer high-level As resistance to cells when overexpressed.[43] *ACR3* encodes a 46 kDa protein with 10 potential membrane-spanning helices[32,33] and deletion

of *ACR3* sensitizes cells to As(III) and As(V) but not to Sb(III).[33] Based on the fact that Acr3p is not able to contribute to As(V) tolerance without the activity of the As(V) reductase Acr2p, it was suggested that Acr3p is a specific As(III) export protein.[43] Indeed, cells expressing multiple copies of the *ACR3* gene accumulate little As(III),[33] whereas the *acr3*Δ mutant exhibits reduced As(III) efflux.[44] The mechanism by which Acr3p transports As(III) is not understood, but the lack of an ATP-binding cassette in the Acr3p sequence suggests that As(III) export may be coupled to the membrane potential.[29] The activity of Acr3p is controlled at the level of transcription; *ACR3* expression is strongly induced by As(III), As(V) and to a lesser extent by Sb(III). Metalloid-stimulated expression of *ACR3* requires the AP-1-like transcription factor Yap8p (also called Acr1p or Arr1p).[45-47] Yap8p is a homodimer that predominantly resides in the nucleus, where it binds to the *ACR3* promoter both in untreated and As(III)-exposed cells. In response to As(III), Yap8p is activated and induces expression of *ACR3*.[46,48]

The fact that *ACR3* expression is stimulated by Sb(III) may suggest that As(III) is not the sole substrate for Acr3p. However, yeast cells lacking *ACR3* are not Sb(III) sensitive[33] and an Acr3p homologue from the *Bacillus subtilis ars* operon confers resistance only to arsenicals.[31] On the other hand, genetic data suggests that Acr3p might contribute to Sb tolerance under certain conditions,[34] so the specificity of Acr3p is not entirely clear.

The yeast cadmium factor 1 (Ycf1p) constitutes the major pathway of toxic metal sequestration in the vacuole. The *YCF1* gene was isolated in a screen for genes conferring cadmium resistance to cells when present in multiple copies. Cells that overexpress *YCF1* exhibit elevated cadmium resistance, while the *ycf1*Δ mutant is hypersensitive to this metal.[49] Ycf1p is an ATP-binding cassette (ABC) transporter that shares a high degree of sequence similarity to the multidrug-associated protein MRP1.[49] Ycf1p catalyzes ATP-dependent transport of a range of glutathione-conjugated metals and xenobiotics into the vacuole.[50,51] Ycf1p also confers resistance to As(III) and Sb(III): *ycf1*Δ is sensitive to As(III) and Sb(III) and the protein is required for transport of the $As(GS)_3$ complex into yeast vacuoles.[34,44] Hence, Acr3p and Ycf1p represent two distinct and parallel metalloid detoxification pathways with different specificities.

How Ycf1p is regulated by metals and xenobiotics is not well understood. Expression of the *YCF1* gene is not induced to any large extent by metal(loid)s. Instead, it appears to be tightly regulated at the posttranslational level. Ycf1p is phosphorylated at various residues: phosphorylation of ser908 and thr911 has a positive effect,[49,52] whereas phosphorylation of ser251 has a negative effect on transport.[53] Ycf1p activity is also stimulated by Tus1p, a guanine nucleotide exchange factor.[54] Yeast cells also possess a B export protein; Bor1p is a plasma membrane protein that confers tolerance by decreasing intracellular B levels.[55]

Metalloid Transport in Parasites

Leishmaniasis and Human African Trypanosomiasis (or sleeping sickness) are parasitic diseases that affect millions of people in the tropics and subtropics and current treatment involves antimonial and organoarsenical drugs (see also the chapter by Mukhopadhyay and Beitz in this book). *Leishmania major* has been shown to take up As(III) and Sb(III) through the aquaglyceroporin LmAQP1.[18,56] Expression of LmAQP1 in a number of drug resistant parasites restores their As(III) and Sb(III) sensitivity and transport assays demonstrated that this sensitivity is caused by increased metalloid uptake. It is striking that overexpression of *LmAQP1* overcomes drug resistance no matter what the mechanism of resistance. Disruption of one of the two *LmAQP1* alleles in *L. major* resulted in increased resistance to Sb(III).[56] Moreover, *AQP1* RNA levels were downregulated in Sb(III) and Pentostam (a pentavalent antimonial)-resistant promastigotes of several *Leishmania* species.[57] In addition, downregulation of LmAQP1 mRNA can be a cause of resistance.[58] Hence, the *Leishmania* aquaglyceroporin AQP1 plays a major role in drug resistance, and modulation of this channel by pharmacological agents may be a stratagem to reverse drug resistance in the field. Trypanosomes also possess aquaglyceroporins and their role in metalloid transport and drug resistance is currently under investigation. Finally, arsenic trioxide and antimony trichloride have been tested for their antimalarial properties.[59] Interestingly, antimony trichloride was shown to

be effective in the nanomolar range, suggesting that the aquaglyceroporin PfAQP of the malarial parasite *Plasmodium falciparum* may catalyze metalloid transport.

One MRP family member from protozoan parasites involved in metalloid detoxification has been characterized.[60,61] *Leishmania* PgpA catalyzes transport of As(III) and Sb(III) conjugated to glutathione or trypanothione, a glutathione-spermidine conjugate that is the major intracellular thiol in trypanosomids such as *Leishmania*. PgpA is localized in membranes that are close to the flagellar pocket, the site of endocytosis and exocytosis in this parasite. Hence, PgpA is an intracellular ABC transporter that confers As(III) and Sb(III) resistance by sequestration of the metal-thiol conjugates.[60]

Conclusion

This short chapter has described the identification of aquaporins as major routes of uptake of As and other metalloids, first in bacteria and then in yeast. These unicellular organisms are still excellent model systems for understanding transport pathways in higher organisms. From what was first learned in *E. coli* and S. *cerevisiae*, we now know that metalloids are taken into most, if not all organisms by aquaglyceroporins—bacteria, fungi, parasites, plants and animals, including humans. To quote the Nobel Prize-winning biologist Jacques Monod, "*what is true of E. coli is true of the elephant*, *except more so*." He meant us.

Acknowledgements

The work in the Rosen lab is supported by NIH grants GM55425 and AI043428 and work in the Tamás lab is supported by the Swedish Research Council (VR).

References

1. Bhattacharjee Y. Toxicology. A sluggish response to humanity's biggest mass poisoning. Science 2007; 315:1659-1661.
2. Meharg AA. Arsenic in rice—understanding a new disaster for South-East Asia. Trends Plant Sci 2004; 9:415-417.
3. Beyersmann D, Hartwig A. Carcinogenic metal compounds: recent insight into molecular and cellular mechanisms. Arch Toxicol 2008; 82:493-512.
4. Rosenberg H, Gerdes RG, Chegwidden K. Two systems for the uptake of phosphate in Escherichia coli. J Bacteriol 1977; 131:505-511.
5. Willsky GR, Malamy MH. Effect of arsenate on inorganic phosphate transport in Escherichia coli. J Bacteriol 1980; 144:366-374.
6. Willsky GR, Malamy MH. Characterization of two genetically separable inorganic phosphate transport systems in Escherichia coli. J Bacteriol 1980; 144:356-365.
7. Rosen BP, Liu Z. Transport pathways for arsenic and selenium: A minireview. Environ Int 2008.
8. Mobley HL, Rosen BP. Energetics of plasmid-mediated arsenate resistance in Escherichia coli. Proc Natl Acad Sci USA 1982; 79:6119-6122.
9. Rosen BP. Restoration of active transport in an Mg^{2+}-adenosine triphosphatase-deficient mutant of Escherichia coli. J Bacteriol 1973; 116:1124-1129.
10. Manoil C, Beckwith J. TnphoA: a transposon probe for protein export signals. Proc Natl Acad Sci USA 1985; 82:8129-8133.
11. Sanders OI, Rensing C, Kuroda M et al. Antimonite is accumulated by the glycerol facilitator GlpF in Escherichia coli. J Bacteriol 1997; 179:3365-3367.
12. Meng YL, Liu Z, Rosen BP. As(III) and Sb(III) uptake by GlpF and efflux by ArsB in Escherichia coli. J Biol Chem 2004; 279:18334-18341.
13. Sanno Y, Wilson TH, Lin EC. Control of permeation to glycerol in cells of Escherichia coli. Biochem Biophys Res Commun 1968; 32:344-349.
14. Richey DP, Lin EC. Importance of facilitated diffusion for effective utilization of glycerol by Escherichia coli. J Bacteriol 1972; 112:784-790.
15. Muramatsu S, Mizuno T. Nucleotide sequence of the region encompassing the glpKF operon and its upstream region containing a bent DNA sequence of Escherichia coli. Nucleic Acids Res 1989; 17:4378.
16. Sweet G, Gandor C, Voegele R et al. Glycerol facilitator of Escherichia coli: cloning of glpF and identification of the glpF product. J Bacteriol 1990; 172:424-430.
17. Preston GM, Carroll TP, Guggino WB et al. Appearance of water channels in Xenopus oocytes expressing red cell CHIP28 protein. Science 1992; 256:385-387.

18. Bhattacharjee H, Rosen BP, Mukhopadhyay R. Aquaglyceroporins and Metalloid Transport: Implications in human diseases. In: Beitz E, ed. Aquaporins. Heidelberg: Springer; 2009; 190:510.
19. Ramirez-Solis A, Mukopadhyay R, Rosen BP et al. Experimental and theoretical characterization of arsenite in water: insights into the coordination environment of As-O. Inorg Chem 2004; 43:2954-2959.
20. Yang HC, Cheng J, Finan TM et al. Novel pathway for arsenic detoxification in the legume symbiont Sinorhizobium meliloti. J Bacteriol 2005; 187:6991-6997.
21. Walz T, Hirai T, Murata K et al. The three-dimensional structure of aquaporin-1. Nature 1997; 387:624-627.
22. Fu D, Libson A, Miercke LJ et al. Structure of a glycerol-conducting channel and the basis for its selectivity. Science 2000; 290:481-486.
23. Bhattacharjee H, Carbrey J, Rosen BP et al. Drug uptake and pharmacological modulation of drug sensitivity in leukemia by AQP9. Biochem Biophys Res Commun 2004; 322:836-841.
24. Jensen MO, Park S, Tajkhorshid E et al. Energetics of glycerol conduction through aquaglyceroporin GlpF. Proc Natl Acad Sci USA 2002; 99:6731-6736.
25. Lu D, Grayson P, Schulten K. Glycerol conductance and physical asymmetry of the Escherichia coli glycerol facilitator GlpF. Biophys J 2003; 85:2977-2987.
26. Lee JK, Khademi S, Harries W et al. Water and glycerol permeation through the glycerol channel GlpF and the aquaporin family. J Synchrotron Radiat 2004; 11(Pt 1):86-88.
27. Sui H, Han BG, Lee JK et al. Structural basis of water-specific transport through the AQP1 water channel. Nature 2001; 414:872-878.
28. Wang Y, Schulten K, Tajkhorshid E. What makes an aquaporin a glycerol channel? A comparative study of AqpZ and GlpF. Structure 2005; 13:1107-1118.
29. Rosen BP. Families of arsenic transporters. Trends Microbiol 1999; 7:207-212.
30. Tisa LS, Rosen BP. Molecular characterization of an anion pump. The ArsB protein is the membrane anchor for the ArsA protein. J Biol Chem 1990; 265:190-194.
31. Sato T, Kobayashi Y. The ars operon in the skin element of Bacillus subtilis confers resistance to arsenate and arsenite. J Bacteriol 1998; 180:1655-1661.
32. Aaltonen EK, Silow M. Transmembrane topology of the Acr3 family arsenite transporter from Bacillus subtilis. Biochim Biophys Acta 2008; 1778:963-973.
33. Wysocki R, Bobrowicz P, Ulaszewski S. The Saccharomyces cerevisiae ACR3 gene encodes a putative membrane protein involved in arsenite transport. J Biol Chem 1997; 272:30061-30066.
34. Wysocki R, Chéry CC, Wawrzycka D et al. The glycerol channel Fps1p mediates the uptake of arsenite and antimonite in Saccharomyces cerevisiae. Mol Microbiol 2001; 40:1391-1401.
35. Liu Z, Shen J, Carbrey JM et al. Arsenite transport by mammalian aquaglyceroporins AQP7 and AQP9. Proc Natl Acad Sci USA 2002; 99:6053-6058.
36. Nozawa A, Takano J, Kobayashi M et al. Roles of BOR1, DUR3 and FPS1 in boron transport and tolerance in Saccharomyces cerevisiae. FEMS Microbiol Lett 2006; 262:216-222.
37. Tamás MJ, Luyten K, Sutherland FC et al. Fps1p controls the accumulation and release of the compatible solute glycerol in yeast osmoregulation. Mol Microbiol 1999; 31:1087-1104.
38. Tamás MJ, Karlgren S, Bill RM et al. A short regulatory domain restricts glycerol transport through yeast Fps1p. J Biol Chem 2003; 278:6337-6345.
39. Thorsen M, Di Y, Tangemo C et al. The MAPK Hog1p modulates Fps1p-dependent arsenite uptake and tolerance in yeast. Mol Biol Cell 2006; 17:4400-4410.
40. Liu Z, Boles E, Rosen BP. Arsenic trioxide uptake by hexose permeases in Saccharomyces cerevisiae. J Biol Chem 2004; 279:17312-17318.
41. Bun-ya M, Shikata K, Nakade S et al. Two new genes, PHO86 and PHO87, involved in inorganic phosphate uptake in Saccharomyces cerevisiae. Curr Genet 1996; 29:344-351.
42. Yompakdee C, Ogawa N, Harashima S et al. A putative membrane protein, Pho88p, involved in inorganic phosphate transport in Saccharomyces cerevisiae. Mol Gen Genet 1996; 251:580-590.
43. Bobrowicz P, Wysocki R, Owsianik G et al. Isolation of three contiguous genes, ACR1, ACR2 and ACR3, involved in resistance to arsenic compounds in the yeast Saccharomyces cerevisiae. Yeast 1997; 13:819-828.
44. Ghosh M, Shen J, Rosen BP. Pathways of As(III) detoxification in Saccharomyces cerevisiae. Proc Natl Acad Sci USA 1999; 96:5001-5006.
45. Bobrowicz P, Ulaszewski S. Arsenical-induced transcriptional activation of the yeast Saccharomyces cerevisiae ACR2 and ACR3 genes requires the presence of the ACR1 gene product. Cell Mol Biol Lett 1998; 3:13-20.
46. Wysocki R, Fortier PK, Maciaszczyk E et al. Transcriptional activation of metalloid tolerance genes in Saccharomyces cerevisiae requires the AP-1-like proteins Yap1p and Yap8p. Mol Biol Cell 2004; 15:2049-2060.

47. Ilina Y, Sloma E, Maciaszczyk-Dziubinska E et al. Characterization of the DNA binding motif of the arsenic-responsive transcription factor Yap8p. Biochem J 2008; 415:467-475.
48. Di Y, Tamás MJ. Regulation of the arsenic-responsive transcription factor Yap8p involves the ubiquitin-proteasome pathway. J Cell Sci 2007; 120:256-264.
49. Szczypka MS, Wemmie JA, Moye-Rowley WS et al. A yeast metal resistance protein similar to human cystic fibrosis transmembrane conductance regulator (CFTR) and multidrug resistance-associated protein. J Biol Chem 1994; 269:22853-22857.
50. Li ZS, Lu YP, Zhen RG et al. A new pathway for vacuolar cadmium sequestration in Saccharomyces cerevisiae: YCF1-catalyzed transport of bis(glutathionato)cadmium. Proc Natl Acad Sci USA 1997; 94:42-47.
51. Li ZS, Szczypka M, Lu YP et al. The yeast cadmium factor protein (YCF1) is a vacuolar glutathione S-conjugate pump. J Biol Chem 1996; 271:6509-6517.
52. Eraso P, Martinez-Burgos M, Falcon-Perez JM et al. Ycf1-dependent cadmium detoxification by yeast requires phosphorylation of residues Ser(908) and Thr(911). FEBS Lett 2004; 577:322-326.
53. Paumi CM, Chuk M, Chevelev I et al. Negative regulation of the yeast ABC transporter Ycf1p by phosphorylation within its N-terminal extension. J Biol Chem 2008; 283:27079-27088.
54. Paumi CM, Menendez J, Arnoldo A et al. Mapping Protein-Protein Interactions for the Yeast ABC Transporter Ycf1p by Integrated Split-Ubiquitin Membrane Yeast Two-Hybrid Analysis. Mol Cell 2007; 26:15-25.
55. Takano J, Kobayashi M, Noda Y et al. Saccharomyces cerevisiae Bor1p is a boron exporter and a key determinant of boron tolerance. FEMS Microbiol Lett 2007; 267:230-235.
56. Gourbal B, Sonuc N, Bhattacharjee H et al. Drug uptake and modulation of drug resistance in leishmania by an aquaglyceroporin. J Biol Chem 2004; 279:31010-31017.
57. Marquis N, Gourbal B, Rosen BP et al. Modulation in aquaglyceroporin AQP1 gene transcript levels in drug-resistant Leishmania. Mol Microbiol 2005; 57:1690-1699.
58. Maharjan M, Singh S, Chatterjee M et al. Role of aquaglyceroporin (AQP1) gene and drug uptake in antimony-resistant clinical isolates of Leishmania donovani. Am J Trop Med Hyg 2008; 79(1):69-75.
59. Gabbiani C, Messori L, Cinellu MA et al. Outstanding plasmodicidal properties within a small panel of metallic compounds: Hints for the development of new metal-based antimalarials. J Inorg Biochem 2008.
60. Légaré D, Richard D, Mukhopadhyay R et al. The Leishmania ABC protein PGPA is an intracellular metal-thiol transporter ATPase. J Biol Chem 2001; 276:26301-26307.
61. Papadopoulou B, Roy G, Dey S et al. Gene disruption of the P-glycoprotein related gene pgpA of Leishmania tarentolae. Biochem Biophys Res Commun 1996; 224:772-778.

Elsbeth Bienert

Chapter 5

Metalloid Transport by Aquaglyceroporins:
Consequences in the Treatment of Human Diseases

Rita Mukhopadhyay* and Eric Beitz*

Abstract

Metalloids can severely harm human physiology in a toxicological sense if taken up from the environment in acute high doses or chronically. However, arsenic or antimony containing drugs are still being used as treatment and are often the sole regime for certain forms of cancer, mainly types of leukemia and diseases caused by parasites, such as sleeping sickness or leishmaniasis. In this chapter, we give an outline of the positive effects of arsenicals and antimonials against such diseases, we summarize data on uptake pathways through human and parasite aquaglyceroporins and we discuss the progress and options in the development of therapeutic aquaporin and aquaglyceroporin inhibitor compounds.

Introduction

Metalloid is a term used in chemistry when classifying the chemical elements. On the basis of their general physical and chemical properties, nearly every element in the periodic table can be termed either a metal or a nonmetal—however a few elements with intermediate properties are referred to as metalloids. In the standard layout of the periodic table, metalloids occur along the diagonal line through the p block from boron to astatine (see also the chapter by Lombi and Holm in this book). Arsenic (As) and antimony (Sb) are considered metalloids and have been used as chemotherapeutic agents from the pre-antibiotic era. Arsenic has been used as a chemotherapeutic agent for more than 2400 years.[1] Thomas Fowler in the 18th century synthesized a bicarbonate-based arsenic trioxide (As_2O_3) solution (Fowler's solution) that was used empirically to treat a variety of diseases during 18th, 19th and 20th centuries.[2] Use of arsenical pastes for skin and breast cancer and arsenous acid for hypertension, bleeding gastric ulcers, heartburn and chronic rheumatism was described in the pharmacological texts of the 1880s.[3] In 1910, Noble laureate Paul Ehrlich developed Salvarsan (dihydroxydiaminoarsenobenzenedihydrochloride), an organic arsenical used to treat syphilis and sleeping sickness (trypanosomiasis). Even today, the As containing drug Melarsoprol is the first line of treatment against sleeping sickness.[4] "Ailing-1", a solution of crude As_2O_3 and herbal extracts of the 1970s from China formed the basis for the treatment of acute promyelocytic leukemia (APL).[1] In 1998 a controlled clinical trial with As_2O_3 showed complete remission of APL, i.e., absence of symptoms.[5] However,

*Corresponding Authors: Eric Beitz—Department of Pharmaceutical and Medicinal Chemistry University of Kiel, Gutenbergstrasse 76, 24118 Kiel, Germany.
Email: ebeitz@pharmazie.unikiel.de;
Rita Mukhopadhyay—Department of Molecular Microbiology and Infectious Diseases, Florida International University College of Medicine, 11200 SW 8th Street, Miami, FL 33199, USA.
Email: rmukhop@fiu.edu

MIPs and Their Role in the Exchange of Metalloids, edited by Thomas P. Jahn and Gerd P. Bienert.

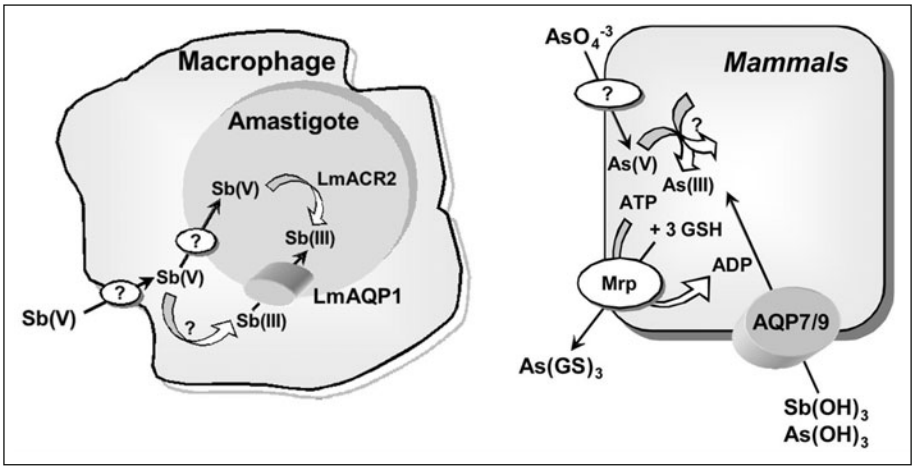


Figure 1. Metalloid transport in mammalian cells and *Leishmania*. AQP3, 7 and 9 have been shown to take up As(III) in mammalian cells. In mammals, Mrp isoforms pump $As(GS)_3$ out of cells. In *Leishmania*, Sb(V) is taken up by macrophages and a portion is reduced to Sb(III), which is then transported into the amastigote by the leishmania aquaglyceroporin LmAQP1. The other portion of Sb(V) is taken into the amastigote and reduced to Sb(III) by LmACR2[68] and perhaps other enzymes such as TDR1.[69] Adapted with permission from Bhattacharjee H, Rosen B, Mukhopadhyay R. Aquaglyceroporine and metalloid transport: Implications in human diseases. In: Beitz E, ed. Handbook of Experimental Pharmacology. Germany: Springer, 2008.

three of eleven of these patients became nonresponsive to As_2O_3 treatment suggesting a close relationship between As resistance and cancer chemotherapy. Sb is a related metalloid to As and pentavalent Sb containing drugs, such as Pentostam and Glucantime are still the first line of treatment for infections with *Leishmania* parasites (leishmaniasis).

Arsenite [As(III)] and antimonite [Sb(III)] are known to be taken up or released by membrane transporters which are ubiquitously present from bacteria to man. In *E. coli* ArsA and ArsB form an ATP-driven efflux pump for As(III) where ArsB is the membrane component and ArsA is the catalytic subunit.[6] Acr3p is a secondary transporter in the plasma membrane of *S. cerevisiae* that extrudes As(III) out of the cell.[7] *S. cerevisiae* also has a parallel pathway for As detoxification. Arsenic is sequestered inside the vacuole as conjugates with glutathione (GSH), i.e., $As(GS)_3$, by Ycf1p (yeast cadmium factor), a p-glycoprotein (PGP) and multi drug resistance protein (MRP-1) homologue.[8] Similar pathways of As transport have been observed in a protozoan parasite *Leishmania* spp. The plasma membrane As(III) transporter is an ATPase which transports As-thiol complexes.[9] We have also demonstrated $As(GS)_3$ transport by PGPA and MRP-1 homologues into vesicles localized near the flagellar pocket in *Leishmania*[10] (Fig. 1). In the soil nematode, *C. elegans* multi drug resistance proteins contribute to As(III) resistance[11] and also in humans homologous proteins have been shown to transport As glutathione (GSH) complexes (Fig. 1). It has been shown that different MRP-1 expression levels in different mouse strains determine the degree of As(III) induced kidney injury.[12] Zaman et al[13] have shown that MRP increases the export of GSH from the cell and this increased export is further elevated in the presence of As(III). Recently, it was demonstrated that chronic exposure to methylated arsenicals stimulates MDR pathways and induces As tolerance in rat liver cells.[14] In contrast, metalloid transport by channels such as aquaglyceroporins is a relatively new concept (see also the chapter by Rosen and Tamás in this book). In this chapter we are going to pursue this aspect of metalloid transport in relation to human diseases such as cancer and infections by human-pathogenic parasites.

Metalloids and Cancer

Arsenic trioxide (As_2O_3) induces potent antitumor effects in vitro and in vivo. It is now being used as a first line of treatment against a certain form of blood cancer, acute promyelocytic leukemia (APL), which is characterized by an excess of immature leukocytes in the bone marrow. At lower doses (<0.5 μmol/L) As_2O_3 induces differentiation into mature cells whereas at higher concentrations it leads to apoptotic cell death.[15-18] Additionally, As_2O_3 can be of potential therapeutic value in cases such as chronic myelogenous leukemia (CML). Here, types of mature leukocytes grow unregulated in the bone marrow and accumulate in the blood. It has been proposed that As_2O_3 can induce apoptosis of CML cell lines[19] and CML leukemic blasts.[20,21] It is also undergoing Phase II clinical trial as stand alone treatment or in combination with other drugs such as melphalan.[22] As_2O_3 is being trialed against solid tumors that are mostly resistant to current therapies.[23] These include liver, pancreatic, gastric, ovarian, cervical, prostate, renal, bladder, lung and neurological (neuroblastoma, glioblastoma multiforme) cancers.[22] In spite of the therapeutic effects of As, it has been classified as a potent human carcinogen or cocarcinogen. Epidemiologic studies from Finland, Taiwan, China, Bangladesh, India, Mexico, South-Western United States and Central and South America have shown that exposure to inorganic As is associated with increased risk of cancers of the skin and internal organs including the urinary bladder, respiratory tract, liver and kidney.[24] The population cancer risk due to As has been suggested to be compatible to environmental tobacco smoke and radon in homes with risk estimates of around 1 per 1000.[25]

The mechanism(s) by which As_2O_3 is able to establish such a wide range of inhibitory effect as an anticancer agent is still unclear. In many cases induction of apoptosis by mitogen-activated protein kinase (MAPK) pathways has been linked. In fact, p38 MAPK has been shown to be altered in various immortal so-called neoplastic cell lines. Pharmacological inhibition of p38 MAPK potentiated As-dependent apoptosis and suppression of growth of leukemia cell lines.[26] A number of studies have suggested that As alters gene expression by modulation of intracellular phosphorylation events and MAPK.[24]

Uptake of Metalloids via Human Aquaglyceroporins

Before As can impair the cellular machinery, it has to enter a cell through an uptake system. As described in other chapters of this book, these uptake systems can very well be aquaglyceroporins. But, how does a cell sense the presence of these metalloids and activate various tolerance systems? MAPKs have also been linked in regulating the expression of aquaporins. It has been shown that hyperosmolar mannitol simulates expression of AQP4 and AQP9 through a p38 MAPK-dependent pathway in rat astrocytes.[27] Hypertonicity-induced AQP1 expression is mediated by the activation of MAPK pathways and a hypertonicity-responsive element in the AQP1 gene.[28] Mitogen-induced cell proliferation was also impaired in AQP3 deficient keratinocytes, with greatly reduced p38 MAPK activity.[29] Recently, a direct link has been established between the regulation of aquaglyceroporins by MAPK and As(III) transport. *S. cerevisiae* Hog1p is a homologue of p38 and the ultimate MAPK of the high osmolarity glycerol pathway.[30] It has been demonstrated that a Hog1p null mutant becomes hypersensitive to As(III)/Sb(III) due to high accumulation of metalloid inside the cells. This occurs because Hog1p-mediated phosphorylation of Fps1p is prevented in a Hog1p null mutant. Fps1p is phosphorylated at thr231 by cytosolic Hog1p which leads to reduced metalloid transport activity by Fps1p.[30] Thus MAPK activation by As(III) in cancer cells could have far reaching consequences. We have shown that human AQP9 has the ability to modulate sensitivity of leukemia cells to the metalloids. When human AQP9 was over expressed in the chronic myelogenous cell line K562 or promyelocytic leukemia cell line HL-60, both became hypersensitive to As(III)/Sb(III) due to higher accumulation of the metalloids. Additionally, we have shown that, vitamin D pretreatment induces expression of AQP9 in HL-60 cells and thereby making them hypersensitive to As(III) and Sb(III) due to excess metalloid accumulation.[31] All trans retinoic acid (ATRA) has also been shown to induce AQP9 expression in HL-60 cells.[32] This could very well be the reason of successful antileukemic regimen of combinatorial therapy of ATRA and As_2O_3 especially in the cases where it was resistant to the

first phase of treatment.[33,34] A correlation between AQP9 expression and sensitivity to As_2O_3 in 11 leukemia cell lines has been observed. Among these cell lines an APL line NB4 was found to express the highest amount of AQP9 and was most sensitive to As_2O_3 treatment. It is intriguing to note that, APL is also clinically the leukemic subtype that responds best to As_2O_3 therapy.[32] These findings were also verified by examining AQP9 expression in a series of acute myeloid leukemia samples, the most common form of adult leukemia (AML). Consistently AQP9 was expressed more in the APL subtypes of AML. This observation was consistent with the high sensitivity of APL to As_2O_3 compared with other AML subtypes.[32] On the other hand, is it possible for cancer cells to become As resistant by down regulating the aquaglyceroporins? Lee et al[35] raised a lung adenocarcinoma cell line (R15) which was 10 times more resistant to As(III) when compared to the wild type (CL3). This cell line does not express any AQP7 or AQP9, only AQP3 which was two fold lower than CL3. It has been observed that R15 overexpresses multi drug resistant related protein (MRP2) and down regulates AQP3. Thus the As resistant cell line reduced the uptake of the drug while inducing the extrusion by an efflux pump. Moreover, overexpression of AQP3 in the human embryonic kidney 293T cells resulted in an increase in both accumulation and sensitivity to As(III). Therefore downregulation of aquaglyceroporin expression may lead to metalloid resistant phenotype.

Metalloids in Protozoan Parasitic Infections

Metalloid containing drugs have been used against protozoan parasitic infections since the pre antibiotic era. As(III) containing drug Melarsoprol and antimonate [Sb(V)] containing drug Pentostam or Glucantime are still the first line of treatments against sleeping sickness and leishmaniasis (one form Kala-Azar), respectively. Human African trypanosomiasis (HAT), or sleeping sickness, constitutes a serious public health threat in Africa, particularly in central Africa, where approximately 60 million persons are at risk to become infected. The disease has reached epidemic proportions in four countries, with a prevalence of over 20% in some areas: Sudan, Uganda, the Democratic Republic of Congo and Angola. It is estimated that 300 000-500 000 persons are infected with trypanosomes.[36] Sleeping sickness develops in two stages, the first involving the hemolymphatic system and the second, the neurological system. Left untreated, sleeping sickness is invariably fatal. There have been no therapeutic advances in more than 40 years. Stage 1 can be treated with Pentamidin and Suramin, but Stage 2 can only be treated with Melarsoprol, a toxic As derivative.[36] The mechanism of action of the drug is not exactly clear. It has been proposed to act on trypanothione, a novel thiol in trypanosomes and other kinetoplastids which is absent in mammals.[37] It also interacts in the glycolytic pathways.[38] Melarsoprol resistance has been linked to diminished drug uptake.[39]

Three aquaglyceroporins have been identified in Trypanosoma brucei—TbAQP1, 2 and 3 which show 40-45% identity to the mammalian AQPs. All three proteins were able to transport water, glycerol and dihydroxyacetone when expressed in *Xenopus* oocytes. In *S. cerevisiae* TbAQPs suppressed hypoosmosensitivity of the *fps1* null mutant. Under iso- and hyperosmotic conditions, these cells constitutively released glycerol, consistent with a glycerol efflux function. Except for urea, TbAQPs were impermeable to other polyols, only TbAQP3 transported erythritol and ribitol.[40] These proteins are expressed differentially at different life cycle stages. TbAQP3 is the major AQP in the logarithmically growing slender blood stream form whereas TbAQP1 is heavily expressed in the stationary phase stumpy bloodstream form. We have identified that all three TbAQPs transport As(III) and Sb(III) very well (unpublished data Mukhopadhyay and Duszenko). However, it is still unclear whether TbAQPs transport Melarsoprol or field isolates of drug resistant parasites actually exhibit down regulation of the AQPs.

Leishmaniasis is endemic in parts of 88 countries across five continents—the majority of the affected countries are in the tropics and subtropics. Approximately 12 million people worldwide are affected by leishmaniasis, while a total of 350 million people are at a risk (http://www.who.int/tdr/diseases/leish/). The disease in humans has been classified in three different forms, each having a broad range of clinical manifestations. Visceral leishmaniasis (VL) is the most severe form of the

disease and is fatal if left untreated. VL is caused by *Leishmania donovani*, *Leishmania infantum* or *Leishmania chagasi* and is characterized by irregular bouts of fever, substantial weight loss, swelling of the spleen and liver and anemia. Approximately 90% of the 500,000 new cases of VL reported annually occur in Bangladesh, Brazil, India, Nepal and Sudan. Cutaneous leishmaniasis (CL) is caused by a variety of species including *Leishmania major*, *Leishmania tropica*, *Leishmania mexicana* and *Leishmania panamensis*. CL is characterized by skin lesions on the exposed parts of the body, such as the face, arms and legs, causing serious disability and leaving the patient permanently scarred. It is the most common form of the disease with 1-1.5 million new cases annually reported worldwide and 90% of all CL cases are reported from Afghanistan, Brazil, Iran, Peru, Saudi Arabia and Syria. Mucocutaneous leishmaniasis (MCL) due to *Leishmania braziliensis* infection produces lesions which can lead to extensive and disfiguring destruction of mucous membranes of the nose, mouth and throat cavities. 90% of MCL cases occur in Bolivia, Brazil and Peru. Additionally, *Leishmania*/HIV co-infection is currently emerging as an extremely serious, new disease and is being considered a real threat in various parts of the world. VL has been widely recognized as an opportunistic infection among persons who are immunosuppressed, particularly in patients infected with human immunodeficiency virus.[41,42] The first line compounds against all forms of leishmaniasis are the two pentavalent antimonials, sodium stibogluconate (Pentostam) and meglumine antimoniate (Glucantime). However, clinical resistance to this treatment is becoming prevalent.[43,44] In fact, more than 50% of VL cases in North-East India are resistant to Pentostam.[45] *Leishmania* resistant to trivalent Sb has also been reported.[46]

Despite being used for several decades, the mode of action of pentavalent antimonials is poorly understood. The possibility of in vivo metabolic conversion of pentavalent Sb(V) to trivalent Sb(III) was suggested more than 50 years ago.[47] This hypothesis was supported by the observation that hamsters infected with *Leishmania garnhami* and then treated with Glucantime, Sb(V), showed similar serum concentrations of Sb(III) and Sb(V).[48] Reduction of Sb(V) to Sb(III) was suggested to be associated with decreasing size and healing of the leishmanial ulcers.[48] Several investigators have shown that Sb(III) is more toxic than Sb(V) to either the promastigote or amastigote forms of different *Leishmania* species.[49-51] Sereno et al demonstrated that axenically grown amastigotes of *L. infantum* were more susceptible to Sb(III) than to Sb(V).[51] However, these amastigotes were found to be poorly responsive to meglumine, Sb(V), compared to amastigotes grown in human macrophages.[52] These results strongly suggested a putative reductase residing within the macrophage, which catalyzes the conversion of Sb(V) to Sb(III). To exert its antiparasitic action, Sb(III) must enter the parasite residing inside the macrophage through a transporter. We have shown that this transporter is an aquaglyceroporin LmAQP1[53] (Fig. 1).

Parasite Aquaglyceroporins Facilitate Metalloid Transport

The *L. major* genome encodes for five AQPs: LmAQP1, LmAQPα, LmAQPβ, LmAQPγ and LmAQPδ. Whereas LmAQP1 shows strong similarity to bacterial AQPs the other *L. major* aquaporins (LmAQP α–δ) are closer to plant AQPs. This is a unique peculiarity of LmAQPs, because other parasitic AQPs known to date are either bacteria- or plant-like and not a mixed population.[54] The roles of the other LmAQPs have not been determined yet. LmAQP1 was expressed in *Xenopus* oocytes and was able to function as a water channel. LmAQP1 belongs to the intermediate class of water channels; its water transport capacity is 65% of that of AQP1, which is a classical water transporter. Interestingly, in contrast to *Plasmodium* and *Trypanosoma* AQPs that are inhibited by mercurials, LmAQP1 water transport was not inhibited by $HgCl_2$, thereby classifying LmAQP1 as a mercurial insensitive water channel. It also transports glycerol, glyceraldehyde and dihydroxyacetone reasonably well. Unlike its homologue, urea transport by this aquaglyceroporin is negligible which we believe to be a protective function as the parasite has to survive in the hostile environment of liver cells.[55] LmAQP1 is the first AQP that was localized to the flagellum of the promastigotes and flagellar pocket, rudimentary flagellum and contractile vacuoles of the amastigotes. LmAQP1 plays an important physiological role in water and solute transport, volume regulation and osmotaxis. This helps the parasite to face the osmotic challenges during a swim towards the proboscis, i.e.,

the sucking mouthpart, of the sandfly and transmission to the vertebrate host.[55] LmAQP1 was also shown to be a metalloid transporter. It was expressed in promastigotes from three different species of *Leishmania.* Each of the transfectants produced substantial amounts of LmAQP1 and became hypersensitive in vitro to As(III) and Sb(III). LmAQP1 expression in a variety of As(III)- or Sb(III)-resistant mutants restored metalloid sensitivity in every strain, independently of the mechanism of resistance. The results of transport studies suggest that this hypersensitivity is caused by an increased rate of uptake of Sb(III) or As(III) in the promastigotes, consistent with increased amounts of the LmAQP1 channel. Disruption of one of the two *LmAQP1* alleles in *L. major* conferred a 10-fold increase in resistance to trivalent Sb compared with the wild type. These results suggest that a major route of entry of trivalent Sb, the activated form of Pentostam, into *Leishmania* is catalyzed by LmAQP1. Importantly, the results demonstrate that a loss of *LmAQP1* can produce resistance and that increased expression of LmAQP1 in drug-resistant parasites can reverse resistance.[53] Additionally, we also over expressed LmAQP1 in drug resistant and sensitive field isolates (*L. donovani*) from India and the intra-macrophageal amastigotes became hypersensitive to Pentostam (unpublished data Mukhopadhyay and Ouellette). We have also shown that downregulation of LmAQP1 leads to drug resistance. *AQP1* mRNA was shown to be significantly decreased in Sb(III)/As(III) resistant *L. major* and *L. tarentolae* cells.[56] Decuypere et al[57] have reported that Pentostam resistant field isolates of *L. donovani* from Nepal showed downregulation of AQP1 leading to reduced uptake of Sb(III). Therefore it is clearly evident in *Leishmania* that LmAQP1 homologues play a major role in cellular physiology and drug resistance which are both novel functions for any aquaglyceroporin.

LmAQP1 shares 32% identity and 50% similarity with the *Plasmodium falciparum* aquaglyceroporin PfAQP. In comparison to the *E. coli* aquaglyceroporin, GlpF, which is a glycerol channel with low water permeability, both LmAQP1 and PfAQP conduct glycerol and water at high rates.[55,58] Beitz et al[59] have shown that glu125 in the extracellular C-loop is critically responsible for the high water permeability of PfAQP. Alteration of glu125 to serine greatly reduces water but not glycerol permeability. The crystal structure of PfAQP indicates that glu125 anchors the C-loop in place by hydrogen bonding with ser200 and thr212 and consequently the neighboring trp124 is hydrogen bonded with arg196 in the selectivity filter. Alteration of glu125 to serine eliminates the stabilization of the C-loop, which in turn disrupts the hydrogen bonding between arg196 and trp124, resulting in increased solvent interaction of arg196 and a higher barrier for passage of water. In contrast, the hydroxyl groups of glycerol have lower polarity than these in water and are not held back similarly.[60]

A topology prediction of LmAQP1 based on the crystal structure of PfAQP indicates that LmAQP1 consists of six membrane spanning helices and two membrane spanning half-helices containing the canonical Asn-Pro-Ala (NPA) motifs. These helices are connected by five loops (A-E). The C-loop of LmAQP1 is slightly longer than PfAQP and has three glutamates at positions 143, 145 and 152; glu152 being at the homologous position to glu125 of PfAQP. arg230 (homologous to arg196 of PfAQP) forms part of the selectivity filter and alteration of this residue severely affects the transport property of LmAQP1.[55] The other residues that are hydrogen bonded to glu125 and arg196 of PfAQP are not conserved in LmAQP1. We have shown that while glu143 and glu145 have no role in LmAQP1 activity, alteration of glu152 to alanine selectively abrogates metalloid permeability but does not affect glycerol transport. We also demonstrated that glu152 is critical for the physiological response of *Leishmania* to osmotic stress conditions.[61] Our experiments indicate that the transport chemistry of metalloid and glycerol might follow distinct mechanisms as glu152 in LmAQP1 is critical for metalloid but not glycerol transport. The crystal structure of PfAQP indicates that alteration of glu125 (homologous to glu152 of LmAQP1) disrupts the hydrogen bonding network around arg196 in the selectivity filter, which results in a higher desolvation penalty for the passage of water.[60] Very likely, alteration of glu152 of LmAQP1 to alanine interferes with the hydrogen bonding of arg230 (homologous to arg196 of PfAQP1), which presents a higher energy barrier to the passage of metalloids. To the best of our knowledge, this is the first report on the molecular dissection of metalloid and glycerol permeability by a single

mutation in an aquaglyceroporin. It is tempting to speculate that this might form the basis of a novel pathway of drug resistance, as *Leishmania* may develop suitable mutations that selectively block the transport of metalloids, but allow the uptake of physiological substrates. This hypothesis needs to be tested on drug-resistant field isolates.

Our observation that a single mutation affects metalloid but not glycerol transport in an aquaglyceroporin may have wider ramifications, especially in the development of As resistant crops. In several regions of the globe, cultivation in As-rich soil and irrigation with As-contaminated water has led to accumulation of high levels of As in rice, wheat, fruits and vegetables.[62] Since plant aquaglyceroporins (nodulin26-like intrinsic proteins (NIPs)) appear to be the major routes of As(III) uptake into plants,[62-65] genetically engineering of NIPs that are permeable to essential metalloids such as boron[66] and silicon,[67] but not to As(III) is a plausible approach towards the creation of low As crops.

Therapeutic Modulation of AQP Permeability

The idea to therapeutically modulate AQP function is documented in the literature for as long as 10 years.[54,70-74] It is thought that inhibition of AQPs may provide a novel regime in the treatment of certain diseases and pathophysiological situations. Currently, the focus is mainly on inhibition of water permeability in the human kidney, the eye, the inner ear and the brain. Respective drugs may be used to treat (i) the state of hyponatraemia by releasing water from the body whereas sodium is retained and, thus, becomes concentrated back to a normal level (drug targets: kidney AQP1 and AQP2),[75] (ii) glaucoma to reduce the pressure in the ocular bulb (target: eye AQP1),[76] (iii) Ménière's disease to control pressure in the endolymph compartment of the inner ear; dysregulation results in vertigo attacks and hearing loss due to the irreparable damage of sensory hair cells (target: inner ear AQP1 and AQP2 in the endolymphatic sac and the stria vascularis)[77,78] and (iv) acute brain edema as derived from the apparent resistance of AQP4 knockout mice against brain swelling after water intoxication or focal ischemia (target: astroglial AQP4 at the blood-brain barrier).[79] Novel findings relate AQPs to cell motility;[80] thus, inhibition may be beneficial in the treatment of certain forms of cancer by blocking metastasis and invasion of healthy tissue. With regard to aquaglyceroporins and metalloid transport it can be envisioned that inhibition of respective AQPs may enhance the susceptibility of targeted cells, e.g., cancer cells to As(V) or Sb(V) treatment by blocking an important As(III)/Sb(III) detoxification pathway. In the case of protozoan parasites blockage of aquaglyceroporins should interfere with metabolism because exchange of the biosynthetic precurser glycerol and of the metabolic waste product ammonia will be impaired. However, a breakthrough in terms of identification of lead structures for the design of small molecules that specifically inhibit AQPs has yet to be reached.

The first and probably only true AQP inhibitor still today is mercury in form of the divalent cation (Hg^{2+}) and as organic mercurial, e.g., *p*-hydroxymercuribenzoate (pHMB).[81] Yet, intolerable toxicity precludes mercury from usage as a therapeutic except in severe cases as the aforementioned form of leukemia. Its general toxocity is due to the highly unspecific mode of action because mercury binds to accessible cysteine sulfhydryl groups of many proteins. Several AQPs contain a cysteine at the channel entry and complexation with mercury occludes the pore.[82] In fact, in 1992 the finding that the newly cloned AQP1 was inhibited by mercury was major evidence that the long-sought water channel was finally identified.[83] Besides unexplained high water permeability of certain epithelia at low Arrhenius activation energy, it had been mercury-inhibition that suggested a proteinaceous water pore in cell membranes. Other cations, i.e., silver (Ag^{+}) and gold (Au^{+}) can also form complexes with pore cysteines in AQPs and block water and solute permeability.[84] The latter metals cannot be expected to be more specific for AQP inhibition than mercury.

Metal ions as AQP inhibitors are of interest only in the setting of a research lab or for toxicology studies. In this regard it was further found that nickel and copper inhibit human AQP3 in the lung and in red blood cells.[85,86] AQP3 is an aquaglyceroporin with intermediate water and high glycerol permeability. If inhaled, nickel blocks AQP3 in the apical membranes of the lung epithelial

cells; the situation is worsened at acidic pH. Inhibition of AQP3 is made responsible for defective water clearance in nickel-induced lung disease.[85] Copper is a trace element in human physiology but after intoxication with higher copper amounts or under certain pathological conditions, such as Wilson disease, copper blood levels rise and can then block AQP3, e.g., in red blood cells.[86] The binding site in AQP3 for nickel and copper is not the cysteine discussed above but involves a histidine residue at the extracellular side of the pore.

Specificity cannot be obtained from single atom ions that bind to a protein. Instead, organic molecules are needed, which interact with several sites in a binding pocket of the protein via electrostatic attraction, hydrogen bonds or lipophilic contact areas. Thinking towards drugs, proper binding constants and specificity are a must. However, further parameters need to comply with pharmaceutical needs, such as sufficient lipophilicity in order to allow for efficient resorption of the compound or metabolic stability in order to reach a suitable half-life. The development of AQP inhibitors has not yet taken the first hurdle, i.e., finding a potent candidate. Current test compounds inhibit AQPs when applied in the high micromolar or even millimolar concentration range. Further, full inhibition has not been achieved; the most effective compounds reduce permeability down to 50-70%. Also, none of the candidates is specific for AQPs.

Positively charged tetraethylammonium (TEA) a known open channel blocker of potassium channels[87] and TEA derivatives with longer alkyl side chains were reported to inhibit AQPs. In the used concentration range of 0.1-10 mM, TEA reduced water permeability to about 60%.[88,89] Amino acid residues in the extracellular loops A and E of AQP1 were identified to contribute to TEA inhibition by site-directed mutagenesis. Exchange of tyr186 in loop E for a phenylalanine abolished the TEA effect[88] as well as mutations of A-loop asparagine 42 or threonine 44 to alanine.[89] Molecular dynamics simulations showed binding of TEA to mainly the latter two residues and negatively charged side chains of two extracellular aspartates.[89] Notably, the efficacy of TEA seems to strongly depend on the test system used for the permeability assay, i.e., *Xenopus* oocytes,[88,89] cultured human cells[90] and yeast.[91] In the yeast system no inhibition of AQP1 by TEA was detectable at all. A potential reason could be differences or even absence of AQP glycosylation in cells from various species. When expressed in yeast, AQP1 is not glycosylated.[91] It is noteworthy that the two residues which are required for TEA inhibition of AQP1 comprise the N-glycosylation site asp42 and a residue of the N-X-S/T glycosylation motif, i.e., thr44.[92] Another coincidence is that only about 50% of the native AQP1 protein becomes glycosylated,[93] a number that fits very well with the maximal inhibition effect. Two other reports challenge the findings of AQP1 inhibition by TEA. One study was carried out in *Xenopus* oocytes expressing human AQP1[94] the other one with native human and mouse erythrocytes as well as rat epithelial cells in culture that all express AQP1 endogenously.[95] In these setups TEA up to 10 mM did not inhibit AQP1. Both references further cannot confirm data for another AQP inhibitor candidate, i.e., acetazolamide, a carbonic anhydrase inhibitor, which was earlier reported to block AQP1 water permeability.[96] These inconsistencies show that TEA and acetazolamide must be regarded as weak AQP1 inhibitors at best. Finally, two recent studies with *Xenopus* ooctyes provide evidence that arylsulfonamides and antiepileptic drugs, such as topiramate and oxcarbazepine, inhibit human AQP4 with IC_{50} values around 10 μM, a concentration that can indeed be established in the plasma.[97,98] As with TEA, inhibition was not complete and reached only 30-70%. Again, this study is challenged by another group which cannot confirm inhibition of AQP1 by the respective compounds.[99]

Why is it so difficult to find good AQP inhibitors? The challenge most likely lies in the typical protein structure of water-specific channels with their narrow 2.8 Å pore constriction (Fig. 2, left panels).[100] This site can only be entered by water molecules, H_2O, which perfectly fit the diameter of the constriction; some AQPs do also pass ammonia next to water. Organic molecules, however, cannot enter the channel region due to their larger size.[101] With regard to the design of an inhibitor molecule this is a major problem because the compound can only bind to domains outside of the actual channel. Figure 2 shows the outer vestibule of the water-specific AQP1 (upper left panel). It is rather shallow and very much exposed to the solvent as seen by the heavy decoration with water molecules (red spheres). An inhibitor molecule would have to compete with water for

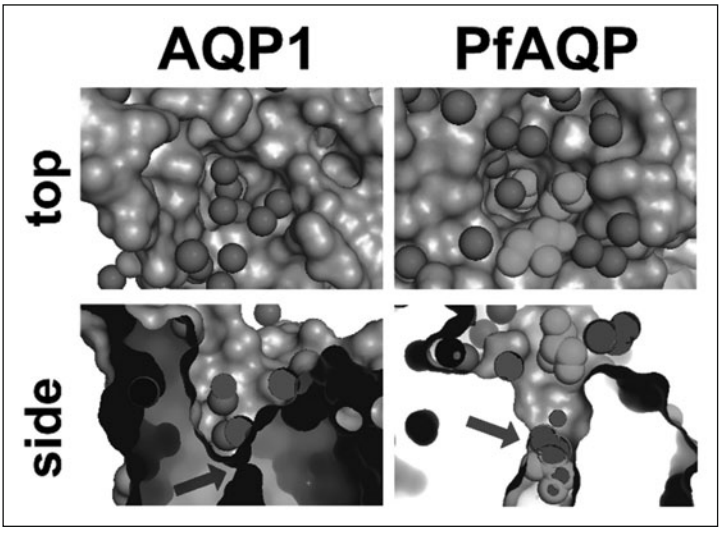


Figure 2. The extracellular vestibules of mammalian water-specific AQP1 (left panels)[100] and the water, solute and metalloid channel PfAQP from the malaria parasite *Plasmodium falciparum* (right panels).[60] Shown are top views as seen from the extracellular side (top) and side views of a section along the channel axis (bottom). The aquaporin surface is drawn in gray and water as well as glycerol molecules that were bound to the crystallized proteins are labeled in red and green, respectively. The blue arrows mark the narrowest pore constriction, i.e., the so-called aromatic/arginine region. Note that the outer vestibules strongly differ between both channels. In AQP1, it is rather shallow and stronger exposed to the solvent water whereas in PfAQP the vestibule is deeper, more pocketlike and harbors bound glycerol molecules. Also, the pore in AQP1 is too narrow to allow organic molecules to enter; the PfAQP structure in turn holds a glycerol right at the constriction site. A color version of this image is available at www.landesbioscience.com/curie.

the mainly polar binding sites. Taking into account the overwhelming difference in abundance of the competing molecules, i.e., 55 molar concentration of water vs micro- or better nanomolar concentrations of a potent inhibitor molecule, the problem becomes evident. The inhibitor must bind stronger to the protein by more than six orders of magnitude.

The situation appears somewhat more promising when looking at aquaglyceroporins including metalloid conducting AQPs. These channels have pore constrictions that are wide enough to accommodate organic molecules, such as formamide, urea, glycerol and longer polyols, as well as the carbonyl compounds dihydroxyacetone and methylglyoxal.[102] Further, the outer vestibule is usually deeper, less occupied by water molecules and has binding sites for glycerol. Figure 2 (right panels) shows the vestibule and pore constriction of the *Plasmodium falciparum* aquaglyceroporin as an example.[58,60] The crystal structure depicts glycerol molecules that are bound to the vestibule (green in Fig. 2) as well as glycerol inside the channel. Under physiological conditions, glycerol concentration ranges between 30-90 μM in human blood.[103] Despite being quite flat the gradient between the extra- and the intracellular space maintains a flux of glycerol from fatty tissue via AQP7 into liver cells for gluconeogenesis via AQP9.[104,105] The intracellular malaria parasite also takes up glycerol from the host blood via red cell AQP3 and its endogenous PfAQP and uses it as a precursor for lipid synthesis.[106] These examples show that aquaglyceroporins efficiently bind and capture glycerol molecules already at micromolar concentrations. If an inhibitor molecule can be designed to interact with the binding sites of two or three glycerol molecules in the AQP vestibule or even occupies sites in the channel pore it is thinkable that the binding constant will be in the desired low micromolar if not nanomolar range. Blockage of protozoan aquaglyceroporins should

directly harm the parasite by depriving it of precursor molecules for biosynthesis, e.g., glycerol,[58] or by obstructing release of metabolic waste products, e.g., cytotoxic ammonia.[107] In this regard, a knockout strain of the murine malaria parasite *Plasmodium berghei* is vital despite lacking its single aquaglyceroporin gene but has a growth rate of only 50% compared to wild-type parasites.[108]

Conclusion

Together, aquaporin-facilitated metalloid transport touches human physiology in several ways: uptake of environmental metalloids with toxicological potential, treatment of certain forms of leukemia and chemotherapy of diseases caused by pathogenic protozoa. Deepening the knowledge of the underlying mechanisms is worthwhile and may in fact lead to novel therapeutic possibilities.

Acknowledgements

This work was supported in part by National Institutes of Health Grant AI58170 to R.M. and by the Deutsche Forschungsgemeinschaft (Be2253) and the European Commission (LSHP-CT-2004-012189) to E.B.

References

1. Klaassen CD. Heavy Metals and Heavy Metal Antagonists. New York: McGraw-Hill; 1996.
2. Kwong YL, Todd D. Delicious poison: arsenic trioxide for the treatment of leukemia. Blood 1997; 89:3487-3488.
3. Aronson SM: Arsenic and old myths. R I Med 1994; 77:233-234.
4. Staff NRC: Arsenic in drinking water: National Academy Press; 1999.
5. Soignet SL, Maslak P, Wang ZG et al. Complete remission after treatment of acute promyelocytic leukemia with arsenic trioxide. N Engl J Med 1998; 339:1341-1348.
6. Bhattacharjee H, Ghosh M, Mukhopadhyay R et al. Arsenic transporters from E. coli to humans. In: Broome-Smith JK, Baumberg S, Sterling CJ, Ward FB, eds. Transport of Molecules Across Microbial Membranes. Leeds: Society for General Micriobiology, 1999; 58:58-79.
7. Wysocki R, Bobrowicz P, Ulaszewski S. The Saccharomyces cerevisiae ACR3 gene encodes a putative membrane protein involved in arsenite transport. J Biol Chem 1997; 272:30061-30066.
8. Ghosh M, Shen J, Rosen BP. Pathways of As(III) detoxification in Saccharomyces cerevisiae. Proc Natl Acad Sci USA 1999; 96:5001-5006.
9. Dey S, Ouellette M, Lightbody J et al. An ATP-dependent As(III)-glutathione transport system in membrane vesicles of Leishmania tarentolae. Proc Natl Acad Sci USA 1996; 93:2192-2197.
10. Legare D, Richard D, Mukhopadhyay R et al. The Leishmania ATP-binding cassette protein PGPA is an intracellular metal-thiol transporter ATPase. J Biol Chem 2001; 276:26301-26307.
11. Broeks A, Gerrard B, Allikmets R et al. Homologues of the human multidrug resistance genes MRP and MDR contribute to heavy metal resistance in the soil nematode Caenorhabditis elegans. EMBO J 1996; 15:6132-6143.
12. Kimura A, Ishida Y, Wada T et al. MRP-1 expression levels determine strain-specific susceptibility to sodium arsenic-induced renal injury between C57BL/6 and BALB/c mice. Toxicol Appl Pharmacol 2005; 203:53-61.
13. Zaman GJ, Lankelma J, van Tellingen O et al. Role of glutathione in the export of compounds from cells by the multidrug-resistance-associated protein. Proc Nat Acad Sci USA 1995; 92:7690-7694.
14. Kojima C, Qu W, Waalkes MP et al. Chronic exposure to methylated arsenicals stimulates arsenic excretion pathways and induces arsenic tolerance in rat liver cells. Toxicol Sci 2006; 91:70-81.
15. Tallman MS. Arsenic trioxide: its role in acute promyelocytic leukemia and potential in other hematologic malignancies. Blood Rev 2001; 15:133-142.
16. Tallman MS, Nabhan C, Feusner JH et al. Acute promyelocytic leukemia: evolving therapeutic strategies. Blood 2002; 99:759-767.
17. Davison K, Mann KK, Miller WH Jr. Arsenic trioxide: mechanisms of action. Semin Hematol 2002; 39:3-7.
18. Shen Y, Shen ZX, Yan H et al. Studies on the clinical efficacy and pharmacokinetics of low-dose arsenic trioxide in the treatment of relapsed acute promyelocytic leukemia: a comparison with conventional dosage. Leukemia 2001; 15:735-741.
19. Perkins C, Kim CN, Fang G et al. Arsenic induces apoptosis of multidrug-resistant human myeloid leukemia cells that express Bcr-Abl or overexpress MDR, MRP, Bcl-2, or Bcl-x(L). Blood 2000; 95:1014-1022.
20. O'Dwyer ME, La Rosee P, Nimmanapalli R et al. Recent advances in Philadelphia chromosome-positive malignancies: the potential role of arsenic trioxide. Semin Hematol 2002; 39:18-21.

21. Chen Z, Chen GQ, Shen ZX et al. Expanding the use of arsenic trioxide: leukemias and beyond. Semin Hematol 2002; 39:22-26.
22. Dilda PJ, Hogg PJ. Arsenical-based cancer drugs. Cancer Treat Rev 2007; 33:542-564.
23. Verstovsek S, Giles F, Quintas-Cardama A et al. Arsenic derivatives in hematologic malignancies: a role beyond acute promyelocytic leukemia? Hematol Oncol 2006; 24:181-188.
24. Simeonova PP, Luster MI. Arsenic carcinogenicity: relevance of c-Src activation. Mol Cell Biochem 2002; 234-235:277-282.
25. Arsenic and Arsenic Compounds: IARC; 1987.
26. Verma A, Mohindru M, Deb DK et al. Activation of Rac1 and the p38 mitogen-activated protein kinase pathway in response to arsenic trioxide. J Biol Chem 2002; 277:44988-44995.
27. Arima H, Yamamoto N, Sobue K et al. Hyperosmolar mannitol simulates expression of aquaporins 4 and 9 through a p38 mitogen-activated protein kinase-dependent pathway in rat astrocytes. J Biol Chem 2003; 278:44525-44534.
28. Umenishi F, Schrier RW. Hypertonicity-induced aquaporin-1 (AQP1) expression is mediated by the activation of MAPK pathways and hypertonicity-responsive element in the AQP1 gene. J Biol Chem 2003; 278:15765-15770.
29. Hara-Chikuma M, Verkman AS. Aquaporin-3 facilitates epidermal cell migration and proliferation during wound healing. J Mol Med 2008; 86:221-231.
30. Thorsen M, Di Y, Tangemo C et al. The MAPK Hog1p modulates Fps1p-dependent arsenite uptake and tolerance in yeast. Mol Biol Cell 2006; 17:4400-4410.
31. Bhattacharjee H, Carbrey J, Rosen BP et al. Drug uptake and pharmacological modulation of drug sensitivity in leukemia by AQP9. Biochem Biophys Res Comm 2004; 322:836-841.
32. Leung J, Pang A, Yuen WH et al. Relationship of expression of aquaglyceroporin 9 with arsenic uptake and sensitivity in leukemia cells. Blood 2007; 109:740-746.
33. Aribi A, Kantarjian HM, Estey EH et al. Combination therapy with arsenic trioxide, all-trans retinoic acid and gemtuzumab ozogamicin in recurrent acute promyelocytic leukemia. Cancer 2007; 109:1355-1359.
34. Zhou GB, Zhang J, Wang ZY et al. Treatment of acute promyelocytic leukaemia with all-trans retinoic acid and arsenic trioxide: a paradigm of synergistic molecular targeting therapy. Phil Trans R Soc Lond 2007; 362:959-971.
35. Lee TC, Ho IC, Lu WJ et al. Enhanced expression of multidrug resistance-associated protein 2 and reduced expression of aquaglyceroporin 3 in an arsenic-resistant human cell line. J Biol Chem 2006; 281:18401-18407.
36. Bouteille B, Oukem O, Bisser S et al. Treatment perspectives for human African trypanosomiasis. Fund Clin Pharmacol 2003; 17:171-181.
37. Fairlamb AH, Henderson GB, Cerami A. Trypanothione is the primary target for arsenical drugs against African trypanosomes. Proc Nat Acad Sci USA 1989; 86:2607-2611.
38. Wang CC. Molecular mechanisms and therapeutic approaches to the treatment of African trypanosomiasis. Ann Rev Pharmacol Tox 1995; 35:93-127.
39. de Koning HP. Transporters in African trypanosomes: role in drug action and resistance. Int J Parasitol 2001; 31:512-522.
40. Uzcategui NL, Szallies A, Pavlovic-Djuranovic S et al. Cloning, heterologous expression and characterization of three aquaglyceroporins from Trypanosoma brucei. J Biol Chem 2004; 279:42669-42676.
41. Choi CM, Lerner EA. Leishmaniasis: recognition and management with a focus on the immunocompromised patient. Am J Clin Dermatol 2002; 3:91-105.
42. Silva ES, Pacheco RS, Gontijo CM et al. Visceral leishmaniasis caused by Leishmania (Viannia) braziliensis in a patient infected with human immunodeficiency virus. Rev Inst Med Trop Sao Paulo 2002; 44:145-149.
43. Faraut-Gambarelli F, Piarroux R, Deniau M et al. In vitro and in vivo resistance of Leishmania infantum to meglumine antimoniate: a study of 37 strains collected from patients with visceral leishmaniasis. Antimicrob Agents Chemother 1997; 41:827-830.
44. Jackson JE, Tally JD, Ellis WY et al. Quantitative in vitro drug potency and drug susceptibility evaluation of Leishmania ssp. from patients unresponsive to pentavalent antimony therapy. Am J Trop Med Hyg 1990; 43:464-480.
45. Sundar S, More DK, Singh MK et al. Failure of pentavalent antimony in visceral leishmaniasis in India: report from the center of the Indian epidemic. Clin Infect Dis 2000; 31:1104-1107.
46. Ouellette M, Borst P. Drug resistance and P-glycoprotein gene amplification in the protozoan parasite. Leishmania. Res Microbiol 1991; 142:737-746.
47. Goodwin LG. Pentostam (sodium stibogluconate); a 50-year personal reminiscence. Trans R Soc Trop Med Hyg 1995; 89:339-341.
48. Lugo de Yarbuh A, Anez N, Petit de Pena Y et al. Antimony determination in tissues and serum of hamsters infected with Leishmania garnhami and treated with meglumine antimoniate. Ann Trop Med Parasitol 1994; 88:37-41.

49. Mottram JC, Coombs GH. Leishmania mexicana: enzyme activities of amastigotes and promastigotes and their inhibition by antimonials and arsenicals. Exp Parasitol 1985; 59:151-160.
50. Roberts WL, Berman JD, Rainey PM. In vitro antileishmanial properties of tri- and pentavalent antimonial preparations. Antimicrob Agents Chemother 1995; 39:1234-1239.
51. Sereno D, Lemesre JL. Axenically cultured amastigote forms as an in vitro model for investigation of antileishmanial agents. Antimicrob Agents Chemother 1997; 41:972-976.
52. Sereno D, Cavaleyra M, Zemzoumi K et al. Axenically grown amastigotes of Leishmania infantum used as an in vitro model to investigate the pentavalent antimony mode of action. Antimicrob Agents Chemother 1998; 42:3097-3102.
53. Gourbal B, Sonuc N, Bhattacharjee H et al. Drug uptake and modulation of drug resistance in Leishmania by an aquaglyceroporin. J Biol Chem 2004; 279:31010-31017.
54. Beitz E. Aquaporins from pathogenic protozoan parasites: structure, function and potential for chemotherapy. Biol Cell 2005; 97:373-383.
55. Figarella K, Uzcategui NL, Zhou Y et al. Biochemical characterization of Leishmania major aquaglyceroporin LmAQP1: possible role in volume regulation and osmotaxis. Mol Microbiol 2007; 65:1006-1017.
56. Marquis N, Gourbal B, Rosen BP et al. Modulation in aquaglyceroporin AQP1 gene transcript levels in drug-resistant Leishmania. Mol Microbiol 2005; 57:1690-1699.
57. Decuypere S, Rijal S, Yardley V et al. Gene expression analysis of the mechanism of natural Sb(V) resistance in Leishmania donovani isolates from Nepal. Antimicrob Agents Chemother 2005; 49:4616-4621.
58. Hansen M, Kun JF, Schultz JE et al. A single, bi-functional aquaglyceroporin in blood-stage Plasmodium falciparum malaria parasites. J Biol Chem 2002; 277:4874-4882.
59. Beitz E, Pavlovic-Djuranovic S, Yasui M et al. Molecular dissection of water and glycerol permeability of the aquaglyceroporin from Plasmodium falciparum by mutational analysis. Proc Nat Acad Sci USA 2004; 101:1153-1158.
60. Newby ZE, O'Connell J, 3rd, Robles-Colmenares Y et al. Crystal structure of the aquaglyceroporin PfAQP from the malarial parasite Plasmodium falciparum. Nat Struct Mol Biol 2008; 15:619-625.
61. Uzcategui NL, Zhou Y, Figarella K et al. Alteration in glycerol and metalloid permeability by a single mutation in the extracellular C-loop of Leishmania major aquaglyceroporin LmAQP1. Mol Microbiol 2008; 70:1477-1486.
62. Tripathi RD, Srivastava S, Mishra S et al. Arsenic hazards: strategies for tolerance and remediation by plants. Trends Biotechnol 2007; 25:158-165.
63. Bienert GP, Thorsen M, Schussler MD et al. A subgroup of plant aquaporins facilitate the bi-directional diffusion of $As(OH)_3$ and $Sb(OH)_3$ across membranes. BMC Biol 2008; 6:26.
64. Isayenkov SV, Maathuis FJ. The Arabidopsis thaliana aquaglyceroporin AtNIP7;1 is a pathway for arsenite uptake. FEBS Lett 2008; 582:1625-1628.
65. Ma JF, Yamaji N, Mitani N et al. Transporters of arsenite in rice and their role in arsenic accumulation in rice grain. Proc Nat Acad Sci USA 2008; 105:9931-9935.
66. Takano J, Wada M, Ludewig U et al. The Arabidopsis major intrinsic protein NIP5; 1 is essential for efficient boron uptake and plant development under boron limitation. Plant Cell 2006; 18:1498-1509.
67. Ma JF, Tamai K, Yamaji N et al. A silicon transporter in rice. Nature 2006; 440:688-691.
68. Zhou Y, Messier N, Ouellette M et al. Leishmania major LmACR2 is a pentavalent antimony reductase that confers sensitivity to the drug pentostam. J Biol Chem 2004; 279:37445-37451.
69. Denton H, McGregor JC, Coombs GH. Reduction of anti-leishmanial pentavalent antimonial drugs by a parasite-specific thiol-dependent reductase, TDR1. Biochem J 2004; 381:405-412.
70. Beitz E, Schultz JE. The mammalian aquaporin water channel family: A promising new drug target. Curr Med Chem 1999; 6:457-467.
71. Oshio K, Binder DK, Bollen A et al. Aquaporin-1 expression in human glial tumors suggests a potential novel therapeutic target for tumor-associated edema. Acta Neurochir Suppl 2003; 86:499-502.
72. Frühbeck G, Catalán V, Gómez-Ambrosi J et al. Aquaporin-7 and glycerol permeability as novel obesity drug-target pathways. Trends Pharmacol Sci 2006; 27:345-347.
73. Jeyaseelan K, Sepramaniam S, Armugam A et al. Aquaporins: a promising target for drug development. Expert Opin Ther Targets 2006; 10:889-909.
74. Papadopoulos MC, Verkman AS. Potential utility of aquaporin modulators for therapy of brain disorders. Prog Brain Res 2008; 170:589-601.
75. Schrier RW. The sea within us: disorders of body water homeostasis. Curr Opin Investig Drugs 2007; 8:304-311.
76. Verkman AS. Role of aquaporin water channels in eye function. Exp Eye Res 2003; 76:137-143.
77. Beitz E, Kumagami H, Krippeit-Drews P et al. Expression pattern of aquaporin water channels in the inner ear of the rat. Hear Res 1999; 132:76-84.
78. Ishiyama G, López IA, Ishiyama A. Aquaporins and Meniere's disease. Curr Opin Otolaryngol Head Neck Surg 2006; 14:332-336.

79. Zador Z, Bloch O, Yao X et al. Aquaporins: role in cerebral edema and brain water balance. Prog Brain Res 2007; 161:185-194.
80. Papadopoulos MC, Saadoun S, Verkman AS. Aquaporins and cell migration. Pflügers Arch 2008; 456:693-700.
81. Agre P, Mathai JC, Smith BL et al. Functional analyses of aquaporin water channel proteins. Methods Enzymol 1999; 294:550-572.
82. Savage DF, Stroud RM. Structural basis of aquaporin inhibition by mercury. J Mol Biol 2007; 68:607-617.
83. Preston GM, Carroll TP, Guggino WB et al. Appearance of water channels in Xenopus oocytes expressing red cell CHIP28 protein. Science 1992; 256:385-387.
84. Niemietz CM, Tyerman SD. New potent inhibitors of aquaporins: silver and gold compounds inhibit aquaporins of plant and human origin. FEBS Lett 2002; 531:443-447.
85. Zelenina M, Bondar AA, Zelenin S et al. Nickel and extracellular acidification inhibit the water permeability of human aquaporin-3 in lung epithelial cells. J Biol Chem 2003; 278:30037-30043.
86. Zelenina M, Tritto S, Bondar AA et al. Copper inhibits the water and glycerol permeability of aquaporin-3. J Biol Chem 2004; 279:51939-51943.
87. Yellen G, Jurman ME, Abramson T et al. Mutations affecting internal TEA blockade identify the probable pore-forming region of a K^+ channel. Science 1991; 251:939-942.
88. Brooks HL, Regan JW, Yool AJ. Inhibition of aquaporin-1 water permeability by tetraethylammonium: involvement of the loop E pore region. Mol Pharmacol 2000; 57:1021-1026.
89. Detmers FJ, de Groot BL, Müller EM et al. Quaternary ammonium compounds as waterchannel blockers. Specificity, potency and site of action. J Biol Chem 2006; 281:14207-14214.
90. Yool AJ, Brokl OH, Pannabecker TL et al. Tetraethylammonium block of water flux in Aquaporin-1 channels expressed in kidney thin limbs of Henle's loop and a kidney-derived cell line. BMC Physiol 2002; 2:4.
91. Wu B, Altmann K, Barzel I et al. A yeast-based phenotypic screen for aquaporin inhibitors. Pflugers Arch 2008; 456:717-720.
92. Preston GM, Jung JS, Guggino WB et al. Membrane topology of aquaporin CHIP. Analysis of functional epitope-scanning mutants by vectorial proteolysis. J Biol Chem 1994; 269:1668-1673.
93. van Hoek AN, Wiener MC, Verbavatz JM et al. Purification and structure-function analysis of native, PNGase F-treated and endo-beta-galactosidase-treated CHIP28 water channels. Biochemistry 1995; 34:2212-2219.
94. Søgaard R, Zeuthen T. Test of blockers of AQP1 water permeability by a high-resolution method: no effects of tetraethylammonium ions or acetazolamide. Pflügers Arch 2008; 456:285-292.
95. Yang B, Kim JK, Verkman AS. Comparative efficacy of HgCl2 with candidate aquaporin-1 inhibitors DMSO, gold, TEA+ and acetazolamide. FEBS Lett 2006; 580:6679-6684.
96. Ma B, Xiang Y, Mu SM et al. Effects of acetazolamide and anordiol on osmotic water permeability in AQP1-cRNA injected Xenopus oocyte. Acta Pharmacol Sin 2004; 25:90-97.
97. Huber VJ, Tsujita M, Yamazaki M et al. Identification of arylsulfonamides as Aquaporin 4 inhibitors. Bioorg Med Chem Lett 2007; 17:1270-1273.
98. Huber VJ, Tsujita M, Kwee IL et al. Inhibition of Aquaporin 4 by antiepileptic drugs. Bioorg Med Chem 2008; 17:418-424.
99. Yang B, Zhang H, Verkman AS. Lack of aquaporin-4 water transport inhibition by antiepileptics and arylsulfonamides. Bioorg Med Chem 2008; 16:7489-7493.
100. Sui H, Han BG, Lee JK et al. Structural basis of water-specific transport through the AQP1 water channel. Nature 2001; 414:872-878.
101. Beitz E, Wu B, Holm LM et al. Point mutations in the aromatic/arginine region in aquaporin 1 allow passage of urea, glycerol, ammonia and protons. Proc Natl Acad Sci USA 2006; 103:269-274.
102. Wu B, Beitz E. Aquaporins with selectivity for unconventional permeants. Cell Mol Life Sci 2007; 64:2413-2421.
103. Pukrittayakamee S, White NJ, Davis TM et al. Glycerol metabolism in severe falciparum malaria. Metabolism 1994; 43:887-892.
104. Kishida K, Shimomura I, Kondo H et al. Genomic structure and insulin-mediated repression of the aquaporin adipose (AQPap), adipose-specific glycerol channel. J Biol Chem 2001; 276:36251-36260.
105. Carbrey JM, Gorelick-Feldman DA, Kozono D et al. Aquaglyceroporin AQP9: solute permeation and metabolic control of expression in liver. Proc Natl Acad Sci USA 2003; 100:2945-2950.
106. Holz GG Jr. Lipids and the malarial parasite. Bull World Health Organ 1977; 55:237-248.
107. Zeuthen T, Wu B, Pavlovic-Djuranovic S et al. Ammonia permeability of the aquaglyceroporins from Plasmodium falciparum, Toxoplasma gondii and Trypansoma brucei. Mol Microbiol 2006; 61:1598-1608.
108. Promeneur D, Liu Y, Maciel J et al. Aquaglyceroporin PbAQP during intraerythrocytic development of the malaria parasite Plasmodium berghei. Proc Natl Acad Sci USA 2007; 104:2211-2216.

Elsbeth Bienert

Chapter 6

Roles of Vertebrate Aquaglyceroporins in Arsenic Transport and Detoxification

Zijuan Liu*

Abstract

Aquaporins are important channel proteins that are responsible for the balance of cellular osmolarity and nutrient transport in vertebrates. Recently, new functions of these ancient channels have been found in the conduction of metalloid arsenic (As). Chronic As exposure through contaminated water and food sources is associated with multiple human diseases and endangers millions of people's health worldwide. Therefore, identification of the As transport pathways is necessary to elucidate the mechanisms of As carcinogenesis. Arsenic detoxification systems have been studied in multiple vertebrates such as mammalian mouse, rat, humans and nonmammalian vertebrates. Multiple transporters and enzymes have been shown to be involved in As translocation and cellular transformation. In these vertebrates, members of aquaglyceroporins, which include AQP7 in kidney and AQP9 in liver, catalyze uptake of inorganic trivalent arsenite [As(III)]. AQP9, the major liver aquaglyceroporin, conducts both inorganic As(III) and organic monomethylarsonous acid [MMA(III)], an intermediate that is generated during the cellular methylation. As a channel that facilitates a downhill movement of substances dependent on the concentration gradient, AQP9 may play an important role in the simultaneous influx of inorganic As(III) from blood to liver and efflux of As metabolite MMA(III) from liver to blood. In this chapter, we will discuss the function of aquaglyceroporins of vertebrates in uptake and detoxification of the metalloid As.

Introduction

Metalloid arsenic (As) contamination became an international public health issue several decades ago, when As-associated diseases became epidemic in some countries. Therefore, researchers have studied the pathways involved in As transport and cellular metabolism. Arsenic uptake is the first step, followed by other cellular transformation processes. Generally speaking, uptake is the rate-limiting step for most of the cellular metabolic pathways. The first uptake pathway for trivalent arsenite [As(III)] was identified in 2002, when it was shown that members of the aquaglyceroporin protein family are also efficient As transporters. Here, we summarize the properties of aquaglyceroporins, the relationship to As exposure and human health as well as the molecular mechanisms of As transport, with emphasis on the evolutionary and physiological significance of the metalloid uptake by these water channel proteins.

Expression of Vertebrate Aquaglyceroporins

The first water channel AQP1 was identified in human erythrocytes in 1992.[1] Since then, additional members in this family were identified in different tissues. In human, 13 aquaporins

*Zijuan Liu—Department of Biological Sciences, Oakland University. 2200 N. Squirrel Rd. Rochester, Michigan 48309, USA. Email: liu2345@oakland.edu

MIPs and Their Role in the Exchange of Metalloids, edited by Thomas P. Jahn and Gerd P. Bienert.

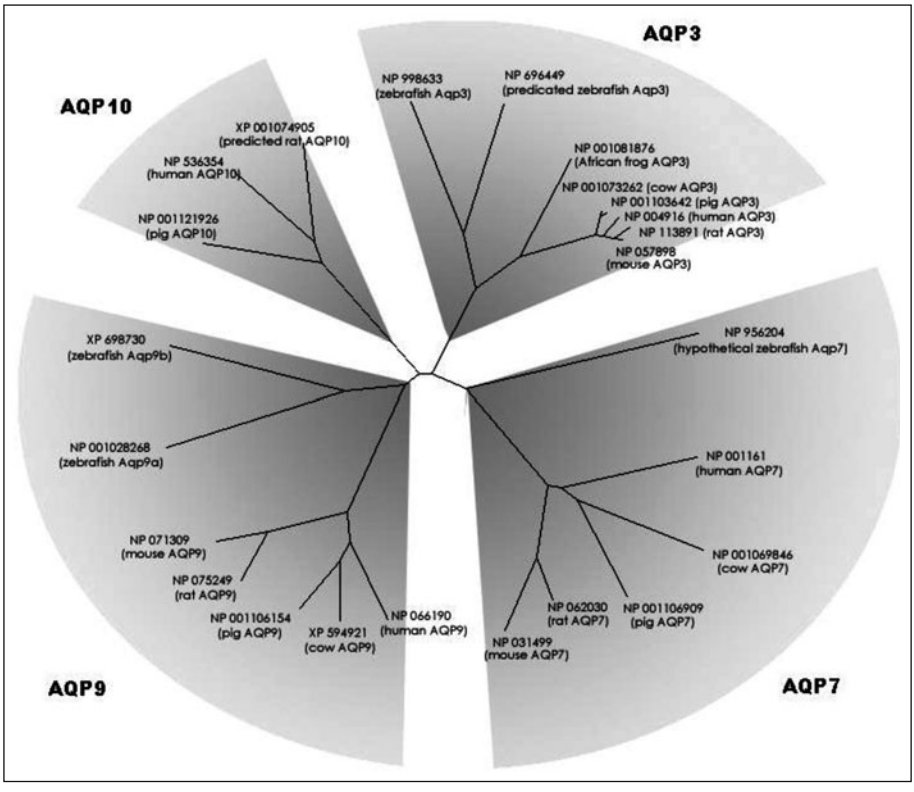


Figure 1. Phylogenetic tree of vertebrate aquaglyceroporins. The sequences of vertebrate aquaglyceroporins were obtained from NCBI. The alignment was performed using ClustalW and the tree was plotted by Treeview Ver1.6.6.

have been identified.[2] A subfamily of isoforms which shares relatively lower sequence similarities with orthodox aquaporins was also discovered and members were termed aquaglyceroporins. This subfamily can translocate larger aliphatic alcohol molecules such as glycerol. Four out of 13 human aquaporins, AQP3, AQP7, AQP9 and AQP10, are aquaglyceroporins and they share 37-45% sequence identity with each other. These channels have different expression patterns in a wide range of tissues. AQP3 is detected in skin, kidney, testis and erythrocytes.[3] AQP7 was initially cloned from rat testis[4] and later was found in adipose tissue, heart and kidney.[5] Both rat and human AQP9 is abundantly expressed in liver.[6] In contrast with rat, human AQP9 is also expressed in peripheral leukocytes and in tissues that accumulate leukocytes, such as lung, spleen and bone marrow.[7] Studies also found that human AQP9 is present in duodenum, jejunum and ileum as well as in brain astrocyte.[8,9] AQP10 is expressed in human duodenum and jejunum.[10] Based on these studies, the expression patterns of these members in different mammals are consistent but have some variability. In zebrafish (*Danio rerio*), as many as seven aquaglyceroporins have been assigned (http://www.ncbi.nlm.nih.gov) which have evolved through gene duplications. They share high sequence similarities (in the range of 72-81%) with the corresponding human members. Of these, Aqp3 is the only member to be functionally studied.[11] A phylogenetic tree is provided here to indicate the sequence homogeneity of four aquaglyceroporins within different vertebrate species, including different mammals, African frog (*Xenopus*) and zebrafish (*Danio rerio*), as shown in Figure 1.

Since glycerol, a substrate of aquaglyceroporins, is a key component of two-thirds of all phospholipids as well as an important metabolite, these channel proteins play important roles in the tissues where they are expressed. For example, AQP7 is an adipose isoform that releases glycerol into the blood stream during lipolysis, whereas AQP9 is a liver isoform that is responsible for the uptake of glycerol.[12,13] The interplay of these two AQPs is critical for gluconeogenesis during starvation.[14]

Although these four mammalian aquaglyceroporins all transport glycerol at a variable amount, they can also take up other substrates. AQP9 seems to be the channel with the broadest substrate specificity. It facilitates many noncharged substrates including carbamides, polyols, purines and pyrimidines.[15] However, the water permeation via AQP9 is quite limited.[14] AQP3, AQP7 and AQP10 can transport water, glycerol and urea.[4,10,16] All of these aquaglyceroporins are able to exclude ions such as hydroxide, hydronium and protons from conductance through the channel.[17]

Importantly, in addition to their physiological roles in nutrient transport, aquaglyceroporins have been shown to facilitate the uptake of the trivalent metalloids As(III) and antimonite [Sb(III)] which in neutral solution mimic glycerol.[18] We will discuss these capacities in details below.

Arsenic Is Both an Environmental Toxin and Human Carcinogen

Arsenic is unevenly distributed geochemically worldwide and an elevated exposure level can endanger human health. The major source of human As exposure is drinking water. In water, inorganic As usually has two oxidation states, trivalent As(III) and pentavalent arsenate [As(V)].[19] As(V) is less toxic than As(III). As(V) usually gets reduced to As(III) in the cytosol of cells.[20,21] The release of As into the aquatic environment is mainly from geochemical sources. However anthropogenic activities, such as the mining and wood processing industries can also lead to regional As contamination.[22] Under these circumstances, the As concentration in water can reach high levels. Chronic As poisoning via drinking water has been reported in many countries including Bangladesh, India, China, Chile and the United States,[23,24] where As in water exceeds the safety limit in many localities. For example, in Bangladesh, over 46 million people drink As-contaminated water that contains as much as 1000-fold more As than the World Health Organization (WHO) and U.S. Environmental Protection Agency (EPA) limit of 10 ppb.[25,26] The As contamination in Bangladesh has been described as "the largest poisoning of a population in history".[27] The exposure of As is associated with many human diseases including cancerous diseases such as skin and bladder cancers and noncancerous diseases such as hyperpigmentation, cardiovascular diseases and diabetes,[28,29] indicating As has no single tissue target. For these reasons, As is classified as the most potent human carcinogen and listed as the number one toxic substance on the superfund list (http://www.atsdr.cdc.gov/cercla/07list.html).

Although drinking water is the major source of human As exposure, also food, especially seafood contributes to As intake. Commercial seafood, including multiple species of fish, clams, crabs and shrimp, contain much higher concentrations of As than terrestrial animals. Fortunately, most of the As in seafood is in the form of organic arsenobetaine (around 80-95%) along with small portions of other organic As species such as arsenocholine and arsenolipids.[30] These organic species are usually not toxic; possibly because of their inability of binding enzymes. However, humans may metabolize them to other As compounds, some of which are toxic.[31,32] Consumption of fish containing nontoxic As compounds may not be harmful to human health in the short-term.[33,34] However, consumption of fish from polluted aquatic environments may be harmful to human health. The safety of these fish for human consumption should therefore be re-evaluated.

Another source of As exposure is rice, a grain on which a large portion of the world population depends on as a staple food. Rice grown in some areas is found to contain multiple species of organic As along with inorganic As,[35] which all have different levels of toxicity. In addition, organic As additives are currently applied in modern feeding industries. For example, the Nitarsone is well used as a feed additive in poultry industry. The organic As is thereafter ingested by human. However, the toxicity and metabolism of many different organic As compounds are not well studied. No

detailed regulations have been made for food As safety worldwide. More investigations are needed to evaluate the safety of these As species in food.

Although As is an environmental toxin and human carcinogen, the mechanisms by which As induces so many different diseases are not well understood. In recent decades, researchers have attempted to understand As carcinogenesis using animal models, including rodents (rats and mice) and larger mammals, such as beagles and monkeys. Transgenic animals were also used to test the carcinogenic effect of arsenicals. In most cases, these models had failed to satisfactorily mimic the actual mechanism of As carcinogenicity.[36] Additional animal models can be an asset for the further elucidation of As carcinogenesis. We are currently using zebrafish as a new model system to study As detoxification. More importantly, to further understand As-induced multiple diseases, the biochemical processes involved in As uptake, efflux and cellular transformations need to be elucidated. Recently, much effort has been made to identify the enzymes that are involved in As detoxification processes. One major finding is the identification of As uptake transporters, which is the very first step that is directly related to As cellular accumulation and toxicity.

Uptake of Organic and Inorganic Arsenic via Aquaglyceroporins

Two inorganic As species exist in ground water at variable ratios, As(III) and As(V). As(III) is the more toxic form.[37] Trivalent As(III), with a p*Ka* of 9.2, is taken up as the neutral hydroxylated species As(III).[37] We have demonstrated that trivalent As(III) is taken up by aquaglyceroporins in prokaryotes and eukaryotes. GlpF, the only aquaglyceroporin in *E. coli*, was shown to be the major channel to facilitate inorganic As(III) uptake.[38] Given to the high sequence similarities between prokaryotic aquaglyceroporins and eukaryotic homologues, the function of four mammalian aquaglyceroporins in As transport have been studied. To study the ability of As(III) transport, rat AQP9 was overexpressed in a yeast strain, HD9, with all As(III) transporters (*fps1*, *ycf1* and *acr3*) being deleted. This yeast is phenotypically more sensitive to As(III) and Sb(III) compared with the control yeast which contains the vector only. In addition, the yeast expressing AQP9 showed efficient As(III) transport in a time-dependent manner while the control yeast exhibited no change above basal level of As(III) uptake. Together these data indicate that rat AQP9 is a channel protein that conducts transport of inorganic trivalent As(III).

Unfortunately, not all mammalian aquaglyceroporins can be expressed in yeast. To understand their function in the metalloid transport, a *Xenopus* oocyte expression system was used. In this system, mRNA is injected into Phase IV to V oocytes. *Xenopus* oocytes have totally ten developmental phases, from Phase I to X. Oocytes at Phase IV and V contain all the machinery necessary for protein synthesis and the protein product is then easily synthesized and maturated. This expression system is currently the most popular system to study channel proteins, including neutral molecular channels and ion channels.[39] Four human aquaporins, AQP3, AQP7, AQP9 and AQP10 were studied by injecting their mRNA into oocytes.[40] The results showed that AQP7 and AQP9 are able to facilitate As(III) uptake efficiently (about 5 fold and 25 fold increases in transport, respectively) while AQP3 showed only limited ability to transport As(III) (about a two fold increase in transport). AQP10 does not conduct As(III) above basal level (Fig. 2). In summary, we have demonstrated that AQP7 from mouse and human (primarily expressed in adipose and kidney) and AQP9 from rat and human (primarily expressed in the liver and testis) facilitate As(III) uptake.[41] Liver isoform AQP9 from both rat and human exhibited a much higher efficiency for As(III) transport than AQP7. This observation is consistent with the fact that As is largely accumulated in liver in many different mammals including human.

Arsenic is metabolized using several cellular pathways. The major enzymatic reaction in As metabolism is As methylation. Elucidation of these metabolic pathways began in the 19th century with studies in microorganisms. Data indicated that As(III) is methylated into various species in a series of steps, producing organic mono-, di- and even tri-methylated As species, such as MMA(III) (monomethylarsonous acid), MMA(V) (monomethylarsonic acid), DMA(III) (dimethylarsonous acid), DMA(V) (dimethylarsonic acid) or trimethylarsin oxide (TMAO).[42] Some (but not all) mammals, including humans, methylate inorganic As and excrete the methylated species,

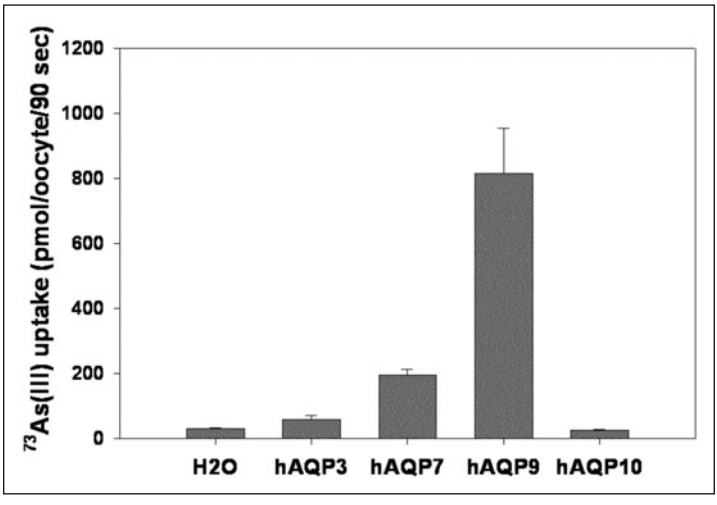


Figure 2. ^{73}As(III) permeability in *Xenopus* oocytes expressing human AQP3, AQP7, AQP9 and AQP10. Oocyte transport of ^{73}As(III) was assayed for 90 seconds. Each bar represents the average of three assays. From left to right, oocytes were injected with H_2O, hAQP3 cRNA, hAQP7 cRNA, hAQP9 cRNA and hAQP10 cRNA. Reproduced with permission from Liu Z et al. Biochem Biophys Res Commun 2004; 316(4):1178-1185;40 ©2004 Elsevier.[40]

predominantly DMA(V), in urine.[43] In early studies, As methylation was originally thought to be considered a detoxification process. However, the in vivo toxicity of inorganic and organic arsenicals is DMA(III)>MMA(III)>As(III)>As(V)>DMA(V)>MMA(V)>TMAO,[44] which means trivalent MMA(III) and DMA(III) are more toxic than inorganic As(V) or As(III).[45,46] Pentavalent DMA(V) and TMAO are approximately a hundred-fold and a thousand-fold less toxic than As(III), respectively.[47] Since methylation of inorganic As activates it to more reactive and toxic forms, elucidating the transport pathway for As methylation products is a topic of considerable importance.

Given to the probable structural similarity between As(III) and MMA(III), it is likely that MMA(III) also has a neutral hydroxylated structure of $CH_3As(OH)_2$. Therefore it is reasonable to study the MMA(III) transport via aquaglyceroporins. The function of AQP9 in the conduction of MMA(III) has been studied in a yeast expression system. In yeast expressing rat AQP9, MMA(III) has been shown to be facilitated more efficiently than As(III). Our results have shown that AQP9 can facilitate MMA(III) transport at a rate three times that of inorganic As(III) and as a result, the AQP9 expressing yeast is more sensitive to MMA(III) than to As(III).[48] Consistent with its toxicity level in yeast, MMA(III) was shown to be more toxic in human than inorganic As(III), probably because of the higher uptake efficiency via aquaglyceroporins.

What is the physiological significance of AQP9's conductivity for both inorganic As and its cellular metabolite? Since AQP9 is a bi-directional channel it can mediate both the influx and efflux of its substrates. This has been demonstrated in our lab using a rat hepatoma cell line MCA7777 which expresses AQP9 (unpublished data). Therefore, we hypothesized that AQP9 is the major uptake pathway for inorganic As(III) originating from water or food into liver, the site of As methylation. AQP9 then conducts one of the methylation products, MMA(III), into the blood stream, where it is then carried to other tissues and eventually to the kidney for excretion into the urine.[48] The whole process is illustrated in Figure 3. According to this hypothesis, AQP9 can serve both functions, accumulation and detoxification, making AQP9 a key target in As homeostasis in human.

Recently, we studied the metalloid uptake by zebrafish aquaglyceroporins. Zebrafish have a large family of aquaporins that includes at least seven members of orthodox aquaporins and seven

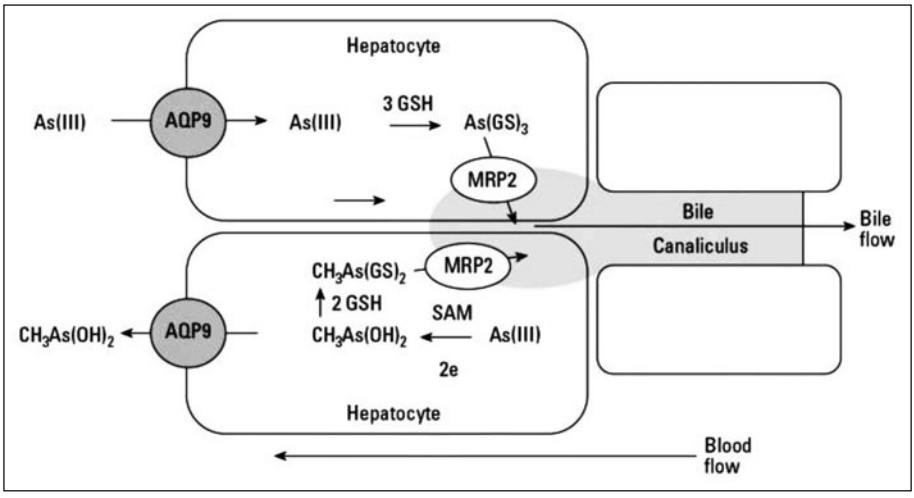


Figure 3. Proposed pathways of trivalent As transport in liver. Abbreviations: GSH: glutathione; SAM: S-adenosylmethionine. Trivalent As in the form of As(III) flows down a concentration gradient from blood into hepatocytes through AQP9, which is the major aquaglyceroporin in liver.[14] In the cytosol of the hepatocyte, As(III) can be either glutathionylated or methylated to MMA(V), which is reduced to MMA(III) ($CH_3As(OH)_2$). $As(GS)_3$ is pumped into bile by the MRP2 and perhaps by other members of the ABC superfamily of ATPases. Alternatively, As(III) can be methylated and reduced to MMA(III), which then flows down its concentration gradient via AQP9 into blood. Reproduced with permission from Liu Z et al. Environ Health Perspect 2006; 114(4):527-31.[48]

members of aquaglyceroporins. They all share high sequence similarity with human homologues, some of which are included in the phylogenetic tree in Figure 1. These channel proteins are believed to play significant roles in osmolarity balance and cold tolerance. However, they may also participate in metalloid As accumulation, as fish are well known and efficient accumulators for multiple metals and metalloids. Recently, we examined the capacity of zebrafish aquaglyceroporins for metalloid uptake. Five out of the seven aquaglyceroporins, Aqp9a, Aqp9b, Aqp3, Aqp3l and Aqp10 all conduct transport of both As(III) and MMA(III). Two proteins, Aqp3l and Aqp9b transport MMA(III) at tenfold higher efficiency than they transport As(III) (unpublished data). These aquaglyceroporins are expressed in varieties of organs and tissues, which explains the extensive As retention in fish (unpublished data). Furthermore, the expression patterns of these zebrafish channels differ from that of human channels (unpublished data); suggesting the divergent evolution has made mammalian AQPs more tissue-specific and less redundant compared with lower vertebrates. The differential expression of these zebrafish AQPs may account for the extensive accumulation of As in a variety of different organs.

As(V) is transported into cells via a different type of pathway than As(III). At physiological pH, pentavalent As(V) is negatively charged. Since As(V) is an analogue of phosphate and is an oxyanion, it is taken up via phosphate transporters in microorganisms. In *E. coli*, two phosphate transporters, *Pit* and *Pst*, catalyze uptake of As(V).[49] In eukaryotic microbes such as *S. cerevisiae*, As(V) is also taken up by phosphate transporters, although these transporters are unrelated to the phosphate transporters in bacterial systems.[50] In mammals, some Na^+/Pi-cotransporters facilitate phosphate in an As(V) sensitive manner, suggesting that they might use As(V) as a substrate.[51,52] Recently, rat Type II phosphate transporters were shown to transport As(V) in an oocyte expression system.[53]

Based on the observations of As(III) uptake by aquaglyceroporins from different vertebrate species, it is obvious that the family of aquaglyceroporins plays important roles in metalloid

translocation in addition to its role in water balance and nutrient transport. What is the evolutionary significance of the As uptake by these nutrition channel proteins? Phylogenetic studies using a variety of MIP proteins indicate that there is a distinct and early separation of aquaporin and aquaglyceroporins. It is hypothesized that the evolution of three mammalian aquaglyceroporins (AQP3, AQP7 and AQP9) and other aquaporins may reflect the two genomic duplication events in vertebrates.[54] Arsenic is not required by living organisms and its transport may not have played a significant role in the evolution of aquaglyceroporins. Arsenic existed in the environment before the appearance of any known life form and has not been shown to be a dietary requirement. Therefore, it is unlikely that the aquaglyceroporins evolved to transport As to positively influence organismal survival rates. Instead, we suggest that because As(III) is a molecular mimic of glycerol it is accidentally taken up by family of aquaglyceroporins. On another hand, as channels that conduct transport dependent on concentration gradient, it is possible that when intracellular As concentration is higher, aquaglyceroporins may facilitate the efflux of As, making the channel play a positive role in As detoxification.

Molecular Mechanisms for Arsenic Translocation by Aquaglyceroporins

The three dimensional structures of several orthodox aquaporins including human aquaporin AQP1 and *E. coli* aquaglyceroporin GlpF have been solved using crystallographic and biochemical methods and their translocation properties for water and glycerol were studied.[55] These channels share some common structural properties. First, they are homotetramers of four distinct water channels. Each monomer consists of six transmembrane alpha-helical domains with cytoplasmically orientated amino and carboxyl termini. Second, their pores are lined by far fewer carbonyl groups which provides less efficient dehydration for ionic solutions while possibly optimizing rapid water permeation. Third, two asparagine-proline-alanine (NPA) signature motifs are located within two highly conserved loop domains which fold back and meet in the center of the channel. These NPA motifs seem to act as filters, one imposing size restrictions and the second creating an electrostatic field that forms an energy barrier to protons and ions. In addition, the narrowest part, which is gated by an arginine residue that immediately follows the second NPA motif and several other hydrophobic residues, imposes a severe restriction so that only one water molecule may pass through the channel at a given time. In comparing the structures of aquaporins and aquaglyceroporins, it is clear that they have different pore diameters. The narrowest part of the pore is gated by a positive charged arginine (arg195 in AQP1 and arg206 in GlpF) and its surrounding hydrophobic region. A substantially larger diameter is observed in GlpF when compared with AQP1.[55] Theoretically, the orthodox water channel can not permeate larger molecules such as As and glycerol due to size exclusion. However, the aquaglyceroporins, which have a larger pore diameter, do not always transport water efficiently. Other molecular mechanisms that are involved in the selectivity for water and glycerol in aquaporins and aquaglyceroporins have been investigated.[56] A small structural change can lead to the transport of different substrates. For example, the water permeation efficiency for human AQP9 is rather limited. It is reported that mutation of two very conserved residues (Y222P/W223L) in an insect aquaporin (AQPcic) can switch the channel selectivity from water to glycerol.[57]

Since the diffusion of As(III) is facilitated by aquaglyceroporins by mimicking glycerol molecules, the translocation mechanisms for these substrates must have shared common features. The molecular mechanisms that are involved in the As selectivity via aquaglyceroporins are not fully clear yet. The studies using rat AQP9 showed that the positive charged arg219 that forms the narrowest part of the pore plays critical roles in the translocation of glycerol, As(III) and MMA(III). A mutation of R219A can lead to the drastic decrease of both glycerol and arsenical transport. However, the mutation of R219K imposed no impact on the channel function, which indicates that a charged residue at the respective position is critical for proper channel function.[48] Meanwhile, the positive charge of arginine also serves as a filter for ions, making this residue an important site for multiple purposes.[58]

Even though these substrates permeate through the same channel in aquaglyceroporins, the transport for inorganic As(III), organic MMA(III) and glycerol by different aquaglyceroporins is

expected to share some minor differences. This has been supported by many observations. First, not all aquaglyceroporins can transport As(III). For example, human AQP10, a channel that transports glycerol, cannot conduct trivalent As(III) according to our study in a *Xenopus* oocyte system.[40] In addition, the transport efficiency for glycerol and As(III) is not proportional; showing the translocation mechanism for these substrates is not exactly the same. Second, some aquaglyceroporins can discriminate between inorganic As(III) and MMA(III), two molecules that have similar conformational structure. With a methyl group which replaces a hydroxyl group, MMA(III) is expected to be larger than As(III). Our studies showed that GlpF from *E. coli* and FPS1 from yeast can transport As(III) but not MMA(III). However, AQP9 conducts MMA(III) at a much higher efficiency than As(III).[48] Recently, a research indicate that a single mutation in extracellular loop of parasite *Leishmania* aquaglyceroporin can discriminate metalloid and glycerol permeation, showing the differences of molecular mechanisms of these substrate transport (see also the chapter by Mukhopadhyay and Beitz in this book).[59]

Although aquaglyceroporins can transport both organic polyols and inorganic As(III), it is not clear if these substrates can inhibit each other. Since they may compete for the same initial binding and translocation sites, it is expected that they will be inhibited by each other. On the other hand, aquaglyceroporins are channel proteins, therefore the binding of the substrates will be transient and the inhibition is not expected to be competitive. So we predict that the transport rate is proportional to the concentration of each substrate.

By molecular mimicry of their natural substrate glycerol, As can be recognized and transported by aquaglyceroporins. The elegant structure of the channels enables an efficient transport for these different substrates. On the other hand, the differential transport for a variety of substrates can be achieved by changes in the pore size, as well as other secondary forces, such as hydrophobic forces and hydrogen bond.

Arsenic Toxicity in Relation of Aquaglyceroporins Regulation

Although As(III) may have more than one uptake pathway, aquaglyceroporins are efficient As transport channels and likely to be responsible for the majority of As(III) uptake in mammalian tissues that express these proteins. Therefore, one would expect that the expression level of these channels will directly correlate to As cellular retention and toxicity. Does environmental As regulate the expression of the aquaglyceroporin channels? There is no direct evidence to show that the expression level of aquaglyceroporins can be regulated by metalloid As. In an As-resistant human lung adenocarcinoma cell line R15, AQP3 was expressed two fold lower.[60] Microarray studies using As(III) treated mammalian cells such as human lymphoblasts,[61] animal tissues such as murine liver[62,63] and murine skin[63] did not locate the expressional changes of any aquaglyceroporins. However, a comprehensive study is still needed to clarify if aquaglyceroporins are regulated by other As compounds with different exposure levels. More vertebrate organs or cells also need to be examined.

As channels evolved for the transport of nutrition, aquaglyceroporins can be regulated under different nutritional conditions. For example, with poor nutritional conditions, such as starvation, the rat liver takes up glycerol for gluconeogenesis. Expression of AQP9 in liver was induced up to 20-fold in rats fasted for 24-96 hours.[14] Thus it is reasonable to conclude that the different nutrition levels of individual humans will affect the As accumulation specifically through the regulation of the expression of liver aquaglyceroporin AQP9. Poor nutritional states such as starvation may increase the AQP9 expression and therefore increase the As(III) uptake and accumulation in liver. In addition, insulin is also found to regulate expression of certain types of aquaglyceroporins, such as kidney AQP7 and liver AQP9.[64] All of these changes are likely to affect As uptake and metabolism indirectly.

In addition, the expression level of aquaglyceroporins is found to be associated with pathological states. For example, a study of AQP9 expression in normal human brain tissues and human brain tumors indicates an increased AQP9 expression level in tumor border.[65] In rat, AQP9 was found to be highly expressed in glioma cells, which suggests an upregulation to counteract the

glioma-associated lactic acidosis by clearance of glycerol and lactate from the extracellular space.[66] In these abnormal physiological states, it is reasonable to state that the As uptake is increased accordingly with an increased expression level of AQP9. Therefore it is reasonable to conclude that individuals at different nutritional and pathological status will have different As tolerance and carcinogenesis. However, there is a lack of corresponding epidemiological studies to support this hypothesis. A more detailed discussion can be found in the chapter by Mukhopadhyay and Beitz in this book.

Perspectives

Aquaglyceroporins are the first transporters that have been identified to facilitate the uptake of metalloid As(III) and MMA(III) in vertebrates. In addition, aquaglyceroporins are important channels for normal cellular function, conducting nutritional molecules such as water, glycerol and urea. These multi-task properties highlight the special importance of this family in both physiological and toxicological research. In the future, it is expected that their structure, regulation and cellular functions in normal physiology and toxin metabolism will be further clarified. These studies may provide us with insight into the molecular mechanisms of As transport and toxicity, as well as to the complete understanding of As carcinogenesis. The stories of aquaglyceroporins will continue.

Conclusion

Multiple aquaglyceroporins(AQPs) from mammals include humans, rodents and fish are able to efficiently transport toxic As, including both environmentally occurring inorganic As(III) and intracellular generated organic As. The bi-directional transport via AQPs highlights the multiple roles of these channels in both As uptake and efflux.

Acknowledgements

We appreciate Mr. Joseph McDermott at Oakland University for his help on the plotting of phylogenetic tree. Thanks are also given to Dr. Sarah Hosch and Ms. Lauren Beene at Oakland University for their critical review and editing of this chapter.

References

1. Preston GM, Carroll TP, Guggino WB et al. Appearance of water channels in Xenopus oocytes expressing red cell CHIP28 protein. Science 1992; 256:385-387.
2. Agre P, King LS, Yasui M et al. Aquaporin water channels—from atomic structure to clinical medicine. J Physiol 2002; 542:3-16.
3. Roudier N, Verbavatz JM, Maurel C et al. Evidence for the presence of aquaporin-3 in human red blood cells. J Biol Chem 1998; 273:8407-8412.
4. Ishibashi K, Kuwahara M, Gu Y et al. Cloning and functional expression of a new water channel abundantly expressed in the testis permeable to water, glycerol and urea. J Biol Chem 1997; 272:20782-20786.
5. Skowronski MT, Lebeck J, Rojek A et al. AQP7 is localized in capillaries of adipose tissue, cardiac and striated muscle: implications in glycerol metabolism. Am J Physiol Renal Physiol 2007; 292:F956-965.
6. Elkjaer ML, Birn H, Agre P et al. Effects of microtubule disruption on endocytosis, membrane recycling and polarized distribution of Aquaporin-1 and gp330 in proximal tubule cells. Eur J Cell Biol 1995; 67:57-72.
7. Ishibashi K, Kuwahara M, Gu Y et al. Cloning and functional expression of a new aquaporin (AQP9) abundantly expressed in the peripheral leukocytes permeable to water and urea, but not to glycerol. Biochem Biophys Res Commun 1998; 244:268-274.
8. Okada S, Misaka T, Matsumoto I et al. Aquaporin-9 is expressed in a mucus-secreting goblet cell subset in the small intestine. FEBS Lett 2003; 540:157-162.
9. Badaut J, Petit JM, Brunet JF et al. Distribution of Aquaporin 9 in the adult rat brain: preferential expression in catecholaminergic neurons and in glial cells. Neuroscience 2004; 128:27-38.
10. Ishibashi K, Morinaga T, Kuwahara M et al. Cloning and identification of a new member of water channel (AQP10) as an aquaglyceroporin. Biochim Biophys Acta 2002; 1576:335-340.

11. Lance SL, Peterson AS, Hagedorn M. Developmental expression of aquaporin-3 in zebrafish embryos (Danio rerio). Comp Biochem Physiol C Toxicol Pharmacol 2004; 138:251-258.
12. Rodriguez A, Catalan V, Gomez-Ambrosi J et al. Role of aquaporin-7 in the pathophysiological control of fat accumulation in mice. FEBS Lett 2006; 580:4771-4776.
13. Hibuse T, Maeda N, Nagasawa A et al. Aquaporins and glycerol metabolism. Biochimica et Biophysica Acta 2006; 1758:1004-1011.
14. Carbrey JM, Gorelick-Feldman DA, Kozono D et al. Aquaglyceroporin AQP9: solute permeation and metabolic control of expression in liver. Proc Natl Acad Sci USA 2003; 100:2945-2950.
15. Tsukaguchi H, Shayakul C, Berger UV et al. Molecular characterization of a broad selectivity neutral solute channel. J Biol Chem 1998; 273:24737-24743.
16. Ishibashi K, Sasaki S, Fushimi K et al. Molecular cloning and expression of a member of the aquaporin family with permeability to glycerol and urea in addition to water expressed at the basolateral membrane of kidney collecting duct cells. Proc Natl Acad Sci USA 1994; 91:6269-6273.
17. Chakrabarti N, Roux B, Pomes R. Structural determinants of proton blockage in aquaporins. J Mol Biol 2004; 343:493-510.
18. Ramirez-Solis A, Mukopadhyay R, Rosen BP et al. Experimental and theoretical characterization of arsenite in water: insights into the coordination environment of As-O. Inorg Chem 2004; 43:2954-2959.
19. Oremland RS, Stolz JF. The ecology of arsenic. Science 2003; 300:939-944.
20. Mukhopadhyay R, Rosen BP, Phung LT et al. Microbial arsenic: from geocycles to genes and enzymes. FEMS Microbiol Rev 2002; 26:311-325.
21. Mukhopadhyay R, Rosen BP. Arsenate reductases in prokaryotes and eukaryotes. Environ Health Perspect 2002; 110:745-748.
22. Abernathy CO, Thomas DJ, Calderon RL. Health effects and risk assessment of arsenic. J Nutr 2003; 133:1536S-1538S.
23. Nordstrom DK. Public health. Worldwide occurrences of arsenic in ground water. Science 2002; 296:2143-2145.
24. Xia Y, Liu J. An overview on chronic arsenism via drinking water in PR China. Toxicology 2004; 198:25-29.
25. Chowdhury UK, Biswas BK, Chowdhury TR et al. Groundwater arsenic contamination in Bangladesh and West Bengal, India. Environ Health Perspect 2000; 108:393-397.
26. Alam MG, Allinson G, Stagnitti F et al. Arsenic contamination in Bangladesh groundwater: a major environmental and social disaster. Int J Environ Health Res 2002; 12:235-253.
27. Smith AH, Lingas EO, Rahman M. Contamination of drinking-water by arsenic in Bangladesh: a public health emergency. Bull World Health Organ 2000; 78:1093-1103.
28. Tchounwou PB, Patlolla AK, Centeno JA. Carcinogenic and systemic health effects associated with arsenic exposure—a critical review. Toxicol Pathol 2003; 31:575-588.
29. Ng JC, Wang J, Shraim A. A global health problem caused by arsenic from natural sources. Chemosphere 2003; 52:1353-1359.
30. Suner MA, Devesa V, Clemente MJ et al. Organoarsenical species contents in fresh and processed seafood products. J Agric Food Chem 2002; 50:924-932.
31. Morton J, Mason H. Speciation of arsenic compounds in urine from occupationally unexposed and exposed persons in the UK using a routine LC-ICP-MS method. J Anal Toxicol 2006; 30:293-301.
32. Schmeisser E, Goessler W, Francesconi KA. Human metabolism of arsenolipids present in cod liver. Anal Bioanal Chem 2006; 385:367-376.
33. Domingo JL, Bocio A, Falco G et al. Benefits and risks of fish consumption Part I. A quantitative analysis of the intake of omega-3 fatty acids and chemical contaminants. Toxicology 2007; 230:219-226.
34. Falco G, Bocio A, Llobet JM et al. Health risks of dietary intake of environmental pollutants by elite sportsmen and sportswomen. Food Chem Toxicol 2005; 43:1713-1721.
35. Zavala YJ, Duxbury JM. Arsenic in rice: I. Estimating normal levels of total arsenic in rice grain. Environ Sci Technol 2008; 42:3856-3860.
36. Wang JP, Qi L, Moore MR et al. A review of animal models for the study of arsenic carcinogenesis. Toxicol Lett 2002; 133:17-31.
37. Rosen BP. Biochemistry of arsenic detoxification. FEBS Lett 2002; 529:86-92.
38. Sanders OI, Rensing C, Kuroda M et al. Antimonite is accumulated by the glycerol facilitator GlpF in Escherichia coli. J Bacteriol 1997; 179:3365-3367.
39. Miller AJ, Zhou JJ. Xenopus oocytes as an expression system for plant transporters. Biochim Biophys Acta 2000; 1465:343-358.
40. Liu Z, Carbrey JM, Agre P et al. Arsenic trioxide uptake by human and rat aquaglyceroporins. Biochem Biophys Res Commun 2004; 316:1178-1185.
41. Liu Z, Shen J, Carbrey JM et al. Arsenite transport by mammalian aquaglyceroporins AQP7 and AQP9. Proc Natl Acad Sci USA 2002; 99:6053-6058.

42. Thomas DJ, Li J, Waters SB et al. Arsenic (+3 oxidation state) methyltransferase and the methylation of arsenicals. Exp Biol Med (Maywood) 2007; 232(1):3-13.
43. Alauddin M, Alauddin ST, Bhattacharjee M et al. Speciation of arsenic metabolite intermediates in human urine by ion-exchange chromatography and flow injection hydride generation atomic absorption spectrometry. J Environ Sci Health A Tox Hazard Subst Environ Eng 2003; 38:115-128.
44. Akter KF, Owens G, Davey DE et al. Arsenic speciation and toxicity in biological systems. Rev Environ Contam Toxicol 2005; 184:97-149.
45. Petrick JS, Ayala-Fierro F, Cullen WR et al. Monomethylarsonous acid (MMA(III)) is more toxic than arsenite in Chang human hepatocytes. Toxicol Appl Pharmacol 2000; 163:203-207.
46. Drobna Z, Waters SB, Devesa V et al. Metabolism and toxicity of arsenic in human urothelial cells expressing rat arsenic (+3 oxidation state)-methyltransferase. Toxicol Appl Pharmacol 2005; 207:147-159.
47. Hirano S, Kobayashi Y, Cui X et al. The accumulation and toxicity of methylated arsenicals in endothelial cells: important roles of thiol compounds. Toxicol Appl Pharmacol 2004; 198:458-467.
48. Liu Z, Styblo M, Rosen BP. Methylarsonous acid transport by aquaglyceroporins. Environ Health Perspect 2006; 114:527-531.
49. Rosenberg H, Gerdes RG, Chegwidden K. Two systems for the uptake of phosphate in Escherichia coli. J Bacteriol 1977; 131:505-511.
50. Bun-ya M, Shikata K, Nakade S et al. Two new genes, PHO86 and PHO87, involved in inorganic phosphate uptake in Saccharomyces cerevisiae. Curr Genet 1996; 29:344-351.
51. Hartmann CM, Wagner CA, Busch AE et al. Transport characteristics of a murine renal Na/Pi-cotransporter. Pflugers Arch 1995; 430:830-836.
52. Xu H, Inouye M, Missey T et al. Functional characterization of the human intestinal NaPi-IIb cotransporter in hamster fibroblasts and Xenopus oocytes. Biochim Biophys Acta 2002; 1567:97-105.
53. Villa-Bellosta R, Sorribas V. Role of rat sodium/phosphate cotransporters in the cell membrane transport of arsenate. Toxicol Appl Pharmacol 2008; 232:125-134.
54. Zardoya R, Ding X, Kitagawa Y et al. Origin of plant glycerol transporters by horizontal gene transfer and functional recruitment. Proc Natl Acad Sci USA 2002; 99:14893-14896.
55. Fu D, Libson A, Miercke LJ et al. Structure of a glycerol-conducting channel and the basis for its selectivity. Science 2000; 290:481-486.
56. Hub JS, de Groot BL. Mechanism of selectivity in aquaporins and aquaglyceroporins. Proc Natl Acad Sci USA 2008; 105:1198-1203.
57. Lagree V, Froger A, Deschamps S et al. Switch from an aquaporin to a glycerol channel by two amino acids substitution. J Biol Chem 1999; 274:6817-6819.
58. de Groot BL, Grubmuller H. Water permeation across biological membranes: mechanism and dynamics of aquaporin-1 and GlpF. Science 2001; 294:2353-2357.
59. Uzcategui NL, Zhou Y, Figarella K et al. Alteration in glycerol and metalloid permeability by a single mutation in the extracellular C-loop of Leishmania major aquaglyceroporin LmAQP1. Mol Microbiol 2008; 70:1477-1486.
60. Lee TC, Ho IC, Lu WJ et al. Enhanced expression of multidrug resistance-associated protein 2 and reduced expression of aquaglyceroporin 3 in an arsenic-resistant human cell line. J Biol Chem 2006; 281:18401-18407.
61. Komissarova EV, Li P, Uddin AN et al. Gene expression levels in normal human lymphoblasts with variable sensitivities to arsenite: identification of GGT1 and NFKBIE expression levels as possible biomarkers of susceptibility. Toxicol Appl Pharmacol 2008; 226:199-205.
62. Chen H, Li S, Liu J et al. Chronic inorganic arsenic exposure induces hepatic global and individual gene hypomethylation: implications for arsenic hepatocarcinogenesis. Carcinogenesis 2004; 25:1779-1786.
63. Ahlborn GJ, Nelson GM, Ward WO et al. Dose response evaluation of gene expression profiles in the skin of K6/ODC mice exposed to sodium arsenite. Toxicol Appl Pharmacol 2008; 227:400-416.
64. Kuriyama H, Shimomura I, Kishida K et al. Coordinated regulation of fat-specific and liver-specific glycerol channels, aquaporin adipose and aquaporin 9. Diabetes 2002; 51:2915-2921.
65. Warth A, Mittelbronn M, Hulper P et al. Expression of the water channel protein aquaporin-9 in malignant brain tumors. Appl Immunohistochem Mol Morphol 2007; 15:193-198.
66. Tan G, Sun SQ, Yuan DL. Expression of the water channel protein aquaporin-9 in human astrocytic tumours: correlation with pathological grade. J Int Med Res 2008; 36:777-782.

Elsbeth Bienert

Chapter 7

Molecular Mechanisms of Boron Transport in Plants:
Involvement of Arabidopsis NIP5;1 and NIP6;1

Kyoko Miwa, Mayuki Tanaka, Takehiro Kamiya and Toru Fujiwara*

Abstract

Understanding of the molecular mechanisms of boron (B) transport has been greatly advanced in the last decade. BOR1, the first B transporter in living systems, was identified by forward genetics using *Arabidopsis* mutants. Genes similar to BOR1 have been reported to share different physiological roles in plants. *NIP5;1*, a member of aquaporins in *Arabidopsis*, was then identified as a boric acid channel gene responsible for the B uptake into roots. *NIP6;1*, the most similar gene to *NIP5;1*, encodes a B channel essential for B distribution to young leaves. In the present chapter, recent advancement of the understanding of molecular mechanisms of B transport and roles of NIP genes are discussed.

Physiological Function of Boron in Plants

Essentiality of Boron in Plants

Essentiality of boron (B) for plants was established more than eighty years ago.[1] B requirements in animals have also been reported in the last several years. Insufficient B reduces plant productivity and reduced quality and quantity of agricultural production are reported in more than 70 countries in the world.[2] For example, the southeast region of China, comprising about 15% of China's land, contains soils with less than 0.25 ppm of water-extractable B, a concentration well below the threshold for normal plant growth.[3]

B deficiency affects the newly growing portions of plants rather than the mature tissues: Major B deficiency symptoms are the cessation of root elongation, reduced leaf expansion and the loss of fertility.[4-6] B deficiency seems to affect cell elongation rather than cell division.[6] Kouchi and Kumazawa observed B deficiency-induced cell swelling in roots and predicted a role of B in cell wall structure.[7] B deficiency also affects membrane function and metabolic activities.[8]

In addition, excess B supply is known to be toxic to plants.[9] Typical visible symptoms of B toxicity include necrosis of marginal regions of leaves, presumably resulting from B accumulation along the transpiration stream. Decreased chlorophyll concentrations, reduced growth and decreased CO_2 fixation were also reported.

In aqueous solution with neutral pH, B is mostly present as boric acid $B(OH)_3$, a weak Lewis acid accepting OH^- group to form the borate ion $B(OH)_4^-$ (*pKa* 9.25, $B(OH)_3 + H_2O = B(OH)_4^- + H^+$). Boric acid reacts with alcohols to form borate esters in a pH-dependent manner and with rapid kinetics.[10] Since the most stable borate esters are formed with *cis*-diols, compounds like ribonucleotides are possible binding targets of boric acid.

*Corresponding Author: Toru Fujiwara—Biotechnology Research Center, The University of Tokyo, Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan. Email: atorufu@mail.ecc.u-tokyo.ac.jp

MIPs and Their Role in the Exchange of Metalloids, edited by Thomas P. Jahn and Gerd P. Bienert.

Rhamnogalacturonan-II Binds Boron

Under conditions of limited B supply, the major portion of B in tobacco cultured cells,[11] squash plants[12] and *A. thaliana* plants[13] has been found in the cell wall fraction, suggesting that the cell wall is the site of B function in cells. Molecular identity of B-binding compounds was reported in the late 1990s. First B-polysaccharide complexes were isolated from cell walls of radish roots by Matoh et al.[14] After the purification steps, which include homogenization, driselase treatment and fractionation, a 7.5 kDa B-polysaccharide complex was purified.[11] B-NMR analysis of this complex indicated the presence of B as tetrahedral 1:2 borate-diol diestel. Kobayashi et al identified the B-polysaccharides as rhamnogalacturonan (RG)-II by glycosyl-linkage analysis and the presence of monosaccharides that are only known to be present in RG-II in the B-polysaccharides fraction.[15] In addition, removal of B by acid-hydrolysis reduced the molecular weight by one-half leading to propose that boric acid links two RG-II molecules. Ishii and Matsunaga also reported B-polysaccharide from cell walls of sugar beet as RG-II.[16]

Dimeric RG-II-B (dRG-II-B) was shown to form in vitro in the presence of boric acid, from monomeric RG-II (mRG-II) isolated from red wine.[17,18] Comparison of glycosyl linkage composition between mRG-II and dRG-II-B formed from mRG-II in vitro, revealed that 1-2,3,3- linked apiosyl residue is present only in dRG-II-B,[17,18] indicating that borate esters are linked to the apiosyl residues.

RG-II is composed of a backbone formed by homogalacturonan (HG, linear 1,4-linked D-galacturonic acid) and four side chains,[19] two of those contain apiosyl residues. Esterification of apiosyl residues was found in the apiofuranosyl residue of the 2-O-methyl-D-xylose-containing side chains in each of the subunits of the dimer, but not in the apiofuranosyl residue in any of the two aceric acid-containing side chains (Fig. 1).[20] This shows that tetrahedral borate diol ester cross-links the apiosyl residues of the same side chains of the two mRG-II subunits. The site of borate esterification is identical between naturally occurring dRG-II-B and the in vitro synthesized one.

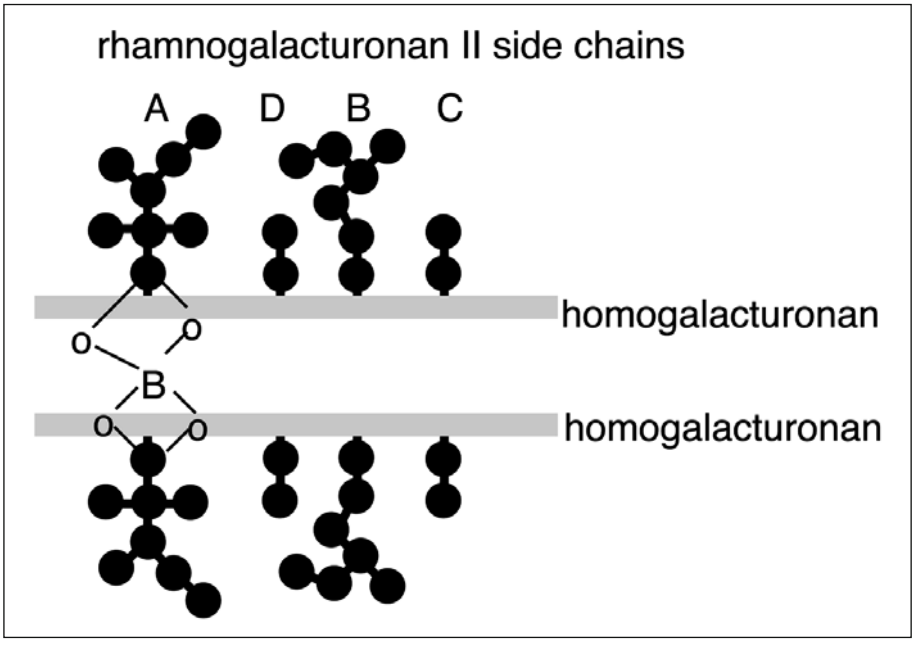


Figure 1. Borate crosslinking of rhamnogalacturonan II. A,B,C, and D represent different side chains of rhamnogalacturonan II. Boron is known to be bound to the side chain A.

Furthermore, it was shown that dimerization of RG-II in vitro was stimulated by the addition of divalent cations such as Sr^{2+}, Pb^{2+}, Ba^{2+}.[18] Kobyashi et al demonstrated that Ca^{2+} was important for dimerization of RG-II and stabilization of dRG-II-B.[21] In addition to the stabilization effect of Ca^{2+} on the dRG-II-B complex, it was also shown that dRG-II-B contains Ca^{2+}. According to the report by Iwai et al, Sr, Ba, La and Pb were detected in the same retention time as dRG-II-B in size-exclusion chromatography (SEC) with inductively coupled plasma-mass spectrometry(ICP-MS) analysis of squash xylem exudates.[22] Therefore, it is likely that not only Ca but also Sr, Ba, La and Pb bind and stabilize dRG-II-B in planta.

Although RG-II binds to B, synthesis of RG-II is not affected by B conditions. Ishii et al showed that B conditions in media did not affect RG-II sugar composition in plants.[23] In the RG-II isolated from B-deficient pumpkin tissues, which is mostly present as a monomeric form of RG-II (mRG-II), the primary structure of RG-II was not altered. This suggests that synthesis of RG-II is not strongly affected by B nutrition.

Involvement of Rhamnogalacturonan-II in B Function

The roles of RGII-B complexes have been studied in the following time. Ishii (2001) demonstrated that B deficiency treatments of pumpkin plants resulted in swollen cell walls and in such cell walls 80-90% of RG-II is present as mRG-II.[23] Application of boric acid converted mRG-II to dRG-II-B and rescued swelling of the cell walls, demonstrating correlation between the mRG-II/dRG-II-B ratio and growth of the plants. It is possible that dimerization of RG-II has a role in maintenance of plant cell walls.

O'Neill et al suggested that efficient formation of dRG-II-B is essential for normal leaf expansion using *A. thaliana* mutant *mur1*.[24] *MUR1* encodes an isoform of GDP-D-mannose-4,6-dehydratase, which catalyze the synthesis of GDP-L-fucose.[25] The content of L-fucose in aerial portion of *mur1* mutant plants is reduced to 2% of those of wild type plants.[26] As fucosyl residues are known to be present in the two side chains of RG-II and xyloglucan in cell walls, both RG-II and xyloglucan are likely to be affected in the *mur1* mutant plants. It was shown that the RG-II in the aerial portion of the *mur1* mutant plants exhibits an abnormal glycosyl composition.[24,27] A similar mutant, *mur2* is also defective in fucose metabolism. In this mutant, xyloglucan fucosyltransferase (AtFUT1) is defective and contents of fucosylated xyloglucan were reduced to 2% of those of the wild type, but contents of L-fucose in RG-II are normal.[28] Thus, by comparing *mur1* and *mur2* mutants, a role of fucose in RG-II can be discussed. The *mur1* mutant plants, but not *mur2* mutant plants, exhibited reduced rosette leaf expansion under normal B conditions. It was also demonstrated that extents of crosslinking of RG-II by borate in *mur1* were lower than those of wild-type plants. The growth defect of *mur1* mutant plants was diminished by supplying high levels of B and under this condition; the extent of crosslinking of RG-II in the mutant plants was close to that in wild type plants. Taken all together, it was suggested that crosslinking of RG-II by borate is essential for normal leaf expansion.[24] It is considered that cross-linking of RG-II by borate establishes a pectic network, which contributes to the strength of cell walls.

This was further supported by Ryden et al (2003), who demonstrated that *mur1* mutant plants showed reduction in strength of hypocotyls as it was observed in *mur2* and *mur3*, xyloglucan glycosyltransferase mutant plants.[29] This phenotype in *mur1* was rescued by addition of higher concentration of B; therefore it was concluded that dRG-II-B was important for tensile strength of cell walls.

RG-II is also suggested to be involved in B signaling. Using antibody against RG-II, Baluska et al demonstrated that dRG-II-B and partially esterified HG were internalized into brefeldin A compartments in meristematic cells of maize roots.[30] This suggests that pectins containing dRG-II-B were internalized via endocytosis. In addition, this internalization of dRG-II-B was inhibited upon B deprivation in maize and wheat root apexes.[31] It was proposed that cell walls are the site of the primary reaction after deprivation of B and that this primary reaction leads to B deficiency responses. This endocytosis-mediated pectin signaling is likely to be a B-deficiency signal from cell wall to cytoplasm.

Roles of B Other Than Binding to RG-II

Although RG-II in plant cell walls is the only compound that is shown to interact with B and is required for the physiological function of B (details are described above), demonstration of essentiality of B in other organisms including bacteria that do not contain RG-II implies the presence of other essential B containing or interacting compounds. The presence of RG-II was also demonstrated in pteridophytes and bryophytes.[19,32] The roles of RG-II in ferns and mosses are not clear at this time and the essentiality of B in these organisms is not reported.

Bassil demonstrated that boronic acids, which compete with boric acid for binding to *cis*-diols, caused the disruption of cytoplasmic strands and cell-to-cell wall detachment in tobacco cultured cells and proposed that B functioned for structure in cytoskeleton.[33]

Iwai et al (2003) reported the presence of unknown B-compounds in xylem sap of squash roots.[22] In SEC/ICP-MS, in addition to the peaks of dRG-II-B and free boric acid, another large peak was found, which corresponded to a borate compound with lower molecular weight than dRG-II-B. Since myo-inositol was detected in high concentration (15.2 $\mu g\ ml^{-1}$) as a free sugar having *cis*-diols, it seemed to be a candidate for a B-binding compound. However, myo-inositol did not form a complex with boric acid at pH 5.5 in vitro (the same condition as xylem sap), suggesting the presence of unknown B-compounds.

Physiological Analysis of B Transport

Three mechanisms of B transport in plants are known: passive diffusion across lipid bilayers, channel-mediated B transport and active B transport by transporters.

Passive Diffusion

In soil solutions at physiological pH, B is present as a noncharged molecule, boric acid $B(OH)_3$. Out of 14 essential elements of plants that are taken up from soil, B is the only element that is taken-up by plants not as ion, but as a noncharged molecule.

Classical physiological analysis up to 1990s indicated that the amount of B uptake is more or less proportional to the B concentration in the medium.[5] This was the basis for the notion that B was transported only by passive diffusion and distributed within the plant body along the transpiration streams. This assumption is supported by the high membrane permeability of boric acid. Raven (1980) calculated the permeability coefficient of boric acid to be in the order of 10^{-6} cm s^{-1} based on the ether-water partition coefficient and suggested that this permeability of boric acid was high enough to account for B uptake and transport in plants.[34] Experimental data also showed that the permeability of boric acid across artificial liposomes consisting of phosphatidylcholine was 4.9×10^{-6} cm s^{-1}, having a good agreement with the calculated values.[35] These authors also demonstrated that the permeability coefficient of boric acid varied from 7×10^{-6} to 9.5×10^{-9} cm s^{-1} depending on sterol composition, type of phospholipids, the presence of head groups, length of fatty acid chains and the pH in the medium.

It was then found, however, that a permeability coefficient of boric acid of plant membranes is orders of magnitude smaller than predicted. Dordas et al experimentally determined the coefficient as 3.9×10^{-7} cm s^{-1} and 2.4×10^{-8} cm s^{-1} for plasma membrane and plasma membrane-depleted vesicles of squash roots, respectively.[36] The permeability coefficient of boric acid was 4.4×10^{-7} cm s^{-1} in the plasma membrane of the charophyte algae *Chara corallina*.[37]

The lower permeability of boric acid in the biological membranes implies that passive diffusion of boric acid across lipid bilayer does not satisfy plant demand. Furthermore, introduction of ICP-MS for elemental analysis of B enabled a highly sensitive determination of B and discrimination of stable isotopes in samples. Consequently, B transport system other than passive diffusion—especially under conditions of the limited supply of B—was reported for the first time in 2000.[38] This is the first suggestion for the presence of B transporters in organisms.

Channel-Mediated B Transport

Dordas et al proposed B transport through channels.[36,39] They first demonstrated that boric acid permeation of the plasma membrane vesicles prepared from squash roots was partially inhibited (30%-39%) by mercuric chloride ($HgCl_2$) and phloretin, nonspecific channel blockers. The inhibition by $HgCl_2$ was reversible by 2-mercaptoethanol, suggesting that B transport at least in part go through proteinaceous channels. In addition, expression of a major intrinsic protein (MIP), maize PIP1, in *Xenopus* oocytes resulted in a 30% increase in B permeability.[36] They also demonstrated the presence of channel-mediated B transport in intact squash plants. Application of the channel inhibitors $HgCl_2$ (either 50 μM or 1 mM), phloretin (250 μM) and 4,4-diisothiocyanoatostilbene-2,2′-disulfonic acid (DIDS)(250 μM) reduced B uptake by 40%, 90%, 44% and 58%, respectively, suggesting possible involvement of channels.[39] Furthermore, addition to the assay media of noncharged compounds with a similar molecular size to boric acid, such as urea, acetamide, formamide, thiourea and glycerol, inhibited B uptake by 54%, 50%, 35%, 53% and 44%, respectively, further supporting the conclusion that boric acid was transported through channels.[39]

"Active" B Transport against Concentration Gradients

To transport B from roots to shoots, B needs to be loaded into xylem vessels. This process, known as xylem loading, is a transport process from symplast to apoplast (xylem). A series of studies demonstrated that B is transported against a concentration gradient under low-B conditions in xylem loading. In some cases, uptake of B from the medium into cells can also be against a concentration gradient. Dannel et al reported that low-B treated sunflower plants showed significantly higher B concentration in root cell saps and xylem exudates than in the medium and root cell saps, respectively.[38] On the other hand, when the plants were precultured in a high B medium, accumulation of B against a concentration gradient was not observed and B concentrations were proportional to those in the media. These results indicate that, under the low B condition, beside passive diffusion system, active transport systems transport B into root cell and xylem vessels against the B concentration gradients. This concentration mechanism of B was inhibited by treatments with low temperature and metabolic inhibitors, suggesting that the B transport system was energy-dependent. In addition, Dannel et al indicated that this transport system followed the Michaelis-Menten kinetics and the K_m and V_{max} value of low-B precultured sunflower roots for B uptake is 15 μM and 30 nmol g root FW^{-1} h^{-1}, respectively.[40] Similar results were reported in the charophyte algae *Chara corallina*.[37] Following one day of B starvation, B uptake into cells within 0 to 10 μM B supply appeared to be a combination of a saturable and a linear component. The saturable component followed the Michaelis-Menten kinetics with an apparent K_m of about 2 μM and V_{max} of about 135 pmol m^{-2} s^{-1}, suggesting the presence of a high affinity B transport system, operating under low B conditions.

In addition to the involvement of transporters in B uptake into roots and xylem loading under limited supply of B, B efflux from root cells under toxic level of B supply was also described. Hayes and Reid proposed borate efflux from roots to be a mechanism of B tolerance in barley.[41] When physiological comparison was performed between the high B-tolerant barley variety Sahara and the B-sensitive variety Schooner, B concentration of roots of Sahara was about half of that of Schooner under 5 mM B treatment. In addition, when plants were exposed to 5 mM B, B uptake into roots in Schooner reached nearly equilibrium state with the external solution within 3 hours, whereas Sahara maintained lower B concentrations in roots, which may lead to the lower B concentrations in xylem and leaves and enhanced B tolerance in Sahara. This suggests that Sahara actively excludes or pumps B out of the cells. Sahara was able to maintain the root B concentration at the low level in a wide range of external B concentrations (0.1 mM to 10 mM) suggesting that the B exclusion system is constitutively present in Sahara. Treatments with sodium azide, a metabolic inhibitor, or with anion channel inhibitors such as ethacrynic acid, A9C, DIDS, CHCA, affected the maintenance of reduced B concentration in Sahara. These results suggested the presence of borate efflux systems mediated by an anion transporter to protect plants from B toxicity. It is now known that this efflux is likely to be mediated by BOT1.[42]

Molecular Mechanisms of B Transport

BOR1, a Transport Protein Responsible for Xylem Loading

B-Deficiency Sensitive Mutant of *Arabidopsis thaliana*, *bor1-1*

As stated above, physiological analysis suggested the presence of a channel as well as a transporter for B. However, no such proteins (channel or transporter) responsible for this transport had been identified. The breakthrough came along with the coincidental isolation of *A. thaliana bor1-1* mutant, which was sensitive to B deficiency.[43] In *bor1-1*, leaf expansion of upper leaves was inhibited when plants were grown with 3 μM boric acid. Wild type plants grew normally at this B concentration. When *bor1-1* plants were supplemented with 30 μM boric acid, leaf expansion was normal, but the plants failed to set seeds. Supply of 150 μM boric acid rescued this sterility. Furthermore, B concentrations in rosette leaves and upper portion of the inflorescences were reduced in *bor1-1* mutant plants compared to those of wild type plants.[43]

To examine the distribution of B, B concentrations were determined in cell saps and water insoluble residues (WIRs) in shoots and roots.[13] In *bor1-1* reduction of B concentration was evident in shoots compared to the roots, indicating that the *bor1-1* mutant is defective in B translocation from roots to shoots. This coincides with the observation that the extent of root growth reduction in *bor1-1* were less than that in shoots.[44] B concentration in cell sap of *bor1-1* shoots was 5% and 65% of those of wild type plants under 3 and 30 μM B supply, respectively, whereas the concentrations in mutant and wild type were similar under 100 μM B supply. B concentrations in WIR of shoots, which are considered to represent cell wall fraction of plants, decreased with low B supply in both genotypes and the extent of reduction was similar in both genotypes, suggesting that B partitioning to WIRs is not affected in the *bor1-1* mutant.

Under low B supply it was also shown that in shoots of *bor1-1*, 60% of RG-II was present as monomeric RG-II whereas in wild type 90% of RG-II was dimerized.[45] Shoot growth retardation was likely to result at least partly from reduced dRG-II-B formation. Since monomeric RG-II derived from *bor1-1* mutant proofed capable of forming dRG-II-B dimers in vitro, reduction of dRG-II-B in *bor1-1* was not likely to be due to a defect in RG-II synthesis or the RG-II structure. Rather, the increase in monomeric RG-II was likely to be caused by a decreased B concentration in shoots of *bor1-1*.[45]

B Transport Properties of *bor1-1*

To understand the cause of reduced B concentration in leaves of *bor1-1*, further physiological analysis was conducted. B concentrations in root cell sap of both wild type plants and the *bor1-1* mutant increased linearly in proportion to the increase of B concentration in the medium.[46] In contrast, B concentrations in xylem saps were clearly different between the wild type and the *bor1-1* mutant. A combination of a linear and a saturating component was present in B concentrations in xylem saps in wild type plants, whereas the saturated curve was not evident in the *bor1-1* mutant. These results demonstrated that *A. thaliana* plants use a passive process in B uptake into root cells and an active mechanism to transport B out of the cell into xylem against a concentration gradient, and that *bor1-1* is defective in xylem loading of B, leading to the decreased B translocation from roots to shoots.

In addition to xylem loading, Takano et al demonstrated that *bor1-1* is defective in preferential B translocation into young leaves.[44] To determine short-term B distribution between old and young rosette leaves, stable isotopes ^{10}B and ^{11}B were used. The plants were grown with supply of ^{11}B and then supplemented with tracer ^{10}B, followed by the determination of ^{10}B concentrations in plant tissues. Proportion of B distribution into young leaves was lower in *bor1-1*, suggesting that BOR1 functions for preferential transport of B to growing portions of shoots.

BOR1 is an Efflux Transporter of Boron

Complementation test and map-based cloning identified *BOR1* At2g47160. BOR1 has a high similarity to anion exchanger proteins on the amino acid sequence level, including the well-characterized Band3 protein in erythrocytes in animals.

BOR1 has putative ten transmembrane regions. When BOR1 fused to GFP was transiently expressed in tobacco cells, GFP fluorescence was observed at the periphery of the cells, suggesting that BOR1 is localized to the plasma membrane.

Given the phenotype of the mutant and the identity of BOR1 as a plasma membrane protein, we assumed that BOR1 might be a B transporter. To examine B transport capability of BOR1, BOR1 was expressed in yeast cells and B transport properties were examined.[46] In *S. cerevisiae*, a gene similar to *BOR1,* YNL275w, now termed as *ScBOR1*,[47] is present. Knock-out of YNL275w increased B concentration in yeast cells by 13 fold compared to the wild type strain after one hour exposure to 0.5 mM boric acid. Expression of *A. thaliana BOR1* in the YNL275w knock-out strain decreased B concentration in cells to 30% of that of the vector control. These results demonstrated that both of YNL275w and BOR1 are capable of decreasing B concentration in cells, suggesting that they are efflux-type B transporters.[46] Although the chemical form of the substrate for BOR1 is not experimentally determined, borate anion is more probable than boric acid from the result of electrophysiology of NaBC1, a human homolog of BOR1.[48]

The characterization of BOR1 proteins represented the first identification of B transporters in biological systems. Most of the transporters in plants were isolated by functional complementation assays in yeast cells and by homology search with the previously known proteins in other organisms. The discovery of BOR1 as the first B transporter in biological systems represents an example of a contribution of the plant membrane transport community to general biology. Genes similar to *BOR1* are present in a wide range of plant species and these *BOR1*-like genes are likely to be B transporters. *BOR1*-like genes in yeast and human were shown to be B transporters.[48]

BOR1 is strongly expressed in root pericycle cells. In transgenic *A. thaliana* lines carrying a promoter *BOR1-GFP* construct, GFP fluorescence was observed predominately in pericycle cells in mature portions of roots. *BOR1* is expressed mainly in cells surrounding the xylem. Considering the *bor1-1* mutant phenotypes, activity of BOR1, subcellular localization and cell-type specificity of expression, it is concluded that BOR1 is an efflux-type B transporter required for xylem loading.[46]

BOR1 Degradation via Endocytosis in Response to High B Supply

In *A. thaliana* plants mRNA accumulation of *BOR1* was not significantly changed under various B conditions,[49] however, accumulation of BOR1 protein decreased under high B supply both in roots and shoots.[50] B translocation from roots to shoots also decreased upon high B supply.[49] These results showed that BOR1 accumulation is regulated at the posttranscriptional level. This regulation was also observed in the transgenic *A. thaliana* lines overexpressing *BOR1-GFP* under the control of cauliflower mosaic virus 35S RNA promoter, a constitutive promoter.[49] In these transgenic lines, mRNA accumulated constitutively, whereas BOR1-GFP was highly accumulated only under the limited supply of B.

Takano et al demonstrated BOR1 degradation via endocytosis. This was the first example of an endocytosis-mediated degradation of a plasma membrane protein in plants.[49] When GFP fluorescence in roots was observed in transgenic lines expressing *BOR1-GFP*, fluorescence was observed in the plasma membrane under low B supply. After transfer to medium containing high B, GFP fluorescence was observed in dot-like structures within one hour and disappeared after two hours. The plasma membrane proteins, GFP-PIP2a and GFP-Lti6b, which are not involved in B transport, stayed at a plasma membrane at constant level irrespective of B conditions, suggesting that the degradation mechanisms that operates under high B supply is specific to BOR1.

Furthermore, BOR1-GFP in dot-like structures was shown to colocalize with endocytosis markers; FM4-64, mRFP-Ara7. Treatment of concanamycin A, a specific inhibitor of V-ATPase, inhibited BOR1-GFP degradation in vacuoles. With treatment of Brefeldin A (BFA), which inhibits exocytosis but not endocytosis, BOR1-GFP was observed in vesicles called BFA compartments both under low B and high B conditions. These observations indicate that BOR1-GFP recycles between plasma membrane and early endosome irrespective of B conditions. Upon high B supply, BOR1 is trafficked to late endosome compartments followed by degradation in vacuoles.

Biological significance of BOR1 degradation upon high B supply is likely for the avoidance of B overaccumulation in aerial portion of the plants under relatively high levels of B supply.

BOR1 Paralogs in *A. thaliana*

The *A. thaliana* genome contains multiple transporter genes for a particular substrate in general. Six *BOR1* paralogs (*BOR2-BOR7*) are present in the *A. thaliana* genome. Accumulation of mRNAs of *BOR2, BOR3, BOR4* and *BOR5* were detected by RT-PCR both in shoots and roots of plants at vegetative stages. However, *BOR6* and *BOR7* transcripts were detected only in flowers.[51] BOR2 is the most similar to BOR1 and shows 91% identity in the amino acid sequence. Disruption of BOR2 inhibited root cell elongation under limited supply of B (Miwa, unpublished). This observation suggests that BOR2 locally transports B into cell walls. It is intriguing that two genes with such high similarities have distinct roles in B transport in plants.

A. thaliana *NIP5;1, a Channel for Boric Acid Mediates B Uptake under B Limitation*

Physiological experiments demonstrated a channel-mediated B transport system in plants as described above. Takano et al identified NIP5;1, a member of the major intrinsic protein (MIP) family as a boric acid channel for efficient B uptake in *A. thaliana* roots and showed its physiological impact on plant growth under B limitation.[52]

MIPs, also known as aquaporins, are membrane proteins with six transmembrane domains. MIPs transport noncharged small molecules such as glycerol, urea or ammonia in addition to water.[53] The NIP (Nod 26-like Intrinsic Protein) subfamily represents one out of seven subfamilies of MIPs in plants.[54] Soybean NOD26 is the founder of this subfamily and is localized to the symbiosome membrane and is capable of transporting water, glycerol and ammonia.[55] It is suggested that NOD26 transports ammonia from rhizobium to plants. On the other hand, subcellular localization and physiological roles of NIP in nonsymbiotic plants were largely unknown until several years ago.

NIP5;1 was identified as a low-B inducible gene through the microarray analysis.[52] RNAs were isolated from wild type *A. thaliana* roots treated with normal or low B for 3 days and *NIP5;1* transcript accumulation was determined. It was found that NIP5;1 transcript accumulation was highly induced upon low B supply. RT-PCR analysis revealed that *NIP5;1* mRNA accumulation in roots increased more than 10 fold at 24 h after the initiation of low B treatment. Re-supply of high B suppressed *NIP5;1* mRNA level back to normal in 24 h. In transgenic *A. thaliana* lines carrying promoter*NIP5;1-GUS*, GUS staining was strongly observed in the root under B limitation. Under high B conditions, GUS staining was only slightly detected, suggesting that NIP5;1 accumulation was regulated by the promoter region used in the experiment.

GFP-NIP5;1 fusion protein was localized to the plasma membrane when GFP-NIP5;1 was transiently expressed in *A. thaliana* protoplasts. When *NIP5;1* was expressed in *Xenopus* oocytes for functional analysis, uptake of boric acid into cells was increased compared to the water-injected oocytes, suggesting that NIP5;1 is a channel for boric acid.[52]

Function of NIP5;1 in plants was investigated with T-DNA insertion lines. The two independent insertion lines *nip5;1-1* and *nip5;1-2* showed severe growth reduction both in shoots and in root cell elongation only under limited supply of B and both grew normally under normal B conditions.[52] Amounts of B uptake into roots were increased in wild type plants under low B supply compared to those under high B supply, whereas this increase of B uptake was not observed in the *nip5;1-1* mutant. These observations demonstrated that NIP5;1 is essential for B uptake into root cells to support normal plant growth under B limitation.[52] Given the similarity to aquaporins, NIP5;1 is likely to transport boric acid according to the concentration gradient and to contribute to satisfy B requirement in shoot and root growth.

Nine NIP members in *A. thaliana* genome are classified into two subgroups with different specificities to substrates. Rice Lsi1, a NIP member (*OsNIP2;1*), belonging to another subgroup present in rice, barley and maize but not in Arabidopsis, is required for the uptake of silicic acid $Si(OH)_4$ in roots (see also the chapter by Ma in this book).[56] Plants are likely to apply NIPs for

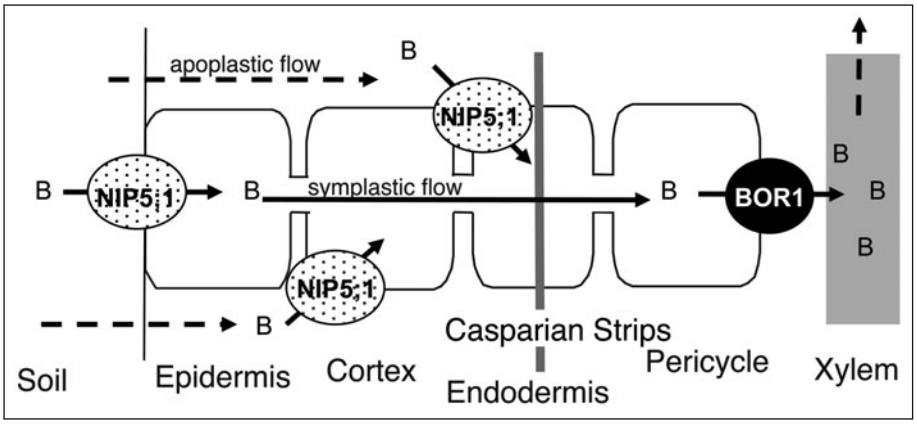


Figure 2. Complementary roles of NIP5;1 and BOR1 in boron (B) transport across root cells for xylem loading. NIP5;1 is a boric acid channel allowing efficient uptake of boric acid to symplast. BOR1 is an efflux transporter expressed in stele and loads B to xylem from symplast.

transport of noncharged small nutrient molecules such as boric acid, ammonia, arsenite, antimonite and silicic acid.[57-59]

Complementary Roles of BOR1 and NIP5;1 in Efficient B Transport under B Limitation

Through the molecular genetic studies using *A. thaliana*, BOR1 and NIP5;1 were identified as an efflux-type B transporter and a channel for boric acid, respectively. Both are required for normal plant growth under limited supply of B. These two types of transporters have differential function in boric acid transport. Under B limitation, BOR1 exports borate out of pericycle cells into xylem against a concentration gradient. NIP5;1 stimulates influx of boric acid from external medium into root cells, because it is expressed in the epidermis and cortex, which represent cell layers distal of the Casparian strips. BOR1 is likely to generate a concentration gradient between root cells and the medium, necessary for B influx into root cells mediated by the channel protein NIP5;1. If this is the case, then BOR1 function is required for efficient transport of boric acid through NIP5;1, because NIP5;1 is a passive channel for boric acid and the B concentration gradient is required for NIP5;1 as a driving force (Fig. 2).

It is intriguing that NIP5;1 expression is regulated at the level of mRNA accumulation, whereas BOR1 accumulation is regulated through protein degradation. It is not clear why plants carry two different systems to regulate the flow of B or how beneficial these differential regulations are for the plants. Moreover, it is hard to imagine the situation in which plants are exposed to abrupt high concentration of B. But, considering that BOR1 is the transporter that generates concentration gradients of B, it might be beneficial for plants to downregulate BOR1 more rapidly than NIP5;1 to rapidly shut off the B flow across the cell.

NIP6;1, *a Channel for Boric Acid Responsible for B Distribution to Leaves under B Limitation*

B in soil is taken up by roots through NIP5;1, a boric acid channel and is loaded into the xylem by BOR1, a borate exporter. The function of *NIP6;1*, the most similar gene to *NIP5;1*, was studied by a reverse genetic approach.[60] NIP6;1 facilitates the rapid permeation of boric acid across the membrane, but is completely impermeable to water. *NIP6;1* transcript accumulation is elevated in response to B deprivation in shoots, but not in roots. *NIP6;1* promoter-*GUS* is predominantly expressed in nodal regions of shoots, especially the phloem region of vascular tissues. Three independently identified T-DNA insertion lines for the *NIP6;1* gene exhibited reduced expansion

of young rosette leaves only under low B conditions. B concentrations decreased in young rosette leaves but not in the old leaves of these mutants. These results strongly suggest that NIP6;1 is a boric acid channel required for proper distribution of boric acid particularly to young developing shoot tissues. [60] NIP6;1 is likely to be involved in xylem-phloem transfer of boric acid at the nodal regions. The water-tight property of NIP6;1 may be important for the boric acid transfer without disturbing transport processes in phloem.[60] It is interesting to note that during evolution, *NIP5;1* and *NIP6;1* were diversified both in terms of the specificity of their expression in plant tissues and water permeation properties of their products, while maintaining their capacity to transport boric acid and their ability to be induced under low B.

Improvement of Plant Growth Property through BOR and NIP Transporters

Low B Tolerant Plants

Polyols including sorbitol have been shown to enhance B translocation. Tao et al introduced an apple cDNA encoding NADP-dependent sorbitol-6-phosphate dehydrogenase (S6PDH), a key enzyme for sorbitol synthesis, into tobacco, not producing sorbitol. S6PDH activity and sorbitol synthesis were confirmed in the transgenic tobacco expressing S6PDH under the control of CaMV 35S promoter.[61] Brown et al revealed that the sorbitol-producing transgenic tobacco line (S11) became tolerant to B deficiency. The transgenic line (S11) showed improvement in plant growth and seed yields under low B condition or especially foliar-application in mature leaves, compared to the wild type tobacco (SR1) and the transgenic line carrying antisense of S6PDH (A4).[62] The transgenic S11 did not exhibited apparent B-deficiency symptoms whereas wild type (SR1) showed flower bud abortion and chlorosis of young leaves under foliar application. Foliar-applied ^{10}B translocation into plant top and seeds were remarkably enhanced in the transgenic S11, suggesting that enhanced B translocation resulted in improved plant growth and seed yields. In addition to this, Bellaloui et al reported enhanced B uptake in the sorbitol-producing transgenic tobacco (S11).[63] Total amount of B uptake and B distribution in meristematic tissues were increased in the S11, compared to the wild type (SR1) and the antisense transgenic line (A4). Interestingly, sorbitol concentrations and contents in the S11 were increased with the increase of B in the growth medium. Enhancement of sorbitol production results in an increase of B translocation from mature leaves to the sink, also in addition to an increased B transport and tolerance to B deficiency. Introduction of S6PDH also stimulated B translocation in rice but growth improvement was not evident.[64] It should also be noted that engineering sugar metabolism may have negative effect on overall growth.[65,66]

Another trial for generation of B-deficiency tolerant plants was to enhance the primary translocation of B from roots to shoots. The growth property of transgenic *A. thaliana* lines expressing BOR1 under the control of CaMV 35S promoter was reported.[67] When *BOR1* was overexpressed in *A. thaliana* plants, improvement of shoot growth and fertility was observed in the transgenic lines under limited supply of B, resulting from the increased B translocation to shoots and shoot apex. It is likely that the function of endogenous BOR1 as a xylem loader of B was enhanced in these transgenic lines. The advantage of this approach is that there is no detrimental effect on plant growth under normal or toxic level of B supply, probably due to the degradation of BOR1 under high B conditions. This is the first report of plants that show improved seed yields under nutrient-deficient conditions as a result of enhanced expression of an essential mineral nutrient transporter. Since BOR1 homologs are present in a wide variety of plant species,[44] this strategy can be useful for other crops and *BOR1* homologs can be used as genetic markers for breeding.

High B Tolerant Plants Can Be Generated through Overexpression of BOR4

B toxicity is a worldwide problem, which impedes food production in semi arid areas. In south Australia, more than 10% wheat yield loss was estimated to be caused by B toxicity. It was reported that overexpression of *BOR4*, a paralog of *AtBOR1*, is capable of conferring extreme tolerance to

high B toxicity.[68] Three homozygous transgenic lines overexpressing AtBOR4 show remarkable improvement of root and shoot growth under 10 mM B conditions, which is lethal for wild type plants. B concentrations in roots and shoots were decreased in these transgenic plant lines with the supply of 3 mM boric acid. There was no significant difference in growth under normal supply of B (30 μM). Overexpression of *BOR4-GFP* reduces B concentration in cells, which are beneficial to maintain growth under high B conditions.

GFP fluorescence of BOR4-GFP was strongly detected in the plasma membrane of the distal side of the root epidermal cells in elongation zone in the transgenic lines expressing BOR4-GFP fusion protein under the control of the *BOR4* promoter.[68] Distal localization of BOR4 is likely to be important for directional B export from roots to soil to prevent B accumulation in xylem and growing cells. Overexpression of BOR4 promoted effective exclusion of B out of the cells, which likely resulted in improvement of growth. BOT1, a homolog of BOR4 was isolated from barley and suggested to be involved in high B tolerance,[44] supporting the importance of this type of B extruder in B tolerance.

Enhancement of B efflux from roots of crop plants is expected to result in improvement of crop productivity on B toxic soils found in a number of regions around the world.

Growth Improvement by Enhanced Expression of NIP5;1

As described above, overexpression of B transporter BOR1 improves shoot growth under low B. However, root growth remains to be similar to that of the wild type plants. Enhancement of expression of *NIP5;1*, but not its overexpression, resulted in improved root elongation under low B conditions in *A. thaliana*. It was found that an *NIP5;1* activation tag line, which have a T-DNA insertion with enhancer sequences near the *NIP5;1* gene, exhibited improved root elongation under low B condition, whereas overexpression of the *NIP5;1* gene by the CaMV 35S promoter resulted in reduced overall growth. These results demonstrate that the cell and tissue specific expression of *NIP5;1* is instrumental for the physiological role of NIP5;1 as a B transporter in low B environment. Overexpression of BOR1 in the *NIP5;1* activation tag line resulted in plants with super tolerance to low B concentration.[69] Furthermore, one of the transgenic lines exhibited improved fertility and short-term B uptake. This represents the first successful improvement of B deficiency tolerance through modification of transporters. This also reveals potentials of enhancing expression of a mineral nutrient channel gene to improve growth under nutrient limiting conditions.

Conclusion

It has been revealed that NIP5;1 and NIP6;1 play important but distinct roles in B uptake and distribution within the plant body, respectively. Recent success in generating a B deficiency tolerant plant by manipulation of *NIP5;1* expression illustrated that NIPs represent targets for plant breeding for enhancement of growth. Future studies are expected to broaden and deepen our understanding of NIPs, which will lead to better technology to generate crops with higher yields in low and/or high B soils.

References

1. Warington K. The effect of boric acid and borax on the broad bean and certain other plants. Ann Bot 1923; 37:629-672.
2. Shorrocks VM. The occurrence and correction of boron deficiency. Plant Soil 1997; 193:121-148.
3. Liu Z, Zhu Q, Tang L et al. Geographical distribution of trace elements-deficient soils in China. Acta Pedol Sin 1982; 19:209-223.
4. Loomis WD, Durst RW. Chemistry and biology of boron. Biofactors 1992; 3:229-239.
5. Marschner H. Mineral Nutritin of Higher Plants, 2nd ed. San Diego: Academic Press, 1995; .
6. Dell B, Huang LB. Physiological response of plants to low boron. Plant Soil 1997; 193:103-120.
7. Kouchi H, Kumazawa K. Anatomical responses of root tips to boron deficiency. III. Effect of boron deficiency on sub-cellular structure of root tips, particularly on morphology of cell wall and its related organelles. Soil Sci Plant Nutr 1976; 22:53-71.
8. Bolanos L, Lukaszewski K, Bonilla I et al. Why boron? Plant Physiol Biochem 2004; 42:907-912.
9. Nable RO, Bañuelos GS, Paull JG. Boron toxicity. Plant Soil 1997; 193:181-198.

10. Power PP, Woods WG. The chemistry of boron and its speciation in plants. Plant Soil 1997; 193:1-13.
11. Matoh T, Ishigaki K, Mizutani M et al. Boron nutrition of cultured tobacco BY-2 cells. 1. Requirement for and intracellular localization of boron and selection of boron and selection of cells that tolerate low levels of boron. Plant Cell Physiol 1992; 33:1135-1141.
12. Hu H, Brown PH. Localization of boron in cell walls of squash and tobacco and its association with pectin (evidence for a structural role of boron in the cell wall). Plant Physiol 1994; 105:681-689.
13. Noguchi K, Dannel F, Heidrun P et al. Defect in root-shoot translocation of boron in Arabidopsis thaliana mutant bor1-1. J Plant Physiol 2000; 156:756-761.
14. Matoh T, Ishigaki KI, Ohno K et al. Isolation and characterization of a boron-polysaccharide complex from radish roots. Plant Cell Physiol 1993; 34:639-642.
15. Kobayashi M, Matoh T, Azuma JI. Two chains of rhamnogalacturonan II are cross-linked by borate-diol ester bonds in higher plant cell walls. Plant Physiol 1996; 110:1017-1020.
16. Ishii T, Matsunaga T. Isolation and characterization of a boron-rhamnogalacturonan-II complex from cell walls of sugar beet pulp. Carbohydr Res 1996; 284:1-9.
17. Pellerin P, Doco T, Vidal S et al. Structural characterization of red wine rhamnogalacturonan II. Carbohydr Res 1996; 290:183-197.
18. O'Neill MA, Warrenfeltz D, Kates K et al. Rhamnogalacturonan-II, a pectic polysaccharide in the walls of growing plant cell, forms a dimer that is covalently cross-linked by a borate ester. In vitro conditions for the formation and hydrolysis of the dimer. J Biol Chem 1996; 271:22923-22930.
19. O'Neill MA, Ishii T, Albersheim P et al. Rhamnogalacturonan II: structure and function of a borate cross-linked cell wall pectic polysaccharide. Annu Rev Plant Biol 2004; 55:109-139.
20. Ishii T, Matsunaga T, Pellerin P et al. The plant cell wall polysaccharide rhamnogalacturonan II self-assembles into a covalently cross-linked dimer. J Biol Chem 1999; 274:13098-13104.
21. Kobayashi M, Nakagawa H, Asaka T et al. Borate-rhamnogalacturonan II bonding reinforced by Ca^{2+} retains pectic polysaccharides in higher-plant cell walls. Plant Physiol 1999; 119:199-204.
22. Iwai H, Usui M, Hoshino H et al. Analysis of sugars in squash xylem sap. Plant Cell Physiol 2003; 44:582-587.
23. Ishii T, Matsunaga T, Hayashi N. Formation of rhamnogalacturonan II-borate dimer in pectin determines cell wall thickness of pumpkin tissue. Plant Physiol 2001; 126:1698-1705.
24. O'Neill MA, Eberhard S, Albersheim P et al. Requirement of borate cross-linking of cell wall rhamnogalacturonan II for Arabidopsis growth. Science 2001; 294:846-849.
25. Bonin CP, Potter I, Vanzin GF et al. The MUR1 gene of Arabidopsis thaliana encodes an isoform of GDP-D-mannose-4,6-dehydratase, catalyzing the first step in the de novo synthesis of GDP-L-fucose. Proc Natl Acad Sci USA 1997; 94:2085-2090.
26. Zablackis E, York WS, Pauly M et al. Substitution of L-fucose by L-galactose in cell walls of Arabidopsis mur1. Science 1996; 272:1808-1810.
27. Reuhs BL, Glenn J, Stephens SB et al. L-Galactose replaces L-fucose in the pectic polysaccharide rhamnogalacturonan II synthesized by the L-fucose-deficient mur1 Arabidopsis mutant. Planta 2004; 219:147-157.
28. Vanzin GF, Madson M, Carpita NC et al. The mur2 mutant of Arabidopsis thaliana lacks fucosylated xyloglucan because of a lesion in fucosyltransferase AtFUT1 Proc Natl Acad Sci USA 2002; 99:3340-3345.
29. Ryden P, Sugimoto-Shirasu K, Smith AC et al. Tensile properties of Arabidopsis cell walls depend on both a xyloglucan cross-linked microfibrillar network and rhamnogalacturonan II-borate complexes. Plant Physiol 2003; 132:1033-1040.
30. Baluska F, Hlavacka A, Samaj J et al. F-actin-dependent endocytosis of cell wall pectins in meristematic root cells. Insights from brefeldin A-induced compartments. Plant Physiol 2002; 130:422-431.
31. Yu Q, Hlavacka A, Matoh T et al. Short-term boron deprivation inhibits endocytosis of cell wall pectins in meristematic cells of maize and wheat root apices. Plant Physiol 2002; 130:415-421.
32. Matsunaga T, Ishii T, Matsumoto S et al. Occurrence of the primary cell wall polysaccharide rhamnogalacturonan II in pteridophytes, lycophytes and bryophytes. Implications for the evolution of vascular plants. Plant Physiol 2004; 134:339-351.
33. Bassil E, Hu H, Brown PH. Use of phenylboronic acids to investigate boron function in plants. Possible role of boron in transvacuolar cytoplasmic strands and cell-to-wall adhesion. Plant Physiol 2004; 136:3383-3395.
34. Raven JA. Short- and long-distance transport of boric acid in plants. New Phytol 1980; 84:231-249.
35. Dordas C, Brown PH. Permeability of boric acid across lipid bilayers and factors affecting it. J Membr Biol 2000; 175:95-105.
36. Dordas C, Chrispeels MJ, Brown PH. Permeability and channel-mediated transport of boric acid across membrane vesicles isolated from squash roots. Plant Physiol 2000; 124:1349-1362.

37. Stangoulis JC, Reid RJ, Brown PH et al. Kinetic analysis of boron transport in Chara. Planta 2001; 213:142-146.
38. Dannel F, Pfeffer H, Römheld V. Characterization of root boron pools, boron uptake and boron translocation in sunflower using the stable isotope ^{10}B and ^{11}B. Aust J Plant Physiol 2000; 156:756-761.
39. Dordas C, Brown PH. Expression of the major intrinsic protein (MIP) PIP1 in Xenopus laevis oocytes. Evidence for channel mediated transport of boric acid in squash (Cucurbita pepo). Plant Soil 2001; 235:95-103.
40. Dannel F, Pfeffer H, Walch-Liu P et al. Plant nutrition-Food security and sustainability of afro-ecosystems, Kluwer Academic Publishers, Dordrecht, 2001;162-163.
41. Hayes JE, Reid RJ. Boron tolerance in barley is mediated by efflux of boron from the roots. Plant Physiol 2004; 136:3376-3382.
42. Sutton T, Baumann U, Hayes J et al. Boron-toxicity tolerance in barley arising from efflux transporter amplification. Science 2007; 318:1446-1449.
43. Noguchi K, Yasumori M, Imai T et al. Bor1-1, an Arabidopsis thaliana mutant that requires a high level of boron. Plant Physiol 1997; 115:901-906.
44. Takano J, Yamagami M, Noguchi K et al. Preferential translocation of boron to young leaves in Arabidopsis thaliana regulated by the BOR1 gene. Soil Sci Plant Nutr 2001; 47:345-357.
45. Noguchi K, Ishii T, Matsunaga T et al. Biochemical properties of the cell wall in the Arabidopsis mutant bor1-1 in relation to boron nutrition. J Plant Nutr Soil Sci 2003; 166:175-178.
46. Takano J, Noguchi K, Yasumori M et al. Arabidopsis boron transporter for xylem loading. Nature 2002; 420:337-340.
47. Nozawa A, Takano J, Kobayashi M et al. Roles of BOR1, DUR3 and FPS1 in boron transport and tolerance in Saccharomyces cerevisiae. FEMS Microbiol Lett 2006; 262:216-222.
48. Park M, Li Q, Shcheynikov N et al. NaBC1 is a ubiquitous electrogenic Na^+-coupled borate transporter essential for cellular boron homeostasis and cell growth and proliferation. Mol Cell 2004; 16:331-341.
49. Takano J, Miwa K, Yuan L et al. Endocytosis and degradation of BOR1, a boron transporter of Arabidopsis thaliana, regulated by boron availability. Proc Natl Acad Sci USA 2005a; 102:12276-12281.
50. Takano J, Miwa K, von Wiren N et al. Regulation of the boron transporter AtBOR1 by boron availability. In Plant nutrition for food security, human health and environmental protection, (Li CJ et al, eds). Beijing, China: Tsinghua University Press, 2005b;138-139.
51. Miwa K, Takano J, Fujiwara T. Roles of BOR1 paralog in boron transport in Arabidpsis thaliana. Plant nutrition for food security, human health and environmental protection. (Li CJ et al, eds). Beijing, China: Tsinghua University Press, 2005;124-125.
52. Takano J, Wada M, Ludewig U et al. The Arabidopsis major intrinsic protein NIP5;1 is essential for efficient boron uptake and plant development under boron limitation. Plant Cell 2006; 18:1498-1509.
53. Tyerman SD, Niemietz CM, Bramley H. Plant aquaporins: multifunctional water and solute channels with expanding roles. Plant Cell Environ 2002; 25:173-194.
54. Danielson JAH, Johanson U. Unexpected complexity of the aquaporin gene family in the moss Physcomitrella patens. BMC Plant Biol 2008; 8:45.
55. Wallace IS, Choi WG, Roberts DM. The structure, function and regulation of the nodulin 26-like intrinsic protein family of plant aquaglyceroporins. Biochim Biophys Acta 2006; 1758:1165-1175.
56. Ma JF, Tamai K, Yamaji N et al. A silicon transporter in rice. Nature 2006; 440:688-691.
57. Ma JF, Yamaji N, Mitani N et al. Transporters of arsenite in rice and their role in arsenic accumulation in rice grain. Proc Natl Acad Sci USA 2008; 105:9931-9935.
58. Kamiya T, Tanaka M, Mitani N et al. NIP1;1, an aquaporin homolog, determines the arsenite sensitivity of Arabidopsis thaliana. J Biol Chem 2009; 284:2114-2120.
59. Kamiya T, Fujiwara T. Arabidopsis NIP1;1 transports antimonite and determines the antimonite sensitivity. Plant Cell Physiol 2009; Plant Cell Physiol 2009; 50:1977-1981.
60. Tanaka M, Wallace IS, Takano J et al. NIP6;1 is a boric acid channel for preferential transport of boron to growing shoot tissues in Arabidopsis. Plant Cell 2008; 20:2860-2875.
61. Tao R, Uratsu SL, Dandekar AM. Sorbitol synthesis in transgenic tobacco with apple cDNA encoding NADP-dependent sorbitol-6-phosphate dehydrogenase. Plant Cell Physiol 1995; 36:525-532.
62. Brown PH, Bellaloui N, Hu H et al. Transgenically enhanced sorbitol synthesis facilitates phloem boron transport and increases tolerance of tobacco to boron deficiency. Plant Physiol 1999; 119:17-20.
63. Bellaloui N, Brown PH, Dandekar AM. Manipulation of in vivo sorbitol production alters boron uptake and transport in tobacco. Plant Physiol 1999; 119:735-742.
64. Bellaloui N, Yadavc RC, Chern MS et al. Transgenically enhanced sorbitol synthesis facilitates phloem-boron mobility in rice. Physiol Plant 2003; 117:79-84.

65. Deguchi M, Koshita Y, Gao M et al. Engineered sorbitol accumulation induces dwarfism in Japanese persimmon. J Plant Physiol 2004; 161:1177-1184.
66. Sheveleva EV, Marquez S, Chmara W et al. Sorbitol-6-phosphate dehydrogenase expression in transgenic tobacco. High amounts of sorbitol lead to necrotic lesions. Plant Physiol 1998; 117:831-839.
67. Miwa K, Takano J, Fujiwara T. Improvement of seed yields under boron-limiting conditions through overexpression of BOR1, a boron transporter for xylem loading, in Arabidopsis thaliana. Plant J 2006; 46:1084-1091.
68. Miwa K, Takano J, Omori H et al. Plants tolerant of high boron levels. Science 2007; 318:1417.
69. Kato Y, Miwa K, Takano J et al. Highly boron deficiency-tolerant plants generated by enhanced expression of NIP5;1, a boric acid channel. Plant Cell Physiol 2009; 50:58-66.

Elsbeth Bienert

Chapter 8

Silicon Transporters in Higher Plants

Jian Feng Ma*

Abstract

Silicon (Si) is the second most abundant element in the Earth's crust and exerts beneficial effects on plant growth and production by alleviating both biotic and abiotic stresses including diseases, pests, lodging, drought and nutrient imbalance. Silicon is taken up by the roots in the form of silicic acid, a noncharged molecule. Recently both influx (Lsi1) and efflux (Lsi2) transporters for silicic acid have been identified in gramineous plants including rice, barley and maize. Lsi1 and its homologs are influx Si transporters, which belong to a Nod26-like major intrinsic protein (NIP) subfamily in the aquaporin protein family. They are responsible for the transport of Si from the external solution to the root cells. On the other hand, Lsi2 and its homologs are efflux Si transporters, belonging to putative anion transporters and are responsible for the transport of Si out of the cells toward the xylem. All influx transporters show polar localization at the distal side. Among efflux transporters, Lsi2 in rice shows polar localization at the proximal side, but that in barley and maize does not show polar localization. The cell-specificity of localization of Si transporters and expression patterns are different between species. Rice Si transporters are also permeable to arsenite.

Introduction

Silicon (Si) is the second most abundant element after oxygen in the Earth's crust. It is essential for animals and diatoms,[1,2] but has not been recognized as an essential element for plant growth, because there is no evidence showing that Si is involved in the metabolism, which is required for the essentiality in higher plants. However, beneficial effect of Si have been observed in a wide range of plant species including dicots and monocots.[3-6]

The effects of Si are characterized by protecting the plant from various biotic and abiotic stresses.[4-7] Silicon enhances resistance of plants to diseases caused by both fungi and bacteria in different plant species such as rice blast, powdery mildew, sheath blight, ring spot, rust, leaf spot and gray leaf spot.[8] Silicon also suppresses insect pests such as stem borer, brown planthopper, rice green leafhopper, whitebacked planthopper and noninsect pests such as leaf spider and mites.[9] Resistance to the damage by wild rabbit in wheat is also enhanced by an increased amount of Si in wheat.[10] Furthermore, Si increases the tolerance of plants to abiotic stresses including salt, metal toxicity, nutrient imbalance, lodging, drought, radiation, high temperature, freezing and UV iridation.[4-7] These effects have been mostly attributed to the deposition of Si at different tissues.[5,6] Silicon deposited beneath the cuticle acts as a physical barrier, which prevents penetration by fungi and insects, increases mechanical strength and reduces transpiration. In addition, soluble Si has been suggested as a modulator of host resistance to pathogens by interacting with several key compounds of plant stress signaling systems and by increasing the synthesis of plant defense compounds.[11-14]

*Jian Feng Ma—Research Institute for Bioresources, Okayama University, Chuo 2-20-1, Kurashiki, 710-0046, Japan. Email: maj@rib.okayama-u.ac.jp

MIPs and Their Role in the Exchange of Metalloids, edited by Thomas P. Jahn and Gerd P. Bienert.

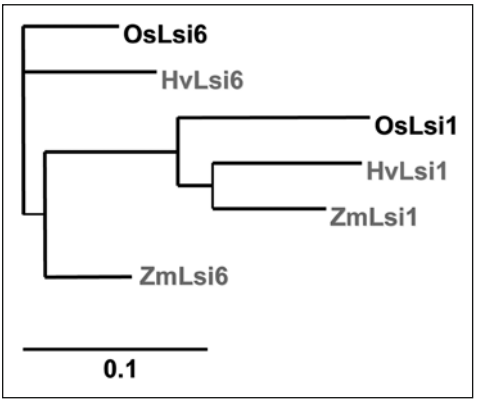


Figure 1. Phylogenetic tree of plant Si influx transporters. Phylogenetic analysis of rice (Os-), maize (Zm-) and barley (Hv-) is performed.

Silicon Transporters

Silicon is taken up by the roots in the form of silicic acid, a noncharged molecule.[15] Recent studies have shown that both influx and efflux transporters are involved in the uptake of Si.[6]

Influx Si Transporters

Influx Si Transporter (OsLsi1) in Rice

Isolation of Rice Si Transporter (OsLsi1)

The first Si transporter (OsLsi1) in higher plants was identified from rice, a typical Si-accumulating species.[16] Rice is able to accumulate Si to over 10% of its dry weight in the shoots and requires a high Si content for healthy growth and high production.[17] The gene encoding the Si transporter was cloned using a rice mutant (*lsi1*, *low silicon 1*) defective in Si uptake, which was isolated by screening mutagenized seeds in a solution containing germanium (Ge).[18] Silicon and Ge are chemically similar and plant roots can not discriminate Ge and Si in terms of uptake. However, in contrast to Si, Ge taken up is toxic to plant, which is characterized by brown spots on the leaves. The gene *Lsi1* is localized on chromosome 2 and consists of five exons and four introns.[16,19] The cDNA of this gene is 1409 bp long and the deduced protein consists of 298 amino acids. Lsi1 belongs to a Nod26-like major intrinsic protein (NIP) subfamily of aquaporin-like proteins. The predicted amino acid sequence has six transmembrane domains and two Asn-Pro-Ala (NPA) motifs, which is well conserved in typical aquaporins. There are several homologs in the gramineous plants including barley and maize (Fig. 1).

A single nucleotide substitution occurred from G in the wild-type to A in the *lsi1* mutant, resulting in an amino acid change from alanine in the wild-type to threonine in the mutant at a position of 132 aa.[16] Alanine at position 132 seems to be a critical residue, because substitution of this amino acid in the mutant significantly alters the conformation according to the modeling of the native and mutant proteins.[16] Thus, the substitution of Thr for ala132 provoked severe steric interactions with val55 and val59 in helix 1 (H1), facilitating a movement of H1. This unfavorable interaction would affect the conformation of asn108, the pore-forming residue.

Expression Pattern of OsLsi1

Lsi1 is constitutively expressed in the roots (Fig. 2A), but its expression is decreased to one fourth by Si supply.[16] Within a root, the expression of *Lsi1* is much lower in the root tip region between 0-10 mm than in the basal regions of the root (>10 mm).[20] Silicon uptake in the root tip region (0 to 10 mm) comprising both the apical meristem and the elongation zone is also much lower than

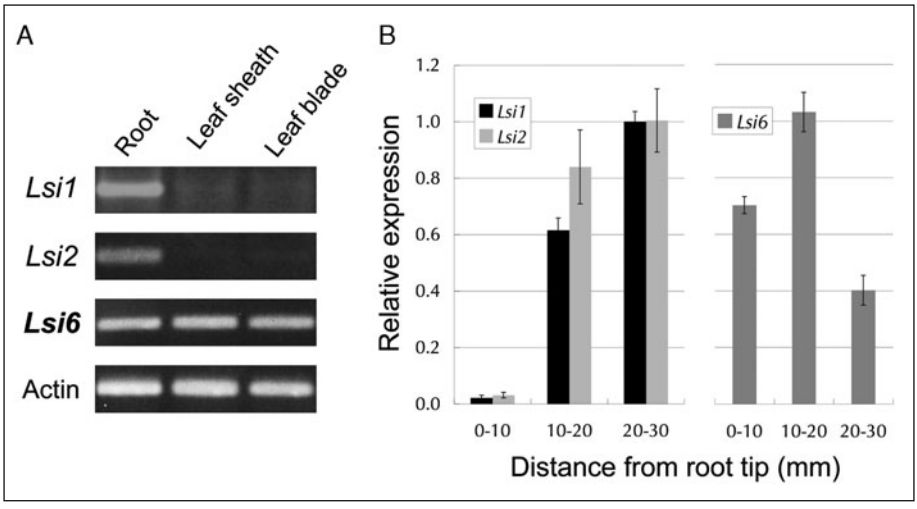


Figure 2. Expression patterns of rice Si transporters. A) Tissue-specific expression pattern of rice Si transporters. B) Spatial expression of rice Si transporters in rice roots. Reproduced from: Yamaji N et al. Plant Cell 2008; 20:1381-1389;[41] ©2008 ASPB.

that in the basal regions (>10 mm from the root tips) (Fig. 2B). These observations indicate that the site of Si uptake is located in the mature regions of the roots rather than the root tips.

The expression of *Lsi1* is transiently enhanced around the heading stage. A previous study showed that 67% of total Si is taken up during the reproductive stage from panicle initiation to heading in rice.[21] Deficiency of Si during this stage results in a significant reduction in the grain yield, suggesting that a high Si uptake during this period is required for producing a high yield. Therefore, the increased expression of *Lsi1* during the heading stage coincides with a high Si requirement during this growth stage.

The expression of *Lsi1* was down-regulated by dehydration stress and abscisic acid (ABA).[20] ABA is known to accumulate in response to water stress. Therefore, the expression of *Lsi1* may be regulated by ABA. In the promoter region of *Lsi1* exists some ABA-responsive motifs. How ABA regulates *Lsi1* expression remains to be examined.

Cellular and Subcellular Localization of OsLsi1

Lsi1 is localized in the main and lateral roots, but not in root hairs.[16] This is consistent with the results of a previous physiological study that root hairs do not play any demonstrable role in Si uptake, but that lateral roots contribute significantly to Si uptake.[22] Furthermore, in rice roots including seminal, lateral and crown roots, the Lsi1 protein is localized at both exodermis and endodermis (Fig. 3A), where the Casparian strips prevents apolplastic transport into the root stele.[16,20] Interestingly, Lsi1 shows polar localization at the distal side of both the exodermis and endodermis cells (Fig. 3A). OsLsi1 is localized to the plasma membrane.[16]

Characteristics of Rice Si Transporter

The characteristics of rice Lsi1 has been investigated by using a *Xenopus* oocyte assay system. Lsi1 shows both influx and efflux transport activity for Si,[16,23] indicating that Lsi1 is a bidirectional transporter although it only functions as an influx transporter in rice roots.[16] This is because silicic acid transported into the root cells from the external solution by Lsi1 is immediately transported out of the cells by another transporter Lsi2 in rice as described below, generating a concentration gradient from the external solution to the root cells. Both the influx and efflux transport activity are inhibited by $HgCl_2$, an inhibitor of aquaporins.[23] The transport activity for Si is unaffected

Table 1. Transport substrates in different groups of NIP in relation to selectivity filter and NPA motif

Group	Selectivity Filter	Selectivity Filter	Selectivity Filter	Selectivity Filter	NPA	NPA	Transport Substrates
	H2	H5	LE1	LE2	NPA1	NPA2	
NIP I	W	V (I)	A	R	NPA	NPA (NPG/ NPV)	water, glycerol, lactic acid, arsenite
NIPII	A	I (V)	G (A)	R	NPS (NPA)	NPV (NPA)	urea, formamide, boric acid. arsenite
NIP III	G	S	G	R	NPA	NPA (NPV)	silicic acid, arsenite

Plant NIPs were classified into three subgroups based on the two NPA motifs and the ar/R selectivity filter formed by four amino acid residues (H2: Helix2; H5: helix5; LE1: loop E1; LE2: loop E2). The NPA motifs and the ar/R residues of each subgroup are indicated in the figure and minor substitutions of those residues were given in the parentheses.

by the low temperature (4°C) treatment. Lsi1 is permeable to water, urea and boric acid, but not to glycerol.[16,23] However, in the presence of equimolar concentrations of urea and boric acid, the transport activity of Si was not or only slightly affected. This indicates that Lsi1 is a highly specific transporter for silicic acid.

However, two recent studies show that Lsi1 is also permeable to arsenite [As(III)].[24] Expression of *Lsi1* in *Xenopus* oocytes and in yeast markedly increased uptake of As(III), but not of arsenate. Mutation of Lsi1 in rice (*lsi1* mutant) resulted in about 60% loss in the short-term (30 min) As(III) influx to roots compared with the wild type rice. These data indicate that As(III) shares the same pathway with Si for the entry into rice root cells, probably because As(III) and silicic acid have a similar size and both are mostly undissociated at pH < 8.

The substrate selectivity of aquaporins is mainly controlled by the ar/R (aromatic/arginine) selectivity filter,[25,26] which is located in the narrowest region on the extra-membrane mouth of the pore. It is formed by four residues one each from helix 2 (H2) and helix 5 (H5), as well as two residues from loop E (LE1 and LE2) typically including aromatic residues, and an Arg residue.[27] The properties of the four residues making up the ar/R selectivity filter appear to govern the substrate specificity of the pore. Based on the ar/R regions of aquaporins, NIPs have been newly divided into three groups, NIP I, II and III (Table 1).[23] NIP I proteins in Arabidopsis have been reported to transport water, glycerol[28] and lactic acid,[29] while NIP II proteins are permeable to larger solutes than NIP I protein, such as urea,[30] formamide,[30] boric acid[31] and As(III).[24] Different from NIP I and NIP II, isoforms of the NIP III subgroup including Lsi1 have a unique selectivity filter, which consists of Gly (G), Ser (S), Gly (G) and Arg (R). It is predicted that the smaller size of the residues form a larger constriction region compared with other NIP groups,[25,32] which allows silicic acid with a larger diameter (4.38 Å) to permeate. However, proteins in NIP I and II groups are also permeable to As(III).[24] Therefore, the exact mechanisms regulating substrate specificity are still unknow.

A number of studies indicated that the activity of both plant and animal aquaporins may be regulated by phosphorylation.[33] Recently, the putative involvement of phosphorylation in the regulation of Lsi1 was investigated using two inhibitors; okadaic acid and K252a as protein phosphatase and protein kinase inhibitors, respectively.[23] However, neither okadaic acid nor K252a affected the transport activity for silicic acid in oocytes. It is possible that, unlike other aquaporins,

phosphorylation is not involved in the regulation of Lsi1 or that phosphorylation could not be seen in the heterologous system. Unlike other minerals, Si does not show excess toxicity to plants because silicic acid auto-polymerizes at higher concentrations. Furthermore, the beneficial effects of Si on plant growth are enhanced with more accumulation of Si. Therefore, it may not be necessary to regulate the activity of Si transporters by phosphorylation.

Based on the localization and transport activity of Lsi1, it is clear that Lsi1 is an influx transporter for Si, which is responsible for the transport of Si from the external solution to the root cells.

Influx Si Transporters in Barley and Maize

Following identification of rice Lsi1(OsLsi1), Si influx transporters have also been identified in maize (ZmLsi1) and barley (HvLsi1).[34,35] ZmLsi1 and HvLsi1 show 82% and 83%, respectively, identity with OsLsi1 at the amino acid level (Fig. 1). Both HvLsi1 and ZmLsi1 show Si influx transport activity like OsLsi1, but their cell-type specificities of localization and expression patterns are different from OsLsi1. HvLsi1 and ZmLsi1 are localized to epidermal, hypodermal and cortical cells.[34,35] Furthermore, the expression levels of both *HvLsi1* and *ZmLsi1* are unaffected by Si.

Efflux Transporter of Silicon

Efflux Si Transporters in Rice

Isolation of Rice Efflux Si Transporter (OsLsi2)

The first efflux transporter gene (*Lsi2*) of Si in rice was also cloned using a novel mutant (*lsi2*) defective in Si uptake.[36] This gene is located on chromosome 3 and consists of two exons and one intron. The open reading frame (ORF) of this gene is 1416 bp long and the deduced protein consists of 472 amino acids. Sequence comparison showed that a single base mutation in the first exon occurred from G in the wild-type to A in the mutant, resulting in an amino acid change from serine in the wild-type to asparagine in the mutant at the 115 aa position. The gene is predicted to encode a membrane protein with 11 transmembrane domains. Lsi2 belongs to a putative anion transporter without any similarity with the Si influx transporter Lsi1.[16,36] There are six full-length homologs in higher plants in the database (http://rapdb.dna.affrc.go.jp/and http://www.arabidopsis.org/), including one in Arabidopsis and five in rice (Fig. 4).

Expression Pattern of OsLsi2

Lsi2 is mainly expressed in the roots as *Lsi1* (Fig. 2A). The mRNA accumulation is constitutive but it is decreased to one fourth by continuous Si supply for three days.[37] Furthermore, there is little accumulation of *Lsi2* transcripts in the root tip (0-10 mm), but much accumulation in the mature parts of the roots (Fig. 2B).[37] The expression of *Lsi2* is also transiently increased around the heading stage.[37] These expression patterns are similar to those of *Lsi1* as described above, suggesting that the expression of *Lsi1* and *Lsi2* may be regulated in a similar manner. Comparison of the promoter region revealed common domains in the *Lsi1* and *Lsi2*.[37] The expression of *Lsi2* is also regulated by ABA as seen for *Lsi1*.

Localization of Rice Si Efflux Transporter OsLsi2

Like Lsi1, Lsi2 is also localized to the exodermis and the endodermis cells of the roots (Fig. 3B). However, in contrast to Lsi1 localized on the distal side, Lsi2 is localized on the proximal side of the exodermis and the endodermis cells.[37] A subcellular study showed that Lsi2 is also localized to the plasma membrane.

Characteristics of Rice Si Efflux Transporter OsLsi2

In *Xenopus* oocytes, Lsi2 did not show influx transport activity for silicic acid but did show efflux transport activity.[37] The efflux of Si is inhibited by a low temperature treatment and by three protonophores; 2,4-dinitrophenol (DNP), carbonylcyanide 3-chlorophenylhydrazone (CCCP) and carbonylcyanide *p*-(trifluoromethoxy)penylhydrazone (FCCP).[37] Furthermore, the efflux activity of Lsi2 is increased at lower external pH values. All these results indicate

Figure 3, viewed on following page. Localization of rice Si transporters. A) Immunostaining of Si influx transporter Lsi1. Lsi1 is localized on the distal side of both exodermis and endodermis. B) Immunostaining of Si efflux transporter Lsi2. Lsi2 is localized on the proximal side of both exodermis and endodermis. C) Double staining of Lsi1 and Lsi2 on exodermis. Green shows Lsi1 and red shows Lsi2. Reproduced from: Ma JF et al. Nature 2006; 440:688-691;[16] ©2006 Nature Publishing Group, and Ma JF et al. Nature 2007; 448:209-211;[37] ©2007 Nature Publishing Group.

Figure 4, viewed on following page. Phylogenetic tree of Si efflux transporter-like proteins. Phylogenetic analysis of rice (Os-), maize (Zm-) and barley (Hv-) is performed.

Figure 5, viewed on following page. Localization of Si efflux transporters in barley (A) and maize (B). Both HvLsi2 and ZmLSi2 are localized to the endodermis without polarity. Reproduced from: Mitani N et al. Plant Cell 2009; 21:2133-2142; ©2009 ASPB.

that different from Lsi1, Lsi2 functions as an active efflux transporter, which is driven by the proton gradient.

Lsi2 is also involved in As(III) uptake.[24] Although direct measurement for the efflux activity of As(III) was not successful, studies with two independent mutants of Lsi2 showed that the mutants have a marked decrease (66-75%) in As accumulation in shoots compared with the respective wild types.[24] As(III) concentration in the xylem sap from the mutants was 73-91% lower than those of their wild type backgrounds. Moreover, addition of Si to the nutrient solution inhibited As(III) transport to the xylem and accumulation in the shoots in the wild type rice, but not in the two *lsi2* mutants.

Efflux Transporters in Barley and Maize

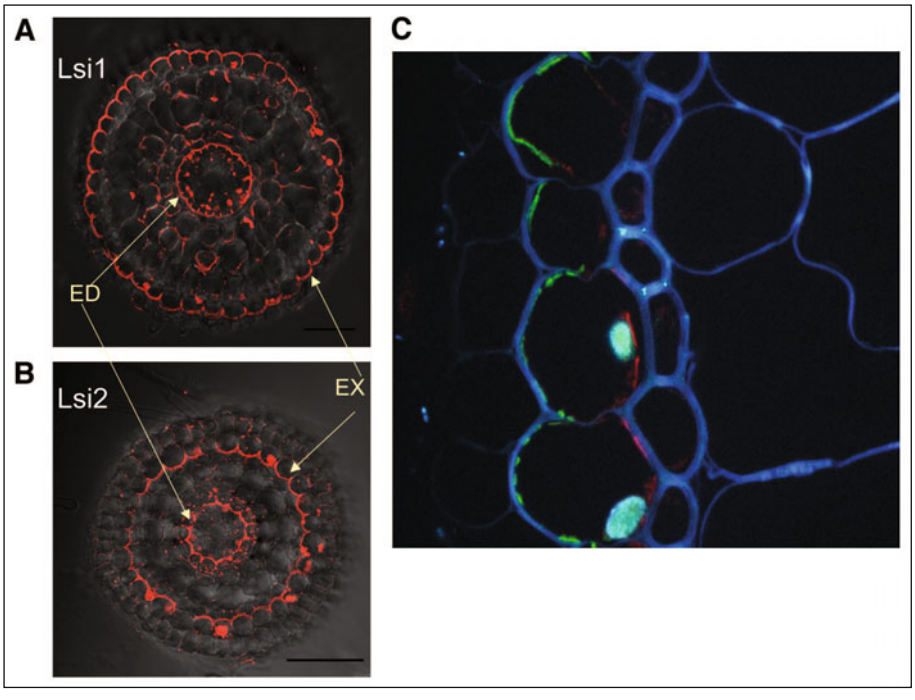
Homologs of rice Lsi2 (OsLsi2) have been identified in barley (HvLsi2) and maize (ZmLsi2).[38] Both share 86% identity with OsLsi2 at amino acid level (Fig. 4). Heterologous expression in *Xenopus* oocytes showed that both ZmLsi2 and HvLsi2 have a Si efflux transport activity like OsLsi2. Furthermore, when HvLsi2 was transformed into a rice mutant (*lsi2*) defective in Si uptake with a construct carrying the *HvLsi2* cDNA under the control of rice *Lsi2* promoter (*Lsi2P-HvLsi2*), the Si uptake was significantly increased in plants carrying *Lsi2P-HvLsi2* compared to the vector control. These findings suggest that like OsLsi2, both ZmLsi2 and HvLsi2 also function as efflux Si transporters.

Both *ZmLsi2* and *HvLsi2* are mainly expressed in the roots, but not in the shoots. Furthermore, the expression is much higher in the basal root regions than that in the root tip region in both maize and barley. Both *ZmLsi2* and *HvLsi2* expressions are down-regulated in response to the Si supply. These expression patterns are similar to that of OsLsi2. Furthermore, there is a significant positive correlation between Si uptake and the *HvLsi2* expression level,[38] indicating that the expression level of *HvLsi2* is a key factor in determining Si uptake in barley. In rice, the expression of both OsLsi1 and OsLsi2 are responsible for genotypic difference in Si uptake.[37] This difference between rice and barley may be attributed to the different regulation of *HvLsi1* and *OsLsi1* expression. *HvLsi1* expression is not affected by Si,[34] whereas OsLsi1 is down regulated by Si.[16]

Both ZmLsi2 and HvLsi2 are located in the plasma membrane.[38] Immunostaining with rabbit anti-ZmLsi2 or anti-HvLsi2 antibodies showed that different from OsLsi2, both ZmLsi2 and HvLsi2 are localized only to the endodermis of roots in maize and barley, respectively. Furthermore, both ZmLsi2 and HvLsi2 do not show polar localization (Fig. 5).[38]

Difference in Si Uptake System between Paddy and Field Crops

So far OsLsi1, HvLsi1 and ZmLsi1 as Si influx transporters and OsLsi2, HvLsi2 and ZmLsi2 as Si efflux transporters have been identified, respectively, from rice, barley and maize. Although the functions of these transporters are similar among different species, they have different localization and expression patterns. In rice, both influx and efflux transporters of Si are polarly



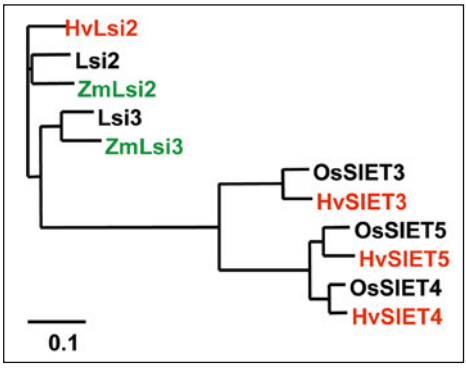
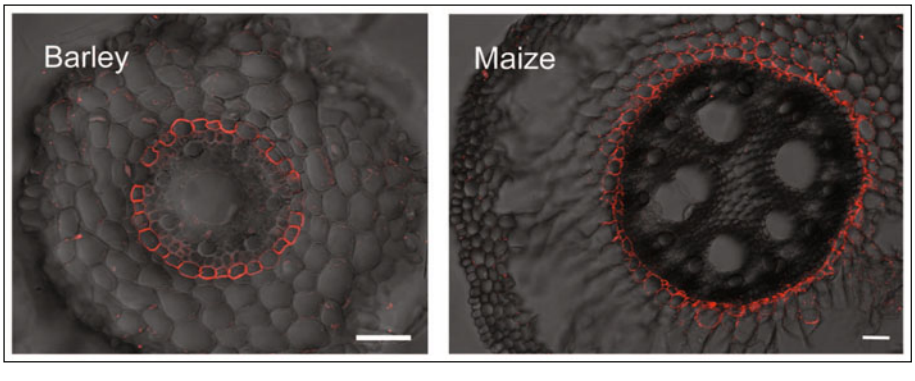


Figure 3, above.

Figure 4, left.

Figure 5, below.

Please see figure legends on previous page.



localized to the same cells of the exodermis and endodermis.[16,37] In contrast, maize and barley influx transporters (ZmLsi1/HvLsi1) and efflux transporters (ZmLsi2/HvLsi2) are located at distinct cells; maize and barley influx transporters (ZmLsi1/HvLsi1) are polarly localized to the epidermal, hypodermal and cortical cells[34,35] and efflux transporters (ZmLsi2/HvLsi2) are nonpolarly localized on the endodermis only (Fig. 6).[38] Therefore, in barley and maize, Si can be taken up from external solution (soil solution) by HvLsi1/ZmLsi1 at different cells including epidermal, hypodermal and cortical cells. In contrast, in rice, Si is only taken up at the exodermal cells by OsLsi1. After being taken up into the root cells, Si is transported to the endodermis by symplastic pathway and then released to the stele by HvLsi2/ZmLsi2 in maize and barley (Fig. 6). By contrast, in rice, Si taken up by OsLsi1 at the exodermal cells is released by OsLsi2 to the apoplast and then transported into the stele by both OsLsi1 and OsLsi2 again at the endodermal cells. These findings indicate that that the uptake system for Si differs between upland crops (barley and maize) and paddy crop (rice).

The difference in the uptake system may be attributed to the root structures observed in rice on the one hand and maize and barley on the other hand. In rice roots, there are two Casparian strips at the exodermis and endodermis, whereas one Casparian strip is usually present at the endodermis of maize and barley roots under nonstressed conditions. Moreover, mature roots in rice have a distinct structure, a highly developed aerenchyma, wherein almost all cortex cells between exodermis and endodermis are destructed. Therefore, Si transported into the exodermis cells by the influx transporter, OsLsi1, has to be released by the efflux transporter, OsLsi2, into the apoplast of a spoke-like structure across the aerenchyma. However, in maize and barley roots, there is no such structure or if any; it is developed poorly. These differences in the localization of transporters and polarity may be one of the reasons for the different Si uptake capacities among species although further work is needed to address this issue.

Silicon Transporters for Xylem Unloading

Silicon transported by Lsi1 and Lsi2 into the stele is then translocated to the shoot by transpirational volume flow through the xylem. More than 90% of Si taken up by the roots is translocated to the shoots.[4] Silicon is present in the xylem at a high concentration, but in the form of monosilicic acid.[39,40] Recently, a transporter (Lsi6), which is responsible for the export of silicic acid from the xylem and subsequently for the distribution of Si, was identified in rice.[41]

Lsi6 is a homolog of Lsi1 (Fig. 1). It also shows transport activity for silicic acid in the oocyte assay.[41] However, different from Lsi1 and Lsi2, Lsi6 is also expressed in the leaf sheaths and leaf blades in addition to the root tips. Lsi6 is localized in the adaxial side of the xylem parenchyma cells in the leaf sheaths and leaf blades (Fig. 7). Knockout of Lsi6 does not affect the uptake of Si by the roots, but affects the silica deposition pattern in the leaf blades and sheaths. The density of silicified dumbbell-shape and motor cells in the knockout line is decreased compared with the wild-type rice. The abaxial epidermis cells are observed to be silicified in the mutant, but infrequently in the wild-type rice.[41] Furthermore, knocking out Lsi6 results in an increased excretion of Si in the guttation fluid. These results suggest that knockout of *Lsi6* results in alteration of the Si pathway to the specific cells. A similar transporter ZmLsi6 has also been identified in maize.[35]

Conclusion

As described above, great progresses have been made in identification of Si transporters in higher plants. So far Lsi1 and Lsi2 have been demonstrated to be involved in Si uptake by the roots, while Lsi6 is necessary for the xylem unloading. However, these transporters are identified from a limited number of plant species including rice, barley and maize. Therefore, cloning of more genes from a wide variety of plant species in future will help to better understand the molecular mechanisms of Si uptake in plants. Furthermore, transfer of Si from the external solution to the specific plant tissues may require additional transporters, which also remain to be identified in the future.

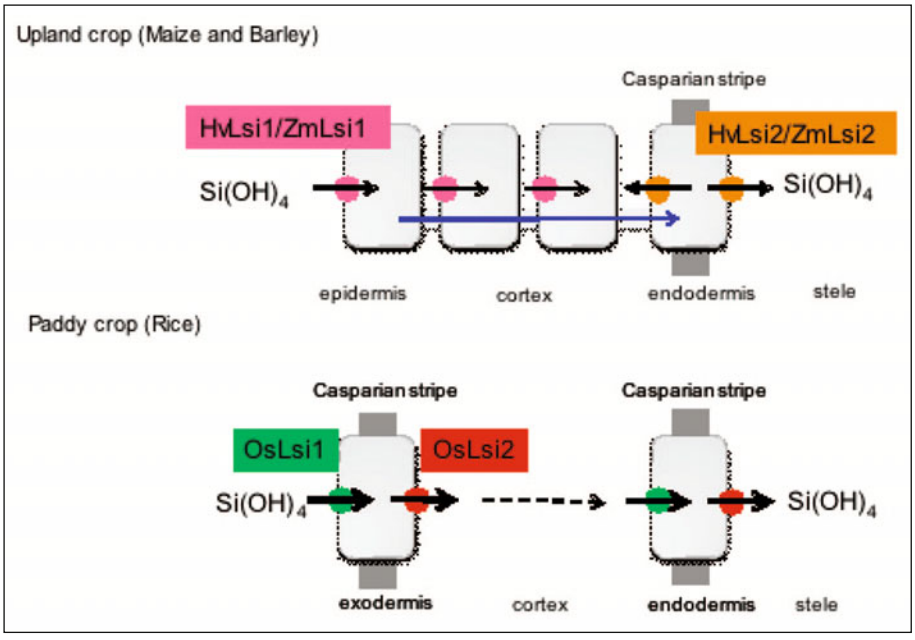


Figure 6. A schematic presentation of Si transport system in upland crop (maize and barley) and paddy crop (rice). In upland crops such as maize and barley, Si as silicic acid is taken up from the external solution by the influx transporter (ZmLsi1/HvLsi1) localized to the distal side of cells in the epidermis and cortex layer and then transferred to the endodermis through symplastic pathway (blue arrows). At the endodermis, the Si is released by an active Si efflux transporter (ZmLsi2/HvLsi2) to the stele. In paddy crop such as rice, Si is taken up from the external solution by OsLsi1 at the distal side and released to the apoplast of aerenchyma by OsLsi2 at the proximal side of the exodermal cells. Silicon is then transported to the stele by OsLsi1 and OsLsi2 at the endodermal cells. Reproduced from: Mitani N et al. Plant Cell 2009; 21:2133-2142; ©2009 ASPB.

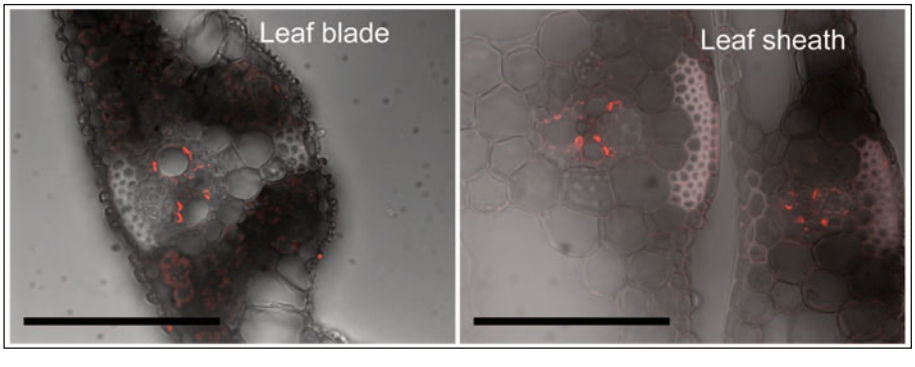


Figure 7. Localization of OsLsi6 in the leaf blade (A) and leaf sheath (B) of rice. OsLsi6 is localized to the adaxial side of the xylem parenchyma cells. Reproduced from: Yamaji N et al. Plant Cell 2008; 20:1381-1389;[41] ©2008 ASPB.

Silicon protects the plants from various stresses, however, most plant species are not able to accumulate Si at levels high enough to be beneficial. Therefore, genetic manipulation of Si transporters may help plants to accumulate more Si, thereby improving the plants ability to overcome biotic and abiotic stresses in future.

Acknowledgements

Work on silicon transporters in the laboratory of M. J. F. is supported by a Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Culture, Sports, Science and Technology of Japan (No. 17078008 to J. F. Ma), the Program of Promotion of Basic Research Activities for Innovative Biosciences (BRAIN) and a grant from the Ministry of Agriculture, Forestry and Fisheries of Japan (Genomics for Agricultural Innovation IPG-006).

References

1. Sripanyakorn S, Jugdaohsingh R, Thompson RPH et al. Dietary silicon and bone health. Nutr Bull 2005; 30:222-230.
2. Lopez PJ, Descles J, Allen AE et al. Prospects in diatom research. Curr Opin Biotech 2005; 16:180-186.
3. Epstein E, Bloom AJ. Mineral Nutrition of Plants: Principles and Perspectives, 2nd ed. New York: John Wiley and Sons, 2006.
4. Ma JF, Takahasi E. Soil, Fertilizer and Plant Silicon Research in Japan. Amsterdam: Elsevier Science, 2002.
5. Ma JF, Yamaji N. Silicon uptake and accumulation in higher plants. Trends Plant Sci 2006; 11:392-397.
6. Ma JF, Yamaji N. Functions and transport of silicon in plants. Cell Mol Life Sci 2008; 65:3049-3057.
7. Ma JF. Role of silicon in enhancing the resistance of plants to biotic and abiotic stresses. Soil Sci Plant Nutr 2004; 50:11-18.
8. Fauteux F, Remus-Borel W, Menzies JG et al. Silicon and plant disease resistance against pathogenic fungi. FEMS Microbio Lett 2005; 249:1-6.
9. Savant NK, Snyder GH, Datnoff LE. Silicon management and sustainable rice production. Advan Agron 1997; 58:151-199.
10. Cotterill JV, Watkins RW, Brennon CB et al. Boosting silica levels in wheat leaves reduces grazing by rabbits. Pest Manag Sci 2007; 63:247-253.
11. Belanger RR, Benhamou N, Menzies JG. Cytological evidence of an active role of silicon in wheat resistance to powdery mildew (Blumeria graminis f. sp tritici). Phytopathology 2003; 93:402-412.
12. Fawe A, Abou-Zaid M, Menzies JG et al. Silicon-mediated accumulation of flavonoid phytoalexins in cucumber. Phytopathology 1998; 88:396-401.
13. Rodrigues FA, Mcnally DJ, Datnoff LE et al. Silicon enhances the accumulation of diterpenoid phytoalexins in rice: A potential mechanism for blast resistance. Phytopathology 2004; 94:177-183.
14. Remus-Borel W, Menzies JG, Belanger RR. Silicon induces antifungal compounds in powdery mildew-infected wheat. Physiol Mol Plant Pathol 2005; 66:108-115.
15. Takahashi E, Hino K. Silicon uptake by plant with special reference to the forms of dissolved silicon. Jap J Soil Sci Manure 1978; 49:357-360.
16. Ma JF, Tamai K, Yamaji N et al. A silicon transporter in rice. Nature 2006; 440:688-691.
17. Tamai K, Ma JF. Reexamination of silicon effects on rice growth and production under field conditions using a low silicon mutant. Plant Soil 2008; 307:21-27.
18. Ma JF, Tamai K, Ichii M et al. A rice mutant defective in active Si uptake. Plant Physiol 2002; 130:2111-2117.
19. Ma JF, Mitani M, Nagao S et al. Characterization of Si uptake system and molecular mapping of Si transporter gene in rice. Plant Physiol 2004; 136:3284-3289.
20. Yamaji N, Ma JF. Spatial distribution and temporal variation of the rice silicon transporter Lsi1. Plant Physiol 2007; 143:1306-1313.
21. Ma JF, Nishimura K, Takahashi E. Effect of silicon on the growth of rice plant at different growth stages. Soil Sci Plant Nutri 1989; 35:347-356.
22. Ma JF, Goto S, Tamai K et al. Role of root hairs and lateral roots in silicon uptake by rice. Plant Physiol 2001; 127:1773-1780.
23. Mitani N, Yamaji N, Ma JF. Characterization of substrate specificity of a rice silicon transporter, Lsi1. Pflugers Archiv-Eur J Physiol 2008; 456:679-686.
24. Ma JF, Yamaji N, Mitani N et al. Transporters of arsenite in rice and their role in arsenic accumulation in rice grain. Proc Natl Acad Sci 2008; 105:9931-9935.

25. Forrest KL, Bhave M. Major intrinsic proteins (MIPs) in plants: a complex gene family with major impacts on plant phenotype. Funct Integr Genomics 2007; 7:263-289.
26. Wallace IS, Roberts DM. Distinct transport selectivity of two structural subclasses of the nodulin-like intrinsic protein family of plant aquaglyceroporin channels. Biochemistry 2005; 44:16826-16834.
27. Ilan B, Tajkhorshid E, Schulten K et al. Electrostatic tuning of permeation and selectivity in aquaporin water channels. Biophys J 2003; 85:2884-2899.
28. Dean RM, Rivers RL, Zeidel ML et al. Purification and functional reconstitution of soybean nodulin 26. An aquaporin with water and glycerol transport properties. Biochemistry 1999; 38:347-353.
29. Choi WG, Roberts DM. Arabidopsis NIP2;1: A major intrinsic protein transporter of lactic acid induced by anoxic stress. J Biol Chem 2007; 282:24209-24218.
30. Wallace IS, Roberts DM. Homology modeling of representative subfamilies of Arabidopsis major intrinsic proteins. Classification based on the aromatic/arginine selectivity filter. Plant Physiol 2004; 135:1059-1068.
31. Takano J, Wada M, Ludewig U et al. The Arabidopsis major intrinsic protein NIP5;1 is essential for efficient boron uptake and plant development under boron limitation. Plant Cell 2006; 18:1498-1509.
32. Wang Y, Schulten K, Tajkhorshid E. What makes an aquaporin a glycerol channel? A comparative study of AqpZ and GlpF. Structure 2005; 13:1107-1118.
33. Agre P, Kozono D. Aquaporin water channels: molecular mechanisms for human diseases. FEBS Lett 2003; 555:72-78.
34. Chiba Y, Yamaji N, Mitani N et al. HvLsi1 is a silicon influx transporter in barley. Plant J 2009; 57:810-818.
35. Mitani N, Yamaji N, Ma JF. Identification of maize silicon influx transporters. Plant Cell Physiol 2009; 50:5-12.
36. Ma JF, Yamaji N, Tamai K et al. Genotypic difference in Si uptake and expression of Si transporter genes in rice. Plant Physiol 2007; 145:919-924.
37. Ma JF, Yamaji N, Mitani N et al. An efflux transporter of silicon in rice. Nature 2007; 448:209-211.
38. Mitani N, Chiba Y, Yamaji N et al. Identification of silicon efflux transporters in maize and barley reveals their different silicon uptake system from rice. Plant Cell 2009; 21:2133-2142.
39. Mitani N, Ma JF, Iwashita T. Identification of silicon form in the xylem of rice (Oryza sativa L.). Plant Cell Physiol 2005; 46:279-283.
40. Casey WH, Kinrade SD, Knight CT et al. Aqueous silicate complexes in wheat, Triticum aestivum L. Plant Cell Envion 2003; 27:51-54.
41. Yamaji N, Mitatni N, Ma JF. A transporter regulating silicon distribution in rice shoots. Plant Cell 2008; 20:1381-1389.

Elsbeth Bienert

Chapter 9

Major Intrinsic Proteins and Arsenic Transport in Plants:
New Players and Their Potential Role

Gerd P. Bienert* and Thomas P. Jahn

Abstract

Arsenic (As) is a toxic and highly abundant metalloid that endangers human health through drinking water and the food chain. The most common forms of As in the environment are arsenate [As(V)] and arsenite [As(III)]. As(V) is a nonfunctional phosphate analog that enters the food chain via plant phosphate transporters. Recently, evidence was provided that uptake of As(III)—the second most abundant As species in soils—is mediated by plant nodulin26-like intrinsic proteins (NIPs), a subfamily of plant major intrinsic proteins (MIPs). Specific NIPs are also essential for the uptake of the metalloids boron and silicon and aquaglyceroporins from microbes and mammals were shown to be the major routes of As uptake. Therefore As(III) transport through MIPs is a conserved and ancient feature. In this chapter we summarize the current view on As transport in plants and address the potential physiological significance of As(III) transport through NIPs.

Introduction

Arsenic (As) and antimony (Sb) are two closely related metalloids that are toxic for all living organisms. Sb is a rather rare element and does usually not reach concentrations high enough to act as a threat for life. In contrast, As is highly bio-available and continuous to enter the biosphere through erosion of rock material, a process that has been strongly accelerated by human activities.[1] Nriagu and Pacyna estimated anthropogenic release of As to 82,000 metric tons per year.[2] Due to its low mobility,[3] As applied through intense irrigation with As-contaminated ground water, is trapped in agricultural soils.

Inorganic As, both trivalent arsenite [As(III)] and pentavalent arsenate [As(V)] are the predominant chemical species occurring in soils (Fig 1). However, soil chemistry[4] as well as microbial activities[5] form the basis for As cycling and derivatization. As(V) respiring bacteria use As(V) as the terminal electron acceptor leading to the formation of As(III). Other bacterial strains oxidize As(III) for energy generation and a variety of microbes methylate As in both oxidation states for detoxification. Methylated As species monomethylarsonic acid [MMA(V)] and dimethylarsenic acid [DMA(V)] have also accumulated in agricultural soils due to the use of these compounds in herbicides, pesticides and defoliants, as well as the use of manure from As fed chickens.[6] Under aerobic conditions, which prevail in well aerated soils, plants are mainly exposed to As(V), whereas

*Corresponding Author: Gerd P. Bienert—Unité de Biochimie Physiologique, Institut des Sciences de la Vie, Université catholique de Louvain, Croix du Sud 5-15, B-1348 Louvain-la-Neuve, Belgium. Email: gerd.bienert@uclouvain.be

MIPs and Their Role in the Exchange of Metalloids, edited by Thomas P. Jahn and Gerd P. Bienert.

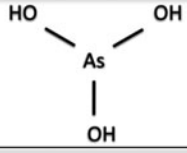
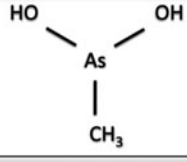
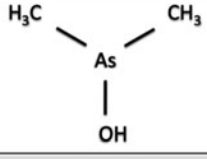
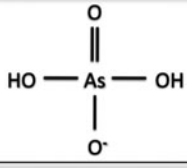
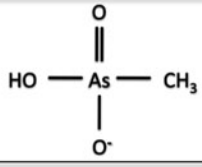
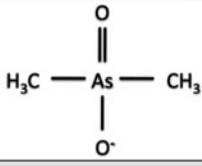
Name Abbreviation	Formula	Toxicity	pKa value
Arsenite As(III)		$LD50_{mouse}$ 4.5 mg/kg	pKa 9.2
Monomethylarsonous acid MMA(III)		$LD50_{hamster}$ 3.6 mg/kg	
Dimethylarsinous acid DMA(III)			
Arsenate As(V)		$LD50_{mouse}$ 14-18 mg/kg	pKa_1 2.3 pKa_2 6.8 pKa_3 11.6
Methylarsonic acid MMA(V)		$LD50_{mouse}$ 1800 mg/kg	pKa_1 3.6 pKa_2 8.2
Dimethylarsinic acid DMA(V)		$LD50_{mouse}$ 10000 mg/kg	pKa_2 6.3

Figure 1. Characteristics of various chemical species of As. Names, abbreviations indicating the chemical valency, chemical structures, median lethal doses (as dose per kilogram of subject body weight) and the pKa values are listed for some As species that are in the primary focus of this book.

under anaerobic, reducing conditions, plants are mainly exposed to As(III). Consequently, water logged soils which are low in oxygen, provide conditions to favour the formation of As(III).

Regardless the oxidation state, both forms of As can be taken up by plants and are toxic for the plant's metabolism. As(V) is a nonfunctional phosphate analog that uncouples oxidative phosphorylation through competition with phosphate. Thereby As(V) inhibits energy-linked reduction of NAD^+, mitochondrial respiration and ATP synthesis. As(V) might also interfere

with enzymes, which are regulated via phosphorylation or depending on phosphate as cofactor in their catalytic cycle.

In plants, phosphorous only occurs in its oxidated form +5 as phosphate. In contrast, As(V) taken up from the soil into the reducing environment inside cells, is reduced to As(III) via As reductases.[7,8] As reductase thus plays a key role in As(V) detoxification because it deviates As metabolism from phosphate metabolism. However, also in its reduced form As(III), As is highly toxic. Toxicity of As(III) is based on its reactivity towards sulfhydryl groups in cysteine residues and the imidazolium nitrogen in histidine residues leading to interferences with general protein function.[9]

Our picture about As in the environment and in plants has changed during the last years. Thirty years ago, As was considered far below toxic limits in edible parts of the plants, even when the crop was grown at conditions of severe phytotoxicity.[10] Since then, we had to realize that the content of As in various products in fact is at dangerous levels. Performing a market survey, Andrew Meharg and coworkers found substantial concentrations of As in rice and rice products in the United States and Japan. Taking statistics for habits of consumers into considerations, they estimated the average daily exposure to inorganic As from eating rice cereals to be at least 0.0042 mg.[11] Processing of rice grain in some cases even leads to further enrichment of inorganic As, which is of major concern in case of rice bran, which has been marketed as super-food to malnourished children in international aid programs.[12] Meharg and coworkers point out that there is no maximum concentration level set for As or its chemical derivates in food stuffs yet.[12]

At the same time, epidemiological studies reported about the risks and consequences connected to As exposure. A tragic story about pollution of drinking water and of agricultural soil caused by irrigation practices in West-Bengal and Bangladesh, has developed into an 'experiment of life'.[13] From here we know that long-term exposure to As also leads to cancer, affecting the lungs, bladder and kidneys [http://www.iarc.fr/].

Today, ingestion of plant products represents the 2nd largest source of As for humans. There is a clear need to react on this problem at global level. This includes agricultural practices, grain processing and food production as well as plant breeding strategies and the use of biotechnology. Safe crops containing low As levels should be available for food production.

In this chapter, we discuss the fundamental processes of As transport and metabolism which are the basis for the ability of different species to either tolerate, accumulate or release As. Understanding the genetic basis of these processes is likely to provide tools in breeding and biotechnological strategies aiming at reducing As in crops. The knowledge may also be crucial in strategies of gross food production and the orientation of markets. Is rice a good choice as staple food in the future? Which crop and variety can be grown at which degree of pollution? Our main focus will be on the role of NIP-aquaporins in mediating transmembrane transport of As(III) in plants.

The Challenge of As Speciation in Plants

Inorganic As(V) and As(III) are the most toxic as well as the most abundant As species in the environment (Fig. 1). Organisms have consequently developed strategies for the detoxification of As, that typically involve incorporation of As into organic compounds which are far less detrimental for cells. An extreme example is arsenobetain a common and abundant As species in fish which is harmless even at concentrations of 200 mg kg^{-1} dry mass.[14]

Because of the highly distinct toxicity of various forms of As, there has been substantial interest in As speciation in plants and plant products. However, speciation of As in plant extracts has remained a challenge. Plant cells are highly compartmentalized by intracellular membranes that separate different microenvironments in close proximity. During cell extraction, compounds that had been compartmentalized in the intact tissues become exposed to conditions that can lead to chemical modifications and consequently erroneous speciation.[15] This is in particular a problem for the speciation of As in various redox states and conjugations.

Reliable data for As speciation and localization came from non-invasive methods using spectroscopy. Such methods revealed large differences between the concentrations of free As(III),

As(V) and thiol-As(III)-complexes in different plant organs of *Pteris vittata, Brassica juncea, Pisum sativum, Prosopis spp*. and *Raphanus sativus*.[3,16-20] The data proof that various plant species differ quite substantially in As speciation and localization, but also demonstrate that As(III), followed by thiol-As(III)-complexes and As(V) are the most prevalent forms of As across various species.

The experimental challenge to dissect the exact localisation and routes of the different As species in space and time have hampered the identification of plant transport proteins responsible for the uptake, extrusion and translocation of diverse As containing compounds.

Transport of As in Plants

In the 1950s, Emanuel Epstein described transport processes across membranes of plant cells by applying terms and processes from enzymology. He found that similar rules applied to enzymes and transporters, provided, the transport process was active, thus either consuming energy in the form of ATP (pumps) or being coupled indirectly to ATP hydrolysis via a concentration gradient across a membrane such as the proton gradient, which to establish costs ATP. Such transport processes reveal Michaelis Menten kinetics and are regarded as saturable, active transport processes. In contrast, transport processes characterized by a linear relationship between substrate concentration and transport activity are considered passive but are potentially of high capacity. Such passive transport across the lipid bilayer of membranes can be based on simple diffusion. However, the diffusion of larger and polar compounds is strongly facilitated by membrane integral protein channels and in particular by major intrinsic proteins (MIPs).

As(V) Uptake

Kinetic analysis and competition between As(V) and the highly similar phosphate suggested that uptake of As(V) is catalyzed by high affinity phosphate transporters.[21-25] Later, a double insertion line of *Arabidopsis thaliana*, lacking the two major phosphate transporters AtPht1;1 and AtPht1;4 showed a clear decrease in sensitivity to externally supplied As(V) when compared to wild type.[26] Another Arabidopsis mutant line, which is impaired in intracellular protein trafficking,[27] is also more resistant to As(V) than wild type. In this mutant, the phosphate transporter Pht1;1 is not trafficked to the plasma membrane but is retained in the ER. These experiments provide genetic evidence for high affinity phosphate transporters being involved in As(V) uptake by plants.

Until recently, phosphate transporters have been in focus for biotechnological strategies to either reduce As content in plants for food production or to increase As uptake for phytoremediation. However, increasing uptake of As(V) is a complex strategy as it implies the need for increased tolerance of the plant to keep up with the increased As content and concomitantly increased stress level. The alternative strategy—to reduce uptake of As(V) in the food chain by down-regulation of phosphate uptake systems—appears counter productive. In agricultural systems, that aim to be highly productive, it is not rational to generate by breading or biotechnology varieties with reduced phosphate uptake capacities, as this would result in phosphorus deficiency and consequently severely limited yields.

As(III) Transport Via NIPs—Gateway for Good and Bad

Uncharged, tetrahedral As(III) is a rather large and polar molecule, thereby possessing characteristics that limit passive diffusion across biological membranes. In rice, uptake of As(III) at low external concentration fitted Michaelis-Menten kinetics suggesting an active uptake process.[28] At higher substrate concentration, however, the influx correlated with As(III) concentration in a linear fashion in agreement with a passive uptake pathway. As opposed to rice, in *Brassica carinata*[29] and the ericoid mycorrhizal fungus *Hymenoscyphus ericae*, uptake kinetics for As(III) strictly displayed linear correlations between the external concentration and influx.[30] Thus it appeared that there are different routes for the transport of As(III) into plants, an active transport system, as measures in rice and a high capacity diffusion-based transport system, that appeared to be widespread from plants to fungi. In organisms such as bacteria, yeast and mammals, aquaglyceroporins had already been identified that channelled As(III) and Sb(III) (see also chapters by Rosen and Tamas as well as Liu in this book).

Aquaglyceroporins are solute channels that together with aquaporins (AQPs) form the super family of major intrinsic proteins (MIPs) (see also the chapter by Danielson and Johanson in this book). In higher plants, all channels of the plant MIP family including PIPs, TIPs, NIPs, HIPs, XIPs and SIPs belong to the AQPs. The only exception in the plant world is PpGip1;1, a MIP from the moss *Physcomitrella patens*, which displays similarity to microbial and mammalian aquaglyceroporins.[31] It seems that aquaglyeroporins had lost their role in higher plants, while other MIPs might have taken over their functions.

The overall structure of MIPs is highly conserved, although the primary sequences vary quite substantially. All MIPs are composed of six transmembrane helices and two membrane embedded loops that form a channel path across the membrane. Two constriction regions are found in the channel path. One is formed by two highly conserved NPA repeats, which—in the folded protein—constitute the centre of the channel path. The second constriction region is located about 6 Å towards the extra-cytoplasmic side and is more variable in sequence. This region, also called the aromatic arginine (ar/R) constriction region, has been demonstrated to be important for substrate selectivity. Among the plant MIPs, the ar/R pore signatures of NIPs share amino acid substitutions with both AQPs and aquaglyceroporins. Additionally, when functionally assayed in a heterologous expression systems some NIPs channel glycerol.[32-35] Therefore NIPs in plants were likely to be the functional analog of aquaglyceroporins in other organisms and were believed to be the group of plant AQPs that had acquired the function analog to that of aquaglyceroporins in microbes and mammals.

A comparison of structural and electrostatic characteristics of glycerol, As(III) and Sb(III) revealed their high similarity.[36] It was concluded that a channel that transports glycerol should also permeate As(III) and Sb(III). The pore diameter in the ar/R selectivity filter of certain NIPs was shown to be even wider than the pore diameter of the aquaglyceroporin GlpF.[37,38] This suggested the passage of larger molecules than glycerol through NIPs. This interpretation was supported by the discovery that members of the NIP subfamily have physiological roles as channels for boric acid $[B(OH)_3]$[39] and silicic acid $[Si(OH)_4]$,[40] two other hydroxylated metalloids (see also the chapter by Miwa et al and the chapter by Ma in this book).

The first molecular evidence to show, that As(III) is transported through plant NIPs came from experiments expressing the channels in yeast and *Xenopus* oocytes.[41-44] Expression of plant NIPs in a yeast triple mutant impaired in their endogenous As transport proteins FpsI, Acr3 and Ycf1 resulted in increased uptake and sensitivity to As(III). Notably, on medium containing As(V), expression of most NIPs significantly improved growth of the mutant yeast. This observation led to the hypothesis that NIPs may serve a function in As tolerance through efflux of As(III) that may build up as a consequence of intracellular As(V) reduction.[41] In any case, NIPs are bi-directionally permeable for As(III) and can therefore play a physiological role in both the influx as well as the efflux of this toxic metalloid.

Genetic Evidence

A forward genetic screen is an elegant method for the identification of gene functions. Forward genetic screens make use of mutant collections—large populations of mutant lines—that have been generated by random mutagenesis using radiation or chemicals as mutagenes. The seeds used for such a screen are obtained from self-pollination of the M1 mutant population to obtain a substantial ratio of lines with the mutated allele present in a homozygote fashion. Under most conditions, loss of gene function results in a growth disadvantage. In forward genetic screens, however, growth conditions are created, in which the loss of function due to mutation of a gene, leads to a growth advantage. Kamiya et al[43] applied a forward genetic screen to identify putative transporters with a role in the uptake of toxic As(III) into roots. As consequence of the loss of gene function, mutants impaired in the uptake of As(III) were expected to show a growth advantage on As(III)-containing medium when compared to wild type. They generated an EMS induced population of Arabidopsis, a treatment that results in random mutagenesis across the chromosomal DNA. Mutants (M1 generation) were self crossed and 30,000 M2 lines of this population were grown on medium containing 15 μM As(III), a concentration which reduced root growth of wild type

plants to 20% of controls grown in the absence of As(III). The authors isolated three independent As(III) tolerant lines, all of which showed to be mutated in the aquaporin AtNIP1;1.[43]

T-DNA insertion collections are an alternative source of material to proof gene function in plants. T-DNA insertion lines are generated by transformation of a transfer DNA containing a resistance gene allowing the easy identification of successfully transformed lines. The randomly inserted T-DNA leads to gene disruption at various places in the genome of the plant. Several Arabidopsis insertion collections have been built over years, where the transferred DNA has been mapped by border-sequencing. Therefore, insertions in specific genes of interest can be purchased for reverse genetic studies. As opposed to the forward genetic study, here the gene is well known, but the physiological role may be completely unknown. The T-DNA insertion line *nip1;1-1* in which the T-DNA was placed in *NIP1;1* showed increased tolerant to As(III) when compared to wild type plants similar to the mutants identified in the forward genetic screen.[43]

Complementary DNAs of wild type *AtNIP1;1* as well as the mutants identified from the EMS collection were generated by PCR and expressed in *Xenopus* oocytes for transport assays. This allowed to directly assay the effect of mutations on the transport of As(III) and confirmed that AtNIP1;1 has a dominant role in As(III) uptake in Arabidopsis.[43]

T-DNA insertion lines for *nip1;2-1, nip3;1-1, nip4;1-1, nip5;1-1, nip6;1-1* and *nip7;1-1*, did not show any significant tolerance to As(III) in the growth medium. This was initially surprising, as AtNIP1;2, AtNIP5;1, AtNIP6;1 and AtNIP7;1 had been shown to be permeable for As(III) when expressed in heterologous systems[41-43] and as AtNIP7;1 appeared to contribute to uptake of As(III) in Arabidopsis.[42]

Uptake and Distribution of As in Rice and the Consequences for the Food Chain

A forward genetic screen in rice resulted in the identification of various transporters for silicic acid (see also the chapter by Ma in this book). Ma and coworkers germinated rice seeds from a mutant population on medium containing germanium hydroxide [$Ge(OH)_3$].[40,45] Germanium is a rare metalloid, chemically similar to silicon (Si) but—like As—toxic to plants at elevated concentration. Thus, similar to the forward genetic screen applying As(III) to Arabidopsis mutant lines, application of $Ge(OH)_3$ to rice mutants allowed the selection of lines defective in $Ge(OH)_3$ uptake. From this screen, low Si mutants *lsi1* and *lsi2* were identified, which suffered from Si deficiency at low external concentration.[40,45] Whereas mapping the gene locus for *lsi1* resulted in the identification of OsNIP2;1, Lsi2 turned out to be a transporter with homology to citrate exporting transporters. Lsi2 is an active transporter that is localized in proximal plasma membrane domains of exodermis and endodermis cells, membrane domains that face the interior of the root, the vascular tissue, which function in translocation of water and nutrients to the shoot. The NIP aquaporin Lsi1/OsNIP2;1 is expressed in the same cells but—after synthesis in the endoplasmic reticulum—targeted to distal plasma membrane domains facing the root surface. Thus, Lsi1/OsNIP2;1 and Lsi2 are expressed in opposite plasma membrane domains in two single-celled cell layers and cooperate in the uptake and translocation of $Si(OH)_4$ to the transpiration stream (see also the chapter by Ma in this book). Active extrusion of $Si(OH)_4$ by Lsi2 creates and maintains a concentration gradient of $Si(OH)_4$, which is the driving force for the diffusion of $Si(OH)_4$ through the NIP aquaporin channel Lsi1/OsNIP2;1, the primary site for uptake of $Si(OH)_4$ into rice roots.

It became rapidly clear that Lsi1/OsNIP2;1 and Lsi2 also transport As(III).[44] Thus Lsi1/OsNIP2;1 and Lsi2 are likely to represent the molecular mechanisms for high capacity and high affinity As(III) uptake systems, respectively, which had been described in earlier physiological studies in rice.[28] OsNIP2;1 mutant plants (*lsi1*) which lack the capacity to take up Si showed significantly reduced As(III) uptake in short-term uptake measurements when compared to wild type plants. Expression of *OsNIP2;1* in rice roots is strongly induced in response to low $Si(OH)_4$ in the soil. In turn, supplementation of soil with silicic acid reduces expression of *OsNIP2;1* and consequently reduces the uptake of As(III). These results suggest that OsNIP2;1 provides the major pathway for As(III) uptake in rice roots.

Measuring short-term uptake, *lsi2* mutant plants showed no difference in As(III) uptake when compared to wild type.[44] In long-term measurements, however, *lsi2* showed a 75% reduction of As in shoots and a 90% reduction As in the xylem sap, indicating that Lsi2 is important for the translocation of As(III) from root to shoot. Another interesting observation in the study of As uptake in these mutants is, that As content in different tissues were quite differentially affected by mutations in *Lsi1* and *Lsi2*. Whereas As content in the xylem sap in *lsi2* was reduced by 90%, total shoot As was only reduced to 75% and strikingly, grain content of As in *lsi2* was still 50% of wild type, suggesting that the remaining 50% must have entered the grain by other means than Lsi2. Field-grown *lsi1* mutant plants had lower concentrations of As in straw but roughly the same As levels in grain and husk when compared to wild type. These observations show that there is a preferential flow and accumulation of As in the grain. Because As in rice in mainly present as As(III) it is tempting to speculate that other NIP isoforms and Lsi2 homologs in rice are involved in the specific pattern of As flow in rice plants. In fact, expression of OsNIP1;1, OsNIP2;2 Lsi6 and OsNIP3;1 in yeast and oocytes[41,44] clearly proofed their capacity to channel As(III) across membranes. OsNIP2;2/Lsi6 is localized in leaf sheath cells surrounding the vascular tissue in leaves and has been ascribed a role in the deposition of silicic acid in leaf cell walls.[46]

Also in Arabidopsis, a number of NIP paralogs namely AtNIP5;1, AtNIP6;1 and AtNIP7;1 proofed to channel As(III) when heterologously expressed in yeast (see Table 1).[41] In vivo studies in Arabidopsis however did not support a role of these NIPs in As(III) uptake, maybe because these isoforms are down regulated in the presence of As(III).[43,44] However, it remains to be tested whether these isoforms play a role in As(III) distribution in these plants.

Rice NIP2;1 Mediates the Uptake of Methylated As Species

Methylation of inorganic As is a mechanism of detoxification in both microbes and mammals. In mammalian cells, As(III) is methylated to various degrees and MMA accounts for a large proportion of total cellular As.[47,48] Methylated As(III) species are primarily formed in the liver and are excreted into the bile and urine (see also the chapter by Liu in this book). Interestingly, AQP9, which is highly expressed in the liver, has a three times higher capacity to channel MMA than inorganic As(III).[49] AQP9 may thus have a function in As detoxification.

Also plants contain relatively large amounts of methylated As species with up to 80% of total As.[6,50] However, despite of a single investigation reporting on methylase activity in leaf extracts from the grass *Agrostis tenuis*,[51] no methylases for As(III) or As(V) have been identified in plants on the molecular level. Other than in microbes and mammals, in plants, methylated As may therefore mainly result from uptake from the soil rather than from synthesis. Methylated As compounds in natural soils originate from microbial activity. However, the former use of As-containing herbicides, pesticides and defoliants has substantially increased the concentration of methylated As species in agricultural soils.

Although inorganic As is taken up more readily than methylated As, plants can contain large amounts of methylated As species.[52-57] This suggests that methylated As species tend to accumulate in plants. It is therefore important to understand, how methylated As species enter the plant.

Kinetic studies on the uptake of MMA and DMA in rice and maize did not provide clear information about the mechanism of transport.[28,58] Among 46 plant species tested, large variations were measured in the absorption and translocation efficiencies for As(V), MMA(V) and DMA(V).[53] Recently, NIP aquaporin Lsi1/OsNIP2;1 in rice, the primary site for the uptake of $Si(OH)_4$, proofed to be the main pathway for uptake of methylated As species.[59] The mutant *lsi1* when exposed to methylated As species in the growth medium contained 80% and 50% less MMA(V) and DMA(V), respectively, when compared to wild type plants.[59] At a large range of concentrations, the uptake of DMA(V) in wild type was about three times higher than in *lsi1* mutant plants. The short-term uptake kinetics of MMA(V) was described by a Michaelis-Menten plus a linear kinetic. However, *lsi2* mutant plants showed no changed uptake capacity of methylated As species compared to the wild type plants. These experiments clearly demonstrate that Lsi1/OsNIP2;1 mediates the influx of methylated As species into rice roots. However, the molecular basis for the high affinity uptake system has not been

Table 1. As(III) transporting aquaporin homologs, comparison of the constriction region and summary of the metalloid transport capacity. Aquaporin homologs tested for transport of As(III) and other metalloids in different test systems. The tetrad of amino acids from transmembrane helix 2 (R1), transmembrane helix 5 (R2) and 2 residues from loop E (R3 and R4) that constitute the selective filter are displayed. The capacities to channel the influx or efflux of As(III) of the tested MIPs in various growth and transport assays are indicated (+, transport; -, no transport; nd, not determined)

Name	Residues Constituting the Selectivity Filter				Arsenite Transport Shown in			Other Transported Metalloids	References
	Rl	R2	R3	R4	Yeast	Oocyte	Homolog System		
AtNIPl;l	W	V	A	R	–	+	+	Antimonite	41,81
AtNIPl;2	W	V	A	R	–	+	–		41,81
AtNIP5;l	A	I	G	R	+	nd	–	Antimonite Boric acid	39,41-43
AtNIP6;l	A	I	A	R	+	nd	–	Antimonite Boric acid	41-43,82
AtNIP7;l	A	V	G	R	+	nd	+/–	Antimonite	41-43
OsNIPl;l	W	V	A	R	–	+	nd		41,44
OsNIP2;l	G	S	G	R	+	+	+	Antimonite Boric acid DMA(V) MMA(V) Silicic acid	40,41,44, 46,59
OsNIP2;2	G	S	G	R	+	+	–	Antimonite Silicic acid	41,44,45
OsNIP3;l	A	A	G	R	nd	+	nd	Boric acid	44,71
OsNIP3;2	A	A	A	R	+	nd	nd	Antimonite	41
LjNIPS;l	A	I	G	R	+	nd	nd	Antimonite	41
LjNIP6;l	T	I	A	R	+	nd	nd	Antimonite	41
rAQP9	F	S	C	R	+	+	nd	Antimonite MMA(III)	41,49,83, 84
hAQP9	F	A	C	R	+	+	nd	Antimonite	83,84
ScFpsI	W	N	T	R	+	nd	+	Antimonite Boric Acid	41,64
hAOP7	F	G	Y	R	nd	+	nd		83,84
EcGlpF	W	G	F	R	+	nd	+	Antimonite	85
SmAqpS	T	A	S	V	nd	nd	+	Antimonite	73
LmAQPl	W	G	Y	R	nd	+	+	Antimonite	86,87

identified. It will be important to characterize the potential role of other NIPs and other putative transporters in the uptake or translocation of MMA(V) or DMA(V) in plants.

Transport of Conjugated As Species in Plants

Inside plants (like in most other organisms) As(V) is reduced by As(V) reductases to the more mobile but more toxic As(III).[7,8,60] Concomitantly, there is an induction of the synthesis of glutathione (GSH) and phytochelatins (polymers of glutathione). Both compounds are able to complex As (thiol-As(III) complexes) and account for an enhanced tolerance towards the metalloid.[61-63] Depending on the plant and the organ, the amount of complexed As(III) varies quite substantially but can account for up to 90% of total As(III).[17,19] In yeast, chelated As(III) is detoxified by sequestration into the vacuole via an ATP Binding Cassette (ABC) transporter.[64] Arabidopsis and rice possess more than 120 members of the ABC transporter family.[65] Although As(III)-thiol-complexes are frequently found in plants, yet there is no molecular evidence for transport of As(III)-thiol-complexes via a plant ABC transporter.

What Do the Different "Omics" Tell Us About NIP-Mediated As Transmembrane Transport?

Sequencing projects for various plant species have allowed the development of new tools for the analysis of protein expression and modification on large scale. Transcriptomics and proteomics are the main approaches, which allow to record global expression and protein modification profiles under adverse environmental conditions and to compare those profiles with those obtained during standard growth condition.

Requejo and Tena studied the effect of exposure of maize to As(V) and As(III) on the proteome of roots and shoots.[9,66] About 10% of the detectable proteins in maize were differentially regulated upon exposure to As. Most of these proteins are involved in cellular homeostasis for redox perturbation. However, no clear hints were provided as to the regulation of proteins involved in transport of As.

A whole-genome oligonucleotide microarray was employed to investigate the transcriptional responses of *Arabidopsis thaliana* plants exposed to As(V).[67] Similar to the study in maize, antioxidant-related genes (i.e., coding for superoxide dismutases and peroxidases) were strongly upregulated suggesting a prominent role in response to As(V). However, none of the Arabidopsis NIPs appeared to be significantly regulated.

Recently, a whole-genome transcriptional analysis in combination with a genetic mapping of loci for As tolerance in two rice varieties provided new insights into the nature of As toxicity and the mechanisms of tolerance.[68,69] The transcriptional analysis focused on genes that are differentially regulated in both, the As(V)-tolerant variety Bala and the As(V)-sensitive variety Azucena. A large number of transcription factors, stress proteins and transporters such as phosphate-, sulphate-, ABC- as well as multidrug and toxic compound extrusion (MATE) transporters showed differential expression. In addition, two MIP isoforms (Os05g14240 = OsTIP2;3 and Os12g10280 = OsNIP3;5) were specifically down regulated in the tolerant variety Bala.[68] Yet, TIPs have not been shown to transport As (Table 1). However, OsNIP3;5 represents an interesting candidate with potential physiological relevance in As transport, although its capacity to channel As(III) remains to be shown.

The concomitant genetic mapping let to a remarkable three-gene model of tolerance which appears to involve an epistatic interplay of three genes, two on chromosome six and one on chromosome ten.[69] Any combination of two of these three genes—inherited from the tolerant Bala parent—leads to As(V) tolerance and increased root growth in the presence of As(V) when compared to lines that received only one or none of the three alleeles. All the three genes seemed to be specific for As(V) tolerance, as they are not found in quantitative trait loci for general root growth.

At one of the loci there were only two genes that are differentially regulated between the two rice varieties under As(V) treatment. These are an aminoacylase-1 (Os06g10770) and the aquaporin OsNIP4;1 (Os06g12310). Both show higher expression levels in roots of the tolerant variety Bala. Further work is needed to substantiate the hypothesis that OsNIP4;1 has a role in As(V) tolerance.

Using a different rice cultivar, Chakrabarty et al[70] reported on a genome wide expression analysis of seedlings exposed to As(III) or As(V). A number of genes that were differentially

regulated under As(V) stress in the study of Norton et al[68] were similarly regulated in this study. Here, OsNIP3;1 (Os10g36924) was down-regulated in response to As(III) but not to As(V). OsNIP3;1 was shown to be permeable for As(III) when expressed in *Xenopus* oocyte.[44] OsNIP3;1 is the closest homolog to AtNIP5;1, both of which were shown to be essential for the uptake of $B(OH)_3$ in Arabidopsis[39] and rice,[71] respectively (see also the chapter by Miwa et al in this book). The data suggest that upregulation of OsNIP3;1 upon boron (B) limitation would lead to increased uptake of toxic As(III). In turn, down-regulation of OsNIP3;1 in the presence of As(III) might represent a molecular response to reduce As(III) uptake under this conditions. It would be interesting to test whether As(III)-responsive DNA binding elements interfere with the transcription of *OsNIP3;1*.

The above-mentioned studies provide accumulating evidence that regulation of NIPs is involved in As tolerance in rice. Expression data indicate that NIPs are usually expressed at very low levels and only present in restricted cell types and tissues. Even though the exact expression for many of these genes is not known yet, the detection of expressional changes in microarray studies may in fact reflect dramatic changes in expression within specific cell types and tissues.

The Physiological Role of NIPs

Arsenic is commonly recognized as a threat for life. The exposure to As results in a number of specific cell responses but also in increased oxidative stress response. Such cross responses may explain the initially unexpected beneficial effects of As on plant growth such as corn roots grown in culture.[72] However, the permeability of MIPs to As(III) has certainly not evolved to serve the uptake of As(III) from soil. Rather it is likely that As(III) permeability is the result of physicochemical similarity between As(III) and other hydroxylated metalloids. Alternatively it is possible that NIPs have evolved to aid plants in As resistance and detoxification. A common strategy amongst different organisms is to reduce As(V) to the more mobile As(III), which is subsequently sequestered or extruded (see also the chapter by Rosen and Tamas in this book). In the bacterium *Sinorhizobium meliloti*, a MIP gene is part of its As resistance operon and constitutes the only transport protein of that organism to extrude As(III).[73] If MIPs do play a role in As detoxification in plants is still a matter of speculation. However, a physiological study in rice and tomato has provided evidence for a high capacity As(III) efflux system in roots.[74] Feeding tomato and rice roots in hydroponic culture with As(V) resulted in uptake, reduction and rapid, subsequent release of As(III) back in the medium. It was shown that As(III) efflux exceeded As accumulation 3 times within 24h of exposure. Such cycling of As involving plant roots may be responsible for the accumulation of As(III), which was measured in the vicinity of plant roots grown in aerobic soil.[75,76] In aerated, aerobic soils, As(III) will eventually enter the As cycle and become reduced back to As(V) (see also the chapter by Lombi and Holm in this book). However, the oxidation of As(III) in the soil is slower compared to the reduction and extrusion by plant roots, potentially establishing a mechanism to reduce the net As content in plants. Some data however strike the involvement of NIPs in the extrusion of As(III): The application of a protonophore and an uncoupler of oxidative phosphorylation inhibited the As(III) efflux in rice,[74] suggesting that an active transport system similar to bacterial ArsB or yeast Acr3p catalyses As(III)-extrusion. However, such inhibitors are not very specific and may have more general negative effects on growth and physiology. Therefore, inhibitor studies only provide limited proof for molecular functions, and the possibility that NIPs are involved in root As(III) extrusion remains a possibility. Passive diffusion of As(III) through NIPs would provide a low-cost, highly efficient mechanism for rapid extrusion of As(III) from the roots to the rhizosphere.

Do MIPs Play a Physiological Role in the Translocation of Toxic Compounds Such as As(III)?

MIPs are channel proteins. The energy for transport of substrates across channels is provided by the concentration gradient across the membrane. As MIPs are bi-directional channels, the direction of transport should be governed by the direction of the gradient across the membrane.

If the substrate is a toxic compound, like it is the case for As(III), the channel may facilitate the release of the compound from the cell, provided the extracellular concentration is lower than the concentration inside. This would indeed be the case for plant roots in aerobic soils, where inside the cell, As(V) is continuously reduced to As(III), which in turn becomes oxidized to As(V) in the soil. NIPs located at the distal cell membrane domains of the epidermis or the endodermis could aid the plants to survive by keeping As efficiently out of the root and the central vascular tissue and to prevent the transport of As(III) to the shoot.

However, if the external concentration of the toxic compound is higher than the intracellular concentration, the channel will facilitate the uptake of the toxic compound. Under changing environmental conditions in the soil as for example under flooding, As(V) will be reduced to As(III). Under such conditions, gating the channel or reduced expression of the channel may provide a means to minimize As(III) influx and uptake in the transpiration stream. Also, the redirection of trafficking of the channel to other cell membrane domains may be a mechanism to regulate As(III) flux under adverse conditions.

Fps1 in yeast is regulated in response to extracellular As(III) via the well-characterized MAPK Hog1p pathway. Here, the N-terminal domain functions as a lid to close the channel and to avoid As(III) uptake.[77] Interestingly, extracellular As(V) did not lead to gating of Fps1p. Therefore in a changing environment with varying redox-potential, this regulation of Fps1p ultimately should results in a preferential flux of As(III) out of the cell.

Alternatively to gating by an intra-molecular, conformational change, a catalytic helper protein could be involved in channel regulation, similar to a mechanism known for a metallochaperon that interacts with the bacterial As(III)-extrusion-pump, ArsAB.[78] Interaction with the metallochaperon increases ArsAB activity.

Regulating Bi-Directional Flux Through MIPs

There are other potential molecular mechanisms that may directly influence the direction of flux through NIPs. Such mechanisms would result in asymmetric influx- and efflux-capacity. Quantitative measurements to proof asymmetrical flux through a channel itself would require the incorporation of purified channels into planar lipid bilayers, with all channel proteins being inserted into the lipid bilayer in the same orientation facing the cytoplasmic side to one and the extracytoplasmic side to the other direction. This would allow to apply concentration gradients of substrates from either side of the channel opening and to quantify the corresponding fluxes across the artificial membranes. Unfortunately, such measurements have not been performed for NIPs and As(III) as substrate. However, some physiological measurements, indeed suggest that NIPs provide a preferential direction of flux out of the cell when expressed in yeast.[41] When compared to rat AQP9, AtNIP5;1 and AtNIP6;1 from Arabidopsis proofed to be very efficient to efflux As(III) upon external addition of As(V). However, yeast expressing the same NIPs suffered less from externally supplied As(III) than yeast expressing rat AQP9. Therefore, the Arabidopsis NIPs seemed to be more efficient in As(III) efflux but less efficient in influx when compared to AQP9. Either, one of the channels must be regulated in the heterologous system, or structural differences of the channel path provide a means to discriminate As(III) from inside versus outside.

Rougé and Barre[79] modelled the chemical environment through NIP isoforms belonging to the functional subgroups NIPII and NIPIII, both of which have been shown to flux As(III) (see Table 1). The model suggested a bipartite/uneven distribution of electric charges along the channel pathway, in which the extracellular vestibule was more electropositively charged and the cytosolic vestibule was more electronegatively charged. These dissimilar physicochemical environments at opposing vestibules of the channel could cause a different accessibility of polar molecules like As(III) or other metalloids each in a specific manner, thereby providing a pathway with preferential direction of flow across the membrane.

In conclusion, it remains to be resolved, if plant NIPs play a physiological role in As(III) detoxification. In other organisms, some of the above mentioned mechanism to regulate MIPs for a directional flux of As(III) do indeed exist, in line with the observation that certain MIPs are

physiologically important for the efflux of hazardous solutes: (i) ScFps1p is gated in response to As(III) but not As(V) allowing intracellular As(III) to efflux from the cell[77] (ii) SmAqpS, from *Sinorhizobium meliloti* is part of the As resistance operon where it has become part of a machinery to efflux toxic As(III)[73] and (iii) the aquaglyceroporin, PfAQP, from *Plasmodium falciparum* was suggested to play a key role in the release of toxic concentrations of NH_3 out of the parasite.[80]

Plant NIPs Transport Trivalent Antimony

Like As, Sb represents a toxic metalloid for all kind of living beings. As opposed to As, however, Sb is very rare in the biosphere (abundance of Sb in the Earth's crust range from 0.2 to 0.5 parts per million). The most important use of Sb is as a hardener in lead for storage batteries, as solders and other alloys and as base material for munitions. Our daily contact to Sb in particular to Sb(III) is probable due to its usage in flame-retardant formulations. These flame-retardant applications include products such as children's clothing, toys, floors, tablecloths, curtains, aircraft and automobile seat covers. More-over pentavalent Sb containing drugs are still the first line of treatment for infections with *Leishmania* parasites (leishmaniasis) (see also the chapter by Mukhopadhyay and Beitz in this book). Apart from mining sites or sites of high munition usage Sb does not reach concentrations high enough to act as a general threat for organisms including plants.

The capacity to mediate the transport of Sb(III) seems to be conserved in NIPs. We observed that expression of a number of As(III)-permeable NIPs from *A. thaliana* (AtNIP5;1, AtNIP6;1 and AtNIP7;1), *L. japonicus* (LjNIP5;1 and LjNIP6;1) and *O. sativa* (Lsi1/OsNIP2;1, OsNIP2;2 and OsNIP3;2) in yeast also increased the sensitivity to Sb(III).[41] Also *nip1;1-1* showed reduced sensitivity to Sb(III),[43] together suggesting that the capacity to mediate the transport of Sb(III) and As(III) is conserved in NIPs (see Table 1). However, expression of various NIP isoforms in yeast did not always lead to the same level of sensitivity towards both metalloids,[41] suggesting that NIPs do discriminate between these highly similar metalloids.

Conclusion

Arsenic in the environment represent a serious global problem. Arsenic enters the food chain via bioaccumulation in crop plants and As(III) is the major form of As in plants. Recent research in plant molecular biology has revealed that NIPs channel As(III) and play a major role in uptake and distribution of As(III) in plants. From biotechnological perspective, this opens the possibility to generate crop varieties with reduced content of As. There is a need to understand the contribution of NIPs in uptake and relocalization of As in various plant organs to bypass the grain as a major destination for the flow of As within the plant. The result of this research is also relevant for the use of plants in strategies to clean up polluted sites. Understanding the molecular mechanism of As accumulator plants such as the fern *Pteris vittata* provide a lesson on how plants can maximize As uptake by simultaneously avoiding toxicity.

Various environmental conditions probably forced plants to develop different pathways for As resulting in a molecular interplay between phosphate transporters, As reductases, NIPs and potentially other players. To implement the recent knowledge in breeding and biotechnology requires further understanding of metalloid transport in plants. NIPs do not only channel As(III) but also hydroxylated forms of essential and beneficial metalloids such as B and Si, respectively. In two of our model plant species, rice and Arabidopsis, we have just begun to understand the complexity of metalloid uptake and redistribution. More genes of the NIP family will have to be cloned and characterized from other species and in particular crop plants and their specific in vivo-roles need to be characterized.

Acknowledgements

Work in the authors laboratories was supported by grants from the Danish Ministery for Science Technology and Innovation 09-065019 to TPJ and from the Marie Curie Actions, FP7-PEOPLE-IEF-2008, Proposal N° 235618 to GPB.

References

1. Meharg AA. Venomous Earth. New York: Macmillan, 2005.
2. Nriagu JO, Pacyna JM. Quantitative assessment of worldwide contamination of air, water and soils by trace metals. Nature 1988; 333:134-139.
3. Aldrich MV, Peralta-Videa JR, Parsons JG et al. Examination of arsenic(III) and (V) uptake by the desert plant species mesquite (Prosopis spp.) using X-ray absorption spectroscopy. Sci Total Environ 2007; 379:249-255.
4. Oscarson DW, Huang PM, Liaw WK. Role of manganese in the oxidation of arsenite by freshwater lake sediments. Clays and Clay Minerals 1981; 29:219-225.
5. Stolz JF, Basu P, Santini JM et al. Arsenic and selenium in microbial metabolism. Annu Rev Microbiol 2006; 60:107-130.
6. Meharg AA, Williams PN, Adomako E et al. Gepgraphical variation in total and inorganic content of polishes (white) rice. Environ Sci Technol 2009; 43:1612-1617.
7. Ellis DR, Gumaelius L, Indriolo E et al. A novel arsenate reductase from the arsenic hyperaccumulating fern Pteris vittata. Plant Physiol 2006; 141:1544-1554.
8. Dhankher OP, Rosen BP, McKinney EC et al. Hyperaccumulation of arsenic in the shoots of Arabidopsis silenced for arsenate reductase (ACR2). Proc Natl Acad Sci USA 2006; 103:5413-5418.
9. Requejo R, Tena M. Proteome analysis of maize roots reveals that oxidative stress is a main contributing factor to plant arsenic toxicity. Phytochemistry 2005; 66:1519-1528.
10. Walsh LM, Sumner ME, Keeney DR. Occurrence and distribution of arsenic in soils and plants. Environ Health Perspectives 1977; 19:67-71.
11. Sun GX, Williams PN, Zhu YG et al. Survey of arsenic and its speciation in rice products such as breakfast cereals, rice crackers and Japanese rice condiments. Environ Intern 2009; 35:473-475.
12. Sun GX, Williams PN, Carey AM et al. Inorganic arsenic in rice bran and its products are an order of magnitude higher than in bulk bran. Environ Sci Technol 2008; 42:7542-7546.
13. Ng JC. Environmental contamination of arsenic and its toxicological impact on humans. Environmental Chemistry 2005; 2:146-160.
14. Branch S, Ebdon L, O'Neill P. Determination of arsenic species in fish by directly coupled high-performance liquid chromatography-inductively coupled plasma mass spectroscopy. J Anal At Spectrom 1994; 9:33-37.
15. Mir KA, Rutter A, Koch I et al. Extraction and speciation of arsenic in plants grown on arsenic contaminated soils. Talanta 2007; 72:1507-1518.
16. Pickering IJ, Prince RC, George MJ et al. Reduction and coordination of arsenic in Indian mustard. Plant Physiol 2000; 122:1171-1177.
17. Pickering IJ, Gumaelius L, Harris HH et al. Localizing the biochemical transformations of arsenate in a hyperaccumulating fern. Environ Sci Technol 2006; 40:5010-5014.
18. Hokura A, Omuma R, Terada N et al. Arsenic distribution and speciation in an arsenic hyperaccumulator fern by X-ray spectrometry utilizing a synchrotron radiation source. J Anal At Sectrom 2006; 21:321-328.
19. Smith PG, Koch I, Reimer KJ. Uptake, transport and transformation of arsenate in radishes (Raphanus sativus). Sci Total Environ 2008; 390:188-197.
20. Castillo-Michel H, Parsons JG, Peralta-Videa JR et al. Use of X-ray absorption spectroscopy and biochemical techniques to characterize arsenic uptake and reduction in pea (Pisum sativum) plants. Plant Physiol Biochem 2007; 45:457-463.
21. Asher CJ, Reay PF. Arsenic uptake by barley seedlings. Aust J Plant Physiol 1979; 6:459-466.
22. Ullrich-Eberius CI, Sanz A, Novacky J. Evaluation of atsenate- and vanadate-associated changes of electtical membrane potential and phosphate transport in Lemna gibba. J Exp Bot 1989; 40:119-128.
23. Meharg AA, Macnair MR. Suppression of the high affinity phosphate uptake system: a mechanism of arsenate tolerance in Holcus lanthanus L. J Exp Bot 1992; 43:519-524.
24. Meharg AA, Naylor J, Macnair MR. Phosphorus nutrition of arsenate-tolerant and nontolerant phenotypes of Velvet grass. J Environ Qual 1994; 23:234-238.
25. Esteban E, Carpena RO, Meharg AA. High-affinity phosphate/arsenate transport in white lupin (Lupinus albus) is relatively insensitive to phosphate status. New Phytologist 2003; 158:165-173.
26. Shin H, Shin HS, Dewbre GR et al. Phosphate transport in Arabidopsis: Pht1;1 and Pht1;4 play a major role in phosphate acquisition from both low- and high-phosphate environments. Plant J 2004; 39:629-642.
27. Gonzalez E, Solano R, Rubio V et al. Phosphate transporter traffic facilitator1 is a plant-specific sEC12-related protein that enables the endoplasmic reticulum exit of a high-affinity phosphate transporter in Arabidopsis. Plant Cell 2005; 17:3500-3512.
28. Abedin MJ, Feldmann J, Meharg AA. Uptake kinetics of arsenic species in rice plants. Plant Physiol 2002; 128:1120-1128.

29. Irtelli B, Navari-Izzo F. Uptake kinetics of different arsenic species by Brassica carinata. Plant and Soil 2008; 303:105-113.
30. Sharples JM, Meharg AA, Chambers SM et al. Mechanism of arsenate resistance in the ericoid mycorrhizal fungus Hymenoscyphus ericae. Plant Physiol 2000; 124:1327-1334.
31. Gustavsson S, Lebrun AS, Nordén K et al. A novel plant major intrinsic protein in Physcomitrella patens most similar to bacterial glycerol channels. Plant Physiol 2005; 139:287-295.
32. Weig A, Deswarte C, Chrispeels MJ. The major intrinsic protein family of Arabidopsis has 23 members that form three distinct groups with functional aquaporins in each group. Plant Physiol 1997; 114:1347-1357.
33. Guenther JF, Roberts DM. Water-selective and multifunctional aquaporins from Lotus japonicus nodules. Planta 2000; 210:741-748.
34. Wallace IS, Wills DM, Guenther JF et al. Functional selectivity for glycerol of the nodulin 26 subfamily of plant membrane intrinsic proteins. FEBS Lett 2002; 523:109-112.
35. Cabello-Hurtado F, Ramos J. Isolation and functional analysis of the glycerol permease activity of two new nodulin-like intrinsic proteins from salt stressed roots of the halophyte Atriplex nummularia. Plant Sci 2004; 166:633-640.
36. Porquet A, Filella M. Structural evidence of the similarity of $Sb(OH)_3$ and $As(OH)_3$ with glycerol: implications for their uptake. Chem Res Toxicol 2007; 20:1269-1276.
37. Wallace IS, Roberts DM. Distinct transport selectivity of two structural subclasses of the nodulin-like intrinsic protein family of plant aquaglyceroporin channels. Biochemistry 2005; 44:16826-16834.
38. Bansal A, Sankararamakrishnan R. Homology modeling of major intrinsic proteins in rice, maize and Arabidopsis: comparative analysis of transmembrane helix association and aromatic/arginine selectivity filters. BMC Struct Biol 2007; 7:27.
39. Takano J, Wada M, Ludewig U et al. The Arabidopsis major intrinsic protein NIP5;1 is essential for efficient boron uptake and plant development under boron limitation. Plant Cell 2006; 18:1498-1509.
40. Ma JF, Tamai K, Yamaji N et al. A silicon transporter in rice. Nature 2006; 440:688-691.
41. Bienert GP, Thorsen M, Schüssler MD et al. A subgroup of plant aquaporins facilitate the bi-directional diffusion of $As(OH)_3$ and $Sb(OH)_3$ across membranes. BMC Biol 2008; 6:26.
42. Isayenkov SV, Maathuis FJM. The Arabidopsis thaliana aquaglyceroporin AtNIP7;1 is a pathway for arsenite uptake. FEBS Lett 2008; 582:1625-1628.
43. Kamiya T, Tanaka M, Mitani N et al. NIP1;1, an aquaporin homolog, determines the arsenite sensitivity of Arabidopsis thaliana. J Biol Chem 2009; 284:2114-2120.
44. Ma JF, Yamaji N, Mitani N et al. Transporters of arsenite in rice and their role in arsenic accumulation in rice grain. Proc Nat Acad Sci USA 2008; 105:9931-9935.
45. Ma JF, Yamaji N, Mitani N et al. An efflux transporter of silicon in rice. Nature 2007; 448:209-212.
46. Yamaji N, Mitatni N, Ma JF. A transporter redgulating silicon distribution in rice shoots. Plant Cell 2008; 1381-1389.
47. Hughes MF, Kenyon EM, Edwards BC et al. Accumulation and metabolism of arsenic. Toxicol Appl Pharmacol 2003; 191:202-210.
48. Kenyon EM, Del Razo LM, Hughes MF. Tissue distribution and urinary excretion of inorganic arsenic and its methylated metabolites in mice following acute oral administration of arsenate. Toxicol Sci 2005; 85:468-475.
49. Liu Z, Styblo M, Rosen BP. Methylarsonous acid transport by aquaglyceroporins. Environ Health Perspect 2006; 114:527-531.
50. Zavala YJ, Gerads R, Gürleyük H et al. Arsenic in rice: II. Arsenic speciation in USA grain and implications for human health. Environ Sci Technol 2008; 42:3861-3866.
51. Wu J, Zhang R, Ross L. Methylation of arsenic in vitro by cell extracts from bentgrass (Agrostis tenuis): effect of acute exposure of plants to arsenate. Func Plant Biol 2002; 29:73-80.
52. Zhao FJ, Ma JF, Meharg AA et al. Arsenic uptake and metabolism in plants. New Phytol 2009; 181:777-794.
53. Raab A, Williams PN, Meharg A et al. Uptake and translocation of inorganic and methylated arsenic species by plants. Environ Chem 2007; 4:197-203.
54. Marin AR, Masscheleyn PH, Patrick WH. The influence of chemical form and concentration of arsenic on rice growth and tissue arsenic concentration. Plant Soil 1992; 139:175-183.
55. Carbonell-Barrachina AA, Aarabi MA, DeLaune RD et al. The influence of arsenic chemical form and concentration on Spartina patens and Spartina alterniflora growth and tissue arsenic concentration. Plant Soil 1998; 198:33-43.
56. Carbonell-Barrachina AA, Burlo F, Valero D et al. Arsenic toxicity and accumulation in turnip as affected by arsenic chemical speciation. J Agric Food Chem 1999; 47:2288-2294.
57. Burló F, Guijarro I, Carbonell-Barrachina AA et al. Arsenic species: Effects on and accumulation by tomato plants. J Agric Food Chem 1999; 47:1247-1253.

58. Abbas MHH, Meharg AA. Arsenate, arsenite and dimethyl arsinic acid (DMA) uptake and tolerance in maize (Zea mays L.). Plant Soil 2008; 304:277-289.
59. Li RY, Ago Y, Liu WJ et al. The rice aquaporin Lsi1 mediates uptake of methylated arsenic species. Plant Physiol 2009; in press DOI:10.1104/pp.109.140350.
60. Duan GL, Zhou Y, Tong YP et al. A CDC25 homologue from rice functions as an arsenate reductase. New Phytol 2007; 174:311-321.
61. Li Y, Dhankher OP, Carreira L et al. Overexpression of phytochelatin synthase in Arabidopsis leads to enhanced arsenic tolerance and cadmium hypersensitivity. Plant Cell Physiol 2004; 45:1787-1797.
62. Picault N, Cazalé AC, Beyly A et al. Chloroplast targeting of phytochelatin synthase in Arabidopsis: effects on heavy metal tolerance and accumulation. Biochimie 2006; 88:1743-1750.
63. Schmoger MEV, Oven M, Grill E. Detoxification of arsenic by phytochelatins in plants. Plant Physiol 2000; 122:793-801.
64. Wysocki R, Chéry CC, Wawrzycka D et al. The glycerol channel Fps1p mediates the uptake of arsenite and antimonite in Saccharomyces cerevisiae. Mol Microbiol 2001; 40:1391-1401.
65. Sugiyama A, Shitan N, Sato S et al. Genome-wide analysis of ATP-binding cassette (ABC) proteins in a model legume plant, Lotus japonicus: comparison with Arabidopsis ABC protein family. DNA Res 2006; 13:205-228.
66. Requejo R, Tena M. Maize response to acute arsenic toxicity as revealed by proteome analysis of plant shoots. Proteomics 2006; 6:156-162.
67. Abercrombie JM, Halfhill MD, Ranjan P et al. Transcriptioal responses of Arabidopsis thaliana plants to As(V) stress. BMC Plant Biology 2008; 8:87.
68. Norton GJ, Lou-Hing DE, Meharg AA et al. Rice-arsenate interactions in hydroponics: whole genome transcriptional analysis. J Exp Bot 2008; 59:2267-2276.
69. Norton GJ, Nigar M, Williams PN et al. Rice-arsenate interactions in hydroponics: a three-gene model for tolerance. J Exp Bot 2008; 59:2277-2284.
70. Chakrabarty D, Trivedi PK, Misra P et al. Comparative transcriptome analysis of arsenate and arsenite stresses in rice seedlings. Chemosphere 2009; 74:688-702.
71. Hanaoka H, Fujiwara F. Channel-mediated boron transport in rice. Plant Cell Physiol 2007; 48:227.
72. Evans G, Evans J, Redman A et al. Unexpected beneficial effects of arsenic on corn roots grown in culture. Environ Chem 2005; 2:167-170.
73. Yang HC, Cheng J, Finan TM et al. Novel pathway for arsenic detoxification in the legume symbiont Sinorhizobium meliloti. J Bacteriol 2005; 187:6991-6997.
74. Xu XY, McGrath SP, Zhao FJ. Rapid reduction of arsenate in the medium mediated by plant roots. New Phytol 2007; 176:590-599.
75. Ultra VUY, Tanaka S, Sakurai K et al. Arbuscular mycorrhizal fungus (Glomus aggregatum) influences biotransformation of arsenic in the rhizosphere of sunflower (Helianthus annuus L.). Soil Sci Plant Nutr 2007; 53:499-508.
76. Vetterlein D, Szegedi K, Ackermann J et al. Competitive mobilization of phosphate and arsenate associated with goethite by root activity. J Environ Qual 2007; 36:1811-1820.
77. Thorsen M, Di Y, Tängemo C et al. The MAPK Hog1p modulates Fps1p-dependent arsenite uptake and tolerance in yeast. Mol Microbiol 2001; 40:1391-1401.
78. Lin YF, Yang J, Rosen BP. ArsD residues Cys12, Cys13 and Cys18 form an As(III)-binding site required for arsenic metallochaperone activity. J Biol Chem 2007; 282:16783-16791.
79. Rougé P, Barre A. A molecular modeling approach defines a new group of Nodulin 26-like aquaporins in plants. Biochem Biophys Res Commun 2008; 367:60-66.
80. Zeuthen T, Wu B, Pavlovic-Djuranovic S et al. Ammonia permeability of the aquaglyceroporins from Plasmodium falciparum, Toxoplasma gondii and Trypansoma brucei. Mol Microbiol 2006; 61:1598-1608.
81. Weig AR, Jakob C. Functional identification of the glycerol permease activity of Arabidopsis thaliana NLM1 and NLM2 proteins by heterologous expression in Saccharomyces cerevisiae. FEBS Lett 2000; 481:293-298.
82. Tanaka M, Wallace IS, Takano J et al. NIP6;1 Is a boric acid channel for preferential transport of boron to growing shoot tissues in Arabidopsis. Plant Cell 2008; 20:2860-2875.
83. Liu Z, Carbrey JM, Agre P et al. Arsenic trioxide uptake by human and rat aquaglyceroporins. Biochem Biophys Res Commun 2004; 316:1178-1185.
84. Liu Z, Shen J, Carbrey JM et al. Arsenite transport by mammalian aquaglyceroporins AQP7 and AQP9. Proc Natl Acad Sci USA 2002; 99:6053-6058.
85. Sanders OI, Rensing C, Kuroda M et al. Antimonite is accumulated by the glycerol facilitator GlpF in Escherichia coli. J Bacteriol 1997; 179:3365-3367.
86. Gourbal B, Sonuc N, Bhattacharjee H et al. Drug uptake and modulation of drug resistance in Leishmania by an aquaglyceroporin. J Biol Chem 2004; 279:31010-31017.
87. Marquis N, Gourbal B, Rosen BP et al. Modulation in aquaglyceroporin AQP1 gene transcript levels in drug-resistant Leishmania. Mol Microbiol 2005; 57:1690-1699.

Elsbeth Bienert

Chapter 10

Major Intrinsic Proteins in Biomimetic Membranes

Claus Hélix Nielsen*

Abstract

Biological membranes define the structural and functional boundaries in living cells and their organelles. The integrity of the cell depends on its ability to separate inside from outside and yet at the same time allow massive transport of matter in and out the cell. Nature has elegantly met this challenge by developing membranes in the form of lipid bilayers in which specialized transport proteins are incorporated. This raises the question: is it possible to mimic biological membranes and create a membrane based sensor and/or separation device?

In the development of a biomimetic sensor/separation technology, a unique class of membrane transport proteins is especially interesting—the major intrinsic proteins (MIPs). Generally, MIPs conduct water molecules and selected solutes in and out of the cell while preventing the passage of other solutes, a property critical for the conservation of the cells internal pH and salt concentration. Also known as water channels or aquaporins they are highly efficient membrane pore proteins some of which are capable of transporting water at very high rates up to 10^9 molecules per second. Some MIPs transport other small, uncharged solutes, such as glycerol and other permeants such as carbon dioxide, nitric oxide, ammonia, hydrogen peroxide and the metalloids antimonite, arsenite, silicic and boric acid depending on the effective restriction mechanism of the protein. The flux properties of MIPs thus lead to the question if MIPs can be used in separation devices or as sensor devices based on e.g., the selective permeation of metalloids.

In principle a MIP based membrane sensor/separation device requires the supporting biomimetic matrix to be virtually impermeable to anything but water or the solute in question. In practice, however, a biomimetic support matrix will generally have finite permeabilities to both electrolytes and non-electrolytes. The feasibility of a biomimetic MIP device thus depends on the relative transport contribution from both protein and biomimetic support matrix. Also the biomimetic matrix must be encapsulated in order to protect it and make it sufficiently stable in a final application. Here, I specifically discuss the feasibility of developing osmotic biomimetic MIP membranes, but the technical issues are of general concern in the design of biomimetic membranes capable of supporting selective transmembrane fluxes.

Introduction

Membranes for separation purposes require high permeability and high selectivity with sufficient mechanical stability to be functional. Reverse osmosis (RO) membranes fulfill these design criteria to a large extent by having relatively high water permeability while maintaining a good salt rejection. RO membranes have found use in drinking water production and waste water treatment for several decades. The driving force in RO operation is pressure, where a force exceeding

*Claus Hélix Nielsen—Department of Physics, Technical University of Denmark, DK-2800 Lyngby, Email: claus.nielsen@fysik.dtu.dk

MIPs and Their Role in the Exchange of Metalloids, edited by Thomas P. Jahn and Gerd P. Bienert.

the osmotic pressure difference across the membrane is applied and convective flux against the osmotic gradient is established. The operating mechanism in RO membranes is based on diffusion of water and small molecular weight organic molecules (non-electrolytes) through the membrane polymer by bonding transiently to the hydrophilic polymer meshwork. Electrolytes (ions) and larger non-electrolytes will not permeate easily due to charge and size exclusion, respectively. Classic RO is a fundamental separation process and in principle it is a realization of the semi-permeable membrane concept as introduced by Nernst a century ago.[1] Solvent (water) passes through the membrane leaving all other solutes behind.

In practice the ability of RO membranes to transport water and certain other molecules while rejecting others is not absolute. The passage of smaller molecules or less charged ions will be greater than the passage of larger molecules or highly charged ions. Thus Ca^{2+} will typically be rejected about three times better than Na^{+}. The passage of dissolved salts through an RO membrane may be as high as 5% as observed for nitrates depending on the particular membrane and on factors such as driving force (pressure), temperature and pH. Also, the closer an organic molecule structure resembles the structure of the RO polymer, the more readily it diffuses through the RO membrane.[2] However some types of RO membranes like sea water reverse osmosis (SWRO) membranes and some nanofiltration (NF) membranes are capable of some metal and metalloid removal.[3]

RO membranes have a biological counterpart—the set of membranes defining boundaries and organelles in living cells. Membrane transport (by e.g., ion channels and transporters) constitutes the basis for all living cells. Cellular membranes are highly specialized complexes of proteins and amphiphilic molecules (mostly lipids). The viability and function of all cells is critically dependent on very controlled fluxes of electrolytes, non-electrolytes and water across their membranes. While neutral gases and to some extent water can cross a protein-free lipid membrane, transmembrane flux of electrolytes is negligible due to the membranes hydrophobic interior. Electrolytes, most notably monovalent and divalent ions, are transported across membranes through highly specialized and ion-selective proteins. Channels facilitate the diffusion along concentration gradients whereas ATP-driven carriers and transporters catalyze uphill transport against concentration gradients. The cells basal need to adjust their osmotic balance (volume) as well as some cell's specific role in water reabsorption (e.g., renal concentration) requires a much larger transport of water than the pure lipid membrane supports. This transport is mediated by specialized water channel proteins—MIPs. In terms of large scale separation, ion channels and water channels are most interesting as their transport is decoupled from conformational changes in the protein—in contrast to the situation for carriers (e.g., the $Na^{+}K^{+}$ ATPase) where the catalytic event (the transfer of a molecule) is coupled to complex changes in protein structure. The result is a 10-1000 fold lower transport capacity in carriers compared to channel proteins. Although biomimetic membranes with transporters have interesting technological potential,[4] I will limit the scope here to MIP channel proteins in biomimetic membranes.

Biological membranes can be seen as a lipid matrix with effective barrier properties in which proteins with very specific properties such as high selectivity and permeability for solutes (ions or water) are incorporated. This leads to the question whether biomimetic membranes can be used in separation/sensor applications. The high water permeability of many MIPs naturally raises the question if these proteins can be used in a water purification device for production of ultra pure water and seawater desalination. This has indeed been suggested[4,5] and attracted commercial interest.[6] Also MIP based up-concentration of contaminants e.g., metalloids as a step in early detection (i.e., at low feed water concentrations) may be feasible.

In this chapter I first briefly define the concept of biomimetic membranes and present some recent developments. Then I discuss issues involved in constructing a biomimetic osmotic type membrane in the form of MIPs embedded in lipid bilayers.

Biomimetic Membranes

The fundamental biomimetic approach is to extract guiding principles from nature in order to provide a basis for creating technological devices. Biomimetic membrane design takes cues from the self-assembly of lipids or other amphiphilic molecules into bilayer membranes and from the

rich repertoire of functions displayed by biomembrane embedded proteins.[4] The overwhelming complexity of a biological membrane with hundreds of lipid species[7] and extensive coupling between membrane components and cytoskeletal elements[8,9] have to some extent been an obstacle in understanding membrane function e.g., the reciprocal coupling in lipid-protein interactions, which means that the bilayer can regulate protein function and vice versa.[10,11] Nevertheless, by combining only a few biomembrane components several biosensors based on biomimetic membrane designs have successfully been developed (for a review see ref. 4).

Since the first reports on formation of bimolecular lipids extensive investigations of the physical properties of these films have provided a detailed picture of bilayer cohesive, elastic and structural properties.[12] At the same time techniques were developed for the reconstitution of proteins starting with incorporation of excitable protein material (voltage gated ion channels) over the first demonstration of single channel activity to the current plethora of reconstituted membrane proteins including receptors, ion channels and transporters.[13] With the introduction of microfluidic chip designs, formation of micron-sized membranes is now possible[14-18] and automation strategies enabling the creation of very large membrane arrays have recently been demonstrated.[19-21]

Recent developments in polymer research have resulted in the concept of polymer membranes formed from amphiphilic copolymers with barrier properties similar to lipid bilayers. Also, protein reconstitution in polymer membranes has been demonstrated.[5,22,23]

Sensor Applications

In most applications of biomimetic membrane biosensors, the membrane serves as a passive matrix for the embedded proteins where the protein typically a receptor can sense a signal and somehow transduce the information. Biosensors based on ion channels, where controlled flow of selected ions constitutes part of the mechanism, can sense low ionic concentrations as comparably few ions are needed to generate pA currents, which are easily detectable using standard voltage-clamp electrophysiological methods.[24] In either case, the biosensors do not require massive flux of matter across the membrane in order to function. Therefore, the membrane matrix can be supported on solid micro-patterned supports such as the smooth surfaces of cleaved mica, silicon or carbon nanotubes either directly or via an intermediate noncovalently bound or covalently tethered cushion for increased stability and portability.[25-32]

The application of supported membranes in the design of biosensors mounted on electro-optical devices is attracting considerable interest. One such example is surface plasmon resonance (SPR) a method that allows for real-time measurements of ligand binding to immobilized proteins[33] and thus opens for the possibility to detect ligand binding to membrane bound (both membrane spanning and membrane adsorbed) proteins. Immunosensing can be seen as a special case of ligand binding sensing. *Staphylococcus* enterotoxin B (SEB) has been detected in milk via a microfluidic system with supported bilayer membranes and biotinylated anti-SEB IgG.[34] A further strategy is to take advantage of the electrical properties of bilayers and use them as insulating surfaces. Any defect in this surface is easily detectable as a change in impedance and as the defect locations create strong nonspecific binding sites, the sensitivity of such a device is high.[35] Impedance analysis on supported lipid bilayers can also be used to dissect the action of channel forming peptides e.g., the bee venom melittin,[36] the potassium specific valinomycin[37] and channel forming proteins e.g., the bacterial outer membrane porin Omp F on the bilayer.[38] This approach has also been used in lipid films where protein-driven energy transduction was realized by incorporation of bacteriorhodopsin (BR) and cytochrome c oxidase (COX)[39] into supported polymer membranes. Recently, approaches based on interconnected inverted micelles formed in two-phase systems (e.g., electrolyte and lipid-alkene solution as the bulk phase) have resulted in very stable systems seemingly well suited for medium- to high-throughput screening of membrane proteins.[40,41] This approach has been extended to simply make the bilayer from an electrolyte drop falling though the lipid-alkane solution with the resulting inverted micelle landing on a lipid monolayer surface formed at the aperture, thereby separating the alkane lipid phase from a lower electrolyte solution and enabling easy buffer exchange and electrical measurements.[42]

Separation Applications

The use of biomimetic membranes in separation applications requires a different approach. Even though a cushioned membrane (see above) on a solid support would have space for some transport on the cushion side, the demand for massive flux across the biomimetic membrane for separation precludes the use of nonporous support material. The extreme alternative—a free standing lipid bilayer[12] with incorporated MIPs (or ion channels) formed across an aperture—is not sufficiently stable to be used in a technological separation device. For planar membranes, this implies that the support material must be sufficiently porous in order to allow substantial vectorial flow. At the same time, the support material needs to be sufficiently dense to support the membrane in the presence of transmembrane pressure gradients. An early attempt to stabilize biomimetic membranes on porous supports included the use of polycarbonate and nitrocellulose filters in various protein reconstitution experiments (for a review see ref. 13). Although protein function could be demonstrated, the stabilization was in fact realized as an impregnation of the support with a lipid-hydrocarbon solvent resulting in lipid structures with complex water transport properties and consequently limited control over non-electrolyte passage. However, recent advances in membrane encapsulation methods and support material development open the possibility of implementing efficient biomimetic separation processes in the form of long-term stabilized membranes with embedded proteins on an industrial scale. Interesting developments in membrane encapsulation have been demonstrated using bacterial S-layer proteins[43-45] or hydrogels,[46-48] and porous polymers[49] may be used as porous support material for stabilization of biomimetic membranes.

Many issues are still not resolved. Simple model proteins notably β-barrel proteins or small peptides are inherently more stable than α-helical bundle proteins. Insertion and stabilization of the latter class of proteins which includes all MIPs and most of the ligand- and voltage-gated ion channels still poses many problems which must be resolved before large scale protein containing membranes can be produced and stored on a commercial basis. Thus a biomimetic device will have to meet design criteria based on both protein-based requirements as well as the requirement for well-defined and stable barrier properties. In addition the protein-matrix complex must be encapsulated in such a way that the encapsulation process does not compromise protein structure and function.

MIP Biomimetic Membranes and Osmotic Processes

MIP Basic Properties

Chapters in this book as well as several recent reviews have nicely summarized many fascinating aspects of MIP structure and function.[50-54] In this section I will present only the basic properties and discuss the permeability properties pertaining to biomimetic water/metalloid transporting membranes. MIPs constitute a family of 24-30 kDa pore forming integral membrane proteins. Since the purification of a red blood cell membrane protein: channel-forming Integral membrane Protein of 28 kDa (CHIP28)[55] and subsequent expression of this protein in *Xenopus* oocytes[56] and liposomes[57] revealing rapid water diffusion along osmotic gradients much has been revealed about this class of proteins for which the term *aquaporins* soon was coined.[58] Thirteen mammalian homologs (denoted AQP0-AQP12) are now known[59] and in total more than 450 members of the MIP family have been identified to date.[60]

The primary sequence of AQP1 reveals the canonical MIP structure in the form of two tandem repeats each containing three transmembrane spanning α-helices (TM1-3). Each tandem repeat contains a loop between TM2 and TM3 with an asparagine-proline-alanine (NPA) signature motif. Biochemical analysis and later crystal structures revealed an hour-glass structure with pseudo two-fold symmetry where the six TM segments surround a central pore structure defined by the two opposing NPA motifs (for a structural and chronological review see ref. 50). Each six TM AQP unit functions as a pore, and the predominant unit-assembly in biological membranes is a tetrameric arrangement.[61] Higher order arrays (aggregates) have been described for AQP4.[62,63] For AQP0 coaxial octamers formed by two juxtaposed tetramers have been proposed.[64]

Based on their permeability properties, mammalian homologs can be classified into two groups: aquaporins and aquaglyceroporins. The *E. coli* model system offers both variants:[65] the orthodox (i.e., 'water only') channel AqpZ[66] and the aquaglyceroporin GlpF.[67] Although some MIPs can be classified as strict water channels (e.g., AQP0, APP4 and AqpZ), it is becoming increasingly clear that many MIPs may have additional permeability properties.[53] For example the nonglycerol transporting AQP1 may transport cations[68] as well as carbon dioxide,[69] nitric oxide[70] and ammonia[71] and AQP6 may transport chloride at low pH.[72] It should, however, be stressed that cation/anion transport by MIPs is an exception. The water and glycerol transporting AQP7, AQP9 and GlpF may also transport metalloids e.g., hydroxylated forms of As and antimony;[73] and GlpF transports urea and glycine in addition to glycerol.[74,75] While the implications of these observations are still under debate,[53,76-78] there is clear evidence that the physiological role of some MIPs stems from both their metalloid and water permeability properties.[78]

In addition to the apparently complex permeability profile, several MIPs display various forms of gating—analogous to the opening and closing of ion channels induced by external stimuli; for ion channels typically in the form of transmembrane potential and/or chemical signals. Although many aspects of aquaporin gating and regulation of their permeability are still unknown, the function of some MIPs has been demonstrated to depend on calmodulin[79,80] phosphorylation[81,82] and pH.[79,83,84]

The application of MIPs and other helical bundle integral membrane proteins (e.g., ion channels) in a biomimetic separation membrane depends on controlling protein stability. Membrane proteins are inherently unstable and may require modifications to ensure effective insertion of stable constitutively active proteins.[85] In addition, the interaction (hydrophobic coupling) between the protein and the biomimetic membrane can affect protein stability and conformational equilibrium.[11,86] In the design of a biomimetic water filtration membrane which is based on MIPs, the issues of protein oligomerization, aggregation, selectivity, regulation and stability must be resolved in order to ensure efficient protein-mediated water filtration.

Considerations Regarding Permeability

A fundamental question to be addressed in the development of biomimetic membrane devices is how much transport is mediated by the proteinaceous pathways inserted into the membrane matrix and how much transport it mediated by the membrane matrix itself? A general answer to this question depends on the solute, the transporting protein and the structure of the support material. Although the phenomenon seems quite simple: solutes and water move in response to transmembrane gradients (electrochemical, hydrostatic and osmotic gradients), the intricate mechanistic details of protein and membrane selectivity have remained a major obstacle for understanding biological membrane transport for more than a century.

The simplicity of the MIP structure as outlined above is only apparent. The structure is the basis of the remarkable ability to transport water molecules and simultaneously reject charged species including protons. Molecular dynamics (MD) simulation based on the experimental structure of AQP1 strongly suggests single-file transport of water molecules through a narrow <3Å pore in which steric and electrostatic factors prevent electrolyte passage.[87] MD simulations have provided values for single channel water osmotic permeabilities p_f. Experimental values for p_f have also been obtained for several MIPs (see Table 1). Theoretical (MD) p_f values may not be accurate as they to some extend depend on the choice of MD force fields. Experimentally obtained single channel values are also characterized by some uncertainties due to the difficulty in quantifying the number of channels in a given preparation. Nevertheless the values listed in Table 1 obtained for various MIPs provide a quantitative basis for designing biomimetic MIP membranes for water separation/purification purposes.

Over the last decade it has become increasingly clear that some MIPs e.g., several plant Nodulin-26-like Intrinsic Proteins (NIPs) are specifically involved in the regulated uptake and distribution of selected metalloids, e.g.,[88-91] (reviewed in refs. 78,92,93). From a separation perspective it is interesting that some NIPs from the thale cress (*Arabidopsis thaliana*) transport boric acid $B(OH)_3$ while possessing a low water permeability (AtNIP5; 1)[89] or no water permeability at all (AtNIP6;1).[90] Also some rice (*Oryza sativa*) NIPs have metalloid transport properties:

Table 1. Aquaporin single channel (subunit) osmotic water permeability constants

	p_f 10^{-14}cm^3/s	Method	Reference
AQP0	0.25	Xenopus Oocytes	135
	0.2	MD[c]	136
AQP1[a]	4.6	Proteoliposomes	137
	5.43	Proteoliposomes	138
	6	Xenopus Oocytes	135
	11.7	Proteoliposomes	57
	10.3	MD[c]	136
AQP2	3.3	Xenopus Oocytes	135
AQP3	2.1	Xenopus Oocytes	135
AQP4	24	Xenopus Oocytes	135
	7.4	MD[c]	136
AQP5	5	Xenopus Oocytes	135
AqpZ	2	Planar bilayers	139
	>10	Proteoliposomes	140
	15.9	MD[c]	136
GlpF	0.7	Planar bilayers	141
	16	MD[c]	136

[a]Also known as Major Intrinsic Protein (MIP); [b]Also known as Channel forming Integral Protein of 28 kDa (CHIP28); [c]Based on a channel length of 16Å.

OsNIP2;1 not only transports $B(OH)_3$ but also silicic acid $Si(OH)_4$, urea and As(III) (see below) and OsNIP3;1 is a boric acid channel required for efficient growth under limited boron conditions.[94] Antimonite Sb(III) is transported by AtNIP7;1[95] and the *E.coli* GlpF.[88]

Of particular interest is the transport of As via MIPs. The toxic and carcinogenic As[96,97] occurs in natural aqueous environments in +5 and +3 oxidation states. The most common inorganic As compounds found in water are arsenite [As(III)] and arsenate [As(V)].[98] Arsenic may be methylated[99] as monomethylarsonic acid MMA(V), monomethylarsonous acid MMA(III), dimethylarsinic scid DMA(V), dimethylarsinous acid DMA(III) and trimethylarsine oxide TMAO. As(III) exists in reduced waters (low oxygen tension) and species may include H_3AsO_3 [As(III)] (pK = 9.23), $H_2AsO_3^-$ (pK = 12.13) and $HAsO_3^{2-}$ (pK = 13.4).[100] At neutral pH As(III) is present as As(III).

As(III) is transported by the mammalian aquaglyceroporins AQP7 and AQP9,[73,101] the aquaglyceroporin homologue AqpS from *Sinorhizobium meliloti*,[102] OsNIP1;1[103] and OsNIP2;1.[104] Also GlpF has been suggested as the basis for As(III) uptake in *E. coli*.[105] The closely related antimonite Sb(III) is also transported by AqpS,[102] AQP9[73] and GlpF.[105] The overlap between As(III), Sb(III) and glycerol transport capability points to some shared properties important for permeation. This is also illustrated by the fact that the methylated form MMA(III) is in fact transported more efficiently than As(III) by rat AQP9.[106]

When compared, As(III), Sb(III) and glycerol share significant physico-chemical properties.[107] As(III) and Sb(III) both adopt a trigonal pyramidal structure with the metalloid atom at the apex,[107,108] and glycerol may adopt a 'retracted' configuration in which the two carbons share the

Table 2. Permeability coefficients for protein-free phosphatidyl choline (PC) membranes

	Permeability Coefficeint cm/s	Remarks	Reference
Water	$4\ 10^{-3}$		115
	$2\ 10^{-3}$	PC:Chol 1:1	115
	$7.5\ 10^{-4}$	PC:Chol 1:8	115
	$8\ 10^{-5}$	ABA BPM[a]	5
Urea	$4\ 10^{-6}$		142
Glycerol	$5.4\ 10^{-6}$		142
Tetraphenyl-phosphonium (TPP^-)	10^{-7}		143
Na^+	10^{-14}		144
Cl^-	10^{-11}		144
H^+/OH^-	10^{-4}-10^{-8}		145
	10^{-9}	PLFE vesicles[b]	118

[a]$PMOXA_{15}$-$PDMS_{110}$-$PMOXA_{15}$ triblock copolymer; [b]PLFE Polar lipid fraction E from *Sulfolobus acidocalarius* membranes.

apex position and the three oxygen atoms defining a triangular surface plane with an area comparable to the corresponding area in As(III) and Sb(III) (~3.2-3.7 $Å^2$). The molecular volumes of As(III), Sb(III) and 'retracted' glycerol are similar (98-118 $Å^3$) which is consistent with all three molecules meeting the same steric constraints during the passage through an AQP. However it should be pointed out that the dipole moment of 'retracted' glycerol (4.07 Debye) is larger than for the metalloids with 2.32 and 2.15 Debye for As(III) and Sb(III), respectively, whereas the hydration energies are higher for the hydroxylated metalloids (~117 kJ mol^{-1}) than for 'retracted' glycerol (~84 kJ mol^{-1}).[107] This illustrates the complex interactions involving water and protein moieties (e.g., carbonyls) between the permeating molecules and the channel. From a biomimetic point this also illustrates the motivation to learn from biological structures and construct de novo channel structures with finely tuned permeation properties based on coordination between the permeating molecules and the proteins.[109,110]

Water, non-electrolyte and electrolyte permeabilities in protein-free lipid bilayers have been investigated intensely over the last 40 years. The permeability for water has been determined both by tracer diffusion measurements yielding the diffusional membrane permeability P_d and by osmotic flow measurements giving the osmotic membrane permeability P_f. Early experimental work reported that $P_f > P_d$ (e.g.,[111]) which could suggest a pore based (single-file) transport mechanism,[112,113] but this is an artifact due to unstirred layers (see also below). There is no evidence for aqueous single-file transport in protein-free bilayers (for a comprehensive review see ref. 114). P_f is a function of bilayer composition.[115] Thus, increasing the cholesterol content decreases the water permeability (see Table 2). Permeabilities for non-electrolytes have always been determined in diffusion measurements[116] and are therefore potentially erroneous due to unstirred layers. Generally, however, permeabilities measured for non-electrolytes are lower than those for water. In general, the barrier for hydrophilic solutes represented by the membrane interior while the barrier for hydrophobic solutes will be the membrane interfacial regions.[116] The high electrical resistance of lipid bilayers is reflected in its very low permeability coefficients for electrolytes i.e., small inorganic anions and cations (see Table 2). The electrolyte permeability can be affected by

amphiphilic anesthetics where physiologically relevant concentrations may increase potassium permeabilities 2-5 fold.[117] Also the membrane structure is important, both ether linked lipids and natural hydrocarbon solvents in the membranes can affect proton permeability.[4,118]

The interaction of As compounds with lipid membranes has been investigated and it appears that thermodynamic parameters for lipids with ethanolamine headgroups change upon exposure to As compounds while choline headgroup lipids are unaffected.[119,120] The effect seems to be related to both direct interactions between polar moieties of the As compounds and the lipids and indirect modification of the interface via changes in water bonding structure in the lipid headgroup region.[121] The water-octanol partitioning, log P which is a measure for molecular hydrophobicity[122] and thus partitioning into the lipid bilayer, reveals that methylation is an important factor for bilayer perturbation induced by As compounds. Both As(III) ($\log P < -3$) and As(V) ($\log P < -7$) are highly hydrophilic compared to DMA(V) ($\log P = 0.36$).[120] Thus in biomimetic designs involving separation/sensing As compounds, the log P (and thus potential bilayer permeation) must be considered in addition to the MIP permeation profiles per se.

Osmotic Processes

The large ratios between MIP-mediated water permeabilities and bilayer ionic permeabilities suggest that it should indeed be possible to construct an osmosis-based biomimetic membrane. MIP-mediated osmoregulation is vital to all living organisms (for recent reviews see refs. 123,124). In pure terms 'osmosis' describes the selective movement of water from regions with high water chemical potential (i.e., a dilute salt solution) to regions with low water chemical potential (i.e., concentrated salt solution). In water treatment/purification this process is typically reversed and realized using RO membranes as mentioned above. RO uses hydraulic pressure to oppose and exceed the osmotic pressure of an aqueous feed solution. Thus in RO the driving force is the applied pressure, whereas in osmosis the osmotic pressure is the driving force. A third osmotic process is called forward osmosis (FO) sometimes also referred to direct osmosis.[125] FO is based on using a draw solution on the permeate side of the membrane as the driving force. The force arises as the draw solution has a higher osmotic pressure than the feed solution. The main advantage of FO is that the hydraulic pressure is low (or even zero). This implies that the supported biomimetic MIP membrane will not be subjected to as high pressures as would be required in typical RO applications and the feed solution is treated 'gently'. In addition the fouling propensity (i.e., the clogging of the membrane) is reduced. Finally, pressure retarded osmosis (PRO)[126] is emerging as a promising process in power generation. PRO uses the osmotic pressure difference between seawater and fresh water to pressurize the saline stream thereby converting the osmotic pressure difference into a hydrostatic pressure capable of driving a turbine.[127]

All of the above mentioned osmotic processes can be described using the relation between volume (water) flux $\overline{J}$ [$cm^3 s^{-1}$] across a membrane separating aqueous Phases 1 and 2 due to osmotic and pressure driving forces:

$$\overline{J}_v = P_f A_M V_w / RT[(P_1 - P_2) + \sum_i \sigma_i(\Pi_{2i} - \Pi_{1i})], \quad (1)$$

where P_f is the membrane permeability [$cm\ s^{-1}$], A_M membrane area [cm^2], V_w the partial molar volume of water [$cm^3 mol^{-1}$], P the pressure [Pa], RT the product of the molar gas constant R [$JK^{-1}\ mol^{-1}$] and temperature T [K], σ_i the reflection coefficient for the i'th solute and $\Pi = \phi RTC$ is the van't Hoff osmotic pressure [Pa] where ϕ is the molar osmotic coefficient and C the concentration.

Let $J_v = \overline{J}_v / A_M$ [$cm\ s^{-1}$] be the volume flux per unit area. In the case of a pressure difference only (i.e., no concentration difference)

$$J_v = P_f V_w (P_1 - P_2)/RT = L_p (P_1 - P_2), \quad (2)$$

where $L_p = P_f V_w / RT$ [$cm^3 N^{-1}\ s^{-1}$] is the hydraulic permeability of the membrane. Thus we may rewrite

$$J_v = L_p[(P_1 - P_2) + RT \sum_i \phi_i \sigma_i (C_{2i} - C_{1i})]. \quad (3)$$

In the case of a concentration difference only (i.e., no pressure difference)

$$J_v = L_p \cdot RT \cdot \sum_i \phi_i \sigma_i (C_{2i} - C_{1i}) = P_f \sum_i \phi_i \sigma_i (C_{2i} - C_{1i}), \quad (4)$$

where $P_f^* = P_f \phi_i \sigma_i$ is the osmotic permeability of the membrane for the *i*'th solute. In the case of an ideal semi-permeable membrane ($\sigma_i = 1$) separating two ideal solutions ($\phi_i = 1$), the osmotic permeability is equal to the membrane permeability and P_f is sometimes in the literature also referred to as the osmotic permeability of the membrane.

Equation (1) can be simplified to describe water transport across an ideal membrane as

$$J_w = L_p(\sigma \Delta\Pi - \Delta P) \quad (5)$$

From this expression we can now directly relate the various osmotic processes to relations between ΔP and ΔΠ. For FO ΔP = 0, for RO ΔP > ΔΠ and for PRO ΔΠ > ΔP (see Fig. 1A,B).

In principle Eqns 1-5 constitute the very basis for osmotic driven water flow through a selectively water permeable membrane.

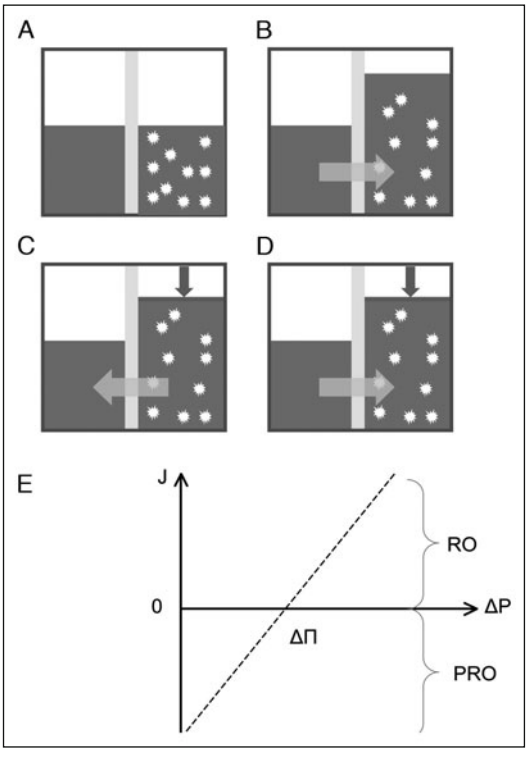


Figure 1. Osmotic membrane processes (after ref. 125). Solvent flow (A) initial condition with water impermeable membrane**.** (B-D): Flow through an ideal semipermeable (biomimetic aquaporin) membrane. B) Forward osmosis (FO), water moves into the concentrated solution. C) Reverse osmosis (RO), water is convectively moving into the diluted solution driven by pressure. D) Pressure retarded osmosis. (PRO): water moves as in (A), but whereas the movement is retarded by external pressure, it still moves into the concentrated solution (from high to low water potential). E) Direction of flow in FO, RO and PRO. For FO there is no hydraulic pressure across the membrane. For RO the applied pressure must exceed the osmotic pressure and convectively drive the water from low to high water potential. For PRO the hydraulic pressure retards the FO process but the hydraulic pressure applied is still less than the osmotic pressure.

Combining Eq (5) with the equation for solute flux J_s:

$$J_s = \omega RT(C_2 - C_1) + J_w(1-\sigma)\bar{C}, \quad (6)$$

where ω is the solute permeability coefficient and $\bar{C}$ is the average concentration of the two solutions on either side of the membrane, we arrive at the Kedem-Katchalsky equations for coupled water and solute movement (eqns 5-6).[128,129] The first term in (6) represents the concentration driven solute movement and the second term describes the solvent drag of the respective solute. The latter as σ describes both the efficacy of the solute to induce the osmotic flow water movement and the drag of the solvent induced by volume movement.

Polarization Effects

The Kedem-Katchalsky framework describes transmembrane transport of homogeneous solutions. Experimentally this can be realized by vigorous mechanical stirring of solutions. Lack of stirring causes formation of concentration boundary layers or unstirred layers as coined by Walther Nernst in 1904[130] on both sides of the membrane, directly adjacent to the membrane surface. The thickness of these layers depends on concentration, density, viscosity and temperature, on diffusive permeability coefficients of the membrane and Rayleigh numbers. This implies that the extent of unstirred layers will have direct influence on the volume fluxes passing across the membrane.

For membranes separating two aqueous solutions there will always be a region of incomplete mixing[131] and for most biological membranes there is only very little stirring at membrane solution interfaces. In the original (oversimplified) approximation by Nernst the unstirred layer was considered as a region with diffusion only and thus no convection. Recently, a more accurate hydrodynamic model for simultaneous diffusion and convection has been described.[132,133] A test of this model has been realized using ion-selective electrodes where the polarization was directly measured in the presence of an osmotic gradient consistent with the hydrodynamic model.[134] For biomimetic MIP membranes the polarization arises as a consequence of water flow through the MIPs. The ions are rejected and build up a concentration gradient in the unstirred layer on the hypotonic side relative to bulk ion concentrations.

The practical realization of the biomimetic membrane requires porous support materials on one or both sides of the biomimetic support matrix. This implies that the solvent (water) filled porous support will effectively act as an unstirred (and hence polarized) layer thereby affecting transmembrane fluxes (see Fig. 2).

This concentration polarization occurs on both sides of the membrane so for example in RO an increased osmotic pressure occurs on the feed side that must be overcome with hydraulic pressure. Alternatively in FO a dilution occurs on the draw side reducing the osmotic driving force. Both cases are examples of external concentration polarization. When the membrane is asymmetrical (see Fig. 2B,C) also internal concentration polarization can occur. These issues must be addressed in any future development of biomimetic MIP membranes, but in principle these issues are not unique to biomimetic membranes. In this respect the biomimetic membrane can be seen as the equivalent to the active layer in conventional RO membranes and the porous support as the equivalent to the support layers in RO membranes (see Fig. 3).

Conclusion

The many outstanding challenges in creating stable, selectively permeable and functional biomimetic membranes for sensor/separation purposes may seem daunting and even discouraging. On the positive side, however, are the recent scientific and technological advances discussed there. First, our increased understanding of the unique permeability properties for MIPs, notably a very high water permeability for some of the orthodox aquaporins and the complex permeation profiles for some MIPs e.g., selectivity for metalloids. Second, the recent progress in membrane stabilization methods opens for the possibility to create membranes with long-term stability. By merging insights from both MIP research and biomimetic membrane design, many potential applications can be envisaged, not only in water purification, but also in sensor devices built on the concept of up-concentrating selected permeants.

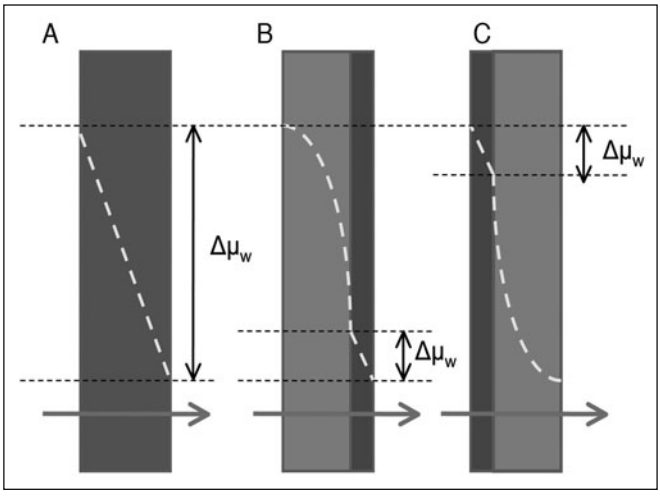


Figure 2. Polarization effects for symmetric and asymmetric membranes. A) In a symmetrical membrane with no unstirred layers osmosis is driven by the full water potential $\Delta\mu_w$. In asymmetrical membranes (e.g., with a biomimetic membrane (dark blue) as the active layer on a porous support (light blue), the effective water potential is changed due to polarization concentration profiles in the unstirred water layer wetting the porous support. Thus in (B) the salt concentration increases up to the active layer, whereas in (C) the salt concentration decreases up to the active layer. A color version of this image is available at www.landesbioscience.com/curie.

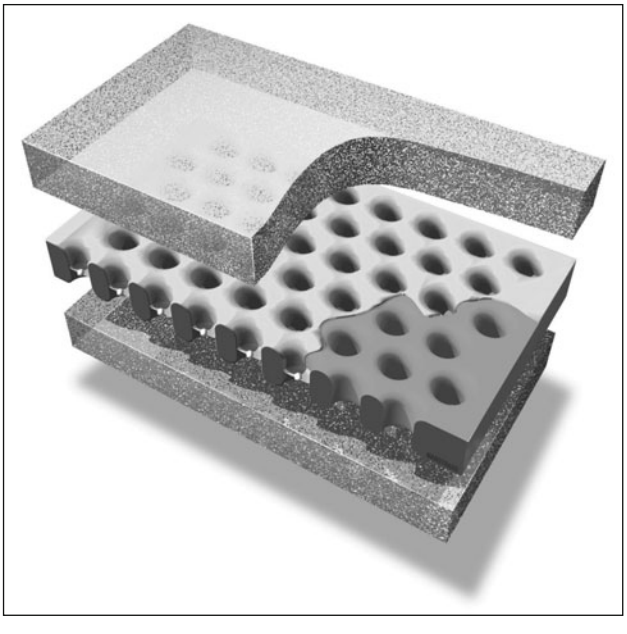


Figure 3. Principal sketch of a biomimetic aquaporin membrane. The aquaporins (white) are embedded in a biomimetic matrix (yellow) formed across a partition (green). Encapsulation material (grey) must be able to support the membrane without compromising transmembrane flow of water. A color version of this image is available at www.landesbioscience.com/curie.

References

1. Nernst W. Zur Theorie des electrischen Reizes. Pfluegers Arch Physiol 1909; 122:275-314.
2. Byrne W. Reverse Osmosis. 2nd ed. Littleton: Tall Oaks Publishing Inc., 2002.
3. Lee S, Lee E, Ra J et al. Characterization of marine organic matters and heavy metals with respect to desalination with RO and NF membranes. Desalination 2008; 221:244-252.
4. Nielsen CH. Biomimetic membranes for sensor and separation applications. Anal Bioanal Chem 2009; 395:607-718.
5. Kumar M, Grzelakowski M, Zilles J et al. Highly permeable polymeric membranes based on the incorporation of the functional water channel protein Aquaporin Z. Proc Natl Acad Sci USA 2007; 104:20719-20724.
6. Mulcahy E. Water Filter. Nano: The Magazine for Small Science 2009; Issue 12:17-19.
7. Dowhan W. Molecular basis for membrane phospholipid diversity: why are there so many lipids? Annu Rev Biochem 1997; 66:199-232.
8. Sackmann E. Physical basis of trigger processes and membrane structures. In: Chapman D, ed. Biological Membranes. London: Academic Press Inc Ltd, 1984; 5:105-143.
9. Vind-Kezunovic D, Nielsen CH, Wojewodzka U et al. Line tension at lipid phase boundaries regulates formation of membrane vesicles in living cells. Biochim Biophys Acta 2008; 1778:2480-2486.
10. Lundbaek JA. Regulation of membrane protein function by lipid bilayer elasticity—a single molecule technology to measure the bilayer properties experienced by an embedded protein. J Phys Condens Matter 2006; 18:S1305-S1344.
11. Nielsen CH. Lipid-protein interactions in biomembranes. In: Bohr HG, ed. Handbook of Molecular Biophysics. Berlin: Wiley, 2009:329-358.
12. Ti Tien H. Bilayer lipid membranes (BML) Theory and practice. 1st ed. New York: Marcel Dekker Inc., 1974.
13. Ottova A, Tien HT. The 40th anniversary of bilayer lipid membrane research. Bioelectrochemistry 2002; 56:171-173.
14. Mayer M, Kriebel JK, Tosteson MT et al. Microfabricated teflon membranes for low-noise recordings of ion channels in planar lipid bilayers. Biophys J 2003; 85:2684-2695.
15. Suzuki H, Tabata KV, Noji H et al. Highly reproducible method of planar lipid bilayer reconstitution in polymethyl methacrylate microfluidic chip. Langmuir 2006; 22:1937-1942.
16. Malmstadt N, Nash MA, Purnell RF et al. Automated formation of lipid-bilayer membranes in a microfluidic device. Nano Lett 2006; 6:1961-1965.
17. Zagnoni M, Sandison ME, Morgan H. Microfluidic array platform for simultaneous lipid bilayer membrane formation. Biosens Bioelectron 2009; 24:1235-1240.
18. Sandison ME, Zagnoni M, Abu-Hantash M et al. Micromachined glass apertures for artificial lipid bilayer formation in a microfluidic system. J Micromech Microeng 2007; 17:S189-S196.
19. Hansen JS, Perry ME, Vogel J et al. Development of an automation technique for the establishment of functional lipid bilayer arrays. J Micromech Microeng 2009; 19:025014.
20. Vogel J, Perry ME, Hansen JS et al. Support structure for biomimetic applications. J Micromech Microeng 2009; 19:025026.
21. Hansen JS, Perry M, Vogel J et al. Large scale biomimetic membrane arrays. Anal Bioanal Chem 2009; 395:719-727.
22. Meier W, Nardin C, Winterhalter M. Reconstitution of channel proteins in (polymerized) ABA triblock copolymer membranes. Angew Chem Int Ed Engl 2000; 39:4599-4602.
23. Ho D, Chu B, Lee H et al. Protein-driven transduction across polymeric biomembranes. Nanotechnology 2004; 15:1084-1094.
24. Reimhult E, Kumar K. Membrane biosensor platforms using nano- and microporous supports. Trends Biotechnol 2008; 26:82-89.
25. Zhang L, Granick S. Dynamical heterogeneity in supported lipid bilayers. MRS Bulletin 2006; 31:527-531.
26. Tanaka M. Polymer-suppoted membranes: Physical models of cell surfaces. MRS Bulletin 2006; 31:513-520.
27. Parikh AN, Groves JT. Materials Science of Supported Lipid Membranes. MRS Bulletin 2006; 31:507-512.
28. Sackmann E, Tanaka M. Supported membranes on soft polymer cushions: fabrication, characterization and applications. Trends Biotechnol 2000; 18:58-64.
29. Sackmann E. Supported membranes: scientific and practical applications. Science 1996; 271:43-48.
30. Rossi C, Chopineau J. Biomimetic tethered lipid membranes designed for membrane-protein interaction studies. Eur Biophys J 2007; 36:955-965.
31. Koper I. Insulating tethered bilayer lipid membranes to study membrane proteins. Mol Biosyst 2007; 3:651-657.

32. Gagner J, Johnson H, Watkins E et al. Carbon nanotube supported single phospholipid bilayer. Langmuir 2006; 22:10909-10911.
33. Löfås S, Johnsson BJ. A novel hydrogel matrix on gold surfaces in surface plasmon resonance sensors for fast and efficient covalent immobilization of ligands. Chem Soc Chem Commun 1990;1526-1528.
34. Dong Y, Scott Phillips K, Chen Q. Immunosensing of Staphylococcus enterotoxin B (SEB) in milk with PDMS microfluidic systems using reinforced supported bilayer membranes. Lab on a Chip 2006; 6:675-681.
35. Steltze M. On the application of supported bilayers as receptive layers for biosensors with electrical detection. J Phys Chem 1993; 97:2974-2981.
36. Becucci L, Leon RR, Moncelli MR et al. Electrochemical investigation of melittin reconstituted into a mercury-supported lipid bilayer. Langmuir 2006; 22:6644-6650.
37. Becucci L, Moncelli MR, Naumann R et al. Potassium ion transport by valinomycin across a Hg-supported lipid bilayer. J Am Chem Soc 2005; 127:13316-13323.
38. Becucci L, Moncelli MR, Guidelli R. Impedance spectroscopy of OmpF porin reconstituted into a mercury-supported lipid bilayer. Langmuir 2006; 22:1341-1346.
39. Ho D, Chu B, Lee H et al. Protein-driven energy tranduction across polymeric biomembranes. Nanotechnology 2004; 15:1084-1094.
40. Holden MA, Needham D, Bayley H. Functional bionetworks from nanoliter water droplets. J Am Chem Soc 2007; 129:8650-8655.
41. Funakoshi K, Suzuki H, Takeuchi S. Lipid bilayer formation by contacting monolayers in a microfluidic device for membrane protein analysis. Anal Chem 2006; 78:8169-8174.
42. Zagnoni M, Sandison ME, Marius P et al. Bilayer lipid membranes from falling droplets. Anal Bioanal Chem 2009; 393:1601-1605.
43. Schuster B, Sleytr UB, Diederich A et al. Probing the stability of S-layer-supported planar lipid membranes. Eur Biophys J 1999; 28:583-590.
44. Schuster B, Sleytr UB. The effect of hydrostatic pressure on S-layer-supported lipid membranes. Biochim Biophys Acta 2002; 1563:29-34.
45. Sleytr UB, Egelseer EM, Ilk N et al. S-Layers as a basic building block in a molecular construction kit. FEBS J 2007; 274:323-334.
46. Jeon TJ, Malmstadt N, Schmidt JJ. Hydrogel-encapsulated lipid membranes. J Am Chem Soc 2006; 128:42-43.
47. Jeon TJ, Poulos JL, Schmidt J. Storable and transportable lipid bilayer membrane precursor. Biophysical Journal Abstracts 2008: 337a.
48. Shim JW, Gu LQ. Stochastic sensing on a modular chip containing a single-ion channel. Anal Chem 2007; 79:2207-2213.
49. Szewczykowski P. Nano-porous materials from diblock copolymers and their membrane application. PhD Thesis. Lyngby: Department of Chemical and Biochemical Engineering, Technical University of Denmark; 2009.
50. Gonen T, Walz T. The structure of aquaporins. Q Rev Biophys 2006; 39:361-396.
51. Fu D, Lu M. The structural basis of water permeation and proton exclusion in aquaporins. Mol Membr Biol 2007; 24:366-374.
52. Ishibashi K. Aquaporin subfamily with unusual NPA boxes. Biochim Biophys Acta 2006; 1758:989-993.
53. Verkman AS. More than just water channels: unexpected cellular roles of aquaporins. J Cell Sci 2005; 118:3225-3232.
54. Nielsen S, Frokiaer J, Marples D et al. Aquaporins in the kidney: from molecules to medicine. Physiol Rev 2002; 82:205-244.
55. Denker BM, Smith BL, Kuhajda FP et al. Identification, purification and partial characterization of a novel Mr 28,000 integral membrane protein from erythrocytes and renal tubules. J Biol Chem 1988; 263:15634-15642.
56. Preston GM, Carroll TP, Guggino WB et al. Appearance of water channels in Xenopus oocytes expressing red cell CHIP28 protein. Science 1992; 256:385-387.
57. Zeidel ML, Ambudkar SV, Smith BL et al. Reconstitution of functional water channels in liposomes containing purified red cell CHIP28 protein. Biochemistry 1992; 31:7436-7440.
58. Agre P, Sasaki S, Chrispeels MJ. Aquaporins: a family of water channel proteins. Am J Physiol 1993; 265:F461.
59. Yasui M. Molecular mechanisms and drug development in aquaporin water channel diseases: structure and function of aquaporins. J Pharmacol Sci 2004; 96:260-263.
60. Sasaki S. Introduction for Special issue for aquaporin : Expanding the world of aquaporins: new members and new functions. Pflugers Arch 2008; 456:647-649.
61. Verbavatz JM, Brown D, Sabolic I et al. Tetrameric assembly of CHIP28 water channels in liposomes and cell membranes: a freeze-fracture study. J Cell Biol 1993; 123:605-618.

62. Yang B, Brown D, Verkman AS. The mercurial insensitive water channel (AQP-4) forms orthogonal arrays in stably transfected Chinese hamster ovary cells. J Biol Chem 1996; 271:4577-4580.
63. Verbavatz JM, Ma T, Gobin R et al. Absence of orthogonal arrays in kidney, brain and muscle from transgenic knockout mice lacking water channel aquaporin-4. J Cell Sci 1997; 110:2855-2860.
64. Palanivelu DV, Kozono DE, Engel A et al. Co-axial association of recombinant eye lens aquaporin-0 observed in loosely packed 3D crystals. J Mol Biol 2006; 355:605-611.
65. Borgnia MJ, Agre P. Reconstitution and functional comparison of purified GlpF and AqpZ, the glycerol and water channels from Escherichia coli. Proc Natl Acad Sci USA 2001; 98:2888-2893.
66. Calamita G, Bishai WR, Preston GM et al. Molecular cloning and characterization of AqpZ, a water channel from Escherichia coli. J Biol Chem 1995; 270:29063-29066.
67. Fu D, Libson A, Miercke LJ et al. Structure of a glycerol-conducting channel and the basis for its selectivity. Science 2000; 290:481-486.
68. Anthony TL, Brooks HL, Boassa D et al. Cloned human aquaporin-1 is a cyclic GMP-gated ion channel. Mol Pharmacol 2000; 57:576-588.
69. Cooper GJ, Boron WF. Effect of PCMBS on CO_2 permeability of Xenopus oocytes expressing aquaporin 1 or its C189S mutant. Am J Physiol 1998; 275:C1481-1486.
70. Herrera M, Hong NJ, Garvin JL. Aquaporin-1 transports NO across cell membranes. Hypertension 2006; 48:157-164.
71. Nakhoul NL, Hering-Smith KS, Abdulnour-Nakhoul SM et al. Transport of NH(3)/NH in oocytes expressing aquaporin-1. Am J Physiol Renal Physiol 2001; 281:F255-263.
72. Yasui M, Kwon TH, Knepper MA et al. Aquaporin-6: An intracellular vesicle water channel protein in renal epithelia. Proc Natl Acad Sci USA 1999; 96:5808-5813.
73. Liu Z, Shen J, Carbrey JM et al. Arsenite transport by mammalian aquaglyceroporins AQP7 and AQP9. Proc Natl Acad Sci USA 2002; 99:6053-6058.
74. Stroud RM, Miercke LJ, O'Connell J et al. Glycerol facilitator GlpF and the associated aquaporin family of channels. Curr Opin Struct Biol 2003; 13:424-431.
75. Stroud RM, Savage D, Miercke LJ et al. Selectivity and conductance among the glycerol and water conducting aquaporin family of channels. FEBS Lett 2003; 555:79-84.
76. Verkman AS. Does aquaporin-1 pass gas? An opposing view. J Physiol 2002; 542:31.
77. Wu B, Beitz E. Aquaporins with selectivity for unconventional permeants. Cell Mol Life Sci 2007; 64:2413-2421.
78. Bienert GP, Schussler MD, Jahn TP. Metalloids: essential, beneficial or toxic? Major intrinsic proteins sort it out. Trends Biochem Sci 2008; 33:20-26.
79. Nemeth-Cahalan KL, Hall JE. pH and calcium regulate the water permeability of aquaporin 0. J Biol Chem 2000; 275:6777-6782.
80. Nemeth-Cahalan KL, Kalman K, Hall JE. Molecular basis of pH and Ca^{2+} regulation of aquaporin water permeability. J Gen Physiol 2004; 123:573-580.
81. Johansson I, Larsson C, Ek B et al. The major integral proteins of spinach leaf plasma membranes are putative aquaporins and are phosphorylated in response to Ca^{2+} and apoplastic water potential. Plant Cell 1996; 8:1181-1191.
82. Johansson I, Karlsson M, Shukla VK et al. Water transport activity of the plasma membrane aquaporin PM28A is regulated by phosphorylation. Plant Cell 1998; 10:451-459.
83. Zeuthen T, Klaerke DA. Transport of water and glycerol in aquaporin 3 is gated by H(+). J Biol Chem 1999; 274:21631-21636.
84. Yasui M, Hazama A, Kwon TH et al. Rapid gating and anion permeability of an intracellular aquaporin. Nature 1999; 402:184-187.
85. Bowie JU. Stabilizing membrane proteins. Curr Opin Struct Biol 2001; 11:397-402.
86. Andersen OS, Koeppe RE, 2nd. Bilayer thickness and membrane protein function: an energetic perspective. Annu Rev Biophys Biomol Struct 2007; 36:107-130.
87. Tajkhorshid E, Nollert P, Jensen MO et al. Control of the selectivity of the aquaporin water channel family by global orientational tuning. Science 2002; 296:525-530.
88. Sanders OI, Rensing C, Kuroda M et al. Antimonite is accumulated by the glycerol facilitator GlpF in Escherichia coli. J Bacteriol 1997; 179:3365-3367.
89. Takano J, Wada M, Ludewig U et al. The Arabidopsis major intrinsic protein NIP5;1 is essential for efficient boron uptake and plant development under boron limitation. Plant Cell 2006; 18:1498-1509.
90. Tanaka M, Wallace IS, Takano J et al. NIP6;1 is a boric acid channel for preferential transport of boron to growing shoot tissues in Arabidopsis. Plant Cell 2008; 20:2860-2875.
91. Kato Y, Miwa K, Takano J et al. Highly boron deficiency-tolerant plants generated by enhanced expression of NIP5;1, a boric acid channel. Plant Cell Physiol 2009; 50:58-66.
92. Rosen BP, Liu Z. Transport pathways for arsenic and selenium: a minireview. Environ Int 2009; 35:512-515.

93. Ludewig U, Dynowski M. Plant aquaporin selectivity: where transport assays, computer simulations and physiology meet. Cell Mol Life Sci 2009.
94. Hanaoka H, Fujiwara F. Channel-mediated boron transport in rice. Plant Cell Physiol 2007; 48:227.
95. Bienert GP, Thorsen M, Schussler MD et al. A subgroup of plant aquaporins facilitate the bi-directional diffusion of $As(OH)_3$ and $Sb(OH)_3$ across membranes. BMC Biol 2008; 6:26.
96. Saha JC, Dikshit AK, Bandyopadhyay M et al. A review of arsenic poisoning and its effects on human health. Crit Rev Env Sci Technol 1999; 29:281-313.
97. Roy P, Saha A. Metabolism and toxicity of arsenic: a human carcinogen. Curr Sci 2002; 82:38-45.
98. Cullen WR, Reimers KJ. Arsenic speciation in the environment. Chem Rev 1989; 89:713-764.
99. Aposhian HV. Enzymatic methylation of arsenic species and other new approaches to arsenic toxicity. Annu Rev Pharmacol Toxicol 1997; 37:397-419.
100. Mok WM, Wai CM. Mobilization of arsenic in contaminated river waters. In: Nriagu JO, ed. Arsenic in the Environment, Part 1, Cycling and Characterization. New York: John Wiley and Sons, 1994.
101. Liu Z, Carbrey JM, Agre P et al. Arsenic trioxide uptake by human and rat aquaglyceroporins. Biochem Biophys Res Commun 2004; 316:1178-1185.
102. Yang HC, Cheng J, Finan TM et al. Novel pathway for arsenic detoxification in the legume symbiont Sinorhizobium meliloti. J Bacteriol 2005; 187:6991-6997.
103. Kamiya T, Tanaka M, Mitani N et al. NIP1;1, an aquaporin homolog, determines the arsenite sensitivity of Arabidopsis thaliana. J Biol Chem 2009; 284:2114-2120.
104. Ma JF, Yamaji N, Mitani N et al. Transporters of arsenite in rice and their role in arsenic accumulation in rice grain. Proc Natl Acad Sci USA 2008; 105:9931-9935.
105. Meng YL, Liu Z, Rosen BP. As(III) and Sb(III) uptake by GlpF and efflux by ArsB in Escherichia coli. J Biol Chem 2004; 279:18334-18341.
106. Liu Z, Styblo M, Rosen BP. Methylarsonous acid transport by aquaglyceroporins. Environ Health Perspect 2006; 114:527-531.
107. Porquet A, Filella M. Structural evidence of the similarity of Sb(OH)3 and As(OH)3 with glycerol: implications for their uptake. Chem Res Toxicol 2007; 20:1269-1276.
108. Pokrovski G, Gout R, Schott J et al. Thermodynamic properties and stoichiometry of As(III) hydroxide complexes at hydrothermal conditions. Geochim Cosmochim Acta 1996; 61:737-749.
109. Sexton LT, Horne LP, Martin CR. Developing synthetic conical nanopores for biosensing applications. Mol Biosyst 2007; 3:667-685.
110. Jeon YJ, Kim H, Jon S et al. Artificial ion channel formed by cucurbit[n]uril derivatives with a carbonyl group fringed portal reminiscent of the selectivity filter of K+ channels. J Am Chem Soc 2004; 126:15944-15945.
111. Cass A, Finkelstein A. Water permeability of thin lipid membranes. J Gen Physiol 1967; 50:1765-1784.
112. Levine SD, Jacoby M, Finkelstein A. The water permeability of toad urinary bladder. II. The value of Pf/Pd(w) for the antidiuretic hormone-induced water permeation pathway. J Gen Physiol 1984; 83:543-561.
113. Mlekoday HJ, Moore R, Levitt DG. Osmotic water permeability of the human red cell. Dependence on direction of water flow and cell volume. J Gen Physiol 1983; 81:213-220.
114. Finkelstein A, ed. Water movement through lipid bilayers, pores and plasma membranes. Theory and Reality. New York: Wiley-Interscience, 1987.
115. Finkelstein A, Cass A. Effect of cholesterol on the water permeability of thin lipid membranes. Nature 1967; 216:717-718.
116. Andersen OS. Permeability properties of unmodified lipid bilayer membranes. Membrane Transport in Biology 1978; 1:369-446.
117. Barchfeld GL, Deamer DW. The effect of general anesthetics on the proton and potassium permeabilities of liposomes. Biochim Biophys Acta 1985; 819:161-169.
118. Gliozzi A, Relini A, Lee-Gau Chong P. Structure and permeability properties of biomimetic membranes of archaeal bolaform tetraether lipids. J Mem Sci 2002; 206:131-147.
119. Suwalsky M, Rivera C, Villena F et al. Arsenite interactions with phospholipid bilayers as molecular models for the human erythrocyte membrane. Biophys Chem 2007; 127:28-35.
120. Jemiola-Rzeminska M, Rivera C, Suwalsky M et al. Interaction of arsenic compounds with model phospholipid membranes. Thermochim Acta 2007; 458:132-137.
121. Suwalsky M, Rivera C, Sotomayor CP et al. Monomethylarsonate (MMAv) exerts stronger effects than arsenate on the structure and thermotropic properties of phospholipids bilayers. Biophys Chem 2008; 132:1-8.
122. Sangster J. Octanol-Water Partition Coefficients Chichester: John Wiley and Sons Ltd, 1997.
123. Zador Z, Stiver S, Wang V et al. Role of aquaporin-4 in cerebral edema and stroke. Handb Exp Pharmacol 2009: 159-170.
124. Spring JH, Robichaux SR, Hamlin JA. The role of aquaporins in excretion in insects. J Exp Biol 2009; 212:358-362.

125. Cath TY, Childress AE, Elimelech M. Forward osmosis: principles, applications and recent developments. J Mem Sci 2006; 281:70-87.
126. Loeb S, Norman RS. Osmotic Power Plants. Science 1975; 189:654-655.
127. Skilhagen SE, Dugstad JE, Aaberg RJ. Osmotic power—power production based on the osmotic pressure difference between waters with varying salt gradients. Desalination 2008; 220:476-482.
128. Kedem O, Katchalsky A. Thermodynamic analysis of the permeability of biological membranes to non-electrolytes. Biochim Biophys Acta 1958; 27:229-246.
129. Kedem O, Katchalsky A. A physical interpretation of the phenomenological coefficients of membrane permeability. J Gen Physiol 1961; 45:143-179.
130. Nernst W. Theorie der Reaktionsgeschwindigkeit in heterogenen Systemen. Z Physk Chem (Leipzig) 1904; 47:52.
131. Schulman JH, Teorell T. On the boundary layer at membrane and monolayer interfaces. Trans Faraday Soc 1938; 34:1337.
132. Levich VG. Physicochemical Hydrodynamics. Englewood Cliffs, NJ, USA.: Prentice-Hall, 1962.
133. Pedley TJ. The interaction between stirring and osmosis. Part 1. J Fluid Mech 1980; 101:843-861.
134. Pohl P, Saparov SM, Antonenko YN. The effect of a transmembrane osmotic flux on the ion concentration distribution in the immediate membrane vicinity measured by microelectrodes. Biophys J 1997; 72:1711-1718.
135. Yang B, Verkman AS. Water and glycerol permeabilities of aquaporins 1-5 and MIP determined quantitatively by expression of epitope-tagged constructs in Xenopus oocytes. J Biol Chem 1997; 272:16140-16146.
136. Hashido M, Kidera A, Ikeguchi M. Water transport in aquaporins: osmotic permeability matrix analysis of molecular dynamics simulations. Biophys J 2007; 93:373-385.
137. Zeidel ML, Nielsen S, Smith BL et al. Ultrastructure, pharmacologic inhibition and transport selectivity of aquaporin channel-forming integral protein in proteoliposomes. Biochemistry 1994; 33:1606-1615.
138. Walz T, Smith BL, Zeidel ML et al. Biologically active two-dimensional crystals of aquaporin CHIP. J Biol Chem 1994; 269:1583-1586.
139. Pohl P, Saparov SM, Borgnia MJ et al. Highly selective water channel activity measured by voltage clamp: Analysis of planar lipid bilayers reconstituted with purified AqpZ. PNAS 2001; 98:9624-9229.
140. Borgnia MJ, Kozono D, Calamita G et al. Functional reconstitution and characterization of AqpZ, the E. coli water channel protein. J Mol Biol 1999; 291:1169-1179.
141. Saparov SM, Tsunoda SP, Pohl P. Proton exclusion by an aquaglyceroprotein: a voltage clamp study. Biol Cell 2005; 97:545-550.
142. Walter A, Gutknecht J. Permeability of small nonelectrolytes through lipid bilayer membranes. J Membrane Biol 1986; 77:255-264.
143. Flewelling RF, Hubbell WL. Hydrophobic ion interactions with membranes. Thermodynamic analysis of tetraphenylphosphonium binding to vesicles. Biophys J 1986; 49:531-540.
144. Hauser H, Oldani D, Phillips MC. Mechanism of ion escape from phosphatidylcholine and phosphatidylserine single bilayer vesicles. Biochemistry 1973; 12:4507-4517.
145. Gutknecht J. Proton/hydroxide conductance through lipid bilayer membranes. J Membr Biol 1984; 82:105-112.

Index

S

T

U

V

W

X