Crystal Structure of a Sulfur Carrier Protein Complex Found in the Cysteine Biosynthetic Pathway of Mycobacterium tuberculosis$^{\dagger,\ddagger}$

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ABSTRACT: The structure of the protein complex CysM–CysO from a new cysteine biosynthetic pathway found in the H37Rv strain of Mycobacterium tuberculosis has been determined at 1.53 Å resolution. CysM (Rv1336) is a PLP-containing β-replacement enzyme and CysO (Rv1335) is a sulfur carrier protein with a ubiquitin-like fold. CysM catalyzes the replacement of the acetyl group of O-acetylseryne by CysO thiocarboxylate to generate a protein-bound cysteine that is released in a subsequent proteolysis reaction. The protein complex in the crystal structure is asymmetric with one CysO protomer binding to one end of a CysM dimer. Additionally, the structures of CysM and CysO were determined individually at 2.8 and 2.7 Å resolution, respectively. Sequence alignments with homologues and structural comparisons with CysK, a cysteine synthase that does not utilize a sulfur carrier protein, revealed high conservation of active site residues; however, residues in CysM responsible for CysO binding are not conserved. Comparison of the CysM–CysO binding interface with other sulfur carrier protein complexes revealed a similarity in secondary structural elements that contribute to complex formation in the ThiF–ThiS and MoeB–MoaD systems, despite major differences in overall folds. Comparison of CysM with and without bound CysO revealed conformational changes associated with CysO binding.

CysM (Rv1336) and CysO (Rv1335) are two proteins that participate in a recently discovered cysteine biosynthesis pathway in Mycobacterium tuberculosis (1). In this pathway CysO thiocarboxylate displaces the acetyl group of O-acetylseryne in a reaction catalyzed by the pyridoxal 5′-phosphate (PLP)$^{1}$ dependent CysM protein. An N → S acyl shift, followed by Mec$^{+}$ (Rv1334) catalyzed hydrolysis generates cysteine (Figure 1). This pathway stands in contrast to the widely used sulfide-dependent cysteine biosynthesis pathway also present in M. tuberculosis (CysK) and may be of importance in the oxidizing environment of the macrophage (2–6) because thiocarboxylates are much more resistant to oxidation than sulfide. Consistent with this, the genes for CysM and CysO are upregulated when the organism is exposed to oxidative stress (7).

Sulfur carrier proteins are structurally homologous to ubiquitin (8) and have now been identified in the biosynthetic pathways for cysteine (1), thiamin (9), molybdopterin (10), and thioquinolobactin (11). Like ubiquitin, the sulfur carrier protein is adenylated at a diglycyl C-terminus by a specific activating protein. The adenylated C-terminus is subsequently converted to a thiocarboxylate, which serves as the sulfide source. Members of the sulfur carrier protein family have diverse binding partners and show little sequence similarity. ThiS is the sulfur carrier protein in thiamin biosynthesis and is activated by ThiF (12). Thiocarboxylated ThiS, deoxy-d-xylulose 5-phosphate, and glycine imine serve as substrates for ThiG in the synthesis of the thiazole moiety of thiamin (13). The sulfur carrier protein MoaD is found in the molybdopterin biosynthetic pathway, and its activating protein is MoeB (14). Crystal structures for the ThiF–ThiS (15), ThiG–ThiS (16), MoeB–MoaD (17), and MoaE–MoaD (18) complexes have been reported. An interesting variation of a sulfur carrier protein is found in the biosynthetic pathway of thioquinolobactin (19). In this system QbsE is the sulfur carrier protein and QbsC is the adenylating protein. QbsE has a diglycyl sequence followed by cysteine and phenylalanine at its C-terminus. Clustered with the genes for these two proteins is a gene for QbsD, a metal-dependent hydrolase, which cleaves the final two amino acids from QbsE generating the diglycyl C-terminus found in all of the other sulfur carrier proteins.

We report here the X-ray crystal structure of the CysM–CysO complex of the cysteine biosynthetic pathway refined to 1.53 Å resolution, the structure of CysM alone at 2.8 Å resolution, and the structure of CysO alone at 2.7 Å resolution. The CysM–CysO complex is asymmetric with one molecule of CysO binding to one end of a CysM dimer. Conformational changes in CysM that occur upon complex formation are described. The CysM–CysO interface is compared to the binding interfaces for ThiF–ThiS, ThiG–ThiS, MoeB–MoaD, MoaE–MoaD, and ubiquitin in complex with the E1-like protein MMS2.

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$^2$ The Brookhaven Protein Data Bank codes for the CysM–CysO complex, the K204A mutant of CysM, and CysO are 3DWG, 3DWI, and 3DWM, respectively.

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$^4$ Abbreviations: PLP, pyridoxal 5′-phosphate; DTT, 1,4-dithiothreitol; rmsd, root-mean-square deviation; OAS, O-acetylseryne; OASS, O-acetylseryne sulfhydrilase.
2 mM MgSO₄, 25 mg/mL FeSO₄, 100 mg/L ampicillin, and reached, followed by induction using 1 mM isopropyl/beta-

MATERIALS AND METHODS

FIGURE 1: The new cysteine biosynthetic pathway in *M. tuberculosis*. All early intermediates are bound to the CysM–CysO complex; however, it is possible that the CysO–thioester adduct is released from CysM before the S–N acyl shift occurs to generate cysteinylated CysO.

Gene Cloning, Overexpression, and Purification. *M. tuberculosis* DNA was a gift from Clifton Barry at the National Institutes of Health. pET-16b and pET-28a plasmids were purchased from Novagen. All plasmid DNA was purified with a DNA miniprep kit from Promega. pET-16b and pET-28a plasmids DNA was a gift from Clifton Barry at the Bioresource Center at Cornell University. DNA fragments as a template for PCR, and sequencing was performed by the Bioresource Center at Cornell University. DNA fragments were purified by agarose gel electrophoresis followed by a QIAquick gel extraction kit from Qiagen. T4 DNA ligase was purchased from New England Biolabs. *Escherichia coli* strain DH5α was used as a recipient for transformation, propagation, and storage. Primers for the CysM plasmid were engineered to introduce *Nde*I and *BamHI* restriction sites at the 5′ and 3′ ends, respectively. The forward primer was 5′-GGG TGA GCG GAG CAT ATG ACA CGA TAC GAC-3′. The reverse primer was 5′-TTC GGA TCC GGC GGA TCC TCA TGC CCA TAG-3′. Primers for the CysO plasmid were engineered to introduce *Nde*I and *XhoI* restriction sites at the 5′ and 3′ ends, respectively. The forward primer was 5′-CCG AGA AAG GCC CAT ATG AAC GTC ACC GTA-3′. The reverse primer was 5′-TCG TGT CAT GTG CTC GAG TCA CCC ACC GGC-3′.

The CysM construct was transformed into B834(DE3) methionine auxotrophic *E. coli* (Novagen) for overexpression. Minimal media used for overexpression of B834(DE3) cells were prepared by dissolving 11.28 g of M9 salts along with 40 mg/L of each amino acid: serine, threonine, asparagine, glutamine, leucine, arginine, valine, phenylalanine, methionine, and lysine. The final media also contained 0.4% glucose, 0.1 mM CaCl₂, 2 mM MgSO₄, 25 mg/mL FeSO₄, 100 mg/L ampicillin, and 10 mM of 10× MEM vitamin solution (Invitrogen). Starter cultures were prepared by growing cells in 20 mL of LB media and then spinning the cells at 4800 g for 5 min. The LB supernatant was decanted and the cell pellet resuspended with 20 mL of minimal media to inoculate 1 L of minimal media. Cells were grown at 37 °C until an OD₆₀₀ of 1.0 was reached, followed by induction using 1 mM isopropyl β-D-thiogalactopyranoside for 16–18 h at 18 °C. Cell pellets were collected by centrifugation at 6400 g for 7 min and placed into a beaker containing 50 mL of a lysis buffer composed of 5 mM imidazole, 0.5 M NaCl, and 20 mM Tris at pH 7.9 to be lysed using sonication. Cell lystate was separated from the insoluble cell particles by centrifugation at 58000 g for 20 min. The supernatant was run over a Ni-NTA affinity column equilibrated with lysis buffer. The column was washed with lysis buffer containing 60 mM imidazole and eluted with lysis buffer containing 1 M imidazole. The eluate was buffer exchanged into 20 mM pH 7.9 Tris and 5 mM 1,4-dithiothreitol (DTT) using size exclusion chromatography columns (Bio-Rad DG) with a molecular mass cutoff of 6 kDa. A centrifugal filter (Amicon Ultra) with a molecular mass cutoff of 10 kDa was used to concentrate the solution by spinning at 5000 g at 4 °C. The final protein concentration was 10–20 mg/mL as measured using the Bradford assay (19). SDS–PAGE analysis showed a purity of greater than 95%. Native CysM and CysO were transformed into BL21(DE3) cells, overexpressed in LB media, and purified as described above, without the use of DTT.

In an attempt to improve crystallization of CysM alone, a putative surface residue in CysM (Lys204) was mutated to alanine using site-directed mutagenesis. Previous studies have shown that mutation of flexible surface residues can lead to reduced surface entropy and improvement in crystal quality (20). A standard PCR protocol using *Pfu* Turbo DNA polymerase per the manufacturer’s instructions (Invitrogen) and *DpnI* (New England Biolabs) to digest the methylated parental DNA prior to transformation was used. The forward primer was 5′-GCA CGT TGC CAA CGT CGC GAT CGT GCG GGG TTC GGC CGC CAC GAT CGC GGA TCC TCA TGC CCA TAG-3′. The reverse primer was 5′-ATG GCC ACC CCG CAC GAT CGC GGA TCC TCA TGC CCA TAG-3′. The forward primer was 5′-TCG TGT CAT GTG CTC GAG TCA CCC ACC GGC-3′. Clones were sequenced by restriction digest for the introduction of a *PvuI* site. A representative clone with the correct restriction pattern was sequenced by the Bioresource Center at Cornell University.

Protein Crystallization and Cryoprotection. A solution of native CysM–CysO was prepared by mixing separate 15 mg/mL solutions each of CysO and CysM in a 2:1 volumetric ratio. This solution was screened using the hanging drop vapor diffusion method. Individual crystals grew in a range of 7–10% PEG 4000 (w/v %), 0.1 M sodium citrate, pH 5.8, and 0.2 M ammonium acetate at 22 °C. Selenomethionine crystals grew in the same conditions with 5 mM DTT. Crystals were green in color and grew to 150 × 150 × 50 μm³ within 2–4 weeks. The K204 mutant was also screened using the hanging drop method with a 10 mg/mL protein.

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solution. Crystals of the K204A mutant grew to approximately $100 \times 40 \times 20 \mu m^3$ in 2–4 weeks using the hanging drop method in 0.1 M HEPES, pH 7.1, and 0.64 M Li$_2$SO$_4$ at 22 °C. Crystals of CysO were grown using the hanging drop method in the presence of the activating protein MoeZ (Rv3206). MoeZ was purified as described previously (1). A solution of native MoeZ–CysO was prepared by mixing separate 10 mg/mL solutions each of MoeZ and CysO in a 1:1 volumetric ratio. Crystals appeared in 0.2 M NaNO$_3$ as colorless thick plates with dimensions of 200 $\times$ 40 $\times$ 40 $\mu m^3$. Preliminary analysis of the X-ray diffraction data showed that these crystals contained only CysO.

Cryoprotection of the CysM–CysO complex and CysM K204A mutant crystals was carried out through sequential dipping into well solutions with increasing concentrations of ethylene glycol. The cryosolution for the selenomethionine derivative and native CysM–CysO crystals contained 9% PEG 4000, 0.1 M sodium citrate, pH 5.8, 0.2 M ammonium acetate, 1 mM DTT, and either 5% or 25% ethylene glycol by volume. Crystals were first looped out of the mother liquor and then placed in the 5% ethylene glycol solution for approximately 10 s, followed by dipping into the 25% ethylene glycol solution for another 10 s. The crystal was then looped out of the 25% solution and plunged into liquid nitrogen for storage. Cryoprotection of the K204A CysM mutant was also done using sequentially increasing concentrations of ethylene glycol. CysM crystals were first dipped into a solution with 5% ethylene glycol, 0.64 M Li$_2$SO$_4$, and 0.1 M HEPES, pH 7.1, followed by dipping into the same cryosolution with 25% ethylene glycol for approximately 10 s each. Crystals of CysO were cryoprotected by dipping into a solution of 0.2 NaNO$_3$, 20% PEG 3350, and 25% PEG 4000 for approximately 10 s before plunging into liquid nitrogen.

**X-ray Data Collection.** Data for the selenomethionine derivative CysM–CysO crystal were collected at the 24-ID-C beamline at the Advanced Photon Source to 2.1 Å resolution. The energy was calibrated with Se foil, and an X-ray fluorescence scan was done on the CysM–CysO crystal to determine the absorption edge. Images were taken using a Quantum ADSC Q315 detector (Area Detectors Systems Corp.) for 720 frames with 1° oscillations in 10° wedges. Native data for the CysM K204A and CysM–CysO complex crystals were collected at the 8-BM beamline at APS using 1° oscillations for 140° and 300°, respectively, using a Quantum ADSC Q315 detector. Data for the CysO crystals were collected using a Rigaku RTP 300 RC rotating copper anode generator operating at 50 kV and 100 mA. A Rigaku RAXIS IV++ image plate detector was used for image detection. Data collection was done in 1° oscillations for a total of 215°. Data collection statistics for all data sets are summarized in Table 1. Integration and scaling of all data was done using the HKL2000 suite of programs (21).

**Table 1: Data Collection Statistics**

| Sample          | SeMet CysOM | native CysOM | K204A CysM | native CysO |
|-----------------|-------------|--------------|------------|-------------|
| Source          | APS 24-ID-C | APS 8-BM     | APS 8-BM   | home        |
| Wavelength (Å)  | 0.97950     | 0.97949      | 0.94980    | 1.54178     |
| Resolution (Å)  | 2.10        | 1.53         | 2.80       | 2.70        |
| Space group     | $P_2_1$     | $P_2_1$      | $P_2_1$    | $P_2_1$     |
| Unit cell parameters | |||| |
| $a$ (Å)         | 56.2        | 55.8         | 72.4       | 87.9        |
| $b$ (Å)         | 80.6        | 80.4         | 85.4       | 62.9        |
| $c$ (Å)         | 91.5        | 89.6         | 98.9       | 36.5        |
| $\beta$ (deg)   | 106.8       | 105.8        | 90         | 114.6       |
| No. of reflections | 45147      | 110648       | 15513      | 5022        |
| No. of unique reflections | 7000       | 633477       | 875722     | 20088       |
| Redundancy      | 14.4 (12.0) | 3.0 (2.5)    | 5.5 (5.3)  | 4.0 (3.0)   |
| Completeness    | 98.9 (92.5) | 96.3 (81.6)  | 99.6 (96.2)| 99.2 (95.4) |
| $R_{int}$ (%)   | 7.8 (30.9)  | 4.6 (21.9)   | 8.0 (24.7) | 14.5 (39.2) |
| $I_{o}$ (%)     | 40.1 (6.6)  | 26.2 (4.0)   | 18.6 (4.1) | 14.5 (3.5)  |

$^a$ Merged with low-resolution data from the SeMet crystal. $^b$ $R_{int} = \sum_{i=1}^{N} I_i - \langle I \rangle / \Sigma I_i$, where $\langle I \rangle$ is the mean intensity of the $N$ reflections with intensities $I$, and common indices $hkl$. 

The final refinement statistics for all data sets and the complex were used as an initial model to refine the CysO only data. All but residues 91–93 were built into the model. The final refinement statistics for all data sets and the
geometries and distances of all final models as evaluated by PROCHECK (29) are shown in Table 2.

**Molecular Modeling of Intermediates.** The program MacroModel, version 9.1 (30, 31) was used to model both the O-acetylsereine (OAS)—PLP adduct 3, the α-aminoacrylate intermediate 5, and intermediate 6. All water molecules were removed from the structure, and the C-terminus of CysO was converted to a thio carbonylate and manually adjusted to point toward the active site. Hydrogen atoms were added where appropriate. The initial position of each intermediate was approximated by overlaying the structure of O-acetylsereine sulhydrolase from *Salmonella typhimurium* containing an external aldime linkage to methionine (32) with CysM and superimposing their respective aldime Cα and carboxylate atoms. Modeling was carried out using a 20 Å shell surrounding the OAS moiety of the adduct, with residues within a 6 Å shell allowed to move and the remaining residues frozen. Some of the key residues in the conformational search included 91-AGG-93 from CysO and 184-GTTGT-188, Lys51, Ser265, and 322-WA-323 from CysM. Torsional rotation was allowed between all atoms within the PLP—OAS adduct connected by a single bond in the conformational search parameters. Modeling was done using the AMBER8 force field (33, 34) and a distance-dependent electrostatic treatment with a dielectric constant of 4.0. The TNCG minimization method was used for the energy minimization of structures generated by conformational search (35). Conformational searching with the α-aminoacrylate intermediate was done in the same way. The final models were manually adjusted to optimize the expected reaction geometry.

**Figure Preparation.** All figures of protein molecules and residues were generated in PyMOL (36).

### RESULTS

**CysM Structure.** The CysM molecule consists of eight α-helices, five 310-helices, and nine β-strands (Figure 2A,B). The protomer has a large and a small domain, each with an αβα sandwich fold. The large domain has a mixed β-sheet with β1|β2|β9|β4|β6|β11|β4|ββl topology, while the smaller domain consists of a three-stranded parallel β-sheet comprised of strands β3, β4, and β5. This topology is consistent with other β-elimination enzymes (37). The interface between CysM molecules consists of loop regions with 60% of the residues being hydrophobic at the dimer interface. Other interactions include a salt bridge between Arg3 and Glu175 and a series of hydrogen-bonding pairs that are listed in Table 3.

**CysO Structure.** The CysO monomer is 93 residues in length and 9.6 kDa in mass. The secondary structure consists of two 310-helices, a mixed four-stranded β-sheet with a β3|β4|β1|β2 topology, and one α-helix inserted between β2 and β3 (Figure 2C,D). This topology describes the β-grasp fold that is seen in the sulfur carrier proteins ThiS (15) and MoaD (17) as well as ubiquitin (38).

**CysM—CysO Complex.** The asymmetric CysM—CysO complex consists of a CysM dimer with a CysO molecule bound to one end (CysMα denotes the protomer interacting with CysO, and CysMβ denotes the unbound protomer) (Figure 2E). It is unclear whether or not the asymmetric complex is an artifact of crystallization. Native analytical gel analysis was inconclusive, and attempts to purify the complex suggest that CysO is weakly bound to CysM (unpublished data). The CysMα and CysMβ protomers show two main differences. First, the loop region spanning residues 211–237 in CysMα contains three 310-helices and extends away from the core of the structure to accommodate CysO binding. CysMβ adopts a more compact structure with an α-helix spanning residues 217–222 and a single 310-helix spanning residues 228–231. Second, the smaller domain comprising strands β3, β4, and β5 and helix α4 is shifted away from the core of the complex structure. Only the conformation of the CysO binding loop in CysMα allows for hydrogen bonds to form between the region connecting β5 and α4 in the smaller domain. The hydrogen bonds that contribute to this shift in CysMβ are between the carboxyl acid side chain from Glu126 and the carbonyl oxygen atom of Ala218 and between the amide nitrogen atom of Gly217 and the carbonyl oxygen atom of Val216.

The comparison between CysO molecules from the complex structure and the CysO alone structure shows that the overall structure remains the same, with a root-mean-square deviation (rmsd) between 89 Ca atom carbons of 0.5 Å. One main difference is that the C-terminus of CysO in the complex is well ordered through several hydrogen bonds with CysM. The absence of CysM binding leads to disorder in the final three residues of CysO alone, which were not modeled into the structure. Twenty-five percent of the accessible surface of CysO is buried in the complex as calculated by the protein—protein interaction server (39). Key hydrogen bonds between CysO and CysM are listed in Table 4. The side chain oxygen atom of Asp65CysO is hydrogen bonded to His271A through a bridging water molecule (subscripts CysO and A refer to CysO and CysMα, respectively). A bridging water molecule is found donating hydrogen bonds to the carbonyl oxygen atoms of both Gly92CysO and Leu183A, and two bridging water molecules form a hydrogen-bonding network connecting the carbonyl oxygen atoms of Val90CysO and Phe226A. Two salt bridges also occur at the protein—protein interface between Arg12CysO and Glu214A and between Asp65CysO and Arg211A.

**Active Site.** A PLP cofactor is found in each protomer of CysM covalently attached to Lys51 through a Schiff base linkage. Figure 3 illustrates key interactions between CysM and PLP. A glycine- and threonine-rich sequence binds the

| Table 2: Refinement Statistics |
|--------------------------------|
| sample | SeMet | native | K204A | native |
|--------|-------|--------|-------|--------|
| no. of non-H atoms | 5529 | 6512 | 4648 | 1290 |
| no. of protein atoms | 5150 | 5454 | 4443 | 1266 |
| no. of water atoms | 349 | 1028 | 160 | 24 |
| no. of ligand atoms | 30 | 30 | 45 | 0 |
| R working (%) | 19.2 | 17.6 | 19.0 | 19.5 |
| R free (%) | 24.3 | 21.7 | 25.6 | 25.2 |

**Table Notes:**

- a $R_{work} = \Sigma|F_o| - |F_c|/\Sigma|F_o|$, where $F_o$ and $F_c$ are the observed and calculated structure factors, respectively.
- b $R_{free}$ was calculated using 5% of all reflections that were excluded at all stages of refinement.

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phosphate group and is characteristic of PLP binding proteins (40). A large area of open space occupied only by water molecules spans the distance between Gly93 of CysO and the PLP molecule. The distance from the Schiff base forming carbon atom of PLP to the closest C-terminal oxygen atom of CysO is 11.3 Å.

**DISCUSSION**

*CysM and Structural Homologues.* Structural homologues were identified using the DALI server (www.ebi.ac.uk/dali), and the top hits were selected for comparison (Table 5). The closest structural homologues are β-elimination enzymes, which use OAS or phosphoserine as a substrate to make cysteine. CysK, the sulfide-dependent cysteine synthase, has rmsd values of 2.4 Å for 281 Cα carbon atoms from CysM_A and 1.8 Å for 278 Cα carbon atoms from CysM_B. *S. typhimurium* O-acetylserine sulfhydrylase (OASS) has rmsd values of 2.5 Å for 287 Cα carbon atoms from CysM_A and 1.9 Å for 283 Cα carbon atoms from CysM_B. The conformation of the CysO binding loop of CysM is the most significant
The conformation of the binding loop in CysM is more similar to the structural homologues than in CysM, which results in the lower rmsd values. Another structural difference is that the C-terminal tail of CysM encompassing residues 300–323 contains two 310 helices and points directly into the active site. Both the CysK and OASS structures lack this N-terminal extension.

### CysM and CysK Active Sites

The PLP binding geometries of CysM and CysK are very similar (Figure 4). The final two residues of CysM, Trp322 and Ala323, point toward the active site of the protein. A π-stacking interaction between Trp322 and Tyr212 helps to position the C-terminus. Residues 218–222, rather than the C-terminus, occupy this region in CysK. These residues correspond to the CysO binding loop of CysM based on sequence alignment. Additionally, there is a molecule of 2-methyl-2,4-pentanediol in CysK corresponding to the region of CysM spanning the C-terminus of CysO and the PLP cofactor. The C-terminus of the CysM K204A mutant also does not point to the active site. However, the CysO binding loop of one of the K204A protomers is seen ordered and has the same conformation as the corresponding residues in CysK.

### CysO and Structural Homologues

Structural homologues were identified using the DALI server and selecting the top hits for comparison (Table 6). This included ThiS, MoaD, and ubiquitin, which were compared to CysO through structural superposition. Each possesses the same topology, with most structural differences found in loop regions.

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**Table 3: Dimeric Interactions**

| A chain | B chain | H-bond atoms |
|---------|---------|--------------|
| Thr2    | Asp172  | OH–Oδ       |
| Tyr4    | Leu16   | NH–O         |
| Tyr4    | Gly18   | O–NH         |
| Asp5    | Gln20   | O–NH         |
| Leu92   | Arg21   | O–NH         |
| Glu111  | Leu301  | NH–O         |
| Leu115  | His256  | O–NH         |
| Tyr116  | Gly259  | OH–O         |
| Asp172  | Arg3    | O–NH         |
| Glu175  | Arg3    | Oe–NH        |
| His256  | Arg91   | O–Ne         |

**Figure 3:** CysM active site with PLP coordinating residues labeled with green carbon atoms.

**Figure 4:** Schematic representation of CysM (A) and CysK (B) binding interactions with the PLP cofactor.

**Table 4: CysM–CysO Hydrogen Bonds**

| CysM | CysO | H-bond atoms |
|------|------|--------------|
| Asn130 | Ala89 | NH–O         |
| Asn130 | Ala91 | Oe–HN        |
| Glu209 | Tyr62 | Oe–HO        |
| Tyr217 | Gly93 | OH–O         |
| Phe226 | Ala89 | NH–O         |
| Arg239 | Asp67 | O–HN         |
| Ser241 | Asp65 | OH–Oδ        |

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**Table 5: CysM Structural Homologues**

| PDB code | Z-score | aligned Cα atoms | rmsd | annotation |
|----------|---------|------------------|------|------------|
| 2Q3B     | 34.8    | 281              | 2.4  | cysteine synthase |
| 1OAS     | 34.2    | 287              | 2.5  | O-acetylserine sulfhydrolase |
| 1WKV     | 32.7    | 278              | 2.0  | O-phosphoserine sulfhydrolase |
| 2TYS     | 27.9    | 274              | 2.7  | tryptophan synthase |
| 1TDJ     | 25.9    | 271              | 2.9  | threonine deaminase |
| 1VT1     | 24.7    | 267              | 3.2  | serine racemase |
| 1PWE     | 23.9    | 264              | 3.4  | serine dehydratase |
| 1F2D     | 22.9    | 262              | 2.7  | 1-aminoacyclopropane-1-carboxylate deaminase |
| 1E5X     | 21.7    | 269              | 3.6  | threonine synthase |
| 1K7L     | 20.7    | 269              | 3.5  | threonine synthase |

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**Table 6: CysO Structural Homologues**

| PDB code | Z-score | aligned Cα atoms | rmsd | annotation |
|----------|---------|------------------|------|------------|
| 1V8C     | 14.9    | 86               | 1.8  | Thermus thermophilus MoaD |
| 1FMA     | 9.5     | 78               | 2.3  | MoaD from MoaE–MoaD complex |
| 1JW9     | 8.9     | 77               | 2.8  | MoaD from MoeB–MoaD complex |
| 2G1E     | 8.9     | 82               | 2.9  | neurabin SAM domain |
| 1XO3     | 8.1     | 80               | 2.9  | Mus musculus Urm1 |
| 2BB5     | 7.9     | 78               | 2.6  | transcobalamin II |
| 1C1Y     | 5.4     | 63               | 2.5  | Ras-binding domain of c-Raf1 |
| 1ZUD     | 5.1     | 58               | 2.6  | ThiS from ThiF–ThiS complex |
| 1TYG     | 4.7     | 59               | 3.2  | ThiS from ThiG–ThiS complex |
| 1ZGU     | 4.4     | 62               | 2.6  | ubiquitin from MMS2–Ub complex |

**CysM and CysK Active Sites.** The PLP binding geometries of CysM and CysK are very similar (Figure 4). The final two residues of CysM, Trp322 and Ala323, point toward the active site of the protein. A π-stacking interaction between Trp322 and Tyr212 helps to position the C-terminus. Residues 218–222, rather than the C-terminus, occupy this region in CysK. These residues correspond to the CysO binding loop of CysM based on sequence alignment. Additionally, there is a molecule of 2-methyl-2,4-pentanediol in CysK corresponding to the region of CysM spanning the C-terminus of CysO and the PLP cofactor. The C-terminus of the CysM K204A mutant also does not point to the active site. However, the CysO binding loop of one of the K204A protomers is seen ordered and has the same conformation as the corresponding residues in CysK.

**CysO and Structural Homologues.** Structural homologues were identified using the DALI server and selecting the top hits for comparison (Table 6). The β-grasp proteins complexed with a binding partner were also used for comparison. This included ThiS, MoaD, and ubiquitin, which were compared to CysO through structural superposition. Each possesses the same topology, with most structural differences found in loop regions.
rather than secondary structural elements (Figure 5). CysO has insertions spanning residues 7–19 and 48–55, which are absent in the homologues. The exception is MoaD, which aligns well with residues 7–19 in CysO. Both CysO and MoaD form a key binding interaction with their respective binding partner in this region. The rmsd values between Ca positions in CysO are 2.6 Å for ThiS for 58 Ca carbon atoms, 2.8 Å for MoaD for 77 Ca carbon atoms, and 2.6 Å for ubiquitin for 62 Ca carbon atoms.

CysM–CysO Interactions and Conserved Residues. The features required for interactions with CysO come from three main sections of the CysM molecule. First is the CysO binding loop encompassing residues 211–237, which contributes the majority of CysO binding interactions. Second is helix α4, which is part of the smaller domain of the CysM molecule. Asn130 from this helix forms one of the key hydrogen bonds that stabilize the C-terminal region of CysO. Third, strand β8 forms hydrogen bonds with the loop that proceeds from the β3 strand of CysO.

In order to determine key CysM residues, sequence alignments were performed using both homologues that utilize sulfur carrier proteins and ones that incorporate sulfur directly from sulfide. Residues Asn81 and Ser265 that bind the PLP cofactor are exclusively conserved in both types. The glycine- and threonine-rich sequence that binds the phosphate group of the cofactor varies slightly in the position of glycine and threonine or serine residues, but overall the motif is conserved. A sequence alignment of 200 nonredundant CysM sequences shows that none of the residues on the CysM surface involved with CysO binding are conserved. When the alignment is restricted to the organisms Mycobacterium vanbaalenii, Mycobacterium smegmatis, Mycobacterium ulcerans, and Mycobacterium flavescens, all of which have both CysO and CysM genes, the CysO binding residues are exclusively conserved. Conservation of the CysM–CysO binding interface is maintained within the Mycobacterium genus but shows greater variation, especially in CysM, for other species.

Comparison of the CysM–CysO Interface with Other β-Grasp Protein Complexes. In order to determine if regions...
of CysM and CysO involved in protein–protein interactions are similar to other \( \beta \)-grasp protein complexes, the structures ThiF–ThiS (15) ThiG–ThiS (16), MoeB–MoaD (17), MoaE–MoaD (10), and ubiquitin in complex with the E2-like protein MMS2 (41) were compared in Figure 6. Table 7 lists several properties of the sulfur carrier proteins and ubiquitin at the protein–protein interface. The percent of hydrophobic residues at the interface ranges from 61% to 72% for the sulfur carrier protein examples, while the ubiquitin–MMS2 structure falls below this range at 58%. The number of hydrogen bonds between sulfur carrier proteins and their binding partners ranges from 9 to 11, but the ubiquitin–MMS2 structure has only 5. This is due to ubiquitin having much less of its accessible surface area in close contact with its binding partner. In the sulfur carrier protein examples, 20–25% of the accessible surface area is in close contact with each binding partner. In ubiquitin, only 13% of the surface makes up the binding interface with MMS2.

Structural alignments were made by superimposing each sulfur carrier protein or ubiquitin complex using only CysO as the template. No structural similarities between CysM, ThiG, MoaE, or MMS2 are observed. However, two \( \beta \)-strands common to CysM, ThiF, and MoeB align when each sulfur carrier protein is overlaid. The strands \( \beta 7 \) and \( \beta 8 \) in CysM align with strands \( \beta 6 \) and \( \beta 7 \) in ThiF and MoeB despite the fact that CysM has a different fold. The regions in the sulfur carrier proteins and ubiquitin that make up most of the binding interface come from strands \( \beta 3 \) and \( \beta 4 \) and the residues following \( \beta 4 \) to the C-terminus. The ubiquitin complex structure has the smallest interface with only residue 42 from strand \( \beta 3 \) and residue 68 from \( \beta 4 \) contributing to hydrogen bonds at the interface.

**Mechanistic Implications.** A mechanistic proposal for the CysO–CysM-catalyzed reaction, based on the extensive mechanistic characterization of the sulfide-dependent cysteine synthase (42), is outlined in Scheme 1. In this proposal, \( \theta \)-acetylserine forms an imine with PLP which then undergoes a deprotonation to give 4. Acetate elimination followed by thiocarboxylate addition to the resulting aminoacrylate gives 6. The reaction is completed by a tautomerization to 7 followed by a transimination reaction to give thioester 8, which then undergoes an N/S acyl shift to give cysteinyl-CysO 9.
minimization, followed by manual optimization, were carried out using MacroModel for both the PLP–OAS adduct 3 and the α-aminoacrylate intermediate 5 in the active site. In the PLP–OAS model (Figure 7A), the carboxylate moiety of OAS accepts a hydrogen bond from the backbone nitrogen atom of Asn81. One oxygen atom of the acetate moiety accepts a hydrogen bond from the hydroxyl group of Tyr212 while the other accepts a hydrogen bond from the amide nitrogen atom of Ala323. In the α-aminoacrylate model (Figure 7B), the carboxylate moiety accepts hydrogen bonds from the hydroxyl group of Tyr212 and the amide nitrogen atom of Ala323. The conformation of the C-terminus of CysO shows the sulfur atom at a distance of 3.7 Å from the β-carbon α-aminoacrylate intermediate 5. Using the ami-
noacylate as a starting point, a model for the subsequent intermediate 6 in which the carbon–sulfur bond has formed was generated (Figure 7C).

The structural and modeling studies are consistent with the mechanistic proposal in Scheme 1. PLP is bound at the active site via an imine with Lys51. The model of the enzyme O-acetylsersine (OAS) complex (Figure 7A) shows Lys51 displaced by the amino group of OAS and that Lys51 could serve as the base involved in the Cα deprotonation. The resulting carbanion is stabilized by delocalization into the PLP cofactor as well as by delocalization into the OAS carboxylate which forms multiple hydrogen-bonding interactions with the enzyme (backbone amides of Asn81 and Thr82, OH of Thr82). The acetate of OAS is reasonably positioned for departure, after planarization of Cα following deprotonation, with the scissile C—O bond perpendicular to the plane of the delocalized π system. This suggests an E1cB mechanism rather than an E2 mechanism for this elimination reaction, which occurs with trans stereochemistry. Acetate is also activated as a leaving group by hydrogen bonding of its carbonyl oxygen to the amide NH of threonine 185 (nonoptimal orientation in the model because Cα is not yet planarized). The thiocarboxylate sulfur is located 5.21 Å from the carbon to which it will add. This large separation suggests that, after acetate departs, the thiocarboxylate group of CysO occupies the acetate binding site and that thiocarboxylate addition is the microscopic reverse of acetate departure. This predicts that the Cβ substitution reaction is occurring with overall retention of stereochemistry.

The model of the aminocarboxylate intermediate (Figure 7B) shows the thiocarboxylate in position to add to the Cβ of the aminocarboxylate (Cβ—S distance = 2.48 Å). The thiocarboxylate occupies the acetate binding site but is not hydrogen bonded to the amide NH of Thr185. This model also predicts that the Cα carbanion, generated after thiocarboxylate addition, is stabilized by delocalization into the cofactor as well as by delocalization into the Cβ carboxylate, which forms hydrogen bonds with the amide NH and the side chain OH of Thr82 and with the backbone nitrogen atom of Asn81. Lys51 is reasonably positioned to protonate this carbanion (Cα—N distance = 4.08 Å).

In the enzyme thioester model (Figure 7C), Lys51 is reasonably positioned to mediate the transamination required for product release. There are no hydrogen-bonding interactions to the thioester carbonyl group whose carbon is held 4.89 Å from the cysteiny1 amino group. This suggests that the final N/S acyl shift is not enzyme catalyzed and occurs after dissociation of the thioester from CysM.

Conclusions. The structure of CysM—CysO is an example of a β-elimination enzyme in complex with a sulfur carrier protein used to generate the amino acid cysteine. CysM is able to bind CysO using a binding loop that is ordered differently in structural comparators and when CysO is unbound. The binding loop contains residues that are only conserved among other species within the Mycobacterium genus that also utilize a sulfur carrier protein for cysteine biosynthesis. The overall fold of CysO is similar to that of other sulfur carrier proteins and ubiquitin. Two β-strands at the interface of CysO and CysM are structurally aligned when compared to the E1-like adenyltransferase proteins ThiF and MoaD. Modeling studies show that the C-terminus of CysO is able to reach the β-carbon of the α-aminocarboxylate intermediate in a conformation favorable for nucleophilic addition and that cysteine formation proceeds by a mechanism that is similar to that used by the sulfide-dependent cysteine synthase.

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