Redox-Linked Gating of Nucleotide Binding by the N-Terminal Domain of Adenosine 5'-Phosphosulfate Kinase*

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Running title: Role of the N-terminal domain in APS kinase

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Background: Adenosine 5’-phosphosulfate kinase (APSK) in plants contains a regulatory disulfide bond.

Results: Analysis of oxidized APSK reveals altered nucleotide binding compared to the reduced enzyme.

Conclusion: The N-terminal domain is responsible for redox-linked structural changes that regulate APSK activity.

Significance: This work provides a molecular basis for understanding reciprocal regulation at a branchpoint in plant sulfur metabolism.

Adenosine 5’-phosphosulfate kinase (APSK) catalyzes the phosphorylation of adenosine 5’-phosphosulfate (APS) to 3’-phosphoadenosine-5’-phosphosulfate (PAPS). Crystallographic studies of APSK from Arabidopsis thaliana revealed the presence of a regulatory intersubunit disulfide bond (Cys86-Cys119). The reduced enzyme displayed improved catalytic efficiency and decreased effectiveness of substrate inhibition by APS compared to the oxidized form. Here we examine the effect of disulfide formation and the role of the N-terminal domain on nucleotide binding using isothermal titration calorimetry (ITC) and steady-state kinetics. Formation of the disulfide bond in AtAPSK inverts the binding affinities at the ATP/ADP and APS/PAPS sites from those observed in the reduced enzyme, consistent with initial binding of APS as inhibitory, and suggests a role for the N-terminal domain in guiding nucleotide binding order. To test this, an N-terminal truncation variant (AtAPSKΔ96) was generated. The resulting protein was completely insensitive to substrate inhibition by APS. ITC analysis of AtAPSKΔ96 showed decreased affinity for APS binding, even though the N-terminal domain does not directly interact with this ligand. Moreover, AtAPSKΔ96 displayed reduced affinity for ADP, which corresponds to a loss of substrate inhibition by formation of an E•ADP•APS dead-end complex. Examination of the AtAPSK crystal structure suggested Arg93 as important for positioning of the N-terminal domain. ITC and kinetic analysis of the R93A mutant also showed a complete loss of substrate inhibition and altered nucleotide binding affinities, which mimics the effect of the N-terminal deletion. These results show how thiol-linked changes in AtAPSK alter the energetics of binding equilibria to control its activity.

Adenosine 5’-phosphosulfate kinase (APSK+) catalyzes the ATP-dependent phosphorylation of APS into 3’-phosphoadenosine-5’-phosphosulfate (PAPS) (1). In prokaryotes and eukaryotes, APSK provides a critical sulfur-containing metabolite for sulfur assimilation and/or the synthesis of a variety of sulfonated compounds (2-8). In plants, APSK provides PAPS for incorporation of sulfate into brassinosteroids, peptide hormones, glucosinolates, and other metabolites (9-11). In Arabidopsis thaliana (thale cress), APSK is necessary for plant reproduction and viability and in vivo studies using T-DNA knockout lines for each of the four A. thaliana APSK (AtAPSK) isoforms demonstrate the metabolic connection between sulfur...
assimilation and glucosinolate biosynthesis (12-14). These studies suggest an important role for APSK in thiol metabolism, but the current understanding of how this protein is biochemically regulated is limited in comparison to the better studied bacterial, fungal, and mammalian homologs (15-20).

Mechanistic studies of the APSK from *Penicillium chrysogenum*, *Escherichia coli*, and *A. thaliana* indicates that the enzyme uses a preferred ordered mechanism of nucleotide binding, in which ATP binds first followed by APS (15-16; 21-22). The APSK from bacteria, fungi, plants, and mammals all display significant substrate inhibition by APS (1, 15-16, 20-23). Isothermal titration calorimetry (ITC) analysis of AtAPSK showed that the affinity for ATP, ATP analogues, and ADP was greater than the affinity for APS binding and that nucleotide binding at the ATP site enhances the $K_d$ for APS by 50-fold (23). Calorimetric analysis of formation of the AtAPSK•ADP•APS dead-end complex implicated in substrate inhibition also revealed a synergistic effect of ADP binding on the affinity for APS. Similar results were reported for *P. chrysogenum* APSK using steady-state kinetics approaches (15).

X-ray crystal structures of AtAPSK, *P. chrysogenum* APSK, and bifunctional ATP sulfurylase-APSK from *Aquilax aeolicus* and *Thiobacillus denitiricans*, and the APSK domain of human PAPS synthetase reveal a canonical $\alpha/\beta$ purine nucleotide binding fold (17-20, 23-26). All of the APSK structures contain a flexible N-terminal domain composed of an unstructured strand (residues 78-89 in AtAPSK) and $\alpha_1$ (residues 90-97 in AtAPSK) ($\textbf{Fig. 1A}$), which is implicated in affecting substrate inhibition by APS in the Arabidopsis enzyme (24) and in the APSK domain of human PAPS synthetase (20). Interestingly, the APSK structure is homologous to other nucleotide/small molecule kinases, such as adenylyl kinase, that are subject to substrate inhibition, but lack an analogous N-terminal domain (27-28). However, the mechanism of substrate inhibition in nucleotide/small molecular kinases has not been thoroughly investigated.

In plants, the N-terminal domain is also implicated in redox control of APSK to coordinate flux between the primary and secondary branches of the sulfur assimilation pathway (24). The primary route provides sulfur for the synthesis of cysteine and glutathione (4-8), whereas PAPS generated in the secondary route is used for the synthesis of other metabolites (9-11). The crystal structure of AtAPSK revealed a disulfide bond formed between Cys86 in the N-terminal domain of one monomer and Cys119 in $\alpha_2$ of the adjoining monomer ($\textbf{Fig. 1A}$) (24). Kinetic analysis of the reduced (AtAPSK$_{\text{RED}}$) and oxidized (AtAPSK$_{\text{OX}}$) forms of the enzyme showed that formation of the disulfide reduced catalytic efficiency and increased effectiveness of substrate inhibition by APS. The functional differences suggest that cellular redox state may act as a novel regulatory feature in the plant APSK (23-24, 29). For example, under oxidative stress conditions, decreased AtAPSK activity may enhance the flow of APS into primary sulfur metabolism to support glutathione synthesis for maintaining redox balance (24).

To elucidate the role of the N-terminal domain in AtAPSK and its contribution to substrate inhibition and redox regulation of activity, ITC was used to examine nucleotide binding to AtAPSK$_{\text{OX}}$, which displayed differential sensitivity to substrate inhibition by APS (24). Extensive analysis of nucleotide binding to the reduced form of AtAPSK was recently described (23) and provides a basis of comparison with the oxidized disulfide-linked form. Here we show that disulfide bond formation in AtAPSK$_{\text{OX}}$ leads to enhanced APS binding in both the presence and absence of either the non-hydrolyzable ATP-analog $\beta,\gamma$-imidoadenosine-5-triphosphate (AMP-PNP) or ADP. Thus, disulfide bond formation alters binding equilibria to disfavor formation of a catalytically productive ternary complex and suggests a role for the N-terminal domain in guiding the ligand binding sequence of AtAPSK. In contrast, truncation of the N-terminal domain (AtAPSKA96) decreased APS binding affinity without altering AMP-PNP binding. Examination of the three-dimensional
structure of AtAPSK suggested that Arg93, which is conserved across the APSK from bacteria, fungi, plants, and animals, is critical for positioning of the N-terminal domain. Kinetic and calorimetric analysis of the R93A mutant is consistent with the proposed role of this residue as a critical anchor for positioning of the N-terminal domain. Based on the results of calorimetric and kinetic experiments, we suggest a model for how the N-terminal domain of AtAPSK modulates APS binding and how redox-linked gating helps determine the order of nucleotide addition.

EXPERIMENTAL PROCEDURES

Reagents. All chemicals and reagents were purchased from Sigma-Aldrich as analytical grade or better. The standard buffer condition for all experiments was 25 mM HEPES, pH 7.5, 200 mM KCl, 5% (v/v) glycerol, and either 1 mM Tris(2-carboxyethyl)phosphine (TCEP) or 5 mM β-mercaptoethanol (BME), unless stated otherwise. Reducing agents prevent disulfide bond formation in AtAPSK, as judged by non-reducing SDS-PAGE (24) of samples taken before and after ITC titrations.

Generation of mutant constructs, protein expression, and purification. For bacterial expression of AtAPSK, the pET-28a-AtAPSKΔ77 bacterial expression construct, which encodes A. thaliana APSK isoform 1 without the plastid localization sequence (residues 1-77) and with an N-terminal hexahistidine tag, was used (30). Generation of the N-terminal truncation mutant AtAPSKΔ96 used pET-28a-AtAPSKΔ77 as template. Forward and reverse primers harboring NheI and EcoRI restriction sites, respectively, were used to PCR-amplify the new versions of the coding region, which were treated with restriction enzymes and sub-cloned into pET-28a. The R93A point mutant used introduced into pET-28a-AtAPSKΔ77 by QuikChange mutagenesis (Agilent). All proteins were overexpressed in E. coli BL21(DE3) and purification by nickel-affinity and gel-filtration chromatographies were done as described previously (23-24). For purification of protein for ITC analysis, all buffers were supplemented with 5 mM BME. After nickel-affinity purification, protein was dialyzed into 25 mM HEPES, pH 7.5, 200 mM KCl, 5% glycerol, and 5 mM dithiothreitol (DTT), then loaded onto a Superdex-200 26/60 HiLoad FPLC size-exclusion column equilibrated in the same buffer. Purified proteins were dialyzed against 25 mM HEPES, pH 7.5, 200 mM KCl, 5% glycerol, and either 1 mM TCEP or 5 mM BME and flash frozen in liquid nitrogen and stored at -80 °C. Protein concentrations of AtAPSKOX and AtAPSKΔ96 were determined spectrophotometrically (31) using calculated extinction coefficients of 15,930 and 21,540 M⁻¹ cm⁻¹, respectively.

Enzyme assays. Determination of steady-state kinetic parameters was performed as previously described using an enzyme-coupled spectrophotometric assay (23-24).

Calorimetric measurements. Isothermal titration calorimetry (ITC) experiments were performed with a VP-ITC calorimeter (Microcal, Inc.). As described above, AtAPSK was isolated and maintained in the reduced form. The oxidized form (i.e., AtAPSKOX) was generated by removal of reducing agent using buffer-exchange in centrifugal spin-filter units (10,000 MW; Amicon) into the standard reaction buffer containing 5 mM trans-4,5-dihydroxy-1,2-dithiane (i.e., oxidized DTT; Sigma). Samples were dialyzed overnight (4 °C) against the same buffer. Stock solutions (100 mM) of ATP, AMP-PNP, ADP, and APS were dissolved in NaOH to attain a pH of 7.5 and stored at -20 °C. AtAPSK complexed with various ligands were generated by incubating protein and 2 mM AMP-PNP, 500 µM ADP, or 2 mM APS overnight (4 °C) followed by a 4 hr equilibration at 17 °C before titrations. Prior to ITC experiments, appropriate dilutions were made with dialysis buffer. Protein and nucleotide solutions were degassed at room temperature before use. Twenty to thirty injections (10 µL) of nucleotide were added into sample solutions containing either AtAPSKOX (15-30 µM) or AtAPSKΔ96 (30-50 µM). Data were analyzed using either an identical binding sites model (eqn 1) or two-site binding model (eqn 2), as follows:
RESULTS

Calorimetric analysis of nucleotide binding to oxidized AtAPSK. Crystallographic and functional analysis of AtAPSK identified an intersubunit disulfide bond between Cys86 and Cys119 that may play a role in regulating enzymatic activity (Fig. 1A; 24). Here we use ITC to examine nucleotide binding to AtAPSKox for comparison with the reduced form of the enzyme (23).

ITC analysis of ATP and ADP binding to AtAPSKox showed exothermic binding with a two-site model best fitting the data (Fig. 2A; Table 1). Comparison of this ITC data with that obtained using AtAPSKred (23) showed that the presence of the Cys86-Cys119 linkage in AtAPSK decreased first site binding affinity for ATP and ADP by 45- and 217-fold, respectively. For the second site, a 9-fold decrease in the affinity for ATP was observed but the affinity for ADP was comparable to the reduced enzyme. In contrast, AtAPSKox displayed an 18-fold tighter binding affinity for APS with a binding isotherm that could be adequately fit to a single-site model (n = 1.95). Previous ITC experiments indicated weaker asymmetric two-site binding to the reduced apoenzyme (23). Overall, formation of the disulfide bond in AtAPSK inverts the binding affinities at the ATP/ADP and APS/PAPS sites from those observed in the reduced enzyme.

Next, ITC experiments were used to determine if pre-formed complexes of AtAPSKox and either phosphonucleotide (AMP-PNP and ADP) or APS altered the binding affinity for APS or phosphonucleotides, respectively (Fig. 2B; Table 2). APS titration of the AtAPSKox•AMP-PNP and AtAPSKox•ADP complexes yielded binding affinities (K1 = 0.64 and 3.70 µM, respectively) that were comparable to those determined for the reduced protein (K1 = 1.50 and 3.30 µM, respectively; 23). Titrations of the AtAPSKox•APS complex with either AMP-PNP (K1 = 37.2 µM) or ADP (K1 = 26.1 µM) showed 12- and 40-fold higher Kd values compared to those of the reduced protein (K1 = 3.10 and 0.65 µM, respectively; 23). These results indicate that disulfide bond formation alters the nucleotide binding equilibria leading to formation of a catalytically productive E•ATP•APS ternary complex and suggest a role for the N-terminal domain in determining the order of ligand addition.

Effect of N-terminal domain deletion (AtAPSKΔ96) on steady-state kinetics. The location of the Cys86-Cys119 disulfide bond in the N-terminal domain of AtAPSK (Fig. 1A-B) and the effects of its formation on nucleotide binding (Fig. 2; Tables 1 & 2) and catalytic activity (23) suggest an important functional role for the N-terminal domain. For example, reduction of the disulfide decreases the Ki of APS substrate inhibition by 15-fold compared to AtAPSKox. To test if removal of the N-terminal domain affects AtAPSK activity, a truncated version of the enzyme lacking residues 77-96 was generated. This construct removes the N-terminal domain through the end of the a1-helix (Fig. 1A). The resulting AtAPSKΔ96 protein displayed only modest changes in kinetic parameters compared to AtAPSKred (i.e., a 3-fold slower turnover rate and a 5-fold higher Km for APS), but completely eliminated the effect of APS substrate inhibition (Table 3). The protein also showed no sensitivity to reducing agents, consistent with the loss of Cys86.

Calorimetric analysis of the effect of N-terminal deletion (AtAPSKΔ96) on nucleotide binding

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Q_i^{\text{tot}} = V_0 \cdot M_i^{\text{tot}} \cdot \frac{((nK_1x)x_1 + (nK_2x_2)){x_2}}{1 + K_1x_1 + K_2x_2(x_2^2)}
\]

where \(Q_i^{\text{tot}}\) is the total heat after the ith injection, \(V_0\) is the calorimetric cell volume, \(M_i^{\text{tot}}\) is the concentration of protein in the cell after the ith injection, \(\Delta H\) is the corresponding enthalpy change to APSK-nucleotide binding, \(n\) is the number of nucleotide binding sites on the APSK dimer, and \(K_1\) is the equilibrium binding constant. In the latter, \(K_1\) and \(K_2\) are the observed binding constants for the first and second sites, \(\Delta H_1\) and \(\Delta H_2\) are the corresponding enthalpy changes upon nucleotide binding to each site. Fitting of data was performed using Origin software.
binding. The effect of the N-terminal truncation on the steady-state kinetics led us to analyze nucleotide binding to the AtAPSKΔ96 apoenzyme and binary nucleotide complexes. ITC experiments showed that binding of ATP and ADP to AtAPSKΔ96 was exothermic and that binding to the first site influenced the second binding event, as a two-site model best fit the observed data (Fig. 3A; Table 4). The $K_d$ values for ATP binding to AtAPSKΔ96 were similar to those determined previously for AtAPSKRED (Table 1; 23). Interestingly, the affinity of AtAPSKΔ96 for ADP (Table 4; $K_1 = 9.4$ μM, $K_2 = 64.3$ μM) was lower in comparison to the full-length reduced enzyme ($K_1 = 0.18$ μM, $K_2 = 4.8$ μM; ref. 23). Titration of AtAPSKΔ96 with APS (Fig. 3A) did not yield sufficient heat signal for analysis and indicates that the truncation reduces the affinity for APS, even though the N-terminal domain does not directly interact with the ligand.

Given the results of the ATP, ADP, and APS titrations with the apoenzyme form of AtAPSKΔ96, ITC analysis ligand binding to nucleotide binary complexes were performed (Fig. 3B-C). ITC experiments demonstrate that formation of the AtAPSKΔ96•AMP-PNP and AtAPSKΔ96•ADP complexes increases affinity for APS (Fig. 3B; Table 4), as binding can now be detected in contrast to the lack of signal for APS titration of the AtAPSKΔ96 apoenzyme (Fig. 3A). For each AtAPSKΔ96 binary complex, the affinity for APS was comparable to the previously reported values for the full-length reduced protein (24). These results suggest the N-terminal region is not required for communication between the ATP/ADP and APS/PAPS binding sites of AtAPSK, as binding of either AMP-PNP or ADP still enhances APS binding as observed in the full-length protein (23).

Although ITC titrations of AtAPSKΔ96 apoenzyme with APS lack significant heat signature (Fig. 3A), the catalytic activity of the enzyme (Table 3) indicates that APS still binds to the enzyme. Pre-incubation of AtAPSKΔ96 with 2 mM APS followed by titration with either AMP-PNP or ADP showed ternary complex formation (Fig. 3C). As observed with the reduced protein (23), AMP-PNP titration of the APS complex showed an endothermic interaction. This variation in binding energetics indicates that structural and/or dynamic changes occur in the active sites of the reduced and oxidized forms of AtAPSK. Formation of an AtAPSKΔ96•APS•nucleotide complexes yielded $K_d$ values for both AMP-PNP and ADP (Table 4) that were comparable to the full-length reduced protein (23); however, the concentrations of APS needed to form the binary complex with AtAPSKΔ96 are likely beyond the physiological range. Overall, ITC analysis of ligand interactions in AtAPSKΔ96 supports an important role for the N-terminal region in nucleotide binding.

**Effect of Arg93 mutation in the N-terminal domain of AtAPSK on activity and nucleotide binding.** The role of the N-terminal domain as an important feature for redox regulation, nucleotide binding, and substrate inhibition by APS led us to re-examine the crystal structure of AtAPSK (24) for other residues involved in positioning of the N-terminal domain. In general, the N-terminal domain of one monomer binds along the surface of the adjacent monomer with interactions mediated primarily through van der Waals contacts (Fig. 1A); however, the region near the disulfide and α1 includes residues that make additional inter-subunit (Tyr126-Asp92) and intra-subunit (Arg93-Asp171) interactions (Fig. 1B). Sequence alignment of the N-terminal region of APSK from a variety of plants, bacteria, fungi, and animals (Fig. 1C) indicate that the arginine corresponding to Arg93 of AtAPSK is highly conserved among these enzymes. In general, either an arginine or a lysine is found at this position in APSK from other enzymes. Based on the crystal structure of AtAPSK (24), Arg93 may play a role in positioning the flexible N-terminal domain along the adjacent monomer surface.

To test the functional role of Arg93, the R93A mutant was generated, expressed, and purified. Substitution of Arg93 with an alanine modestly reduces $k_{cat}$ by 2-fold and increases the $K_m$ for APS by 3-fold (Table 3). Importantly, the R93A mutation eliminates the effect of
substrate inhibition by APS, as observed with AtAPSKΔ96.

ITC experiments with ATP, ADP, and APS as titrants for the AtAPSK R93A mutant behaved similarly to AtAPSKΔ96 (Fig. 4A). Binding of ATP and ADP yielded $K_d$ values similar to those of the N-terminal deletion mutant and displayed differential first and second site binding (Table 5). Negligible heat signal was detected for APS binding to the free enzyme (Fig. 4A). This binding analysis suggests that the effect of the N-terminal domain on nucleotide binding in the reduced form of the protein is largely mediated by Arg93. Titrations of the AtAPSK R93A•AMP-PNP and AtAPSK R93A•ADP complexes with APS showed that formation of complex causes a significant increase in affinity for APS. Thus, the R93A mutant displays a similar ligand binding profile as AtAPSKΔ96 (Fig. 4B; Table 5).

DISCUSSION

APSK controls the flux of sulfate through the secondary sulfur assimilation pathway, which produces a diverse array of metabolites required for normal growth and defense against environmental stress (2-11). All forms of APSK from a variety of organisms studied to date exhibit substrate inhibition by APS (1, 15-16, 20-23), which may provide a mechanism for controlling this enzyme in vivo. Recent identification of a regulatory disulfide bond in AtAPSK links cellular redox state to the degree of substrate inhibition by APS, as well as the rate of catalysis, in plants (23-24, 27). Previous analysis of nucleotide binding to the reduced form of AtAPSK (23) provides a basis of comparison to better understand the molecular underpinnings of redox-regulation and the role of the N-terminal domain in APSK.

In AtAPSK, disulfide bond formation between Cys86 and Cys119 alters the steady-state kinetic behavior of the enzyme from a more active reduced form to a less active oxidized form, which is also more sensitive to substrate inhibition by APS (Table 3) (24). Here we show that oxidation of the disulfide in AtAPSK affects the nucleotide binding equilibria compared to the reduced enzyme (Fig 5). Based on ITC analysis of binary and ternary complex formation in AtAPSKox (Fig. 2; Tables 1 & 2), the binding affinities at the ATP/ADP and APS/PAPS sites are essentially inverted versus those observed in AtAPSKred. The presence of Cys86-Cys119 in AtAPSK reduces the $K_d$ for ATP and ADP by 45- and 217-fold compared to the reduced enzyme, but enhances affinity for APS by 18-fold. Similar effects were also observed using pre-formed binary complexes and addition of the second nucleotide.

These results suggest a mechanism for redox-linked gating of ligand binding at the ATP/ADP and APS/PAPS sites. In AtAPSKred, the ligand binding equilibria favor the catalytically productive sequence of ATP binding first followed by APS addition (Fig. 5). Disulfide formation shifts the binding preference in the apoenzyme to favor APS and reduces the affinity for ATP and ADP binding to either free enzyme or the E•APS complex (Fig. 5). Structurally, the Cys86-Cys119 disulfide cross-links the two monomers in AtAPSK and likely reduces the conformational flexibility of the N-terminal domain (Fig. 1A). Acting like a molecular staple, the disulfide bond may restrict movement of the N-terminal domain to pre-organize the APS binding site by mimicking the overall conformation of the ATP/ADP-bound form of the enzyme, which leads to enhanced APS affinity. The comparable $K_d$ values for APS binding to AtAPSKox and the AtAPSKred•ATP and •ADP complexes suggest structurally similar binding sites for this ligand in different forms of the enzyme. The overall conformation of the oxidized protein may also structurally occlude the ATP/ADP site to decrease affinity for binding of these ligands. Thus, there is a fundamental difference between the oxidized form of AtAPSK and the ATP/ADP bound complexes.

Multiple steady-state kinetic studies of bacterial, fungal, and mammalian APSK, as well as AtAPSKred, suggest that formation of an E•ADP•APS dead-end complex is responsible for substrate inhibition (1, 15-16, 20-23); however, all of these forms lack the regulatory disulfide switch found in the plant APSK. Comparison of ligand binding in the reduced and
oxidized forms of AtAPSK suggests another structural mechanism for substrate inhibition by APS in plants. The restriction in movement of the N-terminal domain in AtAPSKox also strengthens the effectiveness of substrate inhibition by APS.

The effect of disulfide bond formation on activity and ligand binding, and the position of this linkage in AtAPSK, led us to further examine the contribution of the N-terminal domain (residues 77 to 96) to nucleotide binding. Removal of the N-terminal domain in AtAPSK modestly alters both $k_{cat}$ and $K_m$, but completely eliminates substrate inhibition by APS (Table 3), which is similar to the results reported for the human enzyme (20). Although substrate inhibition by APS was previously linked to the N-terminal domain of human APSK (20), here we provide the first experimental evidence for targeted changes in nucleotide binding mediated through the N-terminal domain of an APSK.

Deletion of the N-terminal domain of AtAPSK did not alter either the binding of ATP to free enzyme or the binding of APS to the E•AMP-PNP complex (Fig. 3; Table 4); however, a large effect on APS binding was observed, as no heat signal change was detected in titrations with this ligand to free enzyme. The calorimetric results and the 5-fold increase in $K_m$ for APS (Table 3) suggests that removal of the N-terminal domain results in equilibria that strongly favor the ATP first binding sequence, which retains enhanced APS affinity to the binary complex (Fig. 5). In addition to disrupting APS interaction with the free enzyme, truncation of the N-terminal domain decreased affinity for ADP by 52-fold, although APS binding to the E•ADP complex was unaffected (Fig. 5). Overall, deletion of the N-terminal domain has two major effects on APSK that abolish the substrate inhibition effect of APS: 1) favoring of the catalytically productive ATP first binding sequence and 2) a higher $K_d$ for ADP that weakens formation of the E•ADP•APS dead-end complex.

Earlier work comparing the three-dimensional structures of full-length and an N-terminal truncated versions of human APSK (20) suggested a possible structural basis for differences in substrate inhibition between the two enzyme forms. The structure of human APSK lacking the N-terminal domain revealed an asymmetric dimer with ADP and APS present in one monomer and only ADP in the neighboring monomer, which led to a model that the N-terminal domain is required for formation of a symmetric dimer, which represents the inhibited conformation of the enzyme. However, the binding isotherms observed in our ITC experiments (Fig. 3; Table 4) with AtAPSKΔ96 do not suggest that a greater degree of asymmetry exists in the truncated dimer compared to the full-length reduced protein. Currently, it is unclear how the AtAPSKΔ96 construct alters binding at the APS site, as the N-terminal domain is not in direct contact with the site. Although our results and the human APSK structural studies would imply that removal of the N-terminal domain alters movement of other structural features around the active site that are critical for substrate binding.

Multiple crystal structures of APSK from various organisms show that the N-terminal domain is flexible, as it adopts different conformations in these models (17-20, 23-26). As shown here, the N-terminal domain in AtAPSK is a central feature for regulation of activity by guiding the sequence of nucleotide binding and substrate inhibition. Because positioning of the N-terminal domain of one AtAPSK monomer along the surface of the adjacent monomer (Fig. 1A) appears critical for ligand binding order, we re-examined the AtAPSK crystal structure for additional residues that orient this part of the protein. Structural and sequence comparisons (Fig. 1B-C) suggested that Arg93, may play a role in positioning the flexible N-terminal domain along the adjacent monomer surface by interaction with Asp171 in $\alpha$3. The R93A mutant recapitulated the steady-state kinetic (Table 3) and nucleotide binding parameters observed with AtAPSKΔ96 (Fig. 4; Table 5). As with removal of the entire N-terminal domain, the R93A mutant also abolished substrate inhibition by APS. Interaction between the enzymatic core of one monomer and the N-terminal domain of the
other monomer in APSK appears essential for substrate inhibition and redox regulation.

Overall, our results suggest a two-tiered control of APSK activity in plants. The first mechanism is common across nearly all APSK and centers on positioning of the N-terminal domain over the catalytic site to control nucleotide binding order and to modulate substrate inhibition by APS. Conserved residues in α1 are critical for bending the N-terminal domain along the surface leading toward the active site. In plants, evolution of a second control switch, in the form of a redox-linked disulfide, further tunes the overall catalytic efficiency and substrate inhibition effects for additional regulation between the reduced and oxidized forms of APSK. This potentially provides reciprocal regulation at a metabolic branchpoint, in which oxidation of APS reductase activates flow into the primary sulfur assimilation pathway, while attenuating APSK to decrease the levels of APS directed into the secondary pathway used for sulfonylation of multiple metabolites (24).

Although redox-regulation of proteins through disulfide bonds provides a critical response mechanism to changes in cellular environment (34-35), the structural and energetic basis for how this molecular switches work are often not well understood. AtAPSK provides a rare example for how thiol-linked changes directly alter the energetics of binding equilibria in a protein to control its activity and suggests a likely role for conformational dynamics in these processes.

REFERENCES


**FOOTNOTES**

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4Abbreviations are: AMP-PNP, β,γ-imidoadenosine-5-triphosphate; APS, adenosine-5'-phosphosulfate; APSK, adenosine-5'-phosphosulfate kinase, also known as adenylyl-sulfate kinase, E.C. 2.7.1.25; AtAPSK, *Arabidopsis thaliana* APSK; AtAPSKox, oxidized form of AtAPSK with a disulfide formed between Cys86 and Cys119; AtAPSKΔ96, N-terminal truncation lacking residues 77-96; BME, β-mercaptoethanol; DTT, D/L-dithiothreitol; ITC, isothermal titration calorimetry; PAPS, 3'-phosphoadenosine-5'-phosphosulfate; TCEP, Tris(2-carboxyethyl)phosphine.
FIGURE LEGENDS

Figure 1. Structural overview of AtAPSK. (A) Structure of the AtAPSK•AMP-PNP•APS complex (24). One monomer is shown as a surface rendering (red) and the other as a ribbon diagram with α-helices (blue cylinders) and β-sheets (yellow arrows) indicated. Bound AMP-PNP and APS in the red monomer are represented by yellow and green space-filling models, respectively. The position of the disulfide bond formed between Cys86 of the right dimer and Cys119 of the left dimer is shown by a space-filling model. The alternate conformations of the disulfide observed in the x-ray crystal structure are shown. The N-terminal domain (residues 77-96) of the right monomer extends across the surface of the left monomer and ends at the APS binding site. The position of residue 96 is indicated by an arrow to mark the truncated region in AtAPSKΔ96. (B) AtAPSK N-terminal domain interactions between monomers. Interactions between residues in α1 (blue monomer), α2 (red monomer), and α3 (blue monomer) are indicated by dashed lines. The side-chain and main-chain atoms of Asp92 (α1), Arg93 (α1), Tyr126 (α2) and Asp171 (α3) are shown as stick models. The Cys86-Cys119 disulfide bond is shown as a space-filling model. (C) Sequence alignment of the N-terminal domains of APSK from plants (Arabidopsis, alfalfa, soybean, grape, maize, rice), mosses (Selaginella, Physcomitrella), algae (Chlorella, Chlamydomonas), human, bacteria (E. coli), and fungi (P. chrysogenum, Sacchromyces). Highly conserved residues are highlighted in blue. The residues corresponding to Cys86 and Arg93 of AtAPSK are highlighted in orange and red, respectively. The box indicates residues in α1.

Figure 2. ITC analysis of nucleotide binding to AtAPSKOX. (A) Titration of ADP (□), ATP (■) and APS (○) to AtAPSKOX dimer at 17 °C. In each panel, ITC data (upper panel) is plotted as heat signal versus time. In the lower panel, the integrated heat responses per injection are plotted. The solid lines represent the fit to data using either two-site or one-site binding models (see Table 1). (B) Titration of AtAPSKOX•AMP-PNP (■) and AtAPSKOX•ADP (□) complexes with APS at 17 °C. The solid lines represent the fit to data using either two-site or one-site binding models (see Table 2).

Figure 3. ITC analysis of nucleotide binding to AtAPSKΔ96. (A) Titration of ADP (□), ATP (■), and APS (○) to AtAPSKΔ96 dimer at 17 °C. In each panel, ITC data (upper panel) is plotted as heat signal versus time. In the lower panel, the integrated heat responses per injection are plotted. The solid lines represent the fit to data using a two-site binding model (see Table 4). (B) Titration of AtAPSKΔ96•AMP-PNP (■) and AtAPSKΔ96•ADP (□) complexes with APS at 17 °C. The solid lines represent the fit to data using either two-site or one-site binding models (see Table 4). (C) Titration of the AtAPSKΔ96•APS complex with AMP-PNP (□) and ADP (■). The solid lines represent the fit to data using a two-site binding model (see Table 4).

Figure 4. ITC analysis of nucleotide binding to AtAPSK R93A. (A) Titration of ADP (□), ATP (■), and APS (○) to AtAPSKΔ77 R93A dimer at 17 °C. In each panel, ITC data (upper panel) is plotted as heat signal versus time. In the lower panel, the integrated heat responses per injection are plotted. The solid lines represent the fit to data using a two-site sequential binding model or a single-set of non-interacting sites model (see Table 5). (B) Titration of AtAPSK R93A•AMP-PNP (■) or AtAPSK R93A•ADP (□) complex with APS at 17 °C. The solid lines represent the fit to data using a two-site binding model (see Table 5).

Figure 5. Comparison of binding equilibria in reduced (AtAPSKRED), oxidized (AtAPSKOX), and N-terminal truncated (AtAPSKΔ96) forms of AtAPSK. Open arrows indicated favored equilibria and dashed arrows show events that were not detected (ND) by calorimetry. Values for AtAPSKRED are from previous work (23).
Table 1. Thermodynamic parameters of nucleotide binding to AtAPSKox. All titrations were performed at 17 °C. ITC data were fit to either a one-site binding model (n = number of sites shown below K1 value) or a two-site binding model. For comparison, the previously determined binding constants (K1 and K2) determined for AtAPSKRED (23) are shown in the last column.

<table>
<thead>
<tr>
<th>ligand</th>
<th>K1 (µM)</th>
<th>K2 (µM)</th>
<th>ΔH1 (kcal mol⁻¹)</th>
<th>ΔH2 (kcal mol⁻¹)</th>
<th>AtAPSKRED K1 / K2 (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>56.3 ± 8.2</td>
<td>213 ± 41</td>
<td>-9.5 ± 3.9</td>
<td>-3.1 ± 0.5</td>
<td>1.25 / 24.7</td>
</tr>
<tr>
<td>ADP</td>
<td>39.1 ± 7.3</td>
<td>104 ± 26</td>
<td>-6.2 ± 0.7</td>
<td>-2.7 ± 0.9</td>
<td>0.18 / 82.6</td>
</tr>
<tr>
<td>APS</td>
<td>3.70 ± 0.40</td>
<td>--</td>
<td>-11.5 ± 0.8</td>
<td>--</td>
<td>66.7 / 325</td>
</tr>
</tbody>
</table>

n = 1.95 ± 0.07

Table 2. Thermodynamic parameters of nucleotide binding to AtAPSKox•nucleotide complexes. All titrations were performed at 17 °C with AtAPSKox•nucleotide complexes pre-formed as described in the Experimental Procedures. ITC data were fit to either one-site (n = number of sites shown below K1 value) or two-site binding models.

<table>
<thead>
<tr>
<th>pre-bound nucleotide</th>
<th>ligand</th>
<th>K1 (µM)</th>
<th>K2 (µM)</th>
<th>ΔH1 (kcal mol⁻¹)</th>
<th>ΔH2 (kcal mol⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMP-PNP</td>
<td>APS</td>
<td>0.64 ± 0.02</td>
<td>7.50 ± 0.30</td>
<td>-4.1 ± 0.1</td>
<td>-1.5 ± -0.1</td>
</tr>
<tr>
<td>ADP</td>
<td>APS</td>
<td>3.70 ± 0.80</td>
<td>--</td>
<td>-6.6 ± 0.4</td>
<td>--</td>
</tr>
<tr>
<td>APS</td>
<td>AMP-PNP</td>
<td>37.2 ± 9.4</td>
<td>172 ± 31</td>
<td>4.4 ± 0.8</td>
<td>3.9 ± 0.6</td>
</tr>
<tr>
<td>APS</td>
<td>ADP</td>
<td>26.1 ± 7.4</td>
<td>81.9 ± 4.8</td>
<td>-6.0 ± 1.2</td>
<td>-2.1 ± 0.5</td>
</tr>
</tbody>
</table>

n = 2.10 ± 0.20

Table 3. Steady-state kinetic comparison of AtAPSK forms.

<table>
<thead>
<tr>
<th>Kcat (s⁻¹)</th>
<th>KmAPS (µM)</th>
<th>KιAPS (µM)</th>
<th>kcat / Km (M⁻¹ s⁻¹ x 10⁸)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AtAPSKRED</td>
<td>272 ± 39</td>
<td>0.48 ± 0.41</td>
<td>37.5 ± 6.9</td>
</tr>
<tr>
<td>AtAPSKox</td>
<td>14.1 ± 2.3</td>
<td>0.43 ± 0.26</td>
<td>2.51 ± 0.84</td>
</tr>
<tr>
<td>AtAPSKΔ96</td>
<td>82.3 ± 5.6</td>
<td>2.63 ± 0.50</td>
<td>--</td>
</tr>
<tr>
<td>AtAPSK R93A</td>
<td>136 ± 44</td>
<td>1.72 ± 0.55</td>
<td>--</td>
</tr>
</tbody>
</table>

Average values ± S.E. (n = 3) are shown. Values for reduced and oxidized AtAPSK were previously reported (23) and are provided for comparison.
Table 4. Thermodynamic parameters of nucleotide binding to AtAPSKΔ96•nucleotide complexes. All titrations were performed at 17 °C with AtAPSKΔ96•nucleotide complexes pre-formed as described in the Experimental Procedures. ITC data were fit to either one-site ($n$ = number of sites shown below $K_1$ value) or two-site binding models.

<table>
<thead>
<tr>
<th>pre-bound nucleotide</th>
<th>ligand</th>
<th>$K_1$ (µM)</th>
<th>$K_2$ (µM)</th>
<th>$\Delta H_1$ (kcal mol$^{-1}$)</th>
<th>$\Delta H_2$ (kcal mol$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>--</td>
<td>ATP</td>
<td>1.70 ± 0.40</td>
<td>32.9 ± 8.3</td>
<td>-12.5 ± 3.9</td>
<td>-1.6 ± 0.5</td>
</tr>
<tr>
<td>--</td>
<td>ADP</td>
<td>9.40 ± 0.80</td>
<td>64.3 ± 5.9</td>
<td>-8.8 ± 1.6</td>
<td>-1.5 ± 0.8</td>
</tr>
<tr>
<td>AMP-PNP</td>
<td>APS</td>
<td>3.20 ± 0.90</td>
<td>30.7 ± 5.8</td>
<td>-4.1 ± 0.1</td>
<td>-0.94 ± 0.06</td>
</tr>
<tr>
<td>ADP</td>
<td>APS</td>
<td>4.90 ± 0.90</td>
<td>--</td>
<td>-1.3 ± 0.1</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$n$=1.97 ± 0.10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>APS</td>
<td>AMP-PNP</td>
<td>2.00 ± 0.30</td>
<td>33.0 ± 2.9</td>
<td>3.3 ± 0.7</td>
<td>3.0 ± 0.9</td>
</tr>
<tr>
<td>APS</td>
<td>ADP</td>
<td>0.57 ± 0.07</td>
<td>27.8 ± 4.3</td>
<td>-2.7 ± 0.1</td>
<td>-1.2 ± 0.1</td>
</tr>
</tbody>
</table>

Table 5. Thermodynamic parameters of nucleotide binding to AtAPSK R93A•nucleotide complexes. All titrations were performed at 17 °C with AtAPSK R93A•nucleotide complexes pre-formed as described in the Experimental Procedures. ITC data were fit to either one-site ($n$ = number of sites shown below $K_1$ value) or two-site binding models.

<table>
<thead>
<tr>
<th>pre-bound nucleotide</th>
<th>ligand</th>
<th>$K_1$ (µM)</th>
<th>$K_2$ (µM)</th>
<th>$\Delta H_1$ (kcal mol$^{-1}$)</th>
<th>$\Delta H_2$ (kcal mol$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>--</td>
<td>ATP</td>
<td>3.60 ± 0.20</td>
<td>10.4 ± 1.5</td>
<td>-15.6 ± 1.3</td>
<td>-10.2 ± 2.1</td>
</tr>
<tr>
<td>--</td>
<td>ADP</td>
<td>4.80 ± 0.60</td>
<td>24.4 ± 3.1</td>
<td>-8.2 ± 0.9</td>
<td>-1.8 ± 0.5</td>
</tr>
<tr>
<td>AMP-PNP</td>
<td>APS</td>
<td>0.80 ± 0.10</td>
<td>1.90 ± 0.30</td>
<td>-4.9 ± 0.8</td>
<td>-1.6 ± 0.4</td>
</tr>
<tr>
<td>ADP</td>
<td>APS</td>
<td>5.50 ± 0.40</td>
<td>--</td>
<td>-8.2 ± 1.6</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$n$=2.05 ± 0.02</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 2
Figure 5

Formation of Catalytic Ternary Complex

AtAPSK<sub>RED</sub>  AtAPSK<sub>OX</sub>  AtAPSK<sub>Δ96</sub>

1.25 E•ATP  56.3 E•ATP  1.70 E•ATP
E  E•ATP•APS  E•ATP•APS  E•ATP•APS
66.7 E•APS  3.70 E•APS  ND E•APS
3.10 37.2 2.00

Formation of 'Dead-End' Complex

AtAPSK<sub>RED</sub>  AtAPSK<sub>OX</sub>  AtAPSK<sub>Δ96</sub>

0.18 E•ADP  39.1 E•ADP  9.40 E•ADP
E  E•ADP•APS  E•ADP•APS  E•ADP•APS
66.7 E•APS  3.70 E•APS  ND E•APS
3.30 39.1 0.37
Redox-Linked Gating of Nucleotide Binding by the N-Terminal Domain of Adenosine 5'-Phosphosulfate Kinase
Geoffrey E. Ravilious, Corey S. Westfall and Joseph M. Jez

*J. Biol. Chem.* published online January 15, 2013

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