Proton Configuration in the Ground State and Transition State of a Glutathione Transferase-Catalyzed Reaction Inferred from the Properties of Tetradeca(3-fluorotyrosyl)glutathione Transferase

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The glutathione (GSH) transferases catalyze the addition of the sulfur of GSH to electrophilic functional groups.[1] Most vertebrate GSH transferases (e.g., the class α, μ, and π enzymes) have a tyrosyl residue located near the N-terminus that is important for efficient catalysis. The three-dimensional structures of several isoenzymes in complex with GSH or substrate analogues and products reveal that the hydroxyl group of this residue is within hydrogen-bonding distance of the sulfur of the peptide.[2] The spectral characteristics and kinetic properties of the binary E·GSH complex of the M1-1 (class μ) isoenzyme from rat indicate that the enzyme-bound thiol has a pK_a between 6.2 and 6.5 and suggest that the role of the hydroxyl group of Tyr6 is to provide electrophilic stabilization of the thiolate anion by donating a hydrogen bond (i.e., TyrOH···$^-$SG) in the ground state and transition state for nucleophilic addition.[3] In contrast, others have suggested, on the basis of studies of the class α and π enzymes, that the tyrosyl hydroxyl group has an abnormally low pK_a such that the tyrosinate anion acts as a general base (i.e., TyrO$^-$···HSG) to abstract the proton from the sulfhydryl group.[4] In the latter case, a proton transfer between the thiol and the tyrosinate anion must occur between the ground state and the transition state for nucleophilic addition.

In this paper, we report the preparation and evaluation of the catalytic properties of the tetradeca(3-fluorotyrosyl) mutant [(3-FY)$_{14}$-E] of the M1-1 isoenzyme of GSH transferase, in which all 14 tyrosyl residues have been replaced with 3-fluorotyrosine. The enzyme exhibits spectral and kinetic properties distinct from those of the native enzyme. The results are consistent with the active-site 3-fluorotyrosinate anion acting as a general base in the catalytic mechanism of the mutant enzyme and provide a benchmark for comparison with the native enzyme, where the tyrosyl hydroxyl group provides electrophilic stabilization of the thiolate in the ground state.

Tetradeca(3-fluorotyrosyl) GSH transferase was prepared by heterologous expression of the gene under control of the T7 promoter of the pSW1 expression vector in *Escherichia coli* strain BL21-DE3 grown in minimal media containing no tyrosine. 3-Fluorotyrosine (600 mg/L) was added just prior to induction of transcription with isopropyl β-D-thiogalactoside. The fluorinated enzyme was isolated and purified to homogeneity in a manner similar to that for the native enzyme.[3,5] The extent of incorporation of 3-fluorotyrosine was typically between 95 and 97% by amino acid analysis. The molecular mass of the mutant protein determined by electrospray ionization mass spectrometry was 26 040.2 ± 7.0 amu, within experimental error of the 26 034.8 Da calculated from the gene sequence with 14 3-fluorotyrosyl residues.

The ionization behavior of the 3-fluorotyrosyl residue at position 6 was determined from the pH dependence of the UV−visible difference spectrum (Figure 1) between the (3-FY)$_{14}$-E mutant and the (3-FY)$_{13}$-Y6F-E mutant, where the 3-fluorotyrosyl residue at position 6 is replaced with phenylalanine. The hydroxyl group of the 3-fluorotyrosyl residue exhibited a pK_a = 7.5 ± 0.2 (Figure 1), substantially lower than that observed in a similar titration of the native enzyme (pK_a = 10.2 ± 0.2).[6] Attempts to observe and titrate the thiolate in the binary (3-FY)$_{14}$-E·GSH complex by UV difference spectroscopy with the unliganded enzyme [(3-FY)$_{14}$-E·GSH minus (3-FY)$_{14}$-E] were not successful since, unlike the case with the native enzyme,[3] no difference absorption band for the thiolate could be observed. This fact suggests that the sulfur of GSH bound to the mutant remains protonated in the pH range of 6−9.

The increased acidity of the active site hydroxyl group has a profound effect on the catalytic properties of the mutant enzyme, as illustrated in Figure 2. Although no spectral evidence for the ionization of the thiol in the (3-FY)$_{14}$-E·GSH complex was found, the pH dependence of k_{cat}/K_m^{CDNB} revealed an ionization with an apparent pK_a = 6.0, not too different from that seen in the native enzyme (pK_a = 6.3). The biggest difference between the native and mutant enzymes is the limiting value of k_{cat}/K_m^{CDNB} on the high-pH plateau, which reflects the reactivity of the monoprotonated species (eqs 1 and 2). The value of k_{cat}/K_m^{lim} for the 3-fluorotyrosyl enzyme is 10-fold smaller than that of the native enzyme. Taken together, the differences in spectral and kinetic characteristics of the two monoprotonated species suggest that, while the thiolate anion predominates at high pH in the native enzyme (eq 1), the 3-fluorotyrosinate anion predominates in the reactant state of the mutant enzyme (eq 2).[7]

$$\mathrm{Tyr^6OH{\cdot}GSH} \rightleftharpoons \mathrm{Tyr^6OH\cdots{}^-SG} + \mathrm{H^+} \qquad pK_a = 6.3 \quad (1)$$

$$\mathrm{3\text{-}FTyr^6OH{\cdot}GSH} \rightleftharpoons \mathrm{3\text{-}FTyr^6O^-\cdots HSG} + \mathrm{H^+} \qquad pK_a = 6.0 \quad (2)$$

The two monoprotonated species in eqs 1 and 2 should have quite different fractionation factors in deuterated solvent due to the lower bending and stretching frequencies of the S−L bond and thus may exhibit different kinetic solvent deuterium isotope effects for nucleophilic addition. This expectation is confirmed by the data in Figure 2. The isotope effect on k_{cat}/K_m^{lim} for the native enzyme is small (0.9 ± 0.1) and consistent with that expected for the desolvation of a thiolate anion in going from

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(5) The trideca(3-fluorotyrosyl)-Y6F and trideca(3-fluorotyrosyl)-Y115F mutants of M1-1 were prepared in a similar manner using expression plasmids encoding the Y6F and Y115F mutants.

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(7) That the 3-fluorotyrosyl residue is directly involved in catalysis is apparent from the impaired catalytic properties of the (3-FY)$_{13}$-Y6F mutant, which is 50-fold less active than (3-FY)$_{14}$-E. The only other tyrosyl residue near the active site of M1-1 is Tyr115, located 7.5 Å from the sulfur of GSH. The (3-FY)$_{13}$-Y115F mutant has catalytic properties similar to those of (3-FY)$_{14}$-E toward CDNB, suggesting that the 3-fluorotyrosyl residue at this position does not significantly influence the reactivity of (3-FY)$_{14}$-E·GSH.

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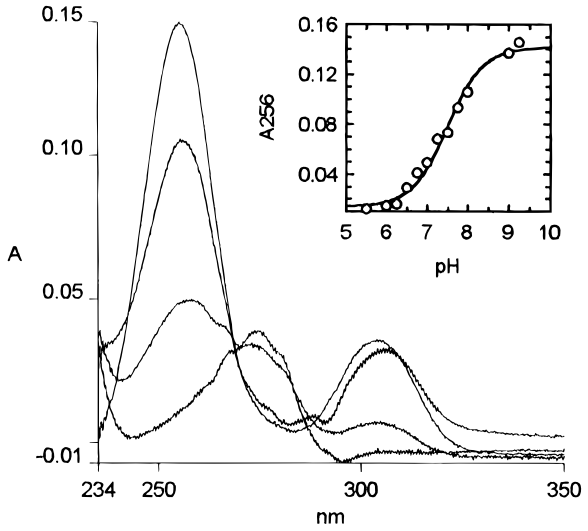

Figure 1. UV−visible difference spectra of $(3\text{-FY})_{14}$-E minus $(3\text{-FY})_{13}$-Y6F-E at pH 5.5, 7.0, 8.0, and 9.25. Concentration of each enzyme was 18 μM active sites. Inset: pH dependence of the appearance of the 3-fluorotyrosinate absorption band at 256 nm in the UV−visible difference spectra. The solid line is a regression fit of the experimental data to the equation $A_{256} = [(A_L + A_H(K_a/H^+)]/(1 + K_a/H^+)$, where $A_L = A_{256}$ at low pH, $A_H = A_{256}$ at high pH, H^+ is the hydronium ion concentration, and K_a is the acid dissociation constant. The pK_a and $\epsilon_{256} = A_H/[E]$ obtained from the regression analysis are 7.5 ± 0.2 and 7900 M^{-1} cm^{-1}, respectively.

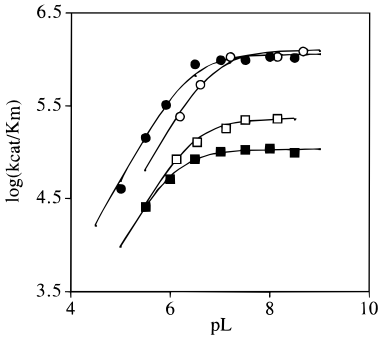

Figure 2. pH dependence of k_{cat}/K_m^{CDNB} for native and mutant enzymes under conditions of saturating GSH. The solid lines are computer fits of the experimental data to the equation $\log(k_{cat}/K_m^{CDNB}) = \log[(k_{cat}/K_m^{lim})/(1 + [H^+]/K_a)]$. The experimental points, values of k_{cat}/K_m^{lim} at high pH, and apparent pK_as derived from the computer fits for the enzymes are as follows: native E in H_2O (●), $k_c/K_m^{lim} = (1.14 \pm 0.09) \times 10^6$ M^{-1} s^{-1}, p$K_a = 6.3 \pm 0.2$; native E in D_2O (○), $k_c/K_m^{lim} = (1.24 \pm 0.12) \times 10^6$ M^{-1} s^{-1}, p$K_a = 6.8 \pm 0.2$; $(3\text{-FY})_{14}$-E in H_2O (■), $k_c/K_m^{lim} = (1.09 \pm 0.03) \times 10^5$ M^{-1} s^{-1}, p$K_a = 6.0 \pm 0.1$; $(3\text{-FY})_{14}$-E in D_2O (□), $k_c/K_m^{lim} = (2.30 \pm 0.09) \times 10^5$ M^{-1} s^{-1}, p$K_a = 6.4 \pm 0.1$.

the reactant state to the transition state, as previously observed with a class α enzyme and in the chemical reaction of GS^-_{aq} with CDNB.[8] In contrast, a large inverse isotope effect of 0.5 ± 0.1 is observed for $(3\text{-FY})_{14}$-E. The most reasonable scenario consistent with an isotope effect of this magnitude is that the proton in the monoprotonated species (eq 2) is associated with

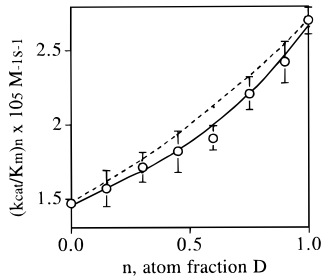

Figure 3. Proton inventory for k_{cat}/K_m^{CDNB} for $(3\text{-FY})^{14}$-E at pL = 7.8. The solid line is a nonlinear regression fit of the experimental data to the Gross−Butler equation for an isotope effect arising from a single reactant state proton, $(k_{cat}/K_m)_n = (k_{cat}/K_m)_o/(1 - n + n\phi^R)$, where $(k_{cat}/K_{m)o} = (1.45 \pm 0.02) \times 10^5$ M^{-1} s^{-1} and $\phi^R = 0.55 \pm 0.01$. The dashed line is that expected for a many-proton fractionation in the reactant state with an isotope effect of 0.55.

the sulfur in the reactant (ground) state ($\phi^R \approx 0.4-0.6$)[9] and is fully transferred to the 3-fluorotyrosyl hydroxyl group in the transition state ($\phi^T \approx 1-1.1$).[9a,b,10] That the observed inverse kinetic solvent deuterium isotope effect in the mutant enzyme is dominated by a single reactant state proton is consistent with the results of a proton inventory for the reaction shown in Figure 3. These data fit the Gross−Butler function describing a single reactant state proton with $\phi^R = 0.55 \pm 0.01$ (solid line) more closely than that expected for a general multiproton solvation isotope effect (dashed line). Similar behavior has been recently observed with the regulatory cysteine residue of yeast pyruvate decarboxylase.[11]

Substitution of a 3-fluorotyrosine residue in the active site of the M1-1 glutathione transferase alters the manner in which the side chain participates in catalysis. The 3-fluorotyrosinate anion appears to act as a general base to abstract the thiol proton between the ground state and transition state, with the proton being essentially fully transferred to the oxygen in the transition state. The catalytic properties of the mutant enzyme, in particular the solvent deuterium isotope effect on the reaction, thus provide a benchmark for distinguishing general base catalysis from the alternative of electrophilic stabilization of the thiolate anion. The rather modest inverse solvent deuterium isotope effect observed for the native class μ enzyme in this work and the class α enzyme studied by Huskey and co-workers[8] is consistent with the active-site tyrosine serving as a surrogate solvent molecule, donating a conventional hydrogen bond to stabilize the reactive thiolate anion, and argues against a general base catalytic role of the tyrosinate anion, as suggested by others.[4]

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(10) The alternatives of a proton in flight in the transition state perpendicular to the reaction coordinate ($\phi^T \approx 0.3-0.6$)[9a,b] or a low-barrier hydrogen bond in the ground state and transition state would be expected to give solvent deuterium isotope effects $\phi^R/\phi^T \geq 1$.

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