Cooperativity and Pseudo-cooperativity in the Glutathione S-Transferase from *Plasmodium falciparum*

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Binding and catalytic properties of glutathione S-transferase from *Plasmodium falciparum* (PfGST) have been studied by means of fluorescence, steady state and pre-steady state kinetic experiments, and docking simulations. This enzyme displays a peculiar reversible low-high affinity transition, never observed in other GSTs, which involves the G-site and shifts the apparent $K_p$ for glutathione (GSH) from 200 to 0.18 ms. The transition toward the high affinity conformation is triggered by the simultaneous binding of two GSH molecules to the dimeric enzyme, and it is manifested as an uncorrected homotropic behavior, termed “pseudo-cooperativity.” The high affinity enzyme is able to activate GSH, lowering its $pK_a$ value from 9.0 to 7.0, a behavior similar to that found in all known GSTs. Using 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole, this enzyme reveals a potential optimized mechanism for the GSH conjugation but a low catalytic efficiency mainly due to a very low affinity for this co-substrate. Conversely, PfGST efficiently binds one molecule of hemin/monomer. The binding is highly cooperative ($n_H = 1.8$) and occurs only when GSH is bound to the enzyme. The thiolate of GSH plays a crucial role in the intersubunit communication because no cooperativity is observed when S-methylglutathione replaces GSH. Docking simulations suggest that hemin binds to a pocket leaning into both the G-site and the H-site. The iron is coordinated by the amidic nitrogen of Asn-115, and the two carboxylate groups are in electrostatic interaction with the e-amino group of Lys-15. Kinetic and structural data suggest that PfGST evolved by optimizing its binding property with the parasitotoxic hemin rather than its catalytic efficiency toward toxic electrophilic compounds.

Cytosolic glutathione S-transferases (GSTs) are a superfamily of multifunctional enzymes involved in the cellular de-
Starting from these preliminary observations, we have investigated the binding and activation of GSH and the kinetic mechanism of the reaction between GSH and the alternative co-substrate, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl). Moreover, the mechanism of interaction of the hemin with \( P^i \text{GST} \) has been elaborated carefully on using pre-steady state, steady state kinetic, and fluorescence experiments. Our results reveal the existence of two distinct cooperative phenomena that modulate the interaction of this enzyme with GSH and with hemin. Structural intersubunit communication in GST superfamily has been demonstrated previously for GSTP1-1, GSTA1-1, and GSTT2-2 (19-25). Our results confirm that cooperativity is a mechanism adopted by a number of GST isoenzymes. Because of this cooperative mechanism, GSTs might be able to modulate interactions with different compounds, thereby responding to specific evolutionary pressures.

**EXPERIMENTAL PROCEDURES**

**Materials**—GSH, 1-chloro-2,4-dinitrobenzene (CDNB), NBD-Cl, 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-F), and hemin were obtained from Sigma-Aldrich.

**Enzyme**—The recombinant \( P^i \text{GST} \) was expressed in *Escherichia coli* and purified as described previously (13). The purified form of \( P^i \text{GST} \) was stored at \(-20\) or \(5\) °C in the presence of \(10\) mM GSH. Under these conditions, the enzyme was stable for a few weeks. After a Sephadex G-25 chromatography and subsequent incubation of the enzyme at \(25\) °C for 12 h, the \(90\)% inactive enzyme (\(P^i \text{GST}\)) was used for reactivation and stopped-flow binding experiments. Protein concentration was calculated assuming an \(\epsilon_{280}\) of 1.1 at \(280\) nm for \(P^i \text{GST}\) on the basis of the amino acid sequence (26). A molecular mass of \(25\) kDa/GST subunit was used in the calculation (14).

**Spectroscopic Detection of GSH Thiolate**—Difference spectra of the GST thiolate bound to \( P^i \text{GST} \) were obtained with a Kontron double beam Uvikon 940 spectrophotometer thermostatted at \(25\) °C. In a typical experiment, \(1\) mM GSH was added to the enzyme (\(15\) \(\mu\)M) in \(0.1\) M potassium phosphate-acetate-borate (50:50:50 mM) buffer, \(pH\) \(7.0\), and the spectra were recorded after 60 min. Spectra were corrected for the contribution of free GSH and free enzyme. The amount of thiolate was calculated by assuming an \(\epsilon_{290}\) of \(5000\) \(M^{-1}\cdotcm^{-1}\). Experiments at different pH values were carried out using the phosphate-acetate-borate (50:50:50 mM) buffer at \(pH\) \(5.0\) and \(7.5\). The \(pK_v\) value of the bound GSH was calculated by fitting the spectral data to Equation 1,

\[
A_{290M} = A_{290} + 10^{[(pK_v - pH) / \Delta pK_v]} \tag{1}
\]

where \(A_{290M}\) is the limiting thiolate absorbance at \(290\) nm and \(A_{290}\) at alkaline pH values.

**Enzymatic Activity**—Standard \(P^i \text{GST}\) activity with CDNB as co-substrate was measured at \(25\) °C in a \(0.1\) M potassium phosphate buffer, \(pH\) \(6.5\), containing \(1\) mM GSH and \(1\) mM CDNB. The activity was assayed spectrophotometrically by following the enzymatic product at \(340\) nm (\(\epsilon = 9600\) \(M^{-1}\cdotcm^{-1}\)). Steady state kinetic experiments with NBD-Cl as co-substrate were performed at \(25\) °C in a \(0.1\) mM sodium acetate buffer, \(pH\) \(5.0\) (\(25\) °C), by varying NBD-Cl from \(0.2\) to \(2\) mM and GSH from \(0.1\) to \(1\) mM over a matrix of \(16\) substrate concentrations. The enzymatic product absorbs at \(419\) nm (\(\epsilon = 14,500\) \(M^{-1}\cdotcm^{-1}\)).

**Fluoride/Chloride Leaving Group Substitution**—Kinetic data were obtained at \(25\) °C and at fixed NBD-Cl or NBD-F (\(0.2\) mM) and GSH (\(1\) mM) concentrations in \(1\) ml (final volume) of \(0.1\) M sodium acetate buffer, \(pH\) \(5.0\), containing suitable amounts of hemin, \(S\), and fixed enzyme concentration (\(2\) \(\mu\)M) in \(\text{H}^1\text{O}\) or \(\text{D}^2\text{O}\). The activity was monitored by following the intrinsic fluorescence of the protein at \(333\) nm. A fresh stock hemin solution was prepared by dissolving hemin chloride in \(100\) mM NaOH and then centrifuging for \(10\) min at \(15,000\) rpm to remove the remaining hemin crystals. Heme concentrations were estimated from the absorbance using \(\epsilon_{365} = 58,400\) \(M^{-1}\cdotcm^{-1}\). This stock hemin solution was diluted to \(0.1\) mM hemin and \(10\) mM NaOH and immediately used. No solubility artifacts were observed in control spectrophotometric experiments carried out under experimental conditions similar to those used for the stopped-flow technique or for the fluorometric experiments described below.

**Isothermic Binding of GSH and Hemin**—Isothermic binding of hemin to \( P^i \text{GST} \) was measured in a single photon counting spectrophotometer (Fluromax, S.A. Instrument, Paris) with a sample holder thermostatted at \(25\) °C. Excitation was at \(295\) nm, and emission was collected at \(333\) nm. The affinity of \( P^i \text{GST} \) (\(2\) \(\mu\)M) for hemin was determined by measuring the perturbation of the intrinsic fluorescence of the protein following the addition of the inhibitor (from \(0.5\) to \(5\) \(\mu\)M) in the presence of \(10\) mM GSH in \(0.1\) M potassium phosphate buffer, \(pH\) \(6.5\). Fluorescence data were corrected both for dilution and inner filter effects. Similarly, the isothermic binding of GSH to \( P^i \text{GST} \) was studied by incubating \( P^i \text{GST} \) (\(2\) \(\mu\)M) with variable amounts of GSH (from \(0.1\) to \(10\) mM) in \(0.1\) M potassium phosphate buffer, \(pH\) \(6.5\). Fluorescence quenching was recorded after \(60\) min. Spectra were corrected for the contribution of free GSH and free enzyme. The data obtained by varying the hemin concentration were analyzed to the Hill equation,

\[
\frac{v/v_{\text{max}}} = \frac{|S|/K_i + |S|} \tag{2}
\]

where \(v\) is the initial velocity observed at a given concentration of hemin, \(v_{\text{max}}\) is the velocity observed in the absence of inhibitor, \(|S|\) is the hemin concentration, and \(n\) is the Hill coefficient at the hemin concentration corresponding to the half-enzyme saturation. Inhibition data were also analyzed with a two-site Adair model for ligand binding to a homodimeric macromolecule,

\[
\frac{v/v_{\text{max}}} = \frac{[S]|K_i + |S| + [S]|K_i + + [S]|K_i + + [S]|K_i} \tag{3}
\]

where \(K_i\) is the dissociation equilibrium constant for hemin binding to the free enzyme, \(aK_i (= K_i)\) represents the dissociation equilibrium constant for hemin to the monoglutathione, and \(a\) is the adimensional interaction parameter coupling the two functionally linked hemin binding sites.

**Docking Simulations**—Docking simulations were performed with the program Autodock 3.0.5 (27) using the \( P^i \text{GST} \) crystal structure in complex with S-hexylglutathione (Protein Data Bank code 1Q4A), after removal of the hexyl moiety. Polar hydrogen atoms were added geometrically, and atom collumns were charged. Docking experiments for hemin were performed with a flexible ligand. Hemin charges were added by the Gasteiger-Marsili method (28). Grids of molecular interactions were calculated in a cubic box, size \(33.375\) Å, grid spacing \(0.375\) Å, centered on the oxygen atom of the tyrosine side chain participating in GSH activation (Tyr-9). Docking was performed 100 times using the Lamarkian genetic algorithm with random starting position and conformation, a population size of 50, standard parameters (29), and a maximum of 250,000 energy evaluations and 27,000 generations. The 100 final docked conformations were ranked according to their binding free energy and clustered using a tolerance of \(3\) Å root-mean-square deviation. The MOLMOL program (30) was used for graphical interpretation and representation of results.

**RESULTS AND DISCUSSION**

**Low-High Affinity Transition**—\( P^i \text{GST} \) shows good stability and constant specific activity only when it is stored at \(1\) mM or higher GSH concentrations. On the contrary, a rapid and biphasic inactivation occurs in GSH-depleted solutions. For example, the specific activity of the enzyme stored in the presence of \(10\) mM GSH drops to \(50\)% in a few minutes after a Sephadex G-25 chromatography (\(t_{1/2} = 3-4\) min), whereas a complete inactivation is reached only after about \(48\) h of incubation at \(25\) °C (\(t_{1/2} = 12\) h). In the presence of traces of GSH, both of the phases become slower, and only the first phase of inactivation can be readily observed (Fig. 1). At \(0.1\) mM GSH concentration,
the 50% inactivation kinetics displays a $t_{1/2}$ of 12 min. The hemi-inactivated enzyme completely recovers the original activity in less than 1 min upon incubation with 10 mM GSH. A complete reactivation is also observed starting from a 90% inactivated enzyme ($PfGST_i$) using either 1 mM GSH (Fig. 1) or 10 mM GSH. In both cases the reactivation is a monophasic process, but a nonlinear dependence of the reactivation rate on GSH concentration may reveal a cooperative phenomenon (Fig. 1, inset). When the enzyme completely loses its original activity, GSH is unable to trigger an efficient reactivation, indicating that a further irreversible process takes place. Interestingly, S-methylglutathione is also able to trigger the activation of $PfGST_i$, and thus the free sulfhydryl group of GSH is not essential for this event. Apart from the possibility that the low-high activity transition may be caused by a structural modification of essential residues involved in catalysis, alternatively, this could be due to structural changes of the G-site and/or of the H-site lowering the affinity for GSH or for the co-substrate. Isothermal binding of GSH to $PfGST_i$, followed on the basis of the intrinsic fluorescence quenching at variable GSH concentrations, indicated that changes of the enzyme affinity for GSH are likely involved in this transition. In fact, a marked sigmoid behavior suggests that GSH binding shifts the enzyme from a low affinity to a high affinity conformation (data not shown). However the fluorescence data did not fit satisfactorily to a cooperative equation, and only an approximate estimation of the affinity constants for GSH has been calculated, i.e. $K_D > 200$ mM for the low affinity conformation and $K_D < 0.2$ mM for the high affinity conformation.
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**Stopped-flow Experiments**—The low-high affinity transition triggered by GSH and the binding mechanism of PfGST have been detailed by means of stopped-flow experiments following the quenching of the intrinsic fluorescence after rapid mixing of PfGST, 30 μM in 0.1 M phosphate buffer, pH 6.5) with GSH at variable concentrations (from 100 to 2 mM) (Fig. 2). Interestingly, k_{obs} values do not increase linearly by increasing GSH concentrations, as expected for a simple bimolecular interaction, but follow a clear exponential behavior. It should be noted that reaction half-times span from ~200 ms to >500 s by changing the final GSH concentrations from 50 to 1 mM, respectively. However, all experimental traces do not fit satisfactorily to a classical positive cooperative mechanism (Fig. 2, inset). Conversely, k_{obs} values increase linearly on the square of GSH concentration (Fig. 3), suggesting the occurrence of a trimolecular interaction. All experimental data obtained from the stopped-flow experiments, taken together with kinetics of enzyme activation and inactivation observed in the presence of GSH or after its removal, have been rationalized on the basis of a more complicated binding mechanism (Scheme 1). GSH binds to the two PfGST subunits of the inactive enzyme (I-I) sequentially, with identical rate constants. Only when both subunits are saturated with GSH does the inactive enzyme rapidly convert into the active dimeric enzyme (A-A). The conversion of the inactive enzyme with GSH bound into the active enzyme apparently is not reversible. The active enzyme becomes inactive only by following Step 4 (in Scheme 1) to give the PfGST-A-I species (which may correspond to the hemi-inactivated enzyme recovered after Sephadex G-25 chromatography). Stopped-flow data fit very well to this mechanism (Fig. 2), and the fitting procedure allowed us to estimate with satisfying precision k_{1}, k_{-1}, k_{2}, and k_{-2} values (Table I). The rate constants for the conversion of the inactive enzyme to the active enzyme (k_{3} and k_{-3}, as well as k_{5}, k_{-5}) were not resolved by the fitting procedure. In these cases, the fitting procedure can only indicate the upper or lower limits of the rate constants. Rate constants for dissociation of GSH from the active enzyme (k_{-4} and k_{-5}) were found experimentally by looking at the rate of the biphasic inactivation due to dilution or removal of GSH from an activated enzyme solution (see Fig. 1). The hysteresis represented by the cyclic reaction in Scheme 1 is due to the very low rate constant for the conversion of the active enzyme into the inactive enzyme in the presence of bound GSH, which causes the apparent irreversibility of reaction 3. Interestingly, the low activity enzyme shows a kinetics of GSH binding (k_{1} and k_{2}) that is thousands of times lower than those observed for Alpha, Pi, and Mu class GSTs (31); conversely, the kinetics of GSH release (k_{-2} and k_{-3}) is similar. Thus, it appears that in the low affinity conformation, the G-site is partially obscured so that GSH cannot interact at a high rate with its protein counterpart. The overall dissociation constant calculated by the stopped-flow data ranges from 200 mM for the low affinity conformation to 0.18 mM for the high affinity state, close to the values calculated by the preliminary analysis of the fluorescence data at equilibrium.

**GSH Activation**—Differential spectrophotometry has been used to verify whether PfGST forces the deprotonation of the bound GSH in the same manner that it occurs in most GSTs (2). These experiments, based on the peculiar absorbance of the thiolate anion at 240 nm, were carried out at different pH values and at fixed 1 mM GSH, a concentration that stabilizes the high affinity conformation. As shown in Fig. 4, the high affinity enzyme lowers the pK_{a} of GSH so that detectable amounts of GS⁻ are found even at neutral pH values. The pH dependence of the absorbance at 240 nm indicates that about 1 mol of thiolate/mol of enzyme subunit is formed at pH 8.5. The apparent pK_{a} of the bound GSH is 7.0, a value 2 units lower than that of the free GSH. Stopped-flow experiments performed by mixing the low affinity enzyme with variable amounts of GSH also indicated that kinetics of thiolate formation are similar to that of the binding process (see Fig. 3); thus, these two events appear synchronous.
and to the Adair equation (Equation 4) (dashed line). Steady state experiments were fitted to the Hill equation (Equation 3) (continuous line).

The data obtained by varying the hemin concentration were fitted to the Hill equation (Equation 3) (continuous line). The data obtained by varying the hemin concentration were fitted to the Hill equation (Equation 3) (continuous line).

Steady state kinetic analysis using NBD-Cl as co-substrate. Steady state experiments were performed at 25 °C and pH 6.5 as described under “Experimental Procedures.” a, NBD-Cl is the variable substrate, and GSH concentrations were fixed at 0.1 mM (●), 0.2 mM (●), 0.4 mM (●), and 1 mM (△). b, GSH was the variable substrate, and NBD-Cl concentrations were fixed at 0.2 mM (●), 0.5 mM (▲), 1 mM (●), and 2 mM (○). Velocities are expressed as ΔA/min at 419 nm.

Fig. 5. Steady state kinetic analysis using NBD-Cl as co-substrate. Steady state experiments were performed at 25 °C and pH 6.5 as described under “Experimental Procedures.” a, NBD-Cl is the variable substrate, and GSH concentrations were fixed at 0.1 mM (●), 0.2 mM (●), 0.4 mM (●), and 1 mM (△). b, GSH was the variable substrate, and NBD-Cl concentrations were fixed at 0.2 mM (●), 0.5 mM (▲), 1 mM (●), and 2 mM (○). Velocities are expressed as ΔA/min at 419 nm.

Fig. 6. Inhibition of PfGST by hemin. Inhibition of PfGST by hemin was studied by mixing 2 μM PfGST with variable amounts of hemin (from 0.5 to 6 μM) as described under “Experimental Procedures.” After about 10 s, 5 mM GSH and 1 mM CDNB were added for activity measurement. The data obtained by varying the hemin concentration were fitted to the Hill equation (Equation 3) (continuous line) and to the Adair equation (Equation 4) (dashed line).

Steady state kinetic analysis using NBD-Cl as co-substrate. Steady state experiments were performed at 25 °C and pH 6.5 as described under “Experimental Procedures.” a, NBD-Cl is the variable substrate, and GSH concentrations were fixed at 0.1 mM (●), 0.2 mM (●), 0.4 mM (●), and 1 mM (△). b, GSH was the variable substrate, and NBD-Cl concentrations were fixed at 0.2 mM (●), 0.5 mM (▲), 1 mM (●), and 2 mM (○). Velocities are expressed as ΔA/min at 419 nm.

Steady state kinetic analysis using NBD-Cl as co-substrate. Steady state experiments were performed at 25 °C and pH 6.5 as described under “Experimental Procedures.” a, NBD-Cl is the variable substrate, and GSH concentrations were fixed at 0.1 mM (●), 0.2 mM (●), 0.4 mM (●), and 1 mM (△). b, GSH was the variable substrate, and NBD-Cl concentrations were fixed at 0.2 mM (●), 0.5 mM (▲), 1 mM (●), and 2 mM (○). Velocities are expressed as ΔA/min at 419 nm.

In Fig. 5, a turnover number of 15 s⁻¹ has been calculated, which indicates a good catalytic efficiency under saturating GSH and NBD-Cl concentrations. From the stoichiometric data presented in Table II, catalytic efficiency under saturating conditions is 2,500-fold higher than that of the corresponding GST, giving rise to a 1,000-fold lower affinity for GSH and NBD-Cl, the kinetic mechanism appears to be ordered, with GSH binding first. The chemical step is probably the rate-limiting event in catalysis. In fact, the substitution of chloride with the better leaving group fluoride in this co-substrate causes a 20-fold increase in the turnover number. This acceleration is similar to the rate increase observed for the spontaneous reaction (18-fold) that is likely rate-limited by a chemical step.

Binding of Hemin—It has been shown that the binding of hemin to GST is highly cooperative, allowing us to evaluate the catalytic competence of this enzyme. On the basis of the reciprocal plots reported in Fig. 5, a turnover number of 15 s⁻¹ has been calculated, which indicates a good catalytic efficiency under saturating GSH and NBD-Cl concentrations. From the double reciprocal plots varying GSH and NBD-Cl, the kinetic mechanism appears to be ordered, with GSH binding first. The chemical step is probably the rate-limiting event in catalysis. In fact, the substitution of chloride with the better leaving group fluoride in this co-substrate causes a 20-fold increase in the turnover number. This acceleration is similar to the rate increase observed for the spontaneous reaction (18-fold) that is likely rate-limited by a chemical step.
action have not been clarified, we obtained further insights using a multidisciplinary approach. Binding of hemin has been studied by means of inhibition and fluorescence experiments at equilibrium and also by a stopped-flow approach following the kinetics of intrinsic fluorescence quenching due to hemin binding. As suggested previously (13, 14), we also observed that no binding occurs in the absence of GSH; thus the high affinity conformation and GSH bound to the G-site are crucial requirements for this interaction. In the presence of 10 mM GSH, inhibition data indicate that binding is not hyperbolic but follows a sigmoid behavior (Fig. 6). Experimental data fit very well to a positive cooperative mechanism with a Hill coefficient of 1.8. By fitting all inhibition data to the Adair equation, we also obtained an estimation of the dissociation constants for the hemin-enzyme complex in the low affinity conformation (K_{D1} = 2.8 \mu M) and in the high affinity conformation (K_{D2} = 0.16 \mu M). Interestingly, replacement of GSH by 10 mM S-methylglutathione changes the binding mechanism. In particular, the process is now hyperbolic and uncooperative with a K_{D} = 2.2 \mu M, close to the value found for the low affinity conformation (data not shown). Thus, the thiolate group of GSH is not involved in a crucial coordination of the iron ion of the ligand but may play an important role in the intersubunit communication triggered by hemin.

Fluorescence data at equilibrium detailed the stoichiometry of this interaction that involves one hemin molecule for one enzyme monomer (Fig. 7). We emphasize that hemin binding causes a dramatic quenching of the intrinsic fluorescence of this protein. In fact, when two hemin molecules are bound to the dimeric enzyme, the fluorescence intensity drops to 3% of its initial value (Fig. 7, inset). Trp-131 is the sole Trp residue present in the structure of PfGST. The strong energy interaction between this group and the hemin molecule indicates that these groups are close enough to be included in the Förster radius, as will be also confirmed by docking simulations (see below).

Stopped-flow experiments detailed the kinetics and possible mechanism of this interaction. By mixing variable amounts of hemin (from 100 to 10 \mu M) to PfGST in the presence of 10 mM GSH, at least two kinetically distinct phases were observed. The first one, accounting for no more than 10% of the total fluorescence quenching, represents a fast mixing artifact that is observed on mixing PfGST with buffer either in the presence or absence of hemin. It was completed in about 50 ms and excluded from the fitting procedure. The second slower and more prominent perturbation is likely because of the formation of the PfGST-hemin complex. The experimental traces were fitted well to the cooperative Scheme 2 (see Fig. 8). The microscopic rate constants given by the fitting procedure are shown in Table II. Interestingly, the affinities of the low affinity and high affinity binding sites (K_{D1} and K_{D2}) calculated by this kinetic approach are close to the corresponding values coming from the inhibition data. Moreover, the origin of the observed cooperativity must be included in a 10-fold decrease of the k_− value for hemin in the high affinity conformation. In other words, in the high affinity conformation, the hemin is more tightly bound to its protein counterpart, whereas the accessibility for the external hemin does not change appreciably in the two conformations.

Docking Simulations—To localize the protein portion and critical residues involved in the hemin binding, docking simulations were performed with the program Autodock (27). The lowest energy model is reported in Fig. 9. The hemin molecule is located in a cleft between the C-terminal region of helix 4 and the N-terminal part of helix 1 and the C-terminal loop. Forty-two of the 50 lowest energy models (of 100 different docking trials) bind to the same site, demonstrating the reliability of the proposed structure. The Autodock program assigns a binding free energy of −12 kcal/mol to the best model. This extremely high affinity is the result of multiple interactions. The protein surface displays a striking complementarity to the hemin molecule, as shown in Fig. 9A. Electrostatic interactions

### Table II

Kinetic parameters for hemin binding to PfGST

| Rate Constants | 10^{-6} M | 10^{-7} M |
|---------------|----------|----------|
| k_1 | 4 ± 2 | 9 ± 3 |
| k_2 | 4 ± 1 | 3 ± 2 |
| k_− | 2 ± 1 | 5 ± 2 |

Fig. 9. Models of the hemin-PfGST complex obtained by the docking simulation. A, contact surface of the PfGST monomer, showing the high complementarity of the binding pocket to the hemin molecule. B, schematic drawing of the hemin binding pocket. Residues interacting with the ligand are highlighted in red. C, ribbon representation of the dimeric structure of the complex, showing that no steric interactions occur between the two hemin molecules (shown in red). GSH is represented in yellow.
further stabilize the docked molecule; as shown in Fig. 9B, specific interactions are present between the side chain of Lys-15 and the carboxylate groups of the hemin molecule and between Asn-112 and the iron atom. Finally, hydrophobic patches are present on the protein surface in correspondence to the aromatic rings of the porphyrin moiety. On the other hand, a direct interaction between the glutathione thiolate and the hemin iron atom is not present, these two atoms being at ~8 Å in the docked structure. It is worth mentioning that the proposed model is compatible with the presence of one hemin molecule/subunit in agreement with the observed 1:1 binding stoichiometry, because no direct interactions are present in the dimeric structure when two ligands are placed in identical positions on each monomer (Fig. 9C).

The docked model provides a rationale for the almost complete quenching of intrinsic fluorescence caused by hemin binding. The single Trp residue of PfGST is located at ~16 Å from both hemin molecules docked to the dimeric P/GST. Hemin is an extremely efficient energy acceptor for tryptophan fluorescence (33); the Förster distance calculated for the spectral features of these two chromophores is 34 Å. Because this interaction radius is much higher than the distance observed in the docked structure, our model is compatible with an almost complete quenching of Trp fluorescence (34).

Conclusions—The existence of intersubunit structural communication in GSTs has been a debated question in the past, and for about 30 years GSTs have been thought to be uncooperative enzymes. A first indication for a cooperative interaction in GSTs has been obtained for GSTP1-1, an isoenzyme that shows positive homotropic behavior by GSH binding after point mutation of crucial residues such as Cys-47 and Lys-54 (25). Later, it was discovered that even the native enzyme triggers both positive and negative homotropic behavior by GSH binding at both high or low temperatures (22). This phenomenon appears to be a particular case of a more general property of GSTP1-1 that uses cooperativity to oppose a complete loss of activity caused by physical or chemical killers (21). Furthermore, half of the site reactivity, an extreme case of negative cooperativity, has been described for the Alpha class GSTA1-1 when it binds GSH (20). This interaction radius, because no direct interactions are present in the molecule/subunit in agreement with the observed 1:1 binding stoichiometry, because no direct interactions are present in the dimeric structure when two ligands are placed in identical positions on each monomer (Fig. 9C).

A completely different cooperativity is observed in the interaction of this enzyme with hemin. This toxic metabolite triggers a true positive homotropic behavior in P/GST, causing a 20-fold increased affinity in the vacant subunit as a consequence of its binding to one subunit. Although all GSTs bind hemin with high affinity, we were not able to find any cooperative activity in the hemin interaction with Alpha, Pi, and Mu GSTs (data not shown). Thus, the existence of a homotropic regulation in P/GST for hemin binding is a peculiar event and suggests an important metabolic functionality, i.e., the protection of the parasite against this toxic compound. Interestingly, P/GST seems to have evolved to interact efficiently with hemin without a parallel optimization of its catalytic competence toward hydrophobic toxic compounds. In fact, the scarce affinity for classical GST substrates is the prominent cause of its very low catalytic activity, stressed in previously published studies (13, 14) and confirmed by all of our present data. Our experiments suggest that P/GST deprotonates the bound GSH efficiently, as observed for the human Alpha, Pi, and Mu GSTs, and also its catalytic competence, evaluated under co-substrate saturating conditions, is similar to that found in the human enzymes. However, the efficient deprotonation of GSH seems to be finalized more to trigger the homotropic binding of hemin rather than to efficiently catalyze the conjugation of GSH to toxic compounds. The disappearance of homotropy when GSH replaces GSH is convincing evidence in this direction. In summary, the discovery of cooperativity and the possible localization of hemin in the crystal structure, as given by docking simulations, may be useful in designing selective inhibitors of P/GST that lower its ability to bind the parasitotoxic hemin.

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