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

INFLUENCE OF A POOR CO-SUBSTRATE ON DYNAMICS AND KINETICS OF HUMAN GLUTATHIONE TRANSFERASE

Anna Maria Caccuri†, Paolo Ascenzi‡, Giovanni Antonini§, Michael W. Parker#, Aaron J. Oakley&&, Ester Chiessi***, Marzia Nuccetelli†, Andrea Battiston†, Anna Bellizia†, and Giorgio Ricci‡‡‡

From the †Department of Biology, University of Rome "Tor Vergata," 00133 Rome, Italy, ‡Department of Pure and Applied Biology, University of L'Aquila, 67010 L'Aquila, Italy, §Department of Pure and Applied Biology, University of L'Aquila, 67010 L'Aquila, Italy, ‡‡‡Department of Chemical Sciences and Technology, University of Rome "Tor Vergata," 00133 Rome, Italy

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Presteady-state and steady-state kinetics of human glutathione transferase P1-1 (EC 2.5.1.18) have been studied at pH 5.0 by using 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole, a poor co-substrate for this isoenzyme. Steady-state kinetics fits well with the simplest rapid equilibrium random sequential bi-bi mechanism and reveals a strong intrasubunit synergistic modulation between the GSH-binding site (G-site) and the hydrophobic binding site for the co-substrate (H-site); the affinity of the G-site for GSH increases about 30 times at saturating co-substrate and vice versa. Presteady-state experiments and thermodynamic data indicate that the rate-limiting step is a physical event and, probably, a structural transition of the ternary complex. Similar to that observed with 1-chloro-2,4-dinitrobenzene (Ricci, G., Caccuri, A. M., Lo Bello, M., Rosato, N., Mei, G., Nico- trava, M., Chiessi, E., Mazzetti, A. P., and Federici, G. (1996) J. Biol. Chem. 271, 16187-16192), this event may be related to the frequency of enzyme motions. The observed low, viscosity-independent $k_{cat}$ value suggests that these motions are slow and diffusion-independent for an increased internal viscosity. In fact, molecular modeling suggests that the hydroxyl group of Tyr-108, which resides in helix 4, may be in hydrogen bonding distance of the oxygen atom of this new substrate, thus yielding a less flexible H-site. This effect might be transmitted to the G-site via helix 4. In addition, a new homotropic behavior exhibited by 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole is found in Cys-47 mutants revealing a structural intersubunit communication between the two H-sites.

The rate-limiting step for the enzymatic conjugation of GSH with 1-chloro-2,4-dinitrobenzene (CDNB)1 by human placental glutathione transferase (GST P1-1) (E.C. 2.5.1.18) has been suggested to be one or more conformational transitions of the ternary complex (1). Kinetic parameters may be related to diffusion-controlled protein motions as $k_{cat}$ and $K_m$ are affected by the co-solvent viscosity. In Cys-47 mutants, the extent of the positive cooperativity toward GSH, $K_{cat}$, and $[S]_{0.5}$ values can be also related to diffusion-controlled motions of protein regions. These results suggest a dynamic scenario in which motions of helix 2 (residues 37–46) may be involved (1). In this paper new findings, obtained by using the alternative co-substrate 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl), indicate that also the flexibility of helix 4 (residues 83–109) may play a relevant role. NBD-Cl is a very active substrate for the Alpha class GSTs ($k_{cat}$ NBD-Cl = $k_{cat}$ CDNB) (2) but a "poor" substrate for the Pi and Mu class isoenzymes having $k_{cat}$ NBD-Cl values only 2–4% of those found with CDNB (2). Steady-state experiments performed at pH 5.0 in the presence of NBD-Cl or CDNB indicate that a minimum rapid equilibrium random sequential bi-bi model is sufficient to describe the catalytic mechanism by GST P1-1, similar to that observed with CDNB at pH 6.5 (3). Presteady-state kinetics, thermodynamic data, and viscosity variation experiments suggest that the rate-limiting step with NBD-Cl is, probably, a structural transition of the ternary complex. Interestingly, intra- and intersubunit communications between active sites are triggered by this new co-substrate. These findings have been tentatively explained on the basis of the x-ray crystal structure.

EXPERIMENTAL PROCEDURES

Materials—7-Chloro-4-nitrobenzo-2-oxa-1,3-diazole was obtained from Fluka AG. 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). Glycerol was a BDH product. GSH and CDNB were purchased from Sigma. The conjugation products between mercaptoethanol and NBD-Cl (EIS-NBD) and that between GST and NBD-Cl (GS-NBD) were synthesized as previously reported (5).

Spectrophotometric Measurements—Steady-state kinetics of GST P1-1 with NBD-Cl as co-substrate were followed spectrophotometrically at 419 nm where the GS-NBD conjugate has its absorption maximum ($\epsilon_{419 \text{ nm}} = 14.5 \text{ M}^{-1} \text{ cm}^{-1}$) (2). Activity with CDNB was measured at 340 nm where the product absorbs ($\epsilon_{340 \text{ nm}} = 9.6 \text{ M}^{-1} \text{ cm}^{-1}$) (6). Spectrophotometric measurements were performed with a double-beam UVICON 940 spectrophotometer (Kontron Instruments) equipped with a cuvette holder thermostatted at 25 °C. Kinetic experiments were done in 1 ml (final volume) of 0.1 M sodium acetate buffer, pH 5.0, containing 5–10 µl of GST P1-1 and variable amounts of substrates. The reaction rates were measured at 0.1-s intervals for a total period of 12 s. Initial rates were determined by linear regression and corrected for the spontaneous reaction. Kinetic data with NBD-Cl were collected by varying NBD-Cl from 10 to 200 µM and GSH from 12 to 500 µM over a matrix of
Data Analysis—

Steady-state Kinetics—

36 substrate concentrations. Inhibition experiments were performed with a 25-substrate concentration matrix in the presence of fixed GSNBD concentrations ranging between 20 and 100 μM. Several sets of V/v versus 1/S plots at various fixed GSNBD concentrations and constant co-substrate concentrations were obtained. The slopes within each set are plotted against the corresponding GSNBD concentration. Ka(NBD-Cl) and Ka(GSH) are the intercepts on the x-axis which are plotted versus the concentration of the fixed substrate. In a random system Ka (slope) varies with the concentration of the fixed substrate (see Fig. 2) (7).

Kinetic data with CDNB were obtained at pH 5.0 by varying CDNB and GSH from 50 μM to 1 mM over a matrix of 36 substrate concentrations. Fluorometric Measurements—

The dissociation constant (K) for substrates and the substrate analogue ETS-NBD was obtained by fluorescence measurements by fitting the data to the simplest model by assuming a single and noncooperative binding site per subunit as shown in Equation 1:

\[ \frac{[B]}{[A]} = \frac{1}{1 + K_s[B]} \]  

(Eq. 1)

where \( [B] \) is the initial concentration of the second substrate and \( [A] \) is the dissociation constant, and \( I \) is the ligand concentration.

The dissociation constant values were used for the analysis of kinetic data that were fitted to a rapid equilibrium random sequential bi-bi model, according to Scheme 1. The parameters to be varied in the fitting procedure were only limited to V_{max} and the coupling factor \( \alpha \), according to Equation 2:

\[ V = \frac{V_{max}[A][B]}{K_{a} + [A] + [B]} \]  

(Eq. 2)

where \( V \) is the initial velocity, \([A]\) and \([B]\) are GSH and NBD-Cl (CDNB) concentrations, and \( K_a \) and \( K_{a}^{NBD-Cl} \) their respective dissociation constants (without the second substrate) obtained by fluorescence experiments. The dissociation constant for fluometry for CDNB is uncertain since its high absorbance at 280 nm and its low affinity for GST; \( K_{a}^{NBD-Cl} \) was then left free to vary in the kinetic fits. \( V_{max} \) is expressed as [product] = [GSH]. Parameters derived from binding data (\( K_a \) and \( K_{a}^{NBD-Cl} \)) and from the fit of kinetic data \( V_{max} \) and \( K_{a}^{NBD-Cl} \) obtained by fluorometry and \( \alpha \) by kinetic fit. Binding and kinetic data were analyzed with a software package WIN-MATLAB (MathWorks South Natick, MA).

Stopped Flow Analysis—

Data from Ref. 12.

Data from Ref. 3.

**RESULTS**

**Steady-state Kinetics—**

Steady-state kinetics with NBD-Cl at pH 5.0 was analyzed and compared with that obtained with CDNB under the same conditions. Thermodynamic constants obtained by fluorometry (Table I) were used for fitting the
kinetic data as reported under the "Experimental Procedures." As already found with CDNB as co-substrate at pH 6.5 (3), the rapid equilibrium random sequential bi-bi model (Scheme 1) or the mathematically equivalent steady-state ordered bi-bi mechanism was a minimum model that describes satisfactorily the experimental data for both co-substrates at pH 5.0 (Fig. 1). A diagnostic procedure to distinguish between these two mechanisms is based on product inhibition studies (7). The pattern of inhibition at pH 5.0 by GS-NBD (Fig. 2) is consistent with a rapid equilibrium random mechanism (7). Data presented in the form of a double-reciprocal plot (Fig. 1) provide evidence of the convergence of the lines above the abscissa axis, showing a coupling factor \( \alpha = 0.036 \) with NBD-Cl and \( \alpha = 0.38 \) with CDNB. These fractional numbers indicate that the affinity for GSH increases in the presence of NBD-Cl about 30 times (and vice versa), but only 3 times in the presence of CDNB (and vice versa) (7). The agreement between fitting procedures and experimental data is evident in Fig. 3 which shows the plots obtained by using Equations 3–5. Furthermore, the results obtained from fluorescence quenching measurements for the binding of a nonreactive analogue of the second substrate (EtS-NBD), in the presence of variable GSH concentrations, indicate that the kinetic synergism between the G-site and H-site must be related to a mutual modulation of the dissociation constants (Fig. 4).

Presteady-state Kinetics—A careful investigation of the presteady-state kinetics of GST P1-1 with NBD-Cl as co-substrate does not confirm our preliminary evidence for an accumulation of GS-NBD product before the attainment of the steady state (12). No evident burst phase is now observed at 419 nm where GS-NBD absorbs strongly (2) (Fig. 5, inset). Some technical problems were responsible for that earlier report; the major problem was an inadequate washing volume for the cell (based on advice by manufacturer). Furthermore, the spectral image of the accumulating species, obtained as described under "Experimental Procedures," is very similar to that of NBD-Cl with a little red shift of about 3 nm (Fig. 5). This spectrum has been assigned to the NBD-Cl complexed enzyme, since a very different spectrum is expected for the Meisenheimer (or \( \alpha \) ) complex which occurs as an intermediate in similar aromatic substitution reactions (13). On the basis of the present data and of the previously reported \( k_{cat} \) independence of the nature of the leaving group (12), we suggest that the rate-limiting step is a physical event, and it must occur between the ternary complex formation and the chemical step, as found for the CDNB/GSH-catalyzed reaction (1). Thermodynamic data also indicate different rate-determining events for the catalyzed and uncatalyzed reactions. In the latter the \( \alpha \)-complex formation was found to be the rate-determining step (12). Calculations from the Arrhenius plots shown in Fig. 6 give an activation energy for the enzymatic NBD-Cl/GSH reaction of about 20 kJ/mmol higher than that for the spontaneous one. This may be a paradoxical result if a coincident rate-limiting step occurs in both reactions.

Cys-47 Mutants Display Cooperativity toward Both GSH and NBD-Cl—When CDNB is the co-substrate, the substitution of Cys-47 by Ala or Ser does not change the \( k_{cat} \) value but lowers the affinity of the G-site for GSH (about 9-fold for the C47S mutant and 14-fold for the C47S/C101S double mutant) and triggers a marked positive cooperativity toward GSH (about 9-fold for the C47S mutant and 14-fold for the C47S/C101S double mutant) and triggers a marked positive cooperativity toward GSH. When CDNB is the co-substrate, the substitution of Cys-47 by Ala or Ser does not change the \( k_{cat} \) value but lowers the affinity of the G-site for GSH (about 9-fold for the C47S mutant and 14-fold for the C47S/C101S double mutant) and triggers a marked positive cooperativity toward GSH (about 9-fold for the C47S mutant and 14-fold for the C47S/C101S double mutant) and triggers a marked positive cooperativity toward GSH. When CDNB is the co-substrate, the substitution of Cys-47 by Ala or Ser does not change the \( k_{cat} \) value but lowers the affinity of the G-site for GSH (about 9-fold for the C47S mutant and 14-fold for the C47S/C101S double mutant) and triggers a marked positive cooperativity toward GSH. When CDNB is the co-substrate, the substitution of Cys-47 by Ala or Ser does not change the \( k_{cat} \) value but lowers the affinity of the G-site for GSH (about 9-fold for the C47S mutant and 14-fold for the C47S/C101S double mutant) and triggers a marked positive cooperativity toward GSH.
complex behavior is found with NBD-Cl. As shown in Table II, the affinity toward both GSH and NBD-Cl decreases 4–10-fold in the mutants while $k_{\text{cat}}$ values are quite unchanged. The homotropic behavior by GSH is again recovered both for C47S mutant and C47S/C101S double mutant (Table II) with Hill coefficients very similar to that obtained in the presence of CDNB (8). Surprisingly, a positive cooperativity is observed toward NBD-Cl with Hill coefficient values of 1.56 and 1.40 for the single and double mutants, respectively (Table II). This reveals a structural intersubunit communication between the two H-sites of the dimeric enzyme which is absent or not detectable in the wild-type and in Cys-47 mutants with CDNB as co-substrate.

Viscosity Effect on Wild Type—In the preceding paper (1) we demonstrated a viscosity dependence of $k_{\text{cat}}$ and $K_m^{\text{GST}}$ values for the GST-catalyzed reaction of CDNB with GSH. This has been interpreted assuming that kinetic parameters depend on some diffusion-controlled motions of the protein. In particular, helix 2 flexibility seems to be involved in determining the G-site affinity, although it is unclear whether it plays a role in the $k_{\text{cat}}$ modulation. By using NBD-Cl as co-substrate, $k_{\text{cat}}$, $K_m^{\text{GST}}$, and $K_m^{\text{NBD-Cl}}$ remain almost unchanged even at a high viscosity value (Table II). These data may be interpreted by means of the x-ray structure of the enzyme (see "Discussion"); moreover, they are a good control to show that the viscosity dependence of $k_{\text{cat}}$ and $K_m^{\text{GST}}$ previously observed in the presence of CDNB (1) is not due to a nonviscosity solvent effect (14).
Influence of Co-substrate on Dynamics/Kinetics of Human GST

TABLE II

Viscosity effect on kinetic parameters of wild-type and Cys-47 mutants

|                           | Wild type | C47S | C47S/C101S |
|---------------------------|-----------|------|------------|
| $K_{\text{NBD-Cl}}^\text{K}$ (µM) | 4         | 4    | 4          |
| $K_{\text{GSH}}^\text{K}$ (µM)    | 8         | 8    | 8          |
| $[S]_{\text{NBD-Cl}}^\text{K}$ (µM) | 15        | 14   | 14         |
| $[S]_{\text{GSH}}^\text{K}$ (µM)   | 35        | 33   | 88         |
| $k_{\text{cat}}$ (s$^{-1}$)     | 1.20      | 0.98 | 1.24       |
| $n_{\text{NBD-Cl}}^\text{H}$       | ND        | ND   | 1.32       |
| $n_{\text{GSH}}^\text{H}$           | ND        | ND   | 1.44       |

FIG. 7. Molecular model of NBD-Cl and GSH in the dimeric GST P1-1. Modeling was performed as described under “Experimental Procedures.” The view is taken down the 2-fold axis of the dimer molecule. Some residues possibly implied in NBD-Cl binding (Tyr-108) and in the synergism between H- and G-sites (Glu-97 and Asp-98) are shown.

![Molecular model of NBD-Cl and GSH in the dimeric GST P1-1](image)

DISCUSSION

Steady-state kinetic data at pH 5.0 with NBD-Cl (or CDNB) as co-substrate are consistent with a rapid equilibrium random sequential bi-bi model (Figs. 1–3). The effects of changing the leaving group (12) and of temperature (Fig. 6) on $k_{\text{cat}}$ and $[S]_{\text{0.5}}^\text{K}$ values which are viscosity-independent (Table II). On the contrary the positive cooperativity toward GSH, as expressed by the Hill coefficient, is markedly lowered by increasing the viscosity (Table II). Thus it appears that the flexibility of helix 2, responsible for the GSH cooperativity in these mutants (1, 8), is still diffusion-controlled in the presence of NBD-Cl as co-substrate. Conversely, the homotropic behavior of NBD-Cl is viscosity-independent (Table II). This new intersubunit interaction between H-sites should involve protein regions different from helix 2.

![Molecular model of NBD-Cl and GSH in the dimeric GST P1-1](image)

![Molecular model of NBD-Cl and GSH in the dimeric GST P1-1](image)

2 During the revision of this paper, according to a reviewer’s suggestion, we checked the effect of non-denaturing urea concentrations (up to 2 M) on $k_{\text{cat}}$ values. With NBD-Cl (in 0.1 M sodium acetate buffer, pH 5.0), $k_{\text{cat}}$ increases up to 200% of the basal value at 2 M urea. No change of $k_{\text{cat}}$ has been observed with CDNB as co-substrate (in 0.1 M potassium phosphate buffer, pH 6.5). By assuming that non-denaturing urea concentrations only lower the internal friction of a protein, its different effect on $k_{\text{cat}}$ values suggests that the internal constraints play a crucial role in determining the structural transition energy barrier only in the presence of NBD-Cl as co-substrate. These findings agree well with the conclusions of this paper.
tion, helix 4 provides a number of important residues close to or in contact with the bound GSH such as Asp-94, Glu-97, and Asp-98 (15). Hence, a structural perturbation due to the interaction of NBD-Cl with Tyr-108, localized on the H-site, may be transmitted to the G-site via helix 4 yielding the observed synergistic effect between the H- and G-sites responsible for the increased affinity toward substrates.

As concerns \( K_m^{\text{GSH}} \), its viscosity-independent value (Table II) seems no more related to diffusion-controlled motions of helix 2 that, on the contrary, are relevant in determining \( K_m^{\text{GSH}} \) in the CDNB/GSH system (1). It is possible that the increased rigidity of the active site due to NBD-Cl binding may be transmitted to helix 2. Alternatively, helix 2 motions may be always diffusion-dependent, but the \( K_m^{\text{GSH}} \) value is mainly determined by GSH interaction with helix 4 promoted by NBD-Cl. The viscosity effect on the Hill coefficients of Cys-47 mutants (Table II) suggests that helix 2 (whose flexibility modulates this parameter (1)) is still diffusion-controlled even in the presence of NBD-Cl.

Preliminary evidence obtained with a mutated GST P1-1, where Tyr-108 is replaced by Phe, confirms the influence of the hydroxyl group of Tyr-108 on \( k_{\text{cat}} \) and \( K_m^{\text{NBD-Cl}} \) as these kinetic parameters increase remarkably and become viscosity-dependent in this mutant.³ Comparison of the crystallographic data and of the catalytic properties toward NBD-Cl in Alpha, Pi, and Mu GST isoenzymes indirectly confirms the above suggestions. Both Pi and Mu class isoenzymes, characterized by low \( k_{\text{cat}} \) and \( K_m^{\text{NBD-Cl}} \) values (2), have equivalently located hydroxyl groups (Tyr-108 and Tyr-115, respectively) which may form a hydrogen bond with the heterocyclic portion of NBD-Cl. On the contrary, Alpha class GST, which displays higher \( k_{\text{cat}} \) and \( K_m^{\text{NBD-Cl}} \) values (2), lacks this hydrogen bond as a Val residue replaces the Tyr residue.

REFERENCES

1. Ricci, G., Caccuri, A. M., Lo Bello, M., Rosato, N., Mei, G., Nicotra, M., Chiesi, E., Mazzetti, A. P., and Federici, G. (1996) J. Biol. Chem. 271, 16187–16192
2. Ricci, G., Caccuri, A. M., Lo Bello, M., Pastore, A., Piemonte, F., and Federici, G. (1994) Anal. Biochem. 218, 463–465
3. Ivanetich, K. M., and Gold, D. D. (1989) Biochim. Biophys. Acta 998, 7–13
4. Lo Bello, M., Battistoni, A., Mazzetti, A. P., Board, P. G., Muramatsu, M., Federici, G., and Ricci, G. (1995) J. Biol. Chem. 270, 1249–1253
5. Lo Bello, M., Pastore, A., Petruzzelli, R., Parker, M. W., Wilce, M. C., Federici, G., and Ricci, G. (1993) Biochem. Biophys. Res. Commun. 194, 804–810
6. Habig, W. H., and Jakoby, W. B. (1981) Methods Enzymol. 77, 398–405
7. Segel, I. H. (1976) Enzyme Kinetics, John Wiley & Sons, New York
8. Lo Bello, M., Lo Bello, M., Caccuri, A. M., Pastore, A., Nuccetelli, M., Parker, M. W., and Federici, G. (1995) J. Biol. Chem. 270, 1243–1248
9. Caccuri, A. M., Ascenzi, P., and Brunori, M. (1988) J. Biol. Chem. 263, 18286–18289
10. Wolf, A. V., Brown, M. G., and Prentiss, P. G. (1985) Handbook of Chemistry and Physics (Weast, R. C., Astle, M. J., and Beyer, W. H., eds) pp. D-219–D-269, CRC Press, Inc., Boca Raton, FL
11. Menegatti, E., Guarnieri, M., Bologna, M., Ascenzi, P., and Amiconi, G. (1985) Biochim. Biophys. Acta 832, 1–6
12. Caccuri, A. M., Ascenzi, P., Lo Bello, M., Federici, G., Battistoni, A., Mazzetti, A. P., and Ricci, G. (1994) Biochim. Biophys. Res. Commun. 200, 1428–1434
13. Graminski, G. F., Zhang, P., Sesay, M. A., Ammon, H. L., and Armstrong, R. N. (1989) Biochemistry 28, 6252–6258
14. Sampson, N. S., and Knowles, J. R. (1992) Biochemistry 31, 8488–8494
15. Rehmeier, P., Dhir, H. W., Ladenstein, R., Huber, R., Lo Bello, M., Federici, G., and Parker, M. W. (1992) J. Mol. Biol. 227, 214–226

³ M. Lo Bello, A. J. Oakley, A. Battistoni, A. P. Mazzetti, M. Nuccetelli, G. Mazzarese, J. Rossjohn, M. W. Parker, and G. Ricci, manuscript in preparation.