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Identification and clarification of the role of key active site residues in bacterial glutathione *S*-transferase zeta/maleylpyruvate isomerase

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ABSTRACT

The maleylpyruvate isomerase NagL from *Ralstonia* sp. strain U2, which has been structurally characterized previously, catalyzes the isomerization of maleylpyruvate to fumarylpyruvate. It belongs to the class zeta glutathione *S*-transferases (GSTZs), part of the cytosolic GST family (cGSTs). In this study, site-directed mutagenesis was conducted to probe the functions of 13 putative active site residues. Steady-state kinetic information for mutants in the reduced glutathione (GSH) binding site, suggested that (a) Gln64 and Asp102 interact directly with the glutamyl moiety of glutathione, (b) Gln49 and Gln64 are involved in a potential electron-sharing network that influences the ionization of the GSH thiol. The information also suggests that (c) His38, Asn108 and Arg109 interact with the GSH glycine moiety, (d) His104 has a role in the ionization of the GSH sulfur and the stabilization of the maleyl terminal carboxyl group in the reaction intermediate and (e) Arg110 influences the electron distribution in the active site and therefore the ionization of the GSH thiolate. Kinetic data for mutants altered in the substrate-binding site imply that (a) Arg8 and Arg176 are critical for maleylpyruvate orientation and enolization, and (b) Arg109 (exclusive to NagL) participates in k_{cat} regulation. Surprisingly, the T11A mutant had a decreased GSH K_m value, whereas little impact on maleylpyruvate kinetics was observed, suggesting that this residue plays an important role in GSH binding. An evolutionary trend in this residue appears to have developed not only in prokaryotic and eukaryotic GSTZs, but also among the wider class of cGSTs.

1. Introduction

Glutathione *S*-transferases (GSTs, EC 2.5.1.18), widely distributed in both eukaryotes and prokaryotes, catalyze the nucleophilic attack by reduced glutathione (GSH) on carbon-, nitrogen- or sulfur-containing electrophilic groups in a wide range of hydrophobic toxic compounds [1]. Cytosolic GSTs (cGSTs) represent the largest GST family and are further classified into more than 10 classes, including the class zeta enzymes. The zeta glutathione *S*-transferases (GSTZs) are highly conserved among prokaryotes and eukaryotes, indicating their importance and central function in cell metabolism [2,3]. When compared with other classes of cGSTs, members of the GSTZs have poor GSH-conjugating activity with standard or conventional GST substrates [2,4,5]. Two primary roles have been elucidated for GSTZs. The first is the GSH-dependent isomerization of maleylacetoacetate to fumarylacetoacetate in the homogentisate pathway, with MaiA from *Aspergillus nidulans*, GSTZ1 from *Homo sapiens* [6] and AtGSTZ1 from *Arabidopsis thaliana* [4] having this function. The second involves conversion of maleylpyruvate to fumarylpyruvate in the gentisate pathway, and NagL from the prokaryotic *Ralstonia* sp. strain U2 [7] is an example. In these reactions, GSH behaves as a cofactor rather than a substrate. Structural information for GSTZs has been obtained for three enzymes: maleylacetoacetate isomerases from humans [8] and *A. thaliana* [9], and a maleylpyruvate isomerase (NagL) from *Ralstonia* sp. U2 [3]. The latter enzyme was co-crystallized with GSH and dicarboxyethyl-glutathione (DCEG, a reaction intermediate analog) and was in the dimeric form that corresponds to the native form of GSTZs. The crystal structures of the former two proteins were in monomeric form. The two eukaryotic maleylacetoacetate isomerases have been subjected to site-directed mutagenesis and characterized, which has generated functional information on their activity [9–12]. However, little is known about the roles of active residues in the reaction carried out by the prokaryotic zeta enzyme. Here we report the results of site-directed mutagenesis experiments designed to probe the active site residues of NagL responsible for GSH and substrate binding, and catalysis during maleylpyruvate isomerization.

Abbreviations: cGST, cytosolic GST; DCEG, dicarboxyethyl-glutathione; GST, glutathione *S*-transferase; GSTZs, class zeta glutathione *S*-transferases; GSH, reduced glutathione.

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2. Materials and methods

2.1. Chemicals

GSH and gentisate were purchased from Sigma Chemical (St. Louis, MO, USA). Maleylpyruvate was prepared by the oxidation of gentisate with a purified *nagI*-encoded gentisate 1,2-dioxygenase from *Ralstonia* sp. U2 as described [13].

2.2. Site-directed mutagenesis

NagL mutants were generated by overlap-extension PCR [14], using primers based on the sequence of the wild-type *nagL* from *Ralstonia* sp. U2 [7]. Full-length DNA sequencing in both directions was performed to confirm the engineered clones.

2.3. Expression and purification of NagL and its mutants

N-terminal His-tagged NagL and its mutants were expressed in *Escherichia coli* BL21 (DE3) pLysS with pET28a (Novagen Inc., Madison, WI, USA). The enzymes were purified using a two-step protocol: Ni^{2+}–nitrilotriacetic acid affinity chromatography followed by gel-filtration chromatography. Gel-filtration chromatography was also used to determine the native molecular masses of the purified proteins [15]. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was employed to determine the subunit composition and molecular weight of the purified proteins.

2.4. Steady-state kinetics

Maleylpyruvate isomerase activity was measured as described [16] with the following modifications. Determinations were set up in triplicate in 96-well plates using a multichannel pipette for simultaneous mixing of the reagents. To determine the substrate- and cofactor-dependent kinetic properties of NagL and its variants, 7–12 different concentrations were prepared for maleylpyruvate and glutathione in 50 mM disodium hydrogen phosphate/sodium dihydrogen phosphate buffer (PB) (pH 8.0). Kinetic parameter were determined for GSH in the presence of a fixed concentration of 1.0 mM maleylpyruvate and for maleylpyruvate in the presence of 100 μM GSH. Excess *nagK*-encoded fumarylpyruvate hydrolase was added to each well and the reactions were initiated by addition of the enzymes. The initial velocities of the enzymatic reactions were determined by measuring the decrease in absorbance at 330 nm due to the disappearance of maleylpyruvate. The molar extinction coefficient of maleylpyruvate was taken to be 13,000 cm^{-1} M^{-1} [17]. The reaction solution without enzyme was used as a negative control. Kinetic data were evaluated by non-linear regression analysis with the Michaelis–Menten equation ($\upsilon = V_{max} \times [S]/(K_m + [S])$), using the Windows-based Origin software package supplied by MicroCal. The catalytic constant, k_{cat}, was calculated using the equation $V_{max} = k_{cat} \times [E]$, where [E] = total enzyme concentration. The protein concentration was determined as described [18].

2.5. Structure-based alignment and phylogenetic analysis of representative cGSTs

Representative cGSTs, including GSTZs, were selected from the protein data bank (PDB). Multiple-sequence alignment and the construction of phylogenetic trees (using the neighbor-joining method) were performed using Mega 3.1 software [19].

3. Results and discussion

3.1. Characterization of NagL and its mutants

A partial structure-based alignment was generated by comparing the sequences of NagL, two maleylacetoacetate isomerases from GSTZs and three members belonging to other representative classes of cGSTs (Supplementary Fig. 1). Based on this alignment and the crystal structures of NagL, 13 potentially active residues were chosen for site-directed mutagenesis. A double mutant, R8A/R176A, was also prepared, and the 14 mutants selected were expressed in soluble form and purified to apparent homogeneity. They all exhibited single bands at the same apparent molecular weight on SDS–PAGE, and the same native molecular masses in gel filtration, as the wild-type. These data suggest that the mutations did not have a significant impact on the overall structure of NagL.

3.2. Steady-state kinetics of NagL and its mutants

Members of GSTZs are thought to have two binding sites, the G-site for GSH and the H-site for the hydrophobic substrate. The former is essentially conserved and shows high specificity for GSH. The latter is distinctive from other classes of cGSTs at the C-terminal region of the enzyme, and varies to accommodate specific electrophilic substrates [3,8,9]. From the steady-state kinetics of the wild-type and mutant NagL enzymes obtained (Table 1), it was found that the residues selected for mutation are important for interactions with the glutamyl, glycine and cysteinyl moieties of the tripeptide glutathione in the G-site and the substrate maleylpyruvate in the H-site. In particular, mutants S9A, H38A, P53A, D102A, Q64A and R110A were found to be completely inactive.

3.3. Active site residues interact with the glutamyl moiety

Asp102 (previously mistaken for Glu102 [3]) and Gln64 in the G-site have been shown in the crystal structure to directly interact with the α-amino group of the glutamyl moiety of glutathione (Fig. 1A). The completely abolished activity of D102A confirmed the primary role of Asp102 in orienting the glutamyl moiety. Mutation of Gln64 to alanine in NagL also generated an inactive enzyme, confirming that Gln64 plays a critical role in glutathione binding. Notably, a highly conserved Glu64 in the *Anopheles dirus* class delta glutathione *S*-transferase D3-3 (equivalent to Gln64 in NagL) was found to be involved in the electron-sharing network that assists the glutamyl moiety of glutathione to function as a catalytic base, accepting the proton from the thiol group to form an anionic glutathione [20]. It was suggested that such a network could be mapped to the same region of all GST classes [21]. As shown in Fig. 1A, Gln49 is thought to interact with Gln64 and Asp102 by hydrogen bonds, and this was confirmed by the significant increase (10-fold) in GSH K_m value observed for Q49A. Surprisingly, this mutant showed a greatly decreased substrate affinity (a 10-fold increase in K_m value) and catalytic activity (a 10-fold decrease in k_{cat} value). This implies the presence of an electron-sharing network in NagL involving both Gln49 and Gln64, since replacing these residues with residues that can not take part in hydrogen-bonding in the electron-sharing network showed reduced enzyme activity [21].

3.4. Active site residues interact with the glycine moiety

It has been shown that the glycine moiety of GSH interacts with Asn108, the main chain nitrogen of Arg110 and NE2 of His38 in NagL [3]. Mutation of His38 to alanine disrupts the hydrogen bond

Table 1
Steady-state kinetic parameters of wild-type NagL and its mutants for the GSH-dependent isomerization reaction at pH 8.0 and room temperature. Enzyme activities were measured at varying concentrations of maleylpyruvate and GSH in 50 mM phosphate buffer, pH 8.0. The reaction was monitored at 330 nm, ε = 13,000 M^{-1} cm^{-1}. NM denotes not measurable (insufficient activity to determine the kinetic parameters when 1 μmol of enzyme was present).

Enzyme	k_m^{MP} (μM)	k_{cat} (s^{-1})	k_{cat}/k_m (M^{-1} s^{-1})	Relative k_{cat}/k_m (%)	k_m^{gsh} (μM)
Wide type	565.81 ± 81.99	101.32 ± 3.74	179,072.45	100	10.68 ± 1.96
R8A	1028.77 ± 161.39	22.59 ± 0.99	21,956.59	12.26	7.20 ± 0.88
S9A	NM	NM	NM	NM	NM
T11A	690.10 ± 109.64	120.87 ± 4.18	175,148.76	97.81	0.78 ± 0.03
H38A	NM	NM	NM	NM	NM
Q49A	6517.99 ± 3118.88	13.45 ± 1.70	2063.35	1.15	101.56 ± 18.91
P53A	NM	NM	NM	NM	NM
Q64A	NM	NM	NM	NM	NM
D102A	NM	NM	NM	NM	NM
H104A	406.68 ± 90.70	11.11 ± 0.59	27,308.88	15.25	30.04 ± 3.77
N108A	345.73 ± 110.11	31.47 ± 4.49	91,018.38	50.83	55.78 ± 13.63
R109A	343.82 ± 69.86	0.54 ± 0.03	1578.97	0.88	503.31 ± 138.04
R110A	NM	NM	NM	NM	NM
R176A	2355.68 ± 1099.87	51.45 ± 6.83	21,841.42	12.20	1.65 ± 0.24
R8A/R176A	3338.92 ± 890.48	1.06 ± 0.08	316.29	0.18	1.51 ± 0.49

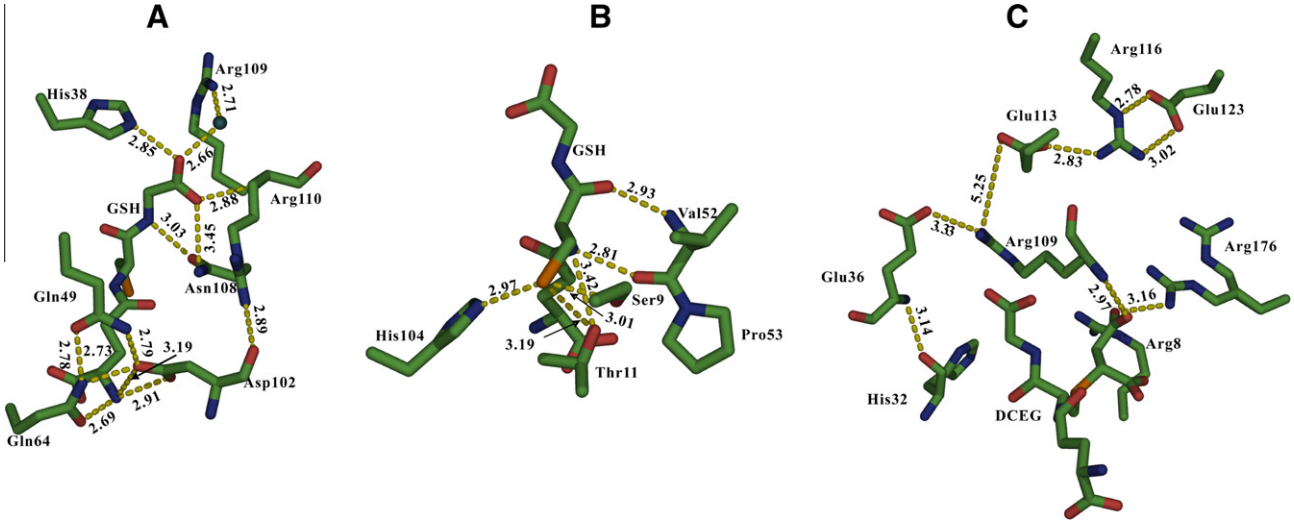

Fig. 1. A model of the active site residues in the crystal structures of maleylpyruvate isomerase NagL, together with glutathione (GSH) and the reaction intermediate analogue dicarboxyethyl-glutathione (DCEG). (A) Active site residues interacting with the glutamyl and glycine moieties of GSH. (B) Active site residues interacting with the cysteinyl moiety of GSH. (C) Active site residues of the hydrophobic-substrate-binding site (H-site) in the NagL–DCEG complex. Carbon atoms are shown in green, oxygen atoms in red, nitrogen atoms in blue, sulfur atom in yellow and water molecule is shown as a ball in black. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

or the salt bridge between His38 (NH1) and the glycine carboxylate, and results in a completely inactive enzyme. This confirms that His38 is important in orienting this carboxylate. Examination of the NagL–GSH complex structure indicated that the glycine carboxylate may be connected to the guanidinium group of Arg109 through water-mediated hydrogen bonds (Fig. 1A). Mutation of Arg109 to alanine resulted in a mutant that showed a dramatic increase (49-fold) in K_m value for GSH, which is likely to be related to the disruption of these interactions. The N108A mutant was also found to exhibit an approximately 5-fold increase in GSH K_m value, which may be due to the disruption of the hydrogen bonds between Asn108 and the glycine moiety (Fig. 1A).

3.5. Active site residues interact with the cysteinyl moiety

The distinctive features of GSTZs include a fingerprint motif of SSCXWRVRIAL, which has been identified from the limited number of available eukaryotic maleylacetoacetate isomerase sequences by the original classification of GSTZs [2]. This motif has the sequence ***S***GTSH***RL******RIAL*** in NagL, where matches with the established motif are shown in bold and italic typeface. Both Ser9 and Thr11 are within the class zeta motif, but Thr11 replaces the conserved cysteine. Ser9 and Thr11, together with His104, lie within hydrogen bonding distance of the glutathione cysteinyl sulfur (Fig. 1B). An S9A mutant was inactive, confirming the important function of this residue in thiolate stabilization. This is consistent with results of mutagenesis experiments with Ser17 in AtGSTZ1 [9] and Ser14 in GSTZ1 [12], which are equivalent to Ser9 in NagL. The T11A mutant was observed to have a similar maleylpyruvate affinity and catalytic efficiency as the wild-type, but exhibited an unexpectedly large decrease (14-fold) in its K_m value for GSH. As Thr11 could provide interactions with the backbone nitrogen and the thiol in the cysteinyl moiety via hydrogen bonding with its hydroxyl group (Fig. 1B), the unexpected increase of the T11A mutant's affinity with GSH may be due to the disruption of these, possibly detrimental, hydrogen bonds. Alternatively, the smaller side chain of the substituted residue may provide additional flexibility allowing GSH to be accommodated more readily, and thereby reducing its apparent K_m value. Although the reason for the large decrease in GSH K_m value remains speculative, this residue may be significant for the rational design of an effective competitive enzyme.

His104 has been implicated in the generation of the thiolate ion by proton abstraction and in stabilizing the ionization of the maleyl terminal carboxyl group in the reaction intermediate [3]. The H104A mutant generated in this study showed a 6.6-fold reduction in catalytic efficiency, largely due to a 9.1-fold decrease in k_{cat}. This evidence therefore supports this prediction. Additionally, the carbonyl oxygen atom and the backbone nitrogen atom of Val52 are within hydrogen bonding distance of the backbone nitrogen atom and the carbonyl oxygen atom of the cysteinyl moiety, respectively, supported by Pro53 in the *cis* conformation (Fig. 1B). Substitution of the highly conserved Pro53 with alanine resulted in a completely inactive mutant enzyme, consistent with results described [22–24]. This suggests that local conformational changes of the Val52-Pro-53 motif, brought about by exchanging the conformational rigidity of Pro53 for the flexible alanine residue, affect the orientation of GSH.

3.6. Positively charged residue Arg110 interacts with GSH

The carboxylate group of the positively charged Arg110 interacts, by hydrogen bonding, with the negatively charged Asp102. Asp102 also interacts directly with the glutamyl moiety of GSH, as discussed earlier (Fig. 1A). The main chain nitrogen of Arg110 is within hydrogen bonding distance of the glycine carboxylate, as shown in Fig. 1A. Mutagenesis of Arg110 to a nonpolar alanine resulted in a completely inactive enzyme. This suggests that the positively charged Arg110, unique among GSTZs to NagL, plays an important role in controlling electronic distribution in the active site and promotes ionization of the sulfhydryl group of GSH and stabilization of the thiolate anion produced.

3.7. Active site residues in the H-site interact with maleylpyruvate

The crystal structure of NagL in the presence of DCEG, a reaction intermediate analogue for GSH-dependent isomerization, indicated that Arg176 may form a hydrogen bond with the adjacent carbonyl of the C4 position of maleylpyruvate, as shown in Fig. 1C. The mutation of the fully conserved Arg176 residue in GSTZs to alanine resulted in an 8-fold decrease in catalytic efficiency and a decrease in substrate affinity (4-fold increase in K_m for maleylpyruvate). This is consistent with results obtained for a mutant at the equivalent residue Arg175 in GSTZ1 [12]. The result suggests that Arg176 plays a critical role in orientating the substrate. The mutant R8A exhibited a nearly 2-fold increase in K_m for maleylpyruvate, indicating a possible salt bridge or hydrogen bond between Arg8 and the pyruvyl moiety of maleylpyruvate, as speculated [3]. The double mutant R8A/R176A exhibited a very large decrease in catalytic efficiency (566-fold decrease in k_{cat}/K_m), mainly due to a 95-fold decrease in k_{cat} and also due to a decrease in substrate affinity (6-fold increase in K_m). Thus, the positively charged residues Arg8 and Arg176 may not only play important roles in substrate binding but also participate in the transfer of electrons to aid the enolization of the C4 carbonyl, which is critical to the isomerization, as described [25]. In addition, this negatively charged oxygen has also been suggested to be stabilized by a hydrogen bond to the main chain nitrogen of Arg109, observed in the structure of the NagL–DCEG complex [3] and shown in Fig. 1C. The R109A mutant demonstrated a slightly lower K_m value for the substrate than the wild-type, but also showed a 140-fold decrease in catalytic efficiency (k_{cat}/K_m), largely due to 200-fold decrease in k_{cat}. This confirms that this positively charged residue has a function in the stabilization of the C4 enolate oxygen. Arg109, which forms a salt-bridge to the side-chain of Glu36, is an integral part of a pocket near the substrate together with the surface-exposed residues His32, Glu36, Glu113, Arg116 and Glu123 (Fig. 1C). Arg109 may be important in maintaining the structural integrity of this pocket. Mutagenesis of the positively charged Arg109 to a smaller and nonpolar alanine disrupted the salt-bridge with Glu36 and rendered the substrate active site more accessible to solvent. This may contribute to the large decrease in k_{cat} observed for this mutant. It is of interest that the GSH K_m values of both the R176A and R8/R176A mutants were reduced in reactions with maleylpyruvate, a phenomenon that was also observed in the mutagenesis study of Arg175 in GSTZ1 [12], indicating that Arg176 has an additional indirect influence on glutathione binding.

3.8. Residue 11 reflects the molecular evolution of cytosolic GSTs

The unexpectedly large decrease (14-fold) in K_m for GSH of the T11A mutant suggested that alanine at this position is more suitable for GSH binding than threonine in NagL. To investigate whether this residue is conserved, a total of 200 sequences homologous to NagL in GSTZs deposited with the UniProtKB protein knowledgebase (www.uniprot.org) from both eukaryotes (100 sequences) and prokaryotes (100 sequences) were analyzed. In prokaryotes, the dominant residue at this position is alanine (>50%), while other residues commonly found are serine, threonine or cysteine. By contrast, cysteine is found to be the dominant residue at the equivalent position in eukaryotic GSTZs (85%). Mutagenesis of this cysteine (Cys16) to alanine in GSTZ1 resulted in a significant increase in GSH K_m value (2-fold for the substrate 2-bromo-3-(4-nitrophenyl)propanoic acid and 12-fold for maleylacetone) [12]. These results suggested that cysteine at this position plays a more important role in GSH binding than alanine in this enzyme, and correlate well with a structural study that indicated that the cysteine residue was within the glutathione binding region [8]. The cysteine was also found to be involved in promoting the proper orientation of GSH, as evidenced by the corresponding C14A mutant in prokaryotic *Sphingomonas* sp. UG30 [26], a member of the GSTZs.

The phylogenetic analysis carried out in this study indicated that while the equivalent residues at position 11 are different among the classes of cGSTs, they are highly conserved within each class (Supplementary Fig. 2). Crystal structures of the GSTs used in the phylogenetic analysis found in the PDB confirmed the proximity of the equivalent residues at position 11 to the positive end of the helix α1 N-terminal in all cases. This indicates a possible role in GSH binding for this residue in different classes of cGSTs. The functional significance of GSH binding of these residues has been revealed by site-directed mutagenesis in a number of members of the cGST family. For example, mutation of the equivalent residue (Arg15) to alanine, histidine, lysine or leucine in the human class alpha glutathione *S*-transferase A1-1 resulted in enzymes with substantially higher K_m values for GSH (12-, 4-, 7- or 13-fold increases observed, respectively), implicating this arginine in GSH binding [27]. Previous mutagenesis studies have also demonstrated that the equivalent residue (Cys10) is key to the binding of GSH in *Ochrobactrum anthropi* class beta glutathione *S*-transferase [28]. Considering that GSH binding and utilization is a distinctive feature of GSTs, it seems likely that the residues important to GSH-binding should be closely related to their function and evolution. The current comparison indicates that variations of the residues equivalent to Thr11 in NagL among the GSTs reflect an evolutionary trend across the entire cGST family.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.05.155.

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