Flexibility of Helix 2 in the Human Glutathione Transferase P1-1
TIME-RESOLVED FLUORESCENCE SPECTROSCOPY*

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Time-resolved fluorescence spectroscopy and site-directed mutagenesis have been used to probe the flexibility of α-helix 2 (residues 35–46) in the apo structure of the human glutathione transferase P1-1 (EC 2.5.1.18) as well as in the binary complex with the natural substrate glutathione. Trp-38, which resides on helix 2, has been exploited as an intrinsic fluorescent probe of the dynamics of this region. A Trp-28 mutant enzyme was studied in which the second tryptophan of glutathione transferase P1-1 is replaced by histidine. Time-resolved fluorescence data indicate that, in the absence of glutathione, the apoenzyme exists in at least two different families of conformational states. The first one (38% of the total population) corresponds to a number of slightly different conformations of helix 2, in which Trp-38 resides in a polar environment showing an average emission wavelength of 350 nm. The second one (62% of the total population) displays an emission centered at 320 nm, thus suggesting a quite apolar environment near Trp-38. The interconversion between these two conformations is much slower than 1 ns. In the presence of saturating glutathione concentrations, the equilibrium is shifted toward the apolar component, which is now 83% of the total population. The polar conformers, on the other hand, do not change their average decay lifetime, but the distribution becomes wider, indicating a slightly increased rigidity. These data suggest a central role of conformational transitions in the binding mechanism, and are consistent with NMR data (Nicotra, M., Paci, M., Sette, M., Oakley, A. J., Parker, M. W., Lo Bello, M., Caccurì, A. M., Federici, G., and Ricci, G. (1998) Biochemistry 37, 3020–3027) and pre-steady state kinetic experiments (Caccurì, A. M., Lo Bello, M., Nuccetelli, M., Nicotra, M., Rossi, P., Antonini, G., Federici, G., and Ricci, G. (1998) Biochemistry 37, 3028–3034) indicating the existence of a pre-complex in which GSH is not firmly bound to the active site.

Cytosolic glutathione transferases (GSTs; EC 2.5.1.18)³ are a family of dimeric isoenzymes which catalyze the addition of the natural tripeptide glutathione (GSH) to a variety of organic compounds which contain an electrophilic center (1), so they have a crucial role in the cellular catabolism and transport of toxic organic compounds (2). They are grouped into at least five isoenzyme classes (Alpha, Mu, Pi, Theta, and Sigma) which have a similar molecular mass (46–48 kDa) but different amino acid sequence and substrate specificity (3–6).

An important contribution to the characterization of these isoenzymes is due to the definition of the three-dimensional structure of representative isoenzymes, obtained by x-ray crystallography (7–12). Despite the low homology of amino acid sequence for GSTs belonging to different classes, all GST isoenzymes show very similar tertiary and quaternary structures and quite identical GSH-binding sites (G-site) (13). Crystallographic data provide details about enzyme-substrate interactions and catalysis. In fact, some snapshots are available which define the apoenzyme structure, the enzyme-GSH complex, the enzyme-product complex, and also the three-dimensional structure of the enzyme in complex with a transition-state analogue (14). Nevertheless, a number of unclarified questions about the catalytic mechanism (i.e. the controversies about the induced fit mechanism and the unexplained mechanism of GSH ionization at the active site) suggest that these “static” pictures are not sufficient to compose a movie which reveals all details of the binding and catalytic events.

Investigations about the dynamics of GSTs may provide the explanatory key for many unsolved questions and, in this context, the human GST P1-1 appears an interesting model. In fact, several pieces of evidence suggest that conformational motions of this enzyme have a crucial role both in the binding of substrates and in the catalytic mechanism. More precisely, the flexibility of helix 2 seems to modulate the G-site affinity for GSH (15); helix 4 motions are possibly involved more directly in the catalytic event which is rate-limited by a structural transition of the ternary complex (16).

In this paper we focus on the dynamics of helix 2 by means of time-resolved fluorescence to probe its relevance in the GSH-binding step. The irregular α-helix 2 (residues 35–46) displays the highest thermal factors of domain 1 and Trp-38 and Lys-44, which reside in this protein segment, are involved in the GSH binding by hydrogen bond interactions (10) (see Fig. 1). Its high flexibility is also suggested by a number of observations including the facile disulfide bond formation between Cys-47 and Cys-101, which appear about 18 Å apart in the crystal structure (17). Moreover helix 2 moves about 4 Å away from helix 4 when GSH binds to the G-site (15). Mutation of Cys-47, which resides at the end of helix 2 and probably modulates its flexibility by an electrostatic interaction with Lys-54, causes an increased mobility of this loop, a lowered affinity for GSH, and

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† The abbreviations used are: GST, glutathione transferase; NATA, N-acetylcysteaminamide.
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Fig. 1. Ribbon representation of the GST P1-1 monomer in complex with GSH. Trp-28 and Trp-38 (red) and GSH (green) are shown using stick representation. This figure was created with the program MOLMOL (37).

strong positive cooperativity toward GSH (18, 19). Similar kinetic changes were recently observed by replacing Gly-41, a possible joint residue for this helix, thus confirming that any critical point mutation perturbing the flexibility on this helix may be crucial for the binding process and for the intersubunit structural communication. Recent data also suggest that the segmental motions involving helix 2 are fast enough to be strongly modulated by the viscosity of the solution (15). However, no data are available for a more precise time scale in which these motions occur as well as for the trajectory of this segment and the thermodynamic parameters which define this flexibility. Another important question is how the movements of this segment have an active role in modulating the interaction of the enzyme with the substrate.

A method currently used to monitor the dynamics of proteins is time-resolved fluorescence spectroscopy. The versatility of this approach to investigate the motions of protein segments is well documented (20) and arises from its intrinsic “kinetic” character. The fluorescence properties of the protein tryptophans are modulated by a number of interactions with the protein environment and with the solvent which occur only in the lifetime of their excited state (about 1 ns). So, fast conformational transitions which occur in or below this time frame and which also involve tryptophan residues can be revealed and studied. Furthermore, the existence of different conformations that interconvert in times slower than the fluorescence lifetime results in a non-exponential intensity decay, and thus can be detected. Recently, time-resolved fluorescence spectroscopy has been applied to study glutathione S-transferase A1-1 from rat (21); since this isoenzyme has a single Trp per subunit, located in the H-site, the intrinsic fluorescence was used to characterize the dynamics of that region. Another fluorescence study focused on the protonation state of Tyr-7 of the same isoenzyme (22). In the case of GST P1-1, two tryptophans are located in each monomer, Trp-28 and Trp-38. The latter is a good intrinsic probe for helix 2 transitions as it resides on this segment and interacts directly with the bound substrate (Fig. 1). By site-directed mutagenesis we expressed the enzyme with Trp-28 replaced by histidine. The mutated enzyme shows kinetic properties and a structure very similar to the native enzyme, so it is a useful model for the present investigation.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis—Mutants of human GST P1-1 were obtained by site-directed mutagenesis, carried out according to a previously described procedure (19). The single-stranded DNA template was generated using plasmid p18seq-1, which contains the full-length nucleotide sequence encoding for GST P1-1 in plasmid pEMBL18 (19). The following oligonucleotide was used as mutagenic primer for W28H: 5′-TCTCTCCTGTTGCTTGC. The nucleotide sequence of this mutant was checked by the dideoxy chain termination method. The mutated 780 base pairs SpH1-Sph1 DNA fragments from plasmid p18seq-1 were subcloned under control of trc promoter in expression plasmids expressing large amounts of recombinant enzyme in the cytoplasm of Escherichia coli cells (23). GST P1-1 and W28H mutant were purified by affinity chromatography on immobilized glutathione (24).

Kinetic Measurements—Kinetic parameters $k_{cat}$ and $K_m$ were determined by using 1-chloro-2,4-dinitrobenzene as co-substrate in 0.1 M potassium phosphate buffer, pH 6.5, as described previously (15).

Fluorescence Spectroscopy—Steady state fluorescence measurements were performed on a SPEX FluorMax photon counting spectrofluorometer (SPEX Industries Inc., Edison, NJ), at 20 °C, with a 4-nm band pass for both excitation and emission. Spectra were corrected for background signal, which was always <1% of the total intensity.

For time-resolved fluorescence measurements, a frequency domain ISS fluorometer (ISS Inc., Champaign, IL) was used. Excitation at 295 nm was provided by a cavity-dumped, externally frequency doubled rhodamine 6G dye-laser (model 700, Coherent), synchronously pumped by a mode-locked Nd-YAG laser (Antares model, Coherent, Palo Alto, CA). The absorbance of the samples at 295 nm was about 0.1–0.2. The enzyme sample was in 0.1 M potassium phosphate buffer, pH 6.5, containing 0.1 mM EDTA. Emission was collected through a WG320 filter (Corion) to remove any scatter light. In the wavelength-dependent lifetime measurements, instead, an emission monochromator (Jobin Yvon Y10, 4-nm band pass) was used. Phase-modulation lifetime measurements were carried out at 12 frequencies from 7 to 200 MHz. Color errors due to photomultiplier response were minimized by the use of reference standard solutions of p-terphenyl (Eastman Kodak, Rochester, NY) in ethanol (lifetime = 1.05 ns). All lifetimes measurements were carried out at “magic angle” configuration. The sample holder was thermostated and was kept at 20 °C, unless where it is otherwise specified.

Phase-modulation data were analyzed with the Globals Unlimited software (25), with different models, including one, two, or three exponential lifetimes, or continuous lifetime distributions of different shapes (see “Results”). Goodness of fit was assessed by the value of minimized $\chi^2$, which was calculated using standard deviations of $\pm 0.02$ ns and $\pm 0.004$ for phase angles and modulation ratios, respectively. Rigorous $\chi^2$-surface error analysis was performed to evaluate uncertainties of recovered fit parameters (26). Statistical evaluation and comparison between the different decay models was carried out using the Schwarz criterion (27).

RESULTS

Kinetic and Structural Properties of W28H Mutant—W28H mutant shows kinetic parameters very similar to those of the native enzyme. At 25 °C and in the presence of 1 mM 1-chloro-2,4-dinitrobenzene as co-substrate (in 0.1 M potassium phosphate buffer, pH 6.5), it has an apparent $K_m$ of $36 \pm 3 \times 10^{-4}$ M $(38 \pm 4$ s$^{-1}$ for wild type) and a $K_m$ value for GSH of $20 \pm 0.2$ mM (0.15 ± 0.01 mM for wild type). Far UV circular dichroism spectrum of the mutant (not shown) overlaps that of the native enzyme, suggesting that mutation does not cause any remarkable change in the structure of the enzyme.

Steady State Fluorescence Spectroscopy—Fig. 2 shows the fluorescence emission spectra (excitation 295 nm) of WT and W28H GST together with that of the reference compound N-acetyl-tryptophan-amide (NATA). The 295-nm excitation wavelength ex-
Judging from the compared with the 5 of the triple exponential and double single-exponential plus a continuous Lorentzian distribution of W28H mutant results from instrumental response, and suggests, in agreement with literature values (28). This rules out the yield of Trp-38 in the mutant, relative to NATA, is 0.30, therefore, if the W28H mutation does not change appreciably the protein concentration (0.1 mg/ml) the total fluorescence intensity is only 0.30 that of the wild type enzyme; there-

### Time-resolved Fluorescence of W28H—Phase-shift and de-modulation data relative to the fluorescence intensity decay of W28H at 20 °C are shown in Fig. 3. Data are analyzed with several decay models (Table I): single, double, and triple lifetime, continuous distributions of lifetimes, with Gaussian, uniform and Lorentzian shape, as well as the combination of a “discrete” lifetime with a continuous distribution and a double continuous distribution.

The single- and double-exponential models, as well as a single continuous distribution do not give an adequate fit of the decay of the W28H mutant (as evident from the high \( \chi^2 \) values). Judging from the \( \chi^2 \) values, the best fit is obtained with a single-exponential plus a continuous Lorentzian distribution of lifetimes. Furthermore, this model has only 4 free parameters, compared with the 5 of the triple exponential and double Lorentzian models, that also achieve good \( \chi^2 \) values. The superiority of the “Lorentzian plus exponential” model is quantified by the Schwarz criterion (27), which calculates a parameter that takes into account both the accuracy of the fit and the number of parameters utilized, and thus compares “non-nested” models with a different number of parameters. Again, the model with an exponential and a distribution gives the best value for this parameter. Finally, the double distribution model gives a near zero width of the distribution centered at a shorter time (0.005 is a lower limit imposed by the fitting program), making this model equivalent to the Lorentzian plus exponential one. Thus a good description of the fluorescence intensity decay of Trp-38 is given by a Lorentzian distribution of lifetimes, centered at 1.7 ± 0.1 ns, with a width of 1.0 ± 0.05 ns, plus a discrete lifetime (\( \tau = 0.42 \pm 0.03 \) ns). The molar fractions associated with the two components are 0.38 ± 0.03 and 0.62 ± 0.03, respectively.

A control measurement of the fluorescence decay of NATA, performed under identical conditions, is accurately described by a single-exponential fit (\( \chi^2 = 1.1 \)), with a value of 2.9 ± 0.1 ns, in agreement with literature values (28). This rules out the possibility that the heterogeneity in the fluorescence decay of W28H mutant results from instrumental response, and suggests that the heterogeneity arises from interaction of the tryptophan residue with the protein matrix. Comparison of the NATA fluorescence lifetime with the average lifetime obtained from the best fit of W28H fluorescence decay \( (0.83 \pm 0.07 \text{ ns}) \) gives a ratio of 0.29 ± 0.03 in perfect agreement with the ratio of steady state intensities. This is a further indication that the model used to describe the fluorescence decay is correct, and also shows that quenching of Trp-38 is only due to dynamic factors.

Even though it is impossible to associate a confidence probability to our model choice, since the so called “F test” can be applied only to “nested models” (27), a strong support to our hypothesis comes from a global analysis of all the time-resolved data collected. As described in the following sections, the fluorescence time decay of W28H GST P1-1 has been measured under various conditions, varying the temperature or the concentration of glutathione in solution. In all the experiments performed the Lorentzian plus exponential model gave the best fit, as judged both by the reduced \( \chi^2 \) and by the Schwarz
Temperature Effect—The fluorescence intensity time decay of W28H is studied as a function of temperature from 5 to 20 °C. Experiments at higher temperature are not carried out; even at 30 °C a small decrease of enzymatic activity is observed during the measurement time (about 30 min), possibly due to the enzyme exposure to laser excitation. The Lorentzian plus exponential model gives the best fit at all temperatures studied. The molar fractions of the two components are unchanged, while the two lifetimes are quenched, as a direct effect of the temperature increase (Table II). The width of the Lorentzian distribution increases with decreasing temperature, while the other component can be adequately described by a discrete lifetime at all the temperatures studied.

Fluorescence Decay-associated Spectra—The fluorescence intensity decay of the two components is also studied as a function of emission wavelength, from 305 to 365 nm, exciting at 295 nm. These wavelength dependent measurements can be used to determine the fluorescence spectra of individual lifetime components. The ideal situation for this kind of analysis is when one can distinguish experimentally a different decay lifetime for each conformer. In that case, one can assume that each lifetime has the same value at all the wavelengths studied (neglecting solvent relaxation problems); the only varying parameters are the fluorescence fractions associated to the lifetimes, that allow a reconstruction of the decay-associated spectra. Unfortunately, this approach cannot be applied in our case: if we assume that the parameters of the Lorentzian distribution and of the discrete lifetime are the same at all the wavelengths, the fit gives parameters comparable to those obtained without the monochromator but the $\chi^2$ values are rather high (Table III, part A). This is due to the fact that the above procedure is not correct for the lifetime distribution. The lifetimes of the conformers that constitute this component are so similar (and so many) that it is impossible to distinguish experimentally almost any distribution (29). Therefore, we performed the global fit of wavelength dependent data with four discrete lifetime: three of them were left free to vary, in order to model the distribution of varying shape, while the fourth one was forced to have the same value at all the wavelengths studied (since the problems described above do not affect the discrete lifetime of our Lorentzian plus exponential model). The results give acceptable $\chi^2$ values and the value for the short component is equal (within the experimental error) to that obtained with the other measurements (Table III, part B). Fit with four discrete components, of which three free and one (τ) linked. fi is the fraction of the total fluorescence relative to lifetime τ. For the errors on the parameters see Table I.

Influence of GSH Binding on the Time-resolved Fluorescence—To check the effect of the GSH binding on the two components of the fluorescence intensity decay, the W28H mutant was studied in the presence of different concentrations of glutathione. The results are shown in Table IV and Fig. 5, and indicate that the substrate quenches the discrete component and increases its molar fraction (not to be confused with the fraction of total fluorescence associated to this lifetime), from 62 to 83% (extrapolating at saturating GSH concentration). Conversely, it widens the Lorentzian distribution, without moving its center. To check for a possible aspecific intermolecular quenching by unbound glutathione, control measurements were performed with NATA; the glutathione concentrations used in the experiments above (up to 5 mM) do not cause any change in the NATA lifetime. Therefore the effects observed on protein fluorescence are caused only by bound glutathione.

Fluorescence quenching by GSH binding was also studied by analyzing the steady state spectra. The percentage of quenching as a function of glutathione concentration is displayed in Fig. 6, where both the data obtained from the average lifetime and the total steady state intensity were included. The two sets of data are comparable within the experimental error and, fitting them globally, a $K_D = 80 \pm 10 \mu M$ is obtained. The spectra also show a shift of their maximum from 344 to 341 nm.

### Table II

| T (°C) | c (ns) | w (ns) | $\alpha_{Lor}$ (ns) | $\tau$ (ns) | $\chi^2$ |
|-------|-------|-------|---------------------|-------|--------|
| 20    | 1.7   | 1.1   | 0.38                | 0.42  | 0.73   |
| 10    | 2.0   | 1.3   | 0.37                | 0.59  | 1.1    |
| 5     | 2.3   | 1.7   | 0.37                | 0.63  | 1.9    |

### Table III

| λ (nm) | $A^a$ | $\lambda$ (nm) | $B^b$ | $\chi^2$ |
|-------|-------|----------------|-------|--------|
| 305   | 0.64  | 11             |       |        |
| 315   | 0.45  | 7.9            |       |        |
| 325   | 0.34  | 1.1            |       |        |
| 335   | 0.30  | 20             |       |        |
| 350   | 0.16  | 13             |       |        |
| 365   | 0.14  | 31             |       |        |

$^a c$, 1.7 ns; w, 1.0 ns; $\tau$, 0.50 ns; $\chi^2$ tot = 13.

$^b \tau$, 0.41 ns; $\chi^2$ tot = 2.3.
by increasing GSH concentration. This is consistent with the finding that the two components observed in the fluorescence intensity decay have different spectra, with the short component (whose fraction increases with GSH concentration) peaked at shorter wavelengths than the other.

**DISCUSSION**

Time-resolved spectroscopy is a well established method to detect conformational heterogeneity in proteins. Tryptophan fluorescence is extremely sensitive to the surroundings of the fluorophore and, if the protein can assume multiple conformations, each of them has, in principle, a different fluorescence lifetime. Therefore conformational heterogeneity is reflected in a nonexponential intensity decay (30–32). In our case, the time behavior of the fluorescence intensity of W28H GST deviates markedly from a simple exponential, indicating the presence of different structures in solution. In more detail, the fluorescence data are consistent with a model with two lifetime components: a discrete lifetime and a continuous Lorentzian distribution of lifetimes. This is the simplest model consistent with all the collected data, but we are aware that the real situation could be even more complex and heterogeneous. According to this analysis, the environment of Trp-38 is sampling two families of conformations (corresponding to the two lifetime components). One of them is associated to a continuous distribution of lifetimes, and therefore must correspond to many (slightly) different structures. The interpretation of the other component is less straightforward, due to possible effects of conformational dynamics. When several substrates are present, any interconversion between them will influence the fluorescence parameters, depending on the relative magnitudes of fluorescence lifetime and interconversion rate. Let us first consider the two extreme cases: if the conformational fluctuations are much slower than the lifetime of the excited state, their effect on the fluorescence parameters can be neglected. If, on the other hand, the interconversion is much faster than the fluorescence lifetime, an averaging effect causes a single exponential decay. In all the intermediate cases, the recovered fluorescence lifetimes and associated fractions depend both on the parameters characteristic of the different conformational substates and on the interconversion rate (31, 33, 34). The decay becomes less heterogeneous as the dynamics becomes faster. Usually, possible effects of excited state dynamics can be detected by varying the temperature (and therefore the interconversion rate). In our experiments, the molar fractions of the two major components remain unchanged at all the temperatures studied. Therefore, the interconversion between them is always much slower than the fluorescence lifetimes (i.e. much slower than 10^{-9} s). On the other hand, the width of the Lorentzian distribution increases at lower temperatures, as a consequence of a slower conformational dynamics. Therefore, the interconversion between the structures that constitute this distribution must happen in times comparable with the fluorescence lifetime. For the discrete lifetime, several interpretations are possible: it could correspond either to a single structure, or to many conformations (with different lifetimes) interconverting very rapidly. The second hypothesis is not likely: lowering the temperature (and therefore slowing down the dynamics) the averaging effect of fast interconversions should have been reduced and the discrete lifetime should have become a distribution with appreciable width. Instead, it remained discrete at all the temperatures studied. Therefore, the discrete component seems to correspond to a single rigid structure (or to a set of conformations so similar to be indistinguishable on the basis of fluorescence lifetimes), even though the alternative hypothesis cannot be definitely discarded. The spectra associated to the two major decay components have a 30-nm difference between their centers of mass (see Fig. 4). This demonstrates that the two families of structures detected by the fluorescence time decay are definitely different also regarding the polarity of the Trp-38 environment (and possibly also its solvent accessibility).

In conclusion, time-resolved fluorescence data indicate that apo-GST is extremely mobile in the surroundings of Trp-38, adopting at least two families of conformations, that interconvert in times slower than 1 ns: one is formed by many slightly different structures, while the other probably corresponds to a more rigid conformation, in which Trp-38 is in a much less polar environment.

After glutathione binding, the two conformational families are still present, although the equilibrium is shifted toward the discrete lifetime component, whose molar fraction (extrapolated at saturating glutathione concentrations) increases to 83%. Furthermore, the fluorescence of this component is quenched, while the average lifetime of the Lorentzian distribution remains unchanged. On the other hand, the distribution becomes wider, as a consequence of a slower interconversion between conformations. Since both lifetime components are influenced

![Decay associated spectra](image)

**Fig. 4. Decay associated spectra.** Decay associated spectra of W28H (T = 20 °C, pH 6.5, λ_{exc} 295 nm). See text for the analysis method. ○, short component; ●, long component; no symbol, total spectrum.

**Table IV**

| C(GSH) | c | w | α_{err} | τ | χ² | Steady state quenching fraction | λ_{peak} | C.o.M. |
|-------|---|---|---------|---|----|---------------------------------|--------|-------|
| 0     | 1.7| 1.1| 0.38    | 0.42| 0.73| 0                               | 344    | 355.0 |
| 0.1   | 1.8| 1.3| 0.20    | 0.40| 0.87| 0.20                            | 343    | 353.9 |
| 0.5   | 1.7| 1.4| 0.25    | 0.32| 1.3 | 0.27                            | 341    | 353.4 |
| 1     | 1.8| 1.4| 0.20    | 0.35| 2.4 | 0.30                            | 341    | 353.1 |
| 5     | 1.7| 1.5| 0.19    | 0.33| 1.0 | 0.30                            | 341    | 353.1 |
in some way by the presence of glutathione, the substrate is likely bound to both conformational families. Anyway, its interaction with the protein is different in the two cases: it causes only an increased rigidity to the flexible, polar component, while it quenches the discrete lifetime fluorescence (either by direct interaction, or by an induced conformational change).

The other consequence of glutathione binding is a shift of the conformational equilibrium toward the structure corresponding to an apolar environment for Trp-38. In this sense we can say that fluorescence data demonstrate the presence of an induced fit in GST P1-1. It is interesting to correlate our results with recent NMR data (35) and pre-steady state experiments (36) regarding the same enzyme. Both strongly suggest that, at saturating GSH concentrations, an equilibrium exists between two forms: the final Michaelis complex \( (E^*zGSH) \) and a pre-complex \( (EzGSH) \) which represents a small fraction of the total enzyme; in the \( EzGSH \) conformation the protein fluorescence is not quenched, and glutathione is flexible and not firmly bound to the active site. The similarities of these results with our data are striking; therefore, we can tentatively associate the single exponential component of the fluorescence intensity decay at saturating substrate concentrations to the tightly bound complex and the Lorentzian distribution of lifetimes to the weakly bound pre-complex.

CONCLUSIONS

The results of this paper may be considered as a first quantitative approach to the conformational dynamics of GST P1-1, and demonstrate the role of structural fluctuations in the substrate binding mechanism. On the basis of the present fluorescence data and of other experimental findings (35, 36), we propose the following hypothesis: helix 2 fluctuates rapidly between two families of conformations (with times slower than nanoseconds but much faster than milliseconds) in the absence of substrate. GSH can enter the active site easily only in the flexible, solvent accessible conformation, forming the weakly bound pre-complex. At this stage, the GSH molecule shows an extended backbone conformation similar to that found in the crystal complex but its glutamyl moiety is not yet firmly anchored to the G-site (35). Then, a much slower conformational transition leads to the Michaelis complex, in which Trp-38 is also quenched. In this scenario, helix 2, which is flexible in the pre-complex and contacts the glycyl end of GSH with Trp-38 and Lys-44, could act as a driver for GSH to find the correct anchorage in the active site. A similar role has been suggested also on the basis of pre-steady state kinetic data (36). We are aware that Scheme 1 is just one of the possible mechanisms findings (NMR, kinetic and fluorescence data), is reported in Scheme 1.

In this scheme \( E_{nq} \) is the flexible non quenched population and \( E_q \) is the quenched conformer. \( k_q \) is the first order rate constant for the transition from \( E_{nq} \) to \( E_q \) and \( k_{-q} \) refers to the inverse reaction. Pre-steady state measurements (36) indicate that the rate-limiting step for protein fluorescence quenching by GSH binding is the transition from the pre-complex \( EzGSH \) to \( E^*zGSH \). Therefore, binding of GSH to \( E_q \) must be kinetically not relevant compared with binding to \( E_{nq} \), and it is not indicated; nevertheless, it could be thermodynamically possible. For the same reason, the interconversion between \( E_q \) and \( E_{nq} \) must be faster than the interconversion after GSH binding.

### Scheme 1

**Helix 2 Flexibility in Human Glutathione Transferase P1-1**

![Scheme 1](image1.png)

**FIG. 5. Effect of GSH binding on the fluorescence time decay.**

Fluorescence intensity decay parameters of W28H as a function of glutathione concentration (T = 20 °C, pH 6.5, \( \lambda_{exc} = 295 \text{ nm} \)). For parameters definitions see Table I.

**FIG. 6. Fluorescence quenching by GSH binding.**

W28H fluorescence quenching as a function of glutathione concentration (T = 20 °C, pH 6.5, \( \lambda_{exc} = 295 \text{ nm} \)). □, quenching efficiency as measured from time resolved fluorescence data; ○, quenching efficiency measured from steady state intensity.
which well fit with experimental data. Molecular dynamics simulations are in progress to define the binding process, more in detail.

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REFERENCES
1. Jakoby, W. B., and Habig, W. H. (1980) in Enzymatic Basis of Detoxification (Jakoby, W. B., ed) Vol. 2, pp. 63–94, Academic Press, New York
2. Armstrong, R. N. (1991) Chem. Res. Toxicol. 4, 131–139
3. Mannervik, B., Alin, P., Guthenberg, C., Jensson, H., Tahir, M. K., Warholm, M., and Jornvall, H. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 7202–7206
4. Meyer, D. J., Coles, B., Pembble S. E., Gilmore, K. S., Fraser, G. M., and Ketserer, B. (1993) Biochem. J. 324, 409–414
5. Meyer, D. J., and Thomas, M. (1995) Biochem. J. 311, 739–742
6. Bueltler, T. M., and Eaton, D. L. (1992) Environ. Carcinog. Ecotoxicol. Rev. 10, 181–203
7. Sinning, I., Kleweyget, G. J., Cowan, S. W., Reinemer, P., Dirr, H. W., Huber, R., Gilliland, G. L., Armstrong, R. N., Ji, X., Board, P. G., Olin, B., Mannervik, B., and Jones, T. A. (1993) J. Mol. Biol. 232, 192–212
8. Ji, X., Zhang, P., Armstrong, R. N., and Gilliland, G. L. (1992) Biochemistry 31, 10169–10184
9. Reinemer, P., Dirr, H. W., Ladenstein, R., Schaffer, J., Gallay, O., and Huber, R. (1991) EMBO J. 10, 1997–2005
10. Reinemer, P., Dirr, H. W., Ladenstein, R., Huber, R., Lo Bello, M., Federici, G., and Parker, M. W. (1992) J. Mol. Biol. 227, 214–226
11. Wilce, M. C. J., Board, P. G., Feil, S. C., and Parker, M. W. (1992) EMBO J. 11, 2133–2143
12. Ji, X., von Rosenvinge, E. C., Johnson, W. W., Tomarev, S. I., Piatigorsky, J., Armstrong, R. N., and Gilliland, G. L. (1993) Biochemistry 32, 12949–12954
13. Jin, X., Armstrong, R. N., and Gilliland, G. L. (1993) Biochemistry 32, 12949–12954
14. Jin, X., Armstrong, R. N., and Gilliland, G. L. (1993) Biochemistry 32, 12949–12954
15. Ricci, G., Caccure, A. M., Lo Bello, M., Rosato, N., Mei, G., Nicotra, M., Chiessi, E., Mazzeiti, A. P., and Federici, G. (1996) J. Biol. Chem. 271, 16187–16192
16. Caccure, A. M., Ascenzi, P., Antonini, G., Parker, M. W., Oakley, A. J., Chiessi, E., Nuccetelli, M., Battistoni, A., Bellizia, A., and Ricci, G. (1996) J. Biol. Chem. 271, 16193–16198
17. Ricci, G., Del Boccio, G., Pennelli, A., Lo Bello, M., Petruzzelli, R., Caccure, A. M., Barra, D., and Federici, G. (1991) J. Biol. Chem. 266, 24409–24415
18. Ricci, G., Lo Bello, M., Caccure, A. M., Pastore, A., Nuccetelli, M., Parker, M. W., and Federici, G. (1995) J. Biol. Chem. 270, 1243–1248
19. Lo Bello, M., Battistoni, A., Mazzaeti, A. P., Board, P. G., Muramatsu, M., Federici, G., and Ricci, G. (1995) J. Biol. Chem. 270, 1249–1253
20. Eftink M. R., (1991) Methods of Biochemical Analysis 35, 127–205
21. Wang, R. W., Bird, A. W., Newton, D. J., Lu, A. Y. H., and Atkins, W. M. (1993) Protein Sci. 2, 2085–2094
22. Dietze, E. C., Wang, R. W., Lu, A. Y. H., and Atkins, W. M. (1996) Biochemistry 35, 6745–6753
23. Battistoni, A., Mazzeiti, A. P., Petruzzelli, R., Muramatsu, M., Federici, G., Ricci, G., and Lo Bello, M. (1995) Protein Express. Purif. 6, 579–587
24. Simons, P., and Vander Jagt, D. L. (1981) Methods Enzymol. 77, 235–237
25. Beechem, J. M., Gratton E., Ameloot M., Kнутson J. R. and Brand L. (1991) in Topics in Fluorescence Spectroscopy. (Lakowicz, J. R., ed) Vol. 2, pp. 241–305, Plenum Press, New York
26. Beechem J. M., and Gratton E. (1988) Proc. SPIE Int. Soc. Opt. Eng. 909, 70–81
27. Landaw, E. M., and Di Stefano, J. J. (1984) Am. J. Physiol. 246, R665–R667
28. Bismuto, E., and Irace, G. (1988) Photochem. Photobiol. 50, 165–168
29. Ferris, F. R., and Gratton E. (1988) Biophys. J. 56, 1185–1196
30. Alcalá, J. R., Gratton E., and Prendergast, F. (1987) Biophys. J. 51, 587–596
31. Alcalá, J. R., Gratton E., and Prendergast, F. (1987) Biophys. J. 51, 957–964
32. Alcalá, J. R., Gratton E., and Prendergast, F. (1987) Biophys. J. 51, 925–936
33. Gratton, E., Alcalá, J. R., and Prendergast, F. (1989) in Fluorescent biomol-ecules (Knutson J. R., ed) pp. 17–32, Plenum Press, New York
34. McGowan, L. P., Yu, H. T., Vela, M. A., Morales, G. A., Shui, L., Franckez, F. R., McLaughlin, M. L., and Barkley, M. D. (1997) J. Phys. Chem. B 101, 3289–3280
35. Nicotra, M., Pacci, M., Sette, M., Oakley, A. J., Parker, M. W., Lo Bello, M., Caccure, A. M., Federici, G., and Ricci, G. (1998) Biochemistry 37, 3020–3027
36. Caccure, A. M., Lo Bello, M., Nuccetelli, M., Nicotra, M., Rossi, P., Antonini, G., Federici, G., and Ricci, G. (1998) Biochemistry 37, 3028–3034
37. Koradi, R., Billeter, M., and Wuthrich, K. (1996) J. Mol. Graph. 14, 51–55