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Pressure-dependent ionization of Tyr 9 in glutathione S-transferase A1-1: Contribution of the C-terminal helix to a "soft" active site

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Abstract

The glutathione S-transferase (GST) isozyme A1-1 contains at its active site a catalytic tyrosine, Tyr 9, which hydrogen bonds to, and stabilizes, the thiolate form of glutathione, GS^-. In the substrate-free GST A1-1, the Tyr 9 has an unusually low pK_a, ~8.2, for which the ionization to tyrosinate is monitored conveniently by UV and fluorescence spectroscopy in the tryptophan-free mutant, W21F. In addition, a short α-helix, residues 208–222, provides part of the GSH and hydrophobic ligand binding sites, and the helix becomes "disordered" in the absence of ligands. Here, hydrostatic pressure has been used to probe the conformational dynamics of the C-terminal helix, which are apparently linked to Tyr 9 ionization. The extent of ionization of Tyr 9 at pH 7.6 is increased dramatically at low pressures ($p_{1/2}$ = 0.52 kbar), based on fluorescence titration of Tyr 9. The mutant protein W21F:Y9F exhibits no changes in tyrosine fluorescence up to 1.2 kbar; pressure specifically ionizes Tyr 9. The volume change, ΔV, for the pressure-dependent ionization of Tyr 9 at pH 7.6, 19 °C, was -33 ± 3 mL/mol. In contrast, N-acetyl tyrosine exhibits a ΔV for deprotonation of -11 ± 1 mL/mol, beginning from the same extent of initial ionization, pH 9.5. The pressure-dependent ionization is completely reversible for both Tyr 9 and N-acetyl tyrosine. Addition of S-methyl GSH converted the "soft" active site to a noncompressible site that exhibited negligible pressure-dependent ionization of Tyr 9 below 0.8 kbar. In addition, Phe 220 forms part of an "aromatic cluster" with Tyr 9 and Phe 10, and interactions among these residues were hypothesized to control the order of the C-terminal helix. The amino acid substitutions F220Y, F220I, and F220L afford proteins that undergo pressure-dependent ionization of Tyr 9 with ΔV values of 31 ± 2 mL/mol, 43 ± 3 mL/mol, and 29 ± 2 mL/mol, respectively. The $p_{1/2}$ values for Tyr 9 ionization were 0.61 kbar, 0.41 kbar, and 0.46 kbar for F220Y, F220I, and F220L, respectively. Together, the results suggest that the C-terminal helix is conformationally heterogeneous in the absence of ligands. The conformations differ little in free energy, but they are significantly different in volume, and mutations at Phe 220 control the conformational distribution.

Keywords: conformational dynamics; hydrostatic pressure; protein volume changes

Glutathione S-transferases comprise a family of xenobiotic-metabolizing enzymes that catalyze the conjugation of the reactive cofactor glutathione with numerous electrophilic compounds. Dimeric GSTs of the alpha-, pi-, and mu-classes possess a catalytically important, evolutionarily conserved tyrosine at each active site (Stenberg et al., 1991; Kolm et al., 1992). This tyrosine modulates the pK_a and the nucleophilic reactivity of the enzyme-bound GSH by stabilizing the thiolate anion, through hydrogen bonding, as Tyr-OH$\cdots^-$SG. The catalytic tyrosine in the substrate-free alpha-class GSTs is "activated" by several nearby residues that lower the pK_a of the phenolic hydroxyl group to ~8.2, from ~10.3 in solution (Atkins et al., 1993; Bjornestedt et al., 1995; Dietze et al., 1996a). Interestingly, the analogous tyrosine in the mu-class GSTs is not "activated," and it exhibits a "normal" pK_a (Parsons & Armstrong, 1996). Although X-ray structures of GSTs from the alpha-, pi-, and mu-classes indicate that the overall structures are very similar, subtle differences clearly are sufficient to lead to changes in catalytic properties (Armstrong, 1994, for review).

One unique structural element found in the alpha-class GSTs is a 14-residue α-helix at the C-terminus of each subunit. This helix provides a "cap" over the active site in the presence of various ligands, but it is disordered in the apo-enzyme; the position of this helix is not well defined crystallographically in the absence of ligands (Fig. 1; Cameron et al., 1995). Moreover, the exact posi-

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Abbreviations: GST, glutathione S-transferase; GSH, glutathione; GS^-, the thiolate anion of glutathione; Tyr-OH, the hydroxyl group on Tyr 9; $p_{1/2}$, the pressure required to afford 50% ionization of Tyr 9; NH_4OAc, ammonium acetate; MeOH, methanol.

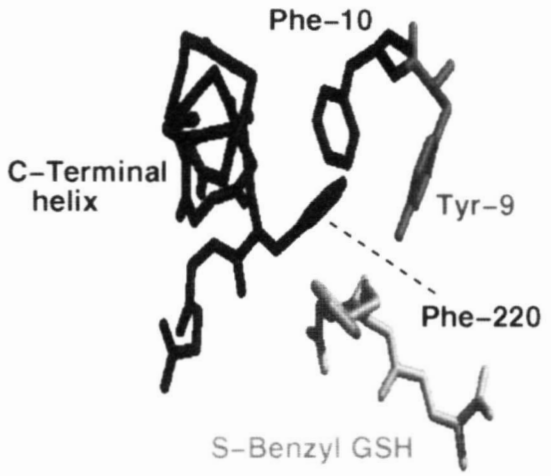

Fig. 1. Location of C-terminal helix in GST A1-1. The C-terminal helix forms a "lid" over the active site, which includes the catalytic Tyr 9. The Phe 220 contained in the helix forms part of an aromatic cluster including Tyr 9 and Phe 10. The S-benzyl GSH is bound at the active site. In the absence of ligands, the location of the C-terminal helix is poorly defined. The model shown is taken from Sinning et al. (1993), and created with MidasPlus.

tions of residues contained in this helix vary with different ligands, when present. In order to obtain insight into the role of active site solvation and conformational dynamics of this helix in the function of GSTs, hydrostatic pressure was used to perturb the distribution of conformational states, as probed by the intrinsic spectroscopic reporter, Tyr 9, of the rat A1-1 isozyme. Because Tyr 9 of the rat A1-1 GST is solvated differentially in the absence and presence of various ligands, and because Tyr 9 has an unusually low pK_a, which distinguishes it spectroscopically from other tyrosines in the protein (Atkins et al., 1993; Dietze et al., 1996b), it provides an ideal probe of catalytically important conformational dynamics of the active site of GST A1-1.

The response of proteins to hydrostatic pressure has been used to study enzyme catalysis (Morild, 1981 for review), protein folding (Vidugiris et al., 1995; Frye et al., 1996), protein–protein interactions (Weber, 1992; Silva et al., 1996, for reviews), and protein–ligand interactions (Di Primo et al., 1990; Coelho-Sampaio & Voss, 1993). The magnitude of the response to pressure is described quantitatively by the derivative of the Gibbs free energy change, ΔG, with respect to pressure p, and volume change ΔV:

$$d(\Delta G)/dp = \Delta V. \quad (1)$$

The decrease in volume of various protein systems upon application of hydrostatic pressure has been attributed to solvent electrostriction at charged sites, and hydration of hydrophobic surfaces, which lead to differential protein–solvent interactions with changing pressure (Weber, 1992; Gross & Jaenicke, 1994). Also, it has been suggested that the efficiency of packing of nonsolvated protein constituents contributes to, and may dominate, the pressure response (Vidugiris et al., 1995; Frye et al., 1996; Gerstein & Chothia, 1996). Regardless of the molecular basis, changes in hydrostatic pressure may reveal molecular insight that is not available from the more common variation of temperature. The response to pressure also provides a quantitative measure of a fundamental thermodynamic parameter for a specific process, ΔV. The specific ionization of Tyr 9 of GST A1-1 by pressure provides a novel example of the utility of this method. An initial characterization of this process is reported here.

Results

Pressure effects on the dimeric structure of GST

Because it is well established that pressure causes dissociation of oligomeric proteins, it was necessary to determine the pressure range in which dimeric rat A1-1 GST dissociated. Previous results (Aceto et al., 1992) indicated that pressure induces dissociation of a pi-class GST irreversibly in a concentration-dependent process. The results were interpreted as a simple "nondeterministic" equilibrium (Erijman & Weber, 1991) between monomer and dimer. Upon pressure-dependent formation of the monomer, the subunits rearranged to inactive, partially structured, species. Notably, at concentrations above 0.1 μM subunits, it was observed that pressures up to 2 kbar did not cause significant dissociation (Aceto et al., 1992).

In order to determine the effect of pressure on the oligomeric state of the rat A1-1 GST, which is a homodimer, tryptophan fluorescence was used as a spectroscopic probe. The rat A1-1 GST has a single tryptophan (Trp 21) in each subunit. This tryptophan is close-packed with hydrophobic residues, and is relatively inaccessible to solvent (Wang et al., 1993). Also, this tryptophan provides a portion of an intrasubunit domain interface between the N-terminal one-third of each subunit and the remaining, largely helical, C-terminal two thirds. It is reasonable to expect that the intrasubunit domain interface would be disrupted upon dissociation of subunits. Therefore, Trp 21 provides a probe of dissociation-dependent structural changes.

Two conspicuous changes in the emission properties of Trp 21 were observed upon an increase in the hydrostatic pressure of samples containing 0.5 μM subunits: the emission intensity decreased to ~88% of the intensity at atmospheric pressure, and the spectral center of mass increased from 343 nm to 349 nm. Most importantly, a clear concentration dependence was observed for the pressure-dependent decrease in intensity, whereas the red-shift in the spectrum demonstrated the same pressure dependence at all protein concentrations studied. The changes in emission intensity are summarized in Figure 2. The lack of concentration dependence for the pressure-induced red-shift of the emission spectrum suggests that this spectral change is due to intramolecular structural changes, without subunit dissociation.

Pressure-dependent dissociation was studied also by measuring enzyme activity of samples left at elevated pressure for fixed time intervals, and then decompressed. As with the decrease in tryptophan fluorescence intensity, no loss in enzyme activity was observed in samples >20 μM subunits that were exposed to 1.0 kbar for 15 min. These results suggest that subunit dissociation does not occur at concentrations above 20 μM subunits and below 1.0 kbar. Importantly, several studies have demonstrated independently that GST monomers are unstable, and they denature rapidly to varying extents once formed (Aceto et al., 1992; Erhardt & Dirr, 1995). Additional experiments are required to characterize fully the effect of pressure on the monomer–dimer equilibrium; they will provide the basis for a separate manuscript.

In contrast to the subunit interface, the ionization of Tyr 9 was found to be remarkably sensitive to pressure in the range from 1 atm to 1.0 kbar. Therefore, the effect of pressure on the ionization of Tyr 9 was studied with concentrations of GST of >20 μM

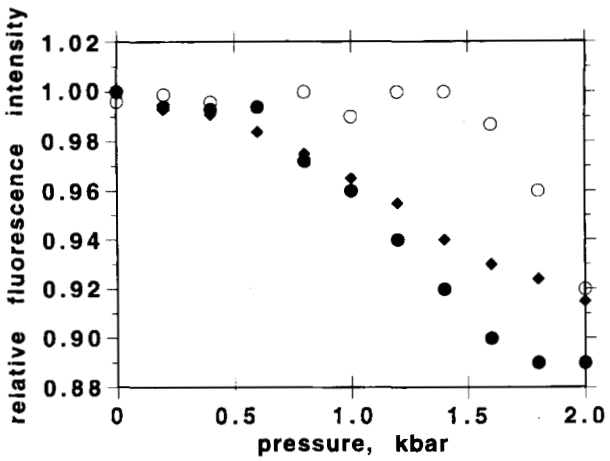

Fig. 2. Pressure-induced changes in the emission properties of Trp 22 in rat GST A1-1. The emission intensity decreases with increasing pressure in a concentration-dependent process. A similar concentration dependence is observed for pressure-dependent loss of enzyme activity. Open circles, 20 μM subunits; closed triangles, 5 μM subunits; closed circles, 0.5 μM subunits. The concentration-dependence is attributed to pressure-induced subunit dissociation below 20 μM subunits and above 1.0 kbar.

subunits, at pressures below 1.0 kbar. Under these conditions, subunit dissociation is negligible.

Pressure-dependent ionization of Tyr 9

As described previously, ionization of Tyr 9 of rat GST A1-1 may be monitored by fluorescence spectroscopy with the tryptophan-free mutant W21F, which retains catalytic properties nearly identical to the wild-type rat A1-1 GST (Atkins et al., 1993; Dietze et al., 1996b). Briefly, tyrosine absorbs maximally at ~225 nm (ϵ = 8,200 M^{-1} cm^{-1}) and ~278 nm (ϵ = 1,350 M^{-1} cm^{-1}), whereas tyrosinate absorbs maximally at ~ 245–256 nm (ϵ = 1,100 M^{-1} cm^{-1}) and ~295 nm (ϵ = 2,350 M^{-1} cm^{-1}). In addition, the fluorescence emission maximum of tyrosine in proteins is typically ~305–320 nm, whereas tyrosinate exhibits an emission maximum at ~345 nm. The fraction of ionized Tyr 9 is determined readily from the fluorescence intensity at emission 345 nm and excitation 295 nm or emission 345 nm and excitation 256 nm (Dietze et al., 1996b). In fact, both methods were used here and yielded identical results. Experiments with 43 μM GST subunits at pH 7.6, 19 °C were performed, and the spectral changes observed with increasing pressure are summarized in Figure 3. At this pH, Tyr 9 is 20% ionized, with a pK_a of 8.2. The excitation spectra reveal the increased deprotonation of Tyr 9 as spectral shoulders at excitation wavelengths 256 nm and 305 nm, emission 348 nm, even at pressure well below 0.6 kbar. Comparison of the W21F mutant with the W21F:Y9F mutant demonstrates clearly that tyrosines other than Tyr 9 do not ionize in the pressure range studied. The extent of ionization was calculated from the ratio of emission intensities at 348 nm for excitation wavelengths 256 nm and 280 nm (I_{256}/I_{280}), and from spectra obtained under conditions with Tyr 9 ionized completely and protonated completely. The extent of ionization at varying pressures is summarized in Figure 4.

For comparison, the pressure-dependent ionization of N-acetyl tyrosine was examined. For these experiments, the pH was 9.5, which yields an initial fraction of tyrosinate comparable to the fraction of tyrosinate of Tyr 9 at pH 7.6. N-acetyl tyrosine exhibits

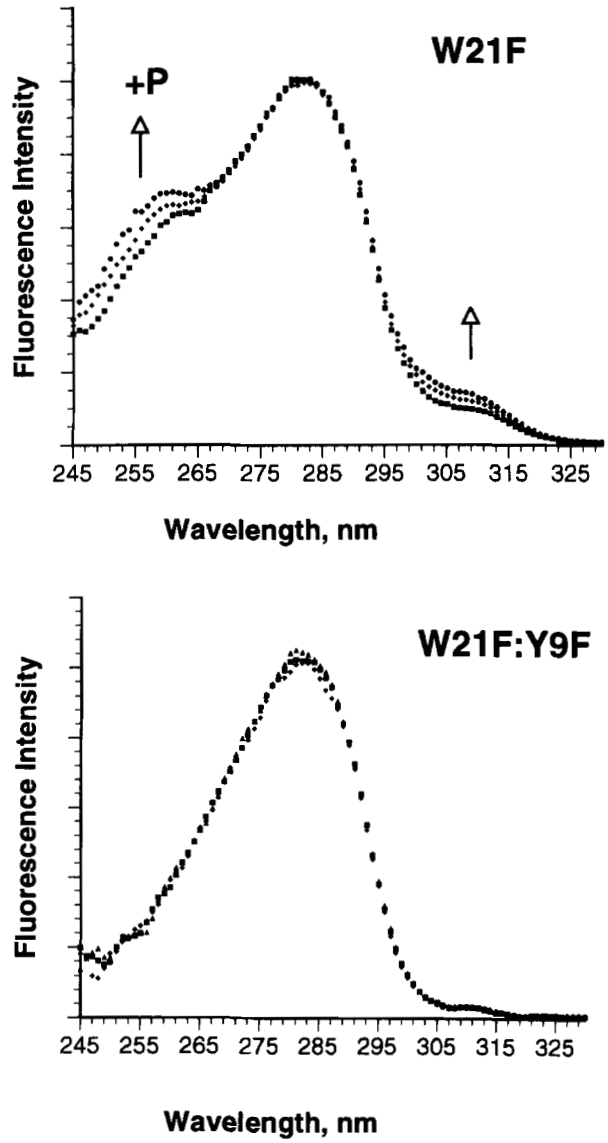

Fig. 3. Pressure-induced changes in the emission properties of Tyr 9 of rat GST A1-1. Normalized excitation spectra are shown for the W21F (top) and the W21F:Y9F (bottom) proteins at 1 atm pressure, 0.3 kbar, and 0.6 kbar. With increasing pressure (+P), spectral shoulders at excitation wavelength 256 nm and 305 nm increase in intensity for the W21F mutant only. Emission was monitored at 348 nm. The fluorescence intensity axis is in arbitrary units. These spectral changes are due to ionization of Tyr 9, as described previously (Dietze et al., 1996b). Ionization of Tyr 9 occurs at low pressures relative to subunit dissociation.

a pressure-dependent ionization as well, but the pressure sensitivity is much less than that observed for Tyr 9. These results are also shown in Figure 4. The carboxylate group present on N-acetyl tyrosine is, in principle, also subject to pressure-dependent changes in ionization state. However, at the pH used in these studies, pH 9.5, the carboxylate ionization is expected to be "pressure silent." Pressure favors the ionized carboxylate anion, relative to the protonated acid (Weber, 1992; Gross & Jaenicke, 1994). At pH 9.5, the carboxylate of N-acetyl tyrosinate is completely ionized and will not undergo additional volume changes due to ionization at this site. Therefore, the volume change is not expected to include a contribution from a change in ionization at the carboxylate.

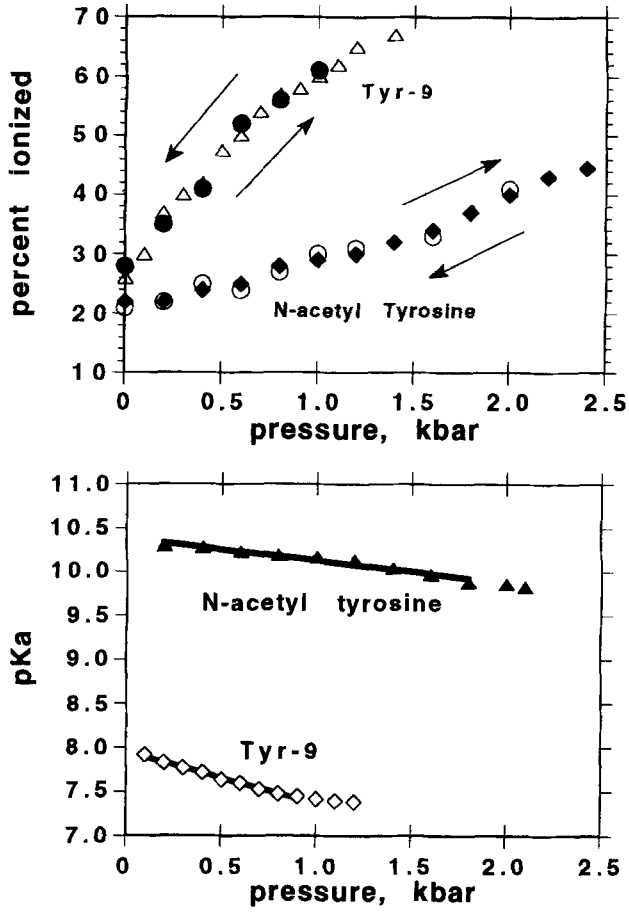

Fig. 4. Extent of ionization versus pressure. Top: Percent of ionization of either Tyr 9 or N-acetyl tyrosine was determined at various pressures, as described in the text. Compression and decompression cycles are shown. Open triangles, W21F GST compression; solid circles, W21F GST decompression; solid diamonds, N-acetyl tyrosine compression; open circles, N-acetyl tyrosine decompression. Bottom: Replot of pK_a versus pressure. The slope of the line yields ΔV, as described in the text. The recovered volume changes for ionization of Tyr 9 (open diamonds) and N-acetyl tyrosine (closed triangles) are −33 and −11 mL/mol, respectively.

The reversibility of the pressure-dependent ionization of Tyr 9 was also examined. After obtaining a limiting high-pressure value for either the W21F GST or N-acetyl tyrosine, the spectra were recorded at each incremental pressure along the decompression curve, and the fraction of tyrosinate-9 or N-acetyl tyrosinate was determined. Ionization processes for both Tyr 9 and N-acetyl tyrosine were completely reversible, without any detectable hysteresis (Fig. 4). After decompression, a second cycle of compression yielded pressure versus extent of ionization curves identical to the first cycle of compression for both N-acetyl tyrosine and Tyr 9 of GST (not shown). The reversibility of the process further demonstrates that the observed ionization of Tyr 9 is not caused by pressure-dependent dissociation of the GST subunits associated with "nonspecific" structural changes leading to denaturation. Under conditions where dissociation of GST subunits has been observed (Aceto et al., 1992; Erhardt & Dirr, 1995), complete recovery of spectral parameters and catalytic activity has not occurred.

It is essential to note that the observed increase in the fraction of tyrosinate-9 with increasing pressure is not due to pressure-induced change of the pH of the system. It is well established that many buffers ionize at elevated pressure, and are characterized by molar volume changes that are comparable in magnitude to molar volume changes for phenol ionization. The pH of Tris buffers, in contrast, is invariant in the pressure range of interest here (Neuman et al., 1973; Gross & Jaenicke, 1994). Additionally, experiments were performed at varying buffer concentrations (5 mM, 10 mM, and 50 mM Tris) without any detectable alteration of the pressure dependence of the Tyr 9 ionization. Also, the experiments with N-acetyl tyrosine demonstrate clearly that the effect of pressure on Tyr 9 is not due to a trivial change in pH of the system.

From the fraction of ionization at each pressure, the pK_a of Tyr 9 may be calculated. In turn, the molar volume change for the process may be obtained from the slope of a plot of pK_a versus pressure, according to Equation 2:

$$d(pK_a)/dp = \Delta V/(2.303)RT, \quad (2)$$

where R and T are the gas constant and temperature, respectively. Although the isothermal compressibilities of proteins have been shown to vary over larger pressure ranges (Gross & Jaenicke, 1994), the values of pK_a for Tyr 9 at variable pressure in the narrow pressure range from 1 atm to 0.8 kbar fit well to a line, between 25% and 80% ionization (Fig. 4). This suggests that the compressibilities of the GST active site and the free amino acid do not change significantly in this range. The molar volume changes associated with ionization of Tyr 9 and N-acetyl tyrosine are obtained from the plots in Figure 3, as described in Materials and methods, and are −33 ± 3 mL/mol and −11 ± 1 mL/mol, respectively, at 292 °K. The $p_{1/2}$ values are 0.52 kbar and 2.6 kbar for ionization of Tyr 9 and N-acetyl tyrosine, respectively. Notably, the volume change obtained for N-acetyl tyrosine is nearly identical to values determined previously for phenol and related compounds (Neuman et al., 1973; Van Eldik et al., 1989), whereas the volume change for ionization of Tyr 9 is on the same order as protein–ligand and subunit dissociation reactions (Gross & Jaenicke, 1994). These results indicate that ionization of the phenolic hydroxyl group of Tyr 9 is not the only source of volume change for the system. Rather, it is likely that pressure-induced conformational change associated with the larger volume change is linked to ionization of the catalytic residue.

Ligand effects on pressure-dependent ionization of Tyr 9

The results with the substrate-free protein suggest that conformational dynamics of the active site structure are linked to the ionization state of Tyr 9. This, in turn, leads to the hypothesis that ligands that alter the populations of these conformations should modulate the pressure dependence of this process. However, previous results have demonstrated that GSH quenches the Tyr 9 fluorescence efficiently, and it cannot be used as a spectral probe in the presence of this cofactor. Similarly, most GSH conjugates lead to a hydrogen bonded Tyr 9 that is spectroscopically indistinguishable from other tyrosines in the protein (Dietze et al., 1996b). However, the S-methyl GSH binds to the active site, without quenching the Tyr 9, which remains partially deprotonated at pH 7.6 (Dietze et al., 1996b). Therefore, the pressure-dependent ionization of Tyr 9 was determined in the presence of a saturating concentration of S-methyl GSH (1.5 mM). This ligand dramatically reduced the apparent compressibility of the Tyr 9 environment. In the pressure range that leads to complete ionization of

Tyr 9 in the ligand-free protein, no pressure-dependent ionization is observed in the presence of S-methyl GSH. At pressures above 0.9 kbar, Tyr 9 deprotonated with increasing pressure in samples containing S-methyl GSH. This pressure is sufficient to cause larger structural changes, subunit dissociation, or ligand dissociation. These experiments do not reveal whether this ionization was due to deprotonation in the presence of S-methyl GSH, or ionization following dissociation of the ligand. The results demonstrate clearly, however, that S-methyl GSH decreases the pressure sensitivity of Tyr 9.

Mutations at Phe 220

The X-ray structure of the ligand-free human A1-1 GST indicates that the C-terminal helix of each subunit, residues 208–222, is "disordered" compared to the ligand-bound structures (Sinning et al., 1993; Cameron et al., 1995). Of the residues in this helix, Phe 220 has been suggested to control the order of the helix, through interactions with bound glutathione conjugates, H-site ligands, and active site residues (Cameron et al., 1995). Although several conservative amino acid differences exist in the C-terminal 14 residues of human and rat A1-1 GSTs, Phe 220 is conserved. Moreover, it has been demonstrated that substitution of Tyr at this position decreases the pK_a of Tyr 9 in the rat apo-enzyme and of bound GSH in the binary complex, presumably through interactions with the aromatic ring of Tyr 9 (Dietze et al., 1996a). Therefore, it was reasonable to hypothesize that mutations at this position would alter the pressure-dependent ionization of Tyr 9. Three mutant proteins were examined, F220Y, F220I, and F220L, in order to determine the importance of the aromatic, phenolic, and hydrophobic side chains at this position. Indeed, amino acid substitutions had marked effects on the sensitivity of Tyr 9 ionization to pressure (Fig. 5). For these experiments, each of the mutant proteins was studied at the same pH, 7.6. The catalytic properties and the spectroscopically determined pK_as of Tyr 9 in each of these mutants will be presented in a separate manuscript. Briefly, the F220I, F220Y, and F220L proteins are each catalytically active and bind to the S-hexyl GSH column used for purification with sufficient affinity. Due to differences in the pK_a of Tyr 9 at atmospheric pressure for these mutants, the pressure curves begin at different extent of ionization.

The F220Y mutant exhibits a ΔV similar to the F220 protein, but the pressure curve is "shifted" significantly to higher pressures, such that the $p_{1/2}$ is 0.61 kbar, compared to 0.56 kbar for the F220 protein. That is, the F220Y protein is more stable to pressure-induced conformational redistribution, whereas the F220I and F220L mutants are more pressure sensitive (Table 1).

One additional mutant provided insight into the effect of pressure on the rat GST A1-1. An F220R mutant was constructed in order to determine the effect of a positive charge in the C-terminal helix. The protein obtained from the standard purification process, however, was a mixture of proteolyzed species. Therefore, electrospray-ionization mass spectrometry was performed in order to determine the sites of proteolytic cleavage (Table 2). Two species were identified unambiguously as residues 1–203 (17%) and 1–216 (28%). The third component (55%) could not be assigned unambiguously based on the recovered mass, but each of the possible species included a cleavage near the C-terminus. Samples containing this mixture exhibited a specific activity that was 4% of wild-type rat GST A1-1, and retained affinity for the S-hexyl GSH-agarose affinity column. Thus, it is likely that these C-terminal truncated mutants retain a dimeric, folded, structure that is similar to the other GST variants used here. However, spectroscopic properties of these samples were completely insensitive to pressure. In the pressure range 0–2 kbar, no formation of tyrosinate was observed. In fact, no change in the excitation or emission properties of tyrosines in these proteins was observed. Importantly, if as little as 10% of the total protein contained in the mixture included a Tyr 9 that was ionized by pressure in this range, it would have been detected by a change in excitation spectra. These results further demonstrate that the pressure-induced ionization of Tyr 9 for wild-type and mutant GSTs involves local effects on the C-terminal helix. Upon truncation of the C-terminal helix before Arg 217, Tyr 9 does not ionize to a measurable extent at any pressure studied here.

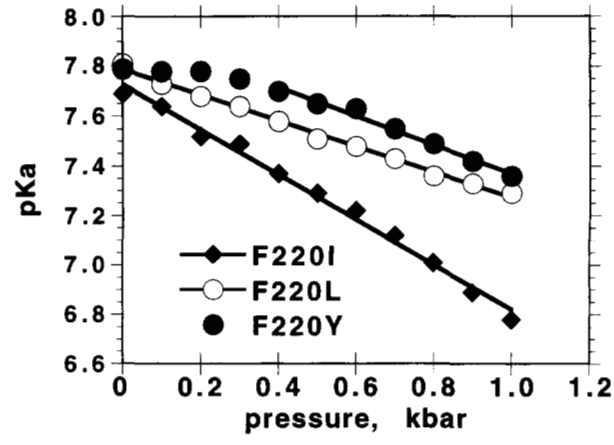

Fig. 5. Effect of mutations at Phe 220. The change in pK_a of Tyr 9 versus pressure is shown for F220Y, F220L, and F220I mutant proteins. The slopes of the lines yield $\Delta V/(2.303RT)$ as described in Materials and methods. Recovered values are summarized in Table 1.

Temperature-dependent ionization of Tyr 9

The temperature dependence of the ionization of Tyr 9 was also studied. In principle, the temperature dependence of the pK_a yields

Table 1. *Summary of the pressure response of mutant proteins*

Protein	pK_a Tyr 9 at 1 atm press[a]	ΔV_c (mL/mol)[b]	$p_{1/2}$ (kbar)[c]	ΔG_c (kcal/mol)[d]
F220	8.1	22 ± 3	0.52 ± 0.04	0.31
F220Y	7.9	20 ± 4	0.61 ± 0.04	0.30
F220I	7.7	32 ± 4	0.41 ± 0.05	0.22
F220L	7.8	18 ± 2	0.47 ± 0.05	0.22
F220/ + S-methyl GSH	7.8	ND	>>1.0	>3.4[e]

[a]Reported pK_as for Tyr 9 were obtained from y-intercepts of plots of pK_a versus pressure. They correlate well with pK_as determined spectroscopically (data to be published in a separate manuscript).

[b]Determined from $\Delta V - \Delta V_{ion}$, where ΔV_{ion} is the experimentally determined value for N-acetyl tyrosine. Reported error is standard error from duplicate experiments.

[c]Determined from the pressure affording 50% ionization of Tyr 9. Reported error is standard error from duplicate experiments.

[d]Determined from $p_{1/2} \times \Delta V_c$.

[e]Determined assuming ΔV_c is ~22 mL/mol, as for ligand-free F220.

Table 2. *Electrospray ionization MS characterization of proteolyzed F220R*

Peptide (residue #s)	Predicted mass (Da)	Observed mass (Da)	Relative abundance (%)
1–216	24,736.81	24,737.50	28
1–203	23,268.13	23,267.00	17
51–214	18,834.95		
50–213	18,834.02	18,835.50	55
37–200	18,834.00		

the ΔH for the reaction according to the van't Hoff equation. In turn, ΔS for the process, derived from the temperature dependence of the reaction, should also reflect contributions of solvent interactions. For N-acetyl tyrosine, increasing the temperature from −5 °C to 35 °C resulted in an increase in pK_a of ~0.8 units. This is consistent with previous studies (Kharakoz, 1986). A van't Hoff plot yielded a linear dependence of pK_a on $1/T$, with a corresponding ΔH of 7.6 ± 0.4 kcal/mol (data not shown, $r^2 = 0.995$). In contrast, however, the pK_a of Tyr 9 was essentially constant over this temperature range. In fact, with increasing temperature, both W21F and W21F:Y9F proteins exhibited an increase in tyrosinate consistent with ~0.3 tyrosine residues becoming deprotonated. Because this effect was not attributable to Tyr 9, information about the active site environment was limited, and further analysis was not performed. The results demonstrate clearly that temperature does not perturb the ionization of Tyr 9 selectively, whereas pressure does.

Discussion

Hydrostatic pressure has been shown to ionize the catalytic Tyr 9 of rat A1-1 GST selectively. As far as we know, selective ionization by pressure of a specific amino acid within an enzyme active site has not been demonstrated previously. Additionally, the observation of this ionization at very low pressures, relative to those typically required to alter protein structure grossly, suggests that the environment of Tyr 9 is extremely flexible. Importantly, hydrostatic pressure does not exert its effects on Tyr 9 by "denaturing" the protein. Presumably, GST A1-1 has evolved to lower the pK_a of Tyr 9, with specific structural features, compared to tyrosine free in solution (Cameron et al., 1995; Dietze et al., 1996a). A pressure-induced denaturation would be expected to cause an increase in the pK_a of Tyr 9, not a further decrease.

It is impossible to interpret directly the magnitude of the observed volume changes upon ionization of Tyr 9 and N-acetyl tyrosine, because the driving force for diminished volume at high pressure in protein systems is understood only qualitatively (Frye et al., 1996). However, the dramatic increase in the magnitude of ΔV for Tyr 9 compared to N-acetyl tyrosine is certainly due to changes in the solvation or the "packing" of active site residues in the protein other than Tyr 9, in addition to electrostriction at the phenolic hydroxyl group. The reasoning behind this assertion is summarized in Figure 6, which provides a useful template for discussion.

The volume change for ionization of phenolic hydroxyl groups at atmospheric pressure, ΔV_{ion}, is −11 to −16 mL/mol, as indicated by results presented here and elsewhere (Neuman & Kauzmann, 1973; Van Eldik et al., 1989). The experimentally derived ΔV obtained for pressure-induced ionization of Tyr 9 is significantly greater, suggesting that an additional process must be invoked, with ΔV_c, as shown in Figure 6. In principle, the total volume change for the two processes that contribute to the ionization of Tyr 9 at high pressure is the sum of the ΔV_{ion} for ionization at atmospheric pressure and the putative conformational change that provides the larger decrease in volume, ΔV_c. On the basis of this scheme, ΔV_c must be −23 mL/mol, assuming that the volume change for ionization of the phenolic hydroxyl group is independent of pressure, and $\Delta V_{ion,p} = \Delta V_{ion}$. The experimental data support the validity of this assumption (Fig. 4). As noted above, a ΔV_c of this magnitude is similar to ΔV values obtained for subunit dissociation and ligand dissociation reactions (Gross & Jaenicke, 1994; Royer, 1995). The results demonstrate that several protein sites must undergo increased solvation, or they "pack" more efficiently to reduce nonsolvated void spaces, with increased pressure, and thus result in a "compressible" site that undergoes a much larger volume change than is expected for generation of a single tyrosinate anion. The recovered ΔV_c values for each of the proteins studied here are summarized in Table 1.

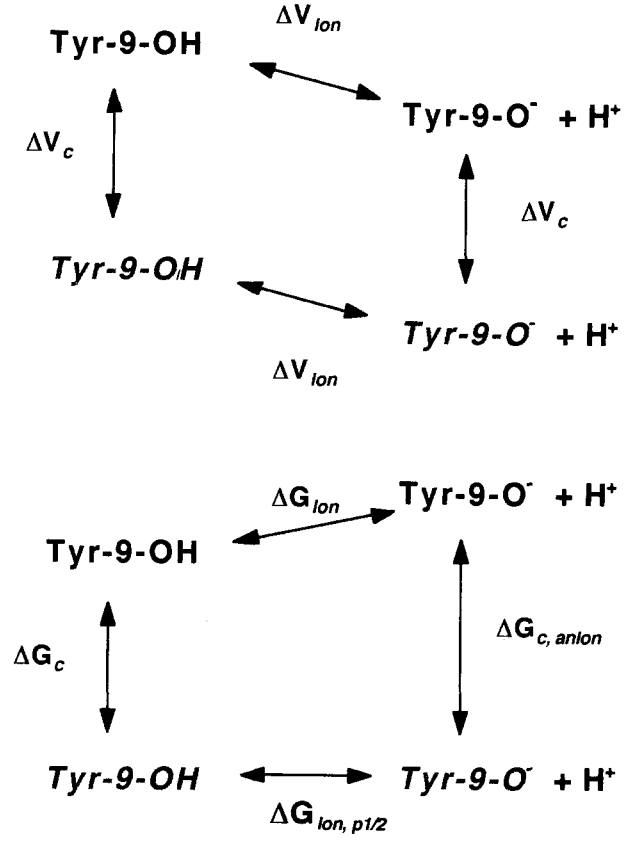

Fig. 6. Volume changes (top) and free energy changes at $p_{1/2}$ (bottom) for pressure-dependent ionization and conformational changes. Pressure-independent ΔV_{ion} for ionization of Tyr 9 is expected to provide only a fraction of the total ΔV measured experimentally. Therefore, a pressure-dependent conformational change is proposed to contribute ΔV_c, which is much larger in magnitude. As long as ΔV_{ion} is pressure independent (see below), ΔV_c is the same for protonated and unprotonated states of Tyr 9. At $p_{1/2}$ and pH 7.6, $\Delta G_{ion,p_{1/2}} = 0$, and $\Delta G_{ion} > 0$, so $\Delta G_c < \Delta G_{c,anion}$ (absolute magnitudes). Pressure and ionization of Tyr 9 favor the same conformational states.

Unlike the ΔV_{ion} values for ionization, the ΔG_{ion} values for ionization clearly vary with pressure, as reflected in the pK_as (see below). An estimate of the free energy change associated with the pressure-induced conformational changes, ΔG_c, is available from $p_{1/2} \times \Delta V_c$. At $p_{1/2}$, $\Delta G_{ion} = 0$, and only the conformational change contributes to ΔG (i.e., $\Delta G = \Delta G_c$.) The recovered ΔG_c values are also summarized in Table 1. As expected for a process that occurs at low pressure, the recovered values emphasize that the conformational change linked to ionization does not require a large change in free energy (<0.5 kcal/mol), but it does occur with a substantial change in system volume. It should also be noted that the ΔG_c values are for the conformational change of the protonated Tyr 9. Because each of the Tyr 9 pK_as of the mutant proteins is decreased relative to the wild type (F220), the free energy change for the analogous conformational change in the ionized tyrosinate, $\Delta G_{c,anion}$, will be larger in magnitude, by an amount determined by the difference in pK_a at atmospheric pressure for the mutants and wild-type proteins. The scheme demonstrates that the conformations in which Tyr 9 is unprotonated are more pressure sensitive than those in which Tyr 9 is protonated.

As in all cases, the thermodynamic experiments presented here would be most informative if they could be correlated to structural models. The spectroscopic data obtained at high pressure do not reveal specific structural changes induced by pressure, other than the ionization of the Tyr 9 hydroxyl group. However, the thermodynamic results are interpreted readily in light of the X-ray structures available for the alpha-class GSTs (Sinning et al., 1993; Cameron et al., 1995). Comparison of X-ray structures of human GST A1-1 in the presence and absence of ligands indicates that addition of GSH conjugates, or hydrophobic electrophiles, has negligible effect on the majority of the protein. However, a difference is observed for the C-terminal 15 residues of each subunit. In the presence of GSH conjugates, or the electrophile ethacrynic acid, these residues form a short α-helix, which forms part of the GSH binding site (Fig. 1; Sinning et al., 1993; Cameron et al., 1995). This helix is not found in mu- and pi-class GSTs, and it has been proposed to contribute to the substrate selectivity of the alpha-class isozymes. Notably, C-terminal truncation mutants (Board & Mannervik, 1991) of a human GST A2-2 have demonstrated the functional importance of the C-terminal helix, as observed here with proteolyzed preparations of the F220R mutant described here. A few residues in this helix make contacts with bound ligands, as well as with Tyr 9, in the human A1-1 GST. However, in the absence of ligands, the electron density is sufficiently low that no coordinates were assigned to these atoms (Cameron et al., 1995). Apparently, the helix backbone is observed crystallographically, but its location is poorly defined. This indicates that many conformational states of similar energy exist for this peptide segment, at least in the crystal environment. The data presented here indicate that some of these states differ significantly in solvation or packing, which in turn leads to large differences in system volume upon redistribution of these states.

These assertions are supported by the results with S-methyl GSH. This ligand effectively converts the "soft" Tyr 9 environment to a "hard" site, which is relatively pressure insensitive. In the presence of S-methyl glutathione, it is likely that the C-terminal helix is "ordered" and fewer conformational states are sampled. With this ligand present, pressure–volume work, in the range of pressures below 1 kbar, does not redistribute the conformational states of these C-terminal resides, as it does in the absence of ligand. Thus, the pressure experiments and the X-ray structures are complementary.

Several possible effects of pressure may explain the observed results. It is possible that the C-terminal helix is well-ordered in the ligand-free rat A1-1 GST, in contrast to the human isozyme, and that pressure causes unfolding of the helix, analogous to a local helix-coil transition. The denatured C-terminal helix would be solvated more completely, and the system volume would be decreased. This scenario is unlikely, because pressure does not disrupt secondary structure at the low pressures used here. In fact, loss of helical structure is not observed, typically, until pressure exceeds 2.0 kbar, conservatively (Gross & Jaenicke, 1994). This model, therefore, is not favored because it requires that the helix is dramatically different from the structural homologue in the human enzyme, and it requires unprecedented sensitivity of secondary structure to pressure.

Pressure could cause the highly disordered helix to "swing away" from the active site, directed into bulk solution with or without further unfolding of the helix, that is, pressure may "truncate" the helix functionally, by destroying its interactions with the remainder of the subunit. By eliminating interactions between the helix and the remainder of the protein, the helix, or the resulting coil, and the "uncovered" active site would both be more completely solvated, and thus the system would occupy a smaller volume. However, based on the results with the proteolyzed F220R mutant, it is unlikely that Tyr 9 would maintain an unusually low pK_a if pressure had these effects on the C-terminal helix. Moreover, the changes in pK_a of Tyr 9 caused by substitutions at Phe 220 indicate that, even in the ligand-free enzyme at atmospheric pressure, the C-terminal helix contributes to the environment of Tyr 9. Pressure apparently changes the nature of the interactions between the C-terminal helix and Tyr 9, but it does not eliminate them.

A third possible source of pressure-induced change in the C-terminal helix arises from relief of solvent-excluded void volume, due to inefficient packing of the helix with the Tyr 9 environment. Apparently, the helix samples many conformations that are nearly isoenergetic, but that differ in system volume, until ligands are bound. Importantly, the pK_a of the catalytic Tyr 9 differs for these conformations. It is interesting to speculate that the C-terminal helix is intact, but dynamic, and inefficiently packed against Tyr 9 in the ligand-free protein. This "molten globule" character of the helix would present a relatively large excluded volume that is compressible, relative to a helix that is more closely packed against the remainder of the subunit including Tyr 9 (Nolting & Sligar, 1993; Frye et al., 1996). Upon compression, the helix may become less dynamic, and pack more efficiently with Tyr 9.

The mutant proteins provide support for the suggestion that the aromatic F220 side chain is important for controlling the position of the C-terminal helix (Cameron et al., 1995). Substitution with aliphatic side chains at this position leads to a decrease in $p_{1/2}$, which suggests that less pressure–volume work is required to induce a change in packing or solvation, relative to the free energy required for the wild-type, F220-containing, helix. Although the calculated ΔG_c values suggest that the differences in free energy between mutants are small, inspection of the data in Figures 3 and 5 indicates that the differences in $p_{1/2}$ values are beyond the experimental error of the measurements. The F220I and F220L substitutions make the C-terminal helix more "sensitive" to pressure. In contrast, the $p_{1/2}$ for the F220Y mutant is higher than each of the other proteins, including the F220 wild type. Apparently, the helix is more stable to pressure, albeit modestly, upon incorporation of the phenolic hydroxyl group. It is interesting to speculate that an additional hydrogen bond has been introduced between the F220Y

phenolic hydroxyl group and another protein group, such as the aromatic ring of Tyr 9 (Dietze et al., 1996a).

Materials and methods

Mutant construction and enzyme purification

The construction of W21F and W21F:Y9F mutant rat A1-1 GSTs, and purification of the expressed proteins have been described previously (Wang et al., 1991; Dietze et al., 1996a). Mutations at Phe 220 were obtained by PCR-based amplification of duplex DNA encoding the C-terminal portion of the rat A1-1 GST gene. The previously described construct pGTB was used as template (Wang et al., 1991). Amplification primers spanned the *Bgl* II or *Sal* I restriction sites within the gene. The oligonucleotide complementary to the *Sal* I site also encoded the desired mutation at position 220. The amplified products were digested with *Bgl* II and *Sal* I and ligated into pGTB digested with the same restriction enzymes. PCR reactions contained in 100 μL total volume 100 pmol of each primer, 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 linearized template, and 1 unit of Vent polymerase (New England Biolabs). Twenty-five amplification cycles were performed, where each cycle was 94 °C for 2 min, 65 °C for 1 min, and 94 °C for 2 min.

High-pressure fluorescence

High-pressure fluorescence experiments were performed with an SLM-Aminco 8100C fluorimeter interfaced with an SLM-Aminco high-pressure spectroscopy cell, HPSC3K, which is essentially identical to the system described by Paladini (1986). Teflon O-rings (Action Grinding, Seattle, Washington) were used instead of the lead O-rings provided by the manufacturer of the HPSCK3 cell. Samples were maintained at constant temperature, as indicated in Results, with a circulating water bath. For experiments in which the ionization of Tyr 9 was monitored with changing pressure, protein samples were 43.5 μM subunits in 20 mM Tris/bis-Tris at pH 7.6, 19 °C. N-acetyl tyrosine samples were 43 μM in the same buffer. Corrected excitation spectra were used to monitor extent of ionization of Tyr 9. The emission wavelength was 348 nm, and the excitation wavelength was scanned from 245 nm to 330 nm. Extent of Tyr 9 ionization was determined from the ratio of fluorescence emission intensities at 348 nm, for excitation wavelengths 280 nm and intensity at 256 nm (I_{256}/I_{280}), as described previously (Atkins et al., 1993; Dietze et al., 1996). Percent ionization of Tyr 9 at each pressure was calculated as

$$[1 - [(I_{256}/I_{280})_{9.5} - (I_{256}/I_{280})_p]/[(I_{256}/I_{280})_{9.5} - (I_{256}/I_{280})_4]] \times 100,$$

where the subscripts 4 and 9.5 refer to pH values at which spectral ratios for fully protonated and fully deprotonated Tyr 9 were obtained, respectively. For each mutant protein, the spectral ratios were determined at the limiting pHs to account for any differences in quantum yield accompanying mutation. All emission and excitation spectra were the average of three emission scans. Values of ΔV were calculated from the rearranged form of Equation 1, $d(pK_a)/dp = \Delta V/(RT2.303)$.

Effects of pressure on Trp 21 were determined from emission spectra of the wild-type protein in the same buffer, with an excitation wavelength of 295 nm. Emission spectra were corrected for "buffer blanks" at each pressure, although pressure had no detectable effect on the observed spectra of "buffer blanks." All slit widths were 8 nm, and all spectra reported or used for analysis were the average of three scans.

The effect of pressure on enzymatic activity was determined by incubating samples of W21F GST at 43 μM subunits at 19 °C, either at atmospheric pressure or 1.0 kbar, for 15 min, in the same Tris/bis-Tris buffer used for spectroscopic experiments. The samples were decompressed rapidly, samples were diluted to 0.1 μM subunits in "standard assay" mix, and the specific activity was determined as described previously (Kubo & Armstrong, 1989).

For experiments involving temperature-dependent ionization of Tyr 9, samples were 43.5 μM GST subunits in 20 mM potassium phosphate buffer, pH 7.6. It was demonstrated experimentally that the pH of the solutions varied by 0.23 pH units over the range −5 °C to 35 °C. Values for ΔH of N-acetyl tyrosine were determined from the slope of the line fit to $d(pK_a)/d(1/T) = \Delta H/(2.303R)$.

Electrospray ionization mass spectrometry

Protein samples were dialyzed against 50 mM NH_4OAc, pH 7.0, and diluted 1:1 with 50% methanol, 49.5% H_2O, 0.5% acetic acid before analysis. LC-MS was performed with a VG 70SEQ Micromass Quatro II Triple Quadrupole mass spectrometer, with a mobile phase of 50% acetonitrile:50% H_2O, at 40 μL/min.

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