Human Glutathione Transferase T2-2 Discloses Some Evolutionary Strategies for Optimization of Substrate Binding to the Active Site of Glutathione Transferases

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Running title: GSH binding to human GSTT2-2

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Rapid kinetic, spectroscopic and potentiometric studies have been performed on human Theta class glutathione transferase T2-2 to dissect the mechanism of interaction of this enzyme with its natural substrate GSH. Theta class glutathione transferases are considered to be older than Alpha, Pi and Mu classes in the evolutionary pathway. Like in the more recently evolved GSTs, the activation of GSH in the human Theta enzyme proceeds by a forced deprotonation of the sulfhydryl group (pKₐ = 6.1). The thiol proton is released quantitatively in solution but, above pH 6.5, a protein residue acts as an internal base. Unlike Alpha, Mu and Pi class isoenzymes, the GSH binding mechanism occurs via a simple bimolecular reaction with $k_{on}$ and $k_{off}$ values at least hundred times lower ($k_{on}$ = $(2.7 \pm 0.8) \times 10^4$ M⁻¹ s⁻¹, $k_{off}$ = $36 \pm 9$ s⁻¹, at 37°C). Replacement of Arg107 by alanine, using site-directed mutagenesis, remarkably increases the pKₐ value of the bound GSH and modifies the substrate binding modality. Y107A mutant enzyme displays a mechanism and rate constants for GSH binding approaching those of Alpha, Mu and Pi isoenzymes. Comparison of available crystallographic data for all these GSTs reveals an unexpected evolutionary trend in terms of flexibility which provides a basis for understanding our experimental results.
INTRODUCTION

The human cytosolic glutathione transferases (GSTs, EC 2.5.1.18) are dimeric proteins grouped into at least four gene independent classes, named Alpha, Mu, Pi and Theta which differ in amino acid sequence, co-substrate specificity and antibody cross-reactivity (for reviews, see refs 1, 2). Despite an inter-class sequence identity of less than 25% between Alpha, Mu and Pi class enzymes and less than 10% to the Theta class GSTs, the three-dimensional fold of these isoenzymes is very similar (3-6). Notable differences for the human Theta enzyme T2-2 are a small and buried active site for GSH and an extra C-terminal extension of about forty residues not found in the other classes (6). Recently a fifth class has been discovered in humans, named Zeta class, showing a serine residue in the active site and high activity towards organic hydroperoxides (7). From an evolutionary point of view, it has been proposed that Alpha, Mu and Pi class GSTs originated from the Theta GST by gene duplication (8). In turn, on the basis of sequence identity at the N-terminus, the Theta GST should might have arisen from the ancestral mitochondrial GST Kappa (9). Alternatively, the Theta class may be only older than Alpha, Pi and Mu GSTs and have all diverged from a common ancestor (7). Recently, we have found that human Alpha, Mu and Pi class GSTs bind GSH by adopting a very similar multi-step mechanism in which the final Michaelis complex is achieved after the formation of a weak pre-complex (10, 11). Does such a mechanism hold for the primitive GSTT2-2? Furthermore, all GSTs activate the substrate by lowering the $pK_a$ value of GSH at the active site, but the peculiar sulfatase reaction catalyzed by hGSTT2-2 could not need the thiolate form of GSH (12). Interestingly, in this old enzyme, Ser11
replaces the Tyr residue found in Alpha, Pi and Mu class GSTs. This aromatic residue contacts the sulfhydryl group of GSH and stabilizes its ionized form (1). Recently, Jemth and Mannervik (13, 14) described some kinetic and binding properties of the rat Theta class GST. They obtained indirect kinetic indications for a forced deprotonation of GSH at the active site, and also proposed Ser11 as the crucial residue involved in this activation. We report here, for the human GSTT2-2, a direct evidence for the ionization of GSH at the active site but, we point out the crucial role of Arg107 in the binding and activation of the substrate. This residue contacts the thiol sulfur of GSH, either directly or through a water molecule (6); replacement of Arg107 by Ala not only alters the pKₐ value of the bound GSH but even changes the binding mechanism of this enzyme. Interestingly, some of the binding properties of the old enzyme seem to be related to the rigidity of selected regions of the protein, indicating a possible evolutionary target in terms of flexibility for the GST superfamily.
EXPERIMENTAL PROCEDURES

Reagents and enzyme preparation - GSH and S-hexylglutathione were Sigma products; sodium 1-menaphthyl sulfate (Msu) was prepared as described by Clapp & Young (15). His tagged recombinant GST2-2 and R107A mutant were expressed in *Escherichia coli* and purified using immobilized metal ion chromatography on a Ni-NTA matrix (Qiagen) as previously described (12, 16).

**GSH binding experiments** - The intrinsic fluorescence of hGSTT2-2 was measured in a single-photon-counting spectrofluorometer (Fluoromax, S.A. Instrument, Paris, France) with a sample holder at 25 °C. Excitation was at 280 nm and emission was at 335 nm. In a typical experiment, fluorescence intensity was measured before and after the addition of suitable amounts of GSH (from 0.02 to 8 mM) to 1.5 µM hGSTT2-2 in 0.1 M potassium phosphate buffer pH 7.0. Experimental data were corrected both for dilution and for inner filter effects and fitted to:

\[
F_L = F_o + (F_{max} - F_o)/(1 + ([S]^{1/2})/([S]^{nH}))
\]

(Eq. 1)

where: \(F_o\) is the protein fluorescence in the absence of GSH, \(F_L\) is the protein fluorescence in the presence of a given amount of GSH, \(F_{max}\) is the protein fluorescence at saturating GSH concentrations and \(nH\) is the Hill coefficient.

**Spectroscopic evidence for GSH ionization** - Difference spectra of GSH thiolate bound to both native and the R107A mutant of hGSTT2-2 were obtained with a Kontron double-beam Uvikon 940 spectrophotometer thermostated at 25 °C. In a typical experiment 1
mM GSH was added to the enzyme (15 µM active sites) in a suitable buffer. From the resulting spectrum, the contributions from free GSH and free enzyme were subtracted. The amount of thiolate was obtained by assuming an ε_{240nm} of 5,000 M⁻¹ cm⁻¹. The pH dependence of the GSH ionization was obtained with the following buffers (0.01M): sodium acetate buffer pH 5.5 and potassium phosphate buffers between pH 6.0 and pH 8.0. pKₐ values were obtained by fitting the data to:

\[ y = \frac{y_{\text{lim}}}{1 + 10^{(pK_a-pH)}} \]  \hspace{1cm} (Eq. 2)

**Potentiometric experiments** - Thiol proton extrusion was detected at 25 °C as reported previously (10). In a typical experiment, performed under a N₂ atmosphere, a GSH solution (10 mM in 0.1M NaCl) was titrated to a fixed pH with 0.1 M NaOH and mixed with the same volume of GSTT2-2 (4 mg/ml in 0.1 M NaCl) at exactly the same pH. Quantitation of the released proton was obtained by suitable addition of 10 mM NaOH up to the initial pH value. A blank was performed at each pH, by replacing GSH with S-hexylglutathione (4 mM in 0.1M NaCl). pKₐ values were obtained by fitting the data to:

\[ y = y_{\text{lim}}(10^{pK_a1}x10^{pH})/(10^{pK_a1}x10^{-pK_a2} + (10^{pK_a1}x10^{pH})+10^{-2pH}) \]  \hspace{1cm} (Eq. 3)

**Stopped-flow experiments** - Rapid kinetic experiments were performed on an Applied Photophysics Kinetic spectrometer stopped-flow instrument equipped with a thermostated 1 cm light path observation chamber. Kinetics of the binding of GSH to wild-type and R107A mutant were studied at 37 °C, by rapid mixing of enzyme (95 µM) and different amounts of GSH (from 0.25 to 12 mM) in carbonate-phosphate-acetate
(50:50:50 µM) buffer pH 7.0. Binding of GSH was monitored by following the increase of the intrinsic fluorescence of the protein. Experimental traces were fitted to a single-exponential decay and pseudo first-order kinetic constants were calculated at different GSH concentrations.

**Binding of GSH as function of pH** - Binding of GSH as function of pH was performed at 37 °C. Wild-type or R107A mutant (95 µM) in carbonate-phosphate-acetate (50:50:50 µM) buffer were rapidly mixed with the same volume of GSH (2.0 mM) dissolved in the same buffer. Experiments were done at different pH values between pH 5.0 and pH 9.0. Binding of GSH was monitored by following the increase of the intrinsic fluorescence of the protein. Experimental traces were fitted to a single-exponential decay and pseudo first-order kinetic constants calculated.
RESULTS

Binding of GSH to hGSTT2-2 - Isothermic binding of GSH to the G-site of hGSTT2-2 has been studied at 25 °C by using the perturbation of the intrinsic fluorescence of the protein upon addition of different GSH concentrations. In the native enzyme, the interaction of the substrate with the active site causes an increase of the tryptophan fluorescence and this is a peculiar finding as other GST isoenzymes display a fluorescence quenching on GSH binding. Binding of GSH is hyperbolic \( (n_H = 1.0) \) with an \( [S]_{0.5} = K_D \) value of \( 0.8 \pm 0.2 \) mM (Fig. 1A). This value is at least four times higher than those found for the more recently evolved Alpha, Pi and Mu class GSTs. This poor affinity for GSH is also reflected by the lack of interaction of this GST with the classical GSH-affinity matrix (12).

Evidence of thiolate formation in hGSTT2-2 and in R107A mutant - Direct demonstration of GSH thiolate formation at the active sites of hGSTT2-2 and of R107A mutant and their dependence on pH have been obtained by differential UV spectroscopy. Fig 1B inset shows a typical thiolate absorption band centered at 240 nm obtained at pH 7.5 by the differential UV spectrum of hGSTT2-2 in the presence of non-saturating GSH concentration (1 mM). Similar thiolate band is obtained with the R107A mutant. Higher GSH concentrations cannot be used because of the large spectral contribution due to the spontaneous ionization of GSH at alkaline pH values. By assuming an \( \varepsilon_{240\text{nm}} \) of 5,000 M\(^{-1}\) cm\(^{-1}\) for the ionized GSH, the limiting value at alkaline pH is 0.48 GS\(^-\) equivalents per hGSTT2-2 active sites when the active site occupancy is about 55 \%. The dependence, at pH 7.0, of the GSH thiolate band at 240 nm on GSH concentration (from 0.1 to 1 mM),
follows an hyperbolic behaviour which overlaps the GSH binding experiments (see Fig 1A) (data not shown). It follows that about 0.9 GSH/active sites are formed in the native enzyme at 25 °C under saturating substrate. The pH dependence of the spectral perturbation at 240 nm gives an apparent pK\textsubscript{a} value for the bound GSH of 6.15 ± 0.10 (Fig 1B), close to the pK\textsubscript{a} values found for Alpha, Mu and Pi class GSTs (10, 11). Mutation of Arg107 remarkably decreases the deprotonation of the substrate and a pK\textsubscript{a} = 7.8 ± 0.2 for the thiol group of the bound GSH is now obtained (Fig 1B), a value 1.6 pH units higher than that calculated for the native enzyme.

*Proton release of upon GSH binding to hGSTT2-2* - The fate of the proton produced from the GSH ionization has been investigated in hGSTT2-2 by a potentiometric approach as described under the “Experimental Procedures” section. In a typical experiment (pH 6.5), a nearly saturating GSH solution (5 mM final concentration) was mixed with hGSTT2-2 (73 µM final concentration), both solutions in the absence of any buffer and titrated to the same pH value. After mixing, a rapid decrease of pH was observed. No pH changes have been found when the GSH analogue S-hexylglutathione replaces GSH. These experiments show that in hGSTT2-2, protons are released upon GSH binding and that they come from the sulfhydryl group of GSH. Back-titration with dilute NaOH allows an estimation of the amount of the released proton. The pH dependence of this event shows a bell-shaped trend (Fig. 2). It is evident that under alkaline conditions at least one protein residue acts as an internal base for proton neutralization. By fixing the pK\textsubscript{a1} = 6.15 (the value obtained for GSH ionization by differential UV spectroscopy), the best fit of the experimental data gives an apparent pK\textsubscript{a2} of 7.32 ± 0.03 for the unknown protein base.
Kinetics of GSH binding to hGSTT2-2 and to R107A mutant - Kinetics of GSH binding was studied at pH 7.0 and 37 °C, by following the increase of protein fluorescence in the milliseconds time-scale by a stopped-flow apparatus. The experimental traces, obtained after rapid mixing of enzyme with increasing GSH concentrations, were well described by single-exponential curves from which the apparent first order rate constants ($k_{obs}$) have been calculated. The native enzyme shows $k_{obs}$ values (65 s$^{-1}$ at 1 mM GSH and 37°C) at least ten times lower than those found for the Alpha, Mu and Pi class GSTs at the same GSH concentration, but at 5 °C (10, 11). Furthermore, unlike that observed in the more recently evolved GSTs, $k_{obs}$ values for hGSTT2-2 follow a linear dependence on GSH concentration within the large range of 0.12 - 6 mM (Fig 3A). These data are well described by Scheme I which shows the binding event as a simple bimolecular interaction to give the Michaelis complex E-GSH.

\[
\begin{align*}
E + GSH & \rightleftharpoons E-GSH \\
& \text{Scheme I}
\end{align*}
\]

Linear regression analysis gives a $k_{on} = (2.7 \pm 0.8) \times 10^4$ M$^{-1}$ s$^{-1}$ and a $k_{off} = 36 \pm 9$ s$^{-1}$. Thus, a dissociation constant ($K_d$) of about 1.3 mM for the E-GSH binary complex formation has been calculated at 37 °C (Fig 3A and Table I). As $k_{on}$ is far from the value expected for a diffusion limited process, a rapid equilibrium between at least two G-site conformations is likely. Only the less populated conformation should be competent for a proper interaction with GSH. The mutant enzyme always displays $k_{obs}$ values higher (five-ten fold) than those observed for the native enzyme at the same temperature and at the same GSH concentration (Fig 3A). For example, at 1 mM GSH, $k_{obs}$ is about 500 s$^{-1}$
in the R107A mutant while is 65 s\(^{-1}\) in the native enzyme. In addition, the dependence of
the observed rate constants on GSH concentration is not linear but now it follows an
hyperbolic behavior. This kinetic trend is similar to that found for Alpha, Mu and Pi class
GSTs (10, 11) and it is diagnostic for a multi-step binding mechanism (see Scheme II).

\[
\begin{align*}
&k_{on} & & k_2 \\
&k_{off} & & k_{2-1}
\end{align*}
\]

Scheme II

E + GSH ⇔ E-GSH ⇔ E\(^{-}\)-GSH

The proposed minimal scheme describes a first fast interaction of GSH with the enzyme
to give a weak pre-complex which is slowly converted into the final Michaelis complex.
Only this final complex is responsible for the fluorescence perturbation at 340 nm. Non-
linear fitting of all experimental data to Scheme II gives a \(k_{on} \geq 1 \times 10^5\) M\(^{-1}\) s\(^{-1}\) (the
angular coefficient of the tangent to the hyperbola at the lowest GSH concentrations) and
a \(k_{off} \geq 140\) s\(^{-1}\). These values are about four fold higher than those calculated for the
native enzyme. The resulting dissociation constant for the pre-complex \((k_{off}/k_{on})\) is 1.4
mM. The microscopic rate constants for the conversion of this pre-complex into the final
Michaelis complex are \(k_2 = 741 \pm 50\) s\(^{-1}\) and \(k_{2-1} = 215 \pm 30\) s\(^{-1}\). The overall dissociation
constant \([\(k_{off}/k_{on}\) x \(k_{2-1}/k_2\)]\) is 0.4 mM, a value lower than that observed for the native
enzyme (see Table I).

**pH dependence of the rate of GSH binding** - The effect of pH on the rate of GSH binding
has also been analyzed. In hGSTT2-2, the \(k_{obs}\) increases at low pH values and at pH 5.0
\((k_{obs} = 110\) s\(^{-1}\)), is about four fold higher than that observed at pH 9.0 \((k_{obs} = 29\) s\(^{-1}\)) (Fig
3B). This suggests that the protonation of one or more protein residues facilitates a
correct interaction with the substrate. In R107A mutant, GSH binds with \(k_{obs}\) values
higher than that shown for the wild-type at the same pH value, but with a similar pH
dependence (Fig 3B). Thus one or more residues, but not Arg107, are involved in the
observed facilitation of GSH interaction at acidic pH values.
DISCUSSION

The first observation coming from the present data is that hGSTT2-2 is able to activate GSH with an efficiency very similar to that shown by the more recently evolved GSTs. In fact, the apparent pKₐ for the sulfhydryl group of the bound GSH is 6.15, a value in the range of those observed for Alpha, Mu and Pi class GSTs (pKₐ = 6.0-6.8). This value, obtained by a direct spectroscopic determination of the thiolate ion, is close to that calculated by kinetic experiments for rat T2-2 enzyme (13). Thus, these findings indicate that the substrate activation is a property acquired early by GSTs and strictly conserved during evolution. The mechanisms for activation and stabilization of the sulfhydryl atom, however, could be different among GSTs: the thiolate is hydrogen bonded to a Tyr residue in the more recently evolved GSTs (1), whereas a similar role is played by a serine residue in the insect Delta class GST previously classified as a Theta-like GST (17). Even hGSTT2-2 shows a Ser residue, and not a Tyr, within hydrogen bonding of the GSH sulfhydryl group (6). The substitution of Ser11 by Ala in the rat GSTT2-2 seems to cause an increase of the GSH pKₐ of 1.3 pH units (14). However, we noted that, in the human Theta GSTT2-2, Arg107 is in hydrogen bonding distance of the main-chain carbonyl of the γ-glutamyl moiety of GSH and forms an interaction with the thiol sulfur of GSH either directly or through a water molecule (6). Subsequent mutagenesis and modelling studies suggested this residue is involved in the sulfatase reaction and in electrostatic substrate recognition (16). The present data indicate that mutation of Arg107 has a remarkable negative effect on the deprotonation of the substrate GSH. In R107A mutant, the apparent pKₐ of the bound GSH shifts from pH 6.1 to pH 7.8. Arg107 could act both as a counterion to promote ionization of the GSH thiolate and
then stabilise the thiolate by direction interaction. This is not the first case of arginine residues being implicated in GSH activation. In hGSTA1-1 (alpha class) Arg15 is within hydrogen-bonding distance of the GSH thiol (18) and its activation/binding role was subsequently confirmed by mutagenesis (19). In hGSTM2-2 kinetic data are consistent with Arg107 playing a role in promoting ionization and binding of GSH (20). In conclusion, on the basis of our data on the human enzyme and of those on the rat GSTT2-2 (13, 14), it is likely that GSH activation is achieved by the synergistic action of at least two residues: Arg107 and Ser11. As for the Alpha, Mu and Pi GSTs, this old enzyme also extrudes quantitatively the thiol proton of GSH from the active site into the surrounding solution, but only up to pH 6.5. Above this value, a protein residue acts as a base in capturing the thiol proton (see Fig. 2). The apparent $pK_a$ of this internal base is 7.3, a value which suggests the involvement of a histidine residue, possibly His 40, which is located close to the bound GSH. A nitrogen atom of the imidazole ring of His 40 is in van der Waals contact of the glycyl moiety of GSH. The capture of the thiol proton at high pH values has been also observed in the Delta GST (17). In that case His 38 and/ or His 50 are probably involved. The ability to release quantitatively the thiol proton in solution at any pH value is probably an evolutionary advantage reached by the Alpha, Mu and Pi class GSTs; during the enzymatic turnover, the proton neutralized by the protein residue in hGSTT2-2 must be released before a new productive cycle can start and this step may limit the overall velocity.

A second aspect we analyzed is the thermodynamic and kinetic efficiency of substrate binding to hGSTT2-2. This old enzyme shows a low affinity for GSH as suggested by an apparent $K_D$ of 0.8 mM, a value at least four times higher than that found in the more recently evolved GSTs. It appears that Alpha, Mu and Pi GSTs are under an
evolutionary pressure in the direction of lower $K_D$ values. This trend is distinctive for enzymes which exhibit $k_{cat}/K_m$ ratios far from the diffusion control limit ($10^8$-$10^{10}$ M$^{-1}$s$^{-1}$) (21). Actually, all GSTs are distant from a perfectly evolved catalyst and hGSTT2-2 shows a specificity constant for GSH of only $10^2$ M$^{-1}$s$^{-1}$. Moreover, a first enzyme on a metabolic pathway (like hexokinase in the glucose metabolism) often evolves towards a lower $K_m$ (or $K_D$) to prevent accumulation of intermediates; this could be the case of GST as the starting enzyme in the mercapturic acid formation.

From a kinetic point of view, the observed rate constants for binding of GSH to the G-site (at 37 °C) are remarkably lower (about ten times) than that observed in the Alpha, Mu and Pi GSTs (at 5°C) (10, 11). Above all, the most striking peculiarity of the Theta class enzyme is the linear dependence of $k_{obs}$ on GSH concentration which is consistent with a single-step binding mechanism i.e. the Michaelis complex is formed by a bimolecular encounter between GSH and enzyme. Is it possible to elucidate the evolutionary pathway GSTs have utilized to optimize their interaction with the substrate? Our data suggests a shifting from a single-step binding mechanism to a multi-step binding process, like as observed in Alpha, Mu and Pi GSTs (10, 11). These younger GSTs are able to interact with GSH through a weak pre-complex, followed by at least one isomerization step which results in a more rapid attainment of the binding equilibrium. This strategy is reminiscent of that used by many enzymes in catalysis where a single specific reaction is normally subdivided into a number of chemical steps with lower activation energies. From a structural point of view it is reasonable to propose that deletion of the extra C-terminal segment, which mostly obscures the G-site of the human Theta class (6), should facilitate the GSH interaction. Furthermore, other important factors must be considered which refer to the dynamics of this enzyme. A plot of the
crystallographic B-factors along the polypeptide chain can give an indication of the relative flexibility of a protein portion compared to other regions. As shown in Fig 4 the Alpha, Mu and Pi GSTs display a similar and well defined flexibility pattern. Several “hot” regions with high mobility can be identified (helix 2 and its flanking regions, C-terminal segment of helix 4, loop between helices 4 and 5 and N-terminal segment of helix 5) separated by a number “cold” segments. The hGSTT2-2 enzyme shows a completely different flexibility as no distinctive “hot” and “cold” regions can be defined (the noise in T2-2 plot is due to the limited resolution of the crystal structure) (Fig 4). It appears that GSTs have utilised flexibility in term of an evolutionary progression. Flexibility explains some of the behavior of the R107A mutant. Arg107 forms a salt bridge with Asp 104 and its replacement by Ala probably increase the structural flexibility of the enzyme. This is a plausible explanation for the remarkable increase of the rate of the GSH binding in the R107A mutant. In addition, replacement of Arg107 changes the binding event from a single-step to a multi-step mechanism (see Fig 3A), as observed in the more recently evolved GSTs. In other words, it appears that this specific substitution in the Theta class enzyme produces an improvement of the kinetic efficiency of GSH binding and the mutant enzyme now resembles the behavior of the more recently evolved GSTs.
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FOOTNOTES

*Both authors equally contributed to the work

The abbreviations used are: GST, glutathione transferase; GSH, glutathione; Msu, 1-
menaphthyl sulfate.

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FIGURE LEGENDS

Fig 1. Binding (A) and GSH thiolate formation in hGSTT2-2 and in the R107A mutant (B).

(A) GSH binding was measured by fluorometry as described under the “Experimental Procedures” section and data were fitted to the Equation (1).

(B) Spectra of GS' in the binary complex with hGSTT2-2 (●) and with R107A mutant (○), were obtained under different pH conditions with 15 µM active sites and 1 mM GSH (see “Experimental Procedures” section). The amount of thiolate formed at the G-site was calculated by assuming an ε_{240nm} of 5,000 M^{-1} cm^{-1}. The solid lines are the best fit of the data to Equation (2). Inset: difference spectrum of the hGSTT2-2-GSH binary complex in 0.01 M potassium phosphate buffer pH 7.5. The S.E.M. for each point does not exceed 5 % and 9% for hGSTT2-2 and R107A mutant respectively.

Fig. 2  pH dependence of the thiol proton release in hGSTT2-2

The concentration of the released thiol proton ([H^+]_{released}) was calculated as described in the “Experimental Procedures” section and normalized to the quantity of active sites. Lines are the best fit of data to Equation (3). Human GSTT2-2 (■); pH dependence of thiol proton release previously reported for the Delta GST ( □) (17). The S.E.M. for each point does not exceed 8 %.
Fig. 3 Kinetics of GSH binding to hGSTT2-2 and to R107A mutant

The observed rate constants ($k_{\text{obs}}$) for binding of GSH to the enzymes were obtained by stopped-flow experiments performed at 37 °C and pH 7.0. With hGSTT2-2, the $k_{\text{obs}}$ show a linear dependence on GSH concentration (panel A -□-) and linear regression analysis gives a $k_{\text{on}} = (2.7 \pm 0.8) \times 10^4$ M$^{-1}$ s$^{-1}$ and a $k_{\text{off}} = 36 \pm 9$ s$^{-1}$ for the equilibrium between GSH and enzyme. With R107A mutant, The $k_{\text{obs}}$ show a non-linear dependence on GSH concentration (panel A -■-). The continuos line is the best fit of the experimental data to Scheme II. The pH dependence of $k_{\text{obs}}$ is shown in panel B for hGSTT2-2 (-○-) and for R107A mutant (-●-). Experiments were performed at fixed GSH concentration (1 mM) and at different pH values between pH 5.0 and 9.0. The S.E.M. for each point does not exceed 4 %.

Fig 4 Mobility profiles of human Alpha, Mu, Pi and Theta class GSTs

The mobility profiles are derived from the crystallographic temperature factors of A1-1 (1GSF) (panel A), M2-2 (1HNA) (panel B), P1-1 (6GSS) (panel C) and T2-2 (1LJR) (panel D) GST isoenzymes.
TABLE I

Microscopic rate constants for the binding of GSH to hGSTT2-2 and R107A mutant. Experiments were performed at 37 °C as described in the “Experimental Procedures” section. Definition of microscopic rate constants are given in Schemes I and II for the wild-type and R107A mutant respectively.

|                | wild-type            | R107A          |
|----------------|----------------------|----------------|
| $k_{on}$       | $(2.7 \pm 0.8) \times 10^4$ M$^{-1}$ s$^{-1}$ | $\geq 1 \times 10^5$ M$^{-1}$ s$^{-1}$ |
| $k_{off}$      | $36 \pm 9$ s$^{-1}$  | $\geq 140$ s$^{-1}$ |
| $k_{off}/k_{on}$ | 1.3 mM              | 1.4 mM         |
| $k_2$          |                      | 741 ± 50 s$^{-1}$ |
| $k_{-2}$       |                      | 215 ± 30 s$^{-1}$ |
| $k_{-2}/k_2$   |                      | 0.29           |
| $(k_{off}/k_{on})(k_{-2}/k_2)$ |                   | 0.40 mM        |

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