The Catalytic Mechanism of Glutathione S-Transferase (GST)

SPECTROSCOPIC DETERMINATION OF THE pKₐ OF TYR-9 IN RAT α-1 GST*

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The rat α-1 glutathione S-transferase (GST) contains a single, non-essential tryptophan and only 8 tyrosines in each subunit. One of these tyrosines, Tyr-9, hydrogen bonds to the substrate glutathione and stabilizes the nucleophilic thiolate anion. Two mutant proteins that allow for the spectroscopic determination of the pKₐ of this catalytic residue have been constructed. The W21F mutant provides a fully active GST with no tryptophans, and the double mutant W21F/Y9F lacks both tryptophan and the active site tyrosine. The intrinsic fluorescence and absorbance properties of these mutants are dominated by tyrosine. Fluorescence emission, fluorescence excitation, and absorbance spectral changes of samples containing the W21F mutant at several pH values in the range 6.8-9.0 reveal a pH-dependent increase in the contribution of tyrosinate. No spectral changes are observed with the W21F/Y9F protein in this pH range. At pH 12.5, both proteins exhibit complete deprotonation of all tyrosines. The pKₐ of Tyr-9 determined from these spectroscopic changes is 8.3-8.5. The changes in absorbance at 250 and 285 nm correspond to titration of 0.95 ± 0.29 tyrosines/subunit in the W21F protein between pH 6.9 and 9.3. Moreover, addition of the inhibitor S-hexylglutathione results in an apparent increase in the pKₐ of Tyr-9. Together, these results indicate that the catalytically active Tyr of GSTs has a pKₐ value that is 1.8-2.0 pKₐ units below tyrosine in solution. It is likely that this decrease in the pKₐ of Tyr-9 contributes to catalysis by altering the equilibrium position of the proton shared between Tyr-9 and GSH, and this active site residue may function as a general base catalyst in addition to a hydrogen bond donor.

The glutathione S-transferases (GST; EC 2.5.1.18) are a family of conjugative enzymes that catalyze the nucleophilic addition of the tripeptide glutathione to xenobiotics, carcinogens, and endogenous lipophilic compounds (1-3). In accord with their physiological role in detoxification, individual cytosolic GST isozymes exhibit broad, overlapping substrate specificities. In addition, levels of GST are elevated in some tumor cells, where they may play a role in drug resistance (4-6). Four gene classes of GSTs, referred to as α, ρ, μ, and δ, are distinguished by their primary amino acid sequence homologies, tissue distribution, and substrate specificities (2, 7). Recently, x-ray crystal structures of ρ- and μ-class GSTs have indicated that the structural topology is very similar for these proteins (8-10). This similarity in structure implies that these enzymes utilize common catalytic mechanisms, and several experimental results support this.

Spectroscopic studies indicate that GSTs effectively lower the pKₐ of the cysteine thiol of glutathione, resulting in formation of the nucleophilic thiolate anion at the active site, at physiological pH (11). Prior to publication of the crystal structures, extensive discussion focused on possible protein-derived electrostatic forces, which might stabilize the thiolate of GSH (8-10, 12, 13). However, these structures do not indicate any obvious source of electrostatic stabilization. Instead, a hydrogen bond is formed between an evolutionarily conserved tyrosine residue and the thiol of GSH. Indeed, replacement of the active site tyrosine by other amino acids dramatically reduces Vₘₐₓ, while minimally affecting the Kₐ for GSH (14-17). Thus, it appears that GSTs from each of these gene classes utilize a common hydrogen bonding scheme to enhance the reactivity of GSH. The structure of the hydrogen-bonded complex has been inferred from the spectroscopic data as Tyr-OH···-SG, with the shared proton residing on the phenolic oxygen, although mechanistic details remain unclear.

It was first appreciated by Ji et al. (10) that the nucleophilicity of the GSH thiolate anion may be modulated by the strength of this hydrogen bond. The equilibrium position of the proton shared between substrate and protein, and the free energy contained in this hydrogen bond, depend on the relative pKₐ values of the active site tyrosine and the thiol of GSH (18). Therefore, the pKₐ of the active site tyrosine is a critical factor controlling the reactivity of GSH, and it has been speculated (8-10) that this tyrosine may have a pKₐ that is lower than tyrosine in solution (pKₐ of free tyrosine is 10.3). By “matching” the pKₐ values of the hydrogen bond donor and acceptor, it is possible to maximize the nucleophilicity of GSH (10, 18). However, the pKₐ of tyrosine at the active site has not been determined for any GST.

In order to directly measure the pKₐ of this active site tyrosine, we have exploited the fact that the rat α-class GST isozyme 1-1 has a single, nonessential tryptophan (Trp-21), unlike most other GSTs, which have 2 or more tryptophans (2). Typically, fluorescence emission and UV absorbance of tryptophan are more intense than tyrosine emission and absorbance in the same protein, hence limiting the use of tyrosine as a spectroscopic reporter (19). This is true for the wild type rat α1-1 isoenzyme, and we have previously characterized the fluorescence properties of Trp-21. Furthermore, the rat α1-1 isozyme contains only 8 tyrosines. Therefore, the active site tyrosine contributes significantly to the total protein spectrum (other GSTs contain 9-14 tyrosine residues). For these reasons, the rat A1-1 GST provides a novel system for combining protein
engineering with spectroscopy to study the ionization behavior of the catalytic tyrosine of GST. This approach has demonstrated that Tyr-9 of rat α1-1 GST has a pKₐ of 8.3–8.5, about 2 pKₐ units below “normal” tyrosine.

**MATERIALS AND METHODS**

Site-directed mutagenesis and protein purification were as described previously, utilizing rat α1-1 GST cloned into the plasmid pKK 2.7 (21, 22). All spectra were recorded in a mixed buffer system containing 25 mM Hepes (U. S. Biochemical Corp.), 25 mM Tris (Sigma) at the pH values indicated in figures. The series of buffers at different pH values was maintained at constant ionic strength by addition of appropriate concentrations of KCl. S-Hexyl-GSH and N-acetyltryptophan were obtained from Sigma. Absorbance spectra were obtained on a Cary 3 double beam spectrophotometer, with 0.34 mg of protein/ml, at a scan rate of 100 nm/min. Difference absorbance spectra were obtained by spectral subtraction. Fluorescence spectra were recorded on an SLM-Aminco 8000C fluorometer in the photon counting mode, thermostated 2 pKa units below “normal” tyrosine.

**RESULTS**

**Experimental Rationale**—Based on the available crystal structures, Trp-21 in the rat α1-1 GST is expected to lie approximately 18–23 Å from the glutathione binding site (8–10). Replacement of Trp-21 with Phe does not significantly alter the catalytic function of the protein (22). Similarly, although the W21F/Y9F mutant has a drastically reduced Vₘₐₓ, it still binds GSH and S-hexyl-GSH with high affinity, as observed for the single mutant Y9F (17). In fact, NMR experiments (23) and preliminary x-ray data indicate that the active sites of GSTs are expected to be excellent structural mimics of the wild type rat α1-1 GST.

Tyrosine residues in proteins exhibit diagnostic W absorptions, and fluorescence changes upon deprotonation of the phenolic oxygen. Specifically, the phenolic chromophore absorbs light at 275–280 nm with an extinction coefficient of 1400 M⁻¹ cm⁻¹, whereas tyrosinate absorbs maximally at 244 and 295 nm, with approximate extinction coefficients of 11,000 M⁻¹ cm⁻¹ and 2500 M⁻¹ cm⁻¹ (19, 24). Additionally, a red-shift is observed in the fluorescence emission of tyrosine upon deprotonation. Tyrosine has a fluorescence emission maximum at 305 nm, whereas tyrosinate has an emission maximum at 345 nm, with a dramatically reduced quantum yield (24, 25). Both decrease in peak emission intensity and a shift to longer wavelengths of the spectral center of mass are expected upon deprotonation of tyrosine.

**Fluorescence Emission Spectra**—At pH 6.8, the fluorescence emission spectra of W21F GST are similar to spectra of the double mutant W21FY9F, although the double mutant has a lower total quantum yield, as expected for a protein with one less tyrosine. Also, the W21F emission spectrum is slightly red-shifted compared to the spectrum of the double mutant, indicating that Tyr-9 contributes a unique spectral component. It is noteworthy that both proteins exhibit relatively red-shifted emission spectra, compared to free tyrosine, with emission maxima at 315–318 nm. This wavelength shift may result from hydrogen bonding of some, or all, of the tyrosines in these proteins. Emission maxima of hydrogen-bonded tyrosines are typically shifted to 310–330 nm (24, 25).

Between pH 6.8 and 9.5, the emission spectrum of W21F GST clearly changes. A decrease in total peak intensity is observed, and the spectrum broadens, predominantly due to a relative increase in intensity on the long wavelength side of the spectrum. In contrast, the spectra of the W21FY9F double mutant are superimposable in this pH range. Normalized emission spectra of both proteins demonstrate a marked decrease in fluorescence intensity and the emission maximum shifts to longer wavelengths. At pH 12.5, the spectra of the both proteins are essentially identical to the spectrum of N-acetyltyrosine. The spectral centers of mass are plotted as a function of pH in Fig. 1. As the pH is increased above 10, the emission spectra of both proteins demonstrate a marked decrease in fluorescence intensity and the emission maximum shifts to longer wavelengths. At pH 12.5, the spectra of the both proteins are essentially identical to the spectrum of N-acetyltyrosine. The spectral centers of mass are plotted as a function of pH in Fig. 2. The spectral changes observed with W21F, which are not seen with the double mutant in the pH range 6.5–8.5, are ascribed to titration of Tyr-9. Curve fitting of the data in Fig. 2 to an equation describing two pH-dependent ionizations affords pKₐ values of 8.3 and 11.7 for the W21F mutant. Control experiments utilizing these spectroscopic criteria yield a pKₐ of 10.6 for N-acetyltyrosine, in excellent agreement with the established value (24, 25). The apparent elevation of the pKₐ of the other tyrosines in W21F and the double mutant is consistent with their participation in hydrogen bonds, as suggested by their red-shifted emission maxima at pH 6.8.

Because tyrosine residues in proteins participate in Förster-type energy transfer, difference emission spectra (W21F minus W21FY9F) may not reflect the emission intensity of Tyr-9 in the W21F protein. However, at pH values ranging from 7.0 to 9.0, difference emission spectra qualitatively demonstrate a peak at 345 nm, with a negative difference emission band at 315 nm (data not shown), as expected if W21F contains a unique tyrosinate in this pH range.

**Fluorescence Excitation Spectra**—Titration of tyrosine is expected to result in a shift in peak intensity of excitation spectra from 280 to 295 nm (24, 25). Normalized excitation spectra of W21F GST and W21FY9F GST at several pH values are shown.
Active Site Tyrosine of Glutathione S-Transferase

**Fig. 2.** Fluorescence emission of W21F and W21F/Y9F glutathione S-transferases at various pH values. The spectral centers of mass, \( v \), obtained from normalized emission spectra are shown for several pH values. Closed circles, W21F; open circles, W21F/Y9F.

**Fig. 3.** Normalized fluorescence excitation spectra of GSTs at various pH values. Top, W21F at pH 7.3, 8.1, 8.8, and 9.4. An increase in the spectral shoulders at 295 and 290 nm is seen with increasing pH. Bottom, W21F/Y9F at the same pH values and at pH 12.5. No spectral changes are observed for the double mutant in the pH range 7.3–9.4. At pH 12.5 the spectra of W21F/Y9F, W21F, and N-acetyltyrosine are identical.

In Fig. 3, there is a dramatic increase in the intensity of a shoulder centered at 295 nm in the W21F protein. In contrast, the spectra of the double mutant at these pH values are superimposable. At higher pH values, each protein exhibits a spectrum consistent with complete titration of all tyrosines, and nearly identical to the spectrum of N-acetyltyrosine (Fig. 3). The relative intensities at 305 nm of the normalized excitation spectra are plotted versus pH in Fig. 4. The W21F mutant affords \( pK_a \) values of 8.4 and 11.6, whereas the double mutant demonstrates a single ionization corresponding to Tyr-9. Curve fitting affords an apparent \( pK_a \) of 8.5.

**Fig. 4.** Fluorescence excitation intensity of W21F and W21F/Y9F GSTs at various pH values. The relative fluorescence intensity at 305 nm of the normalized excitation spectra are plotted as a function of pH. The intensity at 305 nm is normalized to the intensity at 295 nm, at each pH. Closed diamonds, W21F + 200 \( \mu \)M S-hexyl-GSH.

**Fig. 5.** Difference absorbance spectra (W21F minus W21F/Y9F) at several pH values. Top, difference absorbance spectra at pH 6.8, 8.1, and 9.3. Bottom, plot of the ratio \( A_{250}/A_{280} \) as a function of pH. A single ionization corresponding to Tyr-9 is observed. Curve fitting affords an apparent \( pK_a \) of 8.5.

**Absorbance Spectra**—Absolute and difference absorbance spectra (W21F GST minus W21F/Y9F) were obtained at several pH values ranging from pH 6.8 to 11.6. Between pH 6.8 and 9.5, there is a moderate increase in the absorbance of the W21F protein at 295 nm, an increase in absorbance centered at 250 nm, and a concomitant decrease in the absorbance at 280 nm. These spectral changes are absent in the double mutant (data not shown). Difference spectra reveal a prominent peak at 256 nm and a smaller peak at 295 nm as the pH is increased from 6.8 to 9.5, consistent with formation of tyrosinate in the W21F protein (Fig. 5, top). Similar difference absorbance peaks have been observed in other proteins upon deprotonation of tyrosine (26). Two spectral ratios, \( A_{250}/A_{280} \) and \( A_{280}/A_{300} \), provide convenient monitors of the relative amount of tyrosinate/tyrosine at each pH. The \( A_{250}/A_{280} \) ratio is plotted as a function of pH in Fig. 5 (bottom). Curve fitting of these data to an equation has not obtained sufficient data to calculate a \( pK_a \) of Tyr-9 in the presence of inhibitor.

In order to probe the effect of GSH analogues on the ionization of Tyr-9, fluorescence excitation spectra were obtained in the presence of the inhibitor S-hexylglutathione, at several pH values (Fig. 4). Addition of 200 \( \mu \)M S-hexyl-GSH results in an apparent increase in the \( pK_a \) of Tyr-9. S-Hexyl-GSH had no detectable effect on the spectra of the W21F/Y9F mutant at the pH values examined. These results suggest that, in the presence of GSH analogues, the \( pK_a \) of Tyr-9 is higher than in the substrate-free protein, and further demonstrates that the observed spectral effects involve the active site tyrosine. We have not obtained sufficient data to calculate a \( pK_a \) of Tyr-9 in the presence of inhibitor.
describing a single ionization affords a $\Delta pK_a$ value of 8.5 for this spectral titration. For difference spectra, only a single ionization is expected, in contrast to the fluorescence emission spectra (above). As the pH is raised above 10, the tyrosines that are present in both proteins become titrated. However, no further spectral changes resulting from tyrosinate formation are observed because the absorbance due to tyrosinate is "subtracted" in the difference spectra. The $\Delta_{295}/\Delta_{250}$ ratio affords an identical $pK_a$ (data not shown). The changes in absorbance at 295 and 250 nm may be used to calculate the number of tyrosines that are deprotonated with changing pH, using the $\Delta_{295}$ and $\Delta_{250}$ for conversion of tyrosine to tyrosinate. The $\Delta_{295}$ and $\Delta_{250}$ for N-acetyltyrosine were experimentally determined to be 3.94 ms$^{-1}$ cm$^{-1}$ and 2.65 ms$^{-1}$ cm$^{-1}$, respectively, in agreement with previously published values (9). Upon increasing the pH from 6.8 to 9.3, the measured difference absorbance values (W21F - W21F/YSF) correspond to titration of 1.24 and 0.67 tyrosines/subunit GST, using the $\Delta e$ values at 295 and 250 nm, respectively (average $= 0.95 \pm 0.29$). From the absolute spectra of the W21F and W21F/YSF proteins, the number of tyrosines that are titrated upon changing the pH from 6.8 to 13 may be calculated from the $\epsilon_{245}$ of tyrosinate. Using $\epsilon_{245} = 11,000$ cm$^{-1}$ mol$^{-1}$, the calculated number of tyrosines that are deprotonated is 7.36/subunit for the W21F mutant and 6.58/subunit for the double mutant.

**DISCUSSION**

The fluorescence emission spectra, excitation spectra, and absorbance spectra of the rat a1-1 GST mutants yield $pK_a$ values of 8.3-8.5 for the active site tyrosine in the absence of GSH. Given that the $pK_a$ of tyrosine in solution is 10.4, the active site stabilization of the tyrosinate anion may be estimated from the $\Delta pK_a$ to be 2.3 kcal/mol. Previous mutagenesis experiments indicate that the active site tyrosine lowers the $pK_a$ of GSH and Tyr-9 and that the tyrosinate is favored at pH 7.4 (pathway K1K4). In principle, the linked form of the enzyme, even though this form of the enzyme is not predominant at physiological pH, 10% of the GST exists as the deprotonated active site tyrosine. This form of GST may provide general base catalysis for enzyme-mediated deprotonation of GSH. Based on the $pK_a$ values shown, $K_a/K_b > 40$ in order for pathway K1K4 to be favored in the formation of the GS$^-$E complex, where $E$ is enzyme.

**REFERENCES**