The glutathione transferases (GSTs) represent a superfamily of dimeric proteins. Each subunit has an active site, but there is no evidence for the existence of catalytically active monomers. The lock and key motif is responsible for a highly conserved hydrophobic interaction in the subunit interface of pi, mu, and alpha class glutathione transferases. The key residue, which is either Phe or Tyr (Tyr50 in human GSTP1-1) in one subunit, is wedged into a hydrophobic pocket of the other subunit. To study how an essentially inactive subunit influences the activity of the neighboring subunit, we have generated the heterodimer composed of subunits from the fully active human wild-type GSTP1-1 and the nearly inactive mutant Y50A obtained by mutation of the key residue Tyr50 to Ala. Although the key residue is located far from the catalytic center, the $k_{cat}$ value of mutant Y50A decreased about 1300-fold in comparison with the wild-type enzyme. The decrease of the $k_{cat}$ value of the heterodimer by about 27-fold rather than the expected 2-fold in comparison with the wild-type enzyme indicates that the two active sites of the dimeric enzyme work synergistically. Further evidence for cooperativity was found in the nonhyperbolic GSH saturation curves. A network of hydrogen-bonded water molecules, found in crystal structures of GSTP1-1, connects the active sites and the main chain carbonyl group of Tyr50, thereby offering a mechanism for communication between the two active sites. It is concluded that a subunit becomes catalytically competent by positioning the key residue of one subunit into the lock pocket of the other subunit, thereby stabilizing the loop following the helix α2, which interacts directly with GSH.

Glutathione S-transferases (GSTs, EC 2.5.1.18) are members of a superfamily of multifunctional dimeric proteins involved in the cellular detoxification of cytotoxic and genotoxic compounds and in protecting tissues against oxidative damage (1–4). They have also been implicated in the development of resistance of cells and organisms to electrophilic anticancer drugs, pesticides, and herbicides (5–8). Glutathione transferases catalyze the nucleophilic addition of the thiol of reduced glutathione (GSH, γGlu-Cys-Gly) to a wide variety of endogenous and exogenous hydrophobic electrophiles including alkyl and aryl halides, epoxides, quinones, and activated alkenes. The glutathione S-conjugates of these compounds are more polar than facilitating their elimination.

Cytoplasmic mammalian GSTs can be classified, based on amino acid sequences, into at least eight distinct classes: alpha, mu, pi, theta, sigma, kappa, zeta, and omega. The sequence identities between any two members within a class are typically greater than 70% and usually less than 30% between classes (1, 7, 9). The three-dimensional structures of members of six GST classes have been determined by x-ray crystallographic analysis. Each subunit is composed of two domains: an N-terminal domain (domain I) that adopts a thioredoxin-like fold and an all α-helical C-terminal domain (domain II). There are at least two ligand-binding sites per subunit: the glutathione-binding site (G-site), which is very specific for GSH and the hydrophobic substrate-binding site (H-site), which can bind a large variety of different electrophiles (18). The G-site is constructed mainly from residues of the N-terminal domain, whereas the H-site has major contributions from the C-terminal domain. While the functional properties of the amino acid residues making up the G-site of a GST are generally conserved among different classes, the residues forming the hydrophobic substrate-binding pocket vary considerably among different GSTs. Since the structure of the H-site governs the substrate specificity of a particular GST, diversity in the H-site gives the GST family the ability to catalyze reactions toward an exceedingly large number of structurally diverse substrates.

Although all soluble GSTs have very similar overall polypeptide folds, each class exhibits unique features, particularly about the active site and at the C terminus (8, 19, 20). For alpha and theta class enzymes, the C terminus forms a well-defined α-helical structure that shields the active site and plays an important role in catalysis through the control of product release. For the mu class enzymes the C terminus forms an Ω-loop that partially covers the active site. Another salient feature of mu and pi class enzymes is found in the segment that links strand β2 to helix α2. In mu class enzymes, this segment forms an extended solvent-exposed loop (mu-loop) that partially shields the active site. The corresponding segment in pi class enzymes is a short helix that is considered to be part of helix α2 (Fig. 1). Helix α2, which is formed by residues 36–52, is highly flexible in pi class GSTs, and influences the catalytic activity of the enzymes. Although data obtained so far indicate that the flexibility of the highly mobile helix α2 of the pi class enzymes modulates GSH binding and catalysis, it is not clear how the mobility of this helix influences...
the rate of catalysis. Although the substrate binding to one subunit of wild-type GSTP1-1 does not alter the catalytic properties of the other subunit at a physiological temperature, some mutations of amino acid residues forming helix α2 induce cooperativity in GSH binding. Mutants G42A, C48S, and K55A display positive cooperativity (21–23). On the other hand, mutants Y50F (24) and Y50A (the present study) display negative cooperativity of GSH binding. Caccuri et al. (24) suggested that Gly42, Cys48, and Lys55 point mutations induce cooperativity by distorting the conformation of helix α2. The structural effect in one G-site was assumed to be transmitted to the second one through Tyr50 and helix α4, which are located at the dimer interface.

Although each subunit of dimeric GSTs has a G-site and an H-site, which together form the catalytic active center, there is no evidence for the existence of catalytically active monomers in nature. Abdalla et al. (25) succeeded in producing a stable monomeric, but inactive, protein by introducing 10 site-specific mutations at the subunit-subunit interface of GSTP1-1. To date it is not clear whether the dimeric form is functionally essential in catalysis or not. Analyses of crystal structures of pi, mu, and alpha class glutathione transferases (26–28) have provided insights into the major interactions between subunits in the homodimers. Their subunit interfaces involve three types of interactions: polar contacts and hydrogen bonds between electrophilic amino acids, stacking of two symmetrically equivalent arginines (one from each subunit) (7, 28) and hydrophobic interactions including a lock and key motif. In the lock and key motif an aromatic residue (key residue) from domain I in one subunit is wedged into a hydrophobic pocket formed by helices α4 and α5 in domain II of the other subunit (Fig. 1). The lock and key motif is a common feature of pi, mu, and alpha class glutathione transferases and the key residue is either phenylalanine or tyrosine (Tyr50 in hGSTP1-1). Mutagenesis has been used to investigate the importance of the key residue for dimerization and stability of GSTP1-1 (29), GSTA1-1 (30, 31), and GSTM1-1 (32).

The generation of heterodimeric proteins provides a new approach to studying the amino acid residues that are important in subunit interactions and to probe the influence of one subunit on the activity of the other subunit. Tyrosine 50 in GSTP1-1 may play both a structural role as the key residue and a functional role as part of the flexible helix α2. Thus, in the present study we have generated the heterodimer composed of subunits from wild-type GSTP1-1 and mutant Y50A to investigate the importance of residue Tyr50 in dimerization, stability and catalytic activity, and the influence of one essentially inactive subunit on the activity of the neighboring subunit in the heterodimer. The latent cooperativity previously observed in GSTP1-1 was also investigated.

**EXPERIMENTAL PROCEDURES**

Expression and Purification of Wild-type GSTP1-1 and Histidine-tagged Mutant Y50A—Wild-type GSTP1-1 and histidine-tagged mutant Y50A were expressed in *Escherichia coli* strain XL-1 Blue in presence of...
0.2 mm isopropy-1-thio-β-galactopyranoside at 37 °C as described by Kolt et al. (33). The cells were harvested by centrifugation and disrupted by ultrasonication. GSTP1-1 was determined by affinity chromatography as described by Kolt et al. (33) by using S-hexylglutatone-Sepharose 6B as affinity matrix instead of S-hexylglutatone-Sepharose 4B, while the mutant Y50A was purified by using Ni-IMAC, eluted with 0.5 M imidazole in 20 mM phosphate, pH 7.4, containing 1 mM CdNB, 10 mM DTT, and 0.02%, w/v, NaN3, and dialyzed against 10 mM Tris-HCl, pH 7.8, containing 1 mM EDTA, 0.2 mM DTT and 0.02% (w/v) NaN3. The purity of enzymes was analyzed by 20% homogeneous native polyacrylamide gel electrophoresis (PAGE) and isoelectric focusing (IEF) using precast gels (Amersham Biosciences PhastSystem).

**Heterodimer GSTP1/Y50A Preparation and Purification**—The heterodimer GSTP1/Y50A was prepared by mixing 3.4 mg/ml each of GSTP1-1 and mutant Y50A and incubating the mixture in 10 mM Tris-HCl, pH 7.8, containing 3.5 mM GdnHCl and 2 mM DTT at 4 °C overnight with moderate shaking. The buffer constituents of the mixture were then changed by gel filtration to 10 mM Tris-HCl, pH 7.8, containing 0.2 mM NaCl, 1 mM EDTA, 0.2 mM DTT, and 0.02%, w/v, NaN3 (buffer A). GSTP1/Y50A was purified in two consecutive chromatographic steps using glutation-Sepharose 4B and Ni-IMAC. After passing the mixture through glutathione-Sepharose 4B at 10 °C with a flow rate of 0.25 ml/min, the fractions containing GSTP1/Y50A were collected as wash (buffer A), while GSTP1-1 was eluted as eluate (10 mM Tris-HCl, pH 7.8, containing 0.2 mM NaCl, 5 mM S-hexylglutatone, 1 mM EDTA, 0.2 mM DTT, and 0.02%, w/v, NaN3). The wash fraction containing GSTP1/Y50A was passed through Ni-IMAC at 10 °C with a flow rate of 1 ml/min, after dialysis against 20 mM phosphate, pH 7.4, containing 0.5 mM NaCl, 0.085 mM imidazole, 10 mM β-mercaptoethanol and 0.02%, w/v, NaN3 (buffer B). GSTP1/Y50A and mutant Y50A were eluted with 0.2 M and 0.5 M imidazole, respectively in buffer B. Purified GSTP1/Y50A was dialyzed against 10 mM Tris-HCl, pH 7.8, containing 1 mM EDTA, 0.2 mM DTT, and 0.02%, w/v, NaN3 and stored at ~80 °C. The purity of GSTP1/Y50A was found to be homogeneous by 20% native PAGE and IEF.

**Enzyme Assay**—The specific activity of GSTP1-1, GSTP1/Y50A, and mutant Y50A was determined by a spectrophotometric assay in 0.1 M phosphate, pH 6.5, at 30 °C in the presence of 1 mM GSH and 1 mM CDNB (34).

**Determination of Steady-state Kinetic Parameters**—Steady-state kinetics of GSTP1-1, GSTP1/Y50A and mutant Y50A were carried out, at a constant GSH concentration of 10 mM, by varying CDNB concentration from 0.08 to 2 mM or varying PEITC concentration from 0.01 to 0.08 mM. All measurements were performed in triplicate measurements in 0.1 M phosphate, pH 6.5 for CDNB and pH 6.0 for PEITC, at 30 °C. Kinetic parameters were determined by fitting the Michaelis-Menten equation to the experimental data using the SIMFIT package (35).

**Temperature Effect on Cooperativity of GSTP1-1 and GSTP1/Y50A**—Steady-state kinetics for GSTP1-1, GSTP1/Y50A, and mutant Y50A were carried out in triplicate measurements at 20, 30, and 40 °C in 0.1 mM phosphate, pH 6.5, in the presence of 0.33 mM GSH, 1 mM CDNB, and different concentrations of inhibitor. I50 values were calculated as the concentration of inhibitor that gives 50% of the reaction rate of the uninhibited reaction.

**Results**

**Preparation and Purification of GSTP1-1, Mutant Y50A, and GSTP1/Y50A**—GSTP1-1 and histidine-tagged mutant Y50A were expressed in E. coli at 37 °C and purified in a single chromatographic step using S-hexylglutatone-Sepharose 6B and Ni-IMAC columns, respectively. A total yield of 40 mg of protein per liter culture was obtained both with GSTP1-1 and with mutant Y50A. The specific activity of purified GSTP1-1 and mutant Y50A was determined as 100 and 0.004 μmol min⁻¹ mg⁻¹, respectively, using CDNB as the electrophilic substrate.

**Steady state kinetic parameters for GSTP1-1, GSTP1/Y50A, and Y50A mutant**

Vmax, kcat, KM, and Kcat/KM values were calculated at 10 mM GSH and varying electrophilic substrate concentrations as described under “Experimental Procedures.” The Michaelis-Menten equation was fitted to the experimental data with the SIMFIT package. The Vmax is the maximum velocity at saturating substrate concentrations; kcat is the turnover number; kcat/KM is the specificity constant, and the Kcat/KM Michaelis constant, is the substrate concentration corresponding to half-saturation of the enzyme.

| Varied substrate | GSTP1-1 | GSTP1/Y50A | Y50A |
|------------------|--------|-----------|------|
| CDNB             | 157 ± 5| 5.5 ± 0.4 | 0.122 ± 0.09 |
| PEITC            | 110 ± 10| 0.7 ± 0.3 | ND   |

* Parameter values ± S.E. were estimated by nonlinear regression analysis of experimental data.

**Inhibition Studies**—The I50 values for each inhibitor for GSTP1-1, GSTP1/Y50A, and mutant Y50A were determined by measuring the specific activities at 30 °C in 0.1 M phosphate, pH 6.5, in the presence of 1 mM GSH, 1 mM CDNB, and different concentrations of inhibitor. I50 values were calculated as the concentration of inhibitor that gives 50% of the reaction rate of the uninhibited reaction.

**Thermal Stability**—The thermal stability of GSTP1-1 (0.0021 mg/ml) and GSTP1/Y50A (0.078 mg/ml) was examined by incubating the enzyme fractions in 10 mM Tris-HCl, pH 7.8, containing 40 mM GSH, 5 mM DTT, and 0.02%, w/v, NaN3, at 50 °C for different time periods. The enzymatic activity was then measured in 0.1 mM phosphate, pH 6.5, at 30 °C in the presence of 1 mM GSH and 1 mM CDNB.

**TABLE II**

Steady state kinetic parameters for GSTP1-1, GSTP1/Y50A, and Y50A mutant

| Varied substrate | GSTP1-1 | GSTP1/Y50A | Y50A |
|------------------|--------|-----------|------|
| CDNB             | 157 ± 5| 5.5 ± 0.4 | 0.122 ± 0.09 |
| PEITC            | 110 ± 10| 0.7 ± 0.3 | ND   |

* Parameter values ± S.E. were estimated by nonlinear regression analysis of experimental data.

**ND, not determined.**

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substrate (Table I). Histidine-tagged wild type was constructed and kinetically characterized to estimate the histidine-tag effect on kinetic properties of enzyme. The kinetic properties of the histidine-tagged wild type did not differ significantly from unmodified wild-type enzyme (Stenberg et al., Ref. 29). The preparation of heterodimeric GSTP1/Y50A gave a yield 34% of total protein and a specific activity of 1 μmol min⁻¹ mg⁻¹ (Table I). GSTP1-1, GSTP1/Y50A, and mutant Y50A appeared as single bands in IEF gel and 20% homogeneous native PAGE (data not shown).

Steady-state Kinetic Parameters—The steady-state kinetic parameters were determined for wild-type GSTP1-1, GSTP1/Y50A, and mutant Y50A with constant GSH concentration and variable concentrations of the alternative substrates CDNB and PEITC.

As shown in Table II, $K_m^{CDNB}$ of GSTP1/Y50A increased 3-fold and decreased 4-fold in comparison with GSTP1-1 and mutant Y50A, respectively, while $k_{cat}^{PEITC}$ for GSTP1/Y50A was the same as for GSTP1-1. $K_m^{CDNB}$ for GSTP1/Y50A decreased 27-fold and increased 50-fold with reference to GSTP1-1 and mutant Y50A, respectively. Also $k_{cat}^{PEITC}$ for GSTP1/Y50A decreased 140-fold compared with GSTP1-1. $k_{cat}/K_m^{CDNB}$ for the heterodimer decreased 89-fold and increased 200-fold with regard to GSTP1-1 and mutant Y50A, respectively, and $k_{cat}/K_m^{PEITC}$ decreased 122-fold compared with GSTP1-1.

Temperature Effects on GSH as Varied Substrate for GSTP1-1, GSTP1/Y50A, and Mutant Y50A—The mutation at position 50, which is located on the loop forming one wall of the G-site, may distort the conformation of the active site. $k_{cat}$ and $K_m^{GSH}$ values were determined at 20, 30, and 40 °C for GSTP1-1, GSTP1/Y50A, and mutant Y50A.

As shown in an Eadie-Hofstee plot (Fig. 2C), the GSH saturation of mutant Y50A is markedly concave-up at all three temperatures. The GSH saturation of GSTP1/Y50A is slightly concave-up at 30 °C (Fig. 2B). This nonhyperbolic behavior increases at 20 °C and disappears at 40 °C. For GSTP1-1, the GSH binding is hyperbolic from 20 to 30 °C but concave-down at 40 °C (Fig. 2A).

At 30 °C, the $K_m^{GSH}$ value of GSTP1/Y50A is 5 times higher than that of GSTP1-1 but 12 times lower than that of mutant Y50A (Table III). $k_{cat}$ of GSTP1/Y50A at 30 °C temperature is 59 times lower than that of GSTP1-1 but 6 times higher than that of mutant Y50A. By increasing the temperature from 20 to 40 °C, $K_m^{GSH}$ and $k_{cat}$ values of GSTP1-1 and GSTP1/Y50A increase but $K_m^{GSH}$ of mutant Y50A decreases. $k_{cat}$ of mutant Y50A increases with increasing temperature from 20 to 30 °C, but decreases with increasing temperature from 30 to 40 °C.

Temperature Effect on Cooperativity of GSTP1-1 and GSTP1/Y50A—Fig. 2A shows that GSTP1-1 changes from apparent “kinetic” negative cooperativity, to non-cooperativity and positive cooperativity at 20, 30, and 40 °C, respectively, i.e. Hill coefficient values are 0.85, 1, and 1.18, respectively (Table III). On the other hand, GSTP1/Y50A displays negative cooperativity below 40 °C and non-cooperativity at 40 °C (inset of Fig. 2B), i.e. Hill coefficient values are 0.79, 0.86, and 0.97 at 20, 30, and 40 °C, respectively (Table III). Mutant Y50A displays negative cooperativity at all temperatures tested, (inset of Fig. 2C), i.e. Hill coefficient values are 0.66, 0.67, and 0.69 at 20, 30, and 40 °C, respectively (Table III).

Inhibition Studies—The effects of different inhibitors of GSTP1-1, GSTP1/Y50A, and mutant Y50A were determined (Table IV). GSH and CDNB were used as substrates. The difference between $I_m$ values of GSTP1-1 and GSTP1/Y50A is not pronounced for different GSH analogs (Fig. 3), but significant for Cibacron Blue (Table IV). Cibacron Blue is a strongly inhibitory electrophilic substrate analog (36), which probes

*Fig. 2. Eadie-Hofstee plots of wild-type GSTP1-1 (panel A), heterodimer GSTP1/Y50A (panel B), and mutant Y50A (panel C). The insets represent Hill plots of wild-type GSTP1-1 (panel A), heterodimer GSTP1/Y50A (panel B), and mutant Y50A (panel C). Initial velocity and maximum velocity ($V_{max}$) were measured and calculated at constant CDNB concentration of 1 mM and varying GSH concentrations in 0.1 M phosphate, pH 6.5 at 20 ( ), 30 ( ), or 40 °C ( ). Y is the relative velocity ($v/V_{max}$). Measurements were performed in triplicate. Error bars show the S.E.*
other binding sites than do the GSH derivatives; its saturation curves showed no cooperativity. GSTP1/Y50A is less sensitive than GSTP1-1 to all inhibitors. Although GSTP1-1 and GSTP1/Y50A do not demonstrate cooperativity with the different inhibitors, the mutant Y50A displays marked negative cooperativity with GSH derivatives. Thus, the fractional activity of mutant Y50A inhibited by GSH analogs can be modeled with two different $I_{50}$ values apparently accounting for the activity of two subunits with different sensitivities to the inhibitor. Both of these values are higher than those of GSTP1-1 and GSTP1/Y50A. The $I_{50}$ values for $S$-p-bromobenzyl glutathione of GSTP1-1, GSTP1/Y50A, and mutant Y50A increase with increasing temperature, but the mode of cooperativity is not affected by the temperature change (data not shown).

**Fig. 3.** Inhibition curves of different inhibitors on wild-type GSTP1-1 (\(\bullet\)), heterodimer GSTP1/Y50A (\(\square\)), and mutant Y50A (\(\triangle\)). The fractional activity was measured at constant GSH and CDNB concentrations of 1 mM and varying $S$-p-bromobenzyl glutathione (panel A) and glutathione sulfonic acid (panel B) concentrations in 0.1 m phosphate, pH 6.5 at 30 °C. The insets represent the logarithm of the specific activity versus the logarithm of the inhibitor concentration.

**Fig. 3.** Reactivation of GSTP1-1 and GSTP1/Y50A—GSTP1-1 is inactivated in the presence of a denaturant such as GdnHCl, which affects the dissociation of the two subunits. The inactivation was monitored by the loss of enzymatic activity; the specific activity of GSTP1-1 was shown to decrease from 100 to 12.2 $\mu$mol min$^{-1}$ mg$^{-1}$ with increasing GdnHCl concentrations from 0 to 3.5 M (inset of Fig. 4). After removal of GdnHCl, the GSTP1-1 dimer reformed as judged by measuring regain of activity. However, the extent of reactivation depended on the GdnHCl concentration used for denaturation (inset of Fig. 4).
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The concentration of 1 mM after removal of different guanidine-HCl.

The specific activity was measured at constant GSH and type GSTP1-1 (inset) from 1 to a maximum of 15.4 mol min^{-1} mg^{-1} after removal of denaturant. The specific activity of GSTP1/Y50A increased of native wild-type homodimer that takes place after removal of denaturant resulted in an increased to 88.3% at 3.5 M GdnHCl. Attempts to reactivate the activity of wild-type GSTP1-1 before (inset of Fig. 5). GSTP1/Y50A activity increased from 1 to a maximum of 15.6 mol min^{-1} mg^{-1} at 50 °C in presence of 40 mM GSH and 5 mM DTT in 0.1 M phosphate buffer, pH 6.5 for different periods of time.

Fig. 4. Reactivation of heterodimer GSTP1/Y50A and wild-type GSTP1-1 (inset) with different concentrations of guanidine-HCl. The specific activity was measured at constant GSH and CDNB concentration of 1 mM after removal of different guanidine-HCl concentration in 0.1 M phosphate, pH 6.5 at 30 °C. The inset shows the activity of wild-type GSTP1-1 before (dashed line) and after (solid line) removal of denaturant.

The recovery was 100% at less than 1.2 mM GdnHCl, but decreased to 88.3% at 3.5 mM GdnHCl. Attempts to reactivate the heterodimer GSTP1/Y50A after denaturation resulted in an increase of specific activity. This is ascribed to the reformation of native wild-type homodimer that takes place after removal of denaturant. The specific activity of GSTP1/Y50A increased from 1 to a maximum of 15.4 μmol min^{-1} mg^{-1} when concentration of GdnHCl increased from 0 to 2.5 M (Fig. 4).

Thermal Stability—GSTP1-1 activity decreased from 100 to 85.4 μmol min^{-1} mg^{-1} after 180 min of incubation at 50 °C (inset of Fig. 5). GSTP1/Y50A activity increased from 1 to a maximum of 15.6 μmol min^{-1} mg^{-1} after 6 min of incubation at 50 °C and then decreased slowly with the time of incubation until it reached a plateau of 13.8 μmol min^{-1} mg^{-1} after 180 min (Fig. 5). The increase of GSTP1/Y50A activity to 15.6 μmol min^{-1} mg^{-1} is most likely due to reformation GSTP1-1 from GSTP1 monomers through dismutation of the heterodimer GSTP1/Y50A.

**DISCUSSION**

GSTs are dimeric proteins. Although amino acid residues in the dimer interface are not universally conserved in different GSTs classes, a particular lock and key motif is a common feature of the alpha, mu, and pi class enzymes (26–28). To elucidate the significance of the key residue in dimerization and stability of the hGSTP1-1 dimer, Tyr^{50} has been mutated by site-directed mutagenesis (29). Analysis of the Y50A mutant displays the importance of the aromatic group not only on the formation and stability of the dimer, but also on the rate of catalysis. The Y50A and Y50R mutants have more than 1000-fold reduced \( k_{cat} \) values (29) even though the key residue is located far from the catalytic center. Thus, mutant Y50A is one of the most interesting mutants. Because of the low enzymatic activity and the thermal instability of mutant Y50A, the characterization of this mutant is not sufficient to examine the role of an aromatic amino acid side chain as key residue. Thus in the present study the heterodimer GSTP1/Y50A was constructed from wild-type GSTP1-1 and the histidine-tagged mutant Y50A, as a novel approach to studying the influence of the key residue on the rate of catalysis and the cooperativity, and

### Temperature effect on GSH saturation of GSTP1-1, GSTP1/Y50A, and Y50A mutant

\( k_{cat} \), \( K_m \), and \( h \) values were calculated at 1 mM CDNB and varying GSH concentration at 20, 30, and 40 °C as described under “Experimental Procedures.” The experimental data were fitted to the 1:1 Michaelis-Menten and Hill equations as described under “Experimental Procedures.” The \( k_{cat} \) is the turnover number; the \( K_m \) Michaelis constant, is the substrate concentration corresponding to half-saturation of the enzyme; and \( h \) is the Hill coefficient.

| Kinetic parameter \( a \) | 20 °C          | 30 °C          | 40 °C          |
|--------------------------|---------------|---------------|---------------|
| GSTP1-1                  |               |               |               |
| \( k_{cat} \)            | 73.6 ± 0.4    | 105.2 ± 0.37  | 123.7 ± 1.13  |
| \( K_m \)                | 0.16 ± 0.01   | 0.22 ± 0.01   | 0.43 ± 0.03   |
| \( h \)                  | 0.85 ± 0.03   | 1.06 ± 0.05   | 1.18 ± 0.04   |
| GSTP1/Y50A               |               |               |               |
| \( k_{cat} \)            | 1.56 ± 0.01   | 1.8 ± 0.01    | 2.13 ± 0.01   |
| \( K_m \)                | 0.96 ± 0.07   | 1 ± 0.07      | 1.3 ± 0.06    |
| \( h \)                  | 0.79 ± 0.02   | 0.86 ± 0.02   | 0.97 ± 0.02   |
| Y50A                     |               |               |               |
| \( k_{cat} \)            | 0.14 ± 0.003  | 0.29 ± 0.01   | 0.22 ± 0.007  |
| \( K_m \)                | 15 ± 2.1      | 11.8 ± 2.1    | 4.5 ± 0.7     |
| \( h \)                  | 0.66 ± 0.01   | 0.67 ± 0.02   | 0.69 ± 0.02   |

\(^{a}\) Parameter values ± S.E. were estimated by nonlinear regression analysis of the experimental data.

**FIG. 5.** Thermal stability of heterodimer GSTP1/Y50A and wild-type GSTP1-1 (inset) at 50 °C. The specific activity was measured in the standard assay system after incubation of enzyme fractions at 50 °C in presence of 40 mM GSH and 5 mM DTT in 0.1 M phosphate buffer, pH 6.5 for different periods of time.
also to elucidate the influence of one subunit on the activity of the other subunit.

Effect of Key Residue on Dimer Formation and Thermal Stability—The yield of mutant Y50A after bacterial expression (40 mg/liter culture) is the same as the yield of wild-type GSTP1-1. This finding as well as the result of heterodimeriza-
tion, indicate that the key residue is not the strongest contributor to subunit-subunit recognition and dimerization of hGSTP1-1. Optimizing the refolding conditions yielded equal amounts of wild type, heterodimer, and mutant variants. Suboptimal conditions on the other hand, favored the formation of the wild-type homodimer but only to a maximum yield of 37%. Thus, the contribution from tyrosine, in its role as key residue, to the refolding of the native structure seems to be rather small. In this connection, it is noteworthy that the evolutionarily older sigma and theta class GSTs, which lack this structural motif, also form functional dimers. On the other hand, lower thermal stability of heterodimer GSTP1/Y50A (Fig. 5) and mutant Y50A at 50 °C (29) indicates that the aromatic residue in position 50 plays an essential role in stabilizing the dimer.

Effect of Key Residue on Enzymatic Activity—Replacement of Tyr50 with Ala decreases the specific CDNB activity of the enzyme 25,000-fold from 100 μmol min⁻¹ mg⁻¹ for wild-type GSTP1-1 to 0.004 μmol min⁻¹ mg⁻¹ for the Y50A mutant (Table I). If the two subunits of the GSTP1-1 dimer were working independently the expected specific activity value of the heterodimer GSTP1/Y50A would be half of the wild-type specific activity value (50 μmol min⁻¹ mg⁻¹), but the actual value was 1 μmol min⁻¹ mg⁻¹ (1% the wild-type value). A similar low value for GSTP1/Y50A was obtained with the alternative electrophilic substrate PEITC (Table II), which represents an addition reaction rather than the aromatic nucleophilic displacement studied with CDNB. Lowering the specific activity of the heterodimer may be due to failure of dimerization between wild type and mutant monomers, irreversible inhibitory effect of GdnHCl on enzyme activity, or that the two subunits of GSTP1/Y50A work cooperatively. Native PAGE and IEF gels demonstrate the successful dimerization and the purity of GSTP1/Y50A (results not shown). The regain of most of the wild-type activity after removal of GdnHCl shows that there is no remaining inhibitory effect caused by the GdnHCl treatment (inset of Fig. 4). Also the increased specific activity of the heterodimer after GdnHCl treatment indicates heterodimer dissociation and reformation of wild-type enzyme (Fig. 4). Thus the conclusion is that the two subunits of GSTP1/Y50A work synergistically.

Effect of Key Residue on G-site Geometry—Helix α2 and its flanking region (residues 36–52) form one wall of the GSH binding site (Fig. 1). A number of amino acid residues, Trp39, Lys45, Gln52, and the NH group of Leu53, that have H-bond interaction with GSH, contribute to the G-site (37). Chemical modification of the reactive Cys48 (38–40), intramonomer disulfide bond formation between Cys48 and Cys102 (41, 42) and proteolytic cleavage of the peptide bond between Lys45 and Ala46 by trypsin (43), as well as structural evidence, based on x-ray crystallographic and NMR studies (44, 45) demonstrate that this region is highly flexible in the absence of GSH and is stabilized by GSH binding to the active site. Thus any mutation, e.g. Y50A, which disturbs the conformational flexibility of helix α2 and possibly changes the G-site geometry, may be expected to lead to increasing KmGSH values (54-fold increase observed at 30 °C) and a decrease in GSH affinity (Table III). The lack of binding of GSTP1/Y50A and mutant Y50A to the glutathione affinity column as well as inhibition studies of GSTP1-1, mutant Y50A, and GSTP1/Y50A provide further support for the important role of this region in modulation of the affinity for GSH and GSH analogs (Fig. 3 and Table IV). The I50 values of mutant Y50A are higher than those of GSTP1-1, confirming the reduced affinity of mutant Y50A for GSH analogs compared with that of GSTP1-1. The inhibition curve depends not only on the affinity of the active site for the inhibitor but also on the contribution of the subunit to the total enzymatic activity of the heterodimeric enzyme (46). Heterodimer GSTP1/Y50A has two different subunits, one of them is highly active and the other is nearly inactive, thus inhibition...
curves and $I_{50}$ values of GSTP1/Y50A for GSH analogs resemble those of GSTP1-1 (Fig. 3 and Table IV). It is noteworthy that although the catalytically inactive mutated subunit of the heterodimer influences the activity of the wild-type subunit, the mutated subunit does not influence the affinity of the wild-type subunit for GSH analogs. This implies that the effect of the mutation is expressed primarily in catalytic rate and not in binding parameters.

**Effect of Key Residue on the Rate of Catalysis**—The catalytic process of GST P1-1 is dependent on the proximity of the sulphydryl group of GSH to the hydroxyl group of Tyr$^{8}$ as well as to the electrophilic center in the second substrate. In the reaction with CDNB the rate-limiting step of catalysis is a physical event (helix $\alpha_2$ movement dependent) that occurs after GSH and CDNB binding and before $\alpha$-complex formation (47). After binding to the G-site, GSH could be shifted from a pre-catalysis position to a catalysis position that is proximal to the catalytic center. This hypothesis is supported by NMR studies of unliganded and liganded hGSTP1-1, in which it was concluded that after GSH binding to the active site, many structural changes are required to bring the GSH-GSTP1-1 complex to a catalytically active conformation (45). This could be accomplished by the stabilization of helix $\alpha_2$ and positioning of the key residue Tyr$^{50}$ of one subunit inside the lock of the other subunit. The shifting of GSH from the pre-catalysis to the catalysis position may be the rate-limiting step for DNP-SG complex formation. This proposal could also explain why the catalytically active GSTP1-1 must be a dimeric protein. Each subunit needs the lock motif of the other subunit to properly position the key residue and helix $\alpha_2$ and consequently induce productive binding of GSH. Thus, the replacement of Tyr$^{50}$ with Ala may disturb the conformational flexibility of the helix $\alpha_2$ loop and dislocate helix $\alpha_2$ with a change of the G-site geometry in such a way that the necessary shifting of GSH from pre-catalysis to catalysis position is prevented. As a result the $k_{cat}$ values decrease about 1300-fold (Table II).

**The Cooperativity of GSH Binding**—The fact that the specific activity of heterodimer GSTP1/Y50A was lowered to markedly less than half of the value of wild-type GSTP1-1 raised the question of cooperativity between the subunits in the protein. The CDNB and PEITC saturation curves of GSTP1-1, mutant Y50A, and GSTP1/Y50A were well described by the Michaelis-Menten equation, indicating that CDNB and PEITC binding is noncooperative. On the other hand, the GSH saturation curve of GSTP1-1 is temperature-dependent. At 30°C the Michaelis-Menten equation can be fitted to the data, but slightly negative and positive cooperativity is observed at 20°C and 40°C, respectively (Fig. 2A and Table III). The GSH saturation of mutant Y50A displays negative cooperativity to the same extent at all temperatures (Fig. 2C and Table III). Similarly, the negative cooperativity observed with inhibitory GSH derivatives remained at all temperatures investigated. GSTP1/Y50A dis-

| Inhibitor                  | GSTP1-1 | GSTP1/Y50A | Y50A$^{a}$ |
|----------------------------|---------|------------|------------|
| S-Hexyl glutathione         | 22      | 31         | ND$^{b}$   |
| S-p-Bromobenzyl glutathione| 11      | 12         | 32         | 1.6 × 10$^5$ |
| Glutathione sulfonic acid   | 45      | 62         | 201        | 1.1 × 10$^7$ |
| Cibacron blue               | 0.33    | 0.83       | ND         | ND          |

$^{a}$ Two-binding-site function.
$^{b}$ ND, not determined.

**TABLE IV**

**Effect of different inhibitors on GSTP1-1 and GSTP1/Y50A**

$I_{50}$ values were determined by measuring the specific activities in 0.1 M phosphate, pH 6.5, in the presence of 1 mM GSH, 1 mM CDNB and different concentrations of inhibitor at 30°C. $I_{50}$ is the inhibitor concentration that gives 50% of the reaction rate of the uninhibited reaction.
plays negative cooperativity at 20 °C and 30 °C, but displays apparent noncooperativity at 40 °C (Fig. 2B and Table III).

The cooperativity noted in GSH saturation GSTP1-1, mutant Y50A, and GSTP1/Y50A poses two questions. First, how is the GSH binding effect transmitted from one subunit to the neighboring subunit? Available crystal structures of hGSTP1-1 show a strand of water molecules spaced by distances suited for hydrogen bonding (Fig. 6A). The strand extends from the active site in one subunit across the 2-fold symmetry axis to the active site in the second subunit through 21 hydrogen bonds. GSH binding to the active site induces conformational changes, including arranging water molecules into a double-stranded lattice, which is connected to the side-chain of Asp59 and the α-carboxylate of glutathione bound to the active site (Fig. 6B). The positioning of the key residue Tyr50 inside the lock motif leads to stabilization of the helix α2 loop and extending the double-stranded water network to connect with the main-chain carbonyl group of Tyr50. The rearrangement of water molecules into a double-stranded lattice doubles the number of hydrogen bonds involved in this lattice from 20 to 40. All amino acid residues (Tyr8, Arg14, Lys45, Gln52, and Ser66) that interact with glutathione bound to the active site are also engaged in the water network (Fig. 6C). In this regard, thermodynamic data show that overall enthalpy associated with ligand-induced folding is on the order of −120 to −150 kJ/mol (45). The estimated enthalpy for helix α2 folding is −50 to −60 kJ/mol, based on model peptides (48–50). Perhaps part of the difference between the overall enthalpy of ligand-induced folding and that of helix α2 folding is expended on packing of water molecules into a double-stranded lattice between two active sites in the dimer. The water network could help GSH in shifting from the pre-catalysis to the catalysis position. A water network connecting the flexible helix α2 loop and GSH interacting amino acid residues with the α-carboxylate of glutathione, may transfer structural and functional perturbations in one active site to the other active site, including positive or negative cooperativity. In this regard the Y50A mutation that distorts the conformation of the flexible helix α2 loop can also rearrange the water network in such a way that it induces positive or negative cooperativity, or both of them. The suggestion that structured water molecules play a crucial role in the catalytic mechanism can also rationalize the conservation of an aspartic acid (Asp59 in human GST P1-1) in the GST sequences, since this residue is part of the hydrogen-bond lattice connecting the two active sites of the dimeric protein. By the same token, the essential role of the α-carboxylate of the glutamyl group of glutathione can be understood. Thus, an explanation is provided for the essential contributions of the flexible helix α2 loop, Asp59 and the α-carboxylate of glutathione to catalysis, which have previously been difficult to rationalize.

Cooperativity Mechanism and Its Dependence on Temperature, Viscosity, and Mutations—Second, what is the mechanism of cooperativity? Based on results by others (21, 47) and by us, we suggest that wild-type GSTP1-1 at 30 °C allows both the negative and the positive cooperativity conformations to be present at equilibrium in equal concentrations (Fig. 7), and the result is apparent noncooperativity between two subunit of the enzyme. This equilibrium shifts to the positive cooperative conformation with increasing temperature or decreasing the medium viscosity giving positive cooperativity and shifts to the negative cooperative conformation with decreasing temperature or increasing the medium viscosity giving negative cooperativity (Fig. 2A and Table III). The effect of GSH on mutant Y50A, is toward the negative cooperativity conformation which has low GSH affinity, and which is unaffected by temperature change (Fig. 2C and Table III). For the heterodimer GSTP1/

Y50A, the equilibrium is shifted toward the negative cooperativity conformation at 30 °C while the two conformations are equal at 40 °C (Fig. 2B and Table III). Changes in cooperative behavior have also been observed for mutants C48S and C48A, in which the positive cooperativity conformation was predominant but the equilibrium shifted to the negative cooperativity conformation with decreasing of temperature (21), or with increasing of medium viscosity (47). Fig. 7, displaying alternative subunit conformation in equilibrium, is supported by studies of Ricci and coworkers (24) suggesting that wild-type GSTP1-1 as a whole somewhat behaves as a thermodynamic system which obeys the Le Chatelier principle. In fact, whenever a physical factor like temperature or viscosity forces the G-site to assume a low affinity conformation for GSH binding, GSTP1-1 opposes this perturbation by developing positive cooperativity and vice versa. On the other hand, it is noteworthy that the change of cooperativity caused by a mutation may depend on the position of the mutation rather than on the type of amino acid at this position. For example, negative cooperativity was induced by mutation Y50F (24) and Y50A and positive cooperativity was induced by C48S and C48A (21). Thus, further research will be needed to clarify this phenomenon in GSTP1-1.

In conclusion, based on the present study, the key residue is not the strongest contributor in the dimerization of hGSTP1-1 even though it is important for thermal stability of the dimer. Also, helix α2 plays an important role in catalysis through shifting the bound GSH from the pre-catalysis to the catalysis position. Positioning of the key residue of one subunit inside the lock of the other subunit stabilizes helix α2 and shifts bound GSH to the center of catalysis. Thus, a catalytically active monomer does not exist and mutation of the key residue that dislocates helix α2 decreases the rate of catalysis. Furthermore, through GSH binding to the active site water molecules are organized into a double-stranded lattice, which extends between two active sites. These water molecules are suggested to play a crucial role in the catalytic mechanism, and the effect of GSH binding can be communicated from one active site to another through this water network in a cooperative manner.

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