Structural Biology and Regulation of the Plant Sulfation Pathway

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Abstract

In plants, sulfur is an essential nutrient that must be converted into usable metabolic forms for the formation of sulfur-containing amino acids and peptides (primary route) and for the modification of diverse metabolites (secondary route). In plants, the fate of assimilated sulfate depends on the three enzymes - ATP sulfurylase, adenosine-5'-phosphate (APS) reductase, and APS kinase - that form a branchpoint in the pathway. ATP sulfurylase catalyzes the formation of the critical intermediate APS, which can either be used in the primary assimilatory route or be phosphorylated to 3'-phospho-APS (PAPS) for a variety of sulfation reactions. Recent biochemical and structural studies of the branchpoint enzymes in plant sulfur metabolism suggest that redox-regulation may control sulfur partitioning between primary and secondary routes. Disulfide-based redox switches differentially affect APS reductase and APS kinase. Oxidative conditions that promote disulfide formation increase the activity of APS reductase and decreases PAPS production by APS kinase. Here we review recent work on the ATP sulfurylase and APS kinase from plants that provide new insight on the regulation of PAPS formation, the structural evolution of these enzymes in different organisms, and redox-control of this key branchpoint in plant sulfur metabolism.

Introduction

In all organisms, sulfur is a versatile and essential element used in a variety of metabolic pathways. For plants, sulfur, along with nitrogen, phosphorus, and potassium, is a nutrient and a critical determinant of soil quality [1-2]. Plants uptake inorganic sulfate (SO_4^{2-}) from the environment and convert it into physiologically useful forms of sulfur [3-4]. The core sulfur metabolism pathway in plants (**Fig. 1**) reduces sulfate for the production of cysteine, methionine, iron-sulfur clusters, vitamins, molecules that protect against oxidative stresses (i.e., glutathione and phytochelatins), and compounds involved in biological defenses, such as allylsulfur molecules and glucosinolates [5-10]. Additionally, sulfate can be incorporated into 3'-phosphoadenosine-5'-phosphate (PAPS) to provide a sulfate donor for the modification of brassinosteroid and jasmonate hormones, phytosulfokines, phospholipids, and other sulfonated molecules [11-13]. PAPS is also a precursor of 3'-phosphoadenosine-5'-phosphate, which is a retrograde signal that modulates stress gene expression and plant development [14-17].

Plant thiol metabolism begins with the reductive sulfur assimilatory pathway (**Fig. 1**). Three enzymes activate sulfate and reduce it to sulfide [18]. ATP sulfurylase (ATP:sulfate adenylyl transferase) catalyzes the committed step of the pathway to form the high-energy mixed anhydride molecule adenosine-5'-phosphosulfate (APS) and inorganic pyrophosphate (PP_i) from sulfate and ATP [19-21]. Reduction of APS to sulfite (SO_3^{2-}) by APS reductase (5'-adenylylsulfate reductase) requires glutathione as an electron donor [22-23]. In the final step, sulfite reductase converts sulfite to sulfide [24]. Sulfide is then used for formation of cysteine and other peptides in the next stage of plant sulfur metabolism (**Fig. 1**). Cysteine biosynthesis involves formation of O-acetylserine from acetyl-CoA and serine, which is catalyzed by serine acetyltransferase [25]. Next, the pyridoxal-5'-phosphate-dependent enzyme O-acetylserine sulfhydrylase (O-acetylserine(thiol)lyase) generates cysteine from O-acetylserine and sulfide [26]. In addition to its role as a proteogenic amino acid, cysteine is a precursor for methionine biosynthesis [27-28]. Plants also use cysteine for the production of thiol-containing peptides, such as glutathione, a major redox buffer, and phytochelatins, which are synthesized in response to heavy metal exposure [29-32]. In some plants, cysteine is also used to generate specialized glutathione homologs [33].

Although these pathways comprise primary sulfur metabolism in plants, formation of APS by ATP sulfurylase and its subsequent phosphorylation to PAPS by APS kinase constitutes a secondary sulfur metabolic pathway that is critical for the generation of sulfonated metabolites formed by a variety of sulfotransferases [11-13]. A similar diversion of sulfur into PAPS synthesis has been

observed in some pathogenic bacteria, such as *Mycobacterium tuberculosis* [34]. It should be noted that other bacteria, fungi, and humans sequentially use both APS kinase and APS reductase in the primary sulfur assimilation pathway to convert APS into PAPS, which is then further metabolized into sulfite by PAPS reductase [34-36].

Recent work demonstrates an essential role for APS kinase and PAPS in plant reproductive viability and development, as well as the partitioning of sulfur between the primary and specialized pathways [37-41]. Here we summarize recent structural biology studies on the ATP sulfurylase and APS kinase from plants that provide new insight on the biochemical regulation of PAPS formation and redox-control of this key branchpoint in plant sulfur metabolism.

Structure and mechanism of plant ATP sulfurylase

ATP sulfurylase is the metabolic entry point of the sulfur assimilation pathway and catalyzes the energetically unfavorable formation of APS (**Fig. 1**). In plants, multigene families encode multiple ATP sulfurylase isoforms [9-10]. These isoforms are localized to different organelles with the chloroplast providing the primary source of reductive sulfate assimilation [42-43]. Regulation of the plastidic ATP sulfurylases occurs by a sulfur deprivation-inducible microRNA (miR395), which leads to increased translocation of sulfate from the roots to the shoots [44-45]. Moreover, disruption of the ATP sulfurylase 1 in the model plant *Arabidopsis thaliana* (thale cress) results in increased accumulation of sulfate in the leaves, which suggests that this enzyme contributes to metabolic control of sulfur assimilation [45]. Biochemical and structural studies of soybean ATP sulfurylase provide the first molecular insights on this enzyme in plants [20-21].

The 2.48 Å resolution x-ray crystal structure of soybean ATP sulfurylase reveals a dimeric protein with each monomer consisting of two mixed α/β structural domains (**Fig. 2A**) [21]. Binding of APS in the C-terminal domain of each monomer defines the active site (**Fig. 2A-B**). APS binds in a cavity with a channel leading from the α -phosphate of APS to the protein surface (**Fig. 2B**). Multiple interactions anchor APS in the active site with positively-charged residues, including Arg248, His252, His255, and Arg349, providing a possible site for binding of the β - and γ -phosphates of ATP and PP_i during the APS synthesis reaction (**Fig. 2B**). Structural analysis of the soybean ATP sulfurylase also provided a template for interpreting naturally occurring *Arabidopsis* haplotype mutations that affect sulfate assimilation [21].

Steady-state kinetic studies of soybean ATP sulfurylase are consistent with a mechanism in which ATP and APS are the first substrates bound in the forward and reverse reactions, respectively

[20]. Initial velocity experiments indicate a single-displacement mechanism in the APS synthesis reaction with inhibition by chlorate showing competitive inhibition versus sulfate and non-competitive inhibition versus APS. For the reverse reaction (i.e., ATP synthesis), initial velocity analysis also suggests a sequential mechanism with ordered binding of substrates. Ligand binding analysis using isothermal titration calorimetry (ITC) was also consistent with the steady-state kinetic studies [20]. APS bound to free enzyme, but PP_i binding was not detected. Only after formation of the E•APS complex was PP_i binding detected.

Extensive site-directed mutagenesis and steady-state kinetic analysis of 20 point mutants targeting active site residues suggests a chemical reaction mechanism in which nucleophilic attack by the sulfate on the α -phosphate of ATP involves transition state stabilization by Arg248, Asn249, His255, and Arg349 (**Fig. 2C**) [21]. The three-dimensional structure and biochemical studies suggest that ATP sulfurylase overcomes the energetic barrier to APS synthesis by distorting nucleotide structure. Computer-aided docking of ATP and sulfate in the active site (**Fig. 2C**), along with kinetic analysis of site-directed mutants, suggests that Asn249 and Arg349 have potential roles in catalysis. The distorted U-shape of the ATP positions the α -phosphate group for in-line nucleophilic attack by the sulfate group and subsequent formation of APS and release of PP_i. For the energetically favorable reverse reaction, binding of PP_i via interactions with Asn249, His255, and Arg349 allows for facile nucleophilic attack on the α -phosphate of APS to form ATP [21]. Both the forward and reverse substitution reactions employ reactive groups that are in close contact with both substrates to provide stabilizing interactions for a pentavalent transition state.

Structural evolution of ATP sulfurylases: different organization in different organisms

Structural studies of ATP sulfurylases from bacteria, fungi, plants, and humans reveal the conservation of key active site features and the overall monomeric structure; however, these x-ray crystal structures also highlight how nature uses ATP sulfurylase as a 'module' in diverse protein architectures [21, 46-53]. For reductive sulfate assimilation, ATP sulfurylase provides the activated metabolite for this process. In some microbes, sulfate reduction and sulfide oxidation can function in a dissimilatory process to support consumption of sulfate and sulfur compounds for electron transfer processes. Given the retention of the three-dimensional structure of ATP sulfurylase, this activity likely evolved before divergence of the different branches on the tree-of-life.

ATP sulfurylases vary widely in both domain structure and oligomerization (**Fig. 3A-B**), but retain key catalytic and substrate binding residues (**Fig. 3C**). In *Escherichia coli* and *Pseudomonas*

syringae, ATP sulfurylase functions as a heterodimeric protein comprised of a GTPase subunit and a catalytic ATP sulfurylase subunit [46]. In other bacteria, including the thermophiles *Aquifex aeolicus* and *Thermus thermophilus*, the purple sulfur bacterium *Allochromatium vinosum*, and a bacterial symbiont of the vent tubeworm *Riftia pachyptila*, ATP sulfurylase operates either as a monofunctional homodimer or a bifunctional homodimeric PAPS synthetase with ATP sulfurylase and APS kinase domains [47-50]. *Saccharomyces cerevisiae* and *Penicillium chrysogenum* use a homohexameric ATP sulfurylase with each monomer containing an N-terminal ATP sulfurylase domain and a C-terminal APS kinase-like domain, which functions as an allosteric regulation site [51-52]. Although human PAPS synthetase is a homodimer with ATP sulfurylase and APS kinase domains [53], the two isoforms of this enzyme, which are 79% identical in sequence, in humans radically differ in their stability and contributions to PAPS availability in vivo [54]. Sequence comparisons show that the catalytic and substrate binding sites of the ATP sulfurylases from various organisms are highly conserved (**Fig. 3C**). Regions of sequence that correspond to the dimer interface (**Fig. 3C**) tend to display greater sequence variation, which likely leads to structural variations in both oligomerization and domain organization (**Fig. 3B-C**).

Even with this variety of protein structure, genome analysis of sulfate utilization genes suggest complex origins of the enzymes in these pathways, as bioinformatic studies identify both isolated and fused forms of ATP sulfurylase, APS kinase, sulfotransferase, pyrophosphatase, APS reductase, and related regulatory domains in various organisms [55-56].

Structure and mechanism of plant APS kinase

Only recently has the key role of APS kinase and the formation of PAPS (**Fig. 1**) for plant reproduction and viability been established [37-41]. Analysis of T-DNA knockout lines targeting each of the four APS kinase isoforms in *A. thaliana* demonstrated the connection between this branch of sulfur metabolism and glucosinolate biosynthesis [39, 41]. Later analysis of triple knockout lines demonstrated that only isoform 1 maintained wild-type levels of growth and development in Arabidopsis [40]. In addition, measurements of flux through the sulfur assimilation pathway increased when APS kinase activity was reduced, which indicates that altered enzymatic activity partitions sulfur between the primary and secondary pathways [38]. Although APS kinase is critical for reproductive viability in Arabidopsis and competes with APS reductase to partition sulfate between the primary and secondary branches of the sulfur assimilatory pathway, the biochemical function and regulation of the plant APS kinase was poorly understood.

The 1.8-Å resolution x-ray crystal structure of APS kinase from *A. thaliana* in complex with the non-hydrolyzable ATP analog β,γ -imidoadenosine-5'-triphosphate (AMP-PNP), Mg^{2+} , and APS provides a view of the dimeric enzyme (**Fig. 4A**) [57]. The overall fold of each monomer consists of a variable N-terminal region that entwines the adjacent monomer, a α/β -purine nucleotide binding domain, and a small domain that caps both the ATP and APS binding sites. Crystallographic studies of the unliganded and nucleotide bound forms of the *P. chrysogenum* APS kinase show that the capping domain contacts a canonical nucleotide binding P-loop (residues 110-116) and locks substrates into the active site [58-59]. APS binding is mediated through multiple binding contacts, including aromatic residues stacking with the adenine ring and charge-charge interactions to its sulfate and phosphate groups (**Fig. 4B**). Binding of the ATP analog AMP-PNP is mediated by contacts between the phosphates and residues in the P-loop and by a water network centered on the bound Mg^{2+} ion (**Fig. 4B**). The interactions centered on the Mg^{2+} connect the ATP binding site, an active site water network, and Asp138 to organize the site for catalysis. Asp138, the key catalytic residue, contacts the ribose hydroxyl groups of APS to position the 3'-OH proximal to the γ -phosphate group for phosphoryl transfer. In this reaction, Asp138 serves as a general base to abstract a proton from the 3'-OH of APS for nucleophilic attack on the γ -phosphate group of ATP. Although not in direct contact with ligands in the active site, the N-terminal loop (residues 80-84) of the adjacent monomer contacts another loop containing the catalytic aspartate and the side-chain of His84 hydrogen bonds to a water molecule in the network that coordinates the Mg^{2+} ion. Studies on the structurally similar PAPS synthetase from human suggest that the N-terminal loop helps to stabilize active site interactions during the catalytic cycle [60].

Steady-state kinetic analysis of multiple APS kinases from a variety of organisms suggest an ordered kinetic mechanism in which $ATP \cdot Mg^{2+}$ binds first, followed by APS, catalysis, release of PAPS, and release of $ADP \cdot Mg^{2+}$ [61]. ITC experiments examining the energetics of nucleotide binding suggest that structural changes are coordinated for efficient catalysis [62]. ITC analysis of ligand binding to Arabidopsis APS kinase shows that initial binding can occur at either nucleotide site, but that initial interaction at the ATP site is favored and enhances affinity for APS in the second site by 50-fold [62]. The thermodynamics of ATP first binding versus APS first binding imply that active site structural changes may guide the order of nucleotide addition. It appears that binding of $ATP \cdot Mg^{2+}$ organizes the active site for APS binding [62]. In contrast, initial APS binding is possible, but energetically disfavored. Binding of APS may alter movement of the capping domain [62]. Thus, APS binding first has an overall inhibitory effect on the reaction sequence and changes

the energetics that allow for optimal active site conformation. Overall, these studies of APS kinase's kinetic mechanism reveal an important role for the dynamic features of the active site in response to ligand binding [57, 62].

Redox-regulation of plant APS kinase and sulfur metabolism

The x-ray crystal structure of the Arabidopsis APS kinase also revealed a mechanism for redox-regulation that relies on altering the conformational flexibility of the N-terminal domain [57]. The N-terminal domain of each monomer in the dimer lies across the surface of the other monomer, which allows for formation of a disulfide between Cys86 of one monomer and Cys119 of the adjacent monomer (**Fig. 5A**). The location of the Cys86-Cys119 disulfide suggested a possible functional role in controlling enzymatic activity via changes in redox environment [57].

Biochemical analysis of wild-type and mutants lacking the cysteine residues showed that disulfide bond formation was sensitive to redox changes (**Fig. 5B**). Comparison of steady-state kinetic properties under reducing and oxidizing conditions demonstrated that disulfide bond formation reduced catalytic efficiency by 17-fold and increased the effect of APS inhibition by 15-fold [57]. Mutation of either one or both cysteines mimicked the effects of reduction. In addition, redox titrations of the disulfide bond using gel-based and activity-based assays showed a physiologically relevant mid-point potential [57]. Subsequent ITC studies of nucleotide binding demonstrated that the disulfide bond affects the nucleotide binding equilibria compared to the reduced enzyme (**Fig. 5C**) [63]. Formation of the Cys86-Cys119 disulfide increases the K_d for ATP by 45-fold compared to the reduced enzyme and concomitantly enhances the affinity for APS by 18-fold. This is also consistent with the increased sensitivity to substrate inhibition by APS under oxidizing conditions. Moreover, the redox-dependent changes can be triggered via multiple oxidative signals, including photoactivatable atomic oxygen precursors [64].

Based on structural and biochemical studies of ligand binding to the Arabidopsis APS kinase a mechanism for redox-linked gating of ligand binding was proposed [63]. Under reducing conditions, ligand binding favors the catalytically productive addition of ATP followed by APS binding (**Fig. 5C; left**). Under oxidizing conditions, formation of the Cys86-Cys119 linkage connects the N-terminal domain of one monomer onto the surface of the second monomer and alters the binding preference in favor of APS with corresponding decreases in the affinity for ATP binding to either free enzyme or the E•APS complex (**Fig. 5C; right**). This change likely results from altered conformational flexibility.

The redox-control of APS kinase appears to be a specialized feature in plants and mosses. Amino acid sequence comparisons of the APS kinases from plants, mosses, fungi, human, green algae, and cyanobacteria show that the residues corresponding to Cys86 and Cys119 of the *Arabidopsis* enzyme are nearly invariant across homologs from plants and mosses with some algae and yeast retaining Cys119; however, neither cysteine is found in the APS kinase from organisms lacking chloroplasts [57]. In the APS kinase isoforms of plants, the disulfide residues are invariant in plastid-localized isoforms, but can vary in the cytosolic forms [53]. The evolution of redox-control in APS kinases in the green lineage from cyanobacteria (redox-insensitive) to plants (redox-sensitive) involved a series of steps [65]. As cyanobacteria represent the evolutionary origins of the chloroplast in plants, structural and biochemical analysis of the redox-insensitive APS kinase from *Synechocystis* sp. PCC 6803 was used as a starting point to recapitulate the development of redox regulation [65]. Addition of the N-terminal domain feature was the first step around the transition from cyanobacteria to algae with mutations leading to both cysteines in the disulfide occurring later in mosses [65].

The evolution of a redox-control element in APS kinase may be an adaptation to the development of highly redox-active oxygen-generating chloroplasts in the lineage from cyanobacteria to green algae to mosses and 'higher' plants [57]. The evolution of the chloroplast also corresponds to a shift in the organization of the sulfur assimilation pathway from a linear one in cyanobacteria, which uses PAPS as a substrate to produce sulfite, to a branched pathway with PAPS as a secondary branch in the later evolving mosses and plants. Redox-regulation of APS kinase may provide reciprocal control at a key branchpoint in plant sulfur metabolism.

Oxidizing conditions attenuate the activity of APS kinase, but activate APS reductase [66] and the glutathione biosynthesis enzyme glutamate-cysteine ligase (GCL) [29; 67-69]. APS reductase and GCL are both regulated by disulfide bond formation in response to changes in redox environment. Thus, thiol-based control switches may help to coordinate sulfur flow between the primary and secondary metabolic pathways. Under oxidative stress conditions that increase demand for sulfur to support glutathione synthesis as a redox buffer, disulfide bond formation in APS reductase and APS kinase leads to increased and decreased activities, respectively. This would allocate more APS toward the production of sulfide to support cysteine and glutathione synthesis. Moreover, elevated sulfide levels promote formation of the cysteine regulatory complex by serine acetyltransferase and O-acetylserine sulfhydrylase to increase cysteine synthesis rates [6; 70-72]. Thus, both APS reductase and GCL in the primary thiol metabolic pathway would be more active in response to

oxidative stresses and APS kinase activity lowered in the secondary pathway to divert sulfur flow. Additional in planta studies are required to test this hypothesis and the contribution of redox regulation to sulfur partitioning in plants.

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Figure Legends

Figure 1: Overview of the plant sulfur assimilation pathway.

Figure 2: Structure ATP sulfurylase from soybean. (A) Overall structure of the soybean ATP sulfurylase dimer [21]. The N- and C-termini residues of each monomer are indicated. The α -helices and β -strands of one monomer are colored blue and gold, respectively, with α -helices and β -strands of the second monomer colored green and rose, respectively. Secondary structure features along the dimer interface are indicated. The position of APS in each monomer is shown by the space-filling model. (B) Active site of soybean ATP sulfurylase. Dotted lines represent hydrogen bonds between active site residues and bound APS. (C) Proposed reaction mechanism. Modeling of the sulfate ion and the adenine and ribose groups of ATP were initially placed based on the structure of soybean ATP sulfurylase in complex with APS. ATP adopts a distorted conformation that allows for nucleophilic attack by the sulfate ion.

Figure 3: Domain organization and structure of ATP sulfurylases. (A) Dendrogram and domain structure of ATP sulfurylases from a bacterial symbiont of hydrothermal vent tubeworm *Riftia pachyptila* (RpsATPS), soybean (*Glycine max*; GmATPS), human (HsAPSK/ATPS or PAPS synthetase), *Thermus thermophilus* (TtATPS), *Aquifex aeolicus* (AaATPS/APSK), *Penicillium chrysogenum* (PcATPS), *Saccharomyces cerevisiae* (ScATPS), and *Pseudomonas syringae* (PsATPS). For the soybean and human enzymes, representative isoforms are used. The soybean genome encodes four ATP sulfurylase isoforms related by >70% amino acid identity [10]. Likewise, the two human PAPS synthetase isoforms share 79% sequence identity [54]. (B) The corresponding domain structure of each protein is shown with regions corresponding to ATP sulfurylase (purple), APS kinase (yellow), APS kinase-like (green), and GTPase (light blue). (C) Multiple sequence alignment of the ATP sulfurylases from soybean (GmATPS; AF452454) human (HsPAPPS; O43252), *Riftia* symbiont (RpsATPS; Q54506), *S. cerevisiae* (ScATPS, P08563), *P. chrysogenum* (PcATPS, Q12650), *A. aeolicus* (AaATPS, O67174), and *T. thermophilus* (TtATPS, Q5SKH7) is shown with amino acid numbering indicated in parenthesis. Conserved residues are highlighted in orange with residues in white indicating a variation from the conserved sequence. Red highlight denotes active site residues in GmATPS. Light grey boxes indicate regions at the dimer interface of GmATPS; these regions also display large sequence variation across the ATP sulfurylases. Multiple sequence alignment used the MultAlin webpage (<http://multalin.toulouse.inra.fr/multalin/>).

Figure 4: Structure of APS kinase from Arabidopsis. (A) Overall structure of the *A. thaliana* APS kinase dimer. Each monomer is shown as a ribbon diagram and colored in rose and blue, respectively. The positions of AMP-PNP and APS are shown as stick molecules. The N-terminal loop in the rose-colored monomer and the cap domain of the blue-colored monomer are labeled. (B) The active site of Arabidopsis APS kinase. Residues forming the active site and positions of AMP-PNP and APS are shown. The N-terminal loop of the adjacent monomer is in dark gray.

Figure 5: Redox regulation of the APS kinase from Arabidopsis. (A) Structural identification of the thiol-switch in *A. thaliana* APS kinase. One monomer is shown as a surface rendering (red) and the other as a ribbon diagram with α -helices (blue) and β -sheets (yellow) indicated. Bound AMP-PNP (yellow) and APS (green) in the red monomer are shown as space-filling models. The position of the Cys86-Cys119 disulfide bond is also shown as a space-filling model. (B) Redox effects on Arabidopsis APS kinase. SDS-PAGE of wild-type and C86A/C119A enzyme incubated in the absence or presence of either 5 mM reduced DTT (DTT_{red}) or trans-4,5-dihydroxy-1,2-dithiane (DTT_{ox}). (C) Comparison of binding equilibria in reduced ($\text{AtAPSK}_{\text{RED}}$) and oxidized ($\text{AtAPSK}_{\text{OX}}$) forms of Arabidopsis APS kinase. Equilibrium constants (μM) determined by ITC are shown [62-63].

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5