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Crystal structures of mammalian glutamine synthetases illustrate substrate-induced conformational changes, and provide opportunities for drug and herbicide design

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Running title: Structures of mammalian glutamine synthetases

Data deposition: The atomic coordinates and structure factors have been deposited at the Protein Data Bank, www.pdb.org (PDB ID codes 2UU7, 2OJW and 2QC8).

Summary

Glutamine synthetase catalyzes the ligation of glutamate and ammonia to form glutamine, with concomitant hydrolysis of ATP. In mammals, the activity eliminates cytotoxic ammonia, at the same time converting neurotoxic glutamate to harmless glutamine; there are a number of links between changes in glutamine synthetase activity and neurodegenerative disorders such as Alzheimer's. In plants, because of its importance in the assimilation and re-assimilation of ammonia, the enzyme is a target of some herbicides. Glutamine synthetase is also a central component of bacterial nitrogen metabolism, and a potential drug target.

Previous studies had investigated the structures of bacterial and plant glutamine synthetases. In the present publication, we report the first structures of mammalian glutamine synthetases. The *apo* form of the canine enzyme was solved by molecular replacement, and refined to a resolution of 3 Å. Two structures of human glutamine synthetase represent complexes with: a) phosphate, ADP, and manganese, and b) a phosphorylated form of the inhibitor methionine sulfoximine, ADP and manganese; these structures were refined to resolutions of 2.05 Å and 2.6 Å, respectively.

Loop movements near the active site generate more closed forms of the eukaryotic enzymes when substrates are bound; the largest changes are associated with the binding of the nucleotide. Comparisons with earlier structures provide a basis for the design of drugs that are specifically directed at either human or bacterial enzymes. The site of binding the amino acid substrate is highly conserved in bacterial as well as eukaryotic glutamine synthetases, while the nucleotide-binding site varies to a much larger degree. Thus the latter site offers the best target for specific drug design. Differences between mammalian and plant enzymes are much more subtle, suggesting that herbicides targeting glutamine synthetase must be designed with caution.

Keywords: glutamine synthetase, x-ray crystallography, conformational changes, drug design, herbicide

Abbreviations: GS, glutamine synthetase; CfGS, glutamine synthetase from *Canis familiaris*; HsGS, glutamine synthetase from *Homo sapiens*; MtGS, glutamine synthetase from *Mycobacterium tuberculosis*; StGS, glutamine synthetase from *Salmonella typhimurium*; ZmGS, glutamine synthetase from *Zea mays*; MSO, L-methionine-S-sulfoximine; MSO-P, L-methionine-S-sulfoximine phosphate; PPT, phosphinothricin; T_m, melting temperature; r.m.s., root-mean-square; PDB, Protein Data Bank.

Introduction

Glutamine synthetase (GS; EC 6.3.1.2, also known as γ -glutamyl:ammonia ligase) plays an essential role in nitrogen metabolism by catalyzing 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. Links with Alzheimer's plaque formation are well documented; levels of human GS (HsGS, also referred to as GLUL) are, for instance, higher in the cerebral spinal fluid of Alzheimer's patients.¹ GS is also one of the major oxidatively modified proteins in the hippocampus of patients with mild cognitive impairment.² The modification lowers the activity of the enzyme and contributes to glutamate dysregulation and impairment of neuroplasticity during the development of Alzheimer's. Recent studies indicate that GS is essential in early mouse development.³ 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. Because of its importance, the enzyme has been used as a target for herbicides⁴ and considered as a possible target for antimicrobial drugs, as summarized elsewhere.⁵

The ground-breaking work of Eisenberg and coworkers^{6,7,8,9} resulted in a number of structures of *Salmonella typhimurium* GS (StGS) in complex with amino acids (substrates or inhibitors), metals (Mn^{2+} , and thallium ions mimicking the ammonium substrate), and nucleotides (AMP or ADP), as well as a structure with the inhibitor phosphinothricin (PPT) bound together with Mn^{2+} and ADP. These structures correspond to the “taut” (active) state that exists when multiple metal ions are bound. An additional structure represents the GS of *Mycobacterium tuberculosis* (MtGS) with bound AMP,¹⁰ in a “relaxed” (inactive) form that is observed at lower concentrations of metal ions. A more recent report described MtGS in complex with a phosphorylated form of the inhibitor L-methionine-S-sulfoximine (i.e. MSO-P), Mg^{2+} and ATP; this is also a “taut” form of GS.¹¹ The bacterial structures are all

dodecameric, with two hexameric rings of GS subunits stacked onto each other. The first eukaryotic GS structures represented the maize (*Zea mays*) protein (ZmGS) in complexes with: a) MSO-P, Mn^{2+} and ADP, b) MSO, Mn^{2+} and the ATP analogue AMP-PNP, and c) PPT-P, Mn^{2+} and ADP.¹² Interestingly, although the fold of the subunit was similar, the arrangement was in this case that of a decamer, with two pentameric rings stacking. However, structural information concerning the mammalian enzymes has been lacking, limiting our understanding of how they differ from GSs of plants and bacteria.

In the present paper, we report the first structures of mammalian GSs, those from dog (*Canis familiaris*, CfGS) and human (HsGS), alone and in complex with metals, nucleotide and inhibitor. The results provide many new insights that will be useful in the design of novel antibacterial agents and herbicides.

Results

Biochemical characterization

The mammalian GSs appear to be less robust than the bacterial enzymes studied earlier. CfGS is cold sensitive, precipitating when stored on ice, and showing limited stability at 4 °C. HsGS tends to precipitate when concentrated. A series of dye-based fluorescence assays was therefore performed to investigate the effects of various ligands on the stability of HsGS. The melting temperature (T_m) for HsGS in the absence of ligands was 56 °C. Glutamine or glutamate had no appreciable effect on the T_m . Addition of magnesium at concentrations as high as 500 mM caused an increase of ~2 °C. The T_m increased by 4.1 °C and 11.5 °C upon the addition of 1mM of $MnCl_2$ and 1mM ATP, respectively. Addition of $MnCl_2$ and ATP together increased the T_m of HsGS by 15.5 °C. Therefore, both these ligands were added to all subsequent preparations of HsGS prior to concentration. In gel filtration experiments, the enzyme behaved as a single homogeneous species that was not affected by the addition of ATP and Mn^{2+} . CfGS and HsGS were extensively characterized in earlier

work, and shown to have similar catalytic properties.^{13,14} In preliminary kinetic experiments, we found that the same applied to the two proteins studied here.

Overall structures

The structure of the *apo* form of CfGS was solved by molecular replacement, using a pentamer of ZmGS/MnADP/MSO-P (PDB entry code 2D3A)¹² as search model, and refined to a resolution of 3 Å. Statistics for data collection and refinement are summarized in Table 1. The final model consists of three pentamers (Figure 1(a)), two of which are related by a 2-fold non-crystallographic operator, and constitute the biologically relevant decamer (Figure 1(b)). The third pentamer in the asymmetric unit is also part of a decamer, by virtue of a 2-fold crystallographic axis. The decameric assembly is ~90 Å in size measured along the 5-fold axis, and 110 Å along the 2-fold axis. Well-defined electron density is observed for each subunit from Thr3 to Lys372 of the 373-residue native sequence; only residues 70-74 and 300-305 have relatively poor electron density. Temperature factors are generally lower both within and between subunits, as would be expected for a stable oligomer, while those in surface regions are higher, particularly in the relatively unconstrained C-terminal part of the structure (residues 365-372). Subunits A, D, E, H, N, and O have additional weaker density at their C-termini consistent with the His-tag.

The 15 subunits in the asymmetric unit are very similar, with pairwise r.m.s. distances of <0.1 Å between C^α atoms, consistent with the tight NCS restraints used. The structure of a single chain consists of an N-terminal meander (residues 3-24) plus two compact domains. The N-terminal unit (residues 25-112) is generally referred to as the β-grasp domain, and the C-terminal one (residues 113-373) as the catalytic domain. Comparisons of the pentamers in the asymmetric unit reveal that they are also very similar and can be superimposed on each other with r.m.s. distances in the range of 0.2-0.3 Å for equivalent C^α atoms. The N-terminal meander makes contacts with neighboring subunits in the core of the pentamer. Many additional interactions exist between each β-grasp domain and the catalytic domain of its nearest neighbor, further stabilizing the pentamer. Interactions between pentamers are much less

extensive, and involve the loops comprised of residues 150-156 of each subunit. The five loops in one pentamer interact with their 2-fold symmetry related mates of the other pentamer ([Figure 1\(b\) and \(c\)](#)). Most of the interactions are of the van der Waals type, with the exception of the 10 hydrogen bonds that link the main-chain atoms of the Phe154 residues. Also well supported by electron density is a single Mg^{2+} ion in each subunit. Additional strong density elsewhere was modeled as a chloride ion, based on the presence of this anion in the crystallization solutions, and appropriate chelating groups and distances.

The HsGS/MnADP/ P_i structure was solved by molecular replacement, using a subunit of the ZmGS/MnADP/MSO-P (PDB entry code 2D3A)¹² structure as search model; it was subsequently refined to a resolution of 2.05 Å. Statistics related to data collection and refinement are reported in [Table 1](#). The asymmetric unit in this case includes a single pentamer; the decamer is formed as the result of a 2-fold crystallographic axis. The final model comprises residues Ala5-Gly365 of the HsGS sequence (with the exception of residues 302-306, which had no electron density), preceded by a non-native sequence (5-6 residues, LYFQSM) derived from the plasmid vector that is visible in all molecules except chain E. The vector sequence seems to add to the stability of the oligomer *via* inter-subunit and inter-pentameric interactions. Thr142 was disordered in the electron density in chains A and B, because it was displaced by the glutamine of the TEV protease recognition sequence. There are no major differences between the subunits, which superimpose on each other with r.m.s. distances of 0.1-0.3 Å based on C^α atoms. As ATP was included in the crystallization mixture, rather than the observed ADP and phosphate, it is presumed that hydrolysis occurred during the experiment. Also supported by strong electron density in each subunit are four Mn^{2+} ions and one chloride.

The structure of HsGS in complex with MSO-P, ADP and Mn^{2+} was solved by molecular replacement using a pentamer of HsGS/MnADP/ P_i as search model. The final model represents a single decamer, and was refined to 2.6 Å resolution. Statistics for data collection and refinement are given in [Table 1](#). Each protein chain begins

with Asn10 and ends with Gly365; the preceding residues in the construct were not visible in the electron density. The subunits are very similar, and can be superimposed on each other with r.m.s. distances ≤ 0.2 Å based on C $^{\alpha}$ atoms.

Sequence alignment of CfGS and HsGS ([Figure 2](#)) highlights their 97% amino acid sequence identity. It is worth noting, in particular, that there are no insertions or deletions, and so all residue numbering is the same. All sequence differences are found on the surface of the molecule, except for two involving hydrophobic residues in the protein interior. Phe98 and Ile188 of CfGS are replaced by leucine and valine in the HsGS sequence, respectively. All of the changes involve conservative substitutions that are unlikely to affect structure or function.

Active site

A GS decamer has 10 active sites, each located at the interface of two adjacent subunits in the pentameric ring. The N-terminal β -grasp domain of one subunit interacts with the highly curved β -sheet of the C-terminal catalytic domain in the next subunit, so forming a funnel-shaped pocket. The “funnels” face outwards in the pentamer shown in [Figure 1\(a\)](#). Because the two pentamers stack back-to-back ([Figure 1\(b\)](#)), the active sites in the second pentamer are oriented in the opposite direction. They are shifted relative to those of the first by $\sim 35^{\circ}$, i.e. 1/2 of the five-fold rotation component, to give a staggered arrangement.

The most complete picture of interactions within the mammalian GS active site is provided by the HsGS/MnADP/MSO-P structure ([Figure 3](#)). The adenine ring of ADP lies in a primarily hydrophobic pocket bounded by Trp130, Arg262, Tyr336 and Pro208 ([Figure 3\(a\)](#)). The side chains of Trp130 and Arg262 participate in an aromatic-arginine planar stacking interaction, while Pro208 adopts a *cis* conformation. All of these residues are highly conserved in eukaryotic GS sequences. The only hydrogen bonds are provided by the interactions of Ser257 with both N1 and N6 of the adenine moiety ([Figure 3\(b\)](#)). The ribosyl group of ADP, which adopts a C2'-endo conformation, does not make hydrogen bonds with protein ligands. The α -

and β -phosphate groups, however, make a large number of interactions with protein side chains as well as two of the three Mn^{2+} ions in the active site (those designated as n2 and n3, in accordance with earlier conventions).^{6,11} Every polar atom of MSO-P (which is expected to bind at the position of the glutamate substrate) makes one or more interactions with the protein, either directly, or *via* metal-mediated contacts involving all three Mn^{2+} ions (Figure 3(b)). Consistent with previous studies, the phosphate group is attached to the sulfoximido nitrogen of the preferred S enantiomer.^{11,15}

The binding of ADP in the HsGS/MnADP/ P_i structure is identical to that found in HsGS/MnADP/MSO-P. Three Mn^{2+} ions are observed in the same positions, and the free phosphate occupies the site of the phosphate in MSO-P. An additional Mn^{2+} ion makes contacts with Glu196 as well as the inorganic phosphate.

The active site of the CfGS apo structure contains only a single Mg^{2+} (rather than Mn^{2+}) ion, bound at the n1 position.

Conformational changes on ligand binding

Comparison of the CfGS and HsGS/MnADP/MSO-P structures (Figure 4(a)) shows that there are conformational changes near the active site. The largest movements of the main chain involve residues 311-337 of the catalytic domain, together with residues 63-77 of the β -grasp domain of the other subunit, which lie immediately adjacent in the structure. The side chains of residues 70-74 have relatively poor electron density in *apo* CfGS, but become more ordered in the complexed structures. As a rough measure of the magnitude of the changes, the distance between the $\text{C}\alpha$ atoms of residues 75 and 317 is 8.4 Å in CfGS, and 5.8 Å in HsGS/MnADP/MSO-P, a difference of 2.6 Å. Concurrent with these movements is a shift of the helix comprising residues 277-286 and the adjustment of residues 302-307. Inspection of the structures indicates that HsGS/MnADP/ P_i and HsGS/MnADP/MSO-P are most similar to each other, and approximately equally distant from CfGS (Table 2). Using the yardstick introduced above, the distance

between 75 and 317 is 5.8 Å in HsGS/MnADP/P_i, as observed for HsGS/MnADP/MSO-P. We conclude that the active site closes when the nucleotide is bound, and that few additional changes occur on binding of the amino acid substrate (as modeled from the MSO moiety). There are no major allosteric changes within or between the pentamers.

Comparison of mammalian GSs and ZmGS

CfGS and HsGS share ~50% amino acid sequence identity with the maize enzyme, ZmGS, as illustrated in the structure-based sequence alignment presented in [Figure 2](#). While naming conventions for the subunits in the mammalian structures are consistent, we note that chains named as A-E in our structures (in order, when proceeding around a pentameric ring) correspond to chains A, D, E, C and B, respectively, of the ZmGS structures.

Mammalian GSs have a slightly longer N-terminus than ZmGS; this difference lies in the subunit-subunit contact surface within the pentamer. Each subunit-subunit contact area in mammalian GS is thus 200 Å² (~10%) larger than the equivalent one in ZmGS; such effects are likely to be cooperative, and might give rise to a greater stability of the pentamer in the mammalian proteins. Mammalian GSs also have a longer C-terminus, which is on the surface of the molecule, distant from subunit-subunit packing interfaces, and unlikely to have any effects on oligomer stability. Mammalian pentamer-pentamer contacts are comparable to those observed in ZmGS.

There are in fact three structures currently available for ZmGS: a) ZmGS/AMP-PNP/MSO, b) ZmGS/ADP/MSO-P and c) ZmGS/ADP/PPT-P. Three manganese ions were observed in the active site in all cases, with positions equivalent to n1-n3. Residues lining the active sites are also highly conserved. The only notable difference is that Ser187 of the maize enzyme replaces Ala191 in human and dog GS. The serine residue provides a single hydrogen bond to the ribosyl moiety of the nucleotide that is not found in mammalian GSs. We predict that there should be a *cis* proline at Pro204 in ZmGS, as seen for the mammalian proteins; such a feature might easily have been

missed at the lower resolution available for the maize structures (2.63 Å, at best). In [Figure 4\(b\)](#), we compare the conformation of the HsGS/MnADP/MSO-P complex to that of ZmGS/MnADP/MSO-P. The conformations observed in the ZmGS structures are in fact all very similar to both HsGS/MnADP/P_i and HsGS/MnADP/MSO-P, and distinct from that observed for apo CfGS ([Table 2](#)).

Comparisons with bacterial GSs

Structure-based sequence alignments comparing the eukaryotic GSs with the known bacterial GS structures (from *S. typhimurium* and *M. tuberculosis*) are shown in [Figure 2](#). According to this comparison, the mammalian GSs share less than 20% amino acid sequence identity with the bacterial enzymes. Comparisons are complicated by the fact that the bacterial enzymes are dodecameric, rather than decameric. When the catalytic domains of bacterial and mammalian GSs are aligned, it is seen that there are differences in their relationships to the respective N-terminal domains of the same subunit. By virtue of differences in the oligomeric contacts, however, the architecture of each active site is strikingly similar. [Figure 5](#) shows a comparison of the active sites of mammalian and MtGS structures. All residues binding to MSO-P and Mg²⁺/Mn²⁺ are conserved, as are the positions of the three metal ions. By contrast, all side chains lining the nucleotide-binding site are different, and some differences are seen in the positions of main-chain atoms. The bacterial enzymes are shorter at the N-terminal end than mammalian GSs, and this segment is not involved in subunit-subunit contacts in the hexamer. A very long C-terminal extension protrudes from each subunit in the bacterial GSs, and enters the second hexameric ring; no equivalent interaction is seen in the eukaryotic GS structures. In addition, eukaryotic GSs lack a β-hairpin that contributes to the hexamer-hexamer interactions in bacterial enzymes. Thus bacterial hexamer-hexamer contacts are more than 10 times larger than eukaryotic pentamer-pentamer ones. The C-terminal extension of the bacterial GSs also contains the site of the adenylylation (highlighted

in orange in [Figure 2](#)), which is involved in regulation of activity of some prokaryotic enzymes.

Discussion

GS has been proposed to use a two-step reaction mechanism.¹⁶ The first step involves formation of a γ -glutamyl phosphate intermediate. The position of the glutamate substrate is expected to be the same as that of the MSO moiety in the HsGS/MnADP/MSO-P structure. The phosphate group of MSO-P occupies the same position as the phosphate group observed in HsGS/MnADP/P_i; this represents the phosphate that is transferred in the reaction. The three magnesium ions in the active site will thus coordinate the γ -phosphate oxygens of ATP and assist in its transfer to the glutamate. In the second step of the reaction, attack on the intermediate by ammonia yields glutamine and inorganic phosphate. The fourth ion in the HsGS/MnADP/P_i structure lies at or near the ammonium site identified by an earlier structure with bound thallium.¹⁷ This site is filled by the methyl group of the MSO-P in that complex structure.

The active site of *apo* CfGS is more open than is observed in either HsGS/MnADP/P_i or HsGS/MnADP/MSO-P. We conclude that the conformational changes are due to binding the nucleotide, since differences in the sequences of canine and human GSs are few, and at locations unlikely to affect either structure or function. Furthermore, equivalent conformations are observed in all of the subunits in the respective asymmetric units. The importance of nucleotide in determining the conformation of GS is also indicated by the large effects of ATP and Mn²⁺, but not glutamate or glutamine, on the T_m. The movements primarily affect three segments, one from the β -grasp domain, and two from the catalytic domain; one of the latter includes three residues (Arg319, Glu338 and Arg340) that make direct contact with glutamate or metal. Binding of the ATP causes changes in GS that prepare it for binding to glutamate.

The movements in the β -grasp domain involve Asp76, which interacts with Arg319 (e.g. from chains E and A, respectively). The arginine in turn binds to the inorganic phosphate in the HsGS/MnADP/P_i structure, and the phosphate group of MSO-P in the HsGS/MnADP/MSO-P structure. Arg319 is disordered in the CfGS *apo* structure. Asp76 also interacts with Arg324, which moves closer in the complexed structures, allowing it to bind the β -phosphate of the nucleotide. Proper positioning of Asp76 is thus crucial for nucleotide binding. When the A molecules are superimposed, it is seen that the same role is fulfilled by Glu69 in chain E of the maize enzyme, and by Asp68 of chain F in the hexamer of MtGS.

The single magnesium ion seen in the *apo* CfGS structure (n1) is that most dependent on protein contacts; these will favor its binding even in the absence of substrates. The divalent cation will also serve to compensate for the charges on the three acidic residues involved (glutamates 136, 196 and 203). The other two cations are apparently bound only as glutamate and ATP enter the site. A single chloride is bound at a subunit-subunit boundary in both *apo* and complexed structures, and may stabilize the pentamer in the biological setting. Physiologically-relevant levels of chloride have been reported to stabilize the CfGS¹³ and HsGS,¹⁴ and increase the affinity of bovine GS for magnesium or manganese 2-4 fold.¹⁸

In some subunits of the CfGS structure, electron density is observed for the C-terminal His-tag. In each case, a symmetry related molecule is sufficiently close for this segment to bind in a similar fashion within its active site. For instance, the backbone atoms of the His-tag in subunit E interact with residues 55-60 in the edge strand of the β -sheet in a crystallographically-related subunit B; the end of the His-tag lies near where the base of the nucleotide would bind. Similar interactions could have a biological role, for example, in regulation, but further experiments would be needed to test this hypothesis.

Chromosomal mapping identified five homologues of the GS gene in the human genome and revealed their chromosomal location.¹⁹ At least two of the sequences appear to be pseudogenes; the same may be true of two others. The gene that

corresponds to the functional HsGS lies on chromosome 1 and was designated as GLUL. We performed a BLAST search of the human genome, and found a number of GLUL variants. The changes are minor, and all appear to code for active GS enzymes, based on conservation of active site residues. One isoenzyme, for example, has an extra IQLFPLSQLRSL sequence at its C-terminal end, with an intriguing leucine repeat every third residue. Two others represent Phe154Leu and Pro155Thr changes at the pentamer-pentamer interface, which could affect stability of the oligomer. Furthermore, some transcripts of dog brain GS have a 40-residue extension at the N-terminal end; there is an extra exon in the first intron, which is generated by alternative splicing.^{13,20} The variant forms are reported to have slightly different kinetic and physical properties.¹³ Both transcripts are present in all cellular compartments in dog brain, but it is not known if such variants are present in other species; HsGS, at least, has a stop codon at the relevant position. It is not clear what the role of such an extra sequence might be, or indeed whether it might form a part of the folded structure of GS; secondary structure prediction suggests that much of the extension is likely to be disordered. It is highly polar, with nearly half of its residues carrying a charge. A potential physiological role in regulation is intriguing, given the presence of a consensus protein kinase C phosphorylation site near the middle of the single predicted helical segment.

The active agent of several commercially available herbicides, PPT (also known as glufosinate), is a GS inhibitor that, like MSO, binds in the glutamate site. Glufosinate treated plants die primarily as a result of the buildup of ammonia and the cessation of photosynthesis. Use of the herbicide has been combined with the development of resistant crop plants, such as Aventis' Liberty Link series. Since it is vital that normal functions of mammalian GS are not interfered with, comparisons of mammalian and plant GS structures will be important in future herbicide design. Mammalian GSs have a longer N-terminus than ZmGS (Figures 2 and 4), which may add to the stability of the pentamer through slightly larger contact surfaces in the middle of the doughnut-shaped pentameric ring (Figure 1(a)). However, most other

structural features are similar, or not expected to result in functional differences. In particular, comparisons of the active sites of plant GSs with those of mammalian GSs indicate that they are highly similar. In addition to the almost complete conservation of the residues that interact with glutamate, magnesium ions and ATP, residues of the ammonia-binding site are highly conserved. For instance, the equivalent of Val197 is completely conserved in all species, prokaryotic and eukaryotic. This residue makes van der Waals contact with the methyl group of MSO-P in the structure reported here. Therefore, compounds that target the plant GS active site would be expected to inhibit the mammalian enzymes, as well. Humans have some protection from the negative effects of ammonia accumulation, as alternate routes for its removal exist. However, like MSO, the herbicide is neurotoxic, provided that enough crosses the blood-brain barrier.^{21,22} Furthermore, glufosinate is toxic to bacteria, including essential symbionts. Our structures suggest that a safer approach would make use of non-competitive inhibitors that target portions of the enzyme affected by the conformational changes. In general, residues in these regions are much less conserved than those in the active site.

Comparisons with the more distantly related bacterial GSs show that, given the very low sequence identity, the structural conservation in the active site is remarkable (Figure 5). Therefore, the enzymes are expected to have the same reaction mechanism, consistent with our observation that they have comparable levels of activity. Bacterial hexamer-hexamer contacts are much larger than eukaryotic pentamer-pentamer contacts, in line with the observed relative stability of the proteins. Bacterial GS is highly stable, while the mammalian enzymes have a tendency to be unstable or insoluble *in vitro*, as evident here. There is a long C-terminal extension in the bacterial enzymes that includes a tyrosine that can be adenylylated, resulting in lower activity. This segment is missing in the mammalian GSs, and so regulation of this kind is not possible.

The structure-based sequence alignment further illustrates the fact that the bacterial enzymes can exist in two different conformations, which are distinguished

by a three-residue register shift of the β -strand starting at Glu214 (MtGS numbering).¹¹ The residues involved make interactions with glutamate and ATP, and so the relaxed version (boxed in the 1HTO sequence in [Figure 2](#)) is by definition inactive. In the three structures reported here, there is a metal ion present at the n1 site and therefore they are in the taut (active) conformation. In principle, low metal concentration could also cause a strand shift in the eukaryotic enzymes. Since there is a deletion in this region (after Pro199 in mammalian GS), a two-residue register shift would be expected instead of the three-residue one observed in prokaryotic GSs. However, we have no evidence at present that such a strand shift does in fact occur in the mammalian GSs.

Several features are particularly relevant for the design of drugs directed specifically against the bacterial GSs. We emphasize the fact that the relative placement and orientation of glutamate, metals, ATP and ammonia are all extremely similar in the mammalian and bacterial enzymes ([Figure 5](#)). The residues defining the glutamate site are identical, and those binding to metal are almost completely conserved. However, the nature of the nucleotide site is entirely different. Therefore, we suggest that the design of specific inhibitors should focus on the nucleotide-binding site. Although extension of compounds into the metal and/or amino acid binding sites could be useful for reasons of solubility or uptake, these sites do not offer any opportunities for achieving selectivity. Combined with the facts that MtGS is essential,^{23,24,25} and that its extracellular activity is important to the pathogen²⁶ (i.e. drugs need not penetrate the formidable mycobacterial cell wall), prospects for the design of effective anti-tubercular drugs directed at glutamine synthetase seem good.

Experimental procedures

Cloning, protein expression and purification

Plasmid (pET-SGS)¹³ encoding C-terminal His-tagged CfGS was kindly provided by Chankyu Park (KAIST). For protein production, the plasmid was transformed into the *E. coli* BL21DE3 (Star) strain. Cells were grown at 37 °C in LB

medium containing 100 µg/ml ampicillin until A_{600} reached 0.6, at which time expression was induced with 0.5 mM IPTG. After growth for 4 h at 30 °C, cells were harvested by centrifugation, washed with lysis buffer (20 mM Tris-HCl (pH 8.0), 500 mM NaCl, 5 mM imidazole, 10%(v/v) glycerol) and stored at -20 °C. For protein purification, thawed cells were re-suspended in lysis buffer supplemented with 5 µg/ml DNase I and 10 µg/ml RNase A, and lysed by sonication on ice. Soluble CfGS was recovered by high-speed centrifugation and incubated with pre-equilibrated Ni Sepharose[™] 6 Fast Flow (GE Healthcare) for 1 h at 4 °C; the resin was washed and CfGS eluted with 20 mM Tris-HCl (pH 8.0), 500 mM NaCl, and 800 mM imidazole. The elution buffer was then exchanged to 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 5 mM MgCl₂, 1 mM sodium 2-mercaptoethanesulfonate (MESNA) by repeated dilution and concentration using a Vivaspin concentrator (Vivascience) with a 30,000 Da molecular weight cut-off. The protein was concentrated to 9 mg/ml as measured by BioRad Bradford assay and stored at 4 °C.

DNA fragments encoding residues 5-365 and 9-365 of HsGS were PCR-amplified from human muscle cDNA (obtained from the Mammalian Gene Collection at the National Institutes of Health, with entry code BC011852), and cloned into the pNIC-BSA4 vector. The resulting constructs each have a His₆-tag and a TEV protease site at the N-terminus. For protein production, the plasmid was transformed into the *E. coli* BL-21(DE3)gold pRARE2 strain. Cells were grown at 37 °C in a phosphate-buffered Terrific Broth medium²⁷ containing 50 µg/ml kanamycin until A_{600} reached 1.0. At this time the incubation temperature was lowered to 18°C and expression was induced by the addition of 0.5 mM IPTG and continued for 18 hours. Cells were harvested by centrifugation and suspended in lysis buffer (50 mM sodium phosphate (pH 7.5), 10%(v/v) glycerol, 0.5 mM Tris(2-carboxyethyl)phosphine hydrochloride (TCEP), 500 mM NaCl, 10 mM imidazole, plus complete EDTA-free protease inhibitor (Roche Biosciences)). Suspended cells were stored at -80°C until further use. For protein purification, thawed cells were supplemented with 250 U/µl benzonase (Novagen), and lysed by sonication on ice. Soluble protein was recovered

by high-speed centrifugation and filtered through a 0.45 μ m syringe filter. HsGS was purified on a Hi-Trap chelating column followed by a Superdex 200 gel filtration column on the Äkta Express (GE Healthcare). IMAC Bind/Wash Buffer contained 50 mM Na-phosphate, 10%(v/v) glycerol, 0.5 mM TCEP, 500 mM NaCl, and 10 mM imidazole (pH 7.5). IMAC-wash and elution buffers were the same except that the imidazole concentration was raised to 25 and 500 mM, respectively. Gel filtration buffer contained 20 mM HEPES, 300 mM NaCl, 10%(v/v) glycerol, 0.5 mM TCEP (pH 7.5). Fractions containing HsGS were pooled and the TCEP concentration adjusted to 2 mM. ATP and MnCl₂ were added to concentrations of 10 mM each, after which purified HsGS was concentrated using an Amicon Ultra MWCO 10000 concentrator to 40 mg/mL, as measured by BioRad Bradford assay; purified protein was stored at -80 °C.

Fluorescent dye monitored thermal shift assays

25 μ l reactions, performed in a 96-well PCR plate format, contained 5 μ g HsGS and 1000-fold diluted SyproOrange (Molecular Probes) in gel filtration buffer. Various concentrations of the following ligands were tested, alone and in combination: glutamate, glutamine, MgCl₂, MnCl₂, nucleotides and their analogs. Using an iCycler thermocycler (Bio-Rad), the temperature of the reactions was increased from 20°C to 90°C in 0.2 °C/12sec increments, coincident with a fluorescent measurement at each step. The temperature midpoint for the protein unfolding transition (T_m) was extracted from the fluorescence imaging data, using a Boltzmann model and XLfit 4 (ID Business Solutions) as described previously.²⁸

Crystallization, data collection, and processing

Crystals of CfGS were obtained by vapor diffusion in hanging drops at room temperature. A protein solution (9 mg/ml) was mixed with an equal volume of mother liquor consisting of 15%(w/v) polyethylene glycol (PEG) 3350 and 200 mM potassium citrate (pH 8.3). The crystals were further improved by streak-seeding into drops where the protein and precipitant concentrations were reduced by 50%. For X-

ray data collection, crystals were transferred to a cryo-protectant solution of 10%(w/v) PEG3350, 100 mM Tris-HCl (pH 8.5), 100 mM MgCl_2 , 30%(v/v) PEG400, and flash-cooled in liquid nitrogen. A data set extending to 3.0 Å resolution was collected at 100 K on an ADSC Q315r CCD detector at beamline ID14-4 at the European Synchrotron Radiation Facility in Grenoble, France. The data were integrated and scaled using MOSFLM²⁹ and SCALA³⁰ (as implemented in the CCP4 program suite³¹). The crystals belonged to space group C222₁, and the Matthews coefficient³² suggested the presence of three to four pentamers in the asymmetric unit.

Crystals of HsGS in complex with manganese, ADP and phosphate were obtained by vapor diffusion in sitting drops incubated at 277 K. A 20 mg/ml protein solution (containing 10 mM each of ATP and MnCl_2) was mixed with an equal volume of mother liquor consisting of 10%(v/v) isopropanol, 200 mM NaCl and 100 mM HEPES (pH 7.5). For X-ray data collection, crystals were transferred to a cryo-protectant solution of 10%(v/v) isopropanol, 200 mM NaCl and 100 mM HEPES (pH 7.5), 30%(v/v) glycerol, and flash-cooled in liquid nitrogen. A data set extending to 2.05 Å resolution was collected at 100 K on an ADSC Q4r CCD detector at beamline ID14-2 at the European Synchrotron Radiation Facility in Grenoble, France. The data were integrated and scaled using XDS/XSCALE.³³ The crystals belonged to space group C2, and the Matthews coefficient suggested the presence of one pentamer in the asymmetric unit.

A complex of HsGS with manganese, ADP and MSO-P was created *in situ* then crystallized by vapor diffusion in sitting drops. A mixture of 20 mg/ml protein solution (containing 10 mM each of ATP and MnCl_2) and 2 mM MSO was pre-incubated at room temperature for 45 min and then mixed with an equal volume of mother liquor consisting of 1.1 M sodium malonate, 0.5%(w/v) Jeffamine ED-2001 and 0.1 M HEPES (pH 7.0). For X-ray data collection, crystals were transferred to a cryo-protectant solution of 1.1 M sodium malonate, 0.5%(w/v) Jeffamine ED-2001, 0.1 M HEPES (pH 7.0), 30%(v/v) glycerol, and flash-cooled in liquid nitrogen. A data set extending to 2.60 Å resolution was collected at 100 K on an ADSC Q315r

CCD detector at beamline ID29 at the European Synchrotron Radiation Facility in Grenoble, France. The data were integrated and scaled using XDS/XSCALE.³³ The crystals belonged to space group C2, and the Matthews coefficient suggested the presence of two pentamers in the asymmetric unit.

Crystallographic statistics for the three data sets are summarized in [Table 1](#).

Structure determination and refinement

The structure of CfGS was solved by molecular replacement using AMoRe³⁴ as implemented in the CCP4 interface,³⁵ using a pentamer of ZmGS (PDB entry 2D3A¹²) as search model. After refined fitting of three pentamers, the solution was inspected with the graphics program O³⁶ and seen to exhibit good crystal packing. NCS operators relating all the subunits to one monomer of ZmGS structure were obtained using the lsq options in O. Initial crystallographic refinement was performed using the CNS package,³⁷ and strict NCS constraints were maintained within and between the pentamers. The model was first subjected to a simulated-annealing slowcool protocol, followed by cycles of energy minimization and grouped B-factor refinement. In later cycles, REFMAC5³⁸ was instead used for refinement including tight NCS restraints, and individual B-factor refinement. After each refinement cycle, SIGMAA-weighted $2m|F_o|-d|F_c|$ and $m|F_o|-d|F_c|$ maps³⁹ were calculated and used for manual model rebuilding of the A molecule in O; copies were then placed at the positions of the other subunits using the NCS operators. Statistics for the final refined model are given in [Table 1](#).

The structure of HsGS/MnADP/P_i was solved by molecular replacement using MolRep⁴⁰ as implemented in the CCP4 interface, with a subunit of ZmGS (PDB entry 2D3A) as search model. The solution was inspected at the graphics display and seen to exhibit good crystal packing. The initial model was improved and rebuilt using ARP/wARP.⁴¹ REFMAC5 was used in the refinement process including NCS restraints, and individual B-factor refinement. Coot⁴² was used for model building. In the later stages of refinement, TLS parameters⁴³ were included and refined. The TLS groups were selected using the tlsmd server

<http://skuld.bmsc.washington.edu/~tlsmd/>.⁴⁴ Statistics for the final refined model are given in Table 1.

HsGS/MnADP/P_i was used as an initial model for the MSO-P complex structure; the two pentamers of the decamer were placed by molecular replacement using MolRep. Refinement was conducted using REFMAC5 including NCS restraints and individual B-factor refinement. Coot was used for model building. In the later stages of refinement, TLS parameters were included and refined. Statistics for the final refined model are given in Table 1.

Structural comparisons and structure analysis

Structures were compared using the programs O and LSQMAN.⁴⁵ Figures were prepared with Pymol⁴⁶ and ChemDraw (CambridgeSoft Corp).

Protein Data Bank accession codes

Coordinates and structure factor data were deposited in the Protein Data Bank, with accession codes 2UU7, 2OJW, and 2QC8 for the apo CfGS and HsGS/MnADP/P_i and HsGS/MnADP/MSO-P structures, respectively.

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Figure legends:

Figure 1. Overall structures. (a) Cartoon representation of a CfGS pentamer. One subunit is colored from blue to red beginning from the N-terminus. The blue-cyan unit corresponds to the N-terminal β -grasp domain, while the remainder represents the C-terminal catalytic domain. The positions of the n1 magnesium ions (magenta spheres) mark the funnel-shaped active-site cavities that face outwards. The biologically relevant decamer is formed *via* a non-crystallographic or crystallographic 2-fold axis that places the second pentamer ring behind the first. The active sites in the second pentamer are placed between those in the first, and face in the opposite direction. (b) Here, a CfGS decamer is shown from a view 90° away from that in panel (a), illustrating the weaker pentamer-pentamer contacts. Subunits A through E, and F through J, are again colored in the rainbow scheme going from blue to red. A typical interaction at the pentamer-pentamer interface is shown in more detail in panel (c). Residues 150-156 of the 2-fold related subunits are shown, along with the hydrogen bonding interactions between the main-chain atoms of Phe154.

Figure 2. Structure-based sequence alignments. Residues involved in binding of glutamate (red), ATP (blue) and ammonia (green) are highlighted. The adenylylation site found in prokaryotic GSs is highlighted in orange. The residues in the strand shift observed for MtGS are shown boxed.

Figure 3. Active site. (a) HsGS active site with bound ADP, MSO-P and manganese ions. The N-terminal β -grasp domain is colored in blue, while the C-terminal catalytic domain of the adjoining subunit is colored in gold. Electron density of ligands is shown by using the final SIGMAA-weighted $2m|F_o|-d|F_c|$ map contoured at 1σ (0.26 electrons $\text{\AA}^{-3}$). Conserved hydrophobic residues interacting with the adenine ring of ADP, as described in the text, are shown with ball-and-stick representations. (b) Polar interactions with ligands in the HsGS/MnADP/MSO-P structure.

Figure 4. Conformational changes near the active sites of eukaryotic GSs. In each case, the catalytic domain of molecule A and the β -grasp domain of the neighboring subunit are shown in a stereo diagram, after superposition of the respective A molecules using a 0.7 Å cut-off. MSO-P and ADP of HsGS are shown as ball-and-stick models, and manganese ions are depicted by magenta spheres. (a) Superposition of C α -traces of HsGS/MnADP/MSO-P (red carbons) and CfGS (blue carbon atoms). (b) Superposition of C α -traces of HsGS/MnADP/MSO-P (red carbons) and ZmGS/MnADP/MSO-P (green carbons).

Figure 5. Comparison of active sites of mammalian and mycobacterial GSs. The MtGS/MgADP/MSO-P structure (gray) is superposed on that of HsGS/MnADP/MSO-P (gold), as described in Figure 4. MSO-P and ADP of HsGS are shown as ball-and-stick models; the HsGS manganese ions are shown as magenta spheres, while gray spheres represent the magnesium ions of MtGS. For clarity, only residues of HsGS are labeled.

Table 1. Statistics for data collection and refinement.

Data collection^a	CfGS	HsGS/MnADP/P_i	HsGS/MnADP/MSO-P
Cell axial lengths (Å)	183.6, 485.6, 192.2	177.6, 122.6, 126.6	181.2, 126.1, 188.2
Space group	C222 ₁	C2; $\beta = 130.6^\circ$	C2; $\beta = 92.1^\circ$
Resolution range (Å)	30.00-3.00 (3.16-3.00)	40.00-2.05 (2.15-2.05)	40.00-2.60 (2.67-2.60)
Number of reflections measured	619,240	476,341	979,183
Number of unique reflections	168,869	127,761	129,932
Average multiplicity	3.7 (3.6)	3.8 (3.8)	7.5 (7.2)
Completeness (%)	99.0 (98.8)	98.8 (98.6)	99.9 (100.0)
R _{meas} ^b	0.17 (0.41)	0.13 (0.49)	0.12 (0.52)
$\langle I/\sigma(I) \rangle$	7.5 (3.3)	10.3 (3.2)	15.7 (4.1)
Solvent content (%)	62.6	49.1	50.8
Refinement statistics			
Resolution range (Å)	30.00–3.00	40.00-2.05	40.00-2.60
Number of reflections used in working set	166,824	121,373	123,433
No. of reflections for R _{free} calculation	2015	6388	6497
R-value, R _{free} (%)	21.4, 23.1	16.2, 21.2	18.9, 21.6
No. of non-hydrogen atoms	44,025	15,882	28,837
No. of solvent water molecules	-	1390	235
Mean B-factor, protein atoms (Å ²) ^c	43.5	14.2	43.1
Mean B-factor, ligand atoms (Å ²) ^c	-	12.9	44.5
Mean B-factor, Mg ²⁺ /Mn ²⁺ ions (Å ²) ^c	28.0	10.5	42.9

Mean B-factor, solvent atoms (Å ²) ^c	-	22.6	32.4
Ramachandran plot outliers (%) ^d	2.0	0.3	0.3
r.m.s. deviation from ideal bond length (Å) ^e	0.013	0.019	0.015
r.m.s. deviation from ideal bond angle (°) ^e	1.4	1.7	1.6

^aValues in parentheses are for the highest resolution shell.

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

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

^cCalculated using Moleman2.⁴⁷

^dCalculated using a strict-boundary Ramachandran plot.⁴⁸

^eUsing the parameters of Engh & Huber.⁴⁹

Table 2. Structural comparisons.

Structures were compared using molecule A with a cutoff of 0.7 Å in the program LSQMAN. At top/right is the r.m.s. distance in Å. At bottom/left, the number of residues matched is reported; the sequence identity within that region is given in parentheses. Number of residues in the sequence is shown in bold on the diagonal.

Model	CfGS	HsGS/ MnADP/P _i	HsGS/Mn ADP/ MSO-P	ZmGS/MnAD P/ MSO-P	MtGS/MS O-P/ MgADP	StGS/PPT/ MnADP
CfGS	370	0.33	0.30	0.40	0.37	0.40
HsGS/ MnADP/P _i	294 (97.6)	360	0.27	0.35	0.37	0.39
HsGS/MnADP / MSO-P	303 (98)	334 (100)	357	0.35	0.40	0.42
ZmGS/MnAD P/ MSO-P	221 (65)	242 (64)	253 (63)	353	0.40	0.45
MtGS/MSO-P/ MgADP	37 (40.5)	49 (53)	54 (50)	51 (47)	475	0.42
StGS/PPT/ MnADP	40 (45)	35 (40)	39 (38)	38 (47)	180 (58)	468

¹ When superimposing chain A of MtGS on CfGS, residues 113-362 of CfGS superpose with corresponding residues of MtGS, but residues 26-112 from chain E are structurally analogous to residues in chain F of MtGS.

Figure
[Click here to download high resolution image](#)

<i>dog/1-373</i>	1 MATSASSHLNKGIKQVYMSLPQ---GEKV-QAMYIWIDGTGGLRCKTRTLOSEPKGVEELPEWN	61
<i>human/1-373</i>	1 MTTSSASSHLNKGIKQVYMSLPQ---GEKV-QAMYIWIDGTGGLRCKTRTLOSEPKGVEELPEWN	61
<i>maize/1-356</i>	1 -----MACLTDLVNLNLSDTTEKI-IAEYIWIGGSGMDLRSKARTLPGPVTDPKLPKWN	54
<i>M.tuberculosis_2bvc/1-478</i>	1 -----VTEKTPDDVF-KLAK---DEKVEYVDVRFCDLPG-IMQHFTIPAS-AFDKSVFDDGLA	52
<i>M.tuberculosis_1hta/1-478</i>	1 -----VTEKTPDDVF-KLAK---DEKVEYVDVRFCDLPG-IMQHFTIPAS-AFDKSVFDDGLA	52
<i>S.typhimurium/1-469</i>	1 -----MSAEHVL-TMLN---EHVKFVDLRFDTDTKG-KEQHVTIPAH-QVNAEFFEKGK	49
<i>dog/1-373</i>	62 FDGSSTF-QSEGSNSDMYLVPAA--MFRDPFRKDPNKLVLCEVFKY-NRPAETNLRHTCKRIMD	122
<i>human/1-373</i>	62 FDGSSLT-QSEGSNSDMYLVPAA--MFRDPFRKDPNKLVLCEVFKY-NRPAETNLRHTCKRIMD	122
<i>maize/1-356</i>	55 YDGSSTG-QAPGEDSEVILYPQA--IFKDPFRGNNILVMCDCTP-AGEPIPTNKRYSAAKIFS	115
<i>M.tuberculosis_2bvc/1-478</i>	53 FDGSSIRGFQSIHESDMLLPDPETARIDPFRAAKTLNINFFVHDPFTLEPYSRDPRNIARKAEN	117
<i>M.tuberculosis_1hta/1-478</i>	53 FDGSSIRGFQSIHESDMLLPDPETARIDPFRAAKTLNINFFVHDPFTLEPYSRDPRNIARKAEN	117
<i>S.typhimurium/1-469</i>	50 FDGSSIGGWKGINESDMVLMPPDASTAVIDPFFADSTLIIRCDILEPGTLQGYDRDPRSIAKRAED	114
<i>dog/1-373</i>	123 MVS---NQHPWFGMEQYETLMGTDG-----HPFGWPSNGFPG-----PQ	158
<i>human/1-373</i>	123 MVS---NQHPWFGMEQYETLMGTDG-----HPFGWPSNGFPG-----PQ	158
<i>maize/1-356</i>	116 SPEVA--AEEPWYGIQYETLLQKDTN-----WPLGWPIGGFPG-----PQ	154
<i>M.tuberculosis_2bvc/1-478</i>	118 YLISTGIADTAYFGAIAEFYIFDSVSFDSRANGSFYEVDASIGWWNTGAATEADGSPNRGYKVRH	182
<i>M.tuberculosis_1hta/1-478</i>	118 YLISTGIADTAYFGAIAEFYIFDSVSFDSRANGSFYEVDASIGWWNTGAATEADGSPNRGYKVRH	182
<i>S.typhimurium/1-469</i>	115 YLRATGIADTVLFCPEPEFFLFDDIRFGASISGSHVAIDDI EGAWNSSTKY---EGGNKGHRPGV	176
<i>dog/1-373</i>	159 GPYCGVGADKAYGRDIVEAHYRACLYAGIKI---AGTNAEVMP-AQWFFQIGPCEGIDMGDLHW	219
<i>human/1-373</i>	159 GPYCGVGADRAYGRDIVEAHYRACLYAGVKI---AGTNAEVMP-AQWFFQIGPCEGIDMGDLHW	219
<i>maize/1-356</i>	155 GPYCGIGAEEKSFRDIDVAHYKACLYAGINI---SGINGEVMP-GQWFFQVQPSVGISSGDQVW	215
<i>M.tuberculosis_2bvc/1-478</i>	183 KGGFPVAP-NDQYVDLRDKMLTNLINSGFIL---LXGHHIVGS---GGQALINYOQNSLLHAADDQM	243
<i>M.tuberculosis_1hta/1-478</i>	183 KGGFPVAP-NDQYVDLRDKMLTNLINSGFIL---LXGHHIVGS---GGQALINYOQNSLLHAADDQM	243
<i>S.typhimurium/1-469</i>	177 KGGFPVPP-VDSAQDIRSEMCLVMEQMGLVV---LAHHHLVATAGQNIVATRENTMTKKADEIQ	237
<i>dog/1-373</i>	220 VARFILHRVCEDFGVIATFDPKPIPGWNWAGCHTNFSTK-----AMKEENGL-KYIEESIE	275
<i>human/1-373</i>	220 VARFILHRVCEDFGVIATFDPKPIPGWNWAGCHTNFSTK-----AMKEENGL-KYIEEAIE	275
<i>maize/1-356</i>	216 VARYILHRVTEIAGVVTFDPKPIPGWNWAGACHTNFSTK-----SMKEEGGL-EVIEAAIE	271
<i>M.tuberculosis_2bvc/1-478</i>	244 LYKYIKNTAWQNGKTVT FMPKPLFG-DNGSGMHCHQSLWKDGAPLMYDETGYAGLSDTARHYIG	307
<i>M.tuberculosis_1hta/1-478</i>	244 LYKYIKNTAWQNGKTVT FMPKPLFG-DNGSGMHCHQSLWKDGAPLMYDETGYAGLSDTARHYIG	307
<i>S.typhimurium/1-469</i>	238 IYKYVVHNAHRFGKTATFMPKPMFG-DNGSGMHCHMSLAKNGTNLFSGD-KYAGLSEQUALYYIG	300
<i>dog/1-373</i>	276 KLSKRHQYHIRAYDPKGGDLNARRLTGFTSNINDFSAGVANRSGASIRIPRTVQGEEKGYFEDR	340
<i>human/1-373</i>	276 KLSKRHQYHIRAYDPKGGDLNARRLTAFHTSNINDFSAGVANRSGASIRIPRTVQGEEKGYFEDR	340
<i>maize/1-356</i>	272 KKLRLRKHKEHIAAYGE---GNERALTRHITADINTFSWGVANRSGASIRVGRETEQNGKGYFEDR	332
<i>M.tuberculosis_2bvc/1-478</i>	308 GLLHHAPSLLAFTNPT--VNSYKRLVPGYAP I--NLVYSQRNRSGACVRIPITGSNPKAKRLLEFR	368
<i>M.tuberculosis_1hta/1-478</i>	308 GLLHHAPSLLAFTNPT--VNSYKRLVPGYAP I--NLVYSQRNRSGACVRIPITGSNPKAKRLLEFR	368
<i>S.typhimurium/1-469</i>	301 GLVIKHAKEINALANPT--TNSYKRLVPGYAPV--MLAYSARNRSGASIRIPVVA--SPKARRIEVR	360
<i>dog/1-373</i>	341 RPSANCDPF SVTEALIRTCLLNETGDPEFQYKN-----	373
<i>human/1-373</i>	341 RPSANCDPF SVTEALIRTCLLNETGDPEFQYKN-----	373
<i>maize/1-356</i>	333 RPSANMOPYVVTSMIAETIIVWKP-----	356
<i>M.tuberculosis_2bvc/1-478</i>	369 SPDSSGNPYLAFSAML MAGLDG IKNK IEPQAPVDKDL ELPPPEEAASIPQTPTQLSDV IORLEAD	433
<i>M.tuberculosis_1hta/1-478</i>	369 SPDSSGNPYLAFSAML MAGLDG IKNK IEPQAPVDKDL ELPPPEEAASIPQTPTQLSDV IORLEAD	433
<i>S.typhimurium/1-469</i>	361 FPDPAANPYLCFAALLMAGLDG IKNK IHPGEPMDKNL DLPPEEAKIIPQVAGSLEEALNALDLD	425
<i>dog/1-373</i>	-----	
<i>human/1-373</i>	-----	
<i>maize/1-356</i>	-----	
<i>M.tuberculosis_2bvc/1-478</i>	434 HEYLTGGVFTNDLIETWISFKRENEI EPVNIRPHPYEFALYYDV-----	478
<i>M.tuberculosis_1hta/1-478</i>	434 HEYLTGGVFTNDLIETWISFKRENEI EPVNIRPHPYEFALYYDV-----	478
<i>S.typhimurium/1-469</i>	426 REFLKAGGVFTDEAIDAYIALRRE-EDDRVRMTPHPVEFELYYSV-----	469

Figure

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Figure
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(a)

(b)

Figure