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Stability of the domain interface contributes towards the catalytic function at the H-site of class alpha glutathione transferase A1-1

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ABSTRACT

Cytosolic glutathione transferases (GSTs) are major detoxification enzymes in aerobes. Each subunit has two distinct domains and an active site consisting of a G-site for binding GSH and an H-site for an electrophilic substrate. While the active site is located at the domain interface, the role of the stability of this interface in the catalytic function of GSTs is poorly understood. Domain 1 of class alpha GSTs has a conserved tryptophan (Trp21) in helix 1 that forms a major interdomain contact with helices 6 and 8 in domain 2. Replacing Trp21 with an alanine is structurally non-disruptive but creates a cavity between helices 1, 6 and 8 thus reducing the packing density and van der Waals contacts at the domain interface. This results in destabilization of the protein and a marked reduction in catalytic activity. While functionality at the G-site is not adversely affected by the W21A mutation, the H-site becomes more accessible to solvent and less favorable for the electrophilic substrate 1-chloro-2,4-dinitrobenzene (CDNB). Not only does the mutation result in a reduction in the energy for stabilizing the transition state formed in the S_NAr reaction between the substrates GSH and CDNB, it also compromises the ability of the enzyme to form and stabilize a transition state analogue (Meisenheimer complex) formed between GSH and 1,3,5-trinitrobenzene (TNB). The study demonstrates that the stability of the domain–domain interface plays a role in mediating the catalytic functionality of the active site, particularly the H-site, of class alpha GSTs.

1. Introduction

The cytosolic glutathione transferases (GSTs; EC 2.5.1.18) are a large superfamily of homo- and hetero-dimeric proteins that provide defense mechanisms in aerobes against the harmful effects of toxic compounds and oxidative stress [1,2]. Although the canonical GSTs are grouped into various gene classes (alpha, mu, pi, etc.), they share a common fold with each subunit consisting of two structurally distinct domains; a thioredoxin-like domain 1 and an all α-helical domain 2, as shown in Fig. 1 for class alpha. Each subunit has an active site that consists of two adjacent regions, a conserved and highly specific G-site for binding the thiol substrate glutathione (GSH), and a nonpolar H-site that can accommodate a wide spectrum of structurally diverse electrophilic substrates.

The dimeric structures of GSTs together with their conformational stability and dynamic behavior have significant implications for function. A GST dimer is critical for catalysis since both subunits contribute towards the binding of GSH on each subunit [3] and it is a requirement for activity [4]. While the contributions of quaternary interactions between the subunits towards maintaining the functional conformation of the active site [5–11], the cooperative behavior between subunits [9–12], as well as towards the conformational stability [13–23] and conformational dynamics [24,25] of the individual subunits have been studied, the role of the stability of and dynamics at the domain–domain interface in the catalytic functions of GSTs is poorly understood. The interface between the domains is extensive and predominantly hydrophobic. Each active site is located at the domain interface and while the G-site is predominantly constructed from residues in domain 1, the topographically larger H-site involves elements from both domains thus implicating the domain interface in its functionality (Fig. 1). Interactions at the domain interface play an important role in maintaining the stability of GST subunits and dimers [26], and it was recently proposed that the level of functional promiscuity displayed by class alpha GSTs corresponds to the conformational flexibility of their domain interfaces [27].

At least six types of class alpha subunits (A1 to A6) have been identified and they possess a topologically conserved Trp21 in helix 1 of domain 1 that forms a major interdomain contact by interacting with a hydrophobic pocket between helices 6 and 8 in domain 2

Abbreviations: ANS, 8-anilino-1-naphthalene sulfonate; CD, circular dichroism; CDNB, 1-chloro-2,4-dinitrobenzene; EA, ethacrynic acid; GSH, glutathione; GSO_3^-, glutathione sulfonate; GST, glutathione transferase; G- and H-sites, glutathione and hydrophobic substrate binding sites; hGSTA1-1, human glutathione transferase class alpha with two type-1 subunits; TNB, 1,3,5-trinitrobenzene

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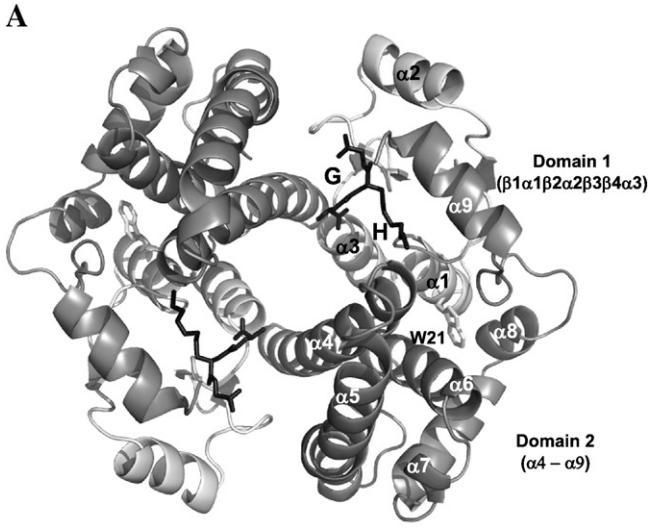

Fig. 1. Structural data of class alpha GSTs. A, ribbon diagram of wild-type hGSTA1-1 (PDB code 1K3L) viewed down the two-fold axis. Trp21 and *S*-hexylglutathione are shown as light and dark grey stick models, respectively. G and H indicate the locations of the G- and H-sites of the active site, respectively. B, aligned sequences of helix 1 in different class alpha subunits types. The conserved tryptophan (Trp21) forming a domain–domain interaction is highlighted in grey and the accession codes for the sequences are indicated.

(Fig. 1). In this study, we investigated the role that this interdomain motif plays in the structure, stability and catalytic function of hGSTA1-1 by replacing its Trp21 with an alanine, and determining the effects of the mutation by spectroscopic, crystallographic, enzyme kinetics and ligand binding methods. While the W21A mutation does not adversely impact the structure of the enzyme, the findings from the study demonstrate that the stability of the domain–domain interface plays a role in mediating the catalytic functionality of the active site, particularly the H-site, of class alpha GSTs.

2. Experimental procedures

2.1. Chemicals

GSH was from ICE Biomedical Inc. (Aurora, Ohio, USA). TFE (99+% grade), 8-anilino-1-naphthalene sulfonate, ethacrynic acid, glutathione sulfonate and 1-chloro-2,4-dinitrobenzene (CDNB) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other reagents were of analytical grade.

2.2. Mutagenesis, protein expression and purification

The plasmid (pKHA1) used for the expression of wild-type hGST A1-1 [28] was a generous gift from Bengt Mannervik (Department of Biochemistry, University of Uppsala, Sweden). Oligonucleotide site-directed mutagenesis, based on the Stratagene Ex-Site mutagenesis method [29], was used to generate the W21A mutant of hGSTA1-1 [30] and DNA sequencing (Inqaba Biotechnical Industries (Pty) Ltd., Pretoria, South Africa) confirmed the presence of the mutation in the plasmid. Wild-type and W21A hGSTA1-1 were expressed in BL21 (DE3) pLysS *Escherichia coli* cells using 1 mM IPTG and purified by CM-Sepharose chromatography [31]. Both proteins were dialyzed against 20 mM sodium phosphate buffer, pH 6.5, containing 1 mM EDTA and 0.02% sodium azide. The concentration of the dimeric proteins was determined at 280 nm using the molar extinction coefficient of 38200 $M^{-1}\,cm^{-1}$ for the wild-type and 27100 $M^{-1}\,cm^{-1}$ for the mutant.

2.3. Spectroscopic methods

Far-UV CD measurements were done at 20 °C in a Jasco model 810 CD spectropolarimeter. Averaged CD signals, corrected for solvent, were converted to mean residue ellipticity [θ] ($^{\circ}cm^2\,dmol^{-1}$),

$$[\theta] = \frac{100 \times \theta}{Cnl}$$

where C is the peptide concentration in millimolar, θ is the measured ellipticity in millidegree, n is the number of residues (17 in this case), and l is the path length (cm). Thermal unfolding was followed by CD at 222 nm from 20 to 80 °C.

Fluorescence measurements were performed at 20 °C in a Hitachi model 850 fluorescence spectrofluorimeter. The intrinsic fluorescence of hGSTA1-1 was measured using an excitation wavelength of 280 nm. The binding of the amphipathic dye ANS to hGSTA1-1 was monitored by fluorescence enhancement using an excitation wavelength of 390 nm [32].

2.4. Protein crystallization, X-ray diffraction and structure solution

Crystals of recombinant W21A-hGSTA1-1 were grown by the hanging drop vapour diffusion method at 293 K using a 24-well microplate. The stock protein concentration was 10 mg/ml in 0.1 M Tris–HCl, pH 7.5, containing 10 mM DTT, 2.5 mM *S*-hexylglutathione, and 0.02% sodium azide. The reservoir buffer was PEG 4000 (18%; w/v) in 0.1 M Tris–HCl, pH 7.5, 10 mM DTT and 0.02% sodium azide. Each hanging drop (4, 6 and 8 μl) comprised of an equal volume of protein stock solution and reservoir buffer. The crystals were harvested, briefly soaked in the reservoir buffer and mounted on a cryo-loop. X-ray diffraction data were collected on a Bruker X8 Proteum system with a Microstar copper rotating-anode generator with Montel 200 optics, a PLATINUM 135 CCD detector, and an Oxford Cryostream Plus system. Crystals were cooled in a stream of nitrogen to 113 K during data collection and images were collected covering an oscillation angle of 0.5° per image. The data sets were processed using APEX and SAINT software (Bruker AXS Inc., Madison, WI, USA).

The structure of W21A-hGSTA1-1 was solved by molecular replacement using PHASER [33], as implemented in the CCP4 suite of programs [34] using wild-type hGSTA1-1 (PDB entry 1K3L; [35]) as the search model. Model refinement was done with REFMAC5 [36] and model building performed with COOT [37]. Solvent molecules were added using COOT [37]. The data collection and refinement statistics are given in Table 1. Stereochemical validation of the model was performed using PROCHECK [38] and MOLPROBITY [39]. PyMol (DeLano Scientific LLC, San Carlos, CA) was used to generate images of the structures.

2.5. Steady-state kinetics

Kinetic parameters were determined at pH 6.5 and 20 °C for the *S*-conjugation reaction between GSH and CDNB using 1–9 nM enzyme [40]. Reaction rates were corrected for non-enzymatic rates. Conditions for determining K_m^{GSH} were 0.05–10 mM GSH and 2 mM CDNB, and for K_m^{CDNB} they were 0.05–2 mM CDNB and 3 mM GSH. For k_{cat}/K_m^{GSH} determinations, CDNB was kept at 2 mM while GSH was varied from 0.02 to 0.1 mM. For k_{cat}/K_m^{CDNB} determinations, GSH was kept at 3 mM while CDNB was varied from 0.01 to 0.2 mM. The data were