Molecular Basis of Cysteine Biosynthesis in Plants

STRUCTURAL AND FUNCTIONAL ANALYSIS OF O-ACETYLSERINE SULFHYDRLASE FROM ARABIDOPSIS THALIANA*

Eric R. Bonner1, Rebecca E. Cahoon, Sarah M. Knapke2, and Joseph M. Jez3

From the Donald Danforth Plant Science Center, St. Louis, Missouri 63132

In plants, cysteine biosynthesis plays a central role in fixing inorganic sulfur from the environment and provides the only metabolic sulfide donor for the generation of methionine, glutathione, phytochelatins, iron-sulfur clusters, vitamin cofactors, and multiple secondary metabolites. O-Acetserine sulphydrylase (OASS) catalyzes the final step of cysteine biosynthesis, the pyridoxal 5′-phosphate (PLP)-dependent conversion of O-acetserine into cysteine. Here we describe the 2.2 Å resolution crystal structure of OASS from Arabidopsis thaliana (AtOASS) and the 2.7 Å resolution structure of the AtOASS K46A mutant with PLP and methionine covalently linked as an external aldimine in the active site. Although the plant and bacterial OASS share a conserved set of amino acids for PLP binding, the structure of AtOASS reveals a difference from the bacterial enzyme in the positioning of an active site loop formed by residues 74–78 when methionine is bound. Site-directed mutagenesis, kinetic analysis, and ligand binding titrations probed the functional roles of active site residues. These experiments indicate that Asn77 and Gln147 are key amino acids for O-acetserine binding and that Thr74 and Ser75 are involved in sulfur incorporation into cysteine. In addition, examination of the AtOASS structure and nearly 300 plant and bacterial OASS sequences suggest that the highly conserved B8A–B9A surface loop may be important for interaction with serine acetyltransferase, the other enzyme in cysteine biosynthesis. Initial protein–protein interaction experiments using AtOASS mutants targeted to this loop support this hypothesis.

Plants assimilate inorganic sulfur as sulfate from the soil or as sulfur dioxide and hydrogen sulfide from the atmosphere (1–3). Incorporation of sulfide into cysteine in chloroplasts, mitochondria, and the cytoplasm is the final metabolic step in environmental sulfur assimilation. Cysteine is the metabolic sulfide donor for all cellular components containing reduced sulfur. In addition to its role in protein structure, cysteine is a precursor of methionine, glutathione, phytochelatins, iron-sulfur clusters, vitamin cofactors, and multiple secondary metabolites.

Two enzymes catalyze the formation of cysteine in plants and bacte-

8 A surface loop may be important

their functional roles of active site residues. These experiments indicate that Asn77 and Gln147 are key amino acids for O-acetserine binding and that Thr74 and Ser75 are involved in sulfur incorporation into cysteine. In addition, examination of the AtOASS structure and nearly 300 plant and bacterial OASS sequences suggest that the highly conserved B8A–B9A surface loop may be important for interaction with serine acetyltransferase, the other enzyme in cysteine biosynthesis. Initial protein–protein interaction experiments using AtOASS mutants targeted to this loop support this hypothesis.

Plants assimilate inorganic sulfur as sulfate from the soil or as sulfur dioxide and hydrogen sulfide from the atmosphere (1–3). Incorporation of sulfide into cysteine in chloroplasts, mitochondria, and the cytoplasm is the final metabolic step in environmental sulfur assimilation. Cysteine is the metabolic sulfide donor for all cellular components containing reduced sulfur. In addition to its role in protein structure, cysteine is a precursor of methionine, glutathione, phytochelatins, iron-sulfur clusters, vitamin cofactors, and multiple secondary metabolites.

Two enzymes catalyze the formation of cysteine in plants and bacte-

8 The abbreviations used are: SAT, serine acetyltransferase; OASS, O-acetserine sulphydrylase; AtOASS, A. thaliana O-acetserine sulphydrylase; SAT4, A. thaliana serine acetyltransferase; NTA, nitritotriacetic acid; PLP, pyridoxal 5′-phosphate; StOASS, S. typhimurium O-acetserine sulphydrylase; FPLC, fast protein liquid chromatography; Mes, 4-morpholineethanesulfonic acid; Mopso, 3-(N-morpholino)-2-hydroxypropanesulfonic acid; r.m.s., root mean square.

9 This work was supported by funds from the Donald Danforth Plant Science Center and United States Department of Agriculture Grant NRI-2005-02518 (to J. M. J.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Present address: Pfizer, Inc., St. Louis, MO 63124.

2 Supported by National Science Foundation–Research Experiences for Undergraduates Grant DBI-024415. Present address: Dept. of Botany and Plant Pathology, Purdue University, West Lafayette, IN 47907.

3 To whom correspondence should be addressed: Donald Danforth Plant Science Center, 975 N. Warson Rd., St. Louis, MO 63132. Tel.: 314-587-1450; Fax: 314-587-1550; E-mail: jjez@danforthcenter.org.
involved in cysteine biosynthesis function and interact with each other, we have determined the three-dimensional structures of AtOASS and the AtOASS K46A mutant. Using these structures as a guide, we examined the role of active site residues in catalysis and ligand binding by site-directed mutagenesis, kinetic analysis, and ligand titration assays. In addition, we test the potential role of a highly conserved loop in AtOASS as a protein-protein interaction site with A. thaliana SAT (AtSAT). These experiments demonstrate the critical role of key amino acids in the OASS active site and provide insight into the molecular basis of cysteine biosynthesis in plants.

**EXPERIMENTAL PROCEDURES**

**Materials—**Integrated DNA Technologies, Inc. (Corvalle, CA) synthesized the oligonucleotides used in this work. The pPCR-Script Amp SK(+) cloning and QuikChange site-directed mutagenesis kits were from Stratagene (La Jolla, CA). The pET28a Escherichia coli expression vector, E. coli BL21(DE3) cells, and E. coli Rosetta(DE3) cells were from Novagen (Madison, WI). Ni²⁺-nitritolactiic acid (NTA) agarose was bought from Qiagen (Valencia, CA). BamHI and then ligating the 1.1-kb DNA fragment into NdeI/BamHI-mated nucleotide sequencing confirmed the fidelity of the AtOASS PCR and the stop codon is in boldface type). The 1.1-kb PCR product was transformed into E. coli BL21(DE3). Transformed cells were grown at 37 °C in Terrific broth containing 50 mM NaCl, 1 mM dithiothreitol and then loaded onto a Superdex-75 size exclusion FPLC column equilibrated in the same buffer without glycerol. Fractions containing AtOASS were concentrated and stored at −80 °C.

His-tagged AtSAT was expressed and purified using a similar procedure, except that E. coli Rosetta(DE3) cells were used for protein expression, and a Superdex-200 size exclusion FPLC column replaced the Superdex-75 column.

**Enzyme Assays—**OASS activity was assayed by measuring the formation of cysteine (27). Standard assay conditions were 100 mM Mops (pH 7.0), 10 mM O-acetylsereine, and 0.25 mM Na₂S in a 0.25-mL reaction volume. Reactions were initiated by the addition of 0.01 μg of AtOASS and incubated for 0, 5, 10, and 15 min at 25 °C. The addition of 50 μL of trichloroacetic acid (20% v/v) final concentration) quenched the reactions. Following centrifugation, 0.25 mL of supernatant was mixed with an equal volume of acetic-ninhydrin reagent. The mixture was heated (95 °C) for 5 min and then cooled on ice for 5 min. Following the addition of 0.5 mL of cold 100% ethanol, the A₅₄₀ was measured. The amount of cysteine in each reaction was determined using a standard curve (0.01–0.5 μmol). Steady-state kinetic parameters were determined by initial velocity experiments, in which product formation was linear over the time monitored (2–20 min) using the above assay with varied concentrations of O-acetylsereine (0–10 mM) at 1 mM Na₂S or varied concentrations of Na₂S (0–15 mM) at 10 mM O-acetylsereine. Substrate concentrations were chosen to avoid substrate inhibition. Mutations that increased Kᵥ for a given substrate were reassayed with concentrations up to 10-fold higher than the Kᵥ value. For both substrates were fitted to the Michaelis-Menten equation, ν = (V_max[S])/(Kᵥ + [S]), using Kaleidagraph (Synergy Software, Reading, PA).

SAT activity was determined by monitoring the formation of O-acetylsereine with a spectrophotometric assay that couples the production of CoA to the generation of 5-thio-2-nitrobenzoate via a disulfide exchange reaction with 5,5’-dithioisobis(2-nitrobenzoate) (28). Assays contained 0.1 mL Hepes (pH 7.5), 0.5 mM 5,5’-dithioisobis(2-nitrobenzoate), 0.1 mM acetyl-CoA, and 5 mM serine. Rates were calculated using the extinction coefficient of 5-thio-2-nitrobenzoate (ε₄₁₂ = 13,600 M⁻¹ cm⁻¹).

**Ligand Binding—**Binding of O-acetylsereine, cysteine, and methionine was measured by monitoring the change in absorbance of the PLP using a Cary Bio300 UV-visible spectrophotometer. Titration of AtOASS (200 μg) with O-acetylsereine (0–220 μM) was performed in 0.1 M Mes (pH 6.0). Titration of AtOASS (200 μg) with cysteine (0–20 mM) or methionine (0–20 mM) was performed in 0.1 M Hepes (pH 7.5). For the AtOASS mutants, appropriate concentrations of ligands were used to saturate the titration curves. The Kᵥ value for each ligand was calculated using the data to a reversible two-state model of binding, ∆A = (A_max[L])/(Kᵥ + [L]), using Kaleidagraph, where ∆A is the change in absorbance at a given wavelength in the presence of ligand at concentration [L]. The data were also plotted as a linear transforms of the same equation (1/∆A versus 1/L).

**Crystallography—**Crystals of AtOASS were grown by the vapor diffusion method in 2-μL hanging drops of a 1:1 mixture of protein (10 mg mL⁻¹) and crystallization buffer (1.8 M ammonium sulfate; 0.1 M Tris, pH 8.0) at 4 °C over a 0.5-mL reservoir. Macroseeding was used to improve crystal size by transferring individual crystals to hanging drops containing a 1:1 mixture of protein (7.5 mg mL⁻¹) and crystallization buffer (1.8 M ammonium sulfate and 0.1 M Tris, pH 8.0). Before freezing...
at 100 K, crystals were washed in 30% glycerol, 1.8 M ammonium sulfate, and 0.1 M Tris (pH 8.0) as a cryoprotectant. All data were collected at 100 K using a Proteum-R Smart 6000 CCD detector connected to a Bruker-Nionis FR591 rotating anode generator. Diffraction intensities were integrated, merged, and scaled using the Bruker Proteum software suite (TABLE ONE). The structure described above. Diffraction intensities were integrated, merged, and scaled using the Bruker Proteum software suite (TABLE ONE).

### RESULTS

#### Three-dimensional Structure

Recombinant AtOASS was expressed in *E. coli* as a hexahistidinetagged protein and purified by nickel affinity and size exclusion chromatography. Digestion with thrombin removed the His tag from the expressed protein. The purified protein was yellow with an absorbance maximum at 412 nm, indicating the presence of PLP as an internal Schiff base in the active site, and displayed a specific activity of 1400 μmol of cysteine produced/min/mg of protein.

The 2.2 Å resolution structure of AtOASS was determined by molecular replacement (TABLE ONE) and reveals a symmetric dimer with each monomer related by a 2-fold crystallographic axis (Fig. 2A). Each monomer consists of two α/β structural domains. The smaller N-terminal domain (residues 45–150) consists of a central four-stranded parallel β-sheet surrounded by four α-helices (α1–α4). The larger C-terminal domain (residues 3–44 and 151–303) folds around a larger C-terminal domain (residues 3–44 and 151–303) folds around a six-stranded β-sheet (β1A–β2A and β7A–β10A) flanked by four α-helices (α5–α8). Residues from the N terminus form the first two strands (β1A–β2A) of the C-terminal β-sheet. An additional α-helix (α9) forms part of the dimerization interface. The dimer interface buries 1380 Å² per monomer or 18.9% of the accessible surface area. The monomer–monomer contacts are formed by multiple hydrogen bonds and hydrophobic interactions.

The overall structure of AtOASS resembles that of the *Salmonella* enzyme (24) with an r.m.s. deviation for the aligned 315 Ca atom pairs of 1.2 Å (Fig. 2B). Structural differences occur in the β1A–β2A and β8A–β9A loops and the C terminus of the two proteins. There is a three-amino acid insertion in the β1A–β2A loop and a six-residue deletion in the β8A–β9A loop of AtOASS compared with StOASS (Fig. 1B). The C terminus of AtOASS extends along the surface of the adjacent monomer, whereas the last 20 amino acids of the StOASS structure were disordered (24).

A search of the Protein Data Bank using DALI (31) showed that AtOASS shares a similar three-dimensional fold with *E. coli* threonine deaminase (32), *S. typhimurium* tryptophan synthase β-subunit (33), *Saccharomyces cerevisiae* threonine synthase (34), *Arabidopsis* threonine synthase (35), Rattus norvegicus serine dehydratase (36), and *Hansenula saturnus* 1-aminoacyclopropane-1-carboxylate deaminase (37). AtOASS

---

### TABLE ONE

|                      | AtOASS       | K46A         |
|----------------------|--------------|--------------|
| Space group          | P4_2_2       | P4_2_2       |
| Cell dimensions      | a = b = 105.2 Å; c = 100.1 | a = b = 105.9 Å; c = 100.2 |
| Resolution           | 15–2.2 Å     | 15–2.7 Å     |
| Reflections (total/unique) | 396,210/26,988 | 83,923/15,981 |
| Completeness (highest shell) | 92.7% (88.1%) | 98.5% (94.9%) |
| Rcryst/ Rfree (%)    | 14.9 (2.2)   | 7.2 (2.0)    |
| Rfree (%)            | 6.45% (39.7%)| 16.2% (36.0%)|
| No. of protein atoms | 2359         | 2324         |
| No. of water molecules | 172          | 72           |
| r.m.s. deviation, bond lengths | 0.006 Å    | 0.008 Å     |
| r.m.s. deviation, bond angles | 1.48°      | 1.53°       |
| Average B-factor, protein/water | 23.0 Å²/29.6 Å² | 25.9 Å²/30.9 Å² |

* a Highest resolution shell: AtOASS, 2.30–2.20 Å; K46A, 2.80–2.70 Å.
* b Rcryst = Σ |Foe| − |Fo|/Σ |Fo|, where |Fo| is the average intensity over symmetry.
* c Rfree = Σ |Foe| − |Fo|/Σ |Fo|, where summation is over the data used for refinement.
* d Rfree is defined the same as Rcryst but was calculated using 5% of data excluded from refinement.

---

**Structure of Arabidopsis O-Acetylserine Sulphydrylase**
is related by 14–21% amino acid sequence identity to these proteins with r.m.s. deviations for the Cα atoms of 2.3–3.0 Å. Since these enzymes require PLP as a cofactor, conservation of the dual α/β-domain structure in each protein maintains the cofactor binding site with structural and sequence variations tailoring substrate recognition and reaction chemistry.

**Active Site Architecture: PLP and Sulfate Binding**

The location of PLP in the AtOASS structure defines the location of the active site (Figs. 2, C and D) with clear electron density for the internal Schiff base between PLP and Lys46 observed (Fig. 2E). In addition to the covalent linkage, Asn77 and Ser269 form hydrogen bonds with the oxygen and nitrogen, respectively, of the pyridine ring. PLP is anchored in the active site by multiple interactions. Hydrogen bonds are formed between Gly181, Thr182, Gly183, Thr185, and the phosphate group. His157 and Thr185 interact with two water molecules, which in turn form hydrogen bonds with the phosphate moiety. The amino acids involved in PLP binding are conserved in both plant and bacterial OASS structures and between the Arabidopsis isoforms (Fig. 1B). The β3B–α2 loop of AtOASS, corresponding to residues 74–78, forms one side of the active site. This loop, which was previously suggested to be involved in substrate binding, adopts the same conformation as observed in the StOASS structure (24). The sequence of this loop (TSGNT) is highly conserved in all plant and bacterial OASS sequences with the exception of the StOASS used for previous crystallization experiments, which replaces the serine with an asparagine (TNGNT) (Fig. 1B). In the AtOASS active site, electron density for a sulfate ion, presumably from the crystallization solution, was observed near the substrate-binding loop. The side-chain hydroxyl group of Thr74 (2.79 Å), the backbone nitrogen of Ser75 (2.77 Å), and the side-chain nitrogen of Gln147 (2.90 Å) interact with the sulfate ion (Fig. 2C).

**Three-dimensional Structure of an External Aldimine Complex**

The sequence difference in the substrate-binding loop of AtOASS and StOASS suggests that the protein-substrate interactions may differ in these enzymes. Efforts to obtain a substrate and/or product analog complex with OASS are complicated by the presence of the active site lysine, which reacts with any external aldimine formed in the active site. To obtain a structure of AtOASS complexed with a ligand in the active site, the AtOASS K46A mutant was generated. The purified mutant protein was inactive for cysteine formation, which was consistent with the catalytic role of the active site lysine (22). An absorbance maximum at 418 nm indicated that PLP had, however, formed a stable external aldime in the active site (21).

Crystals of the AtOASS K46A mutant were obtained, and the 2.7 Å resolution structure of the protein was determined (TABLE ONE). Initial electron density maps unambiguously showed the alanine substitution at position 46 and indicated that PLP formed an external aldime
with an unknown ligand in the active site (Fig. 3A). Given the reaction catalyzed by OASS, glycine and cysteine where modeled in the external aldime linkage, but electron density extending beyond the terminal side-chain atom of either amino acid was observed. For model building and refinement, the external aldime was modeled as a PLP-methionine linkage, since the crystal structure of the StOASS K41A mutant revealed this molecular bond in the active site (25). The overall structures of the wild-type and mutant AtOASS are nearly identical, with an r.m.s. deviation of 0.37 Å². Comparison of the AtOASS K46A mutant, the StOASS, and the StOASS K41A mutant structures shows that the external aldime molecule binds in a similar conformation in the active sites of the plant and bacterial enzymes; however, the substrate-binding loop of AtOASS does not reposition as observed in StOASS (24, 25) (Fig. 3B).

Within the active site of the AtOASS K46A mutant, the side chain of the methionine is oriented toward the active site entrance, and the car-
boxylate group forms contacts with Gln\textsuperscript{147}, Thr\textsuperscript{74}, Asn\textsuperscript{77}, and Thr\textsuperscript{78} (Fig. 3, C and D). These interactions are similar to those observed with the corresponding residues in the StOASS K41A mutant structure (25). In the StOASS structure, Ser\textsuperscript{75} does not interact with the external aldimine, whereas the active site loop of StOASS shifts to place the side chain of an asparagine residue, which corresponds to the serine, within 3 Å of the methionine carboxylate group (25).

**Functional Analysis**

**PLP Binding Site**—An extensive set of conserved hydrogen bond interactions anchor the cofactor in the active site of AtOASS (Fig. 2, C and D). To probe the importance of specific interactions in positioning PLP, amino acid substitutions (H157Q, H157N, T182A, T182S, T185A, T185S, S269A, and S269T) were introduced into AtOASS using site-directed mutagenesis. The mutant proteins were overexpressed, purified, and assayed for cysteine formation (TABLE TWO). Compared with wild-type AtOASS, substitutions of His\textsuperscript{157} and Thr\textsuperscript{182} altered the catalytic efficiency (k\textsubscript{cat}/K\textsubscript{m}) of the enzyme less than 2-fold for either substrate. The decreased reaction rates observed for the S269A and S269T mutants probably result from changes in the position of the pyridine ring, as observed in StOASS (23). Mutation of Thr\textsuperscript{185} to either an alanine or a serine resulted in 400–1600-fold decreases in turnover rates (k\textsubscript{cat}). The absorbance spectra of the T185A and T185S mutants showed a ~10-fold reduction in PLP signal (A\textsubscript{412}) compared with the...
T74A and T74S mutants displayed roughly 15-fold higher catalytic efficiency, as indicated by 105-fold lower catalytic efficiency constant (Kcat) compared to wild-type enzyme, yielding an enzyme with a 105-fold lower catalytic efficiency than wild-type enzyme. Substitution of Asn77 (N77A or N77D) reduces the catalytic efficiency constant (Kcat) by 10-fold.

To determine the effect of the point mutations on ligand binding, titration of wild-type and mutant AtOASS with Na2S was examined for comparison with the wild-type enzyme, indicating that this residue provides a crucial binding interaction with the cofactor.

**Substrate Binding Site**—Although the substrate-binding loop of OASS appears important for cysteine formation, the functional roles of amino acids in this loop remain untested. To examine the contributions of Thr74, Ser75, Asn77, Thr78, and Gln147 to catalysis and ligand binding, we generated the following mutants: T74A, T74S, S75A, S75T, S75N, N77A, N77D, T78A, T78S, Q147A, and Q147E. Using purified recombinant proteins, the kinetic parameters of each AtOASS mutant for O-acetylserine and sulfide were examined for comparison with the wild-type enzyme (TABLE TWO).

**Mutation of Gln147,** the only active site residue not on the substrate-binding loop that contacts methionine in the external aldimine structure, to either an alanine or glutamic acid reduced the catalytic efficiency of AtOASS by 1900–25,400-fold. Incorporation of a negatively charged side chain in the active site (Q147E) decreased the turnover rates more than elimination of the glutamine amide group (Q147A). Although the effects of the Q147E and Q147A mutations were primarily on kcat, the Km value of each mutant for O-acetylserine also increased.

| O-Acetylserine | Na2S |
|----------------|------|
| kcat | km | kcat/km | kcat | km | kcat/km |
| s⁻¹ | mM | s⁻¹ | s⁻¹ | mM | s⁻¹ |
| WT | 1780 ± 280 | 1.4 ± 0.2 | 1,270,000 | 2170 ± 240 | 0.22 ± 0.09 | 9,860,000 |
| T74A | 0.08 ± 0.01 | 1.6 ± 0.2 | 50 | 0.42 ± 0.04 | 3.9 ± 0.3 | 108 |
| T74S | 456 ± 37 | 1.5 ± 0.1 | 304,000 | 1700 ± 270 | 3.2 ± 0.8 | 531,000 |
| S75A | 7.16 ± 0.34 | 1.6 ± 0.2 | 4475 | 20.2 ± 0.9 | 4.6 ± 1.0 | 4390 |
| S75T | 51.5 ± 2.5 | 1.2 ± 0.1 | 42,900 | 82.7 ± 9.2 | 0.21 ± 0.03 | 394,000 |
| S75N | 0.09 ± 0.01 | 2.0 ± 0.3 | 45 | 1.45 ± 0.10 | 5.7 ± 0.6 | 254 |
| N77A | 403 ± 32 | 4.7 ± 0.6 | 85,700 | 155 ± 7 | 0.23 ± 0.03 | 674,000 |
| N77D | 0.34 ± 0.06 | 10.2 ± 1.3 | 33 | 0.73 ± 0.10 | 0.18 ± 0.05 | 4055 |
| T78A | 554 ± 25 | 0.68 ± 0.11 | 815,000 | 537 ± 29 | 0.44 ± 0.08 | 1,220,000 |
| T78S | 775 ± 35 | 0.82 ± 0.14 | 945,000 | 370 ± 26 | 0.30 ± 0.09 | 1,230,000 |
| Q147A | 4.02 ± 0.34 | 4.4 ± 0.3 | 914 | 219.4 ± 7.4 | 1.7 ± 0.2 | 7350 |
| Q147E | 0.19 ± 0.01 | 3.8 ± 1.2 | 50 | 0.22 ± 0.02 | 0.27 ± 0.04 | 815 |
| H157Q | 1055 ± 108 | 0.91 ± 0.14 | 1,160,000 | 1780 ± 51 | 0.30 ± 0.09 | 5,930,000 |
| H157N | 1520 ± 290 | 0.94 ± 0.30 | 1,617,000 | 1990 ± 84 | 0.40 ± 0.10 | 4,975,000 |
| T182A | 889 ± 72 | 1.2 ± 0.1 | 741,000 | 1600 ± 197 | 0.39 ± 0.03 | 4,000,000 |
| T182S | 1660 ± 290 | 1.6 ± 1.0 | 1,038,000 | 1690 ± 181 | 0.22 ± 0.03 | 7,680,000 |
| T185A | 1.07 ± 0.06 | 1.5 ± 0.2 | 713 | 1.44 ± 0.18 | 0.22 ± 0.06 | 6550 |
| T185S | 3.11 ± 0.14 | 1.6 ± 0.3 | 1940 | 5.57 ± 0.82 | 0.36 ± 0.06 | 15,500 |
| S269A | 34.3 ± 4.1 | 0.57 ± 0.20 | 60,200 | 29.5 ± 3.0 | 0.54 ± 0.04 | 54,600 |
| S269T | 572 ± 56 | 0.96 ± 0.16 | 596,000 | 929 ± 35 | 1.6 ± 0.5 | 581,000 |

**Structure of Arabidopsis O-Acetylserine Sulfhydrylase**

OASS is active and SAT is inactive, the location of the wild-type enzyme, indicating that this residue provides a crucial binding interaction with the cofactor.
A loop at the active site entrance suggested this as a potential interaction site with SAT.

Within the β8A-β9A loop of AtOASS, Lys217, His221, and Lys222 were mutated to alanine. These amino acids were chosen for mutagenesis, because polar amino acids are often found at protein interaction hot spots (38–40). Moreover, His221 is highly conserved in the plant and bacterial OASS. The K217A, H221A, and K222A AtOASS mutants were expressed and purified as described for wild-type enzyme to yield proteins lacking the His tag. Each mutant protein catalyzed the formation of

### Table Three

| Ligand binding constants | $K_d$ values were determined as described under “Experimental Procedures” and are expressed as mean ± S.E. for $n = 3$. WT, wild type. |
|--------------------------|----------------------------------------------------------------------------------------------------------------------------------|
|                         | $K_d$ [μM]                                                                                                                      |
|                         | O-Acetylserine | Cysteine | Methionine |
| WT                      | 18.4 ± 2.1     | 0.75 ± 0.1 | 0.70 ± 0.06 |
| T74S                    | 15.6 ± 1.6     | 1.37 ± 0.22 | 1.65 ± 0.12 |
| S75T                    | 14.5 ± 2.0     | 2.19 ± 0.27 | 2.26 ± 0.29 |
| N77A                    | 510 ± 60       | >20      | 8.12 ± 0.60 |
| T78A                    | 29.4 ± 2.8     | 1.72 ± 0.17 | 2.33 ± 0.24 |
| T78S                    | 20.8 ± 2.7     | 0.72 ± 0.13 | 1.63 ± 0.27 |

β9A loop at the active site entrance suggested this as a potential interaction site with SAT.

Within the β8A-β9A loop of AtOASS, Lys217, His221, and Lys222 were mutated to alanine. These amino acids were chosen for mutagenesis, because polar amino acids are often found at protein interaction hot spots (38–40). Moreover, His221 is highly conserved in the plant and bacterial OASS. The K217A, H221A, and K222A AtOASS mutants were expressed and purified as described for wild-type enzyme to yield proteins lacking the His tag. Each mutant protein catalyzed the formation of

![FIGURE 4. Ligand binding titrations of AtOASS.](image)

**A**, titration of AtOASS with O-acetylserine (0–220 μM) to generate the α-aminoacrylate external Schiff base. **B**, variation of $A_{470}$ versus O-acetylserine concentration. **Inset**, double reciprocal plot of the change in $A_{470}$. **C**, titration of AtOASS with cysteine to generate the external Schiff base. **D**, variation of $A_{440}$ versus cysteine concentration. **Inset**, double reciprocal plot of the change in $A_{440}$. **E**, titration of AtOASS with methionine to generate the external Schiff base. **F**, variation of $A_{440}$ versus methionine concentration. **Inset**, double reciprocal plot of the change in $A_{440}$.

**TABLE THREE**

| Ligand binding constants | $K_d$ values were determined as described under “Experimental Procedures” and are expressed as mean ± S.E. for $n = 3$. WT, wild type. |
|--------------------------|----------------------------------------------------------------------------------------------------------------------------------|
|                         | $K_d$ [μM]                                                                                                                      |
|                         | O-Acetylserine | Cysteine | Methionine |
| WT                      | 18.4 ± 2.1     | 0.75 ± 0.1 | 0.70 ± 0.06 |
| T74S                    | 15.6 ± 1.6     | 1.37 ± 0.22 | 1.65 ± 0.12 |
| S75T                    | 14.5 ± 2.0     | 2.19 ± 0.27 | 2.26 ± 0.29 |
| N77A                    | 510 ± 60       | >20      | 8.12 ± 0.60 |
| T78A                    | 29.4 ± 2.8     | 1.72 ± 0.17 | 2.33 ± 0.24 |
| T78S                    | 20.8 ± 2.7     | 0.72 ± 0.13 | 1.63 ± 0.27 |

β9A loop at the active site entrance suggested this as a potential interaction site with SAT.

Within the β8A-β9A loop of AtOASS, Lys217, His221, and Lys222 were mutated to alanine. These amino acids were chosen for mutagenesis, because polar amino acids are often found at protein interaction hot spots (38–40). Moreover, His221 is highly conserved in the plant and bacterial OASS. The K217A, H221A, and K222A AtOASS mutants were expressed and purified as described for wild-type enzyme to yield proteins lacking the His tag. Each mutant protein catalyzed the formation of

![FIGURE 4. Ligand binding titrations of AtOASS.](image)

**A**, titration of AtOASS with O-acetylserine (0–220 μM) to generate the α-aminoacrylate external Schiff base. **B**, variation of $A_{470}$ versus O-acetylserine concentration. **Inset**, double reciprocal plot of the change in $A_{470}$. **C**, titration of AtOASS with cysteine to generate the external Schiff base. **D**, variation of $A_{440}$ versus cysteine concentration. **Inset**, double reciprocal plot of the change in $A_{440}$. **E**, titration of AtOASS with methionine to generate the external Schiff base. **F**, variation of $A_{440}$ versus methionine concentration. **Inset**, double reciprocal plot of the change in $A_{440}$.

**TABLE THREE**

| Ligand binding constants | $K_d$ values were determined as described under “Experimental Procedures” and are expressed as mean ± S.E. for $n = 3$. WT, wild type. |
|--------------------------|----------------------------------------------------------------------------------------------------------------------------------|
|                         | $K_d$ [μM]                                                                                                                      |
|                         | O-Acetylserine | Cysteine | Methionine |
| WT                      | 18.4 ± 2.1     | 0.75 ± 0.1 | 0.70 ± 0.06 |
| T74S                    | 15.6 ± 1.6     | 1.37 ± 0.22 | 1.65 ± 0.12 |
| S75T                    | 14.5 ± 2.0     | 2.19 ± 0.27 | 2.26 ± 0.29 |
| N77A                    | 510 ± 60       | >20      | 8.12 ± 0.60 |
| T78A                    | 29.4 ± 2.8     | 1.72 ± 0.17 | 2.33 ± 0.24 |
| T78S                    | 20.8 ± 2.7     | 0.72 ± 0.13 | 1.63 ± 0.27 |
cysteine with kinetic parameters similar to the wild-type enzyme, indicating that the role of these conserved residues is not the direction of substrates to the active site.

To test whether the K217A, H221A, and K222A mutant proteins interacted with AtSAT, a protein pull-down assay was used. Purified octahistidine-tagged AtSAT (monomer molecular mass = 36.3 kDa) with a specific activity for O-acetylserine formation of 60 μmol of product formed/min/mg of protein was bound to an Ni2+/H9262-NTA column. Following incubation of His-tagged AtSAT with non-His-tagged wild-type, K217A, H221A, or K222A AtOASS (monomer mass of 33.9 kDa), the column was washed to remove unbound protein. Next, bound protein was eluted from the column with imidazole. The wash and elution fractions were analyzed by SDS-PAGE, and SAT activity was assayed.

Fig. 5B shows a comparison of the SDS-PAGE results obtained with the H221A mutant (lanes A and B) and wild-type AtOASS (lanes C and D). After incubation of AtSAT with AtOASS, excess AtOASS was collected from the wash step. The elution fraction contained AtSAT and AtOASS, indicating formation of the cysteine synthase complex. The identities of the two bands in the elution fraction were confirmed by matrix-assisted laser desorption ionization mass spectrometry peptide fingerprinting performed by the Danforth Center Proteomics facility (not shown). The specific activity of AtSAT in this fraction was 400 μmol of product formed/min/mg of protein with low OASS activity (100 μmol of product formed/min/mg of protein) detected. The increase in SAT activity and decrease in OASS activity are consistent with formation of the cysteine synthase complex (6, 41). In contrast, incubation of AtSAT with the H221A AtOASS mutant yielded only AtSAT in the elution fraction with a specific activity of 55 μmol of product formed/min/mg of protein, indicating that the cysteine synthase complex was not formed. Results similar to those obtained for the H221A mutant were also observed with the K217A and K222A AtOASS mutants.

**DISCUSSION**

Compared with the mechanisms of nitrogen and phosphorus uptake and assimilation, plant sulfur metabolism is less well understood at the molecular level, although plants play a central role in the environmental sulfur cycle (2). Within the pathway of sulfur assimilation, OASS catalyzes the production of cysteine and is part of the regulatory system that responds to changes in cellular sulfur supply (5, 6). The crystal structures and functional analysis of AtOASS presented here provide molecular insight into cysteine biosynthesis in plants.

AtOASS, the bacterial OASS, and other enzymes requiring PLP as a cofactor share a common topology in the PLP- and substrate-binding domains (24, 32–37). The overall α/β-domain structure maintains the PLP binding site with sequence variations in the active site diversifying substrate recognition and reaction chemistry. Crystal structures and fluorescence spectroscopy indicate that PLP-dependent enzymes, including OASS, threonine synthase, and serine dehydratase (25, 36, 42–44), shift between open and closed active site conformations.

Detailed studies of StOASS provide a structural model for the reaction catalyzed by the enzyme (45). Crystal structures of uncomplexed (open) and complexed (closed) StOASS formed the basis of this model (24, 25). The reaction mechanism begins with O-acetylserine binding to the resting enzyme, which is in the open conformation. Interaction between the substrate binding loop and the α-carboxylate of bound O-acetylserine (or methionine) was proposed to trigger closure of the active site. In particular, the side chain of an asparagine (Asn69) in StOASS moves 7 Å to interact with the substrate’s carboxylate, locking the substrate in the active site by shifting the position of the loop (25).

The first half-reaction occurs, resulting in the release of acetate. Next, sulfide enters the active site to complete the second half-reaction, yielding cysteine.

Experiments presented here and recent work described by others question the proposed role of the asparagine-substrate interaction as the primary trigger in conformational switching between open and closed active site conformations in OASS. First, the amino acid sequence of the AtOASS active site loop (TSGNT) is highly conserved in all plant and bacterial OASS sequences, whereas the active site loop sequence of StOASS (TNGNT) only occurs in that enzyme (24). Moreover, the S75N AtOASS mutant catalyzes cysteine formation with a 105-fold decrease in catalytic efficiency, indicating that AtOASS and the other OASS enzymes with a serine at this position are functionally different from StOASS. Second, crystal-packing forces probably determine the protein conformation observed in the various three-dimensional structures of OASS. Both wild-type AtOASS and the AtOASS K46A mutant with PLP and methionine linked as an external aldimine crystallized in the open conformation. Likewise, structures of *E. coli* OASS and a mutant version of the same protein adopted open and closed conformations, respectively, in the absence of substrates or analogues bound in the active site (44). Although the asparagine-substrate interaction in StOASS may not act as a general triggering mechanism, the open and closed conformations observed in different OASS structures strongly suggest a functional role for domain movement in the catalytic cycle of the enzyme, as originally proposed.

Extensive studies have examined the chemical reaction of OASS (19), but only limited information is available on the functional roles of residues in the OASS active site. In AtOASS, Lys146 forms a Schiff base linkage to PLP, and multiple interactions anchor the cofactor in the active site (Fig. 2). As observed in StOASS (22), the active lysine of the *Arabidopsis* enzyme is crucial for the catalysis. In addition, Thr185 and Ser269 maintain PLP in a catalytically productive orientation (23), since mutation of either residue affects cysteine synthesis (TABLE TWO).

Based on the AtOASS crystal structures, kinetic analysis, and ligand binding experiments, Asn77 and Gln147 are key residues for O-acetylserine binding. Of the AtOASS mutants assayed, the N77A, N77D, Q147A, and Q147E mutants increased the *Km* value for O-acetylserine (TABLE TWO). Likewise, mutation of Asn77 to an alanine raised the *Kd* for O-acetylserine by 28-fold (TABLE THREE). Moreover, the N77A mutant displayed the largest decreases in binding affinity for cysteine and methionine (TABLE THREE). Although titrations of the N77D, Q147A, and Q147E mutants with O-acetylserine yielded no detectable α-aminoacyl intermediate, these mutants still catalyze cysteine formation with 1000-fold lower turnover rates compared with wild-type enzyme. In the OASS reaction, formation of the α-aminoacyl intermediate is the rate-limiting step, and the intermediate decays with time in the absence of sulfur (20, 46, 47). The lack of detectable intermediate in the N77D, Q147A, and Q147E mutants indicates that the rate of intermediate formation is now slower than the dissociation rate, consistent with an increased *Kf* value for O-acetylserine. These mutants retain the ability to form cysteine at reduced turnover rates, because the addition of sulfide is diffusion-limited (45–47), thus rapidly transforming any α-aminoacyl intermediate formed into product.

Functional analysis of the AtOASS mutants also demonstrates that Thr74 and Ser75 are important active site residues. With regard to sulfide binding, substitution of either amino acid yields mutant enzymes (T74A, T74S, S75A, and S75N) that display more than 10-fold increases in the *Kd* value for sulfide (TABLE TWO). A defined sulfide binding in OASS has not been identified (47); however, the effects of mutating Thr74 and Ser75 suggest that these residues play a role in stabilizing the
transition state of the second half-reaction. The T74A, S75A, and S75N mutations also affect ligand binding, since titrations of these mutant enzymes showed no detectable signal for the α-aminoacrylate intermediate or the external aldimine with cysteine or methionine. As described above, these mutations probably decrease the rate of the first half-reaction, leading to α-aminoacrylate intermediate formation. The T74A mutation eliminates a potential hydrogen bond donor in the active site (Fig. 3, C and D), unlike the T74S mutation, which retains a hydroxyl group at this position and causes minimal changes in binding affinity with O-assay, cysteine, or methionine (TABLE THREE). Although Ser75 does not interact with methionine in the AtOASS K46A crystal structure, in the recently determined structure of E. coli OASS (44), the side-chain hydroxyl group of the corresponding serine contacts the modeled α-aminoacrylate intermediate and sulfur. Thus, the S75A and S75N mutations may disrupt the local structure of the substrate binding loop to affect catalysis when the active site adopts the closed conformation.

The formation of multienzyme complexes provides cells with a means of controlling metabolism by organizing the location of key enzymes (48, 49). In bacteria and plants, SAT and OASS associate to form the cysteine synthase complex, which acts as a molecular sensor in the regulatory circuit that coordinates sulfate assimilation and modulates intracellular cysteine levels (4–6, 50). When the two enzymes are complexed, SAT activity increases and OASS activity decreases. This results in production of O-assay. When intracellular sulfur levels are low, O-assay accumulates, because free OASS is unable to generate cysteine due to a lack of sulfide. Elevated O-assay levels cause the complex to dissociate, thereby down-regulating SAT. Meanwhile, the increased O-assay concentration also activates expression of genes encoding sulfate transporters, OASS, and SAT. This leads to increased sulfur uptake and reduction. As sulfur levels elevate, free OASS begins to catalyze cysteine formation and reduce O-assay levels. This allows association of SAT and OASS, activation of SAT, and resumption of biosynthesis. Yeast two-hybrid (51, 52), gel filtration chromatography (6, 53), and surface plasmon resonance methods (54) demonstrate assembly of this macromolecular complex. Based on these studies, the C terminus of SAT is crucial for interaction with OASS; however, until recently, the region of OASS involved in formation of the cysteine synthase complex has been unclear.

Determination of the three-dimensional structure of the OASS from Haemophilus influenzae complexed with a peptide corresponding to the 10 C-terminal residues of the Haemophilus SAT showed the peptide Haemophilus 8A–9A loop) for providing an Annu. Rev. Plant Physiol. 295, 237–247. 178–1978 Wirtz, M., Droux, M., and Hell, R. (2004) J. Exp. Bot. 55, 1785–1798 Rolland, N., Droux, M., Lebrun, M., and Douce, R. (1993) Arch. Biochem. Biophys. 300, 213–222. Saito, K., Tatsuguchi, K., Takagi, Y., and Murakoshi, I. (1994) J. Biol. Chem. 269, 28187–28192. Rolland, N., Ruffet, M. L., Job, D., Douce, R., and Droux, M. (1996) Eur. J. Biochem. 256, 272–282. Tai, C. H., and Cook, P. F. (2001) Acc. Chem. Res. 34, 49–59. Cook, P. F., Hara, S., Nalabolu, S., and Schnackerz, K. D. (1992) Biochemistry 31, 2298–2303. Schnackerz, K. D., Tai, C. H., Simmons, J. W., Jacobsen, T. M., Rao, G. S., and Cook, P. F. (1995) Biochemistry 34, 12152–12160. Bege, V. S., Kredich, N. M., Tai, C. H., Karsten, W. E., Schnackerz, K. D., and Cook, P. F. (1996) Biochemistry 35, 13485–13493. Daum, S., Tai, C. H., and Cook, P. F. (2003) Biochemistry 42, 106–113. Burkhard, P., Rao, G. S., Hohenester, E., Schnackerz, K. D., Cook, P. F., and Janssion, J. N. (1998) J. Mol. Biol. 283, 121–133. Burkhard, P., Tai, C. H., Ristroph, C. M., Cook, P. F., and Janssion, J. N. (1999) J. Mol. Biol. 291, 941–953. Jez, J. M., Ferrer, J. L., Bowman, M. E., Dixon, R. A., and Noel, J. P. (2000) Biochemistry 39, 809–902. Gaitonde, M. K. (1967) Biochem. J. 104, 627–633. Cook, P. F., and Weddell, R. T. (1977) Arch. Biochem. Biophys. 178, 293–302. Brunger, A. T., Adams, P. D., Clore, G. M., Delano, W. L., Gros, P., Grabe-Kuustelev, R. W., Jiang, J. S., Kuszewski, J. J., Nilges, M., Pannu, N. S., Read, R. J., Rice, L. M., Simonson, T., and Warren, G. L. (1998) Acta Crystallogr. Sect. D 54, 905–921. Jones, T. A., Zou, J. Y., Cowan, S. W., and Kjeldgaard, M. (1993) Acta Crystallogr. Sect. D 49, 148–157. Sander, C., and Schneider, R. (1991) Proteins 9, 56–68. Gallagher, D. T., Gilliland, G. L., Xiao, G., Zondlo, J., Fisher, K. E., Chinchilla, D., and Eisenstein, E. (1998) Structure 6, 465–475. Hyde, C. C., Ahmed, S. A., Padlan, E. A., Miles, E. W., and Davies, D. R. (1988) J. Biol. Chem. 263, 17857–17871. Garrido-Franco, M., Ehlers, S., Messerschmidt, A., MarinKovic, S., Huber, R., Laber, B., Bourenkov, G. P., and Clausen, T. (2002) J. Biol. Chem. 277, 12396–12405. Thomazeau, K., Curien, G., Dumas, R., and Biou, V. (2001) Protein Sci. 10, 638–648. Yamada, T., Komoto, J., Takata, Y., Ogawa, H., Hitot, H., and Takusagawa, F. (2003) Biochemistry 42, 12854–12865. Yao, M., Ose, T., Sugimoto, H., Horiuchi, A., Nakagawa, A., Wakatsuki, Y., Yokoi, D., Murakami, T., Honma, M., and Tanaka, I. (2000) J. Biol. Chem. 275, 34557–34565. Nooren, I. M., and Thornton, J. M. (2003) EMBO J. 22, 3486–3492. Hu, Z., Ma, B., Wolfson, H., and Nussinov, R. (2000) Proteins 39, 331–342. Jones, S., and Thornton, J. M. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 13–20. Saito, K., Yokoyama, H., Noji, M., and Murakoshi, I. (1995) J. Biol. Chem. 270, 16321–16326. McClure, G. D., and Cook, P. F. (1994) Biochemistry 33, 1674–1683. Omi, R., Goto, M., Miyahara, I., Mizuguchi, H., Hayashi, K., Hagamiya, H., and Hirotsu, K. (2002) J. Biol. Chem. 278, 60355–60365. Claus, M. T., Zoccher, G. E., Maier, T. H., and Schulz, G. E. (2005) Biochemistry 44, 8620–8626. Rabeh, W. M., and Cook, P. F. (2004) J. Biol. Chem. 279, 26803–26806. Woehl, E. U., Tai, C. H., Dunn, M. F., and Cook, P. F. (1996) Biochemistry 35,
47. Rabeh, W. M., Alguindigue, S. S., and Cook, P. F. (2005) Biochemistry 44, 5541–5550
48. Hrazdina, G., and Jensen, R. A. (1992) Annu. Rev. Plant Physiol. Plant Mol. Biol. 43, 241–267
49. Winkel, B. S. J. (2004) Annu. Rev. Plant Biol. 55, 85–107
50. Hell, R., and Hillebrand, H. (2001) Curr. Opin. Biotechnol. 12, 161–168
51. Bogdanova, N., and Hell, R. (1997) Plant J. 11, 251–262
52. Wirtz, M., Berkowitz, O., Droux, M., and Hell, R. (2001) Eur. J. Biochem. 268, 686–693
53. Mino, K., Hiraoka, K., Imamura, K., Sakiyama, T., Eisaki, N., Matsuyama, A., and Nakanishi, K. (2000) Biosci. Biotechnol. Biochem. 64, 1874–1880
54. Berkowitz, O., Wirtz, M., Wolf, A., Kuhlman, J., and Hell, R. (2002) J. Biol. Chem. 277, 30629–30634
55. Huang, B., Vetting, M. W., and Roderick, S. L. (2005) J. Bacteriol. 187, 3201–3205