Structural Flexibility Modulates the Activity of Human Glutathione Transferase P1-1

ROLE OF HELIX 2 FLEXIBILITY IN THE CATALYTIC MECHANISM*

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Pre-steady-state and steady-state kinetic studies performed on human glutathione transferase P1-1 (EC 2.5.1.18) with 1-chloro-2,4-dinitrobenzene as co-substrate indicate that the rate-determining step is a physical event that occurs after binding of the two substrates and before the α-complex formation. It may be a structural transition involving the ternary complex. This event can be related to diffusion-controlled motions of protein portions as $k_{cat}/K_m$ linearly increases by raising the relative viscosity of the solution. Similar viscosity dependence has been observed for $K_m^{SSH}$, while $K_m^{DNB}$ is independent. No change of the enzyme structure by viscosogen has been found by circular dichroism analysis. Thus, $k_{cat}$ and $K_m^{SSH}$ seem to be related to the frequency and extent of enzyme structural motions modulated by viscosity. Interestingly, the reactivity of Cys-47 which can act as a probe for the flexibility of helix 2 is also modulated by viscosity. Its viscosity dependence parallels that observed for $K_m^{SSH}$, thereby suggesting a possible correlation between $k_{cat}$, $K_m^{SSH}$, and diffusion-controlled motion of helix 2. The effect of the kinetic parameters of C47S and C47S/C101S mutants confirms the involvement of helix 2 motions in the modulation of $K_m^{SSH}$, whereas a similar role on $k_{cat}$ cannot be ascertained unequivocally. The flexibility of helix 2 modulates also the homotropic behavior of GSH in these mutants. Furthermore, fluorescence experiments support a structural motion of about 4Å occurring between helix 2 and helix 4 when GSH binds to the G-site.

Human placental glutathione transferase P1-1 (GST)† (EC 2.5.1.18) is a dimeric enzyme composed of two identical subunits each containing one binding site for GSH (G-site) and a second binding site for the electrophilic co-substrate (H-site). Inspection of the three-dimensional structure indicates the presence near the G-site of the irregular $\alpha$ helix 2 (residues 37–46) which is exposed to the solvent (1). Lys-44, a part of this helix, is involved in the binding of GSH. At the end of helix 2 is Cys-47 which is probably linked by ion pair formation with Lys-54. This electrostatic interaction seems important for the correct spatial arrangement of the G-site (2); lack of this bond by replacement of Cys-47 or Lys-54 with Ser or Ala lowers the affinity for GSH and triggers a positive cooperativity toward the binding of GSH (3, 4). We therefore suggested that Cys-47 acts as a hinge which limits the extent or frequency of conformational transitions involving helix 2 (3, 4). In its absence, helix 2 would become more flexible and contact the adjacent subunit via helix 4 thereby inducing the observed cooperativity (4). Several pieces of evidence indicate that the irregular $\alpha$ helix 2 is a flexible region even in the wild-type enzyme; it displays the highest temperature factors among all other regions of domain I (5); moreover, it offers the sole point of attack (Lys-44) for the proteolytic cleavage by trypsin (5). Finally, the strongest evidence for large motions of helix 2 is the disulfide bond formation between Cys-101 and Cys-47, which are 18Å apart in the reduced enzyme complexed with S-hexylglutathione (6).

Is the catalytic efficiency of GST P1-1 related, in some way, to flexibility and fluctuations of some portions of the enzyme and, in particular, of the $\alpha$ helix 2? This paper and the following one (7) explore this hypothesis.

It is well known that polymers such as proteins undergo significant fluctuations at room temperature. The x-ray structure of an enzyme represents the average of atomic positions, but atoms and portions of the molecule often exhibit motions of sizable amplitudes about these averages. Obviously, this phenomenon is more emphatic in solution than in the crystal. In a number of enzymes, not only local side chain or loop motions have been observed but also larger scale motions involving helix, domain, and subunit (8). The presence of such fluctuations implies that a protein displays a range of transient conformations, sometimes quite different from the average structure. For a number of enzymes, it has been also demonstrated that these motions play a significant role in the catalytic mechanism (8). In the case of GST, dynamics of this enzyme and its relation to the catalytic mechanism have never been investigated. At present, the static picture of the four isoenzymes (Alpha, Mu, Pi, and Theta) supplied by x-ray data does not allow such correlation. Data in this paper, obtained at pH 6.5 by using 1-chloro-2,4-dinitrobenzene (CDNB) as co-substrate, point out a connection between diffusion-controlled motions of protein regions, in particular of helix 2, and the catalytic parameters of GST P1-1. Evidence is also given that, in solution, the structure of this enzyme in complex with GSH is different from that of the apoenzyme.
EXPERIMENTAL PROCEDURES

Materials—Human placenta GST P1-1 wild-type, C47S mutant, and C47S/C101S double mutant were expressed in Escherichia coli and purified as described previously (4). Glyceral was a BDH product. GSH, CDNB, and 1-fluoro-2,4-dinitrobenzene (FDNB) were purchased from Sigma. CDNB was dissolved in ethanol. S-(2,4-Dinitrophenyl) glutathione (GS-DNP) was synthesized as previously reported (5).

Spectrophotometric Measurements—Steady-state kinetics of GST P1-1 with CDNB as co-substrate was measured at 340 nm where the GS-DNP product absorbs (ε412nm = 9.6 mmol−1 cm−1) (9). Measurements were performed with a double-beam UVICORD 940 spectrophotometer (Kontron Instruments) equipped with a cuvette holder fixed at 25 °C. Kinetic experiments were done in 1 ml (final volume) of 0.1 M potassium phosphate buffer, pH 6.5, containing 0.1–0.3 μg of GST and variable amounts of substrates. The reaction rates were measured at 3-s intervals for a total period of 12 s. Initial rates were determined by linear regression and corrected for the spontaneous reaction. kcat and K<sub>CDNB</sub> values for wild type, and Cys-47 mutants were calculated at fixed GSH concentration (10 mM) and variable CDNB concentrations (from 50 μM to 1 mM). K<sub>CDNB</sub><sup>1000</sup> value for wild type was obtained at fixed CDNB concentration (1 mM) and variable GSH concentrations (from 50 μM to 1 mM). Steady-state kinetic mechanism at pH 6.5 was studied by varying CDNB and GSH from 50 μM to 1 mM over a matrix of 36 substrate concentrations.

Stopped-flow Analysis—Stopped-flow measurements were performed on a stopped-flow spectrophotometer consisting of a High-Tech SHU-51 rapid mixing device thermostatted at 25 °C equipped with a Jasco J 600 spectrophotometer. Light path of the cell was 0.2 cm. Dead-time of the instrument was ~3 ms. Presteady-state analysis was performed by rapid mixing of GST P1-1 (150 μM active sites) in 0.1 M potassium phosphate buffer, pH 6.5, containing 1 mM GSH with an identical volume of 2 mM CDNB in the same buffer. Blank was done in the same conditions without GST. The increase of absorbance at 340 nm was monitored every 0.5 ms.

Role of Helix 2 Flexibility in the Catalytic Mechanism

Cooperativity—The cooperativity of Cys-47 mutants of GST P1-1 toward GSH was assayed by following the dependence of the enzymatic rate upon GSH concentration (from 10 μM to 10 mM) at constant CDNB concentration (1 mM) in 0.1 M potassium phosphate buffer, pH 6.5 (25 °C). Kinetic data were analyzed by the KaleidaGraph (version 2.0.2) (Abelbeck Software) computer program and fitted to a rate equation expressing cooperativity as previously reported (3). The best fit fulfills [GSH]<sup>kcat</sup> and Hill coefficient (nH).

Viscosity Dependence of Kinetic Parameters—The effect of viscosity on kinetic parameters and cooperativity was assayed at 25 °C by using 0.1 M potassium phosphate buffers containing variable glycerol or sucrose concentrations. Viscosity values (η) were calculated as described (22) and randomly controlled with an Ostwald viscometer. Viscosities are reported relative to that of 0.1 M potassium phosphate buffer, pH 6.5 (η<sub>0</sub>). CD spectra of GST P1-1 at pH 6.5 in the presence (η<sub>η</sub>) or absence of glycerol were obtained by means of a Jasco J 600 spectrophotometer.

Reactivity of Cys-47—The reactivity of Cys-47 toward 2,2′-dithiobis(2-nitrobenzoic acid) (DTNB) was assayed at 25 °C by following the increase of absorbance at 412 nm where the thionitrobenzoate ion absorbs (ε412nm = 13.6 mmol−1 cm−1) (10). A typical incubation mixture contained 4.4 μM (active site) GST P1-1 and 0.2 mM DTNB in 0.1 M sodium acetate buffer, pH 5.0. At this pH value only Cys-47 and Cys-101 react with DTNB, but Cys-47 is about 10-fold more reactive than Cys-101 (6); thus, the second-order kinetic constants (k<sub>DTNB</sub>)<sub>cys</sub> calculated from the initial slopes at 412 nm at variable DTNB concentrations (from 0.1 to 100 μM) may be considered as approximate (Cys-101) molecules (10). Similar incubation mixtures were performed on C47S and C101S to calculate k<sub>DTNB</sub><sup>C47S</sup> and k<sub>DTNB</sub><sup>C101S</sup> for Cys-101 and Cys-47, respectively. The second-order kinetic constant for the reaction between free cysteine and DTNB (k<sub>DTNB</sub><sup>Cys</sup>) was obtained in 0.1 M sodium acetate buffer, pH 5.0, at fixed cysteine concentration (10 μM) and variable DTNB (from 0.1 to 0.5 mM). Phosphoryl-first-order kinetic constant for the oxidation involving Cys-47 and Cys-101 (k<sub>cat</sub>)<sub>cys</sub> was obtained by following the inactivation of GST P1-1 (10 μM) incubated at 25 °C in 0.1 M potassium phosphate buffer, pH 8.0, in the presence of 2 μM CuSO<sub>4</sub>. In similar conditions, the pseudo-first-order kinetic constant (k<sub>dt</sub>)<sub>cys</sub> for the oxidation of free cysteine (10 μM) was also calculated.

Fluorescence Measurements—The viscosity effect on the dissociation constant of GST binding (K<sub>cat</sub><sup>CDNB</sup>)<sub>cys</sub> was evaluated by fluorometry. At different concentrations, binding of GST (varied from 10 μM to 1 mM) in 0.1 M potassium phosphate buffer, pH 6.5) was measured by intrinsic fluorescence quenching, as described previously (3). Fluorescence measurements were done with a Perkin-Elmer LS-5 fluorometer with a sample holder at 25 °C. Excitation was at 280 nm and the emission was at 340 nm.

GST labeled with 2-(4′-maleimidylanilino)naphtalene-6-sulfonic acid (MANS) was prepared by reacting 10 μM C47S mutant with 100 μM MANS in 0.1 M potassium phosphate buffer, pH 6.5. After 30 min at 25 °C, the excess of reagent was removed by a G-25 Sephadex column equilibrated with the same buffer. In a typical experiment, different amounts of GST (from 10 to 100 μM) were added to 0.34 μM C47S-MANS protein. Emission spectra were recorded after each addition on a single-photon-counting spectrofluorometer (Fluoromax, S.A. Instruments, Paris, France). The bandwidth of excitation and emission monochromators was D = 4 nm. All measurements were carried out at 25 °C. The efficiency of energy transfer, E<sup>θ</sup>, between donors (Trp-28 and Trp-38) and acceptor (MANS) was evaluated from the relative quantum yield of the donors (11) according to Equation 1

\[ E^\theta = 1 - F_D^0/F_{DA}^0 \] (Eq. 1)

where F<sub>D</sub> and F<sub>DA</sub> are the donors’ quantum yields in absence or in presence of the acceptor. A similar equation was used to evaluate the transfer efficiency in presence of GST, E<sup>θ</sup><sup>cat</sup>.

\[ E^\theta = 1 - F_D^0/F_{DA}^0 \] (Eq. 2)

The above equations may be written in terms of the distances between the donors and the acceptor in the two cases, (R<sup>θ</sup> and R<sup>θ</sup><sup>cat</sup>), respectively as

\[ E^\theta = \omega F_D^0/\alpha F_D^0 + (R^\theta)^2 \] (Eq. 3)

and

\[ E^\theta = \omega F_D^0/\alpha F_D^0 + (R^\theta)^2 \] (Eq. 4)

where the Ω and θ factors depend on chemico-physical parameters (i.e. the overlapping integral, the refraction index, and the relative donors-acceptor dipole orientation). Assuming that GST molecule has a small effect on these factors, Ω = θ = 1 so that the ratio between the Equations 3 and 4 yields the following relationship:

\[ R^\theta = \sqrt{\frac{E^\theta}{E^\theta - 1}} \] (Eq. 5)

From the last equation it is possible to evaluate in which percentage K, the mean distance between donors and acceptors, is affected when the substrate GST is added in solution.

RESULTS AND DISCUSSION

A Physical Process Is the Rate-limiting Step for GST P1-1—Nucleophilic aromatic substitution reactions proceed in solution via formation of a α-complex intermediate. Variation of the leaving group is a diagnostic procedure to define the rate-limiting step. In the uncatalyzed reaction between GSH and CDNB the second-order kinetic constant (k<sub>cat</sub>) increases about 48-fold by substituting the more electronegative F for CI in the aromatic reactant (Table I). In this case the rate-determining event is the formation of the α-complex intermediate. The same
reaction catalyzed by GST P1-1 is quite insensitive to the nature of the leaving group, giving a $k_{\text{cat}}^{\text{CDNB}}/k_{\text{cat}}^{\text{CNDB}}$ ratio of 1.4. Thus, the rate-limiting step of the enzyme-catalyzed reaction seems not to be related to a chemical event, i.e. the formation of the $\alpha$-complex or its decomposition. In the latter case a $k_{\text{cat}}^{\text{CDNB}}/k_{\text{cat}}^{\text{CNDB}}$ ratio < 1 is expected due to the stronger C-F bond. Previously, a physical rate-determining step has been claimed to occur in the reaction between GSH and 4-chloro-3-nitro-1-(trifluoromethyl) benzene catalyzed by the 3–3 isoenzyme from rat liver (Mu class) (12). In that case the product dissociation was suggested to be rate-limiting.

Stopped-flow Analysis—During an enzymatic reaction, the existence of a rate-limiting step occurring after the product formation must be signaled by a burst phase before the attainment of the steady state. This does not occur with GST P1-1, as shown in Fig. 1. This finding and the scarce effect of the leaving group suggest that the rate-limiting step occurs before the $\alpha$-complex formation. Moreover, the steady-state kinetics analysis performed at pH 6.5 (data not shown) confirms that the reaction proceeds by a rapid equilibrium random sequential bi-bi mechanism as already described by Ivanetich and Goold (13). Thus, the rate-limiting step must be localized between the ternary complex formation and the chemical event; it should be one or more conformational transitions occurring after the binding of the substrates.

Viscosity Effect on Kinetic Parameters—The viscosity variation method provides a means for checking the presence of a diffusion-limited component for an enzyme-catalyzed reaction. With very efficient enzymes in which the rate-determining step is the encounter of substrates with the enzyme ($k_{\text{cat}}/k_{\text{m}} = 10^7$–$10^{10}$ M$^{-1}$ s$^{-1}$), the second-order rate constant $k_{\text{cat}}/k_{\text{m}}$ decreases linearly by increasing the microviscosity of the medium as predicted by the Stokes-Einstein relationship (14). Plots of the inverse relative specificity constant $k_{\text{cat}}/k_{\text{m}} / k_{\text{cat}}/k_{\text{m}}$ against the relative viscosity ($\eta/\eta^o$) have slopes ranging from unity to a fractional number depending on whether the reaction is a strictly or partially diffusion-controlled process. Similar viscosity dependence must be observed for enzyme in which the rate-limiting step is a diffusion-controlled product release but, in this case, the unimolecular constant $k_{\text{cat}}$ is affected (12). A different behavior on viscosity has been found for rate-determining events involving diffusion-controlled motions of enzyme portions. As this is an intramolecular event, plots of $k_{\text{cat}}/k_{\text{cat}}$ against $\eta/\eta^o$ usually display slopes <1 because the solvent viscosity effect is damped by an internal friction of protein regions (15). Clearly, the effect of viscosogens on $k_{\text{cat}}$ may vary depending on its accessibility and then on its molecular hindrance. The viscosity effect on GST P1-1 with CDNB as co-substrate may be related to that latter case. Although GST P1-1 is far from a "perfect" enzyme ($k_{\text{cat}}/k_{\text{m}} = 5 \times 10^6$ M$^{-1}$ s$^{-1}$), its kinetic parameters are remarkably affected by viscosity (Fig. 2 and Table II). In the presence of glycerol as co-solvent, $k_{\text{cat}}/k_{\text{cat}}$ increases linearly with $\eta/\eta^o$ giving a slope of about 0.8, while the uncatalyzed reaction is fully viscosity-independent. $K_{\text{GST}}^{\text{GSH}}$ is also affected by glycerol; a linear plot of $K_{\text{m}} / K_{\text{m}}$ against $\eta/\eta^o$ was obtained with a slope of 0.61. On the contrary, no change has been observed for $K_{\text{m}}^{\text{CDNB}}$. The lowering of $K_{\text{m}}^{\text{GSH}}$ by viscosity really reflects a thermodynamic increased affinity; the decrease of $K_{\text{GST}}^{\text{GSH}}$ on viscosity measured by fluorescence quenching experiments, parallels approximately that of $K_{\text{m}}^{\text{GSH}}$ (Table II). A larger viscosogen such as sucrose also lowers both $k_{\text{cat}}$ and $K_{\text{m}}^{\text{GSH}}$ values, but less efficiently (Table II and Fig. 2).

These findings can be interpreted by a static picture in which viscosogens modify "aspecifically" the structure of the enzyme lowering both $k_{\text{cat}}$ and $K_{\text{m}}^{\text{GSH}}$ values. This possibility is not supported by CD spectra of GST P1-1 which, in the absence and in the presence of glycerol ($\eta/\eta^o = 3.8$), are indistinguishable either in the 210–240 or in the 250–300 nm regions (spectra not shown). More likely, we can assume a dynamic scenario in which $k_{\text{cat}}$ and $K_{\text{m}}^{\text{GSH}}$ are modulated by diffusion-controlled motions of one or more parts of the protein. Because of its high flexibility, helix 2 is a good candidate for such a phenomenon.

Reactivity of Cys-47 as Probe for Flexibility of Helix 2—Cys-47 is the most reactive among the four cysteinyl residues present in each subunit. At pH 5.0, DTNB reacts exclusively with Cys-47 and Cys-101, but the reaction with Cys-47 is about 10-fold faster (6). Cys-47 is located at the end of helix 2 with its sulfhydryl function probably linked in an ion pair with

![Fig. 1. Stopped-flow analysis of the enzymatic reaction by GST P1-1.](image)

Fig. 1. Stopped-flow analysis of the enzymatic reaction by GST P1-1. Time course of the enzymatic (a) and spontaneous (b) reactions between GSH and CDNB at pH 6.5 and 25°C. Assay conditions are reported under "Experimental Procedures." Time constant was 0.5 ms. In the figure values every 2 ms are reported.

![Fig. 2. Viscosity effect on kinetic parameters of GST P1-1.](image)

Fig. 2. Viscosity effect on kinetic parameters of GST P1-1. Dependence of the reciprocal of the relative turnover numbers ($k_{\text{cat}}^{\text{GSH}}/k_{\text{cat}}^{\text{GSH}}$) (○) and of the reciprocal of the relative Michaelis constants ($K_{\text{GST}}^{\text{GSH}}/K_{\text{m}}^{\text{GSH}}$) (●) on the relative viscosity ($\eta/\eta^o$) with glycerol as co-solvent. Open symbols represent the experiments with sucrose as co-solvent. Viscosity dependence of the reciprocal of the relative second-order rate constants for the spontaneous reaction between GSH and CDNB at pH 6.5 ($K_{\text{m}}^{\text{CDNB}}/K_{\text{m}}^{\text{CDNB}}$) (■) with glycerol as co-solvent. Kinetic data for the enzymatic and spontaneous reactions were obtained as described under "Experimental Procedures." Slopes of linear fits are reported in Table II. Each experimental point is the mean of three determinations.
Lys-54 and placed in a hydrophobic pocket formed by the main chain atoms of Lys-44 and Gln-51 and side chain atoms of Trp-38 and Leu-52 (2). In the apoenzyme the accessibility of this residue may be related to the flexibility and frequency of spatial fluctuations of helix 2. Thus, we used Cys-47 as an intrinsic probe to assay the influence of viscosogens on diffusion-controlled motions of this protein region. As shown in Table II and Fig. 3, the reactivity of Cys-47 with DTNB is intrinsic probe to assay the influence of viscosogens on diffusion-dependent fluctuations possibly involving helix 2.

A more evident effect by viscosity was observed in the oxidative process in which Cys-47 must fluctuate greatly so as to approach Cys-101 (located on helix 4), which is 18 Å away in the reduced enzyme (1, 6). The pseudo-first-order kinetic constant for this reaction (kcat DTNB GST) is again linearly lowered by increasing viscosity while the oxidation process of free cysteine is viscosity-independent. A linear plot of kcat DTNB GST against r/ξς was obtained with a slope of 1.23 (Table II). This high value probably reflects a cumulative effect of viscosity on two different diffusion-dependent fluctuations possibly involving helix 2 and helix 4. In this context, it may be informative to observe the effect of an increased viscosity on the reactivity of Cys-47 and Cys-101 in the C101S and C47S mutants, respectively. Both Cys-47 and Cys-101 reactivities toward DTNB are remarkably affected by viscosity (Table III), thus suggesting also that helix 4 is involved in diffusion-controlled motions.

Viscosity Effect on Cooperativity of Cys-47 Mutants—Replacement of Cys-47 with Ser or Ala triggers a homotropic effect of an increased viscosity on the reactivity of Cys-47 and Cys-101 in the C101S and C47S mutants, respectively. Both Cys-47 and Cys-101 reactivities toward DTNB are remarkably affected by viscosity (Table III), thus suggesting also that helix 4 is involved in diffusion-controlled motions.

Table II: Viscosity effect on kinetic parameters and on Cys-47 reactivity

| Co-solvent | Glycerol, α | Sucrose, α |
|------------|------------|------------|
| kcat/kcat C47S CDNB | 0.01 ± 0.04 | 0.30 ± 0.02 |
| kcat/kcat GSH | 0.01 ± 0.01 | 0.02 ± 0.01 |
| kcat/kcat CDNB GST | 0.61 ± 0.05 | 0.34 ± 0.08 |
| kcat/kcat GSH | 0.90 ± 0.2 | 0.01 ± 0.1 |
| kcat/kcat CDNB GST | 0.03 ± 0.05 | 0.00 ± 0.01 |
| kcat/kcat GST | 1.23 ± 0.09 | 0.02 ± 0.02 |

*a are the slopes of the dependence of kinetic parameters on r/ξ (Table II) as derived from the best linear fit of the experimental data. kcat DTNB GST represents the second-order kinetic constant for the spontaneous reaction of GST with DTNB at pH 5.0: kcat GST represents the second-order kinetic constant for the reaction between GST and DTNB at pH 5.0 with glycerol as co-solvent (Table II). Kinetic data for enzymatic and spontaneous reactions were obtained as described under "Experimental Procedures." Slopes of linear fits are reported in Table III. Each experimental point is the mean of three determinations.

Viscosity effect on the reactivity of Cys-47. Viscosity dependence of the reciprocal of the relative second-order rate constants (kcat GST /kcat DTNB GST) for the reaction between Cys-47 and DTNB at pH 5.0 with glycerol as co-solvent (●) and sucrose as co-solvent (▲). Viscosity dependence of the reciprocal of the relative second-order rate constants (kcat GST /kcat DTNB GST) for the reaction between free cysteine and DTNB at pH 5.0 with glycerol as co-solvent (●). Data for enzymatic and spontaneous reactions were obtained as described under "Experimental Procedures." Slopes of linear fits are reported in Table II. Each experimental point is the mean of three determinations.

![Graph showing the effect of viscosity on the reactivity of Cys-47](image)

Table III: Viscosity effect on kinetic parameters and on the reactivity of Cys-47 and Cys-101 in some GST mutants

| Mutant | C47S, α | C101S, α | C47S/C101S, α |
|--------|--------|--------|---------------|
| C47S/C101S | 0.66 | 0.87 | 0.94 |
| C47S/C101S | 0.84 | 0.74 | 0.97 |
| C47S/C101S | 0.02 | 0.00 | 0.02 |
| C47S/C101S | 0.61 | 0.71 | 0.97 |

*a are the slopes of the dependence of kinetic parameters on r/ξ (glycerol as co-solvent) as derived from the best linear fit of the experimental data. Kinetic constants were calculated as described under "Experimental Procedures." kcat GST represents the second-order kinetic constant for the reaction between DTNB and Cys-101 (for C47S mutant) or Cys-47 (for C101S mutant) at pH 5.0.

Table IV: Viscosity effect on the positive cooperativity of Cys-47 mutants by GSH

| Relative viscosity | C47S | C47S/C101S |
|-------------------|------|------------|
| nH | 1.43 | 1.56 |
| nH | 1.10 | 1.02 |

*Experiments were performed in triplicate at pH 6.5 with and without glycerol as co-solvent as described under "Experimental Procedures." nH are the Hill coefficients calculated as described under "Experimental Procedures." Values at r/ξ = 1 are drawn from Ref. 3.

Viscosity effect on the positive cooperativity of Cys-47 mutants by GSH.

![Graph showing the effect of viscosity on the positive cooperativity of Cys-47 mutants](image)
GSH-complexed Alpha class GST, rules out this possibility in that case (16). We obtained a different result for GST P1-1 in solution. Evidence for conformational change upon GSH or GSH analogue binding has been previously deduced from the intrinsic fluorescence quenching and from changes in the near-UV CD spectrum (17). A more quantitative evidence for an induced-fit mechanism is now obtained by means of the fluorescent probe (MANS) linked on Cys-101 (helix 4) which is 18 Å from Trp-38 on the mobile helix 2 and 23 Å from Trp-28. These experiments have been performed on C47S mutant as any chemical modification on Cys-47 yields a complete inacti-

vation (18). The C47S-MIANS enzyme has a $k_{cat}$ value of 28 s$^{-1}$ and $K_m$ $\text{GSH}$ value of 13 mM. The fluorescence spectrum of C47S-MIANS is shown in Fig. 4. Upon excitation at 280 nm a residual tryptophanyl emission is present around 340 nm and a major emission peaks at about 425 nm indicating a very efficient energy transfer between the tryptophans and MIANS. When variable amounts of GSH are added in solution, a decrease of the fluorescence at 425 nm is observed with a concomitant increase of the emission at 340 nm (Fig. 4). In Table V both the quantum yields and the transfer efficiencies are reported. According to the Förster theory of energy transfer (11), a decrease in the transfer efficiency corresponds to an increase in the distance R between the donor and the acceptor. Using the equations reported under “Experimental Procedures” we evaluated a ratio $R^*P^* = 1.25$ between the Förster mean distance in presence ($R^*$) or in absence ($R^0$) of GSH. It follows that upon GSH binding the distance between MIANS (helix 4) and tryptophans increases 4.5, 3.5, or 4.0 Å depending whether the energy donors are Trp-28, Trp-38, or both, respectively.

Concluding Remarks—This paper describes the first investigation on GST that relates structural fluctuations of this protein with its catalytic function. The three-dimensional structure of GST P1-1 showing the relative positions of residues referenced in this paper is reported in Fig. 5.

On the basis of presteady-state and steady-state kinetic experiments, the rate-determining step of the catalyzed reaction between GSH and CDNB must be localized between the ternary complex formation and the chemical event. For the sake of simplicity we assume this event(s) to be a single conformational

**TABLE V Fluorometry data**

| Sample       | $\Phi$ (%)$^a$ | $E$ (%)$^b$ |
|--------------|----------------|------------|
| C47S         | 100            |            |
| C47S + GSH   | 86.4           |            |
| C47S-MIANS   | 38.4           | 61.6       |
| C47S-MIANS + GSH | 65.5       | 24.2       |

$^a$ $\Phi$ is the relative intrinsic fluorescence quantum yield.

$^b$ $E$ is the transfer efficiency. Experimental details are reported under “Experimental Procedures.”

In this context, GST P1-1 behaves differently both from the Mu class 4-4 isoenzyme where the $\alpha$-complex formation has been demonstrated to be rate-limiting (19) and from the 3-3 isoenzyme in which the dissociation of products is probably the rate-determining step (12).

The viscosity variation procedure indicates that $k_{cat}$ and $K_m^{GSH}$ are modulated by diffusion-controlled motions of the protein. As the $k_{cat}$ value for GST P1-1 is 76 s$^{-1}$ (Table I), far from that expected for diffusion controlled kinetics, we must assume that the attainment of the kinetically productive conformation is a rare event among a number of fast diffusion-controlled motions. Sucrose, a larger viscosogen, lowers again $K_m^{GSH}$ and $k_{cat}$ values but less efficiently than glycerol. This may be due to a lesser possibility of sucrose to reach the inside protein pockets.

The reactivity of Cys-47, which is located at the end of helix 2, has been used to probe possible diffusional-controlled fluctuations of helix 2. The increase of viscosity by glycerol lowers the reactivity of Cys-47 toward DTNB (Table II). A similar effect by viscosity has been observed in the oxidative process involving Cys-47 and Cys-101 residues. Interestingly, this viscosity effect on Cys-47 reactivity parallels that observed on $K_m^{GSH}$ and $k_{cat}$. The fascinating hypothesis that both $k_{cat}$ and $K_m^{GSH}$ are modulated by diffusion-controlled motions of helix 2.
cannot be unequivocally demonstrated because other parts of the enzyme display diffusion-controlled motions (i.e. helix 4) as suggested by the viscosity-dependence of Cys-101 reactivity in C47S mutant enzyme.

In Cys-47 mutants the increased flexibility of helix 2, which triggers the kinetic and binding cooperativity of GSH, is viscosity-dependent and appears diffusion-controlled. At high \( n/\eta^0 \) values, the positive cooperativity almost disappears for C47S single mutant and C47S/C101S double mutant with a concomitant decrease of \( [S]_{0.5}^{GSH} \) and \( k_{cat} \) values (Tables III and IV). Thus, the suggested relationship between cooperativity and flexibility of helix 2 (3) is further confirmed.

Functionally important dynamics of GST P1-1 were also suggested by an increase in the distance between helix 2 and helix 4 (about 4 Å) upon GSH binding as calculated by means of fluorescence experiments with MIANS.

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