Rational Modulation of the Catalytic Activity of A1-1 Glutathione *S*-Transferase: Evidence for Incorporation of an On-Face (π···HO−Ar) Hydrogen Bond at Tyrosine-9[†]

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ABSTRACT: The alpha-, pi-, and mu-class glutathione *S*-transferases utilize a hydrogen bond between a conserved tyrosine and glutathione (GSH) to stabilize the nucleophilic thiolate anion, as Tyr−OH···$^-$SG. This hydrogen bond is critical for efficient detoxication catalysis. The detailed structure of this hydrogen bond, however, is controlled by active site features which are not conserved across class boundaries. The alpha-class GST A1-1 has a cluster of aromatic residues on one side of the ring of the catalytic tyrosine, Tyr-9. Also, a hydrophobic Met-16 side chain is packed against the edge of the ring of Tyr-9. Molecular modeling and *ab initio* calculations suggested that substitution of Phe-220 with tyrosine could generate an aromatic on-face hydrogen bond (π···HO−Ar) between the ring of Tyr-9 and the hydroxyl group of Tyr-220, and this would lower the pK_a of enzyme-bound GSH. Therefore, Phe-220 was replaced by Tyr in the rat A1-1 isozyme. Also, Met-16 was replaced by Thr in order to investigate the effect of a hydrogen bond donor at the Tyr-9 ring edge. UV spectroscopic titration of GST·GSH and steady-state kinetic analysis indicate that substitution of Tyr at Phe-220 results in a decrease of the pK_a of the cofactor, whereas substitution of Met-16 with Thr results in an increase of this pK_a. Also, the pK_a of Tyr-9 in the absence of substrates was determined directly by fluorescence titration. Substitutions F220Y and M16T resulted in a decrease of 0.5 pK_a unit and an increase of 0.6 pK_a unit, respectively. Together, these results indicate that a weak hydrogen bond between the engineered Tyr-220 side chain and the aromatic ring face of the catalytic Tyr-9 decreases the pK_a of GSH and Tyr-9, and this alters the pH dependence of the enzymatic reaction.

Electrostatic interactions between cations and π-systems may provide appreciable interaction energies in proteins [see Dougherty (1996) for review]. Weak interactions between aromatic rings and neutral hydrogen bond donors also have been suggested to contribute to protein structure and stability (Levitt & Perutz, 1988; Burley & Petsko, 1988). However, the extent to which these "unconventional hydrogen bonds" contribute to protein function remains unclear. For example, an amino/aromatic on-face hydrogen bond has been suggested to contribute to the binding affinity of phosphotyrosine of peptide ligands for the src-SH2 domain, although the energetic contribution of this hydrogen bond has not been determined (Waksman et al., 1992). Also, an on-face hydrogen bond between a phenylalanine ring and the amide side chain of a glutamine in cellular retinoic acid-binding protein (CRBP) has negligible effect on affinity for *all-trans*-retinol (Jamison et al., 1995). Thus, a functional role for such a hydrogen bond in CRBP has not been demonstrated. Similarly, Mitchell et al. (1994) concluded that amino−π hydrogen bonds do not represent a "general" feature of protein structure which has a clear energetic advantage over conventional hydrogen bonds, based on a survey of the Brookhaven Protein Data Base.

However, Liu et al. (1993) suggested that an aromatic hydrogen bond to the catalytically important Tyr-6 of a mu-class glutathione *S*-transferase (GST)[1] modulates, in turn, the hydrogen bond between the hydroxyl group of this tyrosine and the thiolate of the reactive cofactor glutathione (GSH). Indeed, removal of a hydrogen bond donor at the face of Tyr-6 caused an increase in the kinetic pK_a of enzyme-bound GSH. X-ray structures of several "second-sphere" mutants of this GST suggest that residues near Tyr-6 play a functional role in catalysis, by transmitting effects through the aromatic ring of Tyr-6 to the nucleophilic thiolate of GS$^-$ (Xiao et al., 1996). Thus, GSTs provide an opportunity to probe the extent to which aromatic hydrogen bonds contribute to enzymatic function.

The mammalian cytosolic GSTs are represented by the four gene classes, alpha, pi, mu, and theta, which demonstrate relatively low interclass sequence homology. However, X-ray crystal structures of alpha-, pi-, and mu-class GSTs indicate that they share similar overall folding topologies [see Dirr et al. (1994) for review of structures]. Some catalytic elements are conserved in each of these classes of GSTs. For example, the "catalytic" tyrosine referred to above hydrogen bonds to and stabilizes GS$^-$ in each of these classes (this is a "conventional" hydrogen bond involving

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[1] Abbreviations: GST, glutathione *S*-transferase; GSH, glutathione; GS$^-$, the thiolate anion of GSH; CDNB, 1-chloro-2,4-dinitrobenzene. The nomenclature GST A1-1 and GST M1-1 refers to the alpha-class 1-1 and mu-class 1-1 GST isozymes, respectively. In particular, it should be noted that GST M1-1 corresponds to the isozyme previously referred to as GST 3-3.

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the tyrosine hydroxyl, Tyr−OH···$^-$SG, not an aromatic on-face interaction). Spectroscopic (Graminski et al., 1989) and kinetic experiments (Bjornestedt et al., 1995; Wang et al., 1992) have suggested that this hydrogen bond lowers the pK_a of GSH, by stabilizing the nucleophilic thiolate anion at the active site. The importance of this tyrosine in GST catalysis is clear, based on site-directed mutagenesis studies with GSTs from each class (Liu et al., 1992; Stenberg et al., 1991; Kolm et al., 1992). Furthermore, the hydrogen-bonding potential of the active site tyrosine in the alpha- and pi-class GSTs is increased relative to free tyrosine in solution, because it has an unusually low pK_a (Atkins et al., 1993; Bjornestedt et al., 1995; Karshikoff et al., 1993). Apparently, these GSTs share the common catalytic element of a hydrogen bond between the bound GS$^-$ and the phenolic hydroxyl group of an "activated" tyrosine. In all cytosolic GSTs, this hydrogen bond increases the proportion of GS$^-$, relative to GSH, at the active site.

However, the active site features which modulate the hydrogen bond to GS$^-$ are not conserved between GSTs from different classes. For example, the alpha-class GSTs share a conserved arginine which also decreases the pK_a of GS$^-$ via an electrostatic stabilization of the thiolate anion in the binary, GST·GS$^-$, complex. This arginine may modulate also the Tyr-9 phenolic hydroxyl group in the substrate-free enzymes (Garcia-Saez et al., 1994; Wang et al., 1992; Bjornestadt et al., 1995). However, this residue is not conserved in pi- and mu-class GSTs, and therefore, it does not represent a "universal" feature of GST catalysis.

As pointed out above, the mu-class GSTs utilize an on-face hydrogen bond between the catalytic tyrosine and the hydroxyl group of a threonine side chain which is "perched" directly above the center of the aromatic ring (Thr-13 and Tyr-6 in M1-1 GST). The Thr hydroxyl proton apparently forms a hydrogen bond with the π-cloud of Tyr-6 (Liu et al., 1993; Xiao et al., 1996). Interestingly, the alpha- and pi-class GSTs do not share this interaction. The demonstration that the mu-class GST utilizes such an aromatic hydrogen bond as a catalytic device emphasizes the importance of subtle and long-range interactions in enzyme catalysis.

Here, we report a decrease of the pK_a of GSH bound to the active site of rat A1-1 GST following incorporation of an on-face Ar−OH−π hydrogen bond, which is analogous to the interaction in the mu-class 1-1 GST. Successful incorporation of this interaction in the alpha-class GST strongly supports the proposed role of the threonine−tyrosine on-face hydrogen bond in the mu-class GSTs. Moreover, the results suggest that the hydrogen bonds of this type may provide, in favorable cases, a rationale for altering catalytic function of enzymes utilizing aromatic residues in their chemical mechanisms.

MATERIALS AND METHODS

Molecular Modeling. *Ab initio* geometry optimization was performed with Spartan (Wavefunction, Inc., Irvine, CA). Geometry optimization of models was performed with Hartree−Fock theory and the 3-21G* basis set. The coordinates of the non-hydrogen atoms of Tyr-9, *S*-benzyl-GSH, and Phe-220 were obtained from the X-ray structure of the human A1-1 GST (Sinning et al., 1993) and used as templates for the relevant atoms of *p*-cresol, methanethiol, and toluene, respectively. Hydrogen atoms were added to these active site mimics, and geometries were optimized, with the coordinates of heavy atoms fixed, before single point energy calculations of the methanethiol proton affinity, using the 6-31+G* basis set and Gaussian 94, revision B.1 (Frisch et al., 1995). GST structures were visualized with MidasPlus.

Site-Directed Mutagenesis. The site-directed mutants W21F:F220Y and W21F:F220Y:Y9F were constructed by PCR-based amplification of a fragment spanning the *Bgl*II and *Sal*I restriction sites contained in the linearized pKKGTB34-W21F plasmid or the pKKGTB34-W21F:Y9F plasmid template. The plasmid pKKGTB has been described previously (Wang et al., 1989). The sequence of the oligonucleotide primer encoding the amino acid substitution F220Y was 5′-GCAGGGGCCGTCGACGTAAAATTTG-TAAACCTTCCT-3′ and spanned the *Sal*I site. The final PCR product was digested with *Sal*I and *Bgl*II and subcloned into pKKGTB34W21F or pKKGTB34-W21F:Y9F. The W21F:M16T and W21F:M16T:Y9F mutants were prepared by the overlap extension method (Ho et al., 1989), using linearized pKKGTB34-W21F or pKKGTB34-W21F:Y9F as template. Conditions for all PCR reactions were 10 mM $(NH_4)_2SO_4$, 20 mM Tris, pH 8.3, 3 mM $MgSO_4$, 200 μM each dNTP, 0.1% Triton X-100, 10 ng of linearized template, and 100 pmol of each primer in a final volume of 100 μL, with 1 unit of Vent polymerase (New England Biolabs). Cycle profiles were 94 °C for 1 min, 65 °C for 2 min, and 72 °C for 2 min for 25 cycles followed by 10 min at 72 °C. All mutant constructs were validated by DNA sequencing.

Protein Expression, Purification, and Activity. Expression and purification of mutant proteins were as described previously (Dietze et al., 1996). Enzymatic activity was determined by the CDNB assay, as described previously (Kubo & Armstrong, 1989).

Fluorescence Titration. Samples contained 1 μM GST in a mixed buffer of 35 mM Mes/35 mM Tris/35 mM Capso, at various pHs. Titrations were performed as described previously (Atkins et al., 1993; Dietze et al., 1996). pK_a values were determined from the spectral center of mass of emission spectra, as well as from peak intensity at 305 nm in excitation spectra. Titration data were fit to an equation describing a single ionization with Enzfitter. Both approaches yielded the same results.

GSH Thiol Titrations. The pK_a of GSH bound to mutant proteins was determined as described previously for the mu-class GST (Liu et al., 1992). Briefly, samples contained 10 μM GST in the presence or absence of 400 μM GSH in the sample and reference beams, respectively, of a Cary 3E spectrophotometer. Samples contained 50 mM Mes/50 mM Hepes/50 mM Tris/1 mM EDTA and varied in pH between 5.5 and 8.0. Samples were maintained at 25 °C during all measurements.

RESULTS

Rationale. Examination of the crystal structures of the human A1-1 GST indicated that no hydrogen bond donor in the A1-1 GSTs occupies a position analogous to that of Thr-13 in the M1-1 isozyme (Sinning et al., 1993; Cameron et al., 1995). The residue analogous to Thr-13 of the M1-1 enzyme is Met-16 in the rat A1-1 enzyme, and Met-16 is packed against Tyr-9, near the edge of the ring. However,

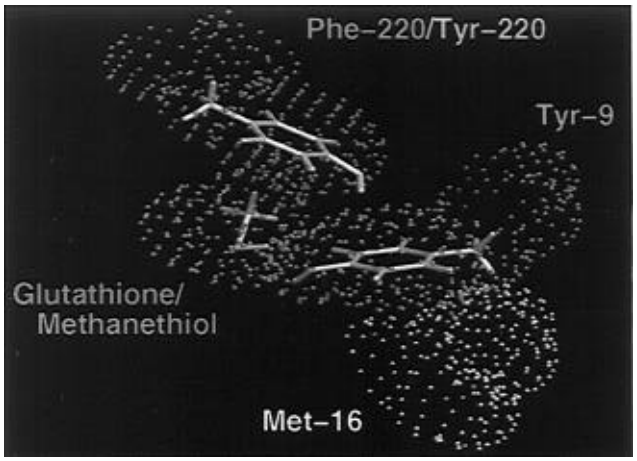

FIGURE 1: Environment of Tyr-9 in A1-1 GST. The model is based on the X-ray structure of the human A1-1 GST (Sinning et al., 1993). The van der Waals radii of Tyr-9 (magenta), Met-16 (yellow), Phe-220 (red), and a portion of GSH including the sulfur and the β-carbon (cyan) are shown. The *ab initio* optimized structures for the *p*-cresol-220, *p*-cresol-9, and methanethiol, which are mimics of Tyr-220, Tyr-9, and GSH, respectively, are shown in gray. The hydroxyl group of *p*-cresol-220 extends into the space between Phe-220 and the face of Tyr-9. In the optimized geometry, the hydroxyl proton of *p*-cresol-220 is 3.02 Å from the ring of *p*-cresol-9. Apparently, no repacking of the Tyr-9 environment is required to form a hydrogen bond between the incorporated Tyr-220 and the Tyr-9 ring.

a striking interaction between Tyr-9 of the A1-1 enzyme and Phe-220 was apparent in the structure with *S*-benzyl-GSH bound at the active site (Sinning et al., 1993) and in the presence of the ethacrynic acid conjugate (Cameron et al., 1995). Specifically, the plane of the aromatic ring of Phe-220 approaches one face of Tyr-9, with the centroid of the Tyr-9 ring approximately 3.5 Å below the ζ-carbon of Phe-220 (Figure 1). The Phe-220 ring axis approaches the plane of Tyr-9 at an angle of $\sim$50°, thus allowing a proton on the hydroxyl group of an incorporated Tyr-220 to overlap with the π-cloud of Tyr-9. That is, Phe-220 provides a well-aimed scaffold for addition of a hydrogen-bonding −OH group, by replacement of Phe-220 with a tyrosine side chain. Importantly, there was ample room for incorporation of a phenolic oxygen atom on the side chain of Phe-220, without significant repacking of the local environment (Figure 1). It was reasonable to postulate, on the basis of the results of Liu et al. (1993), that the conservative replacement of Phe-220 with a tyrosine would afford a structurally intact enzyme with an on-face hydrogen bond between the π-cloud of Tyr-9 and the phenolic hydroxyl group of Phe-220. This hydrogen bond, in turn, would be expected to lower the pK_a of the active site GSH and alter the pH dependence of the conjugation reaction between GS^- and electrophilic substrates.

Notably, Phe-10 also contacts Tyr-9 in the human A1-1 structure, and it is present in the rat homologue. The edge of Phe-10 runs across the same face of Tyr-9 as Phe-220, so that Tyr-9 lies under an "aromatic platform" provided by Phe-10 and Phe-220. Presumably, Phe-10 also affects the electrostatic environment of the catalytic tyrosine. In contrast to Phe-220, however, the aromatic ring of Phe-10 is not situated to allow for an on-face hydrogen bond by incorporation of a phenolic OH group, without significant structural rearrangement. Therefore, we have not attempted to incorporate a hydrogen bond by substitution of Phe-10.

Table 1: Optimized Geometry and Gas-Phase GSH Proton Affinities from Model Calculations

	$GS^-\cdots H-O-$Tyr-9		
model	distance (Å)	proton affinity,[a] GSH (kJ/mol)	Δ energy (kJ/mol)
CH_3S^-		1258.14	
CH_3S^-/*p*-cresol-9	2.265	1114.17	−143.97
CH_3S^-/*p*-cresol-9/toluene-220	2.258	1090.42	−167.72
CH_3S^-/*p*-cresol-9/*p*-cresol-220	2.256	1073.02	−185.12

[a] Single point energy calculation of energy required to remove the proton from GSH. Non-hydrogen atoms of methanethiol and *p*-cresol provide mimics of the relevant atoms of GSH and Tyr-9 and have been constrained on the basis of the coordinates of the A1-1 GST with *S*-benzyl-GSH bound (Sinning et al., 1993). Phe-220/Tyr-220 are mimicked by toluene/*p*-cresol. The reported distances are between the proton on *p*-cresol-9 and the sulfur atom of methanethiol. The distance from the hydroxyl proton of *p*-cresol-220 to the centroid of the ring of *p*-cresol-9 is 3.02 Å.

In addition, the X-ray structure of the ligand-free A1-1 GST indicates that residues in the C-terminal helix (214−222) are not well structured, with electron density at low contour levels (Cameron et al., 1995). Therefore, it was not assumed that an on-face hydrogen bond at Tyr-9 would be achieved in the absence of ligand. A hydrogen bond between Tyr-220 and Tyr-9 was considered most likely to occur in the binary complex GSH·GST, the complex with product conjugate, or the transition state en route to product formation.

In order to determine whether a hydrogen bond between a tyrosine at position 220 and the aromatic ring of Tyr-9 would measurably alter the pK_a of GSH bound at the active site, model *ab initio* calculations were performed using methanethiol, *p*-cresol, and toluene to mimic the thiol of GSH, Tyr-9, and Phe-220, respectively. Models were constructed with these components geometrically constrained using the coordinates of the human A1-1 GST with *S*-benzyl-GSH bound (Sinning et al., 1993). Obviously, these models cannot predict the absolute proton affinities in the protein environment. However, they reasonably reflect the direction of an expected change in pK_a resulting from a specific mutation, assuming the structure is not perturbed. Furthermore, the validity of these models, which utilize Hartree−Fock theory and the 3-21G* and 6-31+G* basis sets, was determined by reproducing the results obtained by Liu et al. (1993) for the mu-class GST. The coordinates of the M1-1 GST residues Tyr-6, Thr-13, and GSH were used to constrain geometrically the mimics *p*-cresol, methanol, and methanethiol, respectively. With the basis sets used here, the difference in energy required to remove the proton from the thiol in the presence and absence of the hydroxyl group analogous to Thr-13 was comparable to the energy reported by Liu et al. (1993), utilizing MP2 theory and the 6-31+G* basis set (7 vs 17 kJ/mol). Thus, the model calculations utilizing the Hartree−Fock theory faithfully reproduce the trend in the mu-class active site, and they may be used qualitatively to predict the effect of an on-face hydrogen bond in the alpha-class GST.

Replacement of the toluene with *p*-cresol mimics the substitution of Phe-220 with Tyr in the alpha-class model. Replacement of the toluene with *p*-cresol resulted in a reduction of the gas-phase pK_a of methanethiol by 2.9 pK_a units, or 17.4 kJ/mol of stabilization. These results are summarized in Table 1. Taken together, the results from

Table 2: Steady-State Kinetic Parameters[a]

protein	V_{max} [nmol/(min·nmol of GST)]	K_M(CDNB) (μM)	K_M(GSH) (μM)
WT	128 ± 7	560 ± 60	370 ± 30
W21F	119 ± 4	520 ± 50	320 ± 30
W21F:F220Y	170 ± 11	410 ± 40	331 ± 19
W21F:M16T	1.4 ± 0.1	620 ± 90	

[a] Assays at pH 6.5, 25 °C.

these models suggested that, in the absence of structural perturbation accompanying mutation, the interaction between Tyr-9 of A1-1 GST and a mutant Tyr-220 hydroxyl group could provide significant stabilization of the GS^- thiolate anion. Because gas-phase interactions "exagerate" electrostatic interactions compared to solution, a much smaller decrease in pK_a would be expected in the mutant protein. Furthermore, the model calculations indicate that the Phe-220 found in the wild-type protein lowers the pK_a of Tyr-9, relative to a protein with "nothing" in this neighborhood. Presumably, the electrostatic interaction between the aromatic rings of Tyr-9 and Phe-220 stabilizes the deprotonated thiolate, thus lowering the pK_a of GSH.

On the basis of these models, Phe-220 was substituted with Tyr in the rat A1-1 GST. This homologue is 75% identical to the human A1-1 enzyme. For comparison, and in order to determine the effect of adding a hydroxyl group at the edge of Tyr-9, Met-16 was replaced independently by Thr. These amino acid substitutions were made in the rat A1-1 mutant protein we have utilized previously to characterize the fluorescence properties of Tyr-9, W21F, which provides the "wild type" for our studies. Therefore, the relevant mutant proteins characterized here are W21F:F220Y, W21F:M16T, W21F:F220Y:Y9F, and W21F:M16T:Y9F. We have described previously the spectroscopic advantages for using the tryptophan-free mutant (Atkins et al., 1993; Dietze et al., 1996), and these advantages were exploited here as well. Importantly, the W21F mutant has essentially identical catalytic and stability properties as the "true" wild-type rat A1-1 GST. Moreover, the pK_a of GSH is identical whether bound to wild type or W21F GST, based on spectroscopic experiments (not shown). Thus, W21F provides a useful wild-type template for examining the role of aromatic interactions at Tyr-9.

Steady-State Kinetics. The W21F, W21F:F220Y, and W21F:M16T mutants were characterized by steady-state kinetic assays using the "standard" CDNB assay. At the standard pH 6.5, only the M16T substitution alters significantly the catalytic behavior. The F220Y substitution has negligible effect on K_M for either GSH or CDNB. The nearly identical K_M values for the two proteins indicate that no significant structural changes is introduced by the F220Y substitution. Because the hydrogen bond between the hydroxyl group of Tyr-9 and the thiolate of GS^- contributes little to the binding affinity of GSH, or to K_M for this cofactor (Liu et al., 1992; Wang et al., 1992), modulation of this hydrogen bond by the putative on-face hydrogen bond from Tyr-9 to Tyr-220 would not be expected to alter K_M for GSH. This was observed experimentally. However, the V_{max} at this pH is increased by 1.4-fold for the F220Y mutant (Table 2). The W21F:M16T protein, in contrast, exhibits a 5-fold decrease in specific activity compared to W21F. In principle,

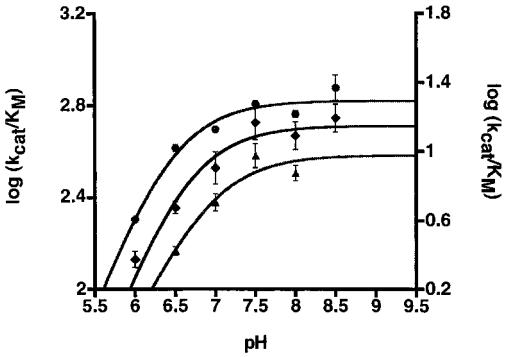

FIGURE 2: pH dependence of k_{cat}/K_M (variable CDNB). The pK_a values recovered from fitting to the equation $\log(k_{cat}/K_M) = \log[C/(1 + [H^+]/K_a)]$, where C is the upper limit of k_{cat}/K_M at high pH, are 6.4 ± 0.1, 6.6 ± 0.1, and 7.0 ± 0.2 for the GSH bound to W21F:F220Y, W21F, and W21F:M16T mutants, respectively. Symbols used are as follows: circles, W21F:F220Y; diamonds, W21F; triangles, W21F:M16T. The left axis applies to the W21F and W21:F220Y mutants, whereas the right axis applies to the W21F:M16T protein.

plots of $\log(k_{cat}/K_M)$ vs pH (saturating GSH, variable CDNB) reveal the pK_a of the thiol nucleophile in the [GST·GSH] binary complex (Liu et al., 1993; Cleland, 1979). Plots of $\log(k_{cat}/K_M)$ vs pH are shown in Figure 2. The kinetic pK_as obtained from fitting the data to the relation $\log(k_{cat}/K_M) = \log[C/(1 + [H^+]/K_a)]$, where C is limiting value of k_{cat}/K_M at high pH (Liu et al., 1993; Cleland, 1979), are 6.6 ± 0.1, 6.4 ± 0.1, and 7.0 ± 0.2 for the W21F, W21F:F220Y, and W21F:M16T proteins, respectively. Thus, the apparent pK_a of GS^- bound at the active site is decreased by 0.2 pK_a unit when Phe-220 is replaced by Tyr, in qualitative agreement with the change expected from the *ab initio* calculations. Notably, this change is outside the limits of experimental error. The kinetic pK_a of GSH bound to the catalytically impaired M16T is 7.0. Presumably, the structure of the W21F:M16T protein is peturbed slightly, and the active site features which lower the pK_a of GSH and Tyr-9 have been compromised.

Spectroscopic Titration of Enzyme-Bound GSH. Direct spectroscopic titration of the GSH by UV absorption spectroscopy was also performed, as described for the wild-type M1-1 isozyme (Liu et al., 1992). The UV absorption band at 239 nm due to thiolate was monitored at nine pH values for W21F, W21F:F220Y, and W21F:M16T, and the resulting titration curves are shown in Figure 3. Direct spectroscopic titration of GSH in the binary complex for the alpha-class GSTs has not been reported previously. Kinetic experiments suggest that the GSH bound to alpha-class GSTs has a higher pK_a (6.9−7.4) than when bound to mu- or pi-class enzymes, which have pK_as of 6.2−6.9. The spectroscopic data support this, as the W21F exhibits a pK_a for GSH of 7.4. Above pH 8.0, the ionization of free GSH results in a large background absorbance, hence limiting the experimentally accessible pH range. For each of the W21F, W21F:F220Y, and W21F:M16T proteins a clear absorbance band due to thiolate at 239−241 nm was observed, with the pH dependences shown in Figure 3. The spectra were nearly identical to those reported for the mu-class GST (Liu et al., 1992) including the weak difference absorbance band at ∼285 nm, attributed to minor conformational change induced by GSH. The intensities of the absorbance bands at 239 nm, along with reported molar absorptivities for thiolate (Lo Bello et al., 1993; Liu et al., 1992), were consistent with

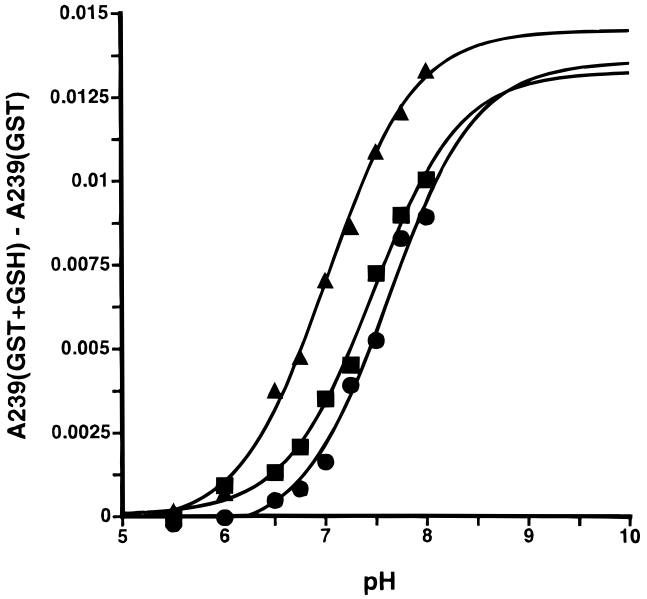

FIGURE 3: Spectral titration of GSH bound to mutant proteins. The absorbance at 239 nm was used to monitor ionization of GSH. Symbols used are as follows: triangles, W21F:F220Y; squares, W21F; circles, W21F:M16T. The recovered pK_a values, based on fitting to a single ionization scheme, are 7.02 ± 0.05, 7.46 ± 0.06, and 7.63 ± 0.09 for the W21F:F220Y, W21F, and W21F:M16T mutants, respectively.

titration of GSH bound to ~60% of the active sites present. The slight differences in absorbance at 239 nm at limiting pH values of the calculated "best fits" are likely due to minor differences in the absorbance properties of the mutant proteins or concentration of the samples.

Most importantly, there was a significant decrease in the pK_a of GSH bound to the W21F:F220Y mutant, compared to the W21F. In contrast, the W21F:M16T mutant demonstrates a marked increase in the pK_a of GSH in the binary complex. The pK_a values of GSH bound to each of these proteins are significantly above the corresponding kinetically determined values, which suggests that additional ionization reactions affect the steady-state catalytic rates. Direct titration of the GSH thiol in each of the M1-1 mutants with altered on-face residues at Tyr-6 was not reported (Liu et al., 1993; Xiao et al., 1996), so comparison of the ΔpK_a for GSH caused by an on-face hydrogen bond in different isozymes, as determined by this method, is not possible.

Fluorescence Titration of Tyr-9. As previously mentioned, it was not obvious that the pK_a of the substrate-free Tyr-9 would be altered by mutation at Phe-220, because residues at the C-terminus are more disordered in the absence of ligand than in the presence of GSH conjugates. In order to examine this possibility, the pK_a of Tyr-9 in the mutant proteins was determined directly from fluorescence excitation and emission spectra, as described previously (Atkins et al., 1993; Dietze et al., 1996). With increasing pH, the tyrosinate which is formed causes a red shift in the emission spectrum, and shoulders centered at 305 and 256 nm in the excitation spectra appear. Thus, emission and excitation spectra were used to determine the pK_a of Tyr-9 for the W21F GST, the W21F:F220Y mutant, and the W21F:M16T protein. The corresponding mutants in which Tyr-9 was replaced with phenylalanine, W21F:F220Y:Y9F and W21F:M16T:Y9F, were also titrated to determine whether tyrosines other than

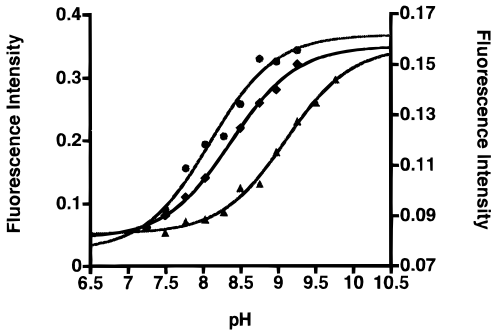

FIGURE 4: Fluorescence titration of Tyr-9. The fluorescence intensity at 305 nm of excitation spectra was used to monitor ionization of Tyr-9. Symbols used are as follows: circles, W21F:F220Y; diamonds, W21F; triangles, W21F:M16T. The pK_a of Tyr-9 is 7.8 ± 0.1, 8.3 ± 0.2, and 9.0 ± 0.2 for W21F:F220Y, W21F, and W21F:M16T, respectively. Mutants with Tyr-9 replaced by Phe exhibit no tyrosine ionization below pH 9.5.

Tyr-9 were contributing to the observed titration curves. In fact, the only Tyr which titrates in the pH range 6.5−9.0 is Tyr-9, as indicated by the lack of spectral change in this pH range in the mutants lacking Tyr-9. The pK_a values recovered from curve fitting of the data (Figure 4) are 7.8 ± 0.1, 8.3 ± 0.2, and 9.0 ± 0.2 for the W21F:F220Y, W21F, and W21F:M16T proteins, respectively. The active site Tyr-9 has a significantly lower pK_a when Phe-220 is replaced with Tyr-220.

DISCUSSION

Studies which have explicitly investigated (experimentally) the role of aromatic hydrogen bonds in protein function have relied mainly on removal by mutagenesis of a proposed hydrogen bond donor at an aromatic ring. The complementary approach, in which a hydrogen bond donor is added to a non-hydrogen-bonded π-cloud, provides a powerful test of the contribution of such hydrogen bonds.

Here we demonstrate with fluorescence and UV spectroscopies, steady-state kinetics, site-directed mutagenesis, and model calculations that residues in contact with Tyr-9 of A1-1 GST modulate the pK_a of this catalytic residue and GSH. Although an X-ray structure of the F220Y mutant is required to confirm the presence of an on-face hydrogen bond, the results provide a compelling argument for its contribution to the properties of the protein. We have considered the possibility that the hydroxyl group on Tyr-220 is recruited to hydrogen bond directly with the GS^- thiolate and lower the pK_a of the latter. However, for this to occur, a significant reorganization of Tyr-9 would be required, because Tyr-9 lies "between" the GS^- thiolate and Phe-220. In light of the experimentally determined kinetic parameters and the pK_as of Tyr-9 and Tyr-220 in this mutant, it is unlikely that such a reorganization has occurred. Based on the direct titration of GSH bound to the W21F and W21F:F220Y proteins, the incorporated hydrogen bond contributes 0.6 kcal/mol of stabilization to the thiolate anion at 25 °C (ΔpK_a = 0.45 unit). A similar $\Delta\Delta G$ is observed for ionization of Tyr-9 upon substitution of Phe-220 with Tyr, based on the direct fluorescence titration of this residue in the absence of ligand. It is not the *magnitudes* of the substitution-induced changes in pK_as of Tyr-9 and GSH which are striking, but rather the *direction* of change. At least 15 mutations have been made in GSTs which *raise* the pK_a of GSH or the active site tyrosine (Liu et al., 1992;

Bjornestadt et al., 1995; Wang et al., 1992; Xiao et al., 1996; data not shown). To our knowledge, the F220Y substitution is the first example of a substitution with a natural amino acid which decreases these pK_as. The mutation-induced decrease in pK_a of GSH was rationally predicted from the available X-ray structures and models for aromatic on-face hydrogen bonds.

These results provide three important points of comparison of the structure−function relationships of GSTs from different classes: (1) The decrease of the pK_a of both Tyr-9 and GSH upon substitution of Phe-220 with tyrosine strongly supports the proposal that a second-sphere on-face hydrogen bond lowers the pK_a of the analogous tyrosine and GSH in the mu-class GSTs (Liu et al., 1993; Xiao et al., 1996). Such a hydrogen bond is not operative in the wild-type alpha-class GSTs. (2) However, the model calculations suggest that the aromatic interactions between Tyr-9 and Phe-220 in the wild-type alpha-class GSTs probably perform a similar function in the alpha-class GSTs. This is based on comparison of the proton affinity of methanethiol hydrogen bonded to *p*-cresol, in the presence and absence of toluene which mimics Phe-220. (3) The spectroscopic titration of GSH bound to the rat A1-1 GST yields a pK_a value of 7.4, which is remarkably different from the previously reported pK_a for GSH bound to mu-class GSTs, pK_a 6.2−6.9 (Graminski et al., 1989; Liu et al., 1992). Conversely, the pK_a reported recently for the active site tyrosine of the ligand-free M1-1 GST (Xiao et al., 1996) is ∼2 pKa units above the pK_a of the corresponding tyrosine in alpha- and pi-class enzymes. Together with the results presented here, then, it is apparent that the mu-class GSTs invest significantly more free energy in the stabilization of the GS^- thiolate than the alpha-class GSTs, which invest equivalent free energy of stabilization to both GS^- and the active site tyrosinate of the ligand-free enzyme. To the extent that these pK_as determine the equilibrium position of the proton shared between GS^- and the active site tyrosines in each GST, these differences clearly indicate that the mechanistic details for catalysis are isozyme-dependent. In this regard, it is interesting to note that recent results (Parsons & Armstrong, 1996) with the M1-1 GST, in which native enzyme was compared to enzyme with all 14 tyrosine residues replaced by 3-fluorotyrosine, demonstrate that a general base mechanism is unlikely. The authors elegantly demonstrated that the shared proton is not transferred from GSH to a tyrosinate-6 during catalysis by the M1-1 GST. In the M1-1 isozyme, however, the pK_a for Tyr-6 (pK_a 10.2) is matched poorly with the pK_a of GSH (pK_a ∼6.3) at the active site. The corresponding pK_as of Tyr-9 (pK_a 8.3) and GSH (pK_a 7.4) at the A1-1 GST active site may result in a hydrogen bond with more tyrosinate character, even if "formal" general base mechanism is not operative. The results presented here indicate that second-sphere effects at the aromatic ring of the catalytic tyrosine in each class of GST may contribute to these mechanistic differences.

The alteration of the pK_a of Tyr-9 in the ligand-free enzyme is particularly interesting. Because the C-terminal residues of the ligand-free A1-1 GST are relatively "unstructured", this alteration of pK_a may be fortuitous. Alternatively, it is possible that the additional hydroxyl group provided by Tyr-220 provides a hydrogen bond to Tyr-9 which increases the "order" of this helix and which includes the on-face hydrogen bond in the absence of ligands. Additional experiments are required to address this possibility.

Finally, these results have implications for protein design and engineering strategies. Although the functional importance of "unconventional" hydrogen bonds which involve aromatic π-clouds has been elusive, these data demonstrate that, under favorable conditions, such hydrogen bonds may be used to alter the catalytic properties of enzymes which include aromatic amino acids in their mechanisms. The energetic contribution of such hydrogen bonds is small, but it is sufficient to alter enzyme catalysis, and aromatic hydrogen bonds may be included in the strategies available for tailoring protein function.

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