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**Structural basis for the inhibition of *Mycobacterium tuberculosis* glutamine synthetase by
novel ATP-competitive inhibitors**

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Running title: ATP-site inhibitors of *M. tuberculosis* glutamine synthetase

Abstract

Glutamine synthetase (EC 6.3.1.2, also known as γ -glutamyl:ammonia ligase) catalyzes the ATP-dependent condensation of glutamate and ammonia to form glutamine. The enzyme has essential roles in different tissues and species, which has led to its consideration as a drug or herbicide target. In the present paper, we describe studies aimed at the discovery of new antimicrobial agents targeting *Mycobacterium tuberculosis*, the causative pathogen of tuberculosis. A number of distinct classes of glutamine synthetase inhibitors with IC_{50} s μ M or better were identified *via* high-throughput screening. A commercially available purine analogue similar to one of the clusters identified (the diketopurines), 1-[(3,4-dichlorophenyl)-methyl]-3,7-dimethyl-8-morpholin-4-yl-purine-2,6-dione, was also shown to inhibit the enzyme, with a measured IC_{50} of $2.8 \pm 0.4 \mu$ M. Two X-ray structures are presented. One is a complex of the enzyme with the purine analogue alone (2.55 Å resolution), and the other includes the compound together with methionine sulfoximine phosphate, magnesium and phosphate (2.2 Å resolution). The former represents a relaxed, inactive conformation of the enzyme, while the latter is a taut, active one. These structures show that the compound binds at the same position in the nucleotide site, regardless of the conformational state. The ATP-binding site of the human enzyme differs substantially, explaining why it has ~60-fold lower affinity for this compound than the bacterial glutamine synthetase. As part of this work, we devised a new synthetic procedure for generating L-(*SR*)-methionine sulfoximine phosphate from L-(*SR*)-methionine sulfoximine, which will facilitate future investigations of novel glutamine synthetase inhibitors.

Keywords: glutamine synthetase, X-ray crystallography, high-throughput screening, drug design, tuberculosis.

Abbreviations: MtGS, glutamine synthetase from *M. tuberculosis*; PA, the purine analogue 1-[(3,4-dichlorophenyl)methyl]-3,7-dimethyl-8-morpholin-4-yl-purine-2,6-dione; MSO, L-methionine-(*SR*)-sulfoximine; MSO-P, L-methionine-(*SR*)-sulfoximine phosphate; NCS, non-crystallographic symmetry; r.m.s., root-mean-square; Cbz, carbobenzyloxy; Bz, benzyl; PDB, Protein Data Bank (<http://www.rcsb.org>).

Introduction

Long thought to be a disease that was “under control”, tuberculosis has reemerged as an even greater global threat. Much of the problem arises from the lengthy (~6 months), and often unpleasant, regimen of drugs needed for a cure, and the drug resistance that results from poor patient compliance. The prognosis for a patient diagnosed with multi-drug resistant tuberculosis is at present worse than for one diagnosed with AIDS, and the situation is expected to worsen in the future. Clearly, new and better drugs are needed.

Glutamine synthetase (GS; EC 6.3.1.2, also known as γ -glutamyl:ammonia ligase) catalyzes the ATP-dependent condensation of glutamate and ammonia to form glutamine. The role of GS in humans depends on the tissue localization. In the brain it regulates the levels of toxic ammonia and converts neurotoxic glutamate to harmless glutamine, while in the liver GS is one of the enzymes responsible for the removal of ammonia. In bacteria and plants, the enzyme is responsible for fixation and re-assimilation of ammonia, allowing glutamine to be used as a nitrogen source for metabolism. Glutamate is also one of the most important charged compounds used by bacteria for osmoregulation. Further, poly-glutamine/glutamate is a key cell wall component and virulence factor of mycobacterial pathogens, accounting for as much as 10% of the dry weight of the cell wall.¹⁻³ GS action has additionally been proposed to prevent the acidification of phagosomes that is required for their fusion with lysosomes, so allowing the mycobacteria to avoid destruction by the macrophages.^{4,5}

Of the four genes encoding GSs in *M. tuberculosis*, only one, *glnA1* (hereafter assumed to encode MtGS), was found to be essential for *in vivo* survival of the bacteria.³ Methionine sulfoximine (MSO) arrests the growth of bacteria by inhibiting extracellular MtGS,⁶ and caused up to one log reduction of colony-forming units in guinea pigs infected with *M. tuberculosis*; its action was synergistic with isoniazid, a current frontline drug.⁷ The structure of MtGS bound to phosphorylated MSO (MSO-P), metals and ADP was reported earlier.⁸

Here we used high-throughput screening to identify novel compounds that inhibit MtGS efficiently. Kinetic studies, as well as X-ray structures of one inhibitor (PA, 1-[(3,4-

dichlorophenyl)methyl]-3,7-dimethyl-8-morpholin-4-yl-purine-2,6-dione, see Figure 1(a)) bound to GS show that, in contrast to previously known inhibitors such as MSO and phosphinothricin, which are glutamate-like, the new compounds enter the nucleotide-binding site. As this site is very different in the enzymes from the two species, it offers better prospects for selective binding to MtGS compared to the human enzyme. Both the high-throughput screening and the structures pave the way for the design of improved inhibitors. As part of this work, we also devised a novel, straightforward synthesis of L-(*SR*)-methionine sulfoximine phosphate (L-(*SR*)-MSO-P, Figure 1(b)), which will provide a useful tool for further work with GSs.

Results

Kinetic parameters--The measured K_m for glutamic acid was 21.6 ± 3.4 mM, and those for ATP and ammonium chloride were 0.50 ± 0.06 mM and 10.8 ± 0.6 mM, respectively, under the conditions of the assay used for screening. The optimal $MgCl_2$ concentration was ~25 mM; higher concentrations led to inhibition. The pH optimum was near 7.5, with HEPES as the best buffer tested. DMSO gave a slight (~15%) activation at a concentration of 2%.

High-throughput screening--To optimize the detection of inhibitor binding, assay conditions (30 mM glutamic acid, 1 mM ATP, 10 mM ammonium chloride, 25 mM $MgCl_2$) were chosen to be near the K_m values for glutamate, ATP and ammonium. Of the 75,000 compounds entering the primary screen, 836 were found to be 'actives' using a criterion of 30% inhibition at a 30- μ M concentration. Of these, 120 had measured IC_{50} s better than 30 μ M. Seventy-five gave satisfactory results in a protein concentration response (to test for possible promiscuity), and so qualified for further work. The inhibitory activity of these compounds was confirmed in an orthogonal assay where the formation of glutamine was measured; 2 of the compounds failed at this point. The 73 remaining compounds were assigned to 11 clusters. These clusters were enriched from the corporate library using near-neighbor analysis, to generate a refined library of 1100 compounds. All of these were rechecked for dose response. In addition, it was demonstrated that the compounds obtained were competing with ATP in the assays. The prioritized clusters,

based on a number of criteria, can be described as: Cluster 1, isoquinoline derivatives; Cluster 2, diarylimidazoles; Cluster 3, pyrazolopyrimidines; Cluster 4, quinoxalines; Cluster 5, aminopurines; Cluster 6, benzothiazoles; Cluster 9, imidazopyridines, Cluster 10, diketopurines; Cluster 14, naphthyridines, and Cluster 23, aminopyrimidines (for representative structures and IC_{50} s, see Supplementary Materials).

PA inhibition -- Substructure searches in databases of commercially available compounds were performed on several of the above clusters, with the purpose of identifying compounds that could be rapidly acquired for X-ray crystallographic studies. Based on these searches, the PA inhibitor (Figure 1(a)) belonging to cluster 10 was identified. Under conditions very similar to those used for high-throughput screening, the measured IC_{50} of MtGS for this compound was $2.5 \pm 0.4 \mu M$. Equivalent tests for the human enzyme (prepared as described previously⁹) resulted in an IC_{50} of $146 \pm 24 \mu M$.

Synthesis of L-(SR)-MSO-P--The synthetic procedure used to prepare the L-(SR)-MSO-P diastereomers from L-(SR)-MSO is described in Figure 2. The amino and carboxylic functions of L-(SR)-MSO were protected in two steps with carbobenzyloxy and benzyl moieties, respectively. Phosphorylation of the diprotected sulfoximine **2** was carried out with *o*-xylylene *N,N*-diethylphosphoramidite according to the procedure reported by Tokutake et al.¹⁰ In the phosphorylation step we did not directly obtain structure **3**, but an intermediate hydroxymethyl benzylhydrophosphoryl compound **3''** carrying three stereocenters was detected and isolated. The open-ring structure of **3''** was supported not only by 1H and ^{13}C NMR data, showing four non-equivalent benzyl methylene groups, but also by ^{31}P NMR experiments (H-decoupled and not H-decoupled). The ^{31}P NMR analysis proved the P-H functionality, confirmed the P-H coupling constant measured in the 1H NMR spectrum ($J = 646$ Hz) and gave the four expected ^{31}P singlet signals for the separate stereoisomers (L-(SS)-**3''**, L-(SR)-**3''**, L-(RS)-**3''** and L-(RR)-**3''**). We assign the structure of **3''** as depicted in Figure 2 and suggest that compound **3'** might exist in equilibrium with **3''**. Addition of aqueous *tert*-butyl hydroperoxide to the reaction mixture gave the desired *N*-phosphoryl sulfoximine **3** in 51% yield. Hydrogenation of compound **3** at

atmospheric pressure, followed by preparative reverse-phase HPLC purification, furnished the target product L-(*SR*)-MSO-P in 54% isolated yield as a diastereomeric 55/45 ratio with no epimerization of the α -carbon detected. L-(*SR*)-MSO-P was fully characterized by ^1H NMR, ^{13}C NMR and high-resolution MS. To the best of our knowledge, this is a new chemical procedure for generation of L-(*SR*)-MSO-P.^{11,12}

MtGS/PA structure--The structure of MtGS bound to PA was solved at 2.55 Å resolution, using the dodecamer from an earlier MtGS apo structure¹³ in molecular replacement. Residues 4-478 were observed for each of the 12 chains in the asymmetric unit, with the exception of residues 63-66 and 405-412; these last were omitted from the model, as the density of the cyclically averaged maps did not allow them to be placed with confidence. Density for residues 58-59 and 334-335 was also very weak. The subunits were restrained using non-crystallographic symmetry (NCS) during the refinement, and with the exception of the loops mentioned above as having poor/weak density, no appreciable differences between them were apparent.

The twelve active sites of the dodecamer are found in long grooves between the subunits. Each site contains clear electron density for the inhibitor. Figure 3(a),(b) shows the site between subunits A and F; in this case, all contacts with the inhibitor are contributed by the A molecule. The interactions are largely non-polar, with only two hydrogen bonds linking inhibitor and protein. The inhibitor's morpholino group stacks near the side chain of His278, and a hydrogen bond links the oxygen of the ring to the side-chain nitrogen of Asn229. The 8-aminopurine-2,6-dione moiety is near the protein main chain at residues 129-133, stacked between the side chains of Phe232 and Arg364. The side chain of Ser280 donates a hydrogen bond to O2 of the diketopurine group. The dichlorophenyl group is positioned further out toward solvent, curving around the ring of Phe232 to lie near Lys361. The plane of the ring is perpendicular to that of nearby Tyr129, and parallel to (although offset from) that of Trp282.

A chloride ion is bound in each subunit, near the interface of the two hexameric rings, but appears to have no role in enzyme function.

MtGS/MSO-P/MgPi/PA structure--The structure of MtGS bound to both PA and MSO-P was solved at 2.2 Å resolution, using the hexamer of the isomorphous MtGS/MSO-P/MgADP complex⁸ as the starting point. A complete dodecamer is formed by virtue of crystallographic symmetry. Residues 3-478 are seen in the electron density maps for each subunit. A breakdown in NCS due to crystal packing causes some variation in structure near residues 403-416. Figure 3(c),(d) shows the active site. In addition to the PA and MSO-P molecules (the latter clearly visible as the active L-(S)-MSO-P¹²), and the added magnesium ions, electron density is seen for a phosphate ion. When the MSO-P sample alone was tested in the assay, phosphate contamination (~5%) was evident, which could arise either from hydrolysis or as an impurity of MSO-P. Since the nitrogen-phosphorus linkage is reported to be highly stable,¹¹ we presume that the latter is the case. Density is also observed for a molecule of PEG, which has no apparent structural or functional significance.

Comparison to MtGS/PA indicates that 368 C α atoms match within a 3.5 Å cutoff, with an r.m.s. difference of 1.0 Å. The most dramatic discrepancy is the β -strand shift noted earlier,¹³ in which residues 217-221 of MtGS/PA align with residues 214-218 of MtGS/MSO-P/MgPi/PA. The structures are back in register by residue 224. Other differences are found near residues 57-67, 180-185, 332-335 and 405-413.

Despite the significant structural changes near the active site, the mode of binding PA is very similar to that seen in the structure without MSO-P, except there is now only a single hydrogen bond linking protein and the inhibitor, that between O2 and Ser280 (Figure 4). Movement of Asn229 compared to the structure without MSO-P results in the loss of the other hydrogen bond. This side chain instead interacts with Glu227 and a water coordinating the n3 metal. The difference is due to the presence of the metal, and the strand shift.

Comparisons to previous MtGS structures--Comparison of the MtGS/PA structure to the previously available relaxed MtGS *apo* structure¹³ indicated that 448 C α atoms (from molecule A) match within an r.m.s. distance of 0.4 Å. The biggest differences are near residues 55 and 324, and involve loops that have very high temperature factors in the 1HTO structure. Binding of PA

opens the active site slightly. For example, the 231-361 C α -C α distance is 13.6 Å in MtGS and 14.3 Å in MtGS/PA. Similarly, the 215-362 distance is 11.2 Å in MtGS, and 12.4 Å in MtGS/PA. Changes near residue 54 have greatest implications for the active site.

The MtGS/MSO-P/MgPi/PA and MtGS/MSO-P/MgADP structures are highly similar, with 468 C α atoms matching within a 0.5 Å cutoff. Comparison of the active sites shows overlap of the inhibitor with the nucleotide (Figure 5(a)). The morpholino group occupies the position of the ADP-ribose. The diketopurine group lies at the location of the purine base, stacked between Phe232 and Arg364, but rotated by ~30° within the same plane. MSO-P and the three magnesium ions occupy equivalent positions in both structures. The phosphate ion in the MtGS/MSO-P/MgPi/PA structure lies at the position of the β -phosphate of ADP. There are some slight movements in the structure near the nucleotide/inhibitor, again associated with an opening of the site when PA is bound (the 231-361 C α -C α distance is 13.3 Å in MtGS/MSO-P/MgADP, and 14.4 Å in MTGS/MSO-P/MgPi/PA). Residues 284-287 and 360-361 lie outside the 0.5-Å cutoff, and reflect small (<1 Å) movements that accommodate the dichlorophenyl group of the inhibitor.

Discussion

Selectivity is a vital consideration in the design of a new drug candidate. Comparison of the active sites of mammalian and mycobacterial GSs earlier⁹ indicated that the nucleotide-binding site offered the best hope for obtaining a compound that would inhibit MtGS, but have no effect on human GS. Therefore, compounds were tested in an assay suited to obtaining inhibitors that bind in this site. High-throughput screening was performed using a 75,000-compound library carefully chosen to represent all of the classes of compounds present in the AstraZeneca corporate library; the screened library also contained a number of kinase inhibitors, to further bias the search toward the ATP site. In this way, a number of compounds were identified with IC₅₀s in the range of nM to μ M; these were furthermore shown to compete with ATP. A commercially available compound (PA) was also found that is similar to one of the clusters identified (cluster

10, the diketopurines), and investigated further. The PA compound was found to have an IC_{50} of $2.5 \pm 0.4 \mu M$.

The crystal structure of an enzyme–inhibitor complex is a powerful tool for designing improved compounds during lead optimization studies. Thus the structure was solved for MtGS in the presence of the PA inhibitor, which showed that binding did indeed occur in the nucleotide site. However, the MtGS/PA structure is a relaxed low pH, metal-free conformation that is not active. It was therefore unclear whether the observed mode of PA binding would correspond to that most important under *in vivo* conditions. In addition, crystallization was not reproducible, and the resolution observed for the ~100 crystals, and more than 1000 conditions, tested was not as good as one would like for a systematic analysis of other inhibitors. Crystals of the taut (active) conformation seen in the MtGS/MSO-P/MgADP structure⁸ were obtained much more reproducibly, and diffracted to higher resolution. The numerous interactions with glutamate, phosphate, and metals are key to stabilizing this form of the protein *in vivo*. However, despite extensive trials including glutamate, etc, we were unable to identify conditions that resulted in a taut conformation in the presence of PA. It was believed that including MSO-P in the crystallization experiments would be advantageous, since the phosphorylated inhibitor binds much more tightly than the substrate glutamate. As MSO-P is not commercially available, it was generated *in situ* for the previous structure; the protein was incubated with MSO and ATP prior to crystallization, so producing MSO-P and ADP. However, displacement of ADP with PA proved prohibitively slow.

Synthesis and isolation of L-(*SR*)-MSO-P proved to be the solution to the problem. The MtGS/MSO-P/MgPi/PA structure represents the taut conformation, and both reproducibility of the crystallization and the resulting resolution were substantially improved. Although the preparation contained a mixture of *S* and *R* forms at the sulfur center, the species bound to MtGS was the active *S* form.^{8,12}

Comparison of the structures of MtGS/PA complexes with and without MSO-P indicated that the inhibitor bound in the same site, and in a very similar way. Minor differences were

attributable to metal binding, and to the β -strand shift that characterizes the relaxed/taut transition. Thus, it is expected that the PA compound will inhibit MtGS, regardless of its conformational state *in vivo*. Further, MSO-P is a generally important tool for crystallization of inhibitors bound to the nucleotide site, which should allow the solution of further structures. A straightforward synthesis of the compound is therefore of wider value, for our and other GS studies.

That nucleotide site-based MtGS inhibitors can reasonably be expected to discriminate strongly against human GS is illustrated in Figure 5(b). The two enzymes bind nucleotide in an extremely similar mode with respect to the catalytic residues, glutamate and metals. The diketopurine and morpholino groups of PA overlay with the base and the ribose moieties of ADP, respectively, in MtGS. In this mode of binding, the dichlorophenyl group of the inhibitor will clash with Trp130 and Arg262 side chains of the human enzyme, and so the compound would not be expected to bind. Tests of PA confirm that it has ~60-fold weaker binding to the human enzyme. The structural differences described above, as well as other architectural features of the MtGS nucleotide-binding site, can be exploited to design potent and selective inhibitors.

Both complexes with PA are slightly (1 Å or less) more open than the corresponding structures in the absence of the inhibitor, but most other structural features are unperturbed. Combined, these structures provide a good starting point for designing inhibitors that will bind even more tightly. An example of such an improvement would be the introduction of new interactions with Glu214, which lies near the morpholino group of the present compound. This and other approaches will be pursued in future studies. One important goal will be to seek compounds that are taken up effectively by macrophages as well as by the bacteria, since compounds that exhibit bacteriocidal effects extracellularly and on phagocytized *M. tuberculosis* may stand the best chance of performing well as anti-mycobacterial drugs.

Materials and Methods

Chemicals--All restriction enzymes were purchased from Boeringer Mannheim, Germany/Bangalore Genei, India. Plasmid pTrec99c was bought from Invitrogen (USA). Monosodium glutamate and glutamine were obtained from Sigma-Aldrich Co. (USA), and PA from InterBioScreen (Moscow, Russia). The piColorlock Gold reagent kit was purchased from Innova Biosciences (UK). Microtiter plates used for screening were from Greiner. The HPLC C18 column was purchased from Waters (USA). The Ni-NTA column (Qiagen) was used for the isolation of His-tagged recombinant MtGS. The molecular weight of the protein was determined using mass spectrometer VG-Quattro (Manchester, England). Tips and plates were purchased from Beckman (USA).

Protein expression and purification--*Escherichia coli* strain GJ4745, lacking adenylyltransferase, was the kind gift of Dr. Gowrishankar, Center for Cellular and Molecular Biology, Hyderabad, India. Protein for structural studies was over-expressed and purified in this strain, using methods described previously.⁸ For high-throughput screening, the *glnA1* gene from the same construct was subcloned into pTrec99c using the XbaI and Hind III sites, and protein was expressed in the GJ4745 strain. Recombinant MtGS obtained in this way was shown to be completely unadenylylated by mass spectrometry, and so fully active.

Assays and library screening--The primary assay was based on the detection of inorganic phosphate produced during the biosynthetic reaction with a PiColorlock Gold reagent kit (Innova Biosciences, UK). K_m values were determined for glutamate, ATP and ammonium, by varying the concentration of the individual substrates while keeping the other substrate concentrations constant. Substrate concentrations were varied in the following ranges: L-glutamate, 5 to 200 mM; ATP, 0.1 to 6 mM; ammonium chloride, 2.5 to 150 mM. The K_m for each of the substrates was calculated using software GraphPad Prism, Version 4.3 (GraphPad Software, Inc., CA, USA). The following assay conditions were standardized: 50 mM HEPES-HCl (pH 7.5) containing 25 mM $MgCl_2$, 10 mM ammonium chloride, 30 mM glutamic acid and 1 mM ATP in a reaction volume of 40 μ L. The reaction was run at ambient temperature (25 °C) with 15 ng

enzyme (subunit concentration 7 nM). The assay was adapted to a 384-well format to run high-throughput screening using a Beckman Coulter laboratory workstation Biomek FX fitted with an ORCA robotic arm. 10,000 compounds could be screened per day.

A concentration of 30 μ M was used for testing compounds (diluted from 10 mM stocks in DMSO, to give a consistent final DMSO concentration of 2%) in a primary screen. Positive controls using MSO, one at a concentration causing 50% inhibition (230 μ M) and the other resulting in 100% inhibition (6 mM), were included in addition to uninhibited and reagent controls. A cut-off of 30% inhibition was used for selection of primary 'actives', which were tested using a 10-point concentration series. Compounds having an IC_{50} of 30 μ M or better were selected as 'hits'. Those having undesirable chemical properties, such as reactive groups, high ClogP or molecular weight more than 500 Da, were then removed. The inhibitory activity of the 'hits' was further confirmed in an orthogonal/secondary assay, based on HPLC (C18 column, Waters Corp, USA). The trinitrophenyl-derivatives of glutamic acid and glutamine were separated using a gradient solvent system consisting of solvent A (20% methanol in 50 mM phosphate buffer, pH 5.5) and solvent B (80% methanol in the same buffer) for 30 min at a flow rate of 1 ml/min. The eluent was monitored at 420 nm. Validated compounds were then grouped into clusters, each of which was enriched from the AstraZeneca corporate library, after which a new round of IC_{50} determination was carried out.

PA kinetic studies--Assays and IC_{50} determination for PA were performed under the conditions described for high-throughput screening, with the small difference that the ammonium chloride concentration was 30 mM, and the final reaction volume was 100 μ l. The incubation was carried out for 1 h at room temperature, after which inorganic phosphate was detected using a PiColorlock Gold reagent kit.

Synthesis of L-(SR)-MSO-P--The new synthetic procedure used to prepare L-(SR)-MSO-P from L-(SR)-MSO is described in the Results section.

Structural studies--For the MtGS/PA structure, equal volumes of mother liquor (0.1 M sodium acetate (pH 5), 0.75 M 1,6-hexanediol) and MtGS solution (10 mg/ml in 20 mM Tris-HCl (pH

7.5), 150 mM NaCl, 5 mM MgCl₂, 2% (v/v) DMSO and 0.2 mM PA) were mixed and allowed to equilibrate by hanging-drop vapor diffusion for five days at 20 °C before seeding. Well-shaped crystals appeared within 24 hours, and were flash-cooled in liquid nitrogen without additional cryoprotectant. A data set extending to 2.55 Å resolution was collected at the European Synchrotron Research Facility, ESRF, on beamline ID14:2. The data were integrated and scaled using XDS and XSCALE.¹⁴ The structure was solved by molecular replacement using the program MOLREP,¹⁵ with the complete dodecamer from PDB entry 1HTO¹³ as a search model. Refinement was carried out with REFMAC5¹⁶, with tight NCS restraints of chains B-L (protein plus bound waters and ligands) to their equivalents in chain A. The sequence was renumbered to reflect that of the native protein, and rebuilt in the program O.¹⁷ Waters observed in most/all of the molecules were added using averaged $|F_o|-|F_c|$ maps. Geometric analysis using MolProbity¹⁸ indicated that 96.7 % of residues were in the most-favored Ramachandran regions, and 0.2 % were Ramachandran outliers. Other statistics for data collection and refinement are summarized in Table 1.

Crystals of the MtGS/MSO-P/MgPi/PA complex appeared under conditions similar to those used for the MtGS/MSO-P/MgADP complex earlier.⁸ Equal volumes of reservoir solution (0.1 M MES (pH 6.8), 0.2 M MgCl₂, 40% (v/v) PEG400) and MtGS protein (30 mg/ml in 0.1 M Tris-HCl (pH 7.5), 125 mM NaCl, 4 mM MgCl₂, 4% (v/v) DMSO, 4 mM MSO-P, 1 mM PA) were equilibrated by hanging-drop vapor diffusion for one day at 20 °C, then streak-seeded; crystals appeared within one day. For X-ray data collection, crystals were directly flash-cooled in liquid nitrogen. A data set extending to 2.2 Å resolution was collected at ESRF on beamline ID14:1, then merged and scaled using XDS and XSCALE. Refinement was carried out with REFMAC5. The MtGS hexamer from PDB entry 2BVC (water molecules and ligands removed) was used directly; rigid-body refinement was followed by rounds of refinement with NCS restraints. In the final round of refinement, NCS was tightly restrained for residues 3-402 and 417-478 (as well as associated waters and other ligands) of chains B-F to their equivalents in chain A, to allow for differences at residues 403-416. Water molecules were added in averaged $|F_o|-|F_c|$ maps.

Geometric analysis using MolProbity indicated that 98.8 % of residues were in the most-favored Ramachandran regions, and there were no Ramachandran outliers. Other statistics for data collection and refinement are summarized in Table 1.

Other methods-- Structures were compared using the programs LSQMAN¹⁹ and O. Figures were prepared with the programs PyMOL (DeLano Scientific [<http://www.pymol.org>]) and ISIS/draw (Symyx Technologies [<https://secure2.mdl.com>]).

Accession numbers

The atomic coordinates and structure factors have been deposited at the Protein Data Bank, www.pdb.org (PDB ID codes 2WHI and 2WGS for the structures with and without MSO-P, respectively).

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Appendix A. Supplementary data, including four figures, provides additional information concerning expression, purification, assays, pH-activity profile and mass spectrometric analysis, as well as structures of high-throughput screening hits and details of the synthesis of L-(SR)-MSO-P.

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Figure legends

Figure 1. Ligand structures. (a) PA, with atom numbering. (b) L-(*SR*)-MSO-P with stereochemical centers labeled.

Figure 2. Synthesis of L-(*SR*)-MSO-P. Reagents and conditions: (a) benzyl chloroformate in 1,4-dioxane, NaHCO₃, H₂O, 0 °C-rt; (b) benzyl bromide, DCHA, DMF, DCM, rt; (c) *o*-xylylene *N,N*-diethylphosphoramidite, 1 *H*-tetrazole, DCM, rt; (d) *t*-BuOOH (70 % aqueous solution), rt; (e) H₂ (1 atm), 10 % Pd/C, MeOH-H₂O-AcOH, rt.

Figure 3. Electron density and protein-ligand interactions. For MtGS/PA, panel (a) shows electron density for ligand in the final SIGMAA-weighted $2m|F_o|-d|F_c|$ map,²⁰ contoured at $1\sigma = 0.22\text{ e}/\text{\AA}^3$, while panel (b) shows the binding of PA in the nucleotide site (important residues of which are colored in cyan). Panels (c) and (d) show corresponding illustrations for the MtGS/MSO-P/MgPi/PA complex; electron density for ligands represents the final map contoured at $1\sigma = 0.30\text{ e}/\text{\AA}^3$. Magnesium ions are represented as magenta spheres.

Figure 4. Comparison of PA binding with and without MSO-P. The carbon atoms of MtGS/PA are colored cyan, while those of MtGS/MSO-P/MgPi/PA have atomic coloring.

Figure 5. Comparisons of PA and nucleotide binding. (a) Carbon atoms of the MtGS/MSO-P/MgPi/PA structure are shown in gold, while those of the MtGS/MSO-P/MgADP structure are shown in gray. (b) In a comparison of the nucleotide-binding sites of MtGS and human GS, MtGS residues together with PA are shown in gold, and human GS residues (together with ADP) are shown in gray.

Table 1. Statistics for data collection and refinement.

Data collection^a	MtGS/PA	MtGS/MSO-P/MgPi/PA
Cell axial lengths (Å)	134.9, 203.2, 230.2	133.2, 228.8, 202.7
Space group	P2 ₁ 2 ₁ 2 ₁	C222 ₁
Molecules in asymmetric unit	12	6
Resolution range (Å)	29.90 - 2.55 (2.65 – 2.55)	40 - 2.20 (2.30 – 2.20)
No. of reflections measured	834,947 (89,498)	601,423 (47,250)
No. of unique reflections	200,628 (21,263)	151,747 (16,701)
Average multiplicity	4.2 (4.2)	4.0 (2.8)
Completeness (%)	97.6 (95.6)	97.2 (86.5)
R _{meas} ^b	9.4 (45.4)	10.9 (33.8)
<I/σ(I)>	12.3 (3.8)	13.4 (3.4)
Wilson B-factor	45.0	31.2
Refinement		
Resolution range (Å)	29.9 - 2.55	39.8 - 2.20
No. of reflections used in working set	194,667	145,335
No. of reflections for R _{free} calculation ^c	10,318	7666
R-value, R _{free} (%)	23.0, 24.6	21.1, 23.1
No. of non-hydrogen atoms	45,804	24,683
No. of solvent molecules	1536	1649
Mean B-factor, protein atoms (Å ²)	42.1	27.2
Mean B-factor, MSO-P (Å ²)	-	24.7
Mean B-factor, phosphate atoms (Å ²)	-	38.5
Mean B-factor, Mg ions (Å ²)	-	25.8
Mean B-factor, PA atoms (Å ²)	47.9	39.1
Mean B-factor, solvent atoms (Å ²)	36.4	28.9
Mean B-factor, chloride atoms (Å ²)	57.8	28.3
Mean B-factor, PEG atoms (Å ²)	-	33.8
Ramachandran plot	2.1	0.9

outliers (%) ^d		
r.m.s. deviation from ideal bond length (Å) ^e	0.010	0.009
r.m.s. deviation from ideal bond angle (°) ^e	1.22	1.12
PDB code	2WGS	2WHI

^a Values in parentheses are for the highest resolution shell.

^b $R_{\text{meas}} = \sum_h (n_h / (n_h - 1))^{1/2} \sum_i |I_{h,i} - \langle I_h \rangle| / \sum_h \sum_i |I_{h,i}|$.

where $\langle I_h \rangle$ is the mean intensity for reflection h and n_h is its multiplicity

^c Brunger, 1992.²¹

^d Calculated using a strict-boundary Ramachandran plot.²²

^e Using the parameters of Engh & Huber.²³

Figure

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(a)

(b)

Supplementary Materials

Structural basis for the inhibition of *Mycobacterium tuberculosis* glutamine synthetase by novel ATP-competitive inhibitors

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Expression and purification of *M. tuberculosis* glutamine synthetase (MtGS) for the high-throughput screening:

Recombinant MtGS protein was expressed in two *Escherichia coli* strains (GJ4745 and BL21-AI, the latter referred to as “wild type” in the following discussion).

Cells were grown at 37 °C to an optical density of 1 (at 600 nm), and induced with 10 mM IPTG for 15 h at 25 °C. Cell pellets were re-suspended in 50 mM HEPES, pH 7.5, 300 mM NaCl, 10% glycerol (Buffer A). PMSF (1 mM) was added to this suspension to inhibit protease activity. The cells were lysed by sonication, after which the lysate was centrifuged at 10,000 x g for 10 min to remove cell debris. The supernatant containing recombinant protein was subjected to ultra-centrifugation (Beckman Instruments) at 60,000 rpm for 1 h to remove contaminating membrane fractions. The clarified supernatant containing expressed protein was mixed with Ni-NTA resin equilibrated with Buffer A (to bind recombinant GlnA1 through its His-tag) for 3 h at 4 °C, then the resin was packed into a column. The column was washed with 150 ml of 50 mM HEPES, pH 7.5, 300 mM NaCl, 50 mM imidazole, to remove unbound/loosely-bound protein molecules. Bound protein was eluted with Buffer A containing 400 mM imidazole, pH 7.5, and collected as 1.5 ml fractions. Fractions chosen on the basis of SDS-PAGE analysis (Figure 1) were pooled and dialysed against the storage buffer (25 mM HEPES, pH 7.5, containing 50 mM NaCl, 10% glycerol, 0.1 mM EDTA) overnight with three changes at 4 °C. The dialyzed protein was stored at –70 °C as small aliquots until used for the assay.

Biochemical analysis of MtGS:

The pH-activity profile for MtGS was determined in HEPES buffer for the pH range 6.5 - 9.5. The enzyme activity, as $V_{\max}$, is plotted against pH in Figure 2, and was analyzed using the XLfit software from IDBS (Guildford, UK).

The functional unit of MtGS is generally accepted to be a dodecamer. The purified protein was run on an ÄKTA-FPLC (GE Healthcare, Uppsala, Sweden) gel filtration column (Superose-6) equilibrated with 25 mM HEPES, pH 7.5, containing 150 mM NaCl and 10% glycerol, to check the aggregation state. The single homogeneous peak suggested that the sample indeed represented a dodecameric species in both of the expression strains used.

Mass spectroscopic analysis of the enzyme expressed in wild type *E. coli* showed a population (~30%) with a difference of 329 Da compared to the enzyme expressed in *E. coli* GJ4745. This corresponds to one AMP residue covalently linked per subunit of the enzyme. The protein expressed in *E. coli* GJ4745 was devoid of adenylation. The adenylylated enzyme had

1/3 the activity of the non-adenylylated protein, and so only the latter was used in the structural and inhibition studies.

Assay and high-throughput screening:

The reaction mixture comprised two aliquots. The first addition (enzyme mix) contained a cocktail of HEPES buffer, MgCl_2 , ammonium chloride, ATP and enzyme, in a 20- μl volume. To start the reaction, 20 μl of glutamic acid solution was added. The reaction was run for 45 min, during which the rates were linear as shown by control experiments. At the end of this time, 10 μl of piColorlock Gold solution (Innova Biosciences, UK) was added to stop the enzyme reaction and develop color. The color was developed for 5 min, then fixed by the addition of 5 ml of stabilizer, as supplied by the manufacturer. The color was subsequently measured at 635 nm (Spectramax, Molecular Devices).

Structures representing the compound classes found to inhibit MtGS are shown in Figure 3.

IC₅₀ determinations:

A 10-point IC₅₀ determination was performed for each inhibitor using serially diluted solutions in DMSO. The control wells contained DMSO at equivalent concentration (2%). The readout was analyzed using the ActivityBase suite (version 5.4.3) provided by IDBS. The IC₅₀ curve obtained for the standard MtGS inhibitor (MSO) is shown in Figure 4.

Secondary/orthogonal assay:

An aliquot of the standard reaction mixture was diluted to 100 ml with 0.1 M phosphate buffer, pH 9, then treated with 2 ml of 5% trinitrobenzenesulfonic acid (TNBS) at 37 °C for 30 min. The sample was then analyzed for trinitrophenyl-derivatives of glutamic acid/glutamine using an HPLC method. Solvent A was 20% methanol in 50 mM phosphate buffer, pH 5.5, and solvent B was 80% methanol in the same buffer. Chromatography was carried out on a C18 column (250 x 4.6 mm) at ambient temperature using a gradient of 0-80% Solvent B over a period of 30 minutes with a flow-rate of 1 ml/min. The eluent was monitored at 420 nm. The concentration of glutamic acid/glutamine present in the reaction mixture was computed from the peak areas of standard curves generated using known quantities of glutamic acid and glutamine.

Preparation and characterization of L-(SR)-methionine sulfoximine phosphate (L-(SR)-MSO-P):

General comments and materials

The L-(SR)-methionine sulfoximine (L-(SR)-MSO) was obtained from Fluka, and the *o*-xylylene *N,N*-diethylphosphoramidite from Sigma-Aldrich. ¹³C NMR analyses of L-(SR)-MSO showed a 60/40 ratio of the diastereomers. All starting materials and reagents were used as received. All purification made use of preparative RP-HPLC-MS, which was performed on a Gilson-Finnigan Thermo Quest AQA system equipped with a Zorbax SB-C8 (5 μm , 21.2 x 150 mm; Agilent Technologies, CA, USA) column at a flow rate of 10 ml/min. The mobile phase consisted of H₂O/MeCN (0.05% HCOOH) and the analyses were run in a gradient mode. The ¹H (400 MHz) and ¹³C (100 MHz) NMR spectra were recorded on a Varian Mercury 400 spectrometer at ambient temperature. The ³¹P NMR spectra were recorded on a Varian Mercury 300 spectrometer with H₃PO₄ (80% in H₂O) as reference. High-resolution mass spectroscopy was performed on a Micromass Q-TOF2 mass spectrometer equipped with an electrospray ion source. Optical rotation was obtained on a Perkin-Elmer 241 polarimeter. Compounds **2**, **3'** and **3** are new compounds. L-(SR)-MSO-P (**4**) is a known compound but without reported spectroscopic characterization data.^{1,2}

NMR data for the L-(SR)-methionine sulfoximine batch used as starting material in the synthesis of L-(SR)-MSO-P: ¹H NMR (400 MHz, D₂O): δ 2.15 to 2.30 (m, 2H, β -proton), 3.02 (s, 3H, CH₃), 3.21 to 3.52 (m, 2H, γ -proton), 3.71 to 3.76 (m, 1H, α -proton); ¹³C NMR (100 MHz,

D₂O) : δ 24.45 (s, C β), 24.49* (s, C β *), 41.22 (s, CH₃), 52.18 (s, C α), 53.20 (s, C γ), 53.25* (s, C γ *), 173.41 (s, C=O).

The N:B ratio between the 1st and the 2nd* diastereoisomers was approximately 60/40.

Synthesis of L-(SR)-benzyl-2-((benzyloxycarbonyl)amino)-4-(methylsulfonimidoyl)-butanoate (2)

L-(SR)-MSO (1.11 mmol, 200 mg) was dissolved in 15 ml of water, and NaHCO₃ (2.22 mmol, 188 mg, 2 equiv) was added. The mixture was stirred at room temperature for 5 min, cooled to 5 °C and a chilled solution of benzyl chloroformate (1.67 mmol, 184 mg, 284 μ l, 1.5 equiv) in 1,4-dioxane (3 ml) was slowly added. The resulting solution was stirred at 0 °C for 1 h and was allowed to warm up to room temperature for 2 days. The reaction mixture was then concentrated *in vacuo* and used in the next step without further purification.

The crude *N*-amino protected L-(SR)-MSO was dissolved in DCM (10 ml) and DMF (10 ml), dicyclohexylamine (2.22 mmol, 403 mg, 2 equiv), and benzyl bromide (2.22 mmol, 380 mg, 2 equiv) were added sequentially. The resulting mixture was stirred at room temperature for 24 h, filtered and concentrated *in vacuo*. The residue was taken up in ethyl acetate and washed with HCl (1M, 3 times), then with a saturated NaCl solution (3 times). The organic layer was dried (MgSO₄), concentrated *in vacuo* and purified with preparative RP-HPLC-MS to give the diprotected L-(SR)-2 (199 mg, 44%) as a white solid. ¹H NMR (400 MHz, CD₃OD): δ 2.10 to 2.24 (m, 1H, β -proton), 2.30 to 2.44 (m, 1H, β -proton), 2.96 (s, 3H, CH₃), 3.13 to 3.28 (m, 2H, γ -protons), 4.35 to 4.45 (m, 1H, α -proton), 5.09 (m, 2H, Bz-protons), 5.19 (m, 2H, Bz-protons), 7.26 to 7.42 (m, 10H, Ar-protons). ¹³C NMR (100 MHz, CD₃OD): δ 25.31 (C β), 25.33* (C β), 41.07 (CH₃), 41.10* (CH₃*), 52.73 (C γ), 52.76* (C γ), 52.98 (C α), 66.64 (CBz), 67.05 (CBz), 127.68 (CAr), 127.89 (CAr), 128.17, 128.22, 128.31, 128.44, 135.93, 136.88 (8C-CAr), 157.42 (C=O), 171.42 (C=O). HRMS (ESI+): calculated for C₂₀H₂₅N₂O₅S (M+1) 405.1484, found 405.1479.

The N:B ratio between the 1st and the 2nd* diastereoisomers was approximately 60/40.

Synthesis of L-(SR)-benzyl-2-((benzyloxycarbonyl)amino)-4-[methyl-N-(o-xylylenephosphoryl)-sulfonimidoyl]butanoate (3)

Diprotected **2** (1.5 mmol, 605 mg) was dissolved in 20 ml of DCM. Next, 1*H*-tetrazole (3.0 mmol, 210 mg, 2 equiv) and *o*-xylylene *N,N*-diethylphosphoramidite (3.0 mmol, 718 mg, 2 equiv) were added. The mixture was stirred for 6 h at room temperature to provide the hydroxyl phosphonite intermediate (**3''**). To this solution *tert*-butyl hydroperoxide (70% aqueous solution, 1.29 ml, 9.0 mol, 6 equiv) was added and the reaction was stirred at room temperature for 40 h. The reaction mixture was evaporated and purified with preparative RP-HPLC-MS to give the L-(SR)-*N*-phosphoryl sulfoximine (**3**) (445 mg, 51%) as a colourless oil.

L-(SS,SR,RS,RR)-benzyl-2-((benzyloxycarbonyl)amino)-4-[methyl-N-(2-(hydroxymethyl)benzylhydrophosphoryl)-sulfonimidoyl]butanoate (3'')

¹H NMR (400 MHz, DMSO-*d*⁶) of **3''**: δ 2.05 to 2.19 (m, 1H, β -proton), 2.21 to 2.33 (m, 1H, β -proton), 3.28 (s, 3H, CH₃), 3.34 to 3.61 (m, 2H, γ -protons), 4.25 to 4.33 (m, 1H, α -proton), 4.55 (app d, 2H, *J* = 5.02 Hz, Bz-protons), 4.95 to 5.19 (m, 6H, Bz-protons), 6.92 (d, 1H, *J* = 646 Hz, H-P), 6.95* (d, 1H, *J* = 646 Hz, H-P*), 7.20 to 7.44 (m, 14H, Ar-protons). 7.87 to 7.94 (m, 1H, NH). ³¹P (300 MHz, DMSO-*d*⁶): δ 5.273, 5.339, 5.395, 5.45. HRMS (ESI+) calculated for C₂₈H₃₄N₂O₈SP (M+1) 589.1774, found 589.1782.

¹H NMR (400 MHz, CD₃OD) of **3**: δ 2.20 to 2.34 (m, 1H, β -proton), 2.44 to 2.56 (m, 1H, β -proton), 3.28 (s, 3H, CH₃), 3.38 to 3.60 (m, 2H, γ -protons), 4.50 (dd, 1H, *J* = 4.89; 9.60 Hz, α -proton), 4.94 to 5.34 (m, 8H, Bz-protons), 7.20 to 7.40 (m, 14H, Ar-protons). HRMS (ESI+): calculated for C₂₈H₃₂N₂O₈SP (M+1) 587.1617 found 587.1629.

The N:B ratio between the 1st and the 2nd* diastereoisomers was approximately 60/40.

Synthesis of L-(SR)-2-amino-4-(methyl-N-phosphonosulfonimidoyl)butanoic acid (L-(SR)-MSO-P)

Purified L-(SR)-N-phosphoryl sulfoximine (**3**) (0.205 mmol, 120 mg) and Pd 10 % on carbon (87 mg, 0.082 mmol Pd, 0.4 equiv Pd) were suspended in 4 ml of a mixture of MeOH, H₂O and AcOH (5:3:2). The air was exchanged with N₂, and the reaction was stirred under H₂ for 2 h at room temperature. The resulting mixture was filtered, evaporated and purified on preparative RP-HPLC-MS to give L-(SR)-methionine sulfoximine phosphate (L-(SR)-MSO-P) as a colourless oil (29 mg, 54 %). $[\alpha]_D^{25} + 8.1$ (c 8.75, H₂O). ¹H NMR (400 MHz, D₂O): δ 2.26 to 2.44 (m, 2H, β-protons), 3.16 (s, 3H, CH₃), 3.39 to 3.58 (m, 2H, γ-protons), 4.05 (t, *J* = 6.4 Hz, 1H, α-proton). ¹³C NMR (100 MHz, D₂O): δ 22.37 (s, Cβ), 22.41* (s, Cβ*), 40.71 (d, ³*J*^{C-P} = 6.61, CH₃), 40.74* (d, ³*J*^{C-P} = 6.61, CH₃*), 50.73 (s, Cα), 50.88 (d, ³*J*^{C-P} = 5.86, Cγ), 50.90* (d, ³*J*^{C-P} = 5.86, Cγ*), 170.47 (s, C=O). HRMS (ESI-): calculated for C₅H₁₂N₂O₆SP (M-1) 259.0154, found 259.0157.

The N:B ratio between the 1st and the 2nd* diastereoisomers was approximately 55/45.

References:

- [1]. For an alternative multi-step method, see: Rowe, W.B., Ronzio, R. A., Meister, A., 1969. Inhibition of glutamine synthetase by methionine sulfoximine. Studies on methionine sulfoximine phosphate, *Biochemistry*, 1969, **8**, 2674–2680.
- [2]. For assignment of the active configuration of L-(S)-MSO, see : Manning, J. M., Moore, S., Rowe, W. B., Meister, A., Identification of L-methionine-S-sulfoximine as the diastereoisomer of L-methionine-SR-sulfoximine that inhibits glutamine synthetase, *Biochemistry*, 1969, **8**, 2681–2685.

Figure 1. SDS-PAGE of different fractions of recombinant MtGS eluted from the Ni-NTA column. Lane 5 contains molecular weight markers. Fractions 1-4 and 6-9 were pooled and dialysed as described in the text. A yield of 102 mg/l of culture (4 g wet cells) was obtained.

Figure 2. pH profile for MtGS activity, using conditions described in the main text.

Figure 3. Representatives of different compound clusters identified by the high throughput screening.

Figure 4. IC₅₀ curve for MSO: A 10-point serially diluted MSO solution in DMSO was used, as described in the text. The enzyme concentration was 7 nM (subunit concentration). Each point was an average from two different experiments. The reaction was run at 25 °C.