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Structural enzymology of sulphur metabolism in *Mycobacterium tuberculosis*

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ABSTRACT

The emergence of multidrug-resistant strains of *Mycobacterium tuberculosis* poses a serious threat to human health and has led to world-wide efforts focusing on the development of novel vaccines and antibiotics against this pathogen. Sulphur metabolism in this organism has been linked to essential processes such as virulence and redox defence. The cysteine biosynthetic pathway is up-regulated in models of persistent *M. tuberculosis* infections and provides potential targets for novel anti-mycobacterial agents, directed specifically toward the pathogen in its persistent phase. Functional and structural characterization of enzymes from sulfur metabolism establishes a necessary framework for the design of strong binding inhibitors that might be developed into new drugs. This review summarizes recent progress in the elucidation of the structural enzymology of the sulphate reduction and cysteine biosynthesis pathways.

1. Introduction

Mycobacterium tuberculosis, the causative agent of tuberculosis (TB), poses a major threat to human health. It is now more than a decade ago since the World Health Organization declared TB "a global health emergency", resulting presently in approximately two million deaths per year. Due to the emergence of multidrug-resistant and extensively drug-resistant strains of *M. tuberculosis* (MDR- and XDR-TB, respectively), it will inevitably become more difficult to treat TB in the future. The need for new drugs to extend the range of TB treatment options is acute. Chemotherapy is further complicated by the ability of *M. tuberculosis* to persist in the lungs of infected individuals for decades by switching to a dormant or latent phase [1], which also induces tolerance to current antibiotics [2,3]. Current estimates by the WHO indicate that about one-third of the world's population is infected with persistent mycobacteria, providing an enormous potential reservoir for further spread of the disease. Reactivation of these dormant bacteria can occur either spontaneously or as the consequence of an immune-compromised state, e.g., HIV infection, resulting in active TB. The lengthy treatment makes patient compliance difficult, and non-compliance is a frequent source of drug-resistant strains [4]. As a consequence, without more effective treatments, the number of infections caused by MDR-TB will probably be difficult to control. Therefore, the development of new antimicrobial drugs with potent anti-TB activity, and new protocols for chemotherapy of the patients with persistent tuberculosis are urgently needed.

In the persistent phase the pathogen is surviving within macrophages [5,6], where it is exposed to oxidative stress and Reactive Nitrogen Intermediates (RNI) as a cellular response to pathogen invasion, leading to oxidation and *S*-nitrosylation of cysteine residues [7]. Bacteria in the group designated as *Actinomycetales*, including mycobacteria do not use glutathione for redox defense, but produce mycothiol (1-d-*myo*-inosityl-2-(*N*-acetyl-cysteinyl) amino-2-deoxy-α-D-glucopyranoside) as their principal low-molecular-mass thiol, which contains a cysteine-derived building block [8]. Thus, the first line of defence of the pathogen against free radicals and hence its long term survival within granulomas is directly linked to the availability of cysteine. The latent phase is further characterized by limited availability to oxygen, and the bacilli switch to anaerobic energy metabolism in order to survive in the phagosome. Not surprisingly gene expression and proteome analysis consistently reveal that genes from the cysteine biosynthetic pathway and nitrogen metabolism are up-regulated in dormancy models [9–14]. These two metabolic processes therefore provide potential targets for novel anti-mycobacterial agents specifically directed toward the pathogen in its persistent phase [15,16]. The essential role of cysteine biosynthesis in *M. tuberculosis* was further emphasized by high density mutagenesis screens showing that mutants carrying transposon insertions in genes related to this pathway are attenuated in macrophages and in a mouse TB-model [17,18].

Since several years we have been involved in a research project that aims to advance understanding, of fundamental and important aspects of the biology of *M. tuberculosis* at the molecular level. Secondly, and most important, it aims to exploit these discoveries

Abbreviations: TB, tuberculosis; PLP, pyridoxal-5-phosphate; OAS, *O*-acetyl-L-serine; OPS, *O*-phospho-L-serine.

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and develop ligands and inhibitors (hit and lead compounds) with the potential to improve therapies of infections by this pathogenic organism. Besides sulphur metabolism, these studies include proteins from nitrogen metabolism [19] and L-alanine dehydrogenase [20]. The latter has been shown to be up-regulated in dormancy models and increased levels of this enzyme have been linked to the generation of alanine for cell wall peptidoglycan biosynthesis [21]. In the following we shall review some of our findings related to the enzymology of sulphur metabolism. Major insights from these studies are the discovery that NirA (Rv2391) is the sulfite reductase operating in the sulphate reduction pathway producing hydrogen sulphide [22] and, simultaneously with others [23] the discovery of an alternative alternative route to cysteine via *O*-phosphoserine rather than *O*-acetyl serine in *M. tuberculosis* [24,25].

2. Cysteine biosynthesis in *M.* tuberculosis

Up to date two independent cysteine biosynthesis pathways have been characterized in this pathogen (Fig. 1). The conventional pathway, analogous to that found in other eubacteria and plants [26] starts from L-serine. Serine acetyltransferase (CysE) converts L-serine into *O*-acetyl-L-serine (OAS) which is metabolized to L-cysteine by the PLP-dependent OAS sulfhydrylase CysK1 [27] through the addition of sulphide provided by the sulphate reduction (APS/PAPS) pathway [22,28]. The alternative CysM dependent pathway uses *O*-phospho-L-serine (OPS) as substrate [23,24] and thiocarboxylated CysO as sulphur source [29]. This route to cysteine appears to be specific to the *Actinomycetales* group including *Mycobacteria*, *Corynebacteria* and *Streptomycetes*.

3. NirA is the sulfite reductase in the sulphate reduction pathway

The sulphur incorporated into cysteine via the pathway starting from *O*-acetyl serine is provided by the APS/PAPS pathway (Fig. 1). The last step, the reduction of sulfite to sulphide is usually catalyzed by a sulfite reductase, however, the identity of the gene encoding the enzyme in the genome of *M. tuberculosis* was unclear. Based on sequence alignments, the protein encoded by the *nirA* (Rv2391) gene was annotated as a member of a family of ferredoxin-dependent sulfite/nitrite reductases. These enzymes are found in archaea, bacteria, fungi and plants (for a review see [30]). Sulfite reductases catalyze the six-electron reduction of sulfite to sulphide, providing reduced sulphur for the biosynthesis of sulphur-containing amino acids and cofactors. Nitrite reductases participate in the assimilation of nitrogen via nitrate in plants and can also act in anaerobic energy metabolism. This class of sulfite/nitrite reductases generally accepts both nitrite and sulfite as substrate, but the particular metabolic function is reflected in pronounced differences in the kinetic parameters. These enzymes contain a unique combination of cofactors, a [Fe_4–S_4] iron sulphur cluster and a siroheme. The *nirA* gene is up-regulated in the dormant phase of *M. tuberculosis* [10,11] and a Himar1 transposon mutagenesis screen further showed that *nirA* is essential [18].

NirA exhibits weak amino acid sequence similarity to bacterial NADPH dependent [Fe_4–S_4] and siroheme containing sulfite reductases, for instance 23% sequence identity to CysI from *Escherichia coli*. Unlike the monomeric ferredoxin-dependent enzymes, the NADPH dependent sulfite reductases are oligomeric complexes consisting of four or eight subunits of the hemoprotein component (CysI) and eight flavoprotein subunits that deliver electrons derived from NADPH to the redox centers of the hemoprotein subunit [31]. In the ferredoxin-dependent enzymes, the electron donor binds transiently, and delivers electrons to the [Fe_4–S_4] cluster, one at a time [32]. The electrons are then transferred to the siroheme, which coordinates the substrate.

The crystal structure of *M. tuberculosis* NirA [22], the first structure of a ferredoxin-dependent sulfite/nitrite reductase, revealed the three-domain architecture of this enzyme, including the locations and binding sites of the two cofactors, a siroheme molecule and [Fe_4–S_4] cluster (Fig. 2). Comparison of the mycobacterial ferredoxin-dependent NirA with NADPH dependent *E. coli* CysI [33] shows that overall structure and the arrangement of the siroheme and [Fe_4–S_4] cofactors are very similar in these two enzymes. In the *E. coli* enzyme the N-terminal 73 amino acids had been proteolytically removed and could thus not be observed in the crystal structure of this enzyme [33]. In NirA this polypeptide segment contributes to the active site architecture and is positioned close to the siroheme molecule. The iron ion of the siroheme is accessible through a narrow channel which most likely is the active site cleft. On the opposite face of the siroheme cofactor, the side chain of an invariant cysteine residue is coordinated to the iron ion and also to one of the iron ions of the [Fe_4–S_4] cluster, thus bridging the two cofactors (Fig. 2). The putative ferredoxin binding site is located close to the [Fe_4–S_4] cluster at the enzyme surface. The electron transport chain thus extends from the external electron donor ferredoxin via the [Fe_4–S_4] cluster and the bridging thiolate to the siroheme, and to the iron-bound substrate molecule, sulfite.

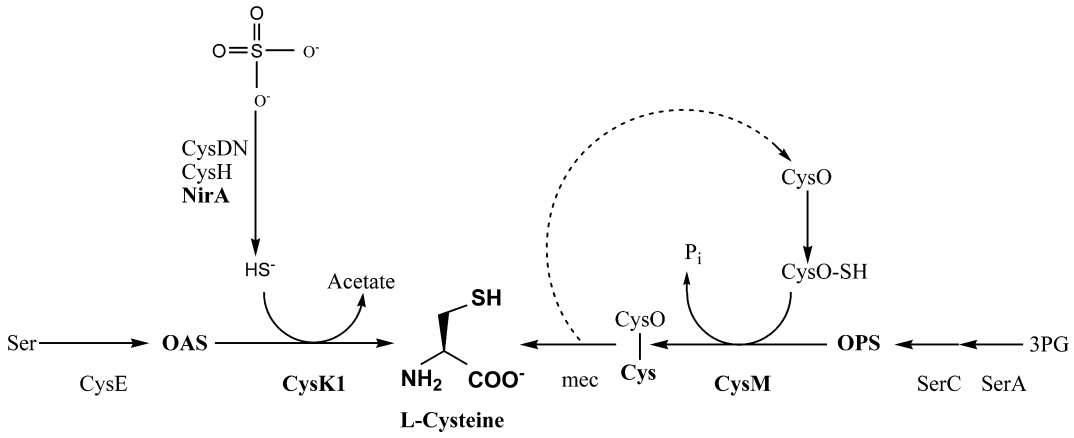

Fig. 1. The two pathways of cysteine biosynthesis in *M. tuberculosis*. In the conventional pathway (left branch) L-serine (Ser) is condensed with an acetyl group to form OAS, which in the next step is converted to cysteine by OAS sulfhydrylase (CysK1). The CysM dependent pathway (right branch) utilizes OPS and thiocarboxylated CysO (CysO-SH) resulting in a CysO–cysteine adduct, which is hydrolysed by the carboxypeptidase mec releasing L-cysteine and regenerating CysO. OPS is synthesized from 3-phosphoglycerate (3PG) in two steps by SerA and SerC.

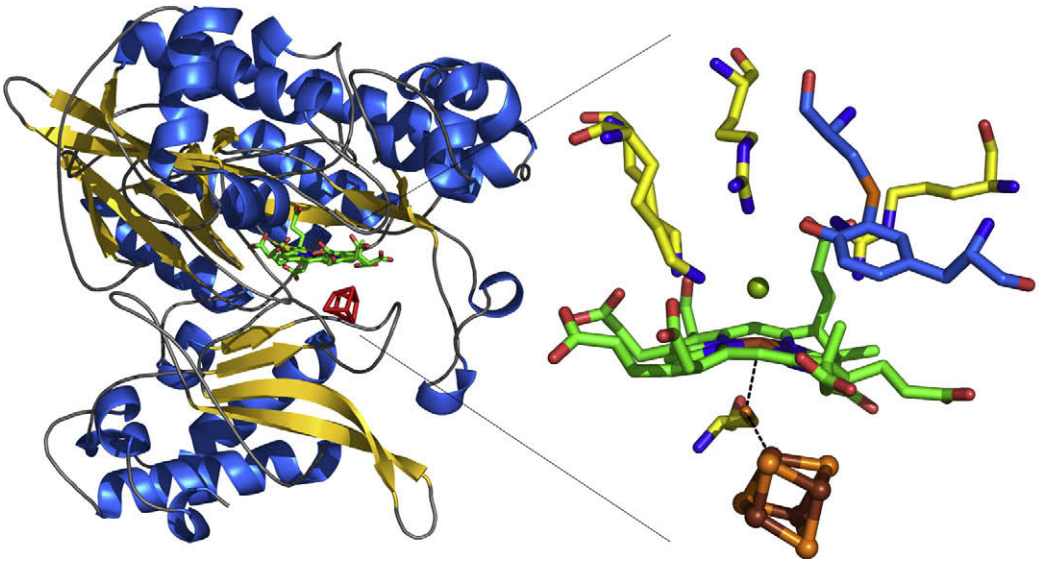

Fig. 2. Left, three-dimensional structure of the ferredoxin-dependent sulfite reductase from the sulphate reduction pathway in *Mycobacterium tuberculosis*. The [Fe_4–S_4] cluster is shown in red and the siroheme molecule in green. Right, close-up of the active site of NirA. The [Fe_4–S_4] cluster is shown in orange, the siroheme molecule in green and active site residues are shown in yellow. Residues Cys161 and Tyr69 which form a covalent bond are shown in blue. The green sphere indicates a bound chloride ion at the position of the substrate binding site.

The crystal structure of the mycobacterial enzyme revealed an unexpected covalent bond between the side chains of Tyr69 and Cys161 in the immediate vicinity of the siroheme cofactor and the substrate binding site. Site-directed mutagenesis of the residues that form this covalent tyrosyl–cysteine linkage, Tyr69 and Cys161, showed that while this bond facilitates catalysis it is not essential for catalytic activity [22]. Mycobacterial NirA is specific for sulfite as substrate, and does not reduce nitrite, identifying it as a sulfite reductase [22,34]. The specificity for sulfite, and the localisation of the *nirA* gene adjacent to the gene coding for the APS reductase CysH suggests that NirA is the sulfite reductase involved in the sulphur reduction pathway, and should therefore more appropriately be annotated as SirA rather than NirA.

4. CysK1 – the *bona fide* OAS sulfhydrylase in *M. tuberculosis*

The conversion of *O*-acetyl serine to cysteine is catalyzed by pyridoxal-5-phosphate (PLP) dependent OAS sulfhydrylases [26]. In many bacteria pyridoxal phosphate (PLP) dependent OAS sulfhydrylase is present as two isoenzymes, denoted CysK and CysM. These isoenzymes show 25–45% identity in amino acid sequence, but exhibit characteristic differences in their substrate specificity with respect to the sulphur donor. CysK uses hydrogen sulphide produced by the sulphate reduction pathway (APS/PAPS-pathway) as sulphur source, while the CysM enzymes characterized so far tend to accept thiosulphate or larger substrates as sulphur donors [35]. The genome of *M. tuberculosis* H37Rv encodes three genes which were annotated as OAS sulfhydrylase (Tuberculist Web Server http://genolist.pasteur.fr/TubercuList/). CysK1 (Rv2334) shows about 37% sequence identity to CysM (Rv1336), whereas the relationship of both isoenzymes to the third isoenzyme (CysK2), encoded by Rv0848, is more distant (26% identity).

Recombinant CysK1 from *M. tuberculosis* has been shown experimentally to be a PLP dependent enzyme that catalyzes the conversion of OAS to cysteine [27]. CysK1 belongs to the family of fold type II PLP enzymes and is similar in structure to other OAS sulfhydrylases [27,36]. Catalysis by CysK1 follows a ping-pong mechanism typical for PLP dependent enzymes [26,27]. The catalytic cycle can be divided into two half-reactions. In the resting state the cofactor forms an internal Schiff base with the invariant catalytic lysine residue. The incoming substrate then forms an external Schiff base with PLP, followed by a β elimination in which acetate is released and a proton is abstracted from the α position, most likely by the lysine side chain [37]. The product of the first half reaction is the α-aminoacrylate intermediate, covalently linked to PLP. The second half reaction starts with a nucleophilic attack of the sulphide (or HS^-) on the β-carbon of the aminoacrylate intermediate and the α carbon is re-protonated resulting in cysteine bound as external Schiff base. Finally the product is released via regeneration of the internal aldimine. Novel insights into the mechanism of the enzyme were obtained by the structure determination of the α-aminoacrylate reaction intermediate by cryo-crystallography [27]. Formation of the aminoacrylate complex is accompanied by a domain rotation resulting in active site closure. The aminoacrylate moiety is bound in the active site via a covalent linkage to the PLP cofactor, that give rise to a shift of the peak in the absorption spectrum by approximately 50 nm. The carboxyl group of the intermediate is anchored to an anion binding pocket of the enzyme by several hydrogen bonds. The Cβ atom of the intermediate is accessible for nucleophilic attack through a narrow channel which may serve as a selectivity filter explaining the preference of CysK1 for small nucleophiles in the second half reaction (Fig. 3A). The catalytic lysine residue is positioned such that it is able to protonate the Cα carbon atom of the aminoacrylate only from the *si*-face resulting in the stereospecific formation of L-cysteine.

5. The C-terminal of CysE inhibits CysK1

CysK1 is competitively inhibited by a four-residue peptide derived from the C-terminal of serine acetyl transferase (CysE) with a K_i of 5 μM [27]. The crystallographic analysis reveals that the peptide binds to the enzyme active site (Fig. 3B), suggesting that CysK1 forms a bi-enzyme complex with CysE, in a manner similar to other bacterial and plant OAS sulfhydrylases as part of the regulation of this pathway [38]. Inhibition by this peptide is sequence specific, i.e., the corresponding peptide derived from CysE from other organisms does not inhibit CysK1 from *M. tuberculosis* [27].

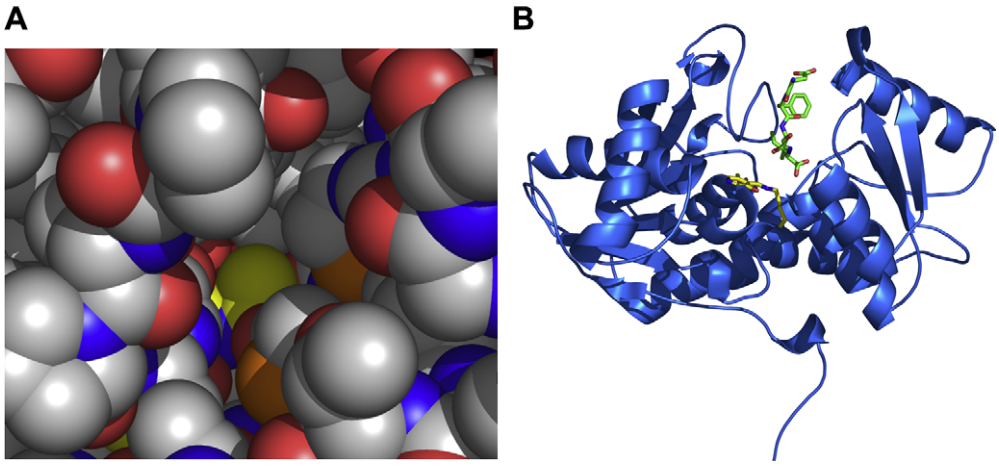

Fig. 3. (A) Substrate channel in the complex of CysK1 with the reaction intermediate α-aminoacrylate. The only atom of the reaction intermediate accessible for the sulphide molecule is the Cβ atom, shown in yellow. The narrow substrate channel serves as selectivity filter resulting in the preference by CysK1 for small molecules such as hydrogen sulphide as sulphur donors. (B) Schematic view of the CysK1 (blue) – peptide inhibitor (green) complex. The peptide, amino acid sequence DFSI, is bound in the active site cleft preventing substrate binding and domain rotation.

Thus, the C-terminal peptide of CysE provides a scaffold for specific inhibition of the mycobacterial enzyme, and the crystal structure of the enzyme–peptide complex forms the basis for the design of improved inhibitors of this enzyme.

6. An alternative cysteine biosynthesis pathway in *M. tuberculosis*

Based on sequence comparisons CysM from *M. tuberculosis* had been annotated as an OAS sulfhydrylase. The crystal structure of the enzyme revealed that CysM belongs to the fold type II family of PLP dependent enzymes [28], and that its overall fold is similar to that of CysK1. In an earlier functional study of CysM it was shown that this enzyme is a sulfhydrylase that uses a novel mode of sulphur delivery [29]. Instead of incorporation of sulphide into the enzyme-aminoacrylate intermediate as other sulfhydrylases, mycobacterial CysM uses a small protein, denoted CysO that is thiocarboxylated at the C-terminus as sulphur source. The enzymatic reaction leads to the formation of a covalent cysteine adduct at the carboxy-terminus of CysO as primary reaction product. In order to complete cysteine formation a Zn(II)-dependent metalloprotease cleaves the CysO–cysteine bond releasing L-cysteine [29].

Functional studies of recombinant CysM from *M. tuberculosis* showed, however, that the enzyme – contrary to previous annotations in sequence databases – is not an OAS sulfhydrylase, but uses *O*-phosphoserine (OPS) as substrate [23,24]. A three-dimensional model of the external aldimine formed between the cofactor and the substrate, based on the crystal structure, revealed an arginine residue at the substrate binding site as a major determinant for recognition of the phosphate group of OPS [24]. Consistent with this model, amino acid replacement of this residue by site-directed mutagenesis results in a major loss in specificity for OPS as substrate. The CysM dependent pathway of cysteine biosynthesis in *M. tuberculosis* thus not only utilizes a protein-bound thiocarboxylate instead of sulphide as sulphur donor [29], but also uses a different sulphur acceptor (Scheme 1). Taken together these studies establish an alternative pathway for cysteine biosynthesis in *M. tuberculosis* independent of *O*-acetyl serine.

In the binary complex of thiocarboxylated CysO with CysM the enzyme was found in a closed conformation with the active site inaccessible to solvent [39]. However, also in the absence of CysO the enzyme can adopt a closed conformation showing considerable structural differences to the closed state obtained in complex with the sulphur delivery protein CysO [25]. In this conformation the C-terminal five amino acids of the enzyme are inserted into the active site cleft, resulting in the complete closure of the substrate binding site (Fig. 4). An enzyme variant where this segment of the polypeptide chain had been removed exhibits decreased lifetime of the α-aminoacrylate reaction intermediate, increased susceptibility to oxidation by small oxidative agents such as hydrogen peroxide,

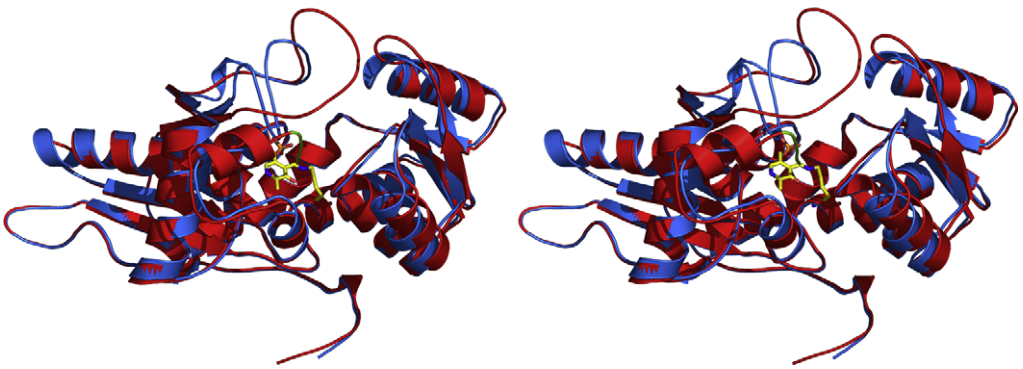

Fig. 4. Stereo figure of the superimposed open (blue) and closed (red) conformations of CysM. The C-terminal segment of CysM, inserted in the active site, is shown in green. The PLP cofactor is shown in yellow.

and partial loss of selectivity towards CysO as sulphur donor. A detailed kinetic comparison of CysM with CysK1 shows that the α-aminoacrylate intermediate is considerably less stable in the latter. CysK1 lacks the C-terminal residues and therefore has an open, albeit narrow, channel leading to the active site [27].

Under the oxidative conditions prevailing in the persistent phase of the bacteria the CysM/CysO pathway appears to be the dominant route to cysteine [17,18]. The higher stability of both substrates, OPS compared to OAS [40] and thiocarboxylates compared to sulphide favour this pathway over the conventional route to cysteine in bacteria under oxidative stress. The reaction cycle of all PLP dependent cysteine synthases includes, however, a relatively unstable, oxidation sensitive reaction intermediate. The increase in stability and resistance of the α-aminoacrylate intermediate in CysM catalysis towards hydrogen peroxide due to the protective C-terminus, not found in most other cysteine synthases, might further favour this pathway of cysteine supply. The important role of this enzyme for *M. tuberculosis* is further emphasized by mutants carrying a transposon insertion in *cysM* which are attenuated in macrophages [17] and in a mouse TB-model [18].

7. Outlook

All major medical organisations including the WHO, research councils and learned societies have identified increasing antibiotic resistance seen in pathogenic microorganisms as an alarming threat to human health. The situation is further aggravated by the fact that most large pharmaceutical companies have, for various reasons, abandoned their bacterial infection programs. The swift pace at which resistance develops in many pathogens requires, however, renewed alliances where medical basic research at academic institutions, government organisations and industry must join forces. Although there are presently more than 20 new compounds against in *M. tuberculosis* in clinical trials this is nearly not enough given the failure rate of drug candidates at this stage. Hence, identification and validation of new targets for chemical intervention as well as development of novel chemistry against "old", well defined targets requires significant efforts both in terms of basic research and drug discovery. Sulphur metabolism has attracted considerable interest as essential for survival, virulence and persistence not only for *M. tuberculosis* but also a number of other pathogenic bacteria [15]. Structural and functional studies of proteins involved in sulphur metabolism provide the basis to explore their potential as possible drug targets. In this endeavour, enzymology and structural biology has an important and decisive role to play also in the future.

Acknowledgments

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