Structural Insights into the Catalytic Mechanism of *Escherichia coli* Selenophosphate Synthetase

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Selenophosphate synthetase (SPS) catalyzes the synthesis of selenophosphate, the selenium donor for the biosynthesis of selenocysteine and 2-selenouridine residues in seleno-tRNA. Selenocysteine, known as the 21st amino acid, is then incorporated into proteins during translation to form selenoproteins which serve a variety of cellular processes. SPS activity is dependent on both Mg^{2+} and K^{+} and uses ATP, selenide, and water to catalyze the formation of AMP, orthophosphate, and selenophosphate. In this reaction, the gamma phosphate of ATP is transferred to the selenide to form selenophosphate, while ADP is hydrolyzed to form orthophosphate and AMP. Most of what is known about the function of SPS has derived from studies investigating *Escherichia coli* SPS (EcSPS) as a model system. Here we report the crystal structure of the C17S mutant of SPS from *E. coli* (EcSPS^{C17S}) in apo form (without ATP bound). EcSPS^{C17S} crystallizes as a homodimer, which was further characterized by analytical ultracentrifugation experiments. The glycine-rich N-terminal region (residues 1 through 47) was found in the open conformation and was mostly ordered in both structures, with a magnesium cofactor bound at the active site of each monomer involving conserved aspartate residues. Mutating these conserved residues (D51, D68, D91, and D227) along with N87, also found at the active site, to alanine completely abolished AMP production in our activity assays, highlighting their essential role for catalysis in EcSPS. Based on the structural and biochemical analysis of EcSPS reported here and using information obtained from similar studies done with SPS orthologs from *Aquifex aeolicus* and humans, we propose a catalytic mechanism for EcSPS-mediated selenophosphate synthesis.

Selenium is an essential nutrient that has been linked to many human diseases (27) related to hormone regulation (35, 38), immune response (16, 42), protection from reactive oxygen species (12), and brain function (4). Deficiencies in selenium intake have been implicated in several health disorders over the past two decades, including cancer (5), cardiovascular disease (3), male infertility (43), asthma (39), and viral infections (29). Selenium-containing proteins, or selenoproteins, are expressed in many organisms and tissues (26, 34, 40). Studies have shown that mutations in some of these selenoproteins can lead to decreased activity resulting in abnormal hormone metabolism (8, 9) and rigid-spine muscular dystrophy (30).

Selenium has been shown to be incorporated into enzymes/proteins either as a selenocysteine (7), which occurs at a specific position in the protein sequence and participates in the protein's catalytic action, or as a selenomethionine, which results from random substitution of selenium in place of sulfur in methionine and exerts no effect on the enzymatic activity of the protein (15). Incorporation of selenocysteine, commonly referred to as the 21st amino acid, is mediated by a UGA codon-directed cotranslation involving four genes, *selA*, *selB*, *selC*, and *selD* (24). The 37-kDa selenophosphate synthetase (SPS) is the gene product of *selD*, which catalyzes the synthesis of selenophosphate (SeP), the selenium donor for the biosynthesis of selenocysteine and the modification of thiouridine to selenouridine in a select class of tRNAs (45). SPS activity is dependent on both Mg^{2+} and K^{+} in catalyzing the following reaction: $ATP + \text{selenide} + \text{water} \rightarrow AMP + P_i + \text{SeP}$.

In this reaction, the gamma phosphate of ATP is transferred to the selenide to form selenophosphate, while ADP is hydrolyzed to orthophosphate and AMP (10, 13). It is still unclear why SPS

hydrolyzes both high-energy bonds of ATP to produce one molecule of selenophosphate. Recently, though, it was proposed that hydrolysis of the second high-energy bond may be required to protect the highly reactive selenophosphate product and to initiate release of the reaction products (46). Studies have shown that residues C17 and K20 are essential for catalysis in *Escherichia coli* SPS (EcSPS) and that mutating them leads to inactivation (18, 19, 24). These studies also suggest that C17 may directly coordinate selenium during catalysis and that K20 may form a phosphorylated intermediate; however, neither interaction has thus far been isolated or demonstrated. Additionally, an unidentified chromophore for SPS that has a characteristic absorbance peak at 315 nm has been reported (47). This peak was found to undergo a red shift in the presence of magnesium and ATP, but not in the presence of nonhydrolyzable ATP analogues.

The crystal structures of SPS from *Aquifex aeolicus* (AaSPS) (17, 28), both native and complexed with AMPCPP (α , β -methyleneadenosine 5'-triphosphate, a nonhydrolyzable ATP analogue), were recently reported. The AaSPS crystal structures provided the first view of the overall structure of SPS, as well as the molecular details of the catalytic machinery of SPS and its interaction with the ATP analogue AMPCPP. It was observed that the glycine-rich N-terminal region of AaSPS underwent a conforma-

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tional change and became more ordered when bound with cobalt metal cofactors (in crystallization buffer) and AMPCPP than the apo structure, leading to the hypothesis that the observed conformational change, ATP binding, and magnesium (native cofactor) binding may function interdependently for catalysis. The AMPCPP molecule was found bound at the proposed catalytic site, nested along the AaSPS dimer interface. As noted by Itoh et al. (17) and observed in our studies as well, four conserved aspartate residues make up the binding site of the phosphate group of the ATP analogue. In addition, several metal ions were found to play an essential role in mediating the binding of the highly electronegative substrate phosphate group. Itoh et al. also proposed a model for direct transfer of selenium to SPS based on docking experiments with their AaSPS crystal structure and the crystal structure of the NifS-like *E. coli* protein CsdB (25).

Crystal structures of human SPS1 (hSPS1) were also recently reported (46), one in complex with AMPCPP and another of a captured intermediate representing the state immediately following ATP hydrolysis, in which both ADP and P_i products were observed in the crystal structure. As with AaSPS, both were found in the closed conformation. Further, a comparison of Na^+ -bound and K^+ -bound crystal structures identified a completely conserved threonine residue hypothesized to be the monovalent cation sensor responsible for the selectivity of K^+ over Na^+ or Li^+ for efficient catalysis of SPS. Mutation of the conserved threonine to an alanine residue nearly abolishes ADP hydrolysis, further demonstrating the importance of the monovalent cation K^+ for catalysis. Together, the AaSPS and hSPS1 structures provide a description of the molecular interactions involved in ATP binding and subsequent hydrolysis. However, the exact mechanism by which SPS synthesizes selenophosphate remains unclear, and more studies, particularly to determine the steps toward selenium transfer and whether a phosphorylated intermediate truly exists, are needed to elucidate the complete reaction mechanism.

The majority of what is known about the function of SPS has come from studies investigating SPS from *E. coli* as a model system. However, crystallizing SPS from *E. coli* has proven very difficult, and the crystal structure has remained unsolved until now. Here we report the crystal structure of the C17S mutant of SPS from *E. coli* (EcSPS^{C17S}) in apo form (not ATP bound). Like AaSPS and hSPS1, EcSPS^{C17S} is a homodimer, with each monomer consisting of N- and C-terminal domains. This dimer form was further verified by sedimentation velocity analysis, and the K_d (dissociation constant) for the dimer, which has not been reported with EcSPS previously, was determined. The glycine-rich N-terminal region (residues 1 through 47) of the apo-EcSPS^{C17S} structure was found to be mostly ordered, with a single magnesium ion bound at the active site of each monomer of the apo structure. We found that mutating each of the conserved aspartate residues that are involved in magnesium binding, along with N87, completely abolished SPS activity, highlighting their essential importance for catalysis in EcSPS and other homologs.

MATERIALS AND METHODS

Cloning and mutagenesis. Crystallization of *E. coli* SPS has proven more difficult than expected. Attempts to crystallize wild-type EcSPS were unsuccessful; however, we were able to grow crystals of the inactive EcSPS^{C17S} mutant. Cloning of EcSPS^{C17S} has previously been reported (19, 24). To simplify protein production for the enzyme kinetics assays, we made a C-terminal His₆ tag for quick purification. We used the follow-

ing primers to PCR amplify EcSPS to have an N-terminal NdeI site and a C-terminal XhoI site: 5'-GATACCATATGAGCGAGAACTCGATTC-3' and 5'-CTATGCTCGAGACGAATCTCAACCATG-3'. The amplified product was then digested with NdeI and XhoI and then ligated into the pET20b vector (Novagen).

Mutagenesis studies were used to investigate the importance of the four conserved aspartate residues (D51, D68, D93, and D227) and N87 in magnesium binding and catalysis. We used the online QuikChange primer design tool (Agilent Technologies) to design primers for the D51A, D68A, N87A, D91A, D227A, and C17S mutants. Sequences were confirmed by DNA sequencing analysis (FDA core facility, NIH, Bethesda, MD).

Protein expression and purification. Expression of wild-type EcSPS and EcSPS^{C17S} has previously been reported (19, 24). Briefly, the EcSPS^{C17S} mutant was transformed into BL21(DE3) cells, plated onto LB-carbenicillin (100 μ g/ml) agar plates, and incubated at 37°C overnight. For activity assays, wild-type and mutant constructs carrying a C-terminal His₆ tag were transformed into T7 Express (New England BioLabs) cells, plated onto LB-carbenicillin (100 μ g/ml) agar plates, and incubated at 37°C overnight. Five-milliliter inoculate cultures (LB-carbenicillin) were then prepared from single colonies and allowed to grow to an optical density at 600 nm (OD_{600}) of ~ 0.6 at 37°C. Then 500 μ l of each inoculate culture was added to 750 ml of LB-carbenicillin (100 μ g/ml) in 2-liter flasks, allowed to grow to an OD_{600} of ~ 0.6 (37°C), and induced with 1 mM IPTG (isopropyl- β -D-thiogalactopyranoside). Cells were harvested after 3 to 4 h by centrifugation at 6,000 rpm (Sorvall SLA-3000 rotor) for 20 min.

Cells were resuspended in lysis buffer (50 mM Tris-HCl [pH 7.5], 200 mM NaCl, 10 mM MgCl₂) supplemented with AEBSF (MP Biomedicals, Inc.) and DNase I (Sigma) and lysed using two passes through a French press at 18,000 lb/in². Lysates were centrifuged at 18,000 rpm (Sorvall SS-34 rotor) for 1 h at 4°C. The purification method for EcSPS^{C17S}, which was expressed without a His₆ tag, has previously been reported (19). For wild-type and active-site mutants carrying the C-terminal His₆ tag, supernatants were run over a 2-ml preequilibrated Ni-agarose (Qiagen) column, washed using 250 ml of wash buffer (50 mM Tris-HCl [pH 7.5], 1 M NaCl, and 20 mM imidazole), and eluted using 30 ml of elution buffer (50 mM Tris-HCl [pH 7.5], 1 M NaCl, and 250 mM imidazole). Eluted protein was then dialyzed in dialysis buffer containing 100 mM Tricine-KOH (pH 7.2), 20 mM KCl, 2 mM dithiothreitol (DTT), and 4 mM MgCl₂. The purity of the proteins was analyzed by SDS-PAGE; the proteins were determined to be >95% pure and much cleaner than proteins purified by previously used methods without the use of a His₆ tag.

Crystallization. We were able to successfully crystallize the C17S mutant of SPS (EcSPS^{C17S}). For crystallization, protein was dialyzed into crystallization buffer consisting of 25 mM Tris HCl [pH 7.5], 200 mM LiCl, and 10 mM DTT overnight and concentrated to 8 mg/ml. Crystallization screening was performed by the hanging-drop vaporization method using a Mosquito (TTP Labtech) crystallization robot, and several leads were optimized with 50 mM Tris-HCl [pH 8.25], 50 mM MgCl₂, and 32% PEG MME 550 (polyethylene glycol monomethyl ether), producing the best crystals.

Data collection and structure determination. Initial screening of the EcSPS^{C17S} crystals was performed using our in-house X-ray source (Rigaku MicroMax-007 HF microfocus X-ray generator; Raxis IV++ detector). Final data sets were collected at the SER-CAT ID-22 beamline at the Advanced Photon Source at Argonne National Laboratory. Data were processed using HKL2000 (33).

The final model of EcSPS^{C17S} was completed by molecular replacement with PHASER within CCP4 (6), using the recently deposited coordinates for SPS from *Aquifex aeolicus* as a search model (PDB code 2ZOD). Subsequent refinement and model building were performed using PHENIX (1) and COOT (11), respectively, including NCS (noncrystallographic symmetry) averaging and TLS (translation/libration/screw) refinement. Final data collection and refinement statistics are summa-

TABLE 1 Data collection and refinement statistics for EcSPS^{C17S}

Parameter ^a	Value(s) ^b for EcSPS ^{C17S}
Data collection statistics	
Space group	P3 ₂ 21
No. of molecules/ASU	2
Unit cell dimensions	
λ, a, b, c (Å)	1.0, 102.347, 102.347, 140.669
α (°)	90
β (°)	90
γ (°)	120
Resolution range (Å)	50–2.25 (2.49–2.25)
Completeness (%)	99.8 (99.8)
Redundancy	7.4 (5.7)
R _{sym} (%)	5.6 (55.5)
I/σ	32.7 (2.8)
Data refinement statistics	
R/R _{free}	0.22/0.27
Wilson B factor (Å ²)	57.23
Average of B factors (Å ²)	68.05
Bond length (Å)	0.009
Bond angle (°)	1.423
No. of water molecules	55
Ramachandran analysis	
Most favored (%)	88.4
Allowed (%)	10.1
Generously allowed (%)	1.3
Disallowed (%)	0.2
PDB ID code	3U0O

^a ASU, asymmetric unit; $R_{\text{sym}} = \sum_{hkl} (|I_{hkl}| - \langle I_{hkl} \rangle) / \sum_{hkl} |I_{hkl}|$, where I_{hkl} is the intensity of a reflection and $\langle I_{hkl} \rangle$ is the average intensity for a set of symmetry-related reflections; $R = \sum_{hkl} (|F_o| - |F_c|) / \sum_{hkl} |F_o|$, where F_o is the observed structure factor amplitude and F_c is the calculated structure factor amplitude; $R_{\text{free}} = \sum_{hkl} (|F_o| - |F_c|) / \sum_{hkl} |F_o|$, where T represents a test set of reflections (5% of the data) which have been omitted from the refinement.

^b The values in parentheses are for the last resolution shell.

rized in Table 1. Root mean square deviation (RMSD) analysis was performed using the STRAP program (14), electrostatic surface potential was calculated and visualized using the APBS plugin (developed by Michael Lerner) in PyMOL (Schrödinger, Inc.), hydrophobicity was calculated using the VASCo program (41) and visualized in PyMOL, and figures were made using PyMOL, Photoshop, and Illustrator (Adobe Systems, Inc.).

Analytical ultracentrifugation. Sedimentation velocity experiments were conducted with a Beckman Optima XL-I analytical ultracentrifuge equipped with a four-place An-60 Ti rotor and cells with 12-mm-path-length, double-sector centerpieces. Proteins were dialyzed into the experimental buffer (50 mM Tris-HCl [pH 7.5], 50 mM KCl), and the dialysis buffer was used as an optical reference. For all experiments, 0.4 ml of the protein sample (2 mg/ml) and dialysis buffer were loaded into sample and reference centerpiece channels, respectively. The rotor was accelerated to 50,000 rpm after thermal equilibrium was reached at 20°C at rest (typically 1 h). Interference and absorbance scans at 280 nm or 230 nm were started immediately after the rotor reached the set speed and were collected until no further sedimentation boundary movement was observed. The apparent sedimentation coefficient distributions were analyzed by the Lamm equation using SEDFIT (37). The final accepted fits had

RMSDs of less than 0.009 units. The observed sedimentation coefficients (weight averages of peaks) were corrected to $s_{20,w}$ values using the appropriate relative viscosity (η/η_0), density (ρ), and partial specific volume ($\bar{v}$) values (see above) as follows: $s_{20,w} = s_{\text{obs}} (\eta/\eta_0) [(1 - \bar{v}\rho_0)/(1 - \bar{v}\rho)]$, where s_{obs} is the observed sedimentation coefficient. The reported $s_{20,w}$ values are in Svedberg units (S).

For sedimentation equilibrium experiments, three double-sector, 12-mm-path-length cells were filled with 0.18 ml of the SPS protein dialyzed into phosphate-buffered saline (PBS) buffer (buffer density $\rho = 1.0071$ g/ml at 4°C). The protein concentrations loaded into each cell were 0.6 μM, 1.2 μM, and 3 μM, respectively. The rotor was cooled to 4°C and initially accelerated to 20,000 rpm for ~1.5 h and then decelerated to 10,000 rpm for sedimentation equilibrium; absorption scans at 230 nm and 4 h intervals were made until equilibrium was attained (24 to 28 h), as evidenced by no time-dependent changes in gradients. The rotor speed was then increased to 20,000 rpm, and absorption scans were restarted. Global fitting of sedimentation equilibrium data obtained at two rotor speeds and three concentrations of SPS to a model for a reversible monomer-dimer association (with fully competent species present) was done using SEDPHAT (36).

Activity assays. UV absorption analyses of EcSPS wild-type and mutant proteins were performed in 100 mM Tricine-KOH [pH 7.2], 20 mM KCl, and 4 mM MgCl₂ on an Agilent 8453 UV-Visible spectrophotometer system with a scan range from 260 nm to 400 nm. For measurement of the selenide-dependent AMP formation from ATP, a modified method based on that of Veres et al. (44) was used. Briefly, the reaction mixture (50 μl) contained 100 mM Tricine-KOH [pH 7.2], 20 mM KCl, 4 mM MgCl₂, 2 mM DTT, 2 mM ATP, 4 μM enzyme, and 1.5 mM sodium selenide, which was added last. The sodium selenide stock solution was prepared as previously described by Klayman and Griffin (20) and stored in an anaerobic chamber (Coy Laboratory Products, Inc.). Reaction mixtures were incubated in the anaerobic chamber for 20 min, terminated by the addition of HClO₄ for 15 min, and then neutralized with KOH (2). The nucleotides were then separated by high-pressure liquid chromatography (HPLC) on an Agilent 1100 series system using a C₁₈ reversed-phase column (5 μm, 250- by 4.6-mm inside diameter; Vydac) equipped with a guard column at a flow rate of 1 ml/min and a mobile phase consisting of 50 mM ammonium bicarbonate, 2 mM tributylamine, and 5% acetonitrile (vol/vol), pH 7.0. Nucleotide peaks were detected at 260 nm, and the relative peak area for each nucleotide was calculated and normalized against C-terminal His₆-tagged wild-type EcSPS activity.

Circular dichroism spectroscopy. Circular dichroism (CD) spectroscopy was performed using a Jasco J-715 spectrometer at 20°C in 50 mM phosphate buffer (pH 7.5) and 50 mM NaCl. Final analysis for each sample was obtained by averaging 10 consecutive scans, and normalization was used to correct for any differences in protein concentration among samples.

Protein structure accession number. The coordinates for the EcSPS^{C17S} structure have been deposited in the Protein Data Bank and assigned the PDB code 3U0O.

RESULTS

The *E. coli* SPS crystal structure. Attempts to crystallize wild-type EcSPS were unsuccessful. Here we report the crystal structure of the C17S mutant of EcSPS (referred to as EcSPS^{C17S} for the remainder of the manuscript), which we found behaved more ideally for crystallization, having much less polymerization at high concentrations than the wild type. EcSPS^{C17S} crystallizes as a homodimer formed by the association of the N-terminal domains of each monomer (Fig. 1 and 2C and D). The EcSPS^{C17S} monomer is composed of an N-terminal domain (residues 1 through 165) and a C-terminal domain (residues 166 through 347) linked by a single pass of the polypeptide chain (Fig. 1). The two domains associate to form the active site, which sits tucked within a long, narrow

FIG 1 The structure of EcSPS^{C17S}. (A) The apo-EcSPS^{C17S} crystal structure showing the EcSPS^{C17S} monomer in ribbon representation, with the N-terminal domain (residues 10 through 165) in green, the C-terminal domain (residues 166 through 347) in gold, and the glycine-rich N-terminal region in red. (B) An electrostatic surface potential representation (± 4 kT/e), with electronegative regions in red and electropositive regions in blue. (C and D) The structures in panels A and B rotated $\sim 60^\circ$ along the x axis. The magnesium cofactor which is located at the active site is shown as a blue sphere.

channel that runs the length of the molecule and is flanked by a glycine-rich N-terminal region (residues 1 through 47). The N-terminal domain is composed largely of a five-stranded β -sheet, which forms most of the dimer interface, and three α -helices, two of which lie adjacent to the β -sheet and form the backside of the substrate binding channel. The C-terminal domain consists of a mixture of α -helices and β -strands, containing a six-stranded β -sheet flanked on three sides by six α -helices.

In chain A, the glycine-rich N-terminal region extends from the top of the molecule and binds along the bottom side along a turn composed of residues 72 through 77 of the N-terminal domain (Fig. 1) and was observed to be mostly ordered as determined both by the presence of strong electron density and by B-factor analysis (Fig. 3C). However, in chain B, the glycine-rich N-terminal region was found to extend away from the binding channel and interacted with a symmetry mate, which we interpreted to be a crystallization artifact. An RMSD of 1.10 Å was calculated for the monomers of the EcSPS^{C17S} dimer, indicating the slight variations between the two structures, primarily along turns and loops and including the glycine-rich N-terminal region. Furthermore, superpositioning of chain A from EcSPS^{C17S} over chain A of AaSPS resulted in a calculated RMSD of 2.54 Å (for 312 aligned residues, 35% sequence identity) and an RMSD of 2.96 Å (for 309 aligned residues, 24% sequence identity) compared to chain A of hSPS1 (Fig. 3A). In addition, the glycine-rich N-terminal region was found in the open conformation (Fig. 3B), similar to what was observed with the apo-AaSPS crystal structure (17).

FIG 2 EcSPS is a dimer in solution. (A) Sedimentation velocity experiment showing that EcSPS was found almost exclusively as a dimer ($\sim 99\%$), with trace amounts of tetramer ($\sim 1\%$) being observed. (B) Sedimentation equilibrium analysis for EcSPS at three different concentrations (0.3 μ M, 1.0 μ M, and 3.0 μ M) and two different speeds (10,000 and 20,000 rpm). A K_d of 170 nM was calculated for the EcSPS dimer under these experimental conditions. (C) The EcSPS^{C17S} dimer structure (side view), showing the N-terminal domain in green, the C-terminal domain in gold, and magnesium cofactors as blue spheres. (D) The EcSPS^{C17S} dimer structure rotated 90° along the x axis (top view) relative to the structure shown in panel C.

FIG 3 Comparison of conformational flexibility in known SPS structures. (A) Alignment of the apo-EcSPS^{C17S} (orange), AaSPS-AMPCPP (green), and hSPS1-ADP (yellow) crystal structures. The location of essential residues C17 and K20 for EcSPS^{C17S} are indicated by yellow and blue spheres, respectively. (B) The structure of apo-EcSPS^{C17S} shown in gray ribbon, highlighting the conformational changes along the glycine-rich N-terminal region of EcSPS^{C17S} (orange), AaSPS (green), and hSPS1 (yellow). For clarity, portions of the N-terminal region have been removed from each structure to best illustrate the conformational change at the active site. (C) The apo-EcSPS^{C17S} structure in B-factor putty representation, where blue represents the most ordered and red the most disordered. The magnesium cofactor is shown as a gray sphere for reference. (D) A surface hydrophobicity representation of the dimer interface of EcSPS^{C17S}, where yellow indicates the most hydrophobic residues.

Within the substrate binding channel, a single magnesium ion was found in each monomer, although the coordination chemistry was different for each. The magnesiums were identified by difference density peaks that were too large for water molecules and were found to have bond lengths and binding properties characteristic of magnesium binding. In chain A, the magnesium (MG1) is coordinated by D91 (bond length of 2.0 Å) and D227 (bond length of 2.2 Å), whereas in chain B, the magnesium (MG2) is coordinated by D51 (bond length of 1.9 Å), D91 (bond length of 2.0 Å), and D227 (bond length of 2.5 Å) (Fig. 4). The bond distances are consistent with magnesium coordination and agree well with those reported for both the AaSPS and hSPS1 crystal structures. As with the AaSPS and hSPS1 crystal structures (17, 46), D68 was also observed participating in magnesium binding in a low-resolution EcSPS-AMP complex structure (data not shown). An electrostatic surface potential map shows that the substrate binding channel is highly electronegative, with few electropositive residues present in or near the active site (Fig. 1B and D).

Oligomeric state of EcSPS is a dimer. To further confirm the oligomeric state of EcSPS in solution, we performed sedimentation velocity experiments which showed that at a monomeric concentration of 54.5 μM, 99% of EcSPS exists as dimers (Fig. 2A). The buried surface area of the dimer interface was calculated to be ~2,000 Å² (~14% of the total surface area) using the online PISA server (21). Using the program VASCo, we calculated the surface

FIG 4 Comparison of magnesium binding sites for EcSPS^{C17S}, AaSPS, and hSPS1. The EcSPS^{C17S} structure is shown in ribbon, and conserved aspartates, which are involved in binding magnesium, in stick representation. In addition, S141, which is conserved and likely involved in stabilizing the adenosine moiety during catalysis, is shown. The location of the magnesium ions found during structure determination are shown as spheres for EcSPS^{C17S} (blue), EcSPS-AMP (magenta), and hSPS1 (green). In addition, the locations of the cobalt metals found within the AaSPS structure, which are thought to mimic magnesium binding, are shown as pink spheres.

FIG 5 Conserved active-site aspartates are required for activity in EcSPS. (A) CD analysis of wild-type His₆ SPS (fully active) and the His₆ SPS mutants from 200 nm to 260 nm, showing that neither the addition of the C-terminal His tag nor any of the mutations had any significant effect on the folding of the enzymes. (B) Summary of the activity assays for EcSPS and the EcSPS mutants. The activity is shown as a percentage of activity normalized against that of the wild-type His₆ EcSPS enzyme. (C) Absorbance measurements monitoring the chromophore peak at ~315 nm. (D) Absorbance measurements monitoring the chromophore peak at ~315 nm in the absence of magnesium (1 mM EDTA), the presence of magnesium (4 mM), and the presence of magnesium (4 mM) and ATP (2 mM).

hydrophobicity of the interface mediating the dimer and found it to be composed largely of hydrophobic residues, as illustrated in Fig. 3D, indicating that EcSPS prefers the dimer state and may not tolerate being a monomer for very long in solution. Given the large buried surface area, we investigated the dimer further by performing sedimentation equilibrium experiments and determined a K_d of 170 nM for the dimer (Fig. 2B).

Mutagenesis and kinetics analysis of EcSPS. We observed that the magnesium ions, thought to be responsible for binding ATP and catalytic function, are coordinated by four completely conserved aspartate residues, D51, D68, D91, and D227. To further investigate their role in catalysis, we mutated each of the conserved aspartates, as well as N87, which we concluded was ideally positioned to interact with the phosphate group of ATP, to alanine. Expressing each of the mutants, along with the wild type and

the C17S mutant as C-terminal His₆-tagged fusion proteins, we performed activity assays by monitoring the conversion of ATP to AMP under anaerobic conditions.

To verify that the mutations and the addition of the His₆ tag did not affect the folding of EcSPS, we first performed CD analysis of all the mutants and the wild-type enzyme and compared them to CD analysis of the EcSPS^{C17S} enzyme used for structure determination (Fig. 5A), which indicated that the mutations and the His₆ tag did not affect folding or secondary structure sensitive to CD analysis. Next, in preparing for the activity assays, we ran UV scans of the enzymes (Fig. 5C) and noticed that the characteristic peak at 315 nm (47) was not present in 100 mM Tricine-NaOH [pH 7.2] buffer, but appeared only after dialysis into 100 mM Tricine-KOH [pH 7.2] buffer. Interestingly, we did not observe the 315-nm peak with the C17S mutant in either buffer, with or

without the His₆ tag, suggesting that both residue C17 of EcSPS and K⁺ are required for the formation of the unidentified chromophore previously observed (47). In addition, the 315-nm peak was also not observed for the D91A mutant. These results obtained in the absence of ATP and selenide also indicate that the formation of this chromophore is independent of ATP and selenide. However, the addition of a nucleotide which contains a hydrolysable gamma phosphate, such as ATP and Mg²⁺, has been shown to induce a red shift of this peak (47). To determine if any of the mutants had a comparable red shift in the presence of ATP and Mg²⁺, we assayed each mutant and compared them to the wild type in the absence of Mg²⁺, in the presence of Mg²⁺, and in the presence of ATP and Mg²⁺. We found that the D51A, D68A, D227A, and N87A mutants, which possess the 315-nm peak, do not exhibit any red shift in the presence of Mg²⁺ and ATP. These results indicate that replacing the aspartate at residues 51, 68, and 227 with alanine or replacing the asparagines at residue 87 with alanine would impair ATP binding or ATP hydrolysis under our experimental conditions (Fig. 5D). While the observed red shift of the 315-nm peak induced by the binding of Mg²⁺ and ATP provides useful information, the nature of this chromophore remains to be determined.

To assay the activity of the EcSPS mutants, we incubated wild-type EcSPS and each of the mutants with ATP and selenide in reaction buffer for 20 min at 37°C under anaerobic conditions and then used HPLC equipped with a C₁₈ column to quantify the hydrolysis of ATP to AMP. To determine whether the C-terminal His₆ tag affected EcSPS activity, we first compared the His₆-tagged wild-type enzyme to that of wild-type EcSPS without any fusion tag. As shown in Fig. 5B, we found that the fusion-tagged protein was fully active compared to the untagged enzyme and that mutating C17 to serine led to complete inactivation, as has been shown previously with untagged enzyme (19). We also observed that mutating each of the four conserved aspartate residues individually to alanine reduced the EcSPS activity to an undetectable level, illustrating the essential requirement of all four aspartates for catalysis under our experimental conditions. Surprisingly, we found that mutating N87 to alanine also completely abolished EcSPS activity. This result was unexpected because we did not observe any direct interactions between residue N87 and Mg²⁺ in our crystal structures. However, based on the AMPCPP-bound AaSPS structure (17), N87 interacts with two water molecules which in turn interact with the γ -phosphate of ATP (Fig. 6). These bound waters may participate in hydrolyzing the gamma phosphate and thus facilitate the formation of selenophosphate. This notion is consistent with the observation that the addition of Mg²⁺ and ATP fails to induce a red shift of the 315-nm peak (Fig. 5D) exhibited by the N87A mutant, since the red shift requires a hydrolyzable gamma phosphate.

DISCUSSION

Structural characterization of EcSPS^{C17S}. The apo crystal structure of EcSPS^{C17S} was found to be a homodimer, characteristic of members of the PurM family and previously observed in both the AaSPS and hSPS1 crystal structures (17, 46). In the EcSPS^{C17S} crystal structure, a single magnesium ion was found at the active site of each monomer, with different coordination chemistry, one being coordinated by D91 and D227 and the other by D51, D91, and D227. Although the catalytic activity of SPS is dependent on both Mg²⁺ and K⁺ (44), no obvious monovalent ions were ob-

FIG 6 Role of N87 during catalysis in EcSPS. Structure of AaSPS (green ribbon) bound with AMPCPP (stick model), showing the interactions of N79 (green stick model) with bridging water molecules to the AMPCPP ligand. Water molecules are shown as red spheres, cobalt ions as blue spheres, and hydrogen bonding interactions as black dashed lines with the associated distances noted. Our results indicate that mutation of this equivalent residue in EcSPS (N87) to alanine may lead to displacement of the interacting water molecules and subsequent destabilization of the ATP phosphate group during catalysis.

served in our structures, despite the presence of lithium in the crystallization buffer. Since the active site of EcSPS^{C17S} was found to be highly electronegative, binding of the highly electronegative phosphate group of the ATP substrate at the active site would likely involve the binding of the Mg-ATP complex and possibly the binding of a second Mg²⁺ and a monovalent metal ion such as K⁺. The binding of Mg²⁺ and K⁺ could provide a charge neutralization effect at the highly negatively charged substrate binding site.

Apo-EcSPS^{C17S} was found in the open conformation, in contrast to the previously reported liganded structures of AaSPS and hSPS1, which were found in a more closed conformation, where the N-terminal glycine-rich region sits in close proximity to the active site. In our structure, the glycine-rich N-terminal region was oriented away from the active site, with both essential residues, C17 and K20, sitting more than 20 Å away from the active-site magnesium (Fig. 3A). A similar observation was seen with the apo-AaSPS crystal structure in the absence of cofactor or substrate. Together, these studies support the hypothesis that the glycine-rich N-terminal region of EcSPS is flexible and becomes more ordered upon binding cofactors and substrate. While the glycine-rich N-terminal region was found to be relatively ordered compared to the rest of the EcSPS^{C17S} structure, magnesium binding alone did not induce a conformational change to the closed conformation. This finding suggests that the conformational change observed in the AaSPS and hSPS1 complex structures can be attributed to the binding of the substrate and substrate analogs found in those structures and not to metal binding alone.

Comparison of the ATP binding sites from EcSPS^{C17S}, AaSPS, and hSPS1 shows overall conservation, as expected. However, an interesting difference between the binding sites involves the residues which serve to bind/stabilize the adenosine moiety of ATP. In AaSPS, two residues (S98 and T133) contribute to binding by forming hydrogen bonds with the adenosine moiety, and in hSPS1, only the second residue, a threonine at position 164

FIG 7 Proposed mechanism for catalysis by EcSPS. EcSPS would first bind Mg^{2+} and K^+ , followed by selenium transfer by either the CsdB-dependent or CsdB-independent (selenide-donor) pathway. Then a conformational change to the closed state would move the Se-charged C17 residue into the proximity of the active site, leading to ATP hydrolysis and the formation of selenophosphate and ADP. Additional energy from ADP hydrolysis is then required for a conformational change back to the open state, which allows the products selenophosphate, orthophosphate, and AMP to be released from the active site. AMP is then able to rebind EcSPS to act as a product inhibitor.

(T164), is conserved. Similar to hSPS1, EcSPS^{C17S} has only a single residue serving the role of stabilizing the adenosine moiety and is only partially conserved. Here a serine at position 141 (S141) is positioned in a position structurally equivalent to that of the conserved threonine (Fig. 4).

Mechanism for EcSPS-mediated selenophosphate synthesis. Based on information obtained from the AaSPS and hSPS1 structures, we propose a mechanism toward catalysis in EcSPS (Fig. 7). In the absence of metal ions and substrates, the glycine-rich N-terminal region (containing active-site residues C17 and K20) is disordered and becomes more ordered upon binding magnesium and possibly potassium ions. Upon ATP binding, the glycine-rich N-terminal region would undergo a conformational change to bring residue C17 into proximity of the catalytic site. Selenide binds to C17 via a selenium-sulfur bond which in turn forms selenophosphate with the gamma phosphate of ATP (22, 32). The proposed order of substrate binding shown in the lower pathway is based on the observation that in *in vitro* experiments, ATP can bind to EcSPS in the absence of selenide, while in the absence of ATP, no significant quantity of selenide-EcSPS was detected (22, 31). However, since the K_m value for selenide is far above the optimal concentration required for cell growth and approached toxic levels, it is believed that *in vivo* a selenium-delivering protein is likely involved in delivering selenium to SPS (32). With such a selenium delivery protein complex, it would be more reasonable for this complex to bind SPS prior to ATP (upper

pathway) because a flexible opened conformation of SPS, which exists prior to ATP binding, would facilitate the interaction between the selenium delivery protein complex and the C17 domain of SPS. To this end, NifS protein from *Azotobacter vinelandii* and the NifS-like proteins in *E. coli*, CsdB, CSD, and IscS, have been shown to deliver selenium from selenocysteine to EcSPS for the synthesis of selenophosphate (23). In addition, Wang et al. (46) proposed that the selenophosphate would remain protected within the active-site cavity until hydrolysis of ADP took place and that the energy of hydrolysis would be used to induce the release of the products selenophosphate, orthophosphate, and AMP.

This mechanism differs from that proposed by Itoh et al. (17), who postulate that ATP binds prior to selenium transfer by the NifS-like protein. However, as shown in the AaSPS and hSPS1 crystal structures, binding of ATP likely induces the closed conformation, which would occlude access to the active site, thereby masking it from interactions with other proteins and restricting conformational flexibility. Furthermore, our mechanism also differs from that of Wang et al. (46) in that while hydrolysis of ADP likely does lead to release of products, we favor the hypothesis that the energy is used for a conformational change from the closed state back to the open state, which has been observed in reported crystal structures, instead of a complete separation of the dimer state, which seems energetically unfavorable given that the dimer interface has a buried surface area of $\sim 2,000 \text{ \AA}^2$ ($\sim 14\%$ of the total surface area). The proposed conformational change to the open

state would be sufficient to allow the products to diffuse away from the active site. This notion is supported by our analysis of the dimer interface, which shows that it is composed largely of hydrophobic residues, and by our analytical ultracentrifugation experiments which show that EcSPS forms a tight dimer. Also, since it has been shown that the K20R mutation retains some activity (18) and since no phosphorylated intermediate has ever been detected, we propose that K20 may play a different role in the formation step of selenophosphate which is likely related to its charge properties and may act to stabilize or orient the phosphate group of ATP, and possibly that of ADP, for catalysis.

In our studies, as well as those with AaSPS and hSPS1, it was observed that four conserved aspartate residues participated in magnesium binding. We found that mutating each of the aspartate residues to alanine completely abolished EcSPS activity. These experiments highlight the importance of the aspartate residues in the binding of magnesium at the active site, which then creates an environment that allows the interaction with the ATP substrate. Similarly, Wang et al. (46) recently identified a conserved threonine residue thought to coordinate the bound monovalent cation; mutating this residue led to nearly complete inactivation of hSPS1. This demonstrates the importance of potassium binding as well. Unexpectedly, we found that mutation of the conserved N87 to alanine also abolished the activity. However, Itoh et al. (17) reported that mutating this conserved asparagine equivalent, N79 in AaSPS, led to an ~3-fold increase in ATP consumption in their assays and concluded that this residue is nonessential. While our EcSPS^{C17S} structure does not show any direct interactions between residue N87 and Mg²⁺, based on the AMPCPP-bound structure of AaSPS (17), we hypothesize that like N79 in AaSPS, N87 would likely play an important role in positioning water molecules to interact with the gamma phosphate of ATP for the synthesis of selenophosphate. This hypothesis is consistent with the data shown in Fig. 5D indicating that the addition of Mg²⁺ and ATP fails to induce a red shift of the 315-nm chromophore exhibited by the N87A mutant, since the red shift requires that the bound nucleotide contain a hydrolyzable gamma phosphate. Nevertheless, it is unclear why mutating this conserved asparagine residue leads to opposite results in AaSPS and EcSPS, and this issue will require further investigation.

In conclusion, EcSPS^{C17S} adopts the same fold as AaSPS and hSPS1 and, similarly, was found as a homodimer in our crystals. We used analytical ultracentrifugation to verify that EcSPS is also a dimer in solution. We have also shown that mutating the four conserved magnesium binding residues, D51, D68, D91, and D227, and the conserved N87 at the active site to alanine completely abolishes AMP production in our activity assays, indicating their essential roles in binding cofactors and substrates during catalysis. Finally, comparison of our unliganded, metal-bound structure (open conformation) to the AaSPS and hSPS1 liganded structures (closed conformations) and the apo AaSPS (open conformation, no ligand, no metals) clearly shows that magnesium binding alone is not sufficient to induce the closed conformation, but instead that ligand binding is also required.

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