The glutathione S-transferase enzymes (GSTs) have a tyrosine or serine residue at their active site that hydro- 

gens to and stabilizes the thiolate anion of glutathi- 
one, GS\(^{-}\). The importance of this hydrogen bond is 
obvious, in light of the enhanced nucleophilicity of GS\(^{-}\) 

versus the protonated thiol. Several A-class GSTs con- 
tain a C-terminal segment that undergoes a ligand-de- 
pendent local folding reaction. Here, we demonstrate 
the effects of the Y9F substitution on binding affinity 
for glutathione conjugates and on rates of the order-disor- 
ter transition of the C terminus in rat GST A1-1. The 
equilibrium binding affinity of the glutathione conju- 
gate, GS-NBD (NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3- 
diazole), was decreased from 4.09 \(\mu M\) to 0.641 \(\mu M\) upon 

substitution of Tyr-9 with Phe. This result was sup- 
ported by isothermal titration calorimetry, with \(K_d\) val- 
ues of 1.51 \(\mu M\) and 0.391 \(\mu M\) for wild type and Y9F, re- 
pectively. The increase in binding affinity for the 

mutant is associated with dramatic decreases in rates 
for the C-terminal order-disorder transition, based on a 
stopped-flow kinetic analysis. The same effects were ob- 
erved, qualitatively, for a second GSH conjugate, GS- 
ethacrynic acid. Apparently, the phenolic hydroxyl 
group of Tyr-9 is critical for orchestrating C-terminal 
dynamics and efficient product release, in addition to its 
role in lowering the \(pK_a\) of GSH.

The importance of protein dynamics and flexibility to 
the energetics of ligand binding is well appreciated and has been 
illustrated in a number of systems (1–5). In many cases, a 
protein-ligand interaction appears to drive a flexible segment 
to fold into a rigid, ligand-bound conformation (6, 7). An anal- 
ogy between global protein folding and ligand binding has been 
only recently described (8–11). The concept of folding funnels, 
where folding progresses via multiple routes rather than a 
single pathway, can be likened to binding where a ligand drives 
an ensemble of local states to a single conformation. However, the 
detailed mechanism of any ligand-dependent, localized 
folding process remains elusive and is probably less well un- 
derstood than global folding (12).

The glutathione S-transferase (GST)\(^1\) isoform A1-1 provides 
an outstanding opportunity to dissect the mechanism of a ligand- 
dependent local folding reaction. The GSTs are a family of 
exenobioc metabolizing enzymes that play a critical role in the 
detoxification of various endogenous and exogenous compounds. The mammalian cytosolic GSTs consist of seven classes based 
on sequence similarity and substrate selectivity: \(\alpha\) (\(\alpha\)), \(\pi\) (\(\pi\)), \(\mu\) 
(\(\mu\)), \(\theta\) (\(\theta\)), \(\kappa\) (\(\kappa\)), \(\sigma\) (\(\sigma\)), and \(\zeta\) (\(\zeta\)) (13–17). The catalytic activity 
of GSTs is based on deprotonation of GSH to form the thiolate 
(GS\(^{-}\)), which is a superior nucleophile compared with the pro- 
tonated thiol. X-ray crystallographic structures and site-di- 
rected mutagenesis studies illustrate that each GST contains a 
conserved tyrosine or serine residue that contributes to thiolate 
formation through a hydrogen bond (OH\(^{-}\)– SG) and reduces the \(pK_a\) of bound GSH. Although mutation at this catalytic site 
dramatically reduces \(k_{\text{cat}}\) or \(V_{\text{max}}\) rates for 1-chloro-2,4-dinitro- 
benzene, \(K_m\) values for the substrate and GSH are not signifi- 
cantly altered (18–24).

The A-class GSTs are unique in that several isoforms contain 
a dynamic C terminus that undergoes a ligand-dependent dis- 
order-to-order transition (25, 26). For example, in the absence 
of a bound hydrophobic ligand, the 15 C-terminal residues of 
hGST A1-1 are invisible crystallographically (25). Although it is 
unclear whether the residues are completely disordered or lie 
in a delocalized helix, the C terminus will be referred to here as 
“disordered” in this unsolvable state. In conjunction, the 
“folded” or “ordered” state refers to the observable helix when a 
GS-product conjugate is bound.

Previously, contributions of the helix to catalysis, ligandin 
function, and ligand dissociation and binding in GST A1-1 have 
been determined (24, 27–31). However, the detailed mechani- 
mism for, and the residues involved in, the ordering of the helix 
have not been elucidated. Hyperbaric experiments have illus- 
trated that the ionization state of Tyr-9 is thermodynamically 
coupled to the dynamics of the C terminus (28). Presumably, 
then, the hydrogen bonding status of Tyr-9 should also be 
energetically linked to the dynamics of the C terminus. There- 
fore, we hypothesized that a hydrogen bond to the phenolic 
oxygen of Tyr-9 provides a mechanism for communication be- 
tween the active site and the C terminus, perhaps through an 
on-face electrostatic interaction between Phe-220 and Tyr-9.

Here, we demonstrate directly that the catalytic Tyr-9 of 
rGST A1-1 controls the ligand-dependent folding of the C ter- 
minus, presumably through stabilization of the transition state 
for this conformational change, as well as the rates of ligand 
binding and dissociation. These results illustrate the impor- 
tance of a second-sphere, indirect interaction between a struc- 
tural element (C terminus) and a remote protein-ligand hydro- 

gen bond (OH–SGR). Moreover, they expand the role of the 

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\(^1\) The abbreviations used are: GST, glutathione S-transferase; rGST, 
GST, derived from rats; hGST, GST, derived from humans; EA, 
ethacrynic acid; GS-EA, the Michael adduct of ethacrynic acid and 
glutathione; GSH, glutathione; GS-NBD, the glutathione conjugate of
catalytic tyrosine in GST A1-1 beyond the previous paradigm, which has been limited to stabilization of the GS\textsuperscript{−} thiolate and catalysis of the chemical conjugation step.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Instrumentation**—GSH, NBD-Cl, and MES were obtained from Sigma. Spectro/Por dialysis membrane was obtained from Spectrum Medical Industries, Inc. (Houston, TX). UV-visible absorbance spectra were recorded on a Cary 3E double beam UV-visible spectrophotometer. \(^1\)H NMR spectra were obtained at 300 MHz using a Varian VXR 300 spectrometer. Low resolution fast atom bombardment and electron ionization mass spectra were obtained using a Micromass 70SEQ tandem hybrid mass spectrometer. Stopped-flow binding and dissociation experiments were performed with a BioLogic SFM4/QFM fluorometer.

**Synthesis of GS-NBD Product Conjugate**—5 mmol of NBD-Cl and 6.25 mmol of GSH were added and dissolved in a 100 ml of a solution of Tris/ethanol (50:50 v/v). After adjusting the pH to 8.1 with NaHCO\textsubscript{3}, the mixture was stirred for 14 h in darkness at room temperature. The reaction mixture was placed at 4 °C, and the product was crystallized in 1 week. The crystals were washed with ice-cold buffer, dried, and stored under vacuum in darkness at 4 °C. Mass spectrometry, NMR, and high performance liquid chromatography were used to confirm the product and its purity.

**Protein Expression and Purification**—The expression and purification of the WT rGST A1-1 protein have been described previously (32). The Y9F mutant was a kind gift from Dr. Anthony Lu (23).

**Equilibrium Dialysis**—Equilibrium dialysis experiments were performed in 0.5-ml Plexiglass cells separated by a dialysis membrane (molecular mass cutoff 12–14 kDa) (33, 34). 1 mM GST (300 \(\mu\)M) was dialyzed against an equal volume of 0.5–50 \(\mu\)M GS-NBD in a 25 °C horizontal shaker water bath. After equilibration for 4 h, samples from each chamber were assayed fluorometrically at \(\lambda_{\text{exc}}\) and \(\lambda_{\text{emi}}\) for the presence of GS-NBD (35–37). Concentrations of free and bound GS-NBD were calculated from a standard curve, and the corresponding equilibrium dissociation constants \((K_d)\) were determined by curve-fitting with the program Enzfitter.

**Gel Filtration**—Gel filtration experiments were performed with two 12-inch G-25 Sephadex columns, aligned in sequence, equilibrated with 100 mM MES buffer, pH 6.5. A 1-ml solution of 18 \(\mu\)M GST and 500 \(\mu\)M GS-NBD was loaded onto the column, and fractions were collected. Each fraction was analyzed by UV-visible absorbance for the presence of GST (\(\lambda_{\text{abs}}\)) and GS-NBD (\(\lambda_{\text{abs}}\text{GS-NBD}\)).

**Isothermal Titration Calorimetry**—Thermodynamic parameters were determined at 25 °C using a CSC 4200 titration calorimeter (Calorimetry Sciences Corporation, Provo, UT). GST solutions (100–200 \(\mu\)M) were titrated with 10-\(\mu\)l injections of GS-NBD (1–2 mM), and the heat flow between the reaction vessel and an isothermal heat sink was monitored. The binding stoichiometry, \(K_a\), and \(\Delta H\) were determined from a nonlinear least squares fitting of the reaction heat by the programs Dataworks and Bindworks.

**GST Binding and Dissociation Kinetics**—Conditions for binding and dissociation experiments were similar to those described previously (29). All reactions were maintained at 15 °C using a circulating water bath. Binding rates were measured by the decrease in protein fluorescence after mixing an equal volume of 2 \(\mu\)M GST and 20–400 \(\mu\)M GS-NBD. Kinetic data were analyzed and fit to a single exponential equation (Equation 1),

\[
y = a \times e^{-k_{\text{obs}} t} + C \quad \text{(Eq. 1)}
\]

where \(a\) is the amplitude of the observed rate constant, \(k_{\text{obs}}\), \(t\) is time in seconds, and \(C\) is the offset constant. Dissociation of protein from the [GST-GS-NBD] complex was followed by the increase in protein fluorescence after rapidly mixing 2 \(\mu\)M complex with an equal volume of 5 mM glutathione sulfonic acid (GS\textsubscript{O} \textsuperscript{−} ) at 15 °C. Dissociation rate constants were obtained directly from a fit to the single exponential equation (Equation 1).

**RESULTS**

As described, we hypothesized on the basis of high pressure experiments that the Tyr-9 hydrogen bond controls the dynamics of the C terminus (28). In turn, we would expect that loss of the hydrogen bond between Tyr-9 and a GS-product conjugate, as in the mutant Y9F, would alter binding affinity as well as rates of ligand binding and dissociation. Equilibrium dialysis was used to determine the binding affinity of WT and Y9F for the synthesized 7-glutathionyl-4-nitrobenzo-2-oxa-1,3-diazole (GS-NBD) product conjugate (Fig. 1). Concentrations of free and bound GS-NBD yielded \(K_d\) values of 4.09 \(\mu\)M and 0.641 \(\mu\)M for WT and Y9F, respectively (Table I). The experimentally observed stoichiometry was 1.2 and 1.1 GS-NBD binding/subunit for WT and Y9F, respectively. As an additional, albeit qualitative method of comparing the affinities of WT and Y9F GST A1-1 for GS-NBD, a gel filtration experiment was performed. In elution fractions containing an equivalent amount of protein (~8 \(\mu\)M), the WT fraction did not contain any detectable GS-NBD, whereas 3.1 \(\mu\)M GS-NBD remained in the Y9F-containing fraction.

Because of the surprising nature of the equilibrium results, isothermal titration calorimetry was used to confirm the binding affinity of Y9F and WT for GS-NBD. Confirming the other measurements, \(K_a\) values determined by isothermal titration calorimetry for WT and Y9F were 1.51 and 0.391 \(\mu\)M, respectively (Table I). Although removal of the hydrogen bond between Tyr-9 and the GS-NBD ligand might be expected to decrease binding affinity, these results clearly demonstrate that ligand binds more tightly in the absence of this hydrogen bond.

**Binding and Dissociation Kinetics**—To directly monitor the C-terminal dynamics of the ligand-bound complex, binding and dissociation rates were measured by stopped-flow fluorescence. A decrease in fluorescence intensity was observed on mixing 2 \(\mu\)M GST with 20–400 \(\mu\)M GS-NBD (Fig. 2, top). The observed rate constant, \(k_{\text{obs}}\), was determined from the single exponential fit to the raw data (Equation 1), and \(k_{\text{obs}}\) was plotted versus GS-NBD concentration, yielding a hyperbolic curve for both WT and Y9F (Fig. 2, bottom). The simplest mechanism that fulfills a hyperbolic plot is a two-step mechanism where GST and GS-NBD rapidly combine to form a precomplex, [GST-GS-NBD] \((k_1)\), which slowly isomerizes to the final complex, [GST-GS-NBD]\textsuperscript{+} \((k_2)\) (Equation 2) (38–41).

\[
k_1 \quad \text{GST + GS-NBD} \rightarrow \text{[GST-GS-NBD]} \quad \text{(Eq. 2)}
\]

In accordance with previous results with the glutathione ethacrynic acid product conjugate (GS-EA), we propose that the initial step, \(k_1\), corresponds to the docking of GS-NBD within the active site to yield [GST-GS-NBD], a complex that has a disordered C terminus. The second step, \(k_2\), corresponds to the folding of the C-terminal helix to yield [GST-GS-NBD]\textsuperscript{+}, the final equilibrium complex (29).

This two-step model is supported by stopped-flow studies with a C-terminal truncation mutant, Δ209–222. In binding experiments with GS-EA, a single, rapid rate was observed on ligand binding to Δ209–222. In contrast, in binding reactions with WT and GS-EA, both rapid and slow binding rates were observed (29). Thus, from our previous studies with GS-EA, it is clear that the second kinetic phase reflects motion of the C terminus. Here, in binding experiments between Δ209–222 and GS-NBD, the kinetic trace was flat. However, the equilibrium fluorescence intensity of a solution of Δ209–222 is higher than that observed in a mixture containing the [Δ209–222-GS-NBD] complex, suggestive that ligand docking occurs but is too
C terminus Dynamics in GST A1-1

In addition to association rates, rates of dissociation were directly obtained from the rate of the increase in intrinsic protein fluorescence after the addition of glutathione sulfonic acid (GSO₃⁻) to an equal volume of the [GST-GS-NBD] complex (Fig. 3). The binding of the trapping agent, GSO₃⁻, caused no fluorescence change; therefore its binding is spectroscopically silent (29). Fluorescence data fit best to the single exponential equation (Equation 1), yielding the dissociation rate, $k_{-2\text{obs}}$, rapid to detect in the stopped-flow experiments (data not shown). According to this two-step mechanism in which the first step is too fast to explicitly measure $k_1$ and $k_{-1}$, the constants $k_{2\text{obs}}$, $k_{-2\text{obs}}$, and $K_2$, where $K_2$ is equal to $k_{-2\text{obs}}/k_{2\text{obs}}$, are determined from the fit of the $k_{\text{obs}}$ plots to (Equation 3) (40).

$$k_{\text{obs}} = k_{-2\text{obs}} + (k_2 \times [\text{GS-NBD}]_m/([\text{GS-NBD}] + K_1))$$  (Eq. 3)

Kinetic constants determined from Equation 3 are found in Table I.

In addition to association rates, rates of dissociation were directly obtained from the rate of the increase in intrinsic protein fluorescence after the addition of glutathione sulfonic acid (GSO₃⁻) to an equal volume of the [GST-GS-NBD] complex (Fig. 3). The binding of the trapping agent, GSO₃⁻, caused no fluorescence change; therefore its binding is spectroscopically silent (29). Fluorescence data fit best to the single exponential equation (Equation 1), yielding the dissociation rate, $k_{-2\text{obs}}$.

Similar to that calculated from (Equation 3), $k_{-2\text{calc}}$ (Table I).

Several interesting results were obtained from the stopped-flow analysis. One informative difference between Y9F and WT is the change in the fluorescence intensity on binding and dissociation (Figs. 2 and 3). In binding and dissociation experiments, the fluorescence intensity change in Y9F is about 70% of WT, as determined by amplitude values (Table I). Although fluorescence intensity changes are difficult to predict, this result is most likely due to the greater fraction of the disordered C terminus for Y9F at equilibrium, 42% versus 27%, as calculated by the $K_c$ ratio.

In addition, and perhaps most striking, substitution at Tyr-9 markedly alters all binding and dissociation constants compared with WT. In the first step, docking ($k_2$), dissociation ($k_{-2}$), or both steps are altered to reduce the equilibrium constant, $K_1$, 5-fold compared with WT. More interestingly, rates of ordering and disordering of the C terminus, $k_2$ and $k_{-2}$, have been significantly reduced in Y9F, 11-fold and 7-fold, respectively. These results are the first to demonstrate the importance of the Tyr-9 to the localized folding of the C terminus and, therefore, to rates of ligand binding and dissociation in GST.

**DISCUSSION**

Here, we have used rGST A1-1 to study the ligand-induced, localized folding of a flexible, dynamic C terminus. Due to the presence of few specific van der Waals contacts between the C-terminal residues and the hydrophobic ligand in crystal structures, the basis for the ligand-driven transition to the crystallographically observable C terminus is not obvious (25, 26). Indeed, the ordering of the C terminus may be driven indirectly through second sphere interactions, as suggested by our hyperbolic results, which indicate a critical role for Tyr-9 (28).

Equilibrium binding experiments, including equilibrium dialysis, gel filtration, and isothermal titration calorimetry, indicate a significant difference in binding affinity for the GS-NBD product conjugate between Y9F and WT (Table I). The
increased binding affinity in the Y9F enzyme was quite striking, as intuitively one would expect that removal of a hydrogen bond would decrease binding affinity. A similar effect was observed in equilibrium binding studies with Y9F hGST A1-1. Allardyce et al. (24) recently found that Y9F hGST A1-1 binds the ligand, S-dinitrophenylglutathione, 20-fold more tightly than WT. ∆H° and ∆S° values also indicate a difference in the thermodynamics of binding to each enzyme. It is striking that the more favorable ∆H° in the Y9F variant comes at an entropic cost, perhaps due to decreased solvent displacement from the active site upon ordering of the C terminus. These thermodynamic results will be discussed in a forthcoming manuscript.

Kinetic binding and dissociation experiments supported our previous observation that ligands bind to rGST A1-1 in a two-step sequence: a docking step, followed by a conformational transition of the C terminus from its disordered to ordered state (Equation 2) (29). Substitution at Tyr-9 alters the rate of both of these steps compared with WT (Table I). The reduction in the equilibrium constant, K1, may be due to a decrease in k-1, an increase in k1, or a combination of the two. Because the docking step is too fast to measure k-1 and k1, we cannot determine how the mutation is affecting this kinetic step of the binding reaction from these studies.

More interesting and relevant to our discussion here are the changes associated with k2 and k-2, which correspond to the order-disorder transition of the C terminus, on substitution of Tyr-9 (Table I). These reductions in rate are not limited to the GS-NBD ligand, as similar results have been obtained with the GS-EA product conjugate. In reactions with GS-EA, the Y9F substitution reduced k-2(calc) by 6-fold, k2 by 8-fold, and K1 by 4-fold. The k-2(calc) could not be determined directly, presumably because GS-EA binds too tightly and is not displaced by the trapping agent, GSO3. Surprisingly, in steady state reactions with hGST A1-1 and EA, kcat was unchanged upon substitution at Tyr-9, in contrast to our results, which suggest a decreased kcat for a Y9F-catalyzed reaction, which is rate-limited by product release (24). The source of these differences is unknown.

The changes in dissociation and binding rates may be due to stabilization of the complexed ground states, destabilization of the transition state, or a combination of the two. A free energy diagram, which accounts for the kinetic terms, provides a useful summary of our results (Fig. 4). The first step of the binding reaction is contained within the diagram for completeness, although we have no information of the actual k1 and k-1 rate constants and have determined only their relative ratios. For simplicity, we assumed that the equilibrium difference between WT and Y9F is entirely realized in k-1, so that k1 is identical for WT and Y9F. On comparison of the ground state energies for the complexes with a disordered and ordered C terminus, [GST-GS-NBD]dis and [GST-GS-NBD]fold the Y9F complexes are 0.9 and 0.6 kcal/mol more stable than the WT complexes, respectively. However, the transition state for the C-terminal isomerization is destabilized in the Y9F complex by 0.5 kcal/mol compared with WT, resulting in a 1–1.5 kcal/mol greater barrier to the C-terminal transition for the ordering (k2) and disordering (k-2) of the C terminus.

X-ray crystal structures give little insight into the mechanism by which the Tyr-9 residue structurally stabilizes the transition state for the order-disorder transition. Tyr-9 does lie in an aromatic cluster with Phe-10 and Phe-220, which compete for the space directly adjacent to Tyr-9 (25, 26). It is interesting to speculate that changes in the hydrogen bond strength at Tyr-9 alter the electrostatic surface of its aromatic ring, thus altering the interaction between the electropositive edge of Phe-10 in the open state and the edge of Phe-220 in the folded state (Fig. 5). This is based on documented changes in the electrostatic potential of the aromatic face of Tyr in different hydrogen bonding states (42, 43). Although this is speculation, our results clearly demonstrate the importance of Tyr-9 to the ligand-induced folding of the C terminus, and they expand its role in rGST A1-1 catalysis. Apparently, the Tyr-9 residue facilitates the localized folding of the C terminus through stabilization of the transition state, thereby significantly affecting rates of ligand binding and dissociation. Because product release is rate-limiting, for a number of reactions catalyzed by the A-class GSTs, this additional role for Tyr-9 has significant functional importance, notwithstanding its role in the deproto- nation of GSH.

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