The Anomalous pKα of Tyr-9 in Glutathione S-Transferase A1-1 Catalyzes Product Release*

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The pKα of the catalytic Tyr-9 in glutathione S-transferase (GST) A1-1 is lowered from 10.3 to 8.1 in the apoenzyme and –9.0 with a GSH conjugate bound at the active site. However, a clear functional role for the unusual Tyr-9 pKα has not been elucidated. GSTA1-1 also includes a dynamic C terminus that undergoes a ligand-dependent disorder-to-order transition. Previous studies suggest a functional link between Tyr-9 ionization and C-terminal dynamics. Here we directly probe the role of Tyr-9 ionization in ligand binding and C-terminal conformation. An engineered mutant of rGSTA1-1, W21F/F222W, which contains a single Trp at the C terminus, was used as a fluorescent reporter of pH-dependent C-terminal dynamics. This mutant exhibited a pH-dependent change in Trp-222 emission properties in agreement with changes in C-terminal solvation or conformation. The apparent pKα values for the conformational transition were 7.9 ± 0.1 and 9.3 ± 0.1 for the apoenzyme and ligand-bound enzyme, respectively, in excellent agreement with the pKα for Tyr-9 in these states. The Y9F/W21F/F222W mutant, however, exhibited no such pH-dependent changes. Time-resolved fluorescence anisotropy studies revealed a ligand-dependent, Tyr-9-dependent, change in the order parameter of Trp-222. However, no pH dependence was observed. In equilibrium and pre-steady-state ligand binding studies, product conjugate had a decreased equilibrium binding affinity (Kd), concomitant with increased binding and dissociation rates, at high pH values. Furthermore, the recovered pKα values for the pH-dependent microscopic rate constants ranged from 7.7 to 8.4, also in agreement with the pKα of Tyr-9. In contrast, the Y9F/W21F/F222W mutant had no pH-dependent transition in Kd or rate constants for ligand binding or dissociation. The combined results indicate that the macroscopic populations of “open” and “closed” states of the C terminus are not determined solely by the ionization state of Tyr-9. However, the rates of transition between these states are faster for the ionized Tyr-9. The ionized Tyr-9 states provide a parallel pathway for product dissociation, which is kinetically and thermodynamically favored. In silico kinetic models further support the functional role for the parallel dissociation pathway provided by ionized Tyr-9.

Glutathione S-transferases (GSTs)1 comprise a superfamily of enzymes that catalyze the nucleophilic conjugation of tripeptide (γ-Glu-Cys-Gly) glutathione (GSH) with a wide variety of electrophilic compounds, including xenobiotics, products of oxidative stress, and endogenous toxins (1, 2). To date, several cytosolic GSTs in mammals have been identified on the basis of sequence similarity and substrate specificity: Alpha (A), Mu (M), Pi (P), Sigma (S), Theta (T), Zeta (Z), Kappa (K), and Omega (O) (3–5). In addition to their detoxification role, GSTs have been implicated in the development of drug resistance toward anticancer therapeutics (6–8).

A structural feature common to nearly all cytosolic GSTs is a conserved hydrogen bond between the hydroxyl group of catalytic Tyr or Ser and the thiol group of GSH. This interaction contributes to the lowering of the pKα of Tyr-9 from ~9.3 in solution to 6.5–7.4 at the active site of various GSTs. In the A-class GSTs, the catalytic Tyr has an unusually low pKα (9, 10). The spectroscopic pKα values of Tyr-9 in the human and rat A1-1 isoforms are 8.1 and 8.3, respectively, compared with 10.3 for Tyr in solution. In the GST A4-4 isomorph, the pKα of the corresponding residue is even lower (11). Upon discovery of the anomalous pKα of Tyr-9, a general base mechanism was considered for the A-class GSTs, wherein Tyr abstracts a proton from GSH prior to attack of the resulting GS− at the electrophiles (9). However, solvent deuterium studies indicate that the catalytic Tyr in GSTM1-1, which has a “normal” pKα does not undergo a general base catalysis (12). Subsequently, kinetic studies on hGSTA1-1 substituted with fluorotyrosoine-9 analogs suggested that the pKα of Tyr-9 in the native enzyme is not sufficiently low to support general base catalysis (13). Thus, a functional role of the anomalous pKα of catalytic Tyr-9 in A-class GST has not been identified.

A structural feature unique to GSTA1-1 is the presence of a dynamic C-terminal helix (residues 208–222) that covers the active site upon binding of ligand at the H-site or both sub-sites of the enzyme (Fig. 1). As described previously (14–16) for hGSTA1-1, wild-type rGSTA1-1, and several mutants, the binding of product GSH conjugate (GS-EA) follows a biphasic mechanism, which includes a bimolecular docking step (k1 and k−1) and a slower unimolecular isomerization step (k2 and k−2) that corresponds to the ordering and closure of the C terminus (Fig. 1). Indirect evidence from several laboratories suggests an intimate link between the ionization state of Tyr-9 and the dynamics of the C terminus (14, 15, 17, 18). One possible implication is that ligands will preferentially bind to or disso-

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‡ The abbreviations used are: GST, glutathione S-transferase; GS−, thiolate anion of glutathione; GSO5−, glutathione sulfonate; MES, 2-(N-morpholino)ethanesulfonic acid; CAPS, 3-(cyclohexylamino)-2-hydroxy-1-propanesulfonic acid; GS-EA, Michael adduct of ethacrynic acid and glutathione; rGST, GST derived from rats; hGST, GST derived from humans.
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were performed with BioLogic SFM/QFM fluorimeter. Steady-state fluorescence of the C-terminal disorder-to-order transition were performed with a 5-cm lens onto a crystal of potassium dihydrogen phosphate. Fluorescence is collected at right angles through a polarizer mounted at 54° to the excitation polarization and then passed through an ISA H-10 monochromator with a 160nm bandpass or through cut-off filters. A Hamamatsu 2809u microchannel plate, amplified by a Minicircuits ZHL-1042A, and a FFD 100 EG & G photodiode provide the start and stop signals, respectively. Constant-fraction discrimination of these signals is performed by a Tennelec TC 455 and time-to-amplitude conversion by an ORTEC 457. Data are stored in a Norland 5500 multichannel analyzer before transfer to and analysis with a PC. The instrument function of this system has a frequency width at half-maximum of 100–150 ps. The polarized fluorescence traces used to obtain fluorescence anisotropy decay parameters are collected to a maximum of 16,000 counts. The peak channel is centered deep in a 5 µM protein, at the indicated pH, in 35 mM MES, 35 mM Tris, 35 mM CAPSO, with either no ligand or 50 µM S-hexyl-GSH.

Global Data Fitting—Time-resolved fluorescence data, acquired at various wavelengths, were subjected to a global fitting procedure found in Spectra Solve™. A local χ² is calculated for each local fit. After all curves are fit (typically 6–11), a global χ² is calculated. The global parameters are varied, and the whole process is repeated to calculate a new global χ². This process is repeated until a minimum is reached for χ². The quality of the fit is determined by visual inspection and residuals.

Site-directed Mutagenesis—The construction of W21F/F222W GSTA1-1 has been described previously (14). The triple mutant Y9F/W21F/F222W was constructed by subcloning the available W21F/F222W insert, which was acquired from Biosearch Technologies, into a Y9F/W21F/F222W plasmid that had been linearized with a similar set of restriction enzymes. Originally, the single and double Y9F mutants were generated by PCR-based amplification of fragment spanning the EcoRI and SalI restriction sites contained in the linearized pH9262 plasmid (19). All mutants employed in the present studies were validated by DNA sequencing.

Protein Expression and Purification—The expression and purification of wild-type rGSTA1-1 and its various mutants have been described previously (20). The purity of each protein was assessed by SDS-PAGE. Enzymatic activity of each mutant protein was assessed as described previously (21).

Determination of Conformational Transition in GSTA1-1—Emission spectra were monitored at excitation wavelength of 295 nm, with slit widths of both monochromators set to 4 nm. In contrast to the previously employed tyrosine titration method (9), the emission properties of tryptophan were exploited to determine the nature of the surrounding environment of this residue. The pH dependence of Trp emission was monitored in 2 µM GST prepared in mixed buffer of 35 mM MES, 35 mM Tris, 35 mM CAPSO at 25 °C. The ρKₐ values were determined by fitting the spectral center of mass, emission peak intensity, relative intensity at 305 nm, and relative intensity at 345 nm versus pH to an equation describing single residue ionization via Enzfitter. Fluorescence titrations were also performed in the presence of 200 µM S-hexylglutathione.

Equilibrium Binding Studies—The equilibrium dissociation constants (Kᵣ) for the Michael addition product GS-EA were determined by the decrease in the fluorescence of W21F/F222W, Y9F/W21F/F222W, and Δ209–222 upon ligand binding. During the titration experiments, serial dilutions of equilibrated [GST-GS-EA] complex, consisting initially of 0.5 µM GST and 100 µM GS-EA in mixed buffer system at pH 6.5 and 9, were made. The steady-state emission of each protein at 25 °C was monitored at an excitation wavelength of 295 nm, using slit widths of 4 nm. The observed fluorescence intensity at each dilution was corrected for the emission intensity of the reference buffer. The mole fraction of GS-EA bound to total protein was determined from a ratio of protein intensity when a ligand is bound and emission intensity of free enzyme. This fraction is further corrected for the dilution factor. Kᵣ values were then derived from Equation 1 via DeltaGraph 4.

where ν is the mole fraction of [GS-EA] bound to protein, and P and C are the upper plateau and lower plateau, respectively, of the ν versus [GS-EA]ᵣ plot.

Ligand Binding and Dissociation Kinetics—Stopped-flow fluorescence studies were performed to determine the rates of ligand binding and dissociation as described in previous studies (14–16, 20). The rate constants for the binding reaction were measured by monitoring the decrease in protein fluorescence after rapid mixing of an equal volume of 2 µM GST and 6.25–100 µM GS-EA at 15 °C and at various pH values. The multicomponent buffer used in these pH-dependent studies was 35 mM MES, 35 mM Tris, 35 mM CAPSO.
measured by monitoring the increase in fluorescence intensity after experiment and the above equation are different from alternatively, a single observed rate, association rates, constants were experimentally observed, the rapid association and dissociation rates, \( k_1 \) and \( k_{-1} \) were fitted to Equation 4 that describes the linear dependence of \( k_{\text{obs1}} \) on GS-EA concentration,

\[
h_{\text{obs1}} = k_{-1} + k_{1} \frac{[\text{GS} - \text{EA}]}{1 + (K_a/[\text{GS} - \text{EA}])}
\]

It should be noted that the \( k_{-1} \) values derived from the binding experiment and the above equation are different from \( k_{-1} \) values that were directly determined from the dissociation experiments. The association and dissociation rates of the slower step of the binding \( k_2 \) and \( k_{-2} \) were determined from a hyperbolic dependence (Equation 5) of \( k_{\text{obs2}} \) on GS-EA concentration,

\[
h_{\text{obs2}} = k_{-2} + k_{2} \frac{[\text{GS} - \text{EA}]}{1 + (K_a/[\text{GS} - \text{EA}])}
\]

Alternatively, a single observed rate, \( k_{\text{obs1}} \), could be plotted against GS-EA concentration to yield a hyperbolic curve fit (Equation 6),

\[
k_{\text{obs1}} = k_{-1} + k_{1} \frac{[\text{GS} - \text{EA}]}{1 + (K_a/[\text{GS} - \text{EA}])}
\]

The dissociation of product from the [GST-GS-EA] complex was measured by monitoring the increase in fluorescence intensity after rapid mixing of equal volume of 2 μM complex and 4 μM of the trapping agent, GS-sulfonate (GSO₄⁻). The dissociation rate constants, \( k_{-1} \) and \( k_{-2} \), were measured from a single (Equation 1) or double (Equation 2) exponential fit to the raw data.

**Kinetic Simulations**—Simulations were performed with Kinetic, using the following expressions (Equation 7) for the time-dependent concentration of relevant species. The specific rate constants and initial concentrations used are indicated under “Results.”

\[
\frac{df_{\text{[Tyr-9-OHL]}}}{dt} = -k_{-1}[\text{[Tyr-9-OHL]}]^{\text{[closed]}} + k_{1}[\text{[Tyr-9-OHL]}]^{\text{[open]}}
\]

\[
\frac{df_{\text{[Tyr-9-OHL]}^{\text{[open]}}}}{dt} = \frac{-(k_{-1} + k_{2})[\text{[Tyr-9-OHL]}^{\text{[open]}}]^{\text{[closed]}} + k_{1}[\text{[Tyr-9-OHL]}^{\text{[open]}}]^{\text{[closed]}}}{1 + (\frac{1}{K_a})}
\]

\[
\frac{df_{\text{[Tyr-9-OHL]}^{\text{[open]}}}}{dt} = \frac{k_{1}[\text{[Tyr-9-OHL]}^{\text{[open]}}]^{\text{[open]}} + k_{-1}[\text{[Tyr-9-OHL]}^{\text{[open]}}]^{\text{[closed]}}}{1 + (\frac{1}{K_a})}
\]

\[
\frac{df_{\text{[L]}^{\text{[open]}}}}{dt} = \frac{k_{1}[\text{[Tyr-9-OHL]}^{\text{[open]}}]^{\text{[open]}} + k_{-1}[\text{[Tyr-9-OHL]}^{\text{[open]}}]^{\text{[closed]}}}{1 + (\frac{1}{K_a})}
\]

**RESULTS**

**Characterization of C-terminal Dynamics by Fluorescence**—Our previous studies on the anomalous pKₐ value of catalytic Tyr-9 in rGSTA1-1 suggest that the ionization state of the tyrosyl hydroxyl group influences the dynamics of the C terminus (20, 18). In human and rat isofoms, the only tryptophan residue in the native GSTA1-1, Trp-21, near the domain-domain interface serves as a probe of global microconformational changes that occur upon ligand binding (22, 23). However, to investigate directly the localized structural changes at the C terminus, a Trp residue was engineered at the ultimate position of rGSTA1-1. The kinetics of ligand binding and dissociation for this mutant, W21F/F222W, are comparable with the true wild-type, indicating that global changes in protein conformation are coupled to local conformational changes at the C terminus (16). Thus, the W21F/F222W serves as a faithful mimic of ligand binding to the wild-type.

In order to determine the relationship between ionization state of Tyr-9 and C-terminal dynamics, the first W21F/F222W mutant was titrated between pH 7 and 10 to monitor the fluorescence emission properties of the terminal Trp. The emission of Trp, which absorbs at 275–285 nm and emits at 340–353 nm, is very sensitive to its local environment and is often used as a direct probe of conformational changes in proteins (23). The changes in the emission spectra of Trp-222 in the site-directed mutants reflect the polarity of the surrounding environment. Fig. 2 summarizes the typical emission spectra for the W21F/F222W and Y9F/W21F/F222W at several pH values. The emission intensity decreases, and the spectrum is red-shifted up to 7 nm with increasing pH in the W21F/F222W mutant. In contrast, the emission intensity of the Y9F/W21F/F222W mutant remained relatively constant, except at the very high pH values, at which the maximal spectral red shift was −2 nm. Clearly, W21F/F222W exhibits a large pH-dependent change in environment only when Tyr-9 is present. From the fluorescence titrations employed, the pKₐ values for this transition could be determined by fitting the spectral peak intensity, relative intensity at 305 nm, or relative intensity at 345 nm against pH via single ionization model. The average pKₐ values recovered from all curve fittings based on intensity change are 7.9 and 10.5 for W21F/F222W and Y9F/W21F/F222W, respectively (Table I).

A more sensitive measurement of polarity at the active site of the enzyme is the pH dependence of spectral center of mass, which closely resembles the trend observed in the pH dependence of peak intensity (Fig. 2). However, the spectral center of mass in W21F/F222W does not reach a plateau after pH 9.5. In fact, the center of mass continues to decrease even after pH 10. Presumably, at high pH, tyrosine residues in the protein become ionized and contribute to the additional spectral red shift as observed in the control Y9F/W21F/F222W mutant. Whereas tyrosine or tyrosinate would not contribute significantly to the
change in intensity at the emission maximum of Trp-222, their spectral signals would contribute to the overall spectral center of mass. Therefore, an accurate pK_a value for the conformational transition based on Trp-222 parameters could not be obtained. Although the spectral center of mass data could not be fitted to an equation yielding a pK_a, they clearly demonstrate a change in Trp polarity in the pH range of 7.5–8.5, which does not occur in the triple mutant. In addition, the overall pK_a recovered from the intensity data of W21F/F222W most likely reflects the pK_a of active site Tyr-9. Although the catalytic Tyr in the triple mutant had been removed, the apparent pK_a of 10.5 is probably due to seven other tyrosines in the protein that become ionized at very high pH.

The binding of S-hexylglutathione (S-hexyl-GSH) to GSTA1-1 has been shown to induce closure of the C terminus, with Tyr-9 pK_a (9) remaining lower than the pK_a of free tyrosine in solution (13, 24, 25). Here the emission spectra of W21F/F222W and Y9F/W21F/F222W were also obtained in the presence of saturating concentrations of S-hexyl-GSH. The S-hexyl-GSH was used because it provides no interference with the emission in fluorescence measurements. If the pH-dependent changes observed with W21F/F222W correlate with ionization of Tyr-9 in the apoenzyme, and if ionization is a major determinant of helix dynamics, then spectral changes would still be observed in the presence of S-hexyl-GSH but at a higher pH. The recovered pK_a values for the conformational transition (Table I) indicate an increase of 1.4 pK_a unit for the ligand-bound double mutant compared with the ligand-free double mutant, in excellent agreement with previously observed ligand-dependent increase in pK_a of Tyr-9. The increase in Tyr-9 pK_a of the conjugate-bound W21F/F222W suggests that, at equilibrium, only ~3% of the complex remains unprotonated at pH 7.5. Also, the triple mutant Y9F/W21F/F222W titrated with ligand at each pH, in order to determine whether the observed pH dependence was related to Tyr-9 ionization. As expected for a mutant with no ionizable group at residue 9, the bound conjugate had little effect on the apparent pK_a for the conformational transition of the triple mutant (Table I).

Together, these spectroscopic studies directly demonstrate for the first time that the local conformational equilibria of the C terminus are pH-dependent in addition to being ligand-dependent. Moreover, this pH dependence exhibits an apparent pK_a equivalent to the pK_a of Tyr-9 (8) in the apo form and pK_a of ~9 for the ligand-bound form, and this dependence is eliminated upon removal of the Tyr-9 phenolic hydroxyl.

**Time-resolved Anisotropy**—The observed pH dependence in steady-state spectral properties of Trp-222 could be due to a pH-dependent shift in the macroscopic equilibrium between closed and open states of the C terminus. Alternatively, there could be a pH-dependent change in the characteristics of the “open” conformation or the “closed,” without a significant change in the relative amount of each. In order to probe these possibilities, time-resolved anisotropy experiments were performed. The anisotropy would be expected to increase upon opening the C terminus to fully expose the C-terminal Trp-222. The anisotropy decay of W21F/F222W was measured for the ligand-free protein at two limiting pH values, 6.5 and 9, in the presence and absence of S-hexyl-GSH, as probes of the open and closed states. Identical experiments were done with the triple mutant in order to assess the contribution of Tyr-9 to any observed effects. The recovered rotational correlation times, and the order parameters, S\(_2\), are summarized in Table II. The data were analyzed globally according to a biexponential decay with a fixed rotational correlation time for the overall protein motion (20 ns). This was based on the assumption that pH or ligand would not change the overall structure sufficiently to alter global protein hydrodynamics. The recovered rotational relaxation time for the whole protein, 20 ns, is consistent with a protein of 48 kDa. In addition, for each sample a fast anisotropy decay component was detected with correlation times ranging from 0.8 to 1.8 ns, clearly reflecting local motion of the C terminus. The results clearly demonstrate the expected increase in the order of Trp-222 upon binding of S-hexyl-GSH, at both pH values. This provides a benchmark for the magnitude of the change in S\(_2\) that is expected for closure of the C terminus. Furthermore, comparison of the double and triple mutants demonstrates that the Tyr-9 hydroxyl group is critical for maintaining the conformational integrity of the C terminus and the ligand-dependent switch. However, the moderate pH dependence observed for the ligand-free double mutant is observed also with the triple mutant. Based on these data, the ionization of Tyr-9 does not shift significantly the equilibrium between open and closed states, in contrast to the ligand, which does.

Rather, the open state, the closed state, or both are different in the ionized versus the un-ionized forms, yielding different steady-state emission spectra. However, these differences do not correspond to changes in the fast local motion of the C-terminal residue expected for closed versus open conformations.

**Equilibrium Binding Affinity**—Equilibrium binding studies were explored as an initial strategy to determine whether the pH-dependent microconformations of the C terminus differentially affected the binding of GSH conjugates. For several substrates, product release is rate-limiting for GSTA1-1 turnover, so the C-terminal conformational status is an important aspect of catalysis. These studies were performed at pH 6.5 and 9 at which the active site Tyr-9 in bound rGSTA1-1 (pK_a ~9.3) is completely protonated and significantly unprotonated (ionized), respectively. The ligand employed in the equilibrium studies was GS-EA, which is a conjugate formed from ethacrynic acid and GSH via Michael addition, and for which we have previously characterized the binding mechanism in detail. Moreover, crystal structures demonstrate that the C-terminal helix is closed and ordered with GS-EA bound. To prevent the proteins from unfolding and the GSH conjugate from hydrolyzing to its parent compounds at highly basic conditions, equilibrium binding studies beyond pH 9 were not pursued. Throughout our studies, the initial stock of GS-EA was kept on ice until use to avoid hydrolysis, which was monitored in a separate experiment via high performance liquid chromatography (data not shown).

The maximal emission intensities of W21F/F222W and Y9F/W21F/F222W at pH 6.5 were measured at 333 and 330 nm, respectively, at varying ratios of GS-EA:protein. In the presence of GS-EA at low pH, these emission maxima blue-shifted to 321 nm, as shown previously (25). At pH 9, only the W21F/F222W mutant exhibited red-shifted emission spectra. Equilibrium ligand binding studies were also performed in the C-terminal truncation mutant, ∆209–222, for comparison. Typical binding isotherms, from which K\(_D\) values were determined, are shown on Fig. 3. The recovered K\(_D\) values are reported in Table III. At pH 6.5, the W21F/F222W mutant yielded a K\(_D\) of 3.3 μM, which is comparable with a previously determined K\(_D\) of wild-type protein (14). The K\(_D\) for GS-EA significantly increased by 2.5-fold at pH 9, indicating lower

### Table I

| Enzyme            | pK_a free | pK_a bound |
|-------------------|-----------|------------|
| W21F/F222W        | 7.9 ± 0.1 | 9.3 ± 0.1  |
| Y9F/W21F/F222W    | 10.5 ± 0.1| 10.8 ± 0.1 |

* Recovered pK_a in the presence of 200 μM S-hexylglutathione.

* Recovered pK_a in the presence of 200 μM S-hexylglutathione.
affinity at equilibrium when Tyr-9 is ionized. In previous studies, the C-terminal truncation mutant, Δ209–222, has been shown to have Tyr-9 pKₐ that is slightly lower than the wild-type (20). Moreover, with an open, solvent-accessible active site, GS-EA was found to bind to the Δ209–222 mutant (Kᵰ values for GS-EA (Table III) were determined from nonlinear regression fits to mole fraction of GS-EA bound to protein values at pH 6.5 and 9 remained essentially unchanged. The lack of pH dependence of Kᵰ values ranging from 8.2 to 8.8 (Fig. 5). From this set of rate constants, calculated equilibrium dissociation constants (Kₐcalc) could be obtained. Of course, the calculated Kᵰ values are pH-dependent, with an apparent pKₐ of 7.7 (Fig. 6). Apparently, all of the recovered pKₐ values (Table IV) reflect the ionization of Tyr-9, further suggesting that ligand docking and dissociation are favored in the open conformation of the enzyme. Due to difficulties in obtaining significant amounts of the Y9F proteins, which bind poorly to GSH affinity column, combined with the need for high protein concentrations in stopped-flow experiments, the kinetics of li-
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Figure 4. Kinetics of GS-EA binding to wild-type rGSTA1-1 (A and B) and Y9F mutant (C). The stopped-flow fluorescence data for GS-EA binding in wild-type and Y9F fit best to double exponential Equation 3 and single exponential Equation 2, respectively. The rate constants \( k_{ob1} \) and \( k_{ob2} \) for wild-type showed linear dependence (Equation 4) and hyperbolic dependence (Equation 5), respectively, on GS-EA concentration \( (R^2 = 0.99 \text{ and } R^2 = 0.92) \). Meanwhile, the single rate constant for Y9F mutant showed hyperbolic dependence (Equation 6) on GS-EA concentration \( (R^2 = 0.98) \). Note that at pH 6 (squares) and pH 9 (circles), the rate constants for GS-EA binding to wild-type rGSTA1-1 showed apparent dependence on pH, although the rate constants for the Y9F mutant were essentially identical at the extreme pH values. Kinetic parameters are reported in Table IV.

Ligand binding and dissociation were determined at only three pH values. In contrast to the wild type, the Y9F mutant indicated no apparent pH dependence of rate constants (Table IV). The apparent calculated \( K_a \) values for GS-EA in Y9F were not significantly different at any given pH (Fig. 6). Clearly, there is no indication that binding and dissociation of GS-EA in Y9F mutant depends on the ionization of a protein residue in this pH range.

It is important to note that the recovered microscopic rate constants at pH 9 are lower limits for the true rate constants when Tyr-9 is fully ionized. At pH 9, the Tyr-9 is \(~50\%\) ionized, based on the experimentally measured \( pK_a \) with GSH-conjugate bound. Presumably, if higher pH values were experimentally accessible, higher rates would have been observed, reflecting the true rates for Tyr-9 in the completely unprotonated form.

Kinetic Simulations—Because the extent of ionization is low for the ligand-bound species, it is reasonable to consider whether the ionized state could have any effect on the rate of ligand binding and dissociation. What magnitude of rate change could be obtained with such a low extent of ionization? In order to relate the pH dependence of the experimentally determined microscopic rate constants to steady-state turnover of the enzyme, kinetic models were constructed, and simulations were performed. The kinetic scheme shown in Fig. 1 was used as a template but was expanded to include a parallel pathway of ligand binding and C-terminal ordering, via the state with unprotonated Tyr-9. The expanded model is shown in Fig. 7 and is oriented such that the reaction starts with the bound state, with the C terminus closed, [Tyr-9-OH]\text{closed}.

The C terminus opens and closes, for the protonated state, with rate constants \( k_{-2} \) and \( k_2 \), as above. For the unprotonated state the analogous rate constants are \( k_{-2'} \) and \( k_2' \). Similarly, the ligand egress and association rate constants are \( k_{-1} \) and \( k_1 \) for the protonated state and \( k_{-1'} \) and \( k_1' \) for the unprotonated state. The rate constants in Table IV for the wild-type enzyme at pH 6 were used directly to model the protonated pathway. The rate equations describing the concentration of each species are provided under “Materials and Methods.” For the unprotonated pathway, each of the rate constants in Table IV for pH 9 was doubled, in order to mimic the expected increase in rate constant at full ionization; based on the \( pK_a \) of 9 in the ligand-bound state, the experimental values are lower limits, as described above. The equilibria defined by each \( pK_a \) value were modeled with a ratio of rate constants that yielded the appropriate ratio of protonated to unprotonated species, at equilibrium pH 7.4, i.e. 3% unprotonated for the closed, ligand-bound species ([Tyr-9-O^-L]\text{closed}, \( pK_a = 9.0 \)), 9% unprotonated for the open ligand bound species ([Tyr-9-O^-L]\text{open}, \( pK_a = 8.5 \)), and 24% unprotonated for the unbound species ([Tyr-9-O^-]; \( pK_a = 8.0 \)). Starting with 1 \( \mu \)M ligand bound, closed, protonated species ([Tyr-9-OH-L]\text{closed}, upper left), in silico stopped-flow simulations were run to compare the time-dependent population of each species in the model and to simulate the experimental observations. The intent of these simulations is not to quantitatively reproduce the experimental results, but rather to qualitatively model the potential effect of Tyr-9 ionization on the rates of ligand dissociation. Unlike the actual experiments, the in silico stopped-flow experiment contains no trapping agent (GSO\text{3}). Still, the time scale for approach to equilibrium in silico, with a half-time of \(~3\) s, is in good agreement with the present and past experimental results (14). Fig. 7A shows the simulated results for decay of the starting species, [Tyr-9-OH-L]\text{closed}, for the simple model with no Tyr-9 ionization (dashed curve), and with the parallel pathway available through the unprotonated species (solid curve). As expected, the decay is faster for the later case. The half-life for the process is 2.4-fold shorter when the unprotonated pathway is available. The results in Fig. 7B demonstrate the time-dependent build up and decay of the protonated, open, ligand-bound intermediate [Tyr-9-OH-L]\text{open}. At its peak concentration, it is slightly less of this intermediate when the parallel unprotonated pathway is available (solid curve) than when it is excluded (dashed curve). This is expected, because the parallel pathway competitively removes some of the available enzyme. Also, the rate of decay of this intermediate is faster when the parallel pathway is available. This is also expected as this intermediate has two pathways through which to decay, rather than one. Fig. 7C shows the build up of the ligand free open state for the protonated species. This behavior is interesting in as much as the rate of accumulation of ligand free [Tyr-9-OH] is faster at early times when the parallel pathway through the unprotonated states is available (solid curve). However, it is slower at later times and reaches a lower equilibrium concen-
When the simulation is run to 20 s, the difference in concentration is exactly 24% (not shown). The fact that the rate of appearance of unbound [Tyr-9-OH] is faster at early times reflects the faster release of ligand when the parallel pathway is available. In fact, Fig. 7 demonstrates this directly. Fig. 7D is the rate of appearance of free ligand. When the parallel pathway is available (solid curve), the free ligand accumulates faster. The half-life for approach to equilibrium is 2.4-fold faster (0.9 versus 2.1 s) when the unprotonated species contribute to the flux. These simulated results match reasonably well with the difference in experimental $k_{obs}$ at pH 6 versus pH 9, which differs by 4-fold. Based on these simulations, it is clear that even a minor extent of ionization of the initial ligand bound, closed, state can allow flux through the faster, unprotonated pathway with a modest increase in the rate of ligand dissociation.

### DISCUSSION

As already noted, there are many indirect data that indicate a functional role for Tyr-9 in ligand binding, which is mediated through the C terminus. For example, a thermodynamic coupling between Tyr-9 ionization and C-terminal dynamics is indicated by our previous pressure studies (9) in which we initially demonstrated a pressure-dependent ionization of Tyr-9, which was sensitive to C-terminal mutations. Also, the rates of ligand binding and dissociation are dramatically affected in the Y9F mutant compared with wild-type (15–17, see below), further implicating a role for Tyr-9 in C-terminal dynamics. However, there has been no direct evidence regarding

### Table IV

| Kinetic parameters for GS-EA binding to rGSTA1-1 wild type and Y9F at variable pH and 15°C |
|---------------------------------------------------------------|
| Wild type                                                   | Y9F                                      |
| pH 6  | pH 6.5  | pH 7  | pH 8  | pH 8.5  | pH 9  | pK_a  |
| $k_1$ μM$^{-1}$ s$^{-1}$  | 3.08   | 2.78  | 4.36  | 3.54   | 8.84  | 8.54  | 8.4   |
| $k_{-1}$ s$^{-1}$           | 24.7   | 23.2  | 38.0  | 37.0   | 74.4  | 106   | 8.8   |
| $K_1$ μM$^{-1}$             | 8.02   | 8.35  | 8.70  | 10.5   | 8.40  | 12.4  | 8.7   |
| $k_c$ s$^{-1}$              | 16.4   | 38.9  | 20.1  | 34.9   | 47.1  | 54.3  | 8.4   |
| $k_{-c}$ s$^{-1}$           | 0.632  | 1.25  | 1.05  | 3.48   | 4.22  | 6.18  | 8.2   |
| $K_c$                        | 0.039  | 0.032 | 0.052 | 0.100  | 0.090 | 0.114 | 7.4   |
| $K_{Dissociation}$ μM$^{-1}$| 0.30   | 0.26  | 0.43  | 0.95   | 0.69  | 1.27  | 7.7   |
| pH 6  | pH 8    | pH 9  |
| $K_1$ μM$^{-1}$             | 9.99   | 5.99  | 9.05  |
| $k_2$ s$^{-1}$              | 0.795  | 0.714 | 0.933 |
| $k_2$ s$^{-1}$              | 0.925  | 0.681 | 0.804 |
| $K_c$                        | 1.16   | 0.894 | 0.862 |
| $K_{Dissociation}$ μM$^{-1}$| 5.37   | 2.92  | 4.19  |

* The pK_a values recovered from each observed rate constant and equilibrium constant as a function of pH.
* $K_1$ is determined from the ratio $k_1/k_{-1}$.
* $K_c$ is determined from the ratio $k_c/k_{-c}$.
* Equilibrium dissociation constants were determined from $K_{Dissociation} = K_c/(k_c/k_{-c} + k_{-c})$.
* $K_1$ and $k_{-2}$ were determined from Equation (6).
4.5-fold greater than $k$ constants use for simulation are as follows: and ligand-free states for the ionized form. See text for details. The rate

of Tyr-9.

Top panels product egress ($A$) time-dependent changes as follows: $24.7 \text{ s}$

binding and release were subsequently affected (26 29). Studies had been on the ionization state of GSH and how its

pH dependence of ligand binding to GSTs, the focus in those

those cases, the rates of ligand binding and release were dependent on whether or not the GSH at the active site of several

GST isoforms was unprotonated. Apparently, the rates of GSH

binding and release were faster at lower pH, where the ligand

is mostly protonated. Importantly, the available crystal struc-

tures clearly indicate ligand-induced closure of the C terminus.

Because release of the product conjugate is rate-limiting for several substrates, these studies reveal new information about

kinetically relevant steps of the reaction cycle. No previous studies have directly investigated the role of Tyr-9 $pK_a$ in the

product dissociation mechanism.

To examine directly the relationship between the ionization state of Tyr-9 and the conformational status of the C terminus,

here we have exploited four approaches as follows: 1) the pH

dependence of conformational dynamics of the C terminus, as

reported by an engineered Trp-222; 2) pH-dependent equilib-

rium binding studies; 3) stopped-flow studies in which binding

and dissociation of ligand were directly measured at variable

pH; and 4) kinetic simulations that mimic the dissociation of a

GSH conjugate, based on experimentally derived parameters.

On the basis of emission properties of Trp-222, the local

environments of the double and triple mutants were indeed different from each other. The decrease in the emission intens-

ty and the red shift in the spectrum of W21F/F222W from pH

7 to 9.5 are indicative of polar, solvent-accessible environment

at higher pH, or some type of conformational change. This

pH-dependent emission profile was lost in the triple mutant

containing Y9F. Moreover, the dependence of Trp-222 spectral

intensity on pH yielded an apparent $pK_a$ of 7.9 in the W21F/

F222W, which is remarkably close to the $pK_a$ for the ionization

of Tyr-9 at the active site. In contrast, the pH dependence of

spectral intensity in the Y9F/W21F/F222W resulted in a $pK_a$ of

10.5, which likely reflects the ionization of other tyrosines in

the protein at the extreme pH values. In fact, no appreciable

Tyr ionization of analogous mutant (Y9F-W21F) was observed

at pH $<11$ (9). Presumably, the C terminus of Y9F/W21F/

F222W mutant is significantly less solvated than W21F/F222W

at pH values above 8.0, suggesting that the C terminus in the

former likely adopts a different conformation. This conclusion

is further supported by similar studies in the presence of ligand

known to induce closure of the C terminus and alter the $pK_a$

of Tyr-9. The increase in Tyr-9 $pK_a$ of W21F/F222W, as a result

of S-hexyl-GSH binding, is not only consistent with our previous

studies but also lends support to our hypothesis that the ion-

ization of Tyr-9 also occurs to a limited extent even with GSH

conjugate bound. Indeed, the pH-dependent spectral properties

of W21F/F222W in complex with S-hexyl-GSH are shifted to

$pK_a$ 9.1, in agreement with the Tyr-9 $pK_a$ in this complex.

Importantly, these are the first spectroscopic data that corre-
late local conformation of the C terminus with ionization of

Tyr-9.

One possible scheme consistent with the steady-state spec-

tral properties of the two mutants is that the C terminus is

open and disordered when Tyr-9 is ionized, and it is closed and

static when Tyr-9 is protonated, corresponding to the two mac-

roconformations observed in crystal structures. However, the

anisotropy decay results are not consistent with this scheme. If

the open and closed states of the C terminus corresponded directly with the ionized and neutral forms of Tyr-9, then a

much larger pH dependence of the fluorescence anisotropy

order parameter would be expected. Obviously, the local

dynamics of the ultimate residue in the C terminus are more

sensitive to addition of ligand than to ionization of Tyr-9 (Table

II). Whereas the anisotropy results nicely confirm the ligand-
dependent disorder-to-order transition observed in the crystal

and results from NMR, the results also indicate that a simple

two-state model, with only open and closed states, is insuf-

ficient. The anisotropy data also confirm the importance of the

Tyr-9 hydroxyl group, in as much as the ligand-dependent

effects are lost upon substitution of Tyr-9 with Phe.

In order to accommodate all of the data, we propose that

there is a conformational distribution of open states and a
distribution of closed states. This is supported by the crystal structures with different ligands bound. Different GSH conjugates, or the inhibitor ethacrynic acid in the absence of GSH, yield readily definable electron density for the C terminus corresponding to the closed macroconformation. However, the exact location of the C terminus is different in each case, defining several possible microconformations within the closed state. Presumably, there are more microconformations sampled in the open state than in the closed, but these experiments do not address that issue. Together, all of the fluorescence data suggest that, whereas the ionization state of Tyr-9 is not the major determinant of the distribution between open and close states (based on anisotropy), it does perturb the local conformational distribution of each state (based on emission properties and kinetic behavior). Alternatively, it is formally possible that even when the majority of the helix is in the closed state, the ultimate Trp residue remains isotropic and indistinguishable from the open state.

Regardless of these details, the equilibrium and pre-steady-state binding experiments indicate a modest, but clear, functional advantage for the ionized form of Tyr-9. To the extent that product release is rate-limiting for many substrates, retention of product conjugates is a kinetic “problem” for GSTA1-1. Furthermore, product inhibition is commonly observed as a result of high affinity of GSH conjugates for the enzyme. The equilibrium binding experiments reveal a modest, 3–4-fold, decrease in affinity of ligand as the Tyr-9 becomes unprotonated. Also, all of the individual rate constants describing the biphaseic binding and dissociation reactions are increased upon deprotonation of Tyr-9. In particular, the microscopic rate constant \( k_{-2} \) which corresponds to the first step of rate-limiting product dissociation, and which we propose corresponds to opening of the helix, is increased by 10-fold upon changing the pH from 6 to 9. Furthermore, although it is experimentally challenging to determine a residue \( pK_a \) by stopped-flow, the variable pH experiments yield \( pK_a \) values remarkably close to that of Tyr-9, and this pH dependence is lost in the triple mutant.

It is instructive to compare these results with a recent study by Armstrong and co-workers (31). They observe a similar modest loss of the 3-fold increase in the \( k_{-2} \) for product release of a mutant relative to wild-type GSTM1-1, based on burst phase analysis of substrate turnover. The increase in the rate of product release is correlated with a disruption of an intramolecular hydrogen bonding network that restricts segmental motion. Upon disruption of these interactions, product release is accelerated modestly. The similar magnitude in rate increase for product release upon alteration of conformational dynamics of the two proteins provides a benchmark for the extent to which diffusive steps are controlled by enzymes; perhaps, very large rate enhancements are inaccessible by fine-tuning ligand binding and dissociation when they are coupled to conformational changes.

The new information obtained from our studies clarifies a functional role for Tyr-9 ionization in GSTA1-1 catalysis. To the extent that the Tyr-9 \( pK_a \) remains low (~9.0) even with a GSH conjugate bound, a small fraction will be unprotonated even at physiological pH. Similarly, our previous results suggest a persistent disordered state, wherein even at saturating ligand and pH 7.4 the C-terminal helix is not completely closed and ordered. Thus, the equilibrium involved yield small populations of deprotonated or disordered helix even at saturating concentration of conjugate. Apparently, several microconformational states are nearly isoenergetic. Our results indicate that several microconformations compose the closed state, and deprotonation of Tyr-9 alters this ensemble in favor of microconformations that promote faster C-terminal opening and release of product. As long as the microconformational states corresponding to the protonated and unprotonated Tyr-9 are in rapid equilibrium, even a small population of the unprotonated form could provide a kinetically favored, parallel, pathway for product release, as depicted in Fig. 7. Based on this, the ionized form of Tyr-9 provides a modest catalytic advantage by lowering the kinetic barrier to C-terminal opening and product release, and by decreasing the extent of product inhibition.

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The Anomalous $pK_a$ of Tyr-9 in Glutathione S-Transferase A1-1 Catalyzes Product Release

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