Plant Aquaporins and CO_2

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Abstract Aquaporins in plants show more abundant and greater diversity than aquaporins in bacteria and animals. This unique characteristic provided versatile tool boxes for plants, dealing with environmental changes, which overcome the disadvantage of immobility. Aquaporins were first known for their function as water channel proteins. Later on, more and more studies showed that other small solutes, i.e., ammonia, glycerol, urea, hydrogen peroxide and metalloids, can also pass through the channel of various aquaporins. Moreover, the function of aquaporins as CO_2 gas channels was studied by several groups (Nakhoul et al. Am J Physiol Cell Physiol 43(2):C543–C548, 1998; Yang et al. J Biol Chem 275(4):2686–2692, 2000; Tholen and Zhu Plant Physiol 156(1):90–105, 2011). In parallel, studies on model reconstituted membranes claim that no such type of channel would be needed due to the high permeability of those model membranes (Missner et al. Proc Natl Acad Sci USA 105(52):E123, 2008a; J Biol Chem 283(37):25340–25347, 2008b). However, experimental data showed the physiological significance of CO_2-conducting channels, particularly in plants. It is generally accepted that plant science presented the first evidence for the physiological relevance and importance of aquaporins as CO_2 transport facilitators (Boron Exp Physiol 95(12):1107–1130, 2010; Terashima and Ono Plant Cell Physiol 43(1):70–78, 2002; Uehlein et al. Nature 425 (6959):734–737, 2003; Heckwolf et al. Plant J 67 (5):795–804, 2011; Uehlein et al. Plant Cell 20(3):648–657, 2008). In this chapter, we discuss the CO_2 diffusion across membranes and the role of plant aquaporins during this process.

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F. Chaumont, S.D. Tyerman (eds.), *Plant Aquaporins*, Signaling and Communication in Plants, DOI 10.1007/978-3-319-49395-4_12

1 Membrane Permeability According to Meyer and Overton

More than a hundred years ago, H. Meyer and C.E. Overton independently worked on the lipid theory of anaesthetic action (Meyer 1899; Overton 1901). They found out that the higher the lipid solubility of an anaesthetic molecule is, the higher is its anaesthetic activity. Basically, these studies showed a direct correlation between the ability of a molecule to dissolve in lipid and its membrane permeability, and consequently, membranes should not impose resistance to diffusion of small hydrophobic molecules. This dependency has been called solubility-diffusion model or Meyer-Overton correlation. The results of transport studies on lipid bilayers are still valid today, and there are only rare exceptions (Missner et al. 2008a). The collection of substances that should comply with the solubility-diffusion model and possess very high membrane permeability contains among others also biologically important gasses like CO_2, O_2, NH_3 and NO (Missner and Pohl 2009). Insertion of special transport proteins would not be able to increase the diffusion rate because it would not be able to reduce the overall resistance of the membrane any further. In addition, the main resistance limiting permeation of small hydrophobic molecules should come from unstirred water layers on both sides of the membrane, drastically reducing the transmembrane concentration (Missner et al. 2008a). The concept of membrane diffusion based on Meyer and Overton has been presented in textbooks ever since.

Summing up, this model can explain how most gasses diffuse across numerous types of membranes. However, some biological membranes evidentially have a very low or no gas permeability at all and challenge the general validity of the solubility-diffusion model.

2 Experimental Findings That Support or Contradict Meyer and Overton

Studies on membrane permeability to various gasses and solutes complying with the Meyer-Overton correlation generally used artificial lipid bilayer membranes as experimental systems (Antonenko et al. 1993; Mathai et al. 2009; Missner et al. 2008a, b; Missner and Pohl 2009). J. Gutknecht and co-workers could show that an artificial bilayer consisting of egg lecithin and cholesterol does not constitute a substantial barrier to the diffusion of CO_2 (permeability coefficient $P_{CO2} \approx 0.35$ cm/s) (Gutknecht et al. 1977). The CO_2 molecule is very hydrophobic and according to Meyer and Overton has high membrane permeability. A comparable situation using a colorimetric approach was found for NH_3 ($P_{NH3} \approx 0.13$ cm/s) (Walter and Gutknecht 1986). P. Pohl and co-workers analyzed the diffusion of NH_3 (Antonenko et al. 1997) and acetate (Antonenko et al. 1993) as well as H_2S (Mathai et al. 2009), across lipid bilayer membranes employing a pH microelectrode technique and again confirmed the solubility-diffusion model.

However, studies that do not comply with the solubility-diffusion model have been performed on biological membranes rather than artificial lipid bilayers. We were able to show that membrane CO_2 permeability of tobacco chloroplast envelopes is around fivefold lower than that of plasma membranes and the variation is even more pronounced when the membrane integral protein composition is altered (Uehlein et al. 2008). The latter finding by itself is not consistent with the model as it shows that membranes can impose resistance to CO_2 diffusion and that this resistance can depend on protein components. In addition the values determined for plant membranes are roughly by a factor of 10^2–10^3 lower than what has been measured on artificial lipid bilayer membranes (Gutknecht et al. 1977; Missner et al. 2008b) and about 10^4-fold lower than what is expected on the basis of theoretical considerations (Missner et al. 2008b; Missner and Pohl 2009).

In mammals, membranes exhibiting unusually low gas permeability were identified, obviously contradicting the common view of gas permeable membranes. Fermentation of non-absorbed nutrients in the colon generates high concentrations of NH_4^+ in the colonic lumen, which is in equilibrium with ammonia (NH_3). NH_3 can easily permeate lipid bilayer membranes (Antonenko et al. 1997; Walter and Gutknecht 1986) and affect transmembrane pH gradients. However, in isolated colonic crypts, it has been observed that cytosolic pH of the epithelial cells does not increase when the NH_3 concentration in the colon lumen is raised but does so immediately when NH_3 concentration is increased on the basolateral side (Singh et al. 1995). Thus, biomembrane permeability to ammonia can vary even within individual cells.

In the colon lumen, some bacteria generate very high CO_2 partial pressures (Rasmussen et al. 1999, 2002). An extreme CO_2 partial pressure can seriously affect the pH of colon epithelial cells, if the CO_2 molecules can freely permeate across the plasma membrane into the cytosol. However, apical membranes of gastrointestinal endothelia exhibit very low CO_2 permeability (Endeward and Gros 2005; Waisbren et al. 1994). Consequently, it was concluded that there must be a permeability barrier to gasses in certain types of membranes. It appears to be quite reasonable from a physiological point of view, if some membranes build up a gas barrier to protect the cells. By comparison, in organs and tissues important for gas exchange, e.g., in lung alveolar endothelia and red blood cells (Endeward et al. 2006b) or photosynthetic cells, membrane CO_2 permeability is very high, in order to guarantee a quick removal of the gaseous waste product or uptake of the substrate.

3 Physiological Significance of CO_2 Diffusion in Plants

The physiological reactions of CO_2 exchange are unequivocally important for most organisms. CO_2 is a waste product from biochemical reactions, while for plants it is the substrate for sugar synthesis (Kaldenhoff 2012). In addition, the concentration gradient between plant cells taking up CO_2 and the atmosphere is less pronounced than it is the case for animals releasing CO_2. Therefore, the physiological study of

CO_2 diffusion in photosynthetic active plants could be more beneficial (Kaldenhoff 2012). Under light-saturating conditions, the photosynthetic capacity is limited by the availability of CO_2 in the chloroplast. Therefore, reduction of the barriers of CO_2 diffusion is becoming the focus of many studies, especially regarding the regulation of stomata and the biochemistry of CO_2 reactions. The complete cellular CO_2 diffusion path from the atmosphere to the site of chemical fixation in the chloroplast stroma is restricted by resistances with different ranges. After diffusing through the stomata and the leaf internal air spaces, atmospheric CO_2 has to pass the cell wall, the plasma membrane, diffuse a short distance through the cytosol, cross the chloroplast envelope and diffuse in the chloroplast stroma (Evans et al. 2009). All these barriers contribute to the so-called mesophyll conductance (g_m) or the mesophyll resistance, which is significant enough to decrease the CO_2 concentrations in the chloroplast and becomes a major limitation in net photosynthesis (Harley et al. 1992; Kaldenhoff 2012).

4 Aquaporin-Facilitated CO_2 Diffusion

During the past 15 years, proteins facilitating the diffusion of CO_2 across membranes have been identified. It has been shown in heterologous model systems that certain aquaporins can facilitate membrane gas transport. First evidence came from research on the human aquaporin 1 (Nakhoul et al. 1998). Since then, hints have accumulated that also other aquaporins may be involved in the facilitation of membrane transport of gasses like CO_2 or NH_3 and maybe O_2. Studies on HsAQP1 showed that it is highly expressed in cells or tissues that require particularly high gas permeability like the lung or erythrocytes (Cooper et al. 2002; Preston and Agre 1991; Verkman et al. 2000). Besides aquaporins, also rhesus blood group antigen-associated glycoproteins (RhAG) that are present in the red blood cell membrane have been shown to transport NH_3 and CO_2 (Endeward et al. 2008; Ripoche et al. 2004).

In the past, *Xenopus* oocytes served as a useful tool to analyze functional properties of aquaporins, as their plasma membranes generally exhibit low water and also low gas permeability (Cooper et al. 2002; Nakhoul et al. 1995). Using them as an expression system, it was shown that HsAQP1 considerably increases the oocyte membrane permeability to both CO_2 and NH_3 (Cooper and Boron 1998; Nakhoul et al. 1998, 2001; Musa-Aziz et al. 2009). The plant aquaporin NtAQP1 in heterologous expression systems as well as in in-vitro systems allows only very low water transport rates. However, it shares the functionally important amino acid residues with HsAQP1 on the basis of sequence comparison and, like HsAQP1, facilitates uptake of CO_2 into *Xenopus* oocytes (Uehlein et al. 2003). Also other aquaporins from other organisms were shown to increase the gas permeability of oocyte membranes (Jahn et al. 2004; Holm et al. 2005; Musa-Aziz et al. 2009). Using *Saccharomyces cerevisiae* as another heterologous expression system, facilitation of CO_2 or NH_3 membrane transport was confirmed for aquaporins from tobacco, maize and wheat (Bertl and Kaldenhoff 2007; Jahn et al. 2004; Loque et al. 2005; Heinen et al. 2014).

Also in native systems, an effect of aquaporin expression on CO_2 membrane diffusion has been shown. Expression of human AQP1 has turned out to facilitate membrane CO_2 transport in red blood cells (Endeward et al. 2006a, b). The tobacco PIP1 aquaporin NtAQP1 is highly expressed in photosynthetically active tissue (Otto and Kaldenhoff 2000). Manipulating its expression level in the plant has a substantial effect on the rate of photosynthesis. Under antisense limited expression of NtAQP1, net photosynthesis is reduced; when NtAQP1 is overexpressed, the photosynthetic performance increases, in each case by around 30–40 % (Uehlein et al. 2003). *Arabidopsis* mutants with a specific knockout of the NtAQP1 ortholog AtPIP1;2 are available. It can be assumed that in these plants the expression of sequence related aquaporin genes is not affected, as it can be the case when the antisense or RNAi technique is applied. Molecular and physiological analyses supported this assumption. Using those mutant plants, the specific importance of AtPIP1;2 for plant physiology can be analyzed, and hints on its molecular function can be deduced. The studies that were performed in this respect indeed confirmed a contribution of the aquaporin in membrane transport of CO_2. Cellular transport of CO_2 was not limited by unstirred layer effects but was dependent on the expression of AtPIP1;2 (Uehlein et al. 2012b). It could be shown that a knockout of AtPIP1;2 expression led to about 40 % reduction in mesophyll conductance to CO_2 and eventually limited photosynthesis. Introduction of the unmutated gene fully complemented the mutated phenotype (Heckwolf et al. 2011).

A similar situation has been observed for membrane diffusion of ammonia. According to the Meyer-Overton rule, NH_3 should easily pass lipid bilayers down its concentration gradient. However, facilitation of ammonia membrane transport in heterologous and native systems has been reported for aquaporins (Nakhoul et al. 2001; Bertl and Kaldenhoff 2007; Holm et al. 2005) as well as special ammonium transporters (Ludewig et al. 2003). Recently the crystal structure of the water- and ammonia-permeable aquaporin AtTIP2;1 revealed a special selectivity filter containing a conserved arginine residue that helps to understand the molecular mechanism of ammonia permeation (Kirscht et al. 2016). In addition it has been shown in zebrafish larvae that an aquaporin can facilitate the membrane diffusion of CO_2, ammonia and water in a physiologically relevant fashion (Talbot et al. 2015).

5 Molecular Mechanism of Aquaporin-Facilitated CO_2 Diffusion

Atomic structure of aquaporins shows high similarity and unique structural elements (see chapter "Structural Basis of the Permeation Function of Plant Aquaporins"). In general, aquaporins consist of six membrane-spanning helical domains with N- and C-termini heading towards the cytosol. Conserved motifs called 'NPA' in loop B and E from opposite sides located inside the membrane form the water-conducting channel (Murata et al. 2000). Experimental data showed that the water channel forms in a single aquaporin monomer; however, aquaporins form

tetramers in the membrane (de Groot et al. 2003). In general, membrane proteins tend to form oligomers not only for stabilization but also for functionality of the protein (Ali and Imperiali 2005). However, the reason why aquaporins tend to form tetramers is still not clear (Strand et al. 2009).

HsAQP1 is an aquaporin that has been closely examined with regard to its function in CO_2 transport. Experimental data gave clear evidence that HsAQP1 can facilitate CO_2 transport in *Xenopus laevis* oocytes (Uehlein et al. 2003; Endeward et al. 2006b). Atomic molecular simulation data based on HsAQP1 crystal structures show that HsAQP1-mediated CO_2 transport via the monomer pores can be expected in membranes with low intrinsic CO_2 permeability (Hub and de Groot 2006). However, it is more likely that CO_2 diffusion is mediated through the central pore formed by the tetramer (Hub and de Groot 2006), because it is lined by hydrophobic amino acid residues and therefore is an ideal path for hydrophobic CO_2 molecules (Wang et al. 2007). Later on, experimental data based on the artificial homo- and heterotetramers of NtPIP1;2 and NtPIP2;1 showed that maximum CO_2 transport rates were obtained when the tetramer consisted of NtPIP1;2 units only. Substitution of two PIP1 by two PIP2 units completely abolished the CO_2 transport capacity. In conclusion, the data showed that tetramer formation is necessary for CO_2 transportation and that a joint structure built by all four units in the tetramer is responsible for this function. It is most likely that the central pore is the respective structure (Otto et al. 2010).

6 In Vitro Studies of CO_2 Diffusion Across Reconstituted Model Membranes

The diffusion of CO_2 across membranes was studied in different systems using different measuring approaches. In early studies, overexpression of aquaporins was done in oocytes, and the measurement of CO_2 diffusion was done with the detection of acidification of the oocytes cytosol in the presence of carbonic anhydrase when subjected to a CO_2 gradient (Nakhoul et al. 1998). Later on, the stopped-flow method was used to detect CO_2 diffusion across yeast cell membranes, in which a CO_2 permeable aquaporin was overexpressed. To guarantee that the membrane transport of CO_2 is the rate limiting step, rather than the conversion reaction of CO_2 to carbonic acid, the yeast cells expressed a carbonic anhydrase. NtAQP1 or NtPIP2;1 was expressed in yeast, and the cells were subjected to functional analysis for intracellular acidification in response to CO_2 uptake. Intracellular acidification was monitored via detecting changes of fluorescein fluorescence. Yeast cells expressing NtAQP1 in addition show faster intracellular decrease in pH compared to control cells and cells expressing NtPIP2;1 (Otto et al. 2010). Similarly, experiments were done using reconstituted proteoliposomes (Prasad et al. 1998; Yang et al. 2000). However, these studies, which employed the stopped-flow technique, were questioned regarding the ability to resolve the rapid kinetics of CO_2 diffusion

across the membrane (Boron et al. 2011). In addition, an ^{18}O exchange mass spectrometric technique was applied to measure the CO_2 diffusion across red blood cells (Itel et al. 2012). Recently, a new method for monitoring CO_2 transport through a lipid bilayer using a micro pH electrode was developed. CO_2 transportation through a two-chamber system that was separated by a lipid bilayer resulted in an acidified region close to the membrane. A micro pH electrode attached to a micromanipulator device was used to monitor this acidification (Uehlein et al. 2012a; Missner et al. 2008b). This tool offered a new instrument to study the steady-state CO_2 diffusion through membranes independent of resolving rapid kinetics and just reliant on the diffusion rate of CO_2. In addition, using non-CO_2-permeable triblock-copolymer membranes, it was possible to reduce the background diffusion, which is quite significant in artificial lipid bilayers, to close to zero (Uehlein et al. 2012a). Further studies examining the effect of sterols as well as non-CO_2-permeable aquaporins on CO_2 diffusion were done using the same method. The development of techniques used for measuring CO_2 diffusion could shed some light on the general principles. Several independent studies revealed that sterols and membrane proteins that are major components of a biological membrane also contribute to the limitation of membrane CO_2 permeability (Itel et al. 2012; Kai and Kaldenhoff 2014; Tsiavaliaris et al. 2015).

7 Conclusions and Perspectives

Taken together, experimental data on membrane gas permeability are available that are not consistent with the Meyer-Overton rule and with theoretical considerations, or the process of CO_2 diffusion across biological membranes is not suitable to apply the Meyer-Overton rule. One possible reason could be that our current view of biological membranes and comparison of their permeability properties to well-established experimental test systems like pure lipid bilayers are not quite correct. Biological membranes are considerably more complex than is reflected by the well-known fluid mosaic model (Singer and Nicolson 1972). They contain integral and associated membrane proteins, as well as membrane regions tightly connected to the cytoskeleton. The protein occupancy in highly specialized membranes reaches up to 75 % (Dupuy and Engelman 2008; Engelman 2005). It ranges between ~23 % for red blood cell membranes (Dupuy and Engelman 2008) and ~70–80 % for chloroplast membranes (Kirchhoff et al. 2008). Therefore, it is not surprising that permeability properties of biological membranes differ greatly from that of artificial lipid bilayers. It may be misleading to expect that findings obtained on pure lipid bilayers and in silico studies directly apply to complex biological membranes. However, one should keep in mind that molecules cannot cross the lipid matrix when the lipid matrix is either replaced by integral membrane proteins or covered by membrane-associated proteins and therefore not accessible (Boron 2010).

The literature shows that membranes differ in CO_2 permeability and that certain aquaporins as well as RhAG proteins are able to transport CO_2 when the background permeability of the membrane in which they reside is low or, in case of special block copolymer membranes, even close to zero. Furthermore, studies show that the gas permeability of biological membranes may decrease with increasing protein content, thus making special transport proteins necessary in cases where high gas permeability is required. There are more physiologically important gasses, namely, NH_3, O_2 and NO, which have to cross membranes in order to be taken up or to be released. Membrane diffusion of CO_2 and NH_3 can be detected by measurement of pH changes, for O_2 and NO carbon fibre electrodes can be used. Understanding the mechanism of gas transport across biological membranes will help understand the physiology of organisms and will provide new arguments for the controversial debate on membrane gas transport. Eventually it will help to understand how CO_2 transport in plants can be improved, and this in turn will help to advance photosynthesis and growth of crop plants. In addition, it will give rise to broader impacts like strategies towards reduction of atmospheric CO_2 concentration and development of technical applications like highly gas-selective membranes for gas purification or sensor technology.

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