Function of Conserved Residues of Human Glutathione Synthetase:

Implications for the ATP-grasp Enzymes

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Running title: Key ATP binding residues of human glutathione synthetase
Abstract

Glutathione synthetase is an enzyme that belongs to the Glutathione synthetase ATP-binding domain-like superfamily. It catalyzes the second step in the biosynthesis of glutathione (GSH) from γ-glutamylcysteine and glycine in an ATP-dependent manner. Glutathione synthetase has been purified and sequenced from a variety of biological sources; still, its exact mechanism is not fully understood. A variety of structural alignment methods were applied and four highly conserved residues of human glutathione synthetase (Glu144, Asn146, Lys305 and Lys364) were identified in the binding site. The function of these was studied by experimental and computational site-directed mutagenesis. The 3D coordinates for several human glutathione synthetase mutant enzymes were obtained using molecular mechanics and molecular dynamics simulation techniques, starting from the reported crystal structure of human glutathione synthetase. Consistent with circular dichroism spectroscopy, our results showed no major changes to overall enzyme structure upon residue mutation. However, semiempirical calculations revealed that ligand binding is affected by these mutations. The key interactions between conserved residues and ligands were detected and found to be essential for enzymatic activity. Particularly, the negatively charged Glu144 residue plays a major role in catalysis.
Introduction

Glutathione synthetase [1-4] catalyzes the second and final step in the biosynthesis of glutathione (GSH)\(^1\) from \(\gamma\)-glutamylcysteine and glycine in an ATP-dependent manner. This process involves formation of an enzyme-bound acyl-phosphate (\(\gamma\)-glutamylcysteinyl-phosphate), followed by attack of the glycine and formation of an enzyme-product complex, which finally dissociates with the release of GSH, ADP and phosphate (\(P_\text{i}\)), equation 1.

Glutathione is present in the majority of living cells and is also the most abundant intracellular thiol. It has a number of vital functions: it protects cells against oxidative damage, facilitates the formation of deoxyribonucleotides, reacts with toxic compounds, participates as a coenzyme for enzymes such as glyoxalase [5] and glutathione-dependent formaldehyde dehydrogenase [6]. Glutathione is also involved in amino acid transport, in metabolism of therapeutic drugs, mutagens and carcinogens, and in the maintenance of protein thiol groups and ascorbic acid in its reduced form [7]. Lowered levels of glutathione have been associated with some diseases, for example, HIV, hepatitis C, type II diabetes, ulcerative colitis, idiopathic pulmonary fibrosis, adult respiratory distress syndrome (ARDS), and cataracts [7].

Substantial attention has been given to human glutathione synthetase because of the biological implications for human patients with hereditary glutathione synthetase deficiency [8]. In generalized glutathione synthetase deficiency, lowered levels of GSH induce an overproduction of \(\gamma\)-glutamylcysteine due to the lack of feedback inhibition of \(\gamma\)-glutamylcysteine synthetase by GSH. Even though \(\gamma\)-glutamylcysteine can compensate for GSH in many aspects of cellular defense against oxidative stress [9], the increased amounts of \(\gamma\)-glutamylcysteine lead to accumulation of 5-oxoproline [8, 10-11]. On the basis of clinical symptoms, patients with glutathione synthetase deficiency can be classified into three
phenotypes: mild, moderate, and severe (or generalized). Patients with mild glutathione synthetase deficiency have hemolytic anemia as their only clinical symptom. Those with moderate glutathione synthetase deficiency usually display symptoms starting from the neonatal period, i.e., metabolic acidosis, 5-oxoprolinuria and hemolytic anemia. Those with severe glutathione synthetase deficiency also develop progressive neurological symptoms such as seizures and psychomotor retardation [12]. The severe form of glutathione synthetase deficiency is caused by mutations in the coding sequence of human glutathione synthetase that lead to a reduction of enzyme activity [13]. However, studies on patients affected by this genetic disorder indicate a residual activity of glutathione synthetase, suggesting that a complete loss of its function is probably lethal [14–15].

Glutathione synthetase has been purified and sequenced from a variety of sources [2, 16–23]. The first highly purified mammalian glutathione synthetase was isolated from rat kidney in 1979 [24] and then was cloned and sequenced in 1995 [25]. In the same year 1995, Gali et al. reported the amino acid sequence for human glutathione synthetase [26]. Currently, the rat and human forms are the most studied mammalian glutathione synthetases. The sequence analysis of the glutathione synthetase enzymes showed that human glutathione synthetase is very similar to rat glutathione synthetase, sharing 88.6% identity, whereas the amino acid sequence comparison to other eukaryotic enzymes revealed a lower identity (18.2–68.8%) [23,27].

The most studied glutathione synthetase enzyme is from E. coli [28–36], which only has 10% sequence identity with the human enzyme [37]. Furthermore, both human and rat glutathione synthetase enzymes are homodimers with 474 amino acids in each unit, while E. coli glutathione synthetase is 158 residues shorter and exists as a tetramer. Despite sharing the same function, the lack of sequence similarity between the two enzymes makes the previous studies of
*E. coli* glutathione synthetase unsuitable for establishing the structure-function relationship of the human enzyme. Extensive kinetic studies of mammalian glutathione synthetase were carried out on the rat and recombinant human enzymes [38-39]. The results suggested that there is a close catalytic dependency between the two substrates of the homodimer, generating a negative cooperativity for binding of γ-glutamylcysteine substrate.

Little was known about the structure of human glutathione synthetase until recently, when Polekhina *et al.* [37] reported its crystal structure. Human glutathione synthetase was co-crystallized with the products – glutathione, ADP, one sulfate ion, which mimics the cleaved γ-phosphate from the ATP, and two magnesium ions, Figure 1. The three-dimensional structure of human glutathione synthetase shows that it belongs to the Glutathione synthetase ATP-binding domain-like superfamily, which consists of enzymes with ATP-dependent carboxylate-amine ligase activity and whose catalytic mechanisms are likely to involve acyl-phosphate intermediates [40]. The ATP-grasp members display a unique nucleotide-binding fold, referred to as a palmate, or ATP-grasp fold.

Even though the catalytic mechanism of human glutathione synthetase is not fully understood, it appears to resemble those of the other ATP-grasp members. Therefore, structural comparison and analysis of members’ binding sites, especially the ATP-binding site, can provide an insight into the catalytic mechanism of human glutathione synthetase. Additionally, structural and molecular properties investigations of human glutathione synthetase – wild type compared to human glutathione synthetase – mutants should reveal significant information about the function of the involved residues in the ATP-grasp superfamily.
Experimental Methods

Materials: Oligonucleotide primers for mutagenesis and sequencing were synthesized by Integrated DNA Technologies, Inc. Restriction enzymes were obtained from New England Biolabs. The Quickchange™ site-directed mutagenesis kit was obtained from Stratagene. γ-Glutamyl-α-aminobutyrate (gluABA) was synthesized as described [24, 41]. IPTG (isopropyl-1-thio-β-D-galactopyranoside) and Lactate dehydrogenase were obtained from Amresco. All other reagents were from Sigma (Sigma Chemicals, MO).

Recombinant DNA methods: N-terminal His tag (6X His) was added to wild type human glutathione synthetase by subcloning human glutathione synthetase from plasmid pT7-7 to pET-15b vector (Novagen) at BamHI and NdeI sites. Mutants were generated using the Quickchange™ kit (Stratagene). The internal primers used for mutants are shown in Table 1. The mutations were confirmed by partial sequencing.

Glutathione synthetase growth and purification: The growth and purification protocol used for the recombinant wild type glutathione synthetase and mutant glutathione synthetase was the same. E. coli BL21DE3 was transformed with pET-15b vector containing glutathione synthetase and grown to an OD_{600} of 1.0 at 37°C in Luria Broth containing ampicillin (100 μg/ml). After the OD_{600} reached 1.0, IPTG (isopropyl-1-thio-β-D-galactopyranoside; 0.8 mM) was added. After induction (4-6 hrs), cells were harvested by centrifugation (4225 × g, 10 min., 4°C) and washed with cold 0.85% NaCl. All purification steps were performed at 4°C. The cells were resuspended in MCAC-0 buffer (Metal Chelate Affinity Chromatography buffer; 20 mM TRIS-Cl, 500 mM NaCl, 10% glycerol, pH 8.0) and disrupted by sonication (three pulses of 2 min. with cooling between pulses). Cell debris was removed by centrifugation (11,950 × g, 20 min). The clear lysate (~25 ml) was applied to a Ni-NTA (Novagen) column previously
equilibrated with MCAC-0 buffer. After loading, the column was washed with MCAC-0 buffer. Non-specific bound proteins were removed by adding MCAC-50 buffer (MCAC-0 buffer plus 50 mM imidazole). Glutathione synthetase enzymes were eluted with MCAC-100 buffer (MCAC-0 buffer plus 100 mM imidazole). The fractions containing human glutathione synthetase was pooled and dialyzed twice against 20 mM TRIS-Cl buffer (pH 8.0 containing 1 mM EDTA; 4L). The purified human glutathione synthetase proteins were pure by SDS/PAGE (at least 99%).

**Enzyme assays and kinetic analysis:** All kinetic analyses were done in duplicates using purified recombinant glutathione synthetase. The enzyme activity was measured at 37°C using a spectrophotometric assay, which couples ADP production to NADH oxidation and is monitored at 340 nm [38-39]. In brief, the standard assay contained buffer (100 mM TRIS-Cl, pH 8.2, 50 mM KCl, 20 mM MgCl₂, 5 mM sodium phospho (enol) pyruvate, 0.2 mM NADH), 10 units of pyruvate kinase (Type III rabbit muscle), 10 units of lactic acid dehydrogenase (Type II rabbit muscle) and glutathione synthetase substrates (see below) (final volume of 0.2 ml) and was initiated by the addition of human glutathione synthetase. For $V_{\text{max}}$ and $K_{\text{cat}}$ determinations, the concentration of ATP, gluABA and glycine were 10, 20 and 10 mM, respectively. Instead of $\gamma$-glutamylcysteine, gluABA was used to avoid the complication of thiol oxidation. The apparent $K_m$ values were determined using the standard assay where two substrates were held at saturating levels, while the third, was varied by about 10 fold around the putative $K_m$ value. Control reactions contained the standard mix minus gluABA. The Michaelis-Menten kinetic equation was used for glycine and ATP data analysis. For gluABA kinetic analysis, the initial velocity ($v$), concentration ($S$) and $V_{\text{max}}$ are substituted into the Adair equation (Equation 2) for negative cooperativity, and the first $K_m$ and alpha ($\alpha$), or interaction factor are calculated. The second ‘estimated’ $K_m$ is obtained by multiplying the first $K_m$ by the interaction factor $\alpha$. The level of
negative cooperativity is assessed by use of the Hill plot (V/V_{max}-V) versus S, to obtain a Hill coefficient, h. A unit of enzyme is defined as the amount that catalyzes 1 μmole of product per minute at 37°C. Protein concentration was determined by the Lowry method [42] using bovine serum albumin as the standard. Kinetic data were plotted and non-linear regression analysis was performed using SigmaPlot software (SPSS Science Inc.).

**Computational Methods**

Crystallographic coordinates of the X-ray structure of human glutathione synthetase (glutathione synthetase, human form), reported to a resolution of 2.10 Å, and those of the other proteins were obtained from the Protein Data Bank (http://www.rcsb.org/pdb/) [43].

The K2 program (http://zlab.bu.edu/k2/), an automated method that uses a genetic algorithm for aligning the 3D structures of proteins, was employed to study the similarities between human glutathione synthetase and all the other members of the ATP-grasp superfamily. The K2 software first aligns the proteins’ secondary structure elements, starting with the more conserved ones, and then the alignment is extended to include any equivalent positions in loops and turns until the best fit is obtained [44].

Identification of the ATP-grasp members was established using the SCOP (Structural Classification of Proteins) database (http://scop.mrc-lmb.cam.ac.uk/scop/index.html) where proteins are clustered into classes, folds, superfamilies, families and domains, which represent hierarchy levels based on their evolutionary and structural relationships [45]. The FSSP (Fold classification based on Structure-Structure alignment of Proteins) database (http://www2.ebi.ac.uk/dali/fssp/fssp.html) [46], another classification tool, was also employed in order to obtain structural neighbors of human glutathione synthetase.
The active sites of the K2 aligned structures were visualized and analyzed with Swiss Pdb-Viewer (http://us.expasy.org/spdbv/) [47], a graphics program for studying macromolecular structure, in connection with the LPC software (Ligand-Protein Contacts) (http://sgedg.weizmann.ac.il/lpc/) [48].

Wild type and mutant 2HGS were modeled with the MOE 2002.03 software (Molecular Operating Environment) [49] using the Amber’94 force field [50]. Starting with the crystal structure coordinates of human glutathione synthetase, the water molecules were removed, hydrogen atoms were added and then the resulting structure was minimized with molecular mechanics. Mutants were built by altering distinct residues of the wild type enzyme, followed by energy minimization, before submitting them to the same molecular dynamics protocol (vide infra) employed for wild type human glutathione synthetase.

All energy minimization procedures comprised three phases: first the steepest descent algorithm was employed (until RMS gradient < 1000), then the conjugate gradient technique (until RMS gradient < 100), and finally truncated Newton method (until RMS gradient < 0.01).

Molecular dynamics simulations with constant NVT and a time step of 0.001 ps were employed in order to find the lowest energy conformation of each structure (wild type and mutants). Initially, the temperature was raised progressively from 0 to 300 K in 1 ps. The system was then equilibrated at 300 K for 1000 ps during which the atomic coordinates were saved every 100 ps. Tests were carried out with different time length simulations before arriving at 1000 ps as the optimal combination of computational efficiency and accuracy. Finally, the 10 saved geometries were energy minimized and the lowest energy conformation among these was stored for further analysis.
Investigations of the active site were carried out for both wild type and mutant human glutathione synthetase enzymes. Hydrogen bonds and atomic distances were computed with the MOE 2002.03 program while interaction enthalpies between the considered constituents of the active site (residues Glu144, Asn146, Lys305, Lys364 and ligands ADP$^{2-}$, SO$_4^{2-}$, 2Mg$^{2+}$) were determined at the semiempirical quantum mechanics level using the Spartan’02 software [51]. The single point enthalpies (heats of formation) of the active site residues and cofactors were computed with the PM3 Hamiltonian [52]. The interaction enthalpy between the two active site elements, denoted in equation 3 as X and Y, was then estimated.

$$\Delta H_f(X \ldots Y) = H_f(X + Y) - \{ H_f(X) + H_f(Y) \}$$ (3)
Results

1. Similarities between Glutathione Synthetase and Members of ATP-Grasp Superfamily

SCOP [45] analysis shows that human glutathione synthetase (PDB code = 2HGS) belongs to the PreATP-grasp superfamily, which is the only member of the PreATP-grasp fold. All members of the PreATP-grasp fold are also members of the ATP-grasp fold. Consequently, human glutathione synthetase is also a member of the Glutathione synthetase ATP-binding domain-like superfamily that is one of the two superfamilies included in the ATP-grasp fold. The Glutathione synthetase ATP-binding domain-like superfamily consists of 51 proteins that are clustered into 6 families. A fold, which has one or more superfamilies, consists of proteins with similar major secondary structures in identical arrangement and topological connections. Superfamilies are subsets of a fold and consist of proteins with low sequence identities, but whose structural and functional features suggest that a common evolutionary origin is probable. Thus, in the SCOP hierarchy, a common fold indicates a major structural similarity, while a common superfamily indicates a probable common evolutionary origin. A common family indicates a clear evolutionary relationship – proteins from the same family share ≈30% or greater sequence identity [45]. However, two families (Succinyl-CoA synthetase and Pyruvate phosphate dikinase) from the Glutathione synthetase ATP-binding domain-like superfamily are slightly more different than the other families, since their members are not included in the PreATP-grasp domain superfamily. The members of the two aforementioned families display the same manner of binding the nucleotide inside the active site, but their similarity to the members of the PreATP-grasp is limited to only two domains (Pyruvate phosphate dikinase) or one domain (Succinyl-CoA synthetase), whereas the members of the other families (Eukaryotic and
Prokaryotic glutathione synthetases, D-Alanine ligase, Biotin carboxylase and Synapsin Ia) have three common domains.

The six most similar proteins to 2HGS were chosen from the PreATP-grasp fold using FSSP database (Table 2). Only those proteins with an FSSP Z score higher than 4 (level of similarity is analogous to 2nd cousins [46] are desired since all structural neighbors display a Z score of at least 2, which means that they belong to the same fold as 2HGS. The PDB codes for the six selected proteins are 1M0W [53], 1GSA [36], 1AUV [54], 1IOW [55], 1B6R [56] and 1EZ1 [57]. Conserved residues in the active site of human glutathione synthetase were identified by using LPC analysis and K2 structural alignment for 2HGS and selected proteins. The LPC analysis listed 40 residues in the active site of 2HGS and each one was verified against the alignment output generated by K2 with each of the 6 proteins just mentioned.

The K2 structural alignments results of 2HGS with the six most structurally similar PreATP-grasp proteins showed that there are only 4 highly conserved residues involved in the ligands binding site: Glu144, Asn146, Lys305 and Lys364 (Figure 2). The K2 alignment of 2HGS with other proteins was extended to all members of the PreATP-grasp domain superfamily and the results confirmed that these 4 residues are conserved. Glu144 is fully conserved all along the series (for both the most similar PreATP-grasp domain superfamily members shown above, as well as for even more structurally disparate members of this superfamily). Lys364 is almost fully conserved in all families, being replaced by Arg only in Carbamoyl phosphate synthetase while Lys305 is replaced by Arg in N5-carboxyaminoimidazole ribonucleotide synthetase, Purt-encoded glycinamide ribonucleotide transformylase and Carbamoyl phosphate synthetase. Asn146 is replaced by Val in Synapsin Ia, Ala in N5-carboxyaminoimidazole ribonucleotide synthetase or Ser in Purt-encoded glycinamide ribonucleotide transformylase. Additional results
for the alignments of the more structurally disparate members belonging to the PreATP-grasp superfamily and SCOP classification of the PreATP-grasp superfamily and the Glutathione synthetase ATP-binding domain-like superfamily are given in the Supplemental Data.

Comparison of human glutathione synthetase with all other members of the PreATP-grasp superfamily shows that Glu144 residue is fully conserved, Lys364 and Lys305 are conserved as regards the charge and Asn146 is backbone-conserved. Our attempt to extend this conclusion to members of the Succinyl-CoA synthetase and Pyruvate phosphate dikinase families, which are classified by SCOP [45] as ATP-grasp members, was not successful. Thus, the active site of 2HGS is highly similar only to members of the PreATP-grasp fold.

2. Comparison of Wild Type and Mutant Glutathione Synthetase

Only four residues (Glu144, Asn146, Lys305 and Lys364) were found to be highly conserved in the active site domain of 2HGS. Thus, it is reasonable to conclude that these four residues are crucial for the biological activity of human glutathione synthetase. This hypothesis was tested experimentally and computationally using site directed mutagenesis of the conserved residues. The Glu144, Asn146, Lys305 and Lys364 residues were replaced with amino acids that cannot interact favorably with the ligands; the structure and catalytic activity of the corresponding mutant enzymes were then compared to wild type.

The functions of the four conserved residues in glutathione synthetase were examined by preparing the human glutathione synthetase mutants using site directed mutagenesis and His-tag purification. The results (Table 3) show that two mutants E144K and N146K had undetectable activity. Five other mutants had very low activities (E144A and N146D, 0.05%; N146A and K364A, 0.1%; and K364E, 0.2% active as wild type human glutathione synthetase). The only
human glutathione synthetase mutants with significant activity were K305A and K305E, with 6.5% and 5% activity of the wild type, respectively.

Two mutant enzymes with sufficient activity (K305A and K305E) were subjected for further kinetic analysis (Table 4). The apparent $K_m$ values for glycine were found the same for the K305E mutant and decreased (~ 7 fold) for the K305A mutant. The apparent $K_m$ values for gluABA for both K305A and K305E were about the same as the wild type human glutathione synthetase, and the mutant enzymes no longer display negative cooperativity. The apparent $K_m$ values for ATP increased dramatically for K305A (10 fold) and K305E (40 fold).

The conformations of the mutants were computed using molecular mechanics and molecular dynamics (MD) simulations and then superimposed on wild type human glutathione synthetase using Swiss Pdb-Viewer. Experimental and calculated results revealed that none of the nine (E144A, E144K, N146A, N146D, N146K, K305A, K305E, K364A and K364E) mutant enzymes adopts a markedly different tertiary structure than that of the wild type human glutathione synthetase. The backbone RMSD calculation applied to structural alignments of mutants with wild type human glutathione synthetase ranges from 1.7 to 2.3 Å, suggesting a high degree of tertiary structure similarity and is consistent with the results of circular dichroism spectroscopy. Since the overall tertiary structure of human glutathione synthetase is not altered by mutation, the reduced activity of the mutant enzymes is probably due to the nature of ligand binding. This hypothesis was explored by studying the interaction between the structural units (the cofactor ligands and the four conserved residues involved in the binding pocket of ATP).

The atomic distance analysis of the human glutathione synthetase structure obtained from MD simulation showed that cofactor ligands ($\text{ADP}^{2-}$, $\text{SO}_4^{2-}$, and $2\text{Mg}^{2+}$) interact with all 4 conserved residues through a network of contacts (Table 5). The 2-D representation of these
contacts is depicted for wild type human glutathione synthetase in Figure 3. The ε-amino group of Lys364 interacts with ADP through three hydrogen bonds: 2.71 Å to N (amino group of ADP), 3.44 Å to N7 (adenine ring of ADP) and 2.12 Å to Oδ (α-phosphate of ADP). The ε-amino group of Lys305 hydrogen bonds to the β-phosphate group of ADP and to the γ-carboxylate group of Glu144. There is one hydrogen bond (2.72 Å) between ε-amino group (Lys305) and Oδ (β-phosphate of ADP) and two hydrogen bonds (1.72 Å and 2.15 Å) between ε-amino group (Lys305) and both Oδ atoms of γ-carboxylate group (Glu144). The γ-carboxylate group of Glu144 also interacts with Mg2+ ion (number 501) using both oxygen atoms, although one is significantly shorter than the other (Mg – O distances are 2.10 Å and 3.83 Å). The β-carbonyl group of Asn146 coordinates the Mg2+ ion (501) within a calculated distance of 2.34 Å. Additionally, the SO42- ion (which mimics the cleaved γ-phosphate) forms a bridge between the two Mg2+ ions. The distances linking the negative O atoms of sulfate and the Mg2+ ions are 2.12 Å, 2.48 Å and 3.51 Å for Mg 501 and 2.08 Å, 2.14 Å and 3.45 Å for Mg 502.

In addition we examined the calculated atomic distances (Table 5) for human glutathione synthetase mutant enzymes using the MD structures. When Glu144 is mutated (Figure 4 A and B), Lys305 and Asn146 side chains are reoriented. The Lys305 side chain moves closer to ADP, forming an additional hydrogen bond (N+\text{-}H---Oδ), while Asn146 rearranges its side chain and forms a hydrogen bond with sulfate ion (N-H---Oδ), which replaces the cleaved γ-phosphate of ATP. Similarly, when Lys364 is mutated (Figure 4 C and D), the side chain of Lys305 moves closer to ADP and forms one additional ionic hydrogen bond of the type N+\text{-}H---Oδ. Furthermore, a change in the position of the Glu144 side chain occurs so that the γ-carboxylate group coordinates more effectively the Mg 501 ion in K364A or coordinates both Mg 501 and 502 ions in K364E structure. Mutation of Lys305 (Figure 4 E and F) shows that Glu144 and
Lys364 side chains are affected. The \( \gamma \)-carboxylate group of Glu144 is closer to both Mg ions resulting in a stronger bond between \( \gamma \)-carboxylate and Mg\(^{2+} \)501 (K305E), as well as additional bonds between the \( \gamma \)-carboxylate and Mg\(^{2+} \)502 (K305A and K305E). On the other hand, the \( \varepsilon \)-amino group of Lys364 is further away from the adenine ring, which causes the loss of two hydrogen bonds relative to the wild type enzyme. For the K305E mutant, the favorable contact between the side chain carbonyl group of Asn146 and Mg\(^{2+} \)(501) ion is lost and an ionic hydrogen bond (N-H---O\(^{\delta-} \)) between the amide group and the sulfate ion is gained. Replacement of Asn146 with Ala or Asp (Figure 4 G and H) gives a weaker hydrogen bond between Lys305 and ADP. However, for the N146A there is a stronger interaction between Glu144 and both Mg\(^{2+} \) ions. In contrast, the N146K mutant (Figure 4 I) forms a second ionic hydrogen bond between Lys305 and ADP and the interaction becomes stronger. Also, the \( \gamma \)-carboxylate group of Glu144 is altered so that there are changes with respect to both Mg\(^{2+} \) (501 and 502), resulting in a significant decrease in the distance between the oxygen atoms and magnesium ions. For the N146K both Mg ions are coordinated by \( \gamma \)-carboxylate group of Glu144. To summarize, this analysis revealed that a single mutation of these conserved residues induces changes in the orientation of the other residues side chains causing deviations in ligand binding.

Semiempirical PM3 calculations were applied to MD structures for both wild type and mutant enzymes to study the interaction enthalpies between conserved residues and cofactor ligands. The cofactor ligands (ADP\(^{2-} \), SO\(_4^{2-} \), and 2Mg\(^{2+} \)) were treated computationally as a single unit because the atomic distance calculations (Table 5.C) show that the interactions between magnesium ions (501, 502) and the sulfate ion, which mimics the cleaved \( \gamma \)-phosphate, remain as it is seen in wild type structure. The results illustrate that interactions between conserved residues and cofactor are sensitive to selected mutations (Figure 5). For example, in the case of
interaction enthalpy between cofactor and residue 144, eight out of nine mutants (E144A, E144K, K364A, K364E, K305A, K305E, N146A and N146K) display a significant change (> 15 kcal/mol) compared to wild type.

The interaction enthalpy between cofactor and residue 146 shows five mutants (E144A, K305E, N146A, N146D and N146K) with a deviation in energy greater than 15 kcal/mol relative to the wild type enzyme. Correspondingly, for two mutants (K364A and K364E) a substantial variation occurs in the interaction enthalpy between cofactor and residue 364 and finally, interaction enthalpy between cofactor and residue 305 show that only one mutant, which is E144K and not K305A or K305E, displays a greater change in energy than 15 kcal/mol.

Consistent with the previous analysis, the PM3 calculations substantiate that each mutated residue alters the interaction enthalpy between itself and the cofactor, and interestingly the mutation also affects the interaction for the other 3 conserved residues.
Discussion

Previous studies [58-60] of the crystal structure of proteins belonging to the PreATP-grasp superfamily have identified several important residues situated near ATP; some of these residues are conserved and some are involved in the ATP binding. However, the structural alignments searches for conserved residues in the PreATP-grasp family included mostly the prokaryotic proteins. The current work is the first study that examines all members of the PreATP-grasp superfamily, including human glutathione synthetase (a eukaryotic member) on the subject of highly conserved residues and their function. We found four residues in the ATP binding site of human glutathione synthetase that are highly conserved in all of the PreATP-grasp superfamily: Glu144, Asn146, Lys305 and Lys364; and thus, the function of these was expected to be essential for catalytic activity. The current modeling and experimental results suggest that the mutations of the four conserved residues of human glutathione synthetase yield enzymes whose activity is not affected by a major change in the tertiary structure of the enzyme, but rather by alteration of the ATP binding site. In a protein, replacement of a charged residue with a neutral or oppositely charged residue is expected to disturb the local electrochemical stability. Our MD simulations of human glutathione synthetase showed that in addition to the mutated residue, the other three conserved residues are also affected with regard to their interaction with the ligands (ADP$^{2-}$, SO$_4^{2-}$, and 2Mg$^{2+}$). Hence, a single point mutation of human glutathione synthetase causes a cascading chain of events that influences the overall binding of the ATP.

Only K305A and K305E human glutathione synthetase mutants show any significant enzyme activity, suggesting that all four conserved residues are critical for enzyme activity. The higher apparent $K_m$ values for ATP for the K305 mutants imply that Lys305 is important for
ATP binding. These findings suggest that mutation of any of the other three conserved residues in human glutathione synthetase is also likely to affect ATP-Mg\(^{2+}\) binding. Further bonding studies, such as iso-thermal microcalorimetry (ITMC) will be useful.

The calculations of interaction enthalpies indicate that the interaction between Lys305 and cofactor is the least sensitive to mutation because Lys305 residue is closer to Glu144 than it is to ADP. Thus, whether Lys305 or the other conserved residues are mutated there is no significant change in interaction enthalpy; however, for the E144K mutation, the interaction enthalpy between Lys305 and ADP increases noticeably as a result of electrostatic repulsion between the two lysine residues (144 and 305). In contrast, the interaction between Glu144 and cofactor is the most sensitive to mutation. The large variations of interaction enthalpies between Glu144 and cofactor ligands for human glutathione synthetase mutants are most likely due to differences in coordination of magnesium (501 and 502) ions by the negatively charged \(\gamma\)-carboxylate group of Glu144.

Our findings suggest that Glu144 is probably very important for the stabilization of the \(\gamma\)-glutamylcysteinyl-phosphate intermediate. Since both magnesium ions are strongly coordinated by the \(\gamma\)-phosphate in the product-complex structure of human glutathione synthetase, it is likely that the acyl-phosphate intermediate coordinates the magnesium ions in a similar manner. The role of Glu144 then, could be to compensate the positive charge around this intermediate and possibly to assist in the ligation of glycine to \(\gamma\)-glutamylcysteine. A reason why both E144K and N146K mutant enzymes exhibit an extremely low catalytic activity is that the Asn146 residue may also help to compensate for the positive charge in the environs of the reaction intermediate.

All four conserved residues are essential for the human glutathione synthetase enzyme, and the counterparts of these conserved residues are presumably essential for the other members
of the PreATP-grasp superfamily. This is especially likely for Glu144 since it is fully conserved in the PreATP-grasp superfamily and participates in the formation of the acyl-phosphate intermediate. Thus, we propose that this residue is necessary for the catalytic function of glutathione synthetase and also for the other enzymes in the superfamily. The charged conserved residues, Lys364 and Lys305, balance the negative charge of ATP and therefore, these residues and their homologous in the PreATP-grasp enzymes are expected to be involved in the optimal orientation of ATP in relation to the carboxyl group of the substrate. The backbone-conserved residue, Asn146, coordinates one of the Mg$^{2+}$ ions and our results suggest that it takes part in catalysis as well. Since few proteins in the PreATP-grasp superfamily uses Ala, Ser or Val residues instead of Asn, it appears that these proteins have a slightly different mechanism for ATP activation than human glutathione synthetase.
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Footnotes

The on-line version of this article contains Supplemental Data (Table S1 and Appendix S1).

1 The abbreviations used are: GSH, glutathione; gluABA, γ-glutamyl-α-aminobutyrate; SCOP, Structural Classification of Proteins; FSSP, Fold classification based on Structure-Structure alignment of Proteins; LPC, Ligand-Protein Contacts; NVT, thermodynamic ensemble where the number of particles, the volume and the temperature are held fixed; PM3, Parametric Method 3; MD, molecular dynamics technique; 2HGS, reported structure of human glutathione synthetase; 1M0W, reported structure of yeast glutathione synthase; 1GSA, reported structure of E. Coli glutathione synthetase; 1IOW reported structure of E. Coli D-ala: D-ala ligase; 1AUV, reported structure of bovine Synapsin Ia; 1B6R, reported structure of E. Coli N5-carboxyaminoimidazole ribonucleotide synthetase; 1EZ1, reported structure of E. Coli pur-t-encoded glycinamide ribonucleotide transformylase.
Figure Legends

Figure 1: Two views of the four conserved residues involved in the ATP binding site.
Front (left) and side (right) view of human glutathione synthetase binding site. The modeled product-complex structure was obtained using the MD technique followed by optimization. Only the ligands and the four conserved residues are presented. The SO$_4$ ion mimics the cleaved $\gamma$-phosphate from the ATP. Note that GSH is distant from the conserved residues.

Figure 2: Structural alignment of conserved residues within PreATP domain.
Each of the 3D structures of 1M0W (purple), 1GSA (red), 1AUV (blue), 1IOW (yellow), 1B6R (gray) and 1EZ1 (cyan) proteins were aligned with 2HGS (green) using the K2 software. The alignment results show only four conserved residues in the active site of human glutathione synthetase: Glu144, Asn146, Lys305 and Lys364.

Figure 3: Wild type 2-D representation of interactions in the binding site of ATP.
Only the four conserved residues and the cofactor ligands are shown for the modeled product-complex structure of human glutathione synthetase. The dashed lines represent hydrogen bonds and/or ionic interactions.
Figure 4: Schematic representation of major differences in substrate-ADP binding upon mutation of the four conserved residues.

Only the four conserved residues and the cofactor ligands were considered. The dashed lines represent hydrogen bonds and/or ionic interactions. Human glutathione synthetase mutants: (A) E144A; (B) E144K; (C) K364A; (D) K364E; (E) K305A; (F) K305E; (G) N146A; (H) N146D; (I) N146K.

Figure 5: Calculated interaction enthalpies between ligands and conserved human glutathione synthetase residues for wild type and each mutant enzyme.

The interaction enthalpies between ligands (ADP$^{2-}$, SO$_4^{2-}$, and 2 Mg$^{2+}$) and individual conserved residues were computed with the PM3 Hamiltonian available in Spartan’02 software, using the single point enthalpies (heats of formation). All calculations are based on the modeled structures that resulted after 1000 ps of molecular dynamics simulations.
Table 1. Primers Used for Site Directed Mutagenesis of Glutathione Synthetase.

| Enzyme | DNA sequence* |
|--------|---------------|
| E144A  | 5'-GCAGAGATGGTGTGTTGACGCATCTGGTTCAGG-3' |
|        | 5'-CCTGAACAGATCGAACATCATCAACATCATCTCGC-3' |
| E144K  | 5'-GCAGAGATGGTGTGCTGGATCTTGTCTGTGGTCAGG-3' |
|        | 5'-CCTGAACAGATCGAACATCATCAACATCATCTCGC-3' |
| N146A  | 5'-GCAGAGATGGTGTCGCATCTGGTTCAGG-3' |
|        | 5'-CCTGAACAGATCGAACATCATCAACATCATCTCGC-3' |
| N146D  | 5'-GCAGAGATGGTGCATTGTGCATCTGGTTCAGG-3' |
|        | 5'-CCTGAACAGATCGAACATCATCAACATCATCTCGC-3' |
| N146K  | 5'-GCAGAGATGGTTTTGGTGTCTGGTTCAGG-3' |
|        | 5'-CCTGAACAGATCGAACATCATCAACATCATCTCGC-3' |
| K305A  | 5'-CTGACCTTCGCGAGTCATCCCAGCGAGC-3' |
|        | 5'-CTGGCTGGGACCTGGAGGATGCAGC-3' |
| K305E  | 5'-CTGCTGGACATCACCTTCAGTCCCAGCCAGC-3' |
|        | 5'-CTGGCTGGGACCTGGAGGATGCAGC-3' |
| K364A  | 5'-CCTCCTCTGGGAGCTGACACAAACCGG-3' |
|        | 5'-CGGTTTGTGCTAGCCACAGAGAGAGG-3' |
| K364E  | 5'-CCTCCTCTGGGAGCTGACACAAACCGG-3' |
|        | 5'-CGGTTTGTGCTAGCCACAGAGAGAGG-3' |

* The underlined bases indicate the nucleotide positions that were changed.
### Table 2. Conserved Residues in the Active Site of 2HGS and Other Closely Related ATP Members.

| Protein name                                  | Species            | PDB ID | FSSP Z score<sup>a</sup> | K2 final score<sup>b</sup> | Conserved residues |
|-----------------------------------------------|--------------------|--------|--------------------------|-----------------------------|--------------------|
| Glutathione synthetase                        | *Homo sapiens*     | 2HGS   | 69.7                     | 10159.6                    | GLU 144, ASN 146, LYS 305, LYS 364 |
| Glutathione synthase                          | *S. cerevisiae*    | 1MOW   | not listed<sup>c</sup>   | 6112.8                     | GLU 146, ASN 148, LYS 324, LYS 382 |
| Glutathione synthetase                        | *E. coli*          | 1GSA   | 9.4                      | 725.6                      | GLU 281, ASN 283, LYS 125, LYS 160 |
| Synapsin Ia                                   | *Bos taurus*       | 1AUV   | 7.0                      | 264.2                      | GLU 386, VAL 388, LYS 225, LYS 269 |
| D-ala D-ala ligase                            | *E. coli*          | 1IOW   | 6.9                      | 433.1                      | GLU 270, ASN 272, LYS 97, LYS 144 |
| N5-Carboxyaminoimidazole ribonucleotide synthetase | *E. coli*          | 1B6R   | 6.5                      | 504.8                      | GLU 238, ALA 240, ARG 80, LYS 120 |
| Purt-Encoded glycinamide ribonucleotide transformylase | *E. coli*          | 1EZ1   | 4.1                      | 282.4                      | GLU 279, SER 281, ARG 114, LYS 155 |

<sup>a</sup> FSSP Z score describes the level of structural similarity relative to 2HGS. Only those with Z score greater than 4 are shown.

<sup>b</sup> The K2 final score is computed by using the number and the root mean square distance of the of aligned residues.

<sup>c</sup> The FSSP Z score for 1M0W is not available yet, but this protein displays the highest level of similarity to 2HGS, based on K2 structural alignment.
Table 3:  \( K_{\text{cat}} \) and Specific Activity for Wild Type Glutathione Synthetase and Mutants.

| Enzyme   | \( K_{\text{cat}} \) (sec\(^{-1}\)) | \( V_{\text{max}} \) (\( \mu \)mol/min/mg) |
|----------|----------------------------------|----------------------------------|
| Wild Type| 6.5                              | 3.72                             |
| E144A    | 0.003                            | 0.002                            |
| E144K    | ND\(^a\)                         | ND\(^a\)                         |
| N146A    | 0.007                            | 0.004                            |
| N146D    | 0.003                            | 0.002                            |
| N146K    | ND\(^a\)                         | ND\(^a\)                         |
| K305A    | 0.423                            | 0.240                            |
| K305E    | 0.336                            | 0.190                            |
| K364A    | 0.008                            | 0.005                            |
| K364E    | 0.015                            | 0.009                            |

\(^a\) ND, non detectable (less than 0.001\( \mu \)mol/min/mg).

Table 4:  Comparison of the Kinetic Parameters of Wild Type Glutathione Synthetase and the Mutants.

| Enzyme   | Glycine (mM) | ATP (mM) | gluABA (mM) | Hill Coefficient \( h \) | Interaction Factor \( \alpha \) |
|----------|--------------|----------|-------------|--------------------------|-----------------------------|
|          |              |          | \( K_{m1} \) | \( \text{est } K_{m2} \) |                            |
| WT (100\%)| 1.75 ± 0.10 | 0.07 ± 0.01 | 0.66 ± 0.10 | 1.50 | 0.80 | 2.28 |
| K305A (6.5\%) | 0.25 ± 0.01 | 0.83 ± 0.01 | 0.34 ± 0.04 | 0.34 | 1.11 | 1.0  |
| K305E (5\%) | 1.96 ± 0.24 | 2.66 ± 0.37 | 0.40 ± 0.06 | 0.14 | 1.20 | 0.35 |

The numbers in the parenthesis are the relative activity of the enzymes. The S.E. on the \( K_{m} \) were calculated from nonlinear regression analysis. The second apparent \( K_{m} \) (\( K_{m2} \)) was estimated as described (Experimental Methods).
Table 5.

A. Hydrogen Bonding and Ionic Interactions Between Conserved Residues and Cofactor Ligands

| Distances (Å)                                      | WILD | E144A | E144K | K364A | K364E | K305A | K305E | N146A | N146D | N146E |
|---------------------------------------------------|------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| H-bonding distance between K364(H⁺) and ADP(O⁻)   | 2.12 | 2.23  | 2.45  | 2.43  | 2.35  | 2.20  | 2.23  | 2.02  |       |       |
| H-bonding distance between K364(H⁺) and ADP(NH₂)  | 2.71 | 2.98  | 3.76  |       | 2.33  | 3.53  |       |       |       |       |
| H-bonding distance between K364(H⁺) and ADP(N7)   | 3.44 | 3.00  | 2.80  | 2.72  | 2.61  | 3.40  |       |       |       |       |
| H-bonding distance between K305(H⁺) and ADP(O⁻)   | 2.72 | 3.02  | 2.47  | 2.57  | 2.86  | 3.26  | 3.93  | 2.71  |       |       |
| Atomic distance between N146(O) and Mg²⁺501        | 2.34 | 2.34  | 2.40  | 2.47  | 2.32  | 2.33  |       | 2.04  |       |       |
| H-bonding distance between N146(H) and SO₄(O⁻)    | 2.29 | 2.66  |       | 3.53  |       |       | 1.89  | 2.89  |       |       |
| Atomic distance between E144(O⁻) and Mg²⁺501       | 2.10 | 2.10  | 2.15  | 2.15  | 2.20  | 2.19  | 2.13  | 2.34  |       |       |
| Atomic distance between E144(O⁻) and Mg²⁺502       | 3.83 | 3.34  | 3.40  | 2.59  | 2.91  | 2.34  |       |       |       |       |

a Distances (Å) in parentheses indicate that only one distance was determined.

b Distances (Å) in parentheses indicate that only one distance was determined.
B. Hydrogen Bonding Interactions Between Conserved Residues

| Distances (Å) | WILD | E144A | E144K | K364A | K364E | K305A | K305E | N146A | N146D | N146K |
|--------------|------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| H-bonding distance between K305(H+) and E144(O-) | 1.72 | 1.75  | 1.64  | 1.86  | 1.77  | 1.71  |       |       |       |       |
|                                                         | 2.15 | 2.14  | 3.55  |       |       |       |       |       | 1.96  |       |

C. Ionic Interactions Between Cofactor Ligands

| Distances (Å) | WILD | E144A | E144K | K364A | K364E | K305A | K305E | N146A | N146D | N146K |
|--------------|------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| Atomic distance between Mg²⁺501 and SO₄(O⁻) | 2.12 | 2.09  | 2.12  | 2.11  | 2.13  | 2.21  | 2.21  | 2.17  | 2.11  | 2.17  |
|                                                         | 2.48 | 2.40  | 2.26  | 2.46  | 2.60  | 2.33  | 2.24  | 2.28  | 2.50  | 2.56  |
|                                                         | 3.51 | 3.31  | 3.26  | 3.66  | 2.90  | 2.70  | 2.75  | 2.59  | 3.71  | 2.75  |
| Atomic distance between Mg²⁺502 and SO₄(O⁻) | 2.08 | 2.12  | 2.12  | 2.13  | 2.14  | 2.18  | 2.17  | 2.16  | 2.12  | 2.09  |
|                                                         | 2.14 | 2.23  | 2.22  | 2.18  | 2.19  | 2.21  | 2.24  | 2.39  | 2.16  | 2.24  |
|                                                         | 3.45 | 3.20  | 3.06  | 3.00  | 2.96  | 2.41  | 2.86  | 2.52  | 3.05  | 2.93  |

a A cut-off of 4 Å and 90º for the donor-H-acceptor was used.

b Interactions for all equivalent hydrogen and/or oxygen atoms were calculated.

Distance calculations are based on the modeled structures that resulted after 1000 ps of molecular dynamics simulations and energy minimization.
Equation 1:

\[ \text{L-}\gamma\text{-Glutamyl-L-cysteine + Glycine + ATP} \xrightarrow{\text{Mg}^{2+}} \text{GSH + ADP + P}_i \]

Equation 2:

\[ v = V_{\text{max}} \left( \frac{[S]}{K_m} + \frac{[S]^2}{\alpha K_m^2} \right) \]

\[ 1 + \frac{2[S]}{K_m} + \frac{[S]^2}{\alpha K_m^2} \]
Figure 2:
Figure 3:
Figure 4:

(A) LYS 305

(LYS 364

ALA 144

ASN 146

Mg$^{2+}$

2.34

2.98

3.02

2.29

(B) LYS 305

LYS 364

LYS 144

ASN 146

Mg$^{2+}$

2.66

2.47

2.45

2.80

3.76
Figure 5:

Interaction enthalpy between cofactor ligands and residue 144

Interaction enthalpy between cofactor ligands and residue 146

Interaction enthalpy between cofactor ligands and residue 364

Interaction enthalpy between cofactor ligands and residue 305
## Table S1. Conserved Residues in the PreATP-grasp Superfamily.

| PDB ID | K2 final score | Conserved residues          |                      |
|--------|----------------|-----------------------------|----------------------|
|        |                | Eukaryotic glutathione synthetase, substrate-binding domain |                      |
| 2HGS   | 10159.6        | GLU 144 ASN 146 LYS 305 LYS 364 |                      |
| 1M0W   | 6112.8         | GLU 146 ASN 148 LYS 324 LYS 382 |                      |
| 1M0T   | 5073.1         | GLU 146 ASN 148 LYS 324 not paired |                      |
|        |                | Prokaryotic glutathione synthetase, N-terminal domain |                      |
| 1GSA   | 725.6          | GLU 281 ASN 283 LYS 125 LYS 160 |                      |
| 1GSH   | 545.2          | GLU 281 ASN 283 LYS 125 LYS 160 |                      |
| 2GLT   | 618.9          | GLU 281 ASN 283 LYS 125 LYS 160 |                      |
| 1GLV   | 615.8          | GLU 281 ASN 283 LYS 125 LYS 160 |                      |
|        |                | D-Ala-D-Ala ligase, N-domain |                      |
| 1IOW   | 433.1          | GLU 270 ASN 272 LYS 97 LYS 144 |                      |
| 1IOV   | 430.7          | GLU 270 ASN 272 LYS 97 LYS 144 |                      |
| 2DLN   | 433.3          | GLU 270 ASN 272 LYS 97 LYS 144 |                      |
|        |                | D-alanine:D-lactate ligase VanA, N-domain |                      |
| 1EHI   | 478.3          | GLU 316 ASN 318 LYS 136 LYS 180 |                      |
| 1E4E   | 353.5          | GLU 305 ASN 307 LYS 133 LYS 171 |                      |
|        |                | Synapsin Ia |                      |
| 1AUV   | 264.2          | GLU 386 VAL 388 LYS 225 LYS 269 |                      |
| 1AUX   | 387.9          | GLU 386 VAL 388 LYS 225 LYS 269 |                      |
|        |                | Glycinamide ribonucleotide synthetase (GAR-syn), N-domain |                      |
| 1GSO   | 224.7          | GLU 286 ASN 288 LYS 105 | not paired |
|        |                | Biotin carboxylase subunit of acetyl-CoA carboxylase, (BC), N-domain |                      |
| 1DV2   | 244.5          | LYS 288 ASN 290 LYS 116 LYS 159 |                      |
| 1DV1-A | 327.0          | GLU 288 ASN 290 LYS 116 not paired |                      |
| 1BNC-A | 314.1          | GLU 288 ASN 290 LYS 116 not paired |                      |
|        |                | N5-carboxyaminoimidazole ribonucleotide synthetase PurK (AIRC), N-domain |                      |
| 1B6R   | 504.8          | GLU 238 ALA 240 ARG 80 LYS 120 |                      |
| 1B6S-D | 569.8          | GLU 238 ALA 240 ARG 80 LYS 120 |                      |
|        |                | Glycinamide ribonucleotide transformylase PurT, N-domain |                      |
| 1EZ1   | 282.4          | GLU 279 SER 281 ARG 114 LYS 155 |                      |
| 1EYZ   | 411.6          | GLU 279 SER 281 ARG 114 LYS 155 |                      |
| 1KJQ   | 456.4          | GLU 279 SER 281 ARG 114 LYS 155 |                      |
| 1KJ9   | 325.3          | GLU 279 SER 281 ARG 114 LYS 155 |                      |
| 1KJ1   | 344.1          | GLU 279 SER 281 ARG 114 LYS 155 |                      |
| 1KJ8   | 422.4          | GLU 279 SER 281 ARG 114 LYS 155 |                      |
| 1KJJ   | 421.1          | GLU 279 SER 281 ARG 114 LYS 155 |                      |
|        |                | Carbamoyl phosphate synthetase (CPS), large subunit PreATP-grasp domains |                      |
| 1A9X-E | 320.8          | GLU 4299 ASN 4301 ARG 4129 ARG 4169 |                      |
| 1BXR-E | 250.9          | GLU 299 ASN 301 ARG 129 ARG 169 |                      |
Alignments of 2HGS with the proteins from PreATP-grasp superfamily, listed below, were generated using the K2 software\(^1\). K2 final score is computed by using the number of aligned residues and the final RMSD.

**Reference:**

\(^1\)Szustakowski, J.D., and Weng, Z. (2000) *Proteins* **38**, 428-440, http://zlab.bu.edu/k2/
Appendix S1: SCOP\(^1\) Analysis of human Glutathione Synthetase

Human glutathione synthetase (PDB code = 2HGS) belongs to the PreATP-grasp domain superfamily (PreATP-grasp domain fold) and also to the Glutathione synthetase ATP-binding domain-like superfamily (ATP-grasp fold). The PreATP-grasp domain fold proteins are included in the ATP-grasp fold common to all PreATP superfamily members. This feature is possible because within the SCOP hierarchy the unit of classification is the protein domain instead of the whole protein. Thus, different regions of a single protein may appear in multiple places in the SCOP database under different folds or, in the case of repeated domains, several times under the same fold\(^2\).

A detailed SCOP lineage and the complete list of proteins included in both superfamilies are shown below (Tables S2 and S3):

### Table S2. Members of the PreATP-grasp domain superfamily

| Family                          | Domain                                      | Protein                                                                 | PDB ID | Species                                      |
|---------------------------------|---------------------------------------------|-------------------------------------------------------------------------|--------|----------------------------------------------|
| Eukaryotic glutathione synthetase, substrate-binding domain | Eukaryotic glutathione synthetase, substrate-binding domain | Human Glutathione Synthetase                                             | 2HGS   | Human (Homo sapiens)                         |
|                                 |                                              | Yeast Glutathione Synthase bound to γ-Glutamyl-Cysteine, AMP-PNP and 2 Magnesium Ions | 1MOW   | Baker's yeast (Saccharomyces cerevisiae)     |
|                                 |                                              | Yeast Glutathione Synthase                                              | 1M0T   |                                              |
| Prokaryotic glutathione synthetase, N-terminal domain | Prokaryotic glutathione synthetase, N-terminal domain | Glutathione synthetase complexed with ADP and glutathione                | 1GSA   | Escherichia coli                            |
|                                 |                                              | Glutathione Synthetase at pH 7.5                                       | 1GSH   |                                              |
|                                 |                                              | Glutathione Synthetase at pH 6.0                                       | 2GLT   |                                              |
|                                 |                                              | Glutathione synthetase at 2.0 Å resolution                               | 1GLV   |                                              |
| D-Alanine ligase N-terminal domain | D-Ala-D-Ala ligase, N-domain                  | Complex of Y216F D-ala: D-ala ligase with ADP and a phosphonyl phosphinate | 1IOW   | Escherichia coli                            |
|                                 |                                              | Complex of D-ala: D-ala ligase with ADP and a phosphonyl phosphonate     | 1IOV   |                                              |
|                                 |                                              | D-alanine: D-alanine ligase at 2.3 Å resolution                          | 2DLN   |                                              |
| D-alanine: D-lactate ligase VanA, N-domain | D-Alanine: D-Lactate Ligase (Lmddl2) |                                                                 | 1EHI   | Leuconostoc mesenteroides                   |
| Family                  | Domain                                  | Protein                                                                 | PDB ID | Species             |
|------------------------|-----------------------------------------|-------------------------------------------------------------------------|--------|---------------------|
| Synapsin Ia            | Synapsin Ia                             | D-Alanyl-D-Lactate Ligase                                               | 1E4E   | Enterococcus faecium|
|                        |                                         | C Domain of Synapsin Ia from bovine brain                              | 1AUV   |                     |
|                        |                                         | C Domain of Synapsin Ia from bovine brain with Ca, ATP-gamma-S bound    | 1AUX   | Caw (bos taurus)    |
| Biotin carboxylase     | Biotin carboxylase (Apo)                 | Biotin Carboxylase subunit of acetyl-CoA carboxylase, (BC), N-domain  | 1DV1   |                     |
|                        | Biotin Carboxylase subunit of acetyl-CoA carboxylase | Biotin Carboxylase subunit of acetyl-CoA carboxylase                    | 1BNC   |                     |
|                        | Biotin Carboxylase, mutant E288K, complexed with ATP | Biotin Carboxylase, mutant E288K, complexed with ATP                   | 1DV2   |                     |
| BC N-terminal domain   | Glycinamide ribonucleotide synthetase (GAR-syn), N-domain | Glycinamide Ribonucleotide Synthetase (Gar-Syn)                        | 1GSO   |                     |
| PurT, N-domain         | N5-Carboxyaminoimidazole ribonucleotide synthetase PurK (AIRC), N-domain | N5-Carboxyamino-imidazole Ribonucleotide Synthetase                     | 1B6R   |                     |
|                        |                                        | N5-Carboxyamino-imidazole Ribonucleotide Synthetase                    | 1B6S   |                     |
| Glycinamide ribonucleotide transformylase PurT, N-domain | Glycinamide Ribonucleotide Transformylase in complex with Mg-ADP | Glycinamide Ribonucleotide Transformylase in complex with Mg-ADP       | 1KJQ   |                     |
|                        |                                        | Purt-Encoded Glycinamide Ribonucleotide Transformylase complexed with Mg-ATP | 1KJ9   |                     |
|                        |                                        | Glycinamide Ribonucleotide Transformylase in complex with Mg-AMPPCP    | 1KJI   | Escherichia coli    |
|                        |                                        | Purt-Encoded Glycinamide Ribonucleotide Transformylase in complex with Mg-ATP and Gar | 1KJ8   |                     |
|                        |                                        | Glycinamide Ribonucleotide Transformylase in complex with Mg-ATP-gamma-S | 1KJJ   |                     |
|                        |                                        | Purt-Encoded Glycinamide Ribonucleotide Transformylase complexed with Mg and AMPPNP | 1EYZ   |                     |
| Family | Domain | Protein | PDB ID | Species |
|--------|--------|---------|--------|---------|
| BC N-terminal domain like | Carbamoyl phosphate synthetase (CPS), large subunit PreATP-grasp domains | Purt-Encoded Glycinamide Ribonucleotide Transformylase complexed with Mg, AMPPNP, and Gar | 1EZ1 | Escherichia coli |
| | | Carbamoyl Phosphate Synthetase: Caught in the act of Glutamine Hydrolysis | 1A9X | |
| | | Carbamoyl Phosphate Synthetase complexed at Cys269 in the Small Subunit with the Tetrahedral Mimic L-Glutamate gamma-Semialdehyde | 1CS0 | |
| | | Carbamoyl Phosphate Synthetase: Small Subunit Mutation C269S | 1C30 | |
| | | Carbamoyl Phosphate Synthetase | 1JDB | |
| | | Carbamoyl Phosphate Synthetase: Small Subunit Mutant C269S with bound Glutamine | 1C30 | |
| | | Inactivation of the Amidotransferase Activity of Carbamoyl Phosphate Synthetase by the Antibiotic Acivicin | 1KEE | |
| | | Carbamoyl Phosphate Synthetase complexed with the Allosteric Ligand Imp | 1CE8 | |
| | | Carbamoyl Phosphate Synthetase complexed with the ATP analog AMPPNP | 1BXR | |
| | | G359F (Small Subunit) Point Mutant of Carbamoyl Phosphate Synthetase | 1M6V | |
Table S3. Members of the Glutathione synthetase ATP-binding domain-like superfamily

| Family                                      | Domain                                   | Protein                                                                 | PDB ID | Species                     |
|---------------------------------------------|------------------------------------------|-------------------------------------------------------------------------|--------|-----------------------------|
| Eukaryotic glutathione synthetase, substrate-binding domain | Eukaryotic glutathione synthetase, substrate-binding domain | Human Glutathione Synthetase                                           | 2HGS   | Human (Homo sapiens)        |
|                                             |                                          | Yeast Glutathione Synthase bound to γ-Glutamyl-Cysteine, AMP-PNP and 2 Magnesium Ions | 1M0W   | Baker's yeast (Saccharomyces cerevisiae) |
|                                             |                                          | Yeast Glutathione Synthase                                             | 1M0T   |                             |
| ATP-binding domain of peptide synthetase    | Prokaryotic glutathione synthetase, N-terminal domain | Glutathione synthetase complexed with ADP and glutathione               | 1GSA   | Escherichia coli            |
|                                             |                                          | Glutathione Synthetase at pH 7.5                                       | 1GSH   |                             |
|                                             |                                          | Glutathione Synthetase at pH 6.0                                       | 2GLT   |                             |
|                                             |                                          | Glutathione synthetase at 2.0 Å resolution                             | 1GLV   |                             |
|                                             | D-Ala-D-Ala ligase, N-domain              | Complex of Y216F D-ala: D-ala ligase with ADP and a phosphonyl phosphinate | 1IQW   | Escherichia coli            |
|                                             |                                          | Complex of D-ala: D-ala ligase with ADP and a phosphonyl phosphonate    | 1IOV   |                             |
|                                             |                                          | D-alanine: D-alanine ligase at 2.3 Å resolution                        | 2DLN   |                             |
|                                             | D-alanine:D-lactate ligase VanA, N-domain | D-Alanine: D-Lactate Ligase (Lmddl2)                                    | 1EHI   | Leuconostoc mesenteroides   |
|                                             |                                          | D-Alanyl-D-Lactate Ligase                                              | 1E4E   | Enterococcus faecium        |
| Synapsin Ia, C-terminal domain              | Synapsin Ia, C-terminal domain           | C Domain of Synapsin Ia from bovine brain                              | 1AUV   | Caw (bos taurus)            |
|                                             |                                          | C Domain of Synapsin Ia from bovine brain with Ca, ATP-gamma-S bound   | 1AUX   |                             |

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| Family                          | Domain                                                                 | Protein                                                                 | PDB ID  | Species          |
|--------------------------------|------------------------------------------------------------------------|------------------------------------------------------------------------|---------|------------------|
| BC ATP-binding domain-like     | Biotin carboxylase subunit of acetyl-CoA carboxylase, (BC), N-domain   | Biotin Carboxylase (Apo)                                               | 1DV1    |                 |
|                                |                                                                        | Biotin Carboxylase subunit of acetyl-CoA carboxylase                   | 1BNC    |                 |
|                                |                                                                        | Biotin Carboxylase, Mutant E288K, complexed with ATP                    | 1DV2    |                 |
| Glycanamid ribonucleotide      | Glycanamid ribonucleotide synthetase (GAR-syn), N-domain               | Glycanamid Ribonucleotide Synthetase (Gar-Syn)                         | 1GSO    | Escherichia coli |
| synthetase (GAR-syn), N-domain |                                                                        |                                                                        |         |                  |
| N5-carboxyaminoimidazole       | N5-Carboxyamino-imidazole Ribonucleotide Synthetase                     | N5-Carboxyamino-imidazole Ribonucleotide Synthetase                     | 1B6R    |                 |
| ribonucleotide synthetase PurK |                                                                        |                                                                        | 1B6S    |                 |
| (AIRC), N-domain               |                                                                        |                                                                        |         |                  |
| Glycanamid ribonucleotide      | Glycanamid Ribonucleotide Transformylase in complex with Mg-ADP        | Glycanamid Ribonucleotide Transformylase in complex with Mg-ADP         | 1KJQ    |                 |
| transformylase PurT, N-domain  |                                                                        |                                                                         | 1KJ9    |                 |
|                                |                                                                        | Glycanamid Ribonucleotide Transformylase in complex with Mg-AMPPCP     | 1KJI    |                 |
|                                |                                                                        |                                                                         | 1KJ8    |                 |
|                                |                                                                        | Purt-Encoded Glycinamid Ribonucleotide Transformylase in complex with Mg-ATP | 1KJ7    |                 |
|                                |                                                                        |                                                                         | 1KJJ    |                 |
|                                |                                                                        | Purt-Encoded Glycinamid Ribonucleotide Transformylase in complex with Mg-ATP-gamma-S | 1EYZ    |                 |
|                                |                                                                        | Purt-Encoded Glycinamid Ribonucleotide Transformylase complexed with Mg and AMPPNP | 1EZ1    |                 |
|                                |                                                                        |                                                                         |         |                 |
| Family                        | Domain                                                                 | Protein                                                                 | PDB ID | Species                        |
|------------------------------|------------------------------------------------------------------------|-------------------------------------------------------------------------|--------|-------------------------------|
| BC ATP-binding domain-like   | Carbamoyl phosphate synthetase (CPS), large subunit PreATP-grasp domains | Carbamoyl Phosphate Synthetase: Caught in the act of Glutamine Hydrolysis | 1A9X   | Escherichia coli              |
|                              |                                                                        | Carbamoyl Phosphate Synthetase complexed at Cys269 in the Small Subunit with the Tetrahedral Mimic L-Glutamate gamma-Semialdehyde | 1CS0   |                               |
|                              |                                                                        | Carbamoyl Phosphate Synthetase: Small Subunit Mutation C269S             | 1C30   |                               |
|                              |                                                                        | Carbamoyl Phosphate Synthetase                                            | 1JDB   |                               |
|                              |                                                                        | Carbamoyl Phosphate Synthetase: Small Subunit Mutant C269S with bound Glutamine | 1C30   |                               |
|                              |                                                                        | Inactivation of the Amidotransferase Activity of Carbamoyl Phosphate Synthetase by the Antibiotic Acivicin | 1KEE   |                               |
|                              |                                                                        | Carbamoyl Phosphate Synthetase complexed with the Allosteric Ligand Imp   | 1CE8   |                               |
|                              |                                                                        | Carbamoyl Phosphate Synthetase complexed with the ATP analog AMPNP        | 1BXR   |                               |
|                              |                                                                        | G359F (Small Subunit) Point Mutant of Carbamoyl Phosphate Synthetase      | 1M6V   |                               |
| Pyruvate phosphate dkinase, N-terminal domain | Pyruvate phosphate dkinase, N-terminal domain                          | Pyruvate Phosphate Dikinase                                              | 1KBL   | Clostridium symbiosum         |
|                              |                                                                        | Pyruvate Phosphate Dikinase with bound Mg-Phosphonopyruvate              | 1KC7   |                               |
|                              |                                                                        | Pyruvate Phosphate Dikinase, at pH 7.0                                   | 1DIK   |                               |
|                              |                                                                        | T453A Mutant of Pyruvate, Phosphate Dikinase                             | 1GGO   |                               |
|                              |                                                                        | R337A Mutant of Pyruvate Phosphate Dikinase                             | 2DIK   |                               |
|                              |                                                                        | K22A Mutant of Pyruvate, Phosphate Dikinase                             | 1JDE   |                               |
|                              |                                                                        | Glycosomal Pyruvate Phosphate Dikinase at 3.0 Å Resolution              | 1H6Z   | Trypanosoma brucei            |
| Family                        | Domain                                                                 | Protein                                                                 | PDB ID | Species                    |
|-------------------------------|------------------------------------------------------------------------|-------------------------------------------------------------------------|--------|----------------------------|
| Succinyl-CoA synthetase, beta-chain, N-terminal domain | Succinyl-CoA synthetase, beta-chain, N-terminal domain | Succinyl-CoA Synthetase                                                 | 1JKJ   |                            |
|                               |                                                                        | Succinyl-CoA Ligase                                                     | 2SCU   |                            |
|                               |                                                                        | Dephosphorylated E. Coli Succinyl-Coa Synthetase                       | 1CQJ   |                            |
|                               |                                                                        | E197Betaa Mutant of E. Coli Succinyl-Coa Synthetase                    | 1JEL   |                            |
|                               |                                                                        | Succinyl-CoA synthetase from Escherichia coli at 2.5 Å resolution   | 1SCU   | Escherichia coli           |
|                               |                                                                        | Complex of ADP and Mg2+ with Dephosphorylated E. coli Succinyl-Coa Synthetase | 1CQI   |                            |
|                               |                                                                        | Dephosphorylated Pig heart, GTP- Specific Succinyl-Coa Synthetase    | 1EUC   | Pig (Sus scrofa)           |
|                               |                                                                        | Phosphorylated Pig heart, GTP-Specific Succinyl-Coa Synthetase      | 1EUD   |                            |

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2 Brenner, S.E., Chothia, C., Hubbard, T.J.P., and Murzin, A.G. (1996) *Methods Enzymol.* **266**, 635-643
