Chapter 12

Sulfite Oxidation in Plants

Robert Hänsch and Ralf R. Mendel*
Department of Plant Biology, Technical University of Braunschweig, Humboldtstrasse 1, D-38106 Braunschweig, Germany

Summary

Sulfite oxidation in plants was a matter of controversial discussion for a long time and still is not finally understood. There is no doubt anymore about the occurrence of sulfite oxidation besides primary sulfate assimilation that takes place in the chloroplast. Sulfate is reduced via sulfite to organic sulfide which is essential for the biosynthesis of S-containing amino acids and other compounds like glutathione. However, it has also been reported that sulfite can be oxidized back to sulfate, e.g. when plants were subjected to SO_2 gas. Work from our laboratory has identified sulfite oxidase as a member of molybdenum-containing enzymes in plants, which seems to be the most important way to detoxify excess of sulfite. In this paper we show how plant cells separate the two counteracting pathways – sulfate assimilation and sulfite detoxification – into different cell organelles. We discuss how these two processes are (co-)regulated and what kind of other sulfite oxidase activities occur in the plant.

I. Sulfur Cycling in Nature

Sulfur is an essential macronutrient for plants, animals and microorganism and plays a critical role in the catalytic and electrochemical functions of biomolecules in the cell. Sulfur is found in the two amino acids cysteine and methionine, in oligopeptides (glutathione and phytochelatins), vitamins and cofactors (biotin, molybdenum cofactor [Moco], thiamine, CoenzymeA, and *S*-adenosyl-Methionine), in phytosulfokin hormones (Matsubayashi, Sakagami 1996) and a variety of secondary products (see

* Corresponding author, Phone: +49 (0) 531 391 5870, Fax: +49 (0) 531 391 8128, E-mail: r.mendel@tu-bs.de

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Leustek 2002). Disulfide bonds between polypeptides mediated by cysteine are very important in protein assembly and structure. Sulfur itself belongs to the chalcogen family; other members of the family are oxygen, selenium, tellurium, and polonium. Because of the electronic status of sulfur, this element can undergo four different oxidation states: β ←6 (sulfate, SO_4^{2-}), β ←4 (sulfite, SO_3^{2-}), ←′ (elemental sulfur S^0) and −2 (sulfide, H_2S), which is important for their biological activity and allows to recycle it in a biogeochemical way including (i) assimilative sulfate reduction, (ii) desulfuration, (iii) oxidation of organic sulfur compounds, and (iv) mineralization of organic sulfur to the inorganic form. Human impact on the sulfur cycle is exerted mainly by producing toxic sulfur dioxide in industry and by motor cars. Sulfur dioxide can be further reduced to sulfide or re-oxidized to sulfate in different enzymatic and non-enzymatic reactions.

II. Sulfate Reduction in Plants

Plants take up sulfate from soil into the roots and translocate it via the xylem to the green parts of the plant where it is stored as the major anionic component of vacuolar sap (Kaiser et al. 1989; Leustek and Saito 1999). Plastids are the limiting organelles where assimilatory sulfate reduction takes place, only cysteine synthesis enzymes are localized in the following three compartments: plastids, cytosol and mitochondria. Sulfate assimilation starts with the activation by ATP to adenosine-5′-phosphosulfate (5′-adenylylsulfate [APS]) and further conversion via sulfite to the final sulfide and requires one ATP and eight electrons coming from reduced glutathione (Hell 1997; Bick and Leustek 1998). Sulfide is later coupled to *O*-acetyl-Ser to form cysteine (see recent reviews Leustek 2002; Droux 2004; Saito 2004). Details are given in chapters I.1 and I.2 in this book.

Sulfate itself can be also covalently bound to a variety of compounds, a process termed sulfation which also begins with APS synthesis. APS is then phosphorylated by APS-kinase to form 3′-phosphoadenosine-5′-phosphosulfate (PAPS) which is used as a sulfuryl donor by a variety of sulfotransferases forming a sulfate ester bond. The most prominent example for this class of compounds formed by sulfation are glucosinolates that function as insect feeding deterrents produced by different species in the *Brassicaceae* family. Here, glucosinolates contain two forms of sulfur in different oxidation states: the reduced form is a thioether derived from cysteine, whereas the oxidized form is a sulfamate which comes from the sulfation pathway (Leustek 2002). For further details see chapter I.6 in this book.

Abbreviations: Moco – Molybdenum cofactor; GFP – green fluorescent protein; PTS – peroxisomal targeting sequence; SO – sulfite oxidase

III. The Ambivalent Nature of Sulfite: an Important but Toxic Intermediate

Sulfite plays an important role in the reductive and oxidative sulfur metabolism in pro- and eukaryotes. In plants, sulfite (SO_3^{2-}) with an oxidation state of β ←4 is the first important intermediate in the reduction pathway of sulfur originating from sulfate. Here, sulfite is also the starting point for the formation of sulfur lipids (Yu et al. 2002). Some microorganisms use sulfite as sole electron source. Sulfite is the key intermediate in the oxidation of reduced sulfur compounds to sulfate and the major product of most dissimilatory sulfur-oxidizing prokaryotes (Kappler and Dahl 2001). In animals, it is known since 1953 (Heimberg et al. 1953) that sulfate is produced from sulfite in an enzymatic reaction. Sulfite oxidases (SO) catalyzes the reaction $SO_3^{2-} + H_2O \rightarrow SO_4^{2-} + 2H^+ + 2e^-$, which is the terminal step in the oxidative degradation of cysteine and methionine.

Deficiencies of SO lead to major neurological abnormalities and early death in the studied animals and humans (Calabrese et al. 1981; Kisker et al. 1997; Garrett et al. 1998). In the mammalian system, SO is localized in the intermembrane space of mitochondria (Cohen et al. 1972) where electrons derived from sulfite can passes via the enzyme's heme domain on to cytochrome *c*, the physiological electron acceptor.

Sulfite is also known for many years to damage plants (Hill and Thomas 1933; Moyer and Geo 1935), for review see Peiser and Yang (1985) and Heber and Hüve (1998). As nucleophilic agent, sulfite is able to attack diverse substrates (Peiser and Yang 1985), where it opens S-S bridges. This reaction – so-called sulfitolysis – can cause inactivation of proteins when incubated with sulfite

(Ziegler 1974) or even when plants are exposed to high concentrations of SO_2 gas (Tanaka et al. 1982). Sulfitolysis of oxidized thioredoxin can interfere with the regulation of enzymes of the Calvin cycle (Würfel et al. 1990). These effects cause severe reduction in plant growth. The susceptibility to SO_2 may vary considerably between the different species and depends on combinations of duration and dosage of SO_2, but also on physiological and environmental factors (Rennenberg 1984). There are two systems discussed to control the internal SO_2 concentration: (i) control of uptake of the gas by the laminar boundary layer, the cuticle or the guard cells, and (ii) the rate of its metabolic conversion and translocation. In plants, theoretically there are two possibilities to handle SO_2: (i) to feed it into the assimilation stream of sulfur for producing of cysteine, methionine or other reduced sulfur compounds, or (ii) to re-oxidize it to sulfate.

As gaseous substance, SO_2 enters plant tissues mainly via their stomata (Rennenberg and Polle 1994; Rennenberg and Herschbach 1996) and is transformed into sulfite and/or bisulfite ions on the wet surface of guard cells and in the cytoplasmic fluid, which results in a proton generation:

$$SO_2 + H_2O \rightarrow [SO_2 \cdot H_2O] \rightarrow HSO_3^- + H^+ \leftrightarrow SO_3^{2-} + 2H^+$$

The guard cells are able to respond to different levels of SO_2 with stomatal closing or opening (Rao and Anderson 1983). In acidic environments, HSO_3^- is prevailing, while under alkaline conditions in the chloroplasts, SO_2 is chemically converted into SO_3^{2-} (Heber et al. 1987). The flow of SO_2 between the gaseous phase of the intercellular space and the liquid phase of the apoplast and/or cytosol seems to be continuous.

When applied as the major source of sulfur at non-toxic dosages, i.e. in soils that do not fulfill the needs for sulfate, biomass production depends on the supply of SO_2 or other volatile sulfur-compounds in the air (summarized in Rennenberg 1984). Furthermore, the amount of SO_2 taken up by the leaves can regulate the sulfur uptake by the roots (Herschbach and Rennenberg 2001). However, when the uptake of SO_2 exceeds a certain threshold that differs from species to species, toxicity effects occur that lead to growth problems of the plant (Linzon 1978). Here, active detoxification of sulfite is necessary for the survival of the whole plant. Until recently, metabolic conversion was interpreted to mean reductive detoxification leading to sulfide that is used to produce cysteine (Heber and Hüve 1998). This process is well understood because it forms part of the sulfate assimilation pathway. But is has been also reported that sulfite can be oxidized to sulfate. Upon foliar application of labeled $^{35}SO_2$ this gas is rapidly metabolized in the light and also in dark, with sulfate as the main end product (Garsed and Read 1977; Van der Kooij et al. 1997). The possibility to explain these results by oxidative conversion of sulfite to sulfate was largely neglected because this step would counteract the assimilatory pathway. Yet, experimental data were accumulating that needed further explanations (Rennenberg et al. 1982).

IV. Sulfite Oxidase Activities in Plants

For decades, occurrence and nature of a sulfite oxidizing activity in higher plants were controversially discussed as shown in the following history.

- Already in 1944, Thomas and coworkers showed high concentrations of sulfate in SO_2-treated alfalfa and sugar beet (Thomas et al. 1944).
- Fromageot described sulfite oxidation by oat roots (Fromageot et al. 1960).
- Apoplastic peroxidases of barley leaves can efficiently detoxify sulfite: after infiltration of sulfite-containing buffer through the stomata, sulfate could be extracted in increasing am ounts over time from the apoplastic washing solution (Pfanz et al. 1990).
- Later, apoplastic peroxidases were discussed to oxidize sulfite using H_2O_2 and different phenolic compounds (Pfanz and Oppmann 1991).
- Miszalski and Ziegler suggested a non-enzymatic oxidation of sulfite, initiated (i) by superoxide anions formed on the reduction site of the electron transport system in chloroplasts, (ii) by free radicals such as OH^-, or (iii) by H_2O_2 (Miszalski and Ziegler 1992).
- Intact chloroplasts isolated from spinach (*Spinacia oleracea*) fed with radioactively labeled sulfite showed a sulfite oxidation activity (Dittrich et al. 1992). This reaction was discussed to proceed via a radical chain reaction involving light-dependent photosynthetic electron transport which was found to be enhanced by light and to be sensitive to inhibitors of the photosynthetic electron transport (Dittrich et al. 1992). However, in addition to this

non-enzymatic light-dependent sulfite oxidation there should also be an enzymatic reaction because sulfite oxidation could also be detected in the dark.

- Jolivet et al. (1995a, 1995b) described a sulfite oxidizing activity associated with isolated thylakoid membranes that was not induced through the photosynthetic radical-dependent oxidation chain reaction. A protein preparation gave activities 50 times higher than in crude extract of spinach leaves, and SDS-PAGE analysis showed four major protein bands (65, 53, 36 and 33 kDa), that were discussed to represent either different subunits of an even more complex enzyme or to be contaminating bands because an *in gel*-staining assay was not successful (Jolivet et al. 1995a).

All publications presented describe sulfite oxidizing activities as non-enzymatic or enzymatic reactions in the apoplastic space or in steps associated with the light-dependent photosynthetic electron transport or other unknown reactions in chloroplasts. Yet, the main problem was still unsolved: a chloroplast-localized sulfite oxidizing activity would counteract sulfate assimilation residing in the same organelle. How could a plant cell regulate these two conflicting pathways in one and the same compartment? The discussion of this obvious problem became an unexpected turn when we viewed sulfite oxidation from the point of eukaryotic molybdenum metabolism.

In mammals, SO is well studied: it is an enzyme containing molybdenum in the active site and is localized in the intermembrane space of mitochondria (Cohen et al. 1972). It is a two-domain protein consisting of a molybdenum domain and a heme domain and it is responsible for detoxifying sulfite in the course of amino acid decomposition. By screening an *A. thaliana* cDNA library using the amino acid sequence of human (XP_006727) or chicken SO (P07850), we identified plant SO as the fourth plant enzyme containing molybdenum (Eilers et al. 2001). The isolated full-length cDNA of *Arabidopsis*-SO has a single open reading frame of 1182 bp encoding a protein of 393 amino acids (43.3 kDa) with 47% identity to the primary sequence of the molybdenum cofactor-domain of chicken SO. However, the sequence for the heme domain known from animal SO was lacking in this plant clone and was also absent in the genomic region. The genomic sequence showed a single open reading frame with 11 introns located on chromosome III. High stringency hybridization of *Arabidopsis* genomic DNA with the isolated cDNA clone as probe demonstrated that the gene encoding for the *Arabidopsis*-SO (At-*so*) is single copy gene.

The alignment of molybdenum cofactor-domains of SOs from different sources with *Arabidopsis*-SO demonstrated considerable overall homology, identifying these enzymes as members of a common family (Eilers et al. 2001). Plant SO turned out to be conserved among higher plants because antibodies raised against *Arabidopsis*-SO detected a dominantly cross-reacting protein of about 45 kDa in a wide range of species belonging to a variety of both herbaceous (dicots and monocots) and woody (e.g. poplar) plants (Eilers et al. 2001). In *Arabidopsis*, SO shows a constitutive expression in all tissues tested and also over the day without any pronounced diurnal rhythm (Hänsch et al. 2006). Hence one can conclude that plant SOs are widely distributed among higher plants and are expressed as a housekeeping gene. Recently, a SO-specific sequence was also detected in the genome of the green alga *Chlamydomonas reinhardtii* (Emilio Fernandez, personal communication) and in the moss *Physcomitrella patens* (Ralf Reski, personal communication).

V. Biochemical Properties of Plant Sulfite Oxidase (EC 1.8.3.1)

For recombinant expression, the isolated *Arabidopsis*-cDNA was cloned into an expression vector allowing the expression and purification as His-tagged protein from *E. coli*. This protein exhibited a sulfite-dependent SO activity when using ferricyanide as artificial (Eilers et al. 2001) or oxygen as natural electron acceptor (Hänsch et al. 2006). No activity was found with cytochrome *c* as electron acceptor as expected, since the heme domain known to mediate electron transfer between the molybdenum cofactor-domain and cytochrome *c* in rat hepatic SO is missing in the plant enzyme. HPLC analysis of the oxidation product of the molybdenum cofactor confirmed its pterin nature as found in animals. And also the spectroscopic properties of recombinant plant SO identified it as member of the general SO family: on the basis of the UV-visible absorption and the EPR signature it was evident that the molybdenum centre of *Arabidopsis*-SO is fundamentally

similar to that of the vertebrate proteins (Eilers et al. 2001; Hemann et al. 2005).

The Km-value of 22.6 µM for sulfite using oxygen as electron acceptor was in the same range as shown for the artificial acceptor ferricyanide determined to be 33.8 µM which is in the range as found for rat SO (Eilers et al. 2001; Hänsch et al. 2006). When plant SO uses molecular oxygen as terminal electron acceptor, the question arises what could be the second end product besides of sulfate? This second reaction product turned out to be hydrogen peroxide (H_2O_2). We showed this by two different methods: (i) Nag et al. (2000) described the specific formation of a yellow-orange peroxo-disulfatotitanate(IV)-complex $[Ti(O_2)(SO_4)_2]^{2-}$ from the hydroxylcation $[Ti(OH)_2(H_2O)_4]^{2+}$ in the presence of H_2O_2 and its detection at 405 nm, and (ii) the fluorescent dye lucigenin is known from Rost et al. (1998) to react specifically with H_2O_2 but not with other reactive oxygen species. Both assays were positive for the plant SO (Hänsch et al. 2006). However, adding low amounts of catalase to both the titanate-complex assay and the fluorescent-dye assay abolished H_2O_2 accumulation completely.

VI. Plant Sulfite Oxidase is a Peroxisomal Enzyme

Analysis of SO in 17 plant species *in silico* revealed that all plant SO-proteins possess a C-terminal peroxisomal targeting sequence (Nakamura et al. 2002). The C-terminal SNL-tripeptide of the *Arabidopsis*-protein (Eilers et al. 2001) is very similar to the C-terminal amino acid motif serine-lysine-leucine (SKL) which is the consensus peroxisomal targeting sequence 1 (PTS1) and which is sufficient to direct polypeptides to peroxisomes *in vivo* in plants, animals and yeast. This non-cleaved tripeptide motif, consisting of a small, a basic and a hydrophobic residue or a variant thereof, resides at the extreme C-terminus and occurs in the majority of peroxisomal matrix proteins (Hayashi et al. 1996; Mullen et al. 1997). Plant PTS1 motifs apparently exhibit more sequence variability as compared to accepted signals in animals (Mullen et al. 1997).

Having the *Arabidopsis*-SO clone at hands (Eilers et al. 2001) and making use of antiserum that we generated against plant SO we finally answered the question of its subcellular localization. Antibodies directed against plant SO were applied for histochemical studies by transmission electron microscopy. Immunogold experiments performed on ultrathin sections of *Arabidopsis thaliana* leaves and of protoplast-derived micro-colonies of *Nicotiana plumbaginifolia* demonstrated for both species that gold labels were exclusively located in peroxisomes, and only a few were observed in other organelles or the cytoplasm (Nowak et al. 2004). To validate these results, we generated GFP::SO fusion constructs, transferred the genes via particle gun into tobacco leaves and monitored transient expression by confocal laser scanning microscopy. A punctuate fluorescence pattern was observed. The overlay of chlorophyll autofluorescence demonstrates that GFP was clearly excluded from the chloroplasts. To distinguish between peroxisomes and mitochondria we performed double transformation experiments with different fluorescent proteins and excluded mitochondria as targets by counterstaining with MitoTracker-Red (Nowak et al. 2004). Thus, independent lines of experimental evidence unequivocally demonstrate that plant SO is a peroxisomal enzyme.

A shared feature of all peroxisomes is their ability to metabolize hydrogen peroxide (H_2O_2), consequently protecting the rest of the cell from this toxic byproduct (Johnson and Olsen 2001). Our studies identified oxygen as the new final electron acceptor thereby generating H_2O_2 as reaction product in addition to sulfate which might explain why plant SO is localized in peroxisomes while animal SO occurs in mitochondria where it uses cytochrome c as electron acceptor. H_2O_2 is a highly reactive molecule that can be decomposed by peroxisomal catalase. Yet, there is another possible way for removing H_2O_2: In clouds and rain droplets, H_2O_2 was identified as one of the most effective non-enzymatic oxidants for HSO_3^- (Clegg and Abbatt 2001). Under our experimental conditions, sulfite did not spontaneously oxidize to sulfate, however the addition of physiological concentrations of H_2O_2 in the micromolar range led to the conversion of sulfite into sulfate (Hänsch et al. 2006). So we suggest that in the case of high sulfite concentrations in the plant cell, the production of H_2O_2 by SO can help to detoxify further sulfite molecules by a non-enzymatic reaction subsequent to enzymatic sulfite oxidation, thus increasing sulfite

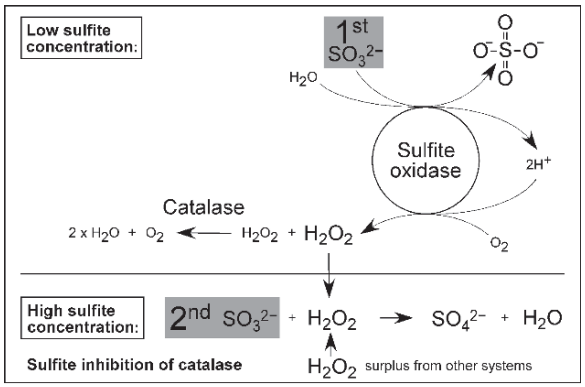

Fig. 1. Proposed interaction of plant SO and catalase. Plant SO oxidizes of sulfite and generates equimolar amount of H_2O_2. At low sulfite concentrations, all H_2O_2 formed will be immediately degraded by peroxisomal catalase. But at high sulfite concentrations, however, catalase will be inhibited by sulfite. The H_2O_2 molecule generated by the plant SO reaction can non-enzymatically oxidize a second molecule of sulfite (according to Hänsch et al. 2006).

removal (Fig. 1). And this makes sense because it has been shown previously that peroxisomal catalase is inhibited when leaves were treated with sulfite (Veljović-Jovanović et al. 1998) – the half-maximal inhibition was below of 500 µM sulfite. Here, on one hand the plant SO could play a role for protecting this important enzyme from sulfite damage and on the other hand: excess of sulfite will inhibit the catalase and the increasing H_2O_2 can help to reduce toxic sulfite. Hence we assume that SO could possibly serve as "safety valve" to detoxify excess amounts of sulfite and protect the cell from sulfitolysis.

VII. Compartmentalization of Sulfur Metabolism

Cells solved the problem of having two important conflicting pathways by separating them into different compartments. This rule holds also true for sulfur metabolism: sulfate assimilation takes place in the chloroplasts whereas sulfite detoxification by the SO is peroxisomally localized. However, peroxisomes seem to be not the only known sulfite-oxidizing organelles. Although the peroxisomal molybdoenzyme SO (EC 1.8.3.1) is the only biochemically and genetically characterized SO, there is still sulfite oxidation going on in the cell. In non-green suspension cultures of mutants lacking the molybdenum cofactor and therefore also peroxisomal SO, the sulfite oxidizing capacity of the cell extract does not go down to zero but to 40% of the wildtype-level (Eilers et al. 2001). The origin of this residual activity remains unclear.

How do the two pathways of chloroplast-based sulfate assimilation and peroxisomal sulfite oxidation interact and how are they co-regulated? Chloroplasts and peroxisomes are closely associated within the plant cell which is the basis for photorespiration where intermediates are crossing back and forth between these two organelles and mitochondria (Buchanan et al. 2000). Obviously, this association forms the basis for the rapid and efficient metabolic channeling of the two toxic metabolites: sulfite and H_2O_2. Finally, another cell-compartment seems to be involved in this metabolic process as well: the end product of sulfite oxidation – sulfate – is stored in the vacuole or could be transported out of the cell. The internal sulfate reserve in the vacuoles may buffer the flux of sulfate through the plant. While the nature of a tonoplast sulfate influx transporter is still unsolved (Buchner et al. 2004), recently Kataoka et al. (2004) could demonstrate SULTR4-type vacuole transporters to facilitate the efflux of sulfate. Sulfate uptake into chloroplast is described to be mediated by the same group 4 transporter family (for review see Leustek 2002 and chapter I.2 in this book). But for peroxisomes only one porin is known as transport system for a variety of different inorganic and organic anions (Reumann et al. 1998), which could principally assist sulfate or sulfite transport.

In the future, more information on the subcellular transport of SO_2, sulfite and sulfate will sharpen our view of the complex regulatory interaction between chloroplasts and peroxisomes and thus will shed more light on the fate of sulfite during assimilatory or dissimilatory processes.

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