Cysteine Synthase (CysM) of Mycobacterium tuberculosis Is an O-Phosphoserine Sulphydrylase

EVIDENCE FOR AN ALTERNATIVE CYSTEINE BIOSYNTHESIS PATHWAY IN MYCOBACTERIA

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The biosynthesis of cysteine is a crucial metabolic pathway supplying a building block for de novo protein synthesis but also a reduced thiol as a component of the oxidative defense mechanisms that appear particularly vital in the dormant state of Mycobacterium tuberculosis. We here show that the cysteine synthase CysM is, in contrast to previous annotations, an O-phosphoserine-specific cysteine synthase. CysM belongs to the fold type II pyridoxal 5′-phosphate-dependent enzymes, as revealed by the crystal structure determined at 2.1-Å resolution. A model of O-phosphoserine bound to the enzyme suggests a hydrogen bonding interaction of the side chain of Arg220 with the phosphate group as a key feature in substrate selectivity. Replacement of this residue results in a significant loss of specificity for O-phosphoserine. Notably, reactions with sulfur donors are not affected by the amino acid replacement. The specificity of CysM toward O-phosphoserine together with the previously established novel mode of sulfur delivery via thiocarboxylated CysO (Burns, K. E., Baumgart, S., Dorrestein, P. C., Zhai, H., McLafferty, F. W., and Begley, T. P. (2005) J. Am. Chem. Soc. 127, 11602–11603) provide strong evidence for an O-phosphoserine-based cysteine biosynthesis pathway in M. tuberculosis that is independent of both O-acetylserine and the sulfate reduction pathway. The existence of an alternative biosynthetic pathway to cysteine in this pathogen has implications for the design strategy aimed at inhibition of this metabolic route.

In the dormant phase Mycobacterium tuberculosis is surviving within granulomas in the lungs of infected individuals where the environment is characterized by hypoxia, nutrient starvation, and oxidative stress (1–3). The latter involves nitrogen monoxide and its spontaneously formed derivatives (reactive nitrogen intermediates), which are used by phagocytes to kill internalized bacteria (4). The prime targets of reactive nitrogen intermediates are cysteine and tyrosine side chains (5). Bacteria in the group actinomycetales involving Streptomyces, Corynebacteria, and Mycobacteria produce mycothiol (1-d-myoinosyl-2-(N-acetyl-cysteinyl) amino-2-deoxy-α-D-glucopyranoside) as their principal low molecular mass thiol, which has a crucial role in maintaining redox balance and contains a cysteine-derived cysteamine moiety (6). Thus the first line of defense of the pathogen against free radicals and hence its long term survival within granulomas are directly linked to the availability of cysteine. Gene expression and proteome analysis of various models of dormant M. tuberculosis also consistently identified genes involved in sulfur metabolism as up-regulated (7–9). Inhibition of cysteine biosynthesis therefore appears to be a potential target for novel antibacterial agents specifically directed toward this pathogen in its persistent phase (10, 11).

De novo l-cysteine biosynthesis in plants, eubacteria, and some archaea starts from l-serine and acetyl-CoA that are condensed to O-acetyl-l-serine (OAS)3 by serine acetyltransferase, denoted CysE in M. tuberculosis (Scheme 1). In a second step this metabolite is converted into l-cysteine by OAS sulfhydrilase (OASS) (12, 13) (Scheme 2). In many bacteria pyridoxal phosphate (PLP)-dependent OASS is present as two isoenzymes, denoted CysK and CysM (14). These isoenzymes show 25–45% identity in amino acid sequence but exhibit characteristic differences in their substrate specificity with respect to the sulfur donor. CysK uses hydrogen sulfide produced by the sulfate reduction pathway as a sulfur source, whereas the CysM enzymes characterized so far tend to accept thiosulfate or larger substrates as sulfur donors (15–17).

The genome of M. tuberculosis H37Rv encodes three genes that were annotated as OASS. Biochemical and structural studies of CysK1 (Rv2334) established that this enzyme is an O-acetyl serine sulfhydrilase, using OAS and sulfide as substrates (18), whereas the function of CysK2 (Rv0848) remains to be elucidated. In an earlier functional study of CysM (Rv1336),

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4 The abbreviations used are: OAS, O-acetyl-l-serine; OPSS, O-phosphoserine sulfhydrilase; PLP, pyridoxal 5′-phosphate.
it was shown that this enzyme is a sulfhydrylase that uses a novel mode of sulfur delivery (15). Instead of incorporation of sulfide into the enzyme-aminoacrylate intermediate as in other OASSs, MtbCysM uses a small protein, denoted CysO (Rv1335) that is thiocarboxylated at the C terminus as sulfur source (15). The reaction leads to the formation of a covalent cysteine adduct at the C terminus of CysO as primary reaction product. To complete cysteine formation, a zinc-dependent metalloprotease (Rv1334) cleaves the CysO-cysteine bond, releasing L-cysteine. The genes coding for CysM, CysO, and the metalloprotease are located on the same transcriptional unit in the genome (15). Mutants carrying a transposon insertion in cysM were shown to be attenuated in macrophages (19) and in a mouse tuberculosis model (20).

In this paper we show that recombinant PLP-dependent CysM from M. tuberculosis, contrary to annotations in sequence data bases, is not an OAS sulfhydrylase but uses O-phosphoserine (OPS) as sulfur acceptor. A three-dimensional model of the external aldimine formed between the cofactor and the substrate, based on the 2.1 Å crystal structure reported here, suggests an arginine residue at the substrate binding site as a major determinant for recognition of the phosphate group of OPS. Consistent with this model, amino acid replacement of this residue by site-directed mutagenesis results in a major loss in catalytic activity with OPS as substrate. The CysM-dependent pathway of cysteine biosynthesis in M. tuberculosis thus not only utilizes a protein-bound thiocarboxylate instead of sulfide as demonstrated earlier (15) but is also independent of OAS as a sulfur acceptor (Scheme 1).

**EXPERIMENTAL PROCEDURES**

**Gene Cloning and Site-directed Mutagenesis**—The DNA sequence coding for CysM from M. tuberculosis (Rv1336) was amplified by PCR and cloned in the expression vector pET28a (Novagen) using upstream NdeI and downstream HindIII sites, resulting in a cleavable six-histidine tag at the N terminus (expression construct pET-His6CysM9). In this construct, thrombin cleavage results in a recombinant protein with three additional amino acids (Gly-Ser-His) at the N terminus. As a consequence of the cloning procedure, the second amino acid in the CysM sequence (Thr) is changed to alanine.

**SCHEME 1.** The two independent pathways of cysteine biosynthesis in M. tuberculosis. In the conventional (left branch) pathway L-serine (Ser) is condensed with an acetyl group to form OAS, which in the next step is converted to cysteine by OASS (CysK1, Rv2334). The CysM (Rv1336)-dependent pathway utilizes OPS (this work) and the CysO (Rv1335)-thiocarboxylate (CysO-SH), resulting in the CysO-Cys adduct that is hydrolyzed by the carboxypeptidase mec (Rv1334) releasing L-cysteine and regenerating CysO (15). OPS is synthesized from 3-phosphoglycerate (3PG) in two steps by SerA (serA1, Rv2996c; serA2, Rv0728c) and SerC (Rv0884c). B, organization of the operons encoding CysE, CysK1 from the OAS specific pathway (left), and CysM, CysO, and mec from the OPS-specific pathway (right) in the M. tuberculosis genome.

**SCHEME 2.** Schematic representation of the reaction catalyzed by PLP-dependent sulfhydrylases. The reaction shown is that for CysM, with OPS as substrate and CysO-thiocarboxylate as a sulfur donor. In the typical OAS sulfhydrylases the substrate is OAS, and sulfide acts as sulfur donor.
The R220A mutant variant was prepared using the QuikChange site-directed mutagenesis (Stratagene) approach starting from pET-His6-CysM9 as template. CysO (Rv1335) was cloned in the pTYB2 (New England Biolabs) vector with upstream NdeI and downstream SmaI sites. This construct results in a translational fusion with the vector-supplied inteine and chitin-binding domain and produces the native protein sequence after the inteine-catalyzed self-cleavage.

**Protein Production and Purification—Escherichia coli BL21(DE3) carrying the construct pET-His6-CysM9 was cultivated in 1.6 liters of LB medium supplemented with kanamycin (30 μg/ml) at 21 °C. At an A600 value of 0.7–0.8 pyridoxal was added to a concentration of 10 mM, and gene expression was induced by the addition of isopropyl β-D-thiogalactopyranoside to 0.1 mM. After ~24 h the cells were harvested and then resuspended in a buffer consisting of 10 mM Tris-HCl, pH 8.0, 300 mM NaCl, and 10 mM imidazole. The cells were disrupted by freeze/thaw cycles, lysozyme, and DNase I treatment followed by sonication. The clarified lysates were loaded on a nickel-nitrilotriacetic acid column (Qiagen) with a column volume of 1.8 ml. After extensive washing with 10 mM imidazole, the remaining proteins were eluted with an imidazole step gradient. Pure His6-CysM eluted in fractions containing 100–200 mM imidazole. Fractions containing His6-CysM were pooled, loaded on a Sephadex G-25 desalting column (GE Healthcare), and eluted in a buffer of 25 mM Tris-HCl, pH 8.0, 150 mM NaCl. The N-terminal His6 tag was removed by thrombin cleavage. Residual noncleaved His-CysM was retained by passage through a nickel-nitrilotriacetic acid column in the presence of 10 mM imidazole. The flow-through containing the cleaved CysM protein was pooled and loaded on a Superdex-200 column pre-equilibrated with 25 mM Tris-HCl, pH 8.0, 150 mM NaCl. This procedure resulted in homogeneous CysM as verified by SDS-PAGE. The CysM protein was concentrated to 21.2 mg/ml (determined according to Bradford using a bovine serum albumin standard) and purified on a chitin affinity column (Thermofisher Scientific) with a column volume of 10 ml by an Amicon centrifugation device with a 10-kDa molecular mass cut-off. Aliquots of the protein preparations were flash-frozen in liquid nitrogen and stored at −80 °C until further use. The CysM R220A mutant was expressed and purified in the same way as the wild type enzyme.

Native CysO was produced in *E. coli* and purified on a chitin affinity column according to the recommendations of the manufacturers of the chitin affinity beads and pTYB2 vector (New England Biolabs). Thiocarboxylated CysO was prepared as described previously (21).

**Spectrophotometry—UV-visible absorbance spectra of recombinant CysM were recorded at an enzyme concentration of 28.1 μM in 25 mM Tris-HCl, pH 8.0, 150 mM NaCl buffer using a Jasco V-65 spectrophotometer. Spectra of the covalent aminoacrylate complex were obtained immediately after the addition of 1.0 mM O-phosphoserine to the enzyme solution.

**Enzyme Activity Measurements—**Recombinant CysM was assayed for O-phosphoserine and O-acetylserine sulphydrylase activity spectrophotometrically at 560 nm by monitoring the formation of cysteine using the acid-ninhydrine method (22). The reactions were carried out in a total volume of 2.0 ml in 50 mM HEPES buffer at pH 7.4 at 37 °C. The reaction mixture contained O-acetyl-l-serine or O-phospho-l-serine at final concentrations of 5 mM, respectively, and 5.0 μg of enzyme.

The reactions were started by the addition of sodium sulfide to a concentration of 4 mM (stock solution of 400 mM sodium sulfide in 1 mM NaOH) to the assay mixtures. Aliquots of 250 μl were taken at various time points, and the reaction was stopped by the addition of trichloroacetic acid (final concentration, 16.6%). Following centrifugation, the amount of product was determined spectrophotometrically using a standard curve for cysteine in the range 0.04–0.8 mM. Specific activities were derived from the linear part of the time curves, and each measurement was carried out in triplicate.

**Determination of Kinetic Parameters for the First Half-reaction—**The first half-reaction using O-phosphoserine as substrate was followed by a stopped flow spectrophotometer by monitoring the appearance of the aminoacrylate intermediate at 463 nm in 100 mM Tris-HCl buffer at pH 7.5 in 100-μl reaction volumes. Substrate concentrations were 0.025, 0.05, 0.1, 0.2, 0.4, 0.8, 1.0, 2.0, 2.5, 3.0, and 4.0 mM with an enzyme concentration of 12 μM. Because of the slower reaction rates, the reaction with O-acetylsersine as substrate concentration range (1.0–6.0 mM) was recorded in a spectrophotometer at 463 nm in 100-μl reaction volumes at an enzyme concentration of 23.8 μM in 100 mM Tris-HCl buffer at pH 7.5.

The kinetic analysis of the individual half-reactions was carried out using the formalism described by Cook and co-workers (14, 23). First order rate constants (kobs) were derived from an exponential fit to the recorded spectrophotometric data. The first order rate constants were plotted against substrate concentrations and fitted to a linear equation to obtain the second order rate constants (kmax/Ks).

**Conversion of the Aminoacrylate Intermediate—**The CysM-aminoacrylate intermediate was prepared prior to the measurements by incubating 600 μM of enzyme and 1.0 mM of O-phosphoserine in 100 mM Tris-HCl, pH 7.5, buffer for 2 min, followed by desalting using a NAP-5 column (GE Healthcare) pre-equilibrated with the reaction buffer 100 mM Tris-HCl, pH 7.5, to remove nonreacted substrate. The ability of potential sulfur donors to react with the aminoacrylate was examined initially by the addition of CysO-thiocarboxylate (60 μM), Na2S (8.0 mM), and sodium thiosulfate (5 mM), respectively, to a solution of 16 μM enzyme-intermediate complex. Decomposition of the aminoacrylate complex was monitored spectrophotometrically at 463 nm.

The time course of the second half-reaction with CysO-thiocarboxylate (concentrations of 15, 22.5, 30, and 60 μM) was followed using a stopped flow spectrophotometer. The reaction with hydrogen sulfide (0.125, 0.25, 0.5, 1.0, 1.5, 2.5, and 4.0 mM) was recorded in a similar way in a spectrophotometer. In both cases the reaction was monitored by the disappearance of the aminoacrylate intermediate at 463 nm in 100 mM Tris-HCl buffer at pH 7.5 in 100-μl reaction volumes. The derivation of the kinetic parameters was performed in the same way as for the first half-reaction.

**Mass Spectrometry—**Samples of CysO-thiocarboxylate (5 μg) were removed directly after the CysO-thiocarboxylate purification step and after the completion of the reaction with the CysM aminoacrylate. Both samples were diluted in 10 mM HEPES, pH 7.4, 150 mM NaCl, 5% glycerol, 0.75 mM Tris(2-carboxyethyl)phosphine, 2.5% acetonitrile, and 0.05% formic acid and analyzed using a mass spectrometer.
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Characterization of M. tuberculosis CysM—Recombinant MtbCysM was produced and purified to electrophoretic homogeneity using a hexahistidine tag at the N terminus that was subsequently removed by thrombin cleavage. Gel filtration chromatography of purified samples of MtbCysM suggested that the enzyme is a dimer in solution, similar to other members of this enzyme family. Recombinant CysM has a yellow color and shows the typical absorption spectrum of PLP-dependent enzymes (30). The absorbance peak at 412 nm (Fig. 1) is char-

![Graph](image)

**FIGURE 1.** UV-visible spectra of the internal aldimine complex of CysM (solid line) and the reaction intermediate formed after the addition of the substrate OPS to 1.0 mM concentration (dashed line). The spectra were recorded at 28.1 μM enzyme concentration in a buffer containing 25 mM Tris-HCl, pH 8.0, 150 mM NaCl.

of 0.263 and an R factor of 54.0%, with two subunits in the asymmetric unit. The relative position and orientation of these monomers corresponded well to the expected dimer. To monitor the behavior of the refinement process, 5% of the x-ray data were removed for the calculation of Rfree. The initial cycles of restrained refinement using Refmac5 (27) resulted in a drop of the R factor by 9.8%. The correctness of the molecular replacement solution was confirmed by electron density for the cofactor, including the covalent linkage to the active site lysine residue appearing at the expected positions.

Manual rebuilding of the model was carried out with the program Coot (28), based on sigma-A weighted 2Fo −Fc and Fo −Fc electron density maps (29). Examination and adjustment of the model was interspersed with rounds of refinement using Refmac5 (27). Water molecules were added based on peak heights, shape of the electron density, temperature factor, and capability to form hydrogen bonds with surrounding protein residues and/or other water molecules. The final model contains the two chains of the CysM dimer, amino acid residues 3–313 from chain A, residues 1–316 of chain B, two PLP molecules, and 221 water molecules. Residues 124–130 and 211–226 in chain B and the last residues in the C termini of both chains did not have defined electron density and were omitted from the model. Details of the refinement and protein models are given in Table 1. The crystallographic data have been deposited with Protein Data Bank accession code 3DKI. The figures were made using the program Pymol. The sequence alignment was made with the program Align.

**TABLE 1**

Statistics of data collection and refinement

The numbers in parentheses are for the highest resolution shell.

| CysM | Data collection | Refinement |
|------|----------------|------------|
| **Resolution** | Beamline: ID14.4, ESRF | Resolution (Å) 2.1 (2.10–2.21) |
| **Space group** | P2_1_2_1 | Multiplicity 3.1 (3.2) |
| **Unit cell** | 74.4, 89.2, 98.8 | Rmerge (%) 7.1 (45.3) |
| **No. of subunits in asymmetric unit** | 2 | Mean I/σ(I) 13.9 (2.6) |
| **No. of reflections** | Overall 120,368 (17,873) | Completeness (%) 98.8 (99.7) |
| **Special details** | Unique 38,431 (5613) | Wilson B factor (Å²) 28.1 |

**RESULTS**

acid and loaded to a CapLC system™ and Q-Tof™ quadrupole/orthogonal acceleration time-of-flight mass spectrometer (Waters Corp., Manchester) using the method described by Sundqvist et al. (24). The spectra were combined and deconvoluted to zero charged ions with the maximum Entropy™ 1 algorithm in Masslynx software (Waters Corp., Milford, MA).

Crystallization and Data Collection—Crystallization screening was carried out using the vapor diffusion method and a Phoenix crystallization robot. After extensive screening and optimization, the diffraction quality crystals of CysM were obtained by equilibrating a mixture of 2 μl of protein solution (21.2 mg/ml) and 2 μl of reservoir solution (0.1M Tris-HCl, pH 7.25–7.5, 0.1M K2HPO4, 4.3M NaCl) against the reservoir solution in sitting drops. The crystals appeared after 24–48 h and were transferred to a solution containing 25 mM Tris-HCl, pH 7.5, 25 mM K2HPO4, 1.1M NaCl, and 30% PEG5K MME before flash-freezing in liquid nitrogen.

X-ray data to 2.1 Å were collected at the Beamline ID14/4 of the European Synchrotron Radiation Facility (Grenoble, France) at 110 K. The x-ray data were processed and scaled with the programs MOSFLM and SCALA from the CCP4 suite (25). The crystals belong to the space group P2_1_2_1 with the cell dimensions a = 74.4 Å, b = 89.2 Å, and c = 98.8 Å. The statistics of the data set are given in Table 1.

Molecular Replacement and Crystallographic Refinement—The structure was determined by molecular replacement using the program MOLREP (26). The coordinates for one subunit of CysK1 from M. tuberculosis (Protein Data Bank accession code 2Q3B) (18) with the cofactor PLP and the solvent atoms omitted were used as the search model. The best solution had a score of 0.263 and an R factor of 54.0%, with two subunits in the asymmetric unit. The relative position and orientation of these monomers corresponded well to the expected dimer. To monitor the behavior of the refinement process, 5% of the x-ray data were removed for the calculation of Rfree. The initial cycles of restrained refinement using Refmac5 (27) resulted in a drop of the R factor by 9.8%. The correctness of the molecular replacement solution was confirmed by electron density for the cofactor, including the covalent linkage to the active site lysine residue appearing at the expected positions.

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Recent studies have shown that thiocarboxylated cysteine (CysO) can act as a sulfur donor in biological processes, such as hydrogen sulfide, thiosulfate, thio-nitro-bensoate, and other sulfur-containing compounds. The Journal of Biological Chemistry has published research on MtbCysM, a putative O-phosphoserine sulfhydrylase, which catalyzes the reaction of O-phosphoserine (OPS) with CysO-thiocarboxylate to form cysteine and phosphate.

In this study, the authors investigated the specificity of MtbCysM towards various sulfur donors and acceptors. They compared the turnover rates for OPS and O-phosphoserine (OAS) with CysO-thiocarboxylate as the acceptor, and with sulfide as the sulfur donor. The kinetic parameters for the individual half-reactions were determined using the method of Gaitonde (22). The specific activities were measured at different temperatures, and the data were evaluated using the formalism described by Cook et al. (14, 23).

**TABLE 2: Specific activities of sulfhydrylases with sulfide as sulfur source**

| Enzyme | Source | Sulfur acceptor | Turnover number | Temperature | Reference |
|--------|--------|-----------------|-----------------|-------------|-----------|
| CysM   | M. tuberculosis | OPS             | 0.1             | 37          | This work |
| CysM   | M. tuberculosis | OAS             | ND              | 37          | This work |
| CysK   | M. tuberculosis | OAS             | 211             | 30          | Ref. 18   |
| CysK   | Salmonella typhimurium | OAS | 280             | 25          | Ref. 31   |

The reactions were followed spectrophotometrically by monitoring the appearance or disappearance of the aminoacrylate intermediate at 463 nm. The data were evaluated using the formalism described by Cook et al. (14, 23).

**FIGURE 2:** Upper panel, mass spectrum of CysO-thiocarboxylate (9574 Da). The peak with the lower mass corresponds to native CysO (9557 Da). Lower panel, mass spectrum of the covalent CysO-cysteine adduct produced in the second half-reaction (9659 Da).

MtbCysM is able to form the aminoacrylate intermediate with both substrates, OAS and OPS, however, at considerably different rates. The addition of OPS to CysM results in immediate formation of the aminoacrylate external aldimine with the characteristic absorption bands at 317 and 463 nm (Fig. 1). An identical spectrum was obtained after the addition of OAS; however, the reaction took more than 40 min to complete, indicating a much slower reaction rate with this substrate. The second order rate constants ($k_{max}/K_a$) determined for the first half-reaction with the substrates OAS and OPS show a more than 600-fold difference in favor of OPS (Table 3).

We then examined the influence of alternative sulfur donors such as hydrogen sulfide, thiosulfate, thio-nitro-bensoate, and CysO-thiocarboxylate on the rate of the reaction. Begley and colleagues (15) have shown that thiocarboxylated CysO can act as a sulfur donor in the MtbCysM catalyzed reaction with OAS. We therefore produced recombinant native CysO and prepared CysO-thiocarboxylate, verified by mass spectrometry (Fig. 2), and used it as a reaction partner in the second half-reaction. The addition of CysO-thiocarboxylate to a solution of MtbCysM aminoacrylate intermediate led to fast decomposition of the intermediate (supplemental Fig. S1). The second half-reaction occurs with CysO-thiocarboxylate and with sodium sulfide as sulfur donors, whereas thiosulfate, the substrate for E. coli CysM (16, 17), or thio-nitro-bensoate (data not shown) failed to react with the aminoacrylate intermediate in the case of MtbCysM (supplemental Fig. S1). The product of the reaction with CysO thio-carboxylate was identified by electrospray mass spectrometry as the expected CysO-cysteine adduct (Fig. 2), whereas the reaction product with sulfide as nucleophile was identified as thioneine using the acid-ninhydrine reagent. The second half-reaction is significantly faster with CysO-thiocarboxylate as substrate compared with Na$_2$S (supplemental Fig. S1). The second order rate constant ($k_{max}/K_a$) for the reaction of the enzyme-aminoacrylate intermediate with the CysO-thiocarboxylate is more than 1000-fold higher than with sulfide as sulfur donor (Table 3).

**Three-dimensional Structure of M. tuberculosis CysM**—As one step in the characterization of CysM and to provide a future framework for inhibitor design, we also determined the three-dimensional structure of the internal aldimine of MtbCysM by...
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FIGURE 3. A, cartoon representation of the subunit structure of MtbCysM. Helices are colored in blue, strands are shown in brown, and the PLP cofactor is shown as a stick model. B, surface representation of MtbCysM showing the deep cleft at the entrance to the active site. The PLP is shown in stick, and the aldimine bond of the cofactor to the catalytic lysine residue is accessible for the substrate.

FIGURE 4. A, superposition of the OAS-specific MtbCysK1 and the OPS-specific MtbCysM active sites. CysK1 residues are shown in yellow, CysM residues shown in olive, and the polypeptide chain of MtbCysM is shown providing the framework of the active site cleft. Arg220 and Tyr152 are indicated. B, model of the external aldimine formed between OPS and PLP at the active site of MtbCysM. The observed positions of the side chain of Tyr152 and Arg220 in the crystal structure are shown in blue, and the modeled energy-minimized positions in the complex are in orange. Note that only a minor conformational change is required to optimize PLP-OPS-enzyme interactions in the complex.

protein crystallography. The asymmetric unit of the crystals contains a dimer of CysM, and the subunits are related by a molecular 2-fold axis. Each of the subunits consists of two domains, reflecting the typical fold of type II PLP enzymes (32, 33), with one pyridoxal-phosphate cofactor covalently bound to an invariant lysine residue (Lys51) at the active site (Fig. 3A). The N-terminal domain (residues 46–153) contains a parallel β-sheet sandwiched by four α-helices, two of which are part of the dimer interface, and one α-helix located on the opposite side of the sheet forming part of the active site entrance. The C-terminal domain consists of residues 11–45, including a unique insertion of eight amino acids (27–34), and residues 154–323. This domain is built up by a six-stranded mixed β-sheet sandwiched by four α-helices, two on each side.

Structural studies of members of this enzyme family have revealed several conformations of the enzyme, an open conformation (34–37), a closed conformation with bound substrates or reaction intermediates (18, 38), and an inhibited form with chloride bound at an allosteric site (39). MtbCysM was crystallized in the open conformation, and structural comparisons of the subunit to known structures of related sulfhydrylases typically result in root mean square deviations for the Cα positions of 1.2–1.3 Å, for instance 1.3 Å for 277 equivalent residues for M. tuberculosis CysK1 (18) and 1.2 Å based on 286 equivalent residues for E. coli CysM (37). A similar root mean square deviation value was obtained for the structural superimposition with O-phosphoserine sulfhydrylase from Aeropyrum pernix (40), 1.7 Å based on 272 equivalent residues. It is noteworthy that the archaeal enzyme carries an additional domain of 80 residues at the N terminus. These root mean square deviation values reflect levels of amino acid identities in primary sequences, obtained from sequence alignments, 36.8% to M. tuberculosis CysK1, 38.3% E. coli CysM, and 22.7% to O-phosphoserine sulfhydrylase (supplemental Fig. S2).

The entrance of the active site (Fig. 3B) is formed by the loops comprising residues 100–108, 124–132, and 210–229. These loops are defined in chain A of MtbCysM, but the segments 124–130 and 211–226 lack defined electron density in chain B, indicating flexibility in the absence of bound substrate. The superposition of the OAS-specific MtbCysK1 and the OPS-specific MtbCysM highlights several residues that are different in the two active sites, in particular Arg220 which might be involved in substrate binding (Fig. 4A).

The PLP-binding Site—The enzyme active site is located in a cleft formed between the two domains within one subunit, and the active site is accessible via a channel that extends from the enzyme surface to the cofactor (Fig. 3B). Pyridoxal-5’-phosphate is bound via a covalent linkage to the side chain of Lys51. The pattern of PLP-enzyme interactions is identical in the two subunits of MtbCysM and very similar to those previously seen in related OASS enzymes (34–36, 41).

Modeling of the OPS External Aldimine in the Active Site of MtbCysM—Based on the crystal structures of MtbCysM and the M. tuberculosis CysK1-aminoacrylate intermediate (18), a model of the PLP-OPS external aldimine complex was built into the active site of MtbCysM chain A. Only minor conformational changes of amino acid side chains were required to accommodate the substrate in the active site pocket (Fig. 4B). Arg220 is in a suitable position for hydrogen bonding with the phosphate, and additional possible hydrogen bonding interactions of the phosphate group are to the hydroxyl group of Tyr152 and the side chain of Thr185.

CysM R220A Mutant—To probe the potential role of Arg220 in the recognition of the OPS substrate by MtbCysM, we replaced this residue by alanine using site-directed mutagenesis. The purified R220A mutant showed an absorption spec-
trum identical to wild type CysM with an absorption band at 412 nm reflecting the Schiff base between Lys$^{51}$ and PLP. Formation of the aminoacrylate intermediate from OAS in the mutant is, however, severely compromised, with an $\sim700$-fold slower rate (Table 3). Notably, neither the rate of aminoacrylate formation using OAS as substrate nor the rate of the second half-reaction with sulfide or with thiocarboxylated CysO as sulfur donors are seriously affected by the mutation, indicating a specific role for this residue in the first half-reaction with OPS (Table 3).

**DISCUSSION**

*CysM from *M. tuberculosis* Is an O-Phosphoserine Sulfhydrylase*—The in vitro data of *Mtb*CysM clearly identify the enzyme as a phosphoserine sulfhydrylase, with a very minor activity as an OAS sulfhydrylase that most likely is too low to be relevant in a metabolic context. In agreement with the findings of Begley and colleagues (15), we also show that the most efficient and probably natural sulfur donor is thiocarboxylated CysO. The reaction with sulfide is slow compared with *bona fide* OAS enzymes where the rate of the second half-reaction was determined as $>1000 \text{ s}^{-1}$ (23). This observation indicates that the aminoacrylate intermediate is not as exposed to spontaneous nucleophilic attack as in OASS-A (CysK) enzymes. It remains, however, unclear at present how the enzyme controls access to the thiocarboxylated C-terminal of CysO while at the same time preventing reaction with sulfide.

**Structural Basis for Switch in Substrate Specificity in the OASS/OPSS Family**—Molecular modeling and amino acid sequence comparisons of members of the OAS family have identified a conserved arginine residue in the active site that possibly interacts with the phosphate group of the substrate OPS. Amino acid replacement by site-directed mutagenesis yielded a mutant with an almost complete loss of substrate preference for OPS. A similarly located arginine side chain (Arg$^{297}$) in the OPSS from the archa *A. pernix* was shown to be important for the OPS activity of the archal enzyme (40). The amino acid sequence comparisons did not reveal any other amino acid differences between the OASSs on the one hand and the OPSSs on the other hand that unambiguously can be related to the switch in substrate preference between these enzymes. It is noteworthy that amino acid replacement at position 220 did not affect the reaction of the mutant with the sulfur donor; i.e. Arg$^{220}$ is specifically involved in recognition of the OPS substrate.

**Implications for Cysteine Biosynthesis in Mycobacteria**—The present schemes of cysteine biosynthesis in mycobacteria (Scheme 1) all assume OAS to be the metabolite that provides the carbon skeleton of the cysteine product. Two pathways have been described to provide the sulfur source. The sulfate reduction pathway leads to the formation of sulfide that is incorporated into OAS by CysK1 (18). An additional pathway of sulfur incorporation has been described recently by the demonstration that CysM can utilize thiocarboxylated CysO as a sulfur source (15). The identification of CysM as a phosphoserine sulfhydrylase presented here establishes, however, an alternative route of cysteine formation in *M. tuberculosis* that is not dependent on OAS (Scheme 1). Our results show that the

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