Thioredoxin-like domain of human κ class glutathione transferase reveals sequence homology and structure similarity to the θ class enzyme

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Abstract

Glutathione transferases (GSTs) are a superfamily of enzymes that play a vital functional role in the cellular detoxification process. They catalyze the conjugation of the thiol group of glutathione (GSH) to the electrophilic groups of a wide range of hydrophobic substrates, leading to an easier removal of the latter from the cells. The κ class is the least studied one among various classes within the superfamily. We report here the expression, purification, and crystal structure of human κ class GST (hGSTK), which has been determined by the multiple-isomorphous replacement method and refined to 1.93 Å resolution. The overall structure of hGSTK is similar to the recently reported structure of κ class GST from rat mitochondrion. Each subunit of the dimeric hGSTK contains a thioredoxin (TRX)-like domain and a helical domain. A molecule of glutathione sulfinate, an oxidized product of GSH, is found to bind at the G site of each monomer. One oxygen atom of the sulfinio group of GSF forms a hydrogen bond with the hydroxyl group of the catalytic residue Ser16. The TRX-like domain of hGSTK shares 19% sequence identity and structure similarity with human θ class GST, suggesting that the κ class of GST is more closely related to the θ class enzyme within the GST superfamily. The structure of the TRX-like domain of hGSTK is also similar to that of glutathione peroxidase (GPx), implying an evolutionary relationship between GST and GPx.

Keywords: glutathione transferase; crystal structure; active site; glutathione sulfinate; thioredoxin-like domain; glutathione peroxidase

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Abbreviations: GST, glutathione transferase; hGSTK, human κ class GST; rGSTK, rat mitochondrial κ class GST; GSH, glutathione; GSF, glutathione sulfinate; TRX, thioredoxin; CDNB, 1-chloro-2,4-dinitrobenzene; DTT, dithiothreitol; MIR, multiple-isomorphous replacement; NCS, noncrystallographic symmetry; RMS, root-mean-square; GPx, glutathione peroxidase; PEG, polyethylene glycol.

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Glutathione transferases (GSTs, EC 2.5.1.18), formerly known as glutathione S-transferases, are a superfamily of enzymes that play a vital role in cellular detoxification process. GSTs catalyze the conjugation of the thiol group of glutathione (GSH), the tripeptide γ -Glu-Cys-Gly, to the electrophilic groups of a wide range of hydrophobic substrates, resulting in greater solubility and easier removal of the hydrophobic substrate from the cells (Mannervik and Danielson 1988; Pickett and Lu 1989; Coles and Ketterer 1990; Armstrong 1991; Tsuchida and Sato 1992; Wilce and Parker 1994; Sheehan et al. 2001). GSTs have been the focus of considerable interest with regard to resistance toward drugs, insecticides, herbicides,

and antibiotics. GSTs may also be involved in the intracellular storage and transport of a variety of hydrophobic, nonsubstrate compounds (Oakley et al. 1999). GSTs were reported to exhibit glutathione peroxidase (GPx, EC. 1.11.1.9) activity (Hurst et al. 1998; Jowsey et al. 2003), and human κ class GST (hGSTK) was recently identified to be localized in peroxisomes (Morel et al. 2004).

GSTs have been divided into an ever-increasing number of classes based on their amino acid sequence homology in combination with other criteria, such as tertiary structure similarity, substrate specificity, and immunological identity (Mannervik et al. 1992; Sheehan et al. 2001). GSTs generally share greater than 60% sequence identity within a class and less than 30% among distinct classes. Over the past years the three-dimensional structures of soluble GSTs in several classes have been reported (Ji et al. 1992, 1995; Dirr et al. 1994; Cameron et al. 1995; Rossjohn et al. 1998; Board et al. 2000; Polekhina et al. 2001). GSTs function as a dimeric enzyme, and the subunit structure adopts a similar canonical fold consisting of an N-terminal domain, assuming a topology similar to the thioredoxin (TRX) fold and a C-terminal domain comprising several α -helices (Sheehan et al. 2001). The κ class is the least studied one among various classes within the superfamily. Recently, the structure of κ class GST from rat mitochondrion (rGSTK) in complex with GSH was reported, which shows a folding topology different from that of the other GST classes (Ladner et al. 2004).

hGSTK is a homodimer; each monomer consists of 226 amino acids with a molecular mass of 26 kDa. hGSTK shares ~70% sequence identity with both rat and mouse κ class GSTs (Pemble et al. 1996; Morel et al. 2004). We report here the expression, purification, and crystal structure of hGSTK, which has been determined by the multiple-isomorphous replacement (MIR) method and refined to 1.93 Å resolution. The structure of rGSTK at 2.5 Å resolution was reported when the structure refinement of hGSTK was nearly complete. The overall structure of hGSTK is similar to that of rGSTK. At the active site of hGSTK, GSH was found to be in an oxidized state, namely glutathione sulfinate (GSF). Sequence alignment and structure comparison of the TRX-like domain of hGSTK with those of other classes of GSTs indicate that the κ class is more closely related to the θ class than to the other classes within the GST superfamily. The structure comparison of hGSTK with GPx is also discussed.

Results

Overall structure of hGSTK

The crystal structure of hGSTK in complex with GSF, refined to 1.93 Å resolution, yielded a final *R*-factor of

0.179 and a free *R*-factor of 0.203, respectively. The final structure model contains 1 homodimer of hGSTK, 2 GSF molecules, and 179 water molecules. Each subunit consists of 218 amino acid residues, and the N-terminal three residues and the C-terminal five residues could not be located due to weak electron density. The side chains of three residues (Met196 in both subunits and His75 in one subunit) exhibit dual conformations. Analysis of the stereochemistry of the protein model using the program PROCHECK (Laskowski et al. 1993) shows that 90.6% and 9.4% of the residues are in the most favored and additional allowed regions of the Ramachandran plot, respectively. The refinement statistics are summarized in Table 1. Figure 1, A and B, shows the overall structure of hGSTK.

hGSTK shares high sequence homology with rat and mouse GSTK (69.5% and 71.2% sequence identity, respectively, without insertion or deletion) (Morel et al. 2004), and the secondary structure of hGSTK is substantially the same as that of rGSTK (Ladner et al. 2004). The hGSTK monomer comprises two domains (Fig. 1B). Domain I is composed of residues 4–52 and 184–221, with a TRX-like fold (Martin 1995) that is characterized by a $\beta\alpha\beta$ ($\beta_1\alpha_1\beta_2$) motif and a $\beta\beta\alpha$ motif ($\beta_3\beta_4\alpha_{10}$) linked by an α -helix (α_2) to form a four-stranded β -sheet surrounded by three α -helices. Domain II is composed of residues 60–178, which folds as seven α -helices (α_3 – α_9). Domain II is inserted between the $\beta\alpha\beta$ and $\beta\beta\alpha$ motifs of domain I. The two domains are connected together by two short linkers.

The tertiary structure of hGSTK is also similar to that of rGSTK. The major differences between the two structures are located in the regions of Asn53–Pro60 and Pro84–Lys94. The root-mean-square (RMS) deviation between the two structures is ~0.7 Å for all C α atoms, and is ~0.5 Å if the aforementioned two regions were omitted in the superposition. In hGSTK residues Asn53–Pro60 form an exposed loop containing a short 3_{10} helix (Pro55–Leu59); the corresponding part in rGSTK forms a β -turn. In hGSTK, the residues Pro84–Lys94 form a β -turn (Pro84–Phe87) and an α -helix (α_4 , Asp86–Cys93); the corresponding region in rGSTK forms an irregular α -helix. Both regions show low-temperature factors in hGSTK but high temperature factors in rGSTK (Ladner et al. 2004).

Dimer interface

The two subunits of the hGSTK dimer are related by a twofold noncrystallographic symmetry (NCS) axis (Fig. 1A). Superposition of the two subunits yields an RMS deviation of 0.22 Å for all C α atoms, indicating that the two subunits are in general identical. The dimer interface buries 1386 Å² solvent-accessible surface area of

Table 1. Statistics of diffraction data and structure refinement

	Native (form A)	Native (form B)	Hg (OAc) ₂ Derivative (form B)	K ₂ Pt(CN) ₄ Derivative (form B)
Diffraction data statistics				
Space group	<i>C</i> 2	<i>P</i> 2 ₁ 2 ₁ 2	<i>P</i> 2 ₁ 2 ₁ 2	<i>P</i> 2 ₁ 2 ₁ 2
Unit cell parameters				
<i>a</i> (Å)	98.28	224.86	224.16	224.35
<i>b</i> (Å)	118.27	88.16	88.03	88.10
<i>c</i> (Å)	52.59	53.95	53.87	53.88
β (°)	102.67			
No. of monomers/asymmetric unit	2	4	4	4
Resolution (Å)	1.86	2.40 ^a	2.30	2.80
No. of unique reflections	48,426	42,746	46,959	25,931
<i>R</i> _{merge} (%) ^b	4.7 (38.3) ^c	19.4 (25.5) ^a	10.9 (23.8) ^c	11.9 (22.6) ^c
Completeness (%)	99.3 (93.9) ^c	99.0 (99.9) ^a	96.9 (83.7) ^c	95.9 (99.1) ^c
$\langle I/\sigma(I) \rangle$	6.9 (1.3) ^c	2.0 (1.9) ^a	5.4 (2.1) ^c	5.6 (2.9) ^c
ΔF_{iso} (%) ^d			14.9	9.1
ΔF_{ano} (%) ^e			4.3	3.4
Refinement statistics				
No. of amino acid residues	436			
No. of GSF	2			
No. of solvent molecules	179			
<i>R</i> factor (%) ^f	17.9			
Free <i>R</i> factor (%)	20.3			
Rms deviation				
Bond length (Å)	0.005			
Bond angles (°)	1.1			
Mean temperature factors (Å ²)				
Main-chain atoms	35			
Side-chain atoms	39			
GSF	25			
Solvent	44			
Luzzati atomic positional error (Å)	0.2			

^a For native crystal form B, X-ray data were collected to 2.4 Å resolution, but the data in the resolution range higher than 3.0 Å were not used. The numbers in parentheses correspond to the data in the resolution shell 3.02–3.25 Å.

^b $R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of the i th observation of reflection hkl and $\langle I(hkl) \rangle$ is the mean intensity for reflection hkl from multiple measurements.

^c The numbers in parentheses correspond to the data in the highest resolution shell (1.86–1.93 Å, 2.30–2.38 Å, and 2.80–2.90 Å for native crystal from A, Hg derivative, and Pt derivative, respectively). For native crystal form A, the data in the shell (1.86–1.93 Å) were not used for refinement, and $\langle I/\sigma(I) \rangle$ in the shell (1.93–2.01 Å) is 1.8.

^d Isomorphous difference ratios between the heavy-atom derivatives (*Fph*) and the native (*Fp*) were calculated at 3 Å resolution. $\Delta F_{\text{iso}} = \langle ||Fph| - |Fp|| \rangle / \langle (|Fph| + |Fp|)/2 \rangle$.

^e Anomalous difference ratios of the heavy-atom derivatives were calculated at 3 Å resolution. $\Delta F_{\text{ano}} = \langle ||Fph(+)| - |Fph(-)|| \rangle / \langle (|Fph(+)| + |Fph(-)|)/2 \rangle$.

^f *R* factor = $\sum ||Fo| - |Fc|| / \sum |Fo|$.

each subunit, corresponding to 13% of the surface area of the monomer. The dimer interface is dominated by hydrophobic interactions between residues from domain I of one subunit and domain II of the other, similar to the other classes of GSTs. In addition, a total of 19 salt bridges and hydrogen bonds are formed across the subunit interface, and two NCS-related aspartic acids (Asp201) make stacking interactions with each other at the dimer interface.

Structure of the active site

The physiological substrate GSH is bound at a hydrophilic cleft, designated as the G-site. Similar to the

G-sites in other GSTs, the G-site in hGSTK is formed primarily by structural elements of the TRX-like domains from both subunits, including α 1, a loop connecting β 4 and α 10, and the two small linkers connecting the TRX-like domain and the α -helical domain. The G site is located in the dimer interface region and is shielded from solvent by the other subunit.

In the hGSTK structure there is strong electron density at the G-site of each subunit, which shows unambiguously that the bound substrate has an oxidized thiol group (Fig. 2A). In other words, the bound substrate is GSF, the sulfinated GSH. The GSF molecule is well ordered, with a mean B value of 25 Å² (Table 1), and

Figure 1. Overall structure of the dimeric hGSTK. Two GSF molecules are shown as red ball-and-stick models. (A) View perpendicular to the twofold NCS axis (thick arrow), showing the butterfly-like shape of the dimer. The α -helices and β -sheets are shown in green and in blue, respectively. (B) View showing the binding cleft of the H-site. Domains I and II are shown in green and in blue, respectively. These diagrams were prepared using the program SETOR (Evans 1993).

is bound in an extended fashion in the G-site. The two GSF molecules of a dimer face each other (Fig. 1A). The amide groups of the γ -glutamyl moieties of the two GSFs are 5.5 Å apart, which is the shortest distance between the two GSFs, and the carboxyl groups of the glycyl moieties point to each other with a distance of about 6–7 Å. This binding mode is similar to those observed in the structures of other GSTs in complexes with GSH or its analogs.

The bound GSF makes extensive hydrophilic (both hydrogen bonding and salt bridge) interactions with several residues nearby, which are conserved within the κ class. The γ -glutamyl moiety interacts with both the main chain and side chain of Ser200, the side chains of Asp201 and Arg202* (* denotes the residue of the adjacent subunit). The side chain of the glycyl moiety makes two direct interactions with the side chains of Asn53 and Lys62* and an indirect interaction with the side chain of Lys62 via a water molecule. The main-chain amide and carbonyl groups of the cysteinyl moiety form two hydrogen bonds with the main chain of Leu183. One oxygen atom of the sulfinoyl group of the oxidized cysteinyl moiety is hydrogen bonded with the side chain O γ atom of Ser16 and the main-chain amide group of Tyr18, and the other oxygen atom forms hydrogen bonds with two water molecules (Fig. 2B). The Ser16 O γ atom forms an additional hydrogen bond to the Ser19 O γ atom. The sulfur atom of GSF is 3.7 Å away from the Ser16 O γ atom, slightly beyond hydrogen-bonding distance.

Near the G-site there is an H-site for binding the hydrophobic substrate (Fig. 1B). In hGSTK, residues from α_3 , α_4 , and α_6 of domain II form the H-site, and several residues from α_1 (along with the β -turn preceding it), α_2 , and the loop connecting α_9 and β_3 also make certain contributions to the H-site. Compared with rGSTK (Ladner et al. 2004), most of the residues forming the H-site are highly conserved in hGSTK.

Discussion

Oxidation state of the GSH substrate

In the rGSTK structure the GSH substrate is in a reduced state containing a thiol group, and the sulfur atom of GSH makes a hydrogen bond (2.9 Å) with the O γ atom of Ser16 (Fig. 2B) (Ladner et al. 2004). Although the crystals of hGSTK were grown in the presence of GSH, the structure of hGSTK reveals that the bound substrate is GSF, an oxidized product of GSH. In order to accommodate the two additional oxygen atoms of GSF, both the side chain of Ser16 and the sulfur atom of GSF in the hGSTK structure are displaced apart by 0.6–0.7 Å, compared with those in the rGSTK structure. Consequently, the hydrogen bond between the sulfur atom of GSF and the O γ

Figure 2. Structure of the catalytic active G-site. (A) SIGMAA-weighted 2Fo-Fc electron density map (1 σ contour level) at the G-site. This diagram was prepared using the program TURBO-FRODO. (B) Hydrogen-bonding interactions of the sulfinoyl group of GSF with Ser16 of hGSTK (left), compared with those of the thiol group of GSH with Ser16 of rGSTK (right). Two water molecules bound to the sulfinoyl group of GSF in hGSTK structure are shown as spheres. Hydrogen bonds are shown as dotted lines. This diagram was prepared using the program SETOR.

atom of Ser16 is disrupted (3.7 Å). Instead, one oxygen atom of the sulfinio group of GSF forms two hydrogen bonds with the Ser16 O γ atom (2.6 Å) and the main-chain amide group of Tyr18 (3.0 Å). The other oxygen atom of the sulfinio group makes hydrogen bonds (2.9 Å) with two water molecules (Fig. 2B). The side chain of Ser16 also forms a hydrogen bond with the O γ atom of Ser19 (2.7 Å), which is also found in the rGSTK structure. This hydrogen-bonding network appears to stabilize the oxidized sulfinio group of GSF.

It was reported that the turnover of the S16A mutant of rGSTK toward its electrophilic substrate 1-chloro-2,4-dinitrobenzene (CDNB) was about 30-fold less efficient than the wild-type enzyme (Ladner et al. 2004), suggesting that Ser16 is essential for the catalysis. In the structure of rGSTK in complex with GSH, the hydroxyl group of Ser16 makes a hydrogen-bonding interaction with the sulfur atom of GSH, suggesting that it is likely protonated to stabilize the thiolate anion in the catalysis. In both the κ and θ classes of GSTs the catalytic Ser residue is strictly conserved, suggesting that these two classes might share a common catalytic mechanism. In most of the other classes of GSTs, including α , μ , and π classes, the catalytic residue is a tyrosine. In the ω class and bacterial β class of GSTs, a conserved cysteine forms a disulfide with the thiol group of GSH (Nishida et al. 1998; Board et al. 2000).

In the hGSTK structure, the bound substrate is in the oxidized state. The first possibility is that GSH is oxidized in the crystallization solution by oxygen in air, and the oxidized product GSF then binds the enzyme, acting as an inhibitor. The chemical reaction from RS $^-$ to RSO $_2^-$ follows a radical-mediated mechanism involving the RSOO $^{\cdot-}$ radical (Oae and Doi 1991).

Besides GSH-conjugating activity, GSTs can also serve as a peroxidase and is present in peroxisomes where oxygen free radicals, hydroxyl radicals, and hydrogen peroxides can be generated, as previously reported (Jowsey et al. 2003; Morel et al. 2004). The primary biological function of peroxidase enzymes is to oxidize a variety of hydrogen donors at the expense of peroxide or molecular oxygen (Forstrom et al. 1978). hGSTK exhibits low activity (0.173 ± 0.06 and $0.015 \pm 0.001 \mu\text{M} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$, respectively) of GSH peroxidase towards cumene hydroperoxide and t-butyl hydroperoxide, respectively (Morel et al. 2004). The kinetic study showed a linear dependence of rate with concentration of 1-palmitoyl-2-(13-hydroperoxy-*cis*-9, *trans*-11-octadecadienoyl)-L-3-phosphatidylcholine (PLPH-OOH) and a catalytic specificity value $K_{\text{cat}}/K_m(\text{PLPC-OOH})$ of 4.2 ($\mu\text{M}^{-1} \cdot \text{S}^{-1}$) for a recombinant α class GST (Hurst et al. 1998). The structure of hGSTK in complex with GSF suggests the second possibility; i.e., hGSTK catalyzes the oxidation of the substrate GSH by exerting its per-

oxidase activity, with oxygen in air as an oxidant, and GSF is the product of the enzymatic reaction, which is bound at the active center. However, the detailed mechanism is unclear.

The κ class of GSTs is closely related to the θ class enzyme

Although all classes of mammalian GSTs consist of a TRX-like domain and a helical domain, the secondary structure topology of the κ class GSTs differs substantially from those of the other classes of GSTs. In the other classes the two typical structural motifs ($\beta\alpha\beta$ motif and $\beta\beta\alpha$ motif) of the TRX-like domain are linked together by a long loop containing an α -helix (α_2), and the C-terminal helical domain consists of a varied number of α -helices (e.g., four α -helices in the π and the μ classes, five in the α class, and six in the θ class). The two domains are connected together by a short linker. However, in the κ class of GSTs the helical domain is inserted between the $\beta\alpha\beta$ and $\beta\beta\alpha$ motifs of the TRX-like domain and contains seven α -helices. The dimer of hGSTK adopts a butterfly-like shape with wide wings (Fig. 1A), while in the other classes there is a deep V-shape crevice in the intersubunit interface of the dimer, which does not exist in the κ class enzymes.

It was previously known that the entire sequence of hGSTK showed an absence of sequence homology with any other class of GST, and rGSTK was reported to have limited identity with the θ class at the N terminus (over residues 6–16) (Harris et al. 1991; Pemble et al. 1996; Morel et al. 2004). However, the sequence alignment of the TRX-like domain between hGSTK and various classes of GSTs indicates that the secondary structural elements of this domain can be aligned reasonably well with each other (Fig. 3A). This secondary structure-based alignment reveals three strictly conserved amino acid residues (Pro184, Asp191, and Gly192 in hGSTK) and four highly conserved residues (Leu10, Tyr12, Leu26, and Leu206 in hGSTK) among various classes of GSTs. Pro184 adopts a conserved *cis*-configuration in all known GST structures (Sheehan et al. 2001). This residue is located in the vicinity of the G site (the shortest distances from Pro184 to both GSF and Ser16 are ~ 4.5 Å in the hGSTK structure) and right after Leu183, the main chain of which forms a pair of hydrogen bonds with the backbone of the cysteinyl moiety of GSF (Fig. 2A). These hydrogen-bonding interactions are conserved in the structures of all GSTs in complexes with GSH or its analogs. It seems likely that the *cis*-configuration of Pro184 is required for maintaining Leu183 at a position favorable for binding the GSH substrate.

Figure 3. Comparison of the TRX-like domain of hGSTK with other GSTs. (A) Sequence alignment of the TRX-like domains between hGSTK and the representatives of other classes of GSTs. The GSTs used in comparison are human θ GST (PDB entry code 1LJR), human α GST (1K3L), human μ GST (1HNA), human π GST (5GSS), and squid sigma GST (1GSQ). The residue numbers of hGSTK are shown on top. Residues strictly conserved in all classes are shown in red, and the residues identical to and conserved with those of hGSTK in blue and orange, respectively. (B) The C α atom superposition of the TRX-like domains of hGSTK (Arg6–Ser42 and Pro184–Gly211 in green) and human θ class GST (Leu3–Val33 and Pro55–Gln79 in pink). This diagram was prepared using the program SETOR.

Moreover, the structure-based alignment of the TRX-like domain indicates that this domain of hGSTK shares 15 identical residues (19%) and additional eight conserved substitutions (10%) with that of human θ class GST (hGSTT), while the identical and conserved residues together are in the range of 15%–19% between hGSTK and the GSTs in any other class (α , μ , π , and σ) (Fig. 3A). Superposition of the TRX-like domains of other classes of GSTs with hGSTK shows that they share structure similarity in this domain except for α_2 , yielding an RMS deviation of 1.8 Å between κ and θ classes for 56 corresponding C α atoms (Fig. 3B) and of 2.0–2.1 Å between κ and either of α , μ , π , and σ classes for 55–57 C α atoms (the calculation is always limited to the $\beta\alpha\beta$ and $\beta\beta\alpha$ motifs in this paper). The conformation of the segment Asp13–Trp20 of hGSTK where the catalytic residue Ser16 is

located is similar to the corresponding region of the θ class and very different from those of the other classes. In addition, both κ and θ classes of GSTs lack some common features found in other classes of GSTs, such as the pronounced V-shape crevice and a “key-and-lock motif” in three-dimensional structures (Sheehan et al. 2001). Both κ and θ classes have a serine residue as the catalytic active site. The enzymatic activity of the κ class GSTs is limited to conjugation with CDNB and ethacrynic acid (Harris et al. 1991), which is also similar to the θ class GSTs. Based on the structural comparison of rGSTK with members of the canonical GST superfamily, Ladner and coworkers (Ladner et al. 2004) proposed that the protein folds of GSTs diverge from a common thioredoxin/glutaredoxin progenitor via parallel evolutionary pathways with a domain insertion for the former and a domain addition

for the latter. Our results further suggest that the κ class is more closely related to the θ class than to the other classes within the GST superfamily.

rGSTK was reported to be more closely related to DsbA, a disulfide bond protein, than to other classes of GSTs (Ladner et al. 2004). The folding topology of DsbA is similar to κ class GSTs, with a TRX-like domain interrupted by a helical domain containing six helices. When the entire sequence of hGSTK is aligned with that of DsbA, $\alpha 2$ – $\alpha 4$ of hGSTK corresponds to a 44-residue deletion in DsbA, and the two complete sequences share 10% identity and 6% conservative changes, approximately half in each domain, indicating that the sequence homology between hGSTK and DsbA is higher for the entire sequence and lower for the TRX-like domain, compared with those between hGSTK and any other class of GSTs. The superposition of the TRX-like domain between hGSTK and DsbA gives an RMS deviation of 2.3 Å for 65 corresponding C α atoms, larger than those between different classes of GSTs.

Structure comparison of hGSTK with GPx

Although hGSTK shows low activity of a peroxidase, it differs from GPx, which belongs to the selenoprotein family and functions to catalyze the reduction of hydroperoxides using GSH as a reducing substrate (Forstrom et al. 1978). GPx is a tetrameric enzyme, and its subunit structure contains a TRX-like fold and the catalytic site is a selenocysteine (Ren et al. 1997). The two types of enzyme, GST and GPx, share no sequence homology, and are dissimilar in overall structure of the entire subunit, but they exhibit similarity in the thioredoxin-like fold structure except for $\alpha 2$, with an RMS deviation of 1.6 Å for 65 C α atoms when hGSTK is superimposed with bovine erythrocyte GPx (Epp et al. 1983). The relative positions of the catalytic residues in the TRX-like folds in the two enzymes (Ser16 in hGSTK and a selenocysteine in the GPx) are well conserved, which accounts for the recently reported results that incorporation of selenocysteine into GSH-specific binding scaffold using auxotrophic expression system converted the *Lucilia cuprina* GST to a selenium-containing enzyme that displayed the GPx activity comparable with that of natural GPx, which provided a proof that GST and GPx were evolved from a common “glutathione-binding protein” ancestor (Yu et al. 2005).

Materials and methods

Protein expression and purification

The cDNA corresponding to hGSTK was obtained from the cDNA library of human CD34+ hematopoietic stem/

progenitor cells (Zhang et al. 2000). The hGSTK gene was cloned into the NdeI and XhoI restriction sites of the pET-22b(+) expression plasmid (Novagen) and fused with a hexahistidine tag at the C terminus. The plasmid was transformed into and expressed in *Escherichia coli* BL21(DE3) strain (Novagen). The cells transformed with the vector were grown overnight at 310 K in 100 mL of LB medium containing ampicillin (0.1 mg/mL). The culture was added to 2 L of LB medium and incubated until OD₆₀₀ reached 0.7. After 3 h of expression induced with 0.4 mM isopropyl- β -D-thiogalactopyranoside (IPTG) at 303 K, the cells were collected by centrifugation at 4000g and suspended in 40 mL of lysis buffer (pH 7.4) containing 50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, 1 mM EDTA, 5 mM β -mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride. The cells were lysed on ice by sonication and the cell debris was precipitated by centrifugation at 15,000g.

The hGSTK protein was purified by affinity chromatography using a nickel-nitrilotriacetic acid-agarose column (Qia-gen). The lysis extract was loaded on the column and then washed with a washing buffer (50 mM NaH₂PO₄, 300 mM NaCl, 50 mM imidazole [pH 7.4]) to elute nonspecific binding proteins. The target protein was eluted with an elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, 500 mM imidazole [pH 7.4]). The fractions containing the hGSTK protein were pooled together and dialyzed extensively against a storage buffer containing 20 mM NaH₂PO₄ (pH 7.2), 20 mM NaCl, 1 mM EDTA, and 1 mM DTT. Reducing SDS-PAGE analysis of the purified protein showed a single band at 26 kDa. Dynamic light scattering analysis indicated that the protein was a homogeneously dispersed homodimer in both the presence and absence of a substrate (data not shown). The purified protein was further concentrated to about 40 mg/mL in the storage buffer for crystallization experiments. All purification steps were carried out at 277 K.

Crystallization and diffraction data collection

Two forms of hGSTK crystals were grown by the hanging drop vapor diffusion method. Crystals of form A grew at 293 K: A 2 μ L protein solution at a concentration of 40 mg/mL (20 mM NaH₂PO₄ [pH 7.2], 20 mM NaCl, 1 mM EDTA, 1 mM DTT, and 1 mM GSH) was mixed with 2 μ L reservoir solution containing 10% PEG8000, 10% PEG1000, and 3% glucose. Crystals of form B grew at 277 K: A 2 μ L protein solution at a concentration of 10 mg/mL was mixed with 2 μ L reservoir solution containing 0.1 M MES (pH 6.0), 10% dioxane, 1.6 M (NH₄)₂SO₄, and 0.01 M trimethylamine.

Native X-ray diffraction data of both crystal forms were collected using a MarCCD detector at Beijing Synchrotron Radiation Facility, to 1.86 Å resolution at 293 K and to 2.4 Å resolution at 100 K for crystals of form A and form B, respectively, and the latter data in the resolution range higher than 3.0 Å were not used because of anisotropy of diffraction. The data were processed using the program AUTOMAR (Klein and Bartels 2000).

To determine the initial phases, two heavy-atom derivatives were prepared by soaking the native crystals of form B in Hg(OAc)₂ and K₂Pt(CN)₄ (0.1 M for 9 h), respectively. The derivative data were collected at 100 K using an in-house Rigaku R-Axis IV++ image-plate detector. Diffraction data processing was performed using the CrystalClear package (Pflugrath 1999). Structure factors of the derivative data were subsequently scaled together with the native data (crystal form B) using the CCP4 program suite (Collaborative Computational Project 1994).

The crystal data and the data collection statistics are summarized in Table 1.

Structure determination and refinement

The initial phases of crystal form B were solved by MIR using the program SOLVE (Terwilliger and Berendzen 1999). The Patterson functions of the two heavy-atom derivatives at 3 Å resolution revealed eight Hg atoms and one Pt atom, yielding an overall Z-score of 21.5 and a mean figure of merit (FOM) of 0.41. The initial phases were further improved by statistical density modification using the program RESOLVE, yielding an overall FOM of 0.75 (Terwilliger 2002). The RESOLVE program automatically built 673 polyaniline residues out of 904 (226×4) residues in an asymmetric unit and successfully located most of the secondary structural elements. Chain was easily traced, and an initial model was built for one of the four subunits of crystal form B using program O (Jones et al. 1991) and TURBO-FRODO (Roussel and Cambillau 1991). This initial model was then used as the search model to solve the phases of crystal form A by molecular replacement using program AmoRe (Navaza 1994), which produced two outstanding peaks, corresponding to the two subunits in an asymmetric unit of crystal form A.

Crystallographic refinement was performed using the program CNS (Brünger et al. 1998) against the native data of crystal form A, since the crystal in this form diffracts better and contains less molecules in an asymmetric unit than crystal form B. A bulk solvent correction was applied throughout the refinement. Manual model building was carried out using TURBO-FRODO based on SIGMAA-weighted difference Fourier maps (2Fo–Fc and Fo–Fc) and composite omit maps. NCS restraints were imposed during the course of refinement up to 2.2 Å resolution, and were released in the later stage of refinement. In the initial difference Fourier maps there was strong residual electron density at the G-site, which was unambiguously interpreted to be GSF. Water molecules were included in the structure model in the late stage of refinement.

Coordinates

The atomic coordinates of the human κ class glutathione transferase have been deposited with the Protein Data Bank under accession code 1YZX.

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