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Adenosine 5'-phosphosulfate reductase – the key enzyme of plant sulfate assimilation

Komise pro obhajoby doktorských disertací v oboru
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CONTENTS

ABSTRACT

Sulfate assimilation is a pathway used by prokaryotes, fungi, and photosynthetic organisms to convert inorganic sulfate to sulfide, which is further incorporated into carbon skeletons of amino acids to form cysteine or homocysteine. In the last decade due to combination of biochemical and genetic methods a big progress in understanding the sulfate reduction in plants was achieved. We have contributed to this progress by resolving the old question of sulfate reduction via bound or free sulfite and the controversy about the reaction mechanism of the enzyme catalyzing the reduction of adenosine 5'-phosphosulfate (APS). The surprising findings of an iron-sulfur cluster bound to the enzyme and stable reaction intermediate with sulfite covalently bound to the protein clearly support a reductase mechanism and thus a correct nomenclature as APS reductase. Very recently, however, we discovered a new isoform of APS reductase in lower plants and algae, which catalyzes reduction of APS without the need for the iron sulfur chemistry.

Numerous investigations revealed that the sulfate assimilation pathway is highly regulated in a demand-driven manner. APS reductase rapidly and strongly responds to most environmental conditions and chemical treatments. We have shown that the enzyme activity is induced by carbohydrates and reduced nitrogen compounds and repressed by nitrogen and carbon starvation. Using control flux analysis we revealed that it possesses a very high control over sulfate assimilation and cysteine synthesis. Regulation of APS reductase is complex involving transcriptional, post-transcriptional and post-translational mechanisms. In this dissertation I present evidence for APS reductase being the key enzyme of the sulfate assimilation pathway. The biochemical mechanism and evolution of the enzyme will be discussed as well as the enzyme regulation in various plants species.

THE DISSERTATION

This dissertation summarises the results of my work and work of my co-workers and collegues on the enzyme adenosine 5‘-phosphosulfate reductase (APR). The work was initiated during my time as a postdoc in the lab of Christian Brunold, the pioneer of plant sulfur research, and later formed the centre of my reserach as assistant professor in Freiburg and now as a project leader at John Innes Institute in Norwich. We have applied biochemical, genetic, and molecular methods on numerous plant species to characterise the enzyme‘s biochemical mechanism, regulation in plants, and evolution, revealing a great variety in the form and regulation of APR among different plant species. I recieved funding from Swiss National Science Foundation, Deutsche Forschungs-gemeinschaft, and British Biolotechnology and Biological Sciences Council. From the results we can conclude that APR is the key enzyme of plant sulfate assimilation, possessing very high control over the pathway.
The dissertation is based on 17 original papers with contributions of a few more. In the first part of the dissertation the biochemical mechanism of the enzyme is outlined, followed by its diversity and evolution. In the second part the regulation of APR is described focussed on its contribution to control of the pathway.

INTRODUCTION

Sulfur is found in nature in different oxidation states in inorganic, organic, and bioorganic forms. For living organisms sulfur is an essential element with many different functions. It is found in reduced form in amino acids, peptides, and proteins, in iron-sulfur clusters, lipoic acid, and other cofactors and in oxidized form as sulfonate group modifying proteins, polysaccharides, lipids, and many natural products. In addition, reduced sulfur compounds,

such as hydrogen sulfide serve as electron donors for chemotrophic or phototrophic growth in a large and diverse group of bacteria and archae, including purple and green sulfur bacteria [1]. On the other hand, oxidized sulfur compounds such as sulfate can function as terminal electron acceptor in respiration to support growth of sulfate reducing bacteria [2]. Plants, yeast, and most prokaryotes cover their demand for reduced sulfur by reduction of inorganic sulfate to sulfide which is afterwards incorporated into organic compounds. Sulfur is the least abundant from the six macronutrients required by plants and was long not considered to limit plant growth, therefore, probably; the sulfur metabolism was less studied in the past. In the last decade a significant progress in understanding the pathway of sulfate assimilation in plants was achieved as documented by numerous reviews [3-7].

To be metabolized, the relatively inert sulfate must be first activated. The activation step, an adenylation to adenosine 5'-phosphosulfate (APS) is catalyzed by ATP sulfurylase (ATPS). APS is reduced by APS reductase (APR) to sulfite. In the second reduction step sulfite is reduced by a ferredoxin dependent sulfite reductase to sulfide. Sulfide is finally incorporated into the amino acid skeleton of *O*-acetyl-L-serine (OAS) forming cysteine (Figure 1) [3,6].

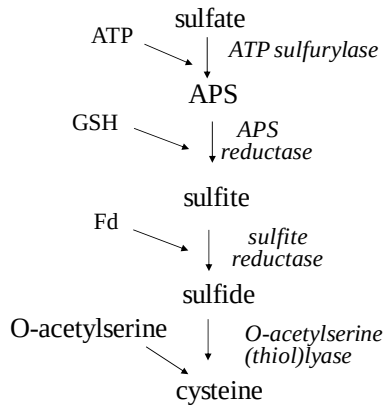

Figure 1. *Assimilatory sulfate reduction pathway in higher plants.*

However, this simple scheme of plant sulfate assimilation has been resolved only recently after many years of controversy about the sulfate reducing step [for a review see 8]. Plants (and algae) were believed

to use a “bound inter-mediate” pathway named after a presumed reaction product of a highly regulated enzyme of the pathway named APS sulfotransferase, sulfite bound to a thiol carrier [9-11]. In addition, a bacteria- and fungi-like pathway dependent on an additional phosphorylation of APS to 3’-phosphoadenosine 5’-phosphosulfate (PAPS) before reduction to sulfite, has been believed to exist in plants [11,12]. The cloning of Arabidopsis cDNA encoding a protein with a plastid targeting peptide, a central part homologous to *E. coli* PAPS reductase, and a C-terminal extension similar to thioredoxin [13,14] was an important starting point for deciphering the mechanism of sulfate reduction in plants.

BIOCHEMICAL CHARACTERIZATION OF APS REDUCTASE

Since the newly identified enzyme from Arabidopsis reduced APS to sulfite, it was named APS reductase (APR), adding to the confusion about the sulfate reducing mechanism [14-16]. The controversy was resolved by Suter et al. [**II**]. We isolated the enzyme described as APS sulfotransferase from *Lemna minor* and cloned the corresponding cDNA. The deduced amino acid sequence was very similar to that of APS reductase from *A. thaliana*. Free sulfite was the only reaction product of the recombinant *Lemna* protein detected under non-oxidizing conditions, while S-sulfo-glutathione was only formed when oxidized glutathione was present in the enzyme assay [**II**]. From these experiments we concluded that (i) APS sulfotransferase and APS reductase were identical enzymes, (ii) the bound sulfite pathway was an artifact due to the presence of oxidized thiols in the reaction assay, and (iii) the enzyme should, therefore, be named APS reductase.

The reductase mechanism of APR was corroborated by identification of a reaction intermediate, with sulfite covalently bound to a cysteine residue of the enzyme [**IV**]. Incubation of recombinant APR with [^{35}S]APS at 4°C

resulted in radioactive labelling of the protein. Analysis of tryptic peptides revealed $^{35}SO_3^{2-}$ bound to the cysteine residue conserved between APS and PAPS reductases [17]. When this cysteine was exchanged to serine, no labelling of APR occurred and the mutant protein lost completely its enzymatic activity [**IV**]. Therefore it seems that the reaction of APR can be divided into two independent steps: a reductive transfer of sulfate to the active cysteine residue and the release of the sulfite by the C-terminal domain. For the first step, which results in binding of sulfite to APR a thiol is not required; it is catalyzed also by the truncated APR lacking the thioredoxin-like domain. Interestingly, the reductase domain from *A. thaliana* APR2 was able to catalyze sulfite production even without additional thioredoxin or glutaredoxin. Only addition of recombinant thioredoxin m but not thioredoxin f enhanced the reaction velocity [**IV**].

The recombinant APR and the enzyme purified from *L. minor* were colored yellow-brown [**II**] indicating the presence of a cofactor, possibly FAD and/or iron-sulfur centre. UV/visible spectra of recombinant APR indicated the presence of an FeS center and, indeed, we found iron and acid-labile sulfide binding to the APR protein [**VI**]. Electron paramagnetic resonance and Mössbauer spectroscopy than confirmed the presence of a diamagnetic $[4Fe\text{-}4S]^{2+}$ cluster as cofactor of plant APS reductase. This cluster is unusual since only three of the iron sites exhibited the same Mössbauer parameters. Although there is no signature for binding an FeS cluster in the sequence of plant APR, the N-terminal part of APR differs from PAPS reductase by the presence of two additional cysteine pairs in the plant enzyme. Only three from these four additional cysteine residues can bind the FeS cluster explaining its extraordinary characteristics [**VI**].

Analysis of prokaryotic genome sequences revealed that many bacteria, such as *Pseudomonas*, *Rhizobium*, or *Bacillus*, which were believed to use the PAPS dependent sulfate reduction, possess genes more similar to plant APR

than to PAPS reductase from *E. coli* and yeast [**VII**]. This raised the question whether the sulfate reduction in these species is really PAPS dependent. Indeed, we and others revealed APS dependent sulfate assimilation in several species of *Rhizobiaceae*, *Pseudomonas*, *Burkholderia*, *Ralstonia*, *Mycobacterium,* and *Bacillus* [**VII,** 18-20]. The major difference between these new APS reductases and *E. coli* PAPS reductase was a presence of two conserved cysteine pairs that bind the FeS cofactor in plant APR [**VII**]. We, and later others, confirmed a presence of an FeS cluster similar to the one found in plant APR in all bacterial APS reductases analyzed [**VII**, 21-23]. These results thus clearly pointed to the presence of the FeS cluster as a determinant of APS vs. PAPS specificity.

An important factor of the characterization of APR is confirmation of its inter- and intracellular localization. APR activity was localized in spinach chloroplasts [24,25] and proplastids of root cells [26]. Accordingly, using immunogold labeling we confirmed the APR protein exclusively in chloroplasts of three *Flaveria* species of different type of photosynthesis [**V**]. Similarly, in the moss *Physcomitrella patens* both APS reductase isoforms (see below) are present in the plastids as shown by GFP fusions [**XIV**]. However, the distribution of the GFP signal derived by the two proteins differed. While APR-B was localized uniformly throughout the plastids, the GFP label of APR was clustered in a spotty pattern [**XIV**]. The same pattern was observed in the green alga *Chlamydomonas reinhardtii* [**XVII**]. Using immuno-localization and electron microscopy the APR labeling was shown to be concentrated around the starch sheath, in close association with the pyrenoid. Notably, the same distribution within *Chlamydomonas* chloroplast has been observed for SiR, which points to a possibility that the two enzymes form a „sulfate reducing complex“ [**XVII**]. This is a very attractive hypothesis, as such multienzyme complex would present the organism with several advantages. Firstly, the assembly of the enzyme would allow for chanelling of reaction intermediates

and increase thus the efficiency of the reactions. Secondly, one of these intermediates, sulfite, is very reactive and cytotoxic, therefore, its confinement in the complex would be a good prevention of cellular damage. The hypothesis of sulfate assimilating enzymes forming a complex is corroborated by finding of *in vitro* interaction of ATPS and APR [27].

The experiments with *Flaveria* indeed confirmed plastidic localization of APR, this was, however, not their primary aim [**V**]. Sulfate assimilation, namely the activity of ATPS and APR, was shown before to be restricted to the bundle sheath cells of C_4 plants [28-30]. Accordingly, we showed that in maize the mRNAs for APR, ATPS, and SiR accumulated in bundle sheath only, whereas the mRNA for O-acetylserine-(thiol)lyase was also detected in mesophyll cells [31]. To address the question whether the exclusive or almost exclusive localization of APR was a pre-requisite or a consequence of C_4 photosynthesis, the distribution of these enzymes was studied in *Flaveria* species with different types of photosynthesis [**V**]. The dicot genus *Flaveria* (*Asteraceae*) is an excellent model to study the evolution of C_4 photosynthesis because, beside C_3 and C_4 species, a relatively large number of C_3-C_4 intermediates occur in this genus [32] and a continuous gradation both in the physiology and biochemistry of photosynthesis exists among *Flaveria* species [33]. However, surprisingly, by northern analysis of cell-specific RNA and *in situ* hybridization ATP sulfurylase and APR mRNA were present at comparable levels in both mesophyll and bundle sheath cells of the C_4 species *Flaveria trinervia*. Also immunogold electron microscopy confirmed the presence of APR protein in chloroplasts of both cell types [**V**]. Therefore, it seems that the localization of assimilatory sulfate reduction in the bundle sheath cells is not ubiquitous among C_4 plants but occurs only in C_4 monocots and is neither a pre-requisite nor a consequence of C_4 photosynthesis.

DIVERSITY AND EVOLUTION OF APS REDUCTASE

The characterization of APR clearly demonstrated its key role in plant sulfate assimilation but it still could not exclude that plants possess the bacterial-like PAPS reductase as predicted before [12]. The analysis of *Arabidopsis* genome did not identify any gene homologous to *E. coli* PAPS reductase other than those encoding the three isoforms of APR [13,14]. Since PAPS reductase in plants may have a completely divergent structure, only analysis of plants lacking APR activity may prove or exclude the PAPS dependent sulfate assimilation. However, no such plants were described, probably because APR is encoded by small multigene families of 2-3 isoforms in most plant species analyzed to date [**VII**,**XVI**, 13,14,34]. To overcome this problem we turned to the moss *Physcomitrella patens*, which in the last few years became an increasingly used model system to study the function of plant genes because of an efficient system of homologous recombination allowing exact gene targeting [35-38].

Analysis of an EST database and Southern analysis revealed that APR is encoded by a single copy gene in this moss. Therefore, in order to find possible alternative routes of sulfate assimilation we disrupted the *APR* gene in *Physcomitrella patens* by homologous recombination [**VIII**]. This resulted in complete loss of the correct transcript and enzymatic activity. Surprisingly however, the knockout plants grew on sulfate as the sole sulfur source and the concentration of thiols in the knockouts did not differ from the wild type plants. Only when exposed to sub-lethal concentration of cadmium, the knockouts were more sensitive than wild type plants and the flux through sulfate reduction, measured by incorporation of [^{35}S]sulfate was about 50% lower than in the wild type. Clearly, a sulfate reducing enzyme other than APR must exist in *P. patens*; this alternative system is, however, not as efficient as APR. Although PAPS reductase activity could not be measured in moss extracts with different thioredoxins as reductants, a gene very similar to bacterial PAPS reductase was found in

P. patens [**VIII**]. The corresponding recombinant protein possessed PAPS reductase activity, seemingly confirming the identity of the gene. However, the APS reduction rate was at least thousand fold higher that that of PAPS, showing that the protein is actually a novel isoform of APS reductase without iron-sulfur cluster [**XIV**]. The protein was therefore named APR-B, to distinguish from the FeS cluster containing APR. The two enzymes differ in affinity to APS, while APR possesses a K_M of 6 µM and is inhibited by APS concentration higher than 20 µM, APR-B performs best at APS concentration of 100 µM. Compared to PpAPR, the APS reductase activity of PpAPR-B was several thousand folds lower at low APS concentrations but at APS concentrations higher than 50 µM the difference was only approx. 200 fold. This could be caused by the better catalytical efficiency due to the presence of cofactor but possibly also due to the fact that APR-B does not possess the Trx-like domain and requires free thioredoxin in the reaction assay [**XIV**]. The reaction conditions of the two enzymes differ significantly, especially the effects of various salts, so that it is possible to design assays to measure them separately in moss extracts [**XIV**,**XV**]. As expected, the APR-B is significantly more stable than APR, which loses 90% activity after 24 h storage at 4°C [**XIV**]. APR and APR-B thus represent an unique pair of enzymes catalyzing the same reaction but one using a FeS cofactor and the other not. Also *APR-B* gene was disrupted by homologous recombination leading to plants without a phenotype at normal growth conditions but sensitive to Cd similar to *ΔAPR* plants [**XV**]. This shows that APS reduction indeed is the sole mechanism of sulfate reduction in plants.

The recent increase in availability of sequence information from various algae and plants enables a wide sampling of taxa for phylogenetic analysis. This allows addressing questions of the origin of plant genes and pathways and metabolic diversity of plants and algae including the sulfate assimilation and APS reductase. The APR and APR-B sequences from different sources are well conserved,

containing 60-80% identical amino acid residues. Since the reductase domains of APR are 22-27% identical to the PAPS reductase from *E. coli* or yeast and share a common active center represented by the amino acid sequence K/R E C G L/I H [8], they can be aligned and their phylogeny analyzed together [**VII, XVI**, 39] (Figure 2). A neighbor-joining tree constructed from APS and PAPS reductase related sequences, retrieved from the Genbank is divided into two major branches [**VII, XVI**]. The first branch contains a cluster of APRs from plant and algae, together with many bacterial APS reductases, the other one is subdivided into clusters comprising fungal PAPS reductases, well-characterized PAPS reductases from enteric bacteria and cyanobacteria, as well as the novel APR-B sequences from lower plants and microalgae. Although it was believed that APS reduction is linked to the presence of the FeS cofactor, the phylogenetic analysis clearly positions the APR-B genes into the same cluster as PAPS reductases and not plant APRs [**XIV**,**XVI**]. Importantly, genes very similar to *Physcomitrella* APR-B were found in the lycophytes *Selaginella lepidophylla* and *S. moellendorffii* (Figure 2). Since lycophytes belong to vascular plants, this finding brought evidence that APR-B is not a result of a single horizontal gene transfer from fungi to moss but played a role in evolution of plant sulfate assimilation over a long period [**XIV**,**XVI**].

Interestingly, the only two genes homologous to APS or PAPS reductase identified in the completely sequenced genome of the diatom *Thalassiosira pseudonana*, which reduces APS with up to 100-fold higher rate than plants ([40], Kopriva S., unpublished) also cluster with the APR-B and do not contain the two Cys pairs. It seems therefore, that the FeS cluster-independent APS reduction is not a peculiarity of several lower plant species, but a widespread mechanism. Thus APR-B had to provide some evolutionary advantage to these species. The reduced cost of sulfate reduction due to the lack of FeS cluster in PAPS reductase is balanced by consumption of one additional ATP per

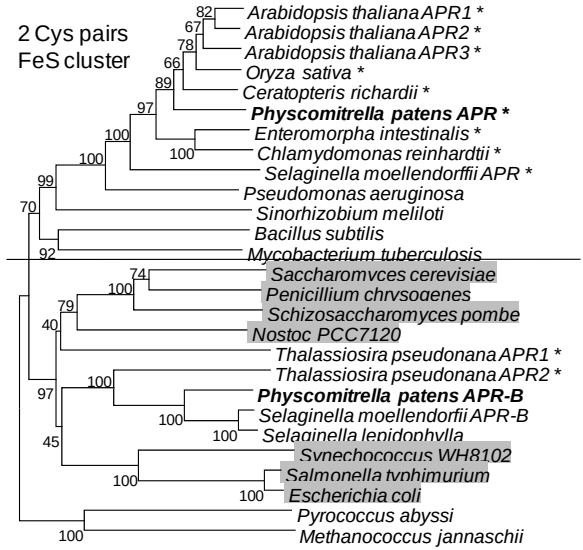

Figure 2. *Neighbor joining tree of APS and PAPS reductase sequences. PAPS reductases are shaded. The horizontal line separates proteins containing the 2 Cys pairs binding FeS cluster. Asterisks mark proteins with thioredoxin-like domain. P. abyssi and M. jannaschii proteins of unknown function but alignable with APS reductase were used as outgroup.*

sulfate reduced. PAPS reductase/APR-B might confer specific advantage in iron limiting environments. Remarkably, we found APR-B like genes in several marine phytoplankton species [**XVI**]. Indeed, iron is often limiting growth of marine algae [41], therefore, they developed strategies to reduce Fe demand. FeS proteins in marine diatoms are frequently substituted by alternative enzymes, e.g. the ferredoxin by flavodoxin [42] or cytochrome c6 by plastocyanin [43]. Thus, the APR-B should be especially advantageous in Fe limiting habitats, such as marine and other aquatic environments. Once the FeS independent sulfate reduction was enabled, changing the substrate specificity to APS reduced the costs even further. The plant

APR gene originated most probably from a fusion between prokaryotical genes for APS reductase and thioredoxin. Since all APRs isolated or cloned from higher plants, as well as the APR from the green algae *Enteromorpha intestinalis* [40], have the same structure, this fusion must have occurred early in the evolution of plants. The close relation of the *Plectonema* APR to plant enzymes also implies that plants obtained the gene for APS reductase from the cyanobacterial endosymbiont. The cyanobacterial gene was then allocated to the plant nuclear genome and supplemented with the sequence encoding the targeting peptide, such as genes coding, e. g., for Calvin cycle enzymes [44]. The hypothesis that APS reductase is an evolutionary predecessor of PAPS reductase is further corroborated by the observations that (i) the PAPS reductase only occurs in γ-proteobacteria and cyanobacteria, whereas APS reductase was confirmed in most taxonomical groups of *Eubacteria*, (ii) dissimilatory sulfate reducing bacteria and green sulfur bacteria possess APS reductase which contains FeS cluster as the cofactor [**VII,**45,46]. Altogether, activated sulfate can be reduced to sulfide by similar but distinct enzyme forms: (i) bacterial and fungal PAPS reductase, (ii) bacterial assimilatory APS reductase, which is thio-redoxin dependent similarly to PAPS reductase, (iii) plant APR, which possesses a glutaredoxin-like C-terminal extension, (iv) lower plant and protozoan APR-B, and (v) bacterial dissimilatory APS reductase (Figure 3).

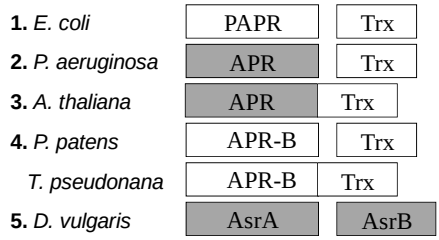

Figure 3. *Types of sulfonucleotide reducing enzymes. Gray colour symbolizes presence of FeS cluster.*

REGULATION OF SULFATE ASSIMILATION

Sulfur containing compounds play essential role in many metabolic processes. Among these compounds, the tripeptide glutathione (GSH) is of major importance [47-49]. It is, therefore, not surprising that the assimilatory sulfate reduction pathway is thoroughly regulated in order to keep the concentration of glutathione and other reduced sulfur compounds stable but also to prevent accumulation of toxic intermediates such as sulfite and sulfide. The pathway is thus well coordinated with the assimilation of carbon and nitrogen and regulated according to the need of the plants for reduced sulfur [3-8]. The first two reaction steps of sulfate assimilation, catalyzed by ATPS and APR are most susceptible to regulation. Because of its position at the beginning of the pathway ATPS was the first target for investigations of regulation of sulfate assimilation [50]. Studies of regulation of sulfate uptake and ATPS by sulfate starvation and GSH led to the formulation of generally accepted hypothesis of demand-driven control of sulfate assimilation [51,52]. In a wide range of other experiments however we showed that APR is more extensively regulated than ATPS and that it is responsible for control of flux through sulfate assimilation. [**I**, **III**, **IX**, **X**, **XI**, **XIII**, **XVII**]. In the next sections, therefore, the regulation of APR will be described in detail.

REGULATION OF APR BY SULFUR COMPOUNDS

In accordance with the concept of demand-driven regulation of sulfate assimilation, APR is regulated by availability of sulfur. APR activity and mRNA levels increase upon sulfur starvation and decreased when sulfate was again available or upon supply of reduced sulfur compounds such as fumigation with SO_2 while other enzymes of sulfate reduction pathway were much less influenced [53-57]. It is not only the sulfate starvation that induces APR, the enzyme activity can also be upregulated by decreasing GSH levels. This is often the case during stress, but can be

achieved by treatment with an inhibitor of GSH biosynthesis L-buthionine-S, R-sulfoximine (BSO) [51,58]. Indeed, in poplar roots, as well as in Arabidopsis root cultures, BSO induced APR mRNA levels and activity [**IX**,58].
In contrast, the enzyme activity and mRNA accumulation is feedback inhibited by cysteine and GSH [**IX,**58,59]. Also other reduced or partially reduced sulfur compounds cause APR activity to decrease. Fumigation with hydrogen sulfide or sulfur dioxide, gases naturally occurring in the atmosphere, supplies plants with reduced sulfur and enables them to reduce the energetically demanding sulfate uptake and reduction [**XII**,53, 60,61]. In the line with observations that APR is the major control point of the reduction pathway, the activity of APR but not of other enzymes is strongly diminished in the fumigated plants [**XII**,53,61]. Interestingly, not all plants respond to the H_2S fumigation in the same way, e.g. *Brassica oleracea* reduces both APR activity and sulfate uptake while in onion the sulfate uptake is not affected [**XII**]. Also the mechanisms of APR regulation seem to be different. On the first sight the regulation appears straightforward as the exposure to reduced sulfur compounds diminishes the APR activity and mRNA levels [**IX**, **XII**] indicating a transcriptional regulation. When protein levels are determined by Western blotting, however, the picture is more complicated. While in *B. oleracea* the APR protein in fumigated plant is reduced similar to the enzyme activity and mRNA levels, in onion it remains high [**XII**]. This means that an unknown post-translational mechanism is responsible for reduction in APR activity in fumigated onion plants.

REGULATION OF APR BY NITROGEN AND CARBON METABOLITES

Cysteine synthesis is a merging point of sulfate assimilation with carbon and nitrogen metabolism. It is therefore not surprising that the three pathways are well coordinated [3,4]. While the interactions between sulfate and nitrate assimilation and nitrate and carbon metabolism have been

long known, the links between sulfate reduction and CO_2 assimilation have been identified only recently.

Decrease in nitrogen availability results in reduced production of amino acids. The demand for cysteine and methionine is thus reduced and correspondingly the sulfate assimilation pathway is downregulated. The same is true in the opposite direction, reduction in sulfate supply leads to decrease in nitrate uptake and assimilation [62]. In *Lemna minor* the activities of ATPS and APR decrease under nitrogen deficient conditions [63]. At the same time, addition of nitrate or ammonia to the medium quickly restored the activity of these two enzymes. On the other hand, addition of ammonia or amino acids (Arg, Asn, Gln) to the nutrient solution caused an 50-110% increase in extractable APR activity in *Lemna* [63, 64]. Addition of ammonium increased the flux through the sulfate assimilation measured as incorporation of ^{35}S in proteins after feeding [^{35}S]sulfate [54]. These results were confirmed with various plant species. In *A. thaliana* withdrawal of nitrogen from nutrition for three days led to a 30% and 50% decrease of APR activity in leaves and roots, respectively, while OAS (thiol)lyase was not affected [**III**]. The decrease of APR activity correlated with decreased mRNA and enzyme levels. The enzyme activity was quickly restored in roots where reduced nitrogen compounds were more effective than nitrate. $^{35}SO_4^{2-}$ feeding revealed that the closer the N-containing compound was to OAS, the higher was the flux through the pathway, making OAS a very good candidate for the molecular signal of the nitrogen status towards sulfur assimilation [**III**]. Also in poplar nitrogen starvation resulted in decrease in transcript levels and activity of APR [65].

Since APR sulfate reduction requires reducing equivalents normaly obtained from photosynthesis, the flux through sulfate assimilation, and correspondingly APR activity are higher during the day than in the night [**I**, 66,67]. With establishment of Arabidopsis as the model plant, the availability of cDNA sequences and antibodies enabled to study the regulation of sulfate assimilation on the level of

enzyme activities, mRNA and protein accumulation, metabolites (Cys and GSH), and fluxes. The first among such studies was our investigation of the control of sulfate assimilation by light [**I**]. We have shown that APR activity undergoes a diurnal rhythm with maximum activity 4 hours after light onset in both shoots and roots and minimum activity at the beginning of the night [**I**]. Regulation by endogenous circadian rhythm is not involved because during prolonged light the activity remained stable and low. The activity decreased to undetectable levels within 32 hours of darkness both in shoots and roots, but was restored to 50% in shoots and to 20% in roots within 8 hours after re-illumination. The changes in APR activity corresponded to changes in mRNA levels of all 3 APR isoforms and APR protein accumulation. $^{35}SO_4^{2-}$ feeding experiments showed that the incorporation of ^{35}S into reduced sulfur compounds *in vivo* was significantly higher in light than in the dark [**I**]. However, since the accumulation of mRNA and protein, as well as enzyme activity increased already during the last 4 hours of the dark period, light cannot be the only factor involved in APR regulation. The obvious candidates for such signal were sugars. Indeed, addition of 0.5% sucrose to the nutrient solution after 38 hours of darkness led to a seven-fold increase of root APR activity during 6 hours. The sucrose-induced increase of APR activity was even quicker than that measured in shoots after reillumination of dark-adapted plants [**I**]. Also addition of 0.5% glucose resulted in a similar increase of APR levels in roots (mRNA, protein and activity) [**XI**]. Treatment of roots with D-sorbitol, D-mannitol, and 2-deoxy-D-glucose did not increase APR activity, indicating that osmotic stress and hexokinase signaling was not involved in APR regulation. Feeding plants with a combination of glucose and OAS resulted in a more than additive induction of APR activity, showing that these metabolites regulate APR independently [**XI**].

In order to study the interconnection of sulfate, nitrogen, and carbon assimilation, *Lemna minor* was used to analyze

the effects of CO_2 omission from the atmosphere and simultaneous application of alternative carbon sources on APR and nitrate reductase (NR) [**X**]. Incubation in air without CO_2 led to a severe decrease in APR and NR activities and mRNA levels, but Rubisco was not considerably affected. Simultaneous addition of sucrose prevented the reduction in enzyme activities but not in mRNA levels. OAS could also attenuate the effect of missing CO_2 on APR, but did not affect NR. When the plants were subjected to normal air after 24 hours pre-treatment in air without CO_2 both APR and NR activities and mRNA levels recovered within the next 24 h. Addition of sucrose and glucose in air without CO_2 also recovered both enzyme activities; OAS again influenced only APR. $^{35}SO_4^{2-}$ feeding showed that treatment in air without CO_2 severely inhibited sulfate uptake and the flux through sulfate assimilation. After re-supply of normal air or addition of sucrose, incorporation of ^{35}S into proteins and glutathione greatly increased. OAS treatment resulted in high labelling of cysteine; the incorporation of ^{35}S in proteins and glutathione was much less increased [**X**]. These results corroborate the tight interconnection of sulfate, nitrate, and carbon assimilation and show that OAS might be the signal from N assimilation towards S assimilation, but most probably not *vice versa*.

REGULATION OF APR BY STRESS

Exposure of plants to various environmental conditions often causes production of reactive oxygen species and oxidative stress. GSH plays a prominent role in plant stress defense as it is the electron donor for regeneration of ascorbate, the primary ROS scavenger [68]. Thus, oxidative stress results in a higher demand for GSH and leads to upregulation of sulfate assimilation [69]. Similarly, demand for GSH is increased due to exposure to heavy metals or xenobiotics, since GSH is the precursor of phytochelatins and used for conjugations by glutathione S-transferases, respectively [70,71].

The increase flux through sulfate assimilation is primarily achieved by an increase of APR activity. The upregulation of APR and ATPS by Cd, as a model heavy metal, was documented in various plant species, including maize, pea, and *Brassica juncea* [34,70,72,73]. This increase in enzymatic activity was accompanied by an increase in the corresponding mRNA levels indicating a transcriptional regulation [34,73]. Another example for induction of APR as a result of oxidative stress is the response of maize to chilling. Maize is generally a chilling sensitive plant, because incubation of maize at low temperature leads to photoinhibition and ROS production. The protection against chilling injury is linked to GSH production, since GSH synthesis is induced by chilling and since maize genotypes with higher GSH content are often chilling tolerant. Accordingly, to provide cysteine for the increased GSH synthesis chilling of maize seedlings at 12°C results in a prominent increase of APR mRNA and activity [31,74]. The involvement of APR in plant stress defense is corroborated by many reports of regulation of APR expression by phytohormones involved in stress signaling. Steady state mRNA levels of APR isoforms are induced by jasmonate [75,76], nitric oxide [77], ethylene and salycilate [**XVII**]. Since also APR activity is induced by jasmonate, everything points again to a transcriptional control of APR by stress [76].

We have, however, challenged this view recently when it became apparent that the regulation of APR is much more complicated [**XVII**]. To dissect the molecular mechanisms of regulation of APR by stress, we analysed Arabidopsis mutants in the stress signaling pathways for the regulation of APR. APR activity and mRNA levels of all three APR isoforms increased three-fold in roots of wild type plants treated with 150 mM NaCl. Since the regulation of APR was not affected in mutants deficient in abscisic acid (ABA) synthesis APR must be regulated by salt stress in an ABA independent manner. In mutants deficient in jasmonate, salicylate, or ethylene signaling APR mRNA levels were

increased upon salt exposure similar to WT plants, showing that these pathways are not involved in regulating APR transcript by salt. Surprisingly, however, APR enzyme activity was not increased by salt in these mutants. This indicates that APR is regulated post-transcriptionally and that the signalling pathways were necesarry to induce such posttranscriptional regulator. The same result was obtained in mutants affected in cytokinin and auxin signaling. Signaling via gibberelic acid on the other hand turned out to be essential for the increase in APR mRNA by salt treatment. These results demonstrate an extensive post-transcriptional regulation of plant APR and reveal that the sulfate assimilation pathway is controlled by a complex network of multiple signals on different regulatory levels [**XVII**].

APS REDUCTASE IN CONTROL OF SULFATE ASSIMILATION

Several characteristics of APR suggest that it is the enzyme controlling the pathway of sulfate assimilation. Firstly, we and others never detected any back reaction of APR, the synthesis of APS from sulfite and AMP, with recombinant assimilatory enzymes from bacteria or plants. Secondly, the enzyme is highly regulated and often it is the only enzyme in the pathway responding to the treatment. In addition, overexpression of APR in plants leads to high accumulation of reduced sulfur compounds causing severe stress, while overexpression of other genes of the pathway produce no visible phenotypes [78-80]. Using two different approaches we firmly established APR as the key enzyme of the sulfate reduction pathway.

We have utilized Arabidopsis root cultures to investigate the changes in flux through sulfate assimilation by thiols. Addition of the thiols into nutrient solution results in decrease in APR mRNA and protein accumulation and also the enzyme activity [**IX**]. Feeding experiments using [^{35}S]sulfate in the presence of 0.5 mM cysteine or GSH showed that both thiols also decreased sulphate uptake

and the incorporation of the radioactivity into cysteine, GSH, and proteins. Since this treatment affected solely APR, but not ATPS, SiR, or OAS (thiol)lyase, the data were subjected to control flux analysis to calculate the control coefficient of APR. Starting from internal sulfate the flux control coefficient of APR is 0.9 (equivalent to 90% of the total control), showing a very high control of the pathway by APR. When the flux is calculated from external sulfate, the control is shared with sulfate uptake. APR thus indeed possesses a high control over the flux through the sulfate reduction pathway [**IX**].

Independent evidence for the key role of APR in controlling plant sulfur homeostasis was obtained by quantitative genetics exploiting Arabidopsis natural variation [**XIII**]. Two ecotypes of Arabidopsis, Bay-0 of European origin and Shahdara isolated in Tadjikistan, differ in sulfate accumulation. Measuring sulfate concentration in 420 recombinant inbred lines generated from crossing of these ecotypes and using quantitative trait analysis two major loci controlling sulfate levels were identified. One of these loci comprised a region on chromosome 1 including the gene encoding APR2 isoform of APS reductase. We have analysed the parental ecotypes and two near isogenic lines (NIL) which differed only in the region around the QTL. Lines containing the Shahdara allele accumulated ca. 25% more sulfate and the foliar APR activity was significantly reduced. Overexpression of the Bay-0 *APR2* allele in Shahdara restored APR activity and reduced sulfate accumulation to normal levels [**XIII**]. Analysis of *apr2* knock-out in Col-0 background revealed a decrease of APR activity to ca. 25% (the remaining activity derived from APR1 and APR3) and an about 50% higher sulfate content in the mutants compared to wild type Col-0 plants. Thus, clearly, reduction of APR activity in the *apr2* plants or in Shahdara leads to accumulation of sulfate [**XIII**]. In order to find the molecular basis for the difference in APR activity between Shahdara and Col-0 or Bay-0 we expressed the APR2 proteins from each ecotype in *E. coli* using the pET

expression system and analysed the kinetics of the recombinant enzymes. APR2 in Shahdara differs from Col-0 and Bay-0 by 3 amino acid substitutions. One of these substitutuions is an exchange of Ala for Glu very close to the active centre in the C-terminal Trx-like domain. We thus predicted that the binding of GSH as electron donor would be affected in the Shahdara enzyme. Indeed, the K_M for GSH increased from 8 mM to 31 mM way above physiological GSH concentration, while K_M for APS was not changed. The V_{max} of the Shahdara enzyme was also reduced, ca. 4,000-fold compared to the enzyme from Col-0 and Bay-0. We have thus brought evidence that a single nucleotide polymorphism in Shahdara results in a crucial amino acid alteration that reduces the enzyme performance several thousand-fold, diminishing thus sulfate reducing capacity in this ecotype and leading to accumulation of sulfate [XIII]. APR2 thus controls sulfate accumulation in Arabidopsis.

CONCLUSIONS

Our investigations of sulfate assimilation pathway, namely the adenosine 5'-phosphosulfate reductase, and application of molecular methods resolved many of the old open questions. The controversy about the "free sulfite" and "bound sulfite" routes was resolved and the responsible enzyme APS reductase was well characterized biochemically. A new isoform of APS reductase has been discovered in lower plants and algae, resulting in discovery of a very interesting case of two enzyme isoforms catalyzing the same reaction with and without an iron-sulfur cluster as coenzyme. The regulation of APS reductase has been well described and shown to be largely driven by demand for reduced sulfur. Most of these results, most significantly including a control flux analysis, indicate that APS reductase is the enzyme possessing the highest control over the flux through sulfate assimilation.

Still, many important issues need to be resolved. The catalytic mechanisms of the two APS reductase will have to be solved to understand the exact role of the iron sulfur cluster. In addition, the molecular mechanisms of regulation of APR are still elusive. There, functional genomics and forwards and reverse genetics approaches are required to dissect the signals and transduction pathways leading to the described regulation of APR. Thus, APR will further remain in the centre of sulfur research.

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SOUHRN

Předkládaná disertace shrnuje výsledky mého výzkumu enzymu 5´-adenyl sulfát reduktázy.
Uvedené výsledky vedou k závěru, že tento enzym je zodpovědný za regulaci asimilace síry rostlinami. Asimilace síry je metabolický pochod používaný bakteriemi, houbami, řasami a rostlinami pro konverzi anorganického síranu na sulfid, který je dále začleněn do aminokyselin cysteinu nebo homocysteinu.
V posledním desetiletí došlo k velkému pokroku v porozumění regulace rostlinné asimilace síry díky kombinaci biochemických a genetických metod, na kterých jsme se, spolu se svými kolegy, velmi významně podíleli vyřešením několika dlouho otevřených problémů. Především tím, zda je síra redukovaná s použitím „vázaného“ nebo volného sulfitu a dále polemikou o reakčním mechanismu enzymu, který katalyzuje redukci 5´-adenyl sulfátu (APS). Biochemickou analýzou rekombinantní rostlinné APS reduktázy jsme nečekaně objevili, že tento enzym obsahuje železo-sirný komplex jako koenzym. Kromě toho jsme charakterizovali stabilní meziprodukt reakce skládající se ze sulfitu kovalentně vázaného na enzym. Podrobná analýza počátečních reakčních produktů ukázala, že dříve popsaný „vázaný sulfit“ je výsledkem chemické reakce mezi skutečným produktem enzymu, volným sulfitem a oxidovaných thiolů, například glutathionu. Tyto výsledky jasně ukazují, že APS je redukován enzymem používajícím mechanismus reduktázy, a proto je jeho správné jméno APS reduktáza. Analýzou pochodu redukce síranu v různých bakteriích a rostlinách jsme zjistili, že mnoho bakterií používá APS reduktázu a ne typický bakteriální enzym PAPS reduktázu a dospěli jsme k závěru, že schopnost používat APS pro redukci síranu je spojeno s přítomností železo-sirného komplexu jako koenzymu. Tato teorie se však nepotvrdila, protože v nedávné době jsme objevili novou izoformu APS reduktázy v nižších rostlinách a mikrořasách, která

katalyzuje redukci APS, má podobnou strukturu, ale neváže železo-sirný komplex.
Celá řada experimentů ukázala, že asimilace síry je silně regulovaná převážně poptávkou po redukované síře. APS reduktáza rychle a silně reaguje na chemické podněty a na většinu změn růstových podmínek. Ve svém výzkumu jsme používali kombinaci fyziologických a molekulárních metod, měřili jsme aktivity enzymů, množství mRNA a proteinů pomocí northern a western blotů, metabolity a navíc i inkorporaci radioaktivity ze síranu do proteinů a thiolů, jako měřítko průchodu pochodem asimilace síry. Naše výsledky ukázaly, že aktivita APS reduktázy je zvýšena přidáním sacharidů a redukovaných sloučenin dusíku a snížena nedostatečností dusíku a kysličníku uhličitého. Regulace APS reduktázy je komplexní, zahrnující regulaci na úrovni transkripce, post transkripční a post translační mechanismy. Biochemické vlastnosti APS reduktázy a vysoký stupeň její regulace naznačují, že tento enzym je velmi důležitý pro kontrolu asimilace sulfátu. Využitím kontrolní metabolické analýzy jsme opravdu ukázali, že APS reduktáza má velmi vysokou kontrolu nad asimilací sulfátu a syntézou cysteinu. Kromě toho jsme metodami kvantitativní genetiky dokázali, že APS reduktáza kontroluje množství sulfátu v listech.
Předkládaná disertace dokazuje, že APS reduktáza je klíčovým enzymem kontrolujícím asimilace sulfátu v rostlinách.