Analysis of the Role of the Active Site Tyrosine in Human Glutathione Transferase A1-1 by Unnatural Amino Acid Mutagenesis

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Received September 10, 1997

A major detoxification pathway used by aerobic organisms involves the enzymatic conjugation of the tripeptide glutathione (GSH) to the electrophilic center of toxic substances by the glutathione transferases (GSTs) (EC 2.5.1.18).[1] These enzymes activate the cysteine thiol group of GSH for nucleophilic addition to a variety of substrates, including aryl halides, α,β-unsaturated aldehydes and ketones, and epoxides. However, despite a large number of biochemical and structural studies,[2] the mechanism by which GSH transferases catalyze these addition reactions remains unclear. The three-dimensional structures have shown that the hydroxyl group of an active site tyrosine residue (Tyr9 for human GST A1-1, class alpha), which is conserved among the majority of known GSH transferases, is within hydrogen-bonding distance of the sulfur of glutathione.[2] In solution, the pK_a of GSH is about 9.0,[3] whereas in the GST A1-1 enzyme−GSH complex the pK_a of the thiol group is 6.2.[4] The tyrosine residue of this complex is believed to stabilize the thiolate of GSH through a hydrogen-bonding interaction (TyrOH···$^-$SG).[5] Alternatively, the abnormally low pK_a of the tyrosyl hydroxyl group may provide a tyrosinate anion which can act as a general base (TyrO$^-$···HSG)[6] to abstract the proton from the sulfhydryl group. To investigate this issue, we have used unnatural amino acid mutagenesis to site-specifically replace Tyr9 in human GST A1-1 with a series of fluorinated tyrosine analogues with pK_a values ranging from 5.3 to 10. The observed values of k_{cat} and the pH dependence of the mutants are relatively unaffected by fluorine substitution with the exception of the 2,3,5,6-tetrafluorotyrosine mutant. The results are consistent with a previously proposed hydrogen-bonding role for Tyr9 in human GST A1-1[4,7] and are compared with previous experimental[8] and theoretical studies[9] of GSTs in which the pK_a of the active tyrosine residue is altered.

A series of fluorinated tyrosine analogues, 2-fluorotyrosine (**1**), 3-fluorotyrosine (**2**), 3,5-difluorotyrosine (**3**), and 2,3,5,6-tetrafluorotyrosine (**4**), was substituted for Tyr9.[10,11] Due to fluorine's relatively small size (van der Waals radius of 1.35 Å relative to 1.2 Å for hydrogen),[12] the steric perturbation resulting from fluorine substitution should be relatively small. Incorporation of the fluorinated tyrosine analogues **1**−**4**, as well as L-tyrosine (**5**), at position 9 of human GST A1-1 was accomplished by in vitro suppression of a Tyr9 → TAG stop mutation with a chemically aminoacylated suppressor $tRNA_{CUA}$ derived from yeast $tRNA^{Phe}$.[13,14] Amino acids **1**−**4** were incorporated with suppression efficiencies ranging from 15 to 25% relative to that for in vitro expression of wild-type (wt) protein. In contrast, when $tRNA_{CUA}$ was omitted from the in vitro reaction or was not aminoacylated, less than 1% full-length GST was produced in comparison to in vitro expression of wt GST. Wild-type and mutant GSTs were purified to homogeneity from 5.0 mL in vitro protein synthesis reactions.[15]

Steady-state kinetic analysis of the wt and mutant enzymes was performed over a pH range of 6.0−8.0 using previously described assay conditions.[16] Interestingly, the increased acidity of the active site hydroxyl group significantly influences the catalytic properties only of the Tyr9 → 2,3,5,6-tetrafluorotyrosine mutant (Figure 1). The limiting value of k_{cat}/K_m^{CDNB} on the high-pH plateau, which is postulated to reflect the reactivity of the monoprotonated species of the complex,[8] declines only an average of approximately 2-fold for the Tyr9 → **1**, Tyr9 → **2**, and Tyr9 → **3** mutants. In contrast, the k_{cat}/K_m^{CDNB} of mutant **4** is reduced roughly 20-fold in the same pH range. The pH dependence of log k_{cat}/K_m for the mutants is also very similar to that of wt enzyme, again with the exception of 2,3,5,6-tetrafluorotyrosine,

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(10) Amino acids **1**, **2**, **4**, and **5** are commercially available. 3,5-Difluorotyrosine was synthesized from 3,5-difluoro-4-methoxybenzaldehyde by the procedure of Kruse et al.[20] The aldehyde was reduced with sodium borohydride followed by bromination of the hydroxyl group with PBr_3. The bromide was subsequently coupled with diethyl acetamidomalonate, and the resulting product was hydrolyzed with 50% HBr to give the desired product: ^{1}H NMR (D_2O) δ 6.70 (2H, d, J = 8.0 Hz), 3.72 (1H, dd, J = 7.4, 5.4 Hz), 2.96 (1H, dd, J = 14.7, 5.2 Hz), 2.83 (1H, dd, J = 14.7, 7.8 Hz); HR MS calcd 218.0629, found 218.0626 (M + 1). It has been shown that only L-amino acids are accepted by the amino acid biosynthetic machinery.[14]

(11) *Handbook of Biochemistry and Molecular Biology: Physical and Chemical Data;* Fasman, G. D., Ed.; CRC Press: Boca Raton, FL, 1976; Vol I, p 314.

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Published on Web 01/01/1998

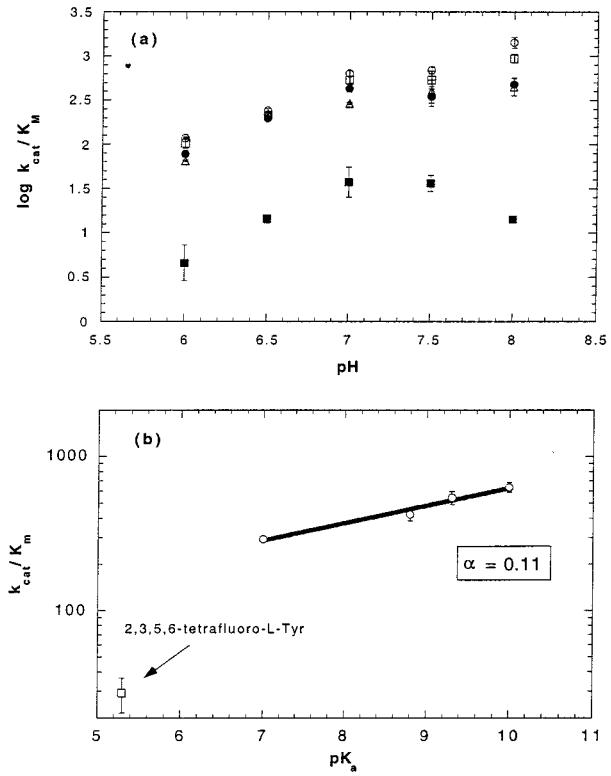

Figure 1. (a) k_{cat}/K_m^{CDNB} as a function of pH for wild-type and mutant forms of GST A1−1: (○) Tyr^9→L-Tyr (**5**, wild-type); (□) Tyr^9→2-fluoro-L-Tyr (**1**); (●) Tyr^9→3-fluoro-L-Tyr (**2**); (△) Tyr^9→3,5-difluoro-L-Tyr (**3**); (■) Tyr^9→2,3,5,6-tetrafluoro-L-Tyr (**4**). (b) Linear free energy correlation between log k_{cat}/K_m^{CDNB} versus pK_a at pH 7.0.[11,21]

indicating that the ionization behavior of the active site residues in these mutants is not very different from that of wt enzyme.

The distinct behavior of the Tyr9 → **4** mutant relative to the other mutants and the similarity in the behavior of the latter and wt GST support a model in which the active site tyrosine and fluorinated analogues **1**−**3** are protonated above the apparent pK_a of the enzyme−substrate complex and stabilize the nucleophilic thiolate anion. The pK_a on the acidic side of the pH profile of the wt enzyme, which appears to be relatively unperturbed in the mutants, has been attributed to ionization of a carboxyl group in glutathione.[7] A linear free energy analysis of log k_{cat}/K_m^{CDNB} versus pK_a for amino acids **1, 2, 3,** and **5** provides a linear relationship (Figure 1b), indicating that k_{cat}/K_m^{CDNB} correlates with the acidity of the proton donor. The value of α (pH = 7.0) from a linear free energy correlation log $k_{cat}/K_m^{CDNB} = \alpha(pK_a) + C$, where α reflects the degree of proton transfer between Tyr9 and GSH, is 0.11. This value indicates that, for **1**−**3** and **5**, the shared proton resides near the phenolic oxygen in the enzyme−GSH complex, which is in agreement with studies upon rat GST M1−1 (class mu)[1d,5] and recent ab initio molecular orbital calculations for the wild-type enzyme−substrate models.[9] These results are inconsistent with the expected behavior of a low-barrier hydrogen bond[17] and suggest that increasing the strength of the TyrOH···$^-$SG hydrogen bond leads to a slight decrease in the nucleophilicity of the thiolate anion.

While our results indicate that the location of the proton can be shifted slightly by changing the pK_a of the active site tyrosine, the shift does not induce a significant change in the behavior of GST until **4** is introduced (Figure 1). This change may be attributable to formation of a tetrafluorotyrosinate GSH pair with a concomitant change in mechanism to general base catalysis. Similar behavior was observed in a recent study in which replacement of all the tyrosine residues in the rat GST M1-1 isozyme ((3-FTyr)$_{14}$-GST) with 3-fluorotyrosine resulted in the formation of a tyrosinate anion−GSH pair.[8] Structural differences between the two proteins may affect the degree of tyrosine fluorination required to form the tyrosinate anion. The decrease in k_{cat}/K_m^{CDNB} at higher pH for the Tyr9 → **4** mutant may result from formation of the thiolate anion, resulting in a repulsive interaction with the tyrosinate anion and structural changes in the active site. Alternatively, introduction of the fourth fluorine substituent in the tetrafluorotyrosine mutant may alter the side chain conformation and the orientation of the hydroxyl group. Indeed, the recent crystal structure of the (3-FTyr)$_{14}$-GST suggests that the 3-fluorine substituent on Tyr 6 may interact both sterically and electronically with the sulfhydryl group of GSH.[18] Finally, the specific activity of **4** (4% wild-type) is similar to the Tyr^9 → Phe mutant, which displays 2−8% of wild-type enzyme activity, depending upon the electrophile.[19] In the latter case, the mutation likely leads to an enzyme−GSH complex in which the proton is located near the sulfur atom of GSH, requiring transfer of the proton to an acceptor in the transition state.

Acknowledgment. This work was supported by grants from the National Institutes of Health (GM49220), the Swedish Research Council for Engineering Sciences, and the Swedish Natural Science Research Council. P.G.S. is a Howard Hughes Medical Institute Investigator and a W. M. Keck Foundation Investigator, and J.S.T. is a Merck Postdoctoral Fellow of the Helen Hay Whitney Foundation.

Supporting Information Available: Table of kinetic data (1 page). See any current masthead page for ordering and Internet access instructions.

JA9731682

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