Role of Active Site Tyrosine in Glutathione S-Transferase: Insights from a Theoretical Study on Model Systems

Ya-Jun Zheng[†] and Rick L. Ornstein*

Contribution from the Environmental Molecular Sciences Laboratory, Pacific Northwest National Laboratory, Richland, Washington 99352

Received May 17, 1996[⊗]

Abstract: *Ab initio* molecular orbital theory was used to examine the role of active site tyrosine in glutathione S-transferases by using appropriate model systems. The location of the key mechanistic proton of the enzyme−glutathione binary complex, O- - -H- - -S, was predicted to be near the phenolic oxygen, which is in agreement with experiments. However, the position of the proton can be manipulated by changing the acidity of the tyrosine, which can be accomplished by either introducing a substituent group to the tyrosine phenol ring or changing the protein environment. Thus, our study seems to have resolved previous confusion as to where the proton is located. The hydrogen bonding between tyrosine and thiolate of glutathione is very strong. On the basis of our present study, we propose that, in the Y6F (Tyr → Phe) mutant, a water molecule replaces the function of the hydroxyl group of the active site tyrosine of the wild-type enzyme. Several lines of evidence in support of the above hypothesis are discussed. The latter hypothesis is reminiscent of the notion of substrate-assisted catalysis.

Introduction

Glutathione S-transferases (GSTs) are a class of detoxifying enzymes that catalyze the conjugation of a tripeptide glutathione (γ-Glu-Cys-Gly, GSH, or HSG) to a wide variety of chemical compounds that have electrophilic groups, compounds including alkylhalides, arylhalides, lactones, epoxides, quinones, esters, and some activated alkenes.[1−6] Tetrachloro-*p*-hydroquinone reductive dehalogenase (TeCH-RD), an enzyme involved in the biodegradation pathway of pentachlorophenol by flavobacteria, is also a glutathione S-transferase.[7] On the basis of sequence similarity, GSTs can be grouped into five gene classes: α, μ, π, σ, and θ. All known cytosolic isoenzymes of GSTs are dimers with a combined molecular weight of about 50 kD; the two subunits can be either identical or nonidentical.

A number of three-dimensional GST crystal structures have been solved, including complexes with substrate (GSH), substrate analogues, or products.[8−16] These crystal structures provide a detailed picture of the active site environment. A conserved active site tyrosine residue has been implicated in catalysis. In solution, the pK_a of GSH is about 9.0,[17] whereas in the enzyme (M1-1, class μ) complex, the pK_a of the thiol group of GSH is between 6.2 and 6.7.[18] The tyrosine residue is believed to stabilize the thiolate of GSH by hydrogen bonding (TyrOH- - -$^-$SG). However, the question remains as to where the proton is actually located. In principle, the location of this important proton could be as follows: TyrOH- - -$^-$SG (**1**), TyrO$^-$- - -HSG (**2**), or (TyrO- - -H- - -SG)$^-$ (**3**). Armstrong and co-workers proposed that the proton is located near the oxygen (**1**),[1,18] while others seem to believe that the proton is near the sulfur atom of the glutathione (**2**).[19−21] The pK_a of Tyr 9 in rat α1-1 GST was measured using spectroscopic techniques to be in the range 8.3−8.5, which is about 1.8−2.0 pK_a units lower than tyrosine in aqueous solution.[19] Studies of covalent

[†] Current address: Department of Chemistry, University of California, Santa Barbara, CA 93106.

* Corresponding author. FAX: (509) 375-6904. E-mail: rl_ornstein@pnl.gov.

[⊗] Abstract published in *Advance ACS Abstracts,* February 1, 1997.

(1) Armstrong, R. N. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1994**, *69*, 1−44.

(2) Dirr, H.; Reinemer, P.; Huber, R. *Eur. J. Biochem.* **1994**, *220*, 645−661.

(3) Wilce, M. C. J.; Parker, M. W. *Biochim. Biophys. Acta* **1994**, *1205*, 1−18.

(4) Rushmore, T. H.; Pickett, C. B. *J. Biol. Chem.* **1993**, *268*, 11475−11478.

(5) Jakoby, W. B.; Ziegler, D. M. *J. Biol. Chem.* **1990**, *265*, 20715−20718.

(6) Mannervik, B.; Danielson, U. H. *CRC Crit. Rev. Biochem.* **1988**, *23*, 283−337.

(7) Orser, C. S.; Dutton, J.; Lange, C.; Jablonski, P.; Xun, L.; Hargis, M. *J. Bacteriol.* **1993**, *175*, 2640−2644.

(8) Reinemer, P.; Dirr, H. W.; Ladenstein, R.; Schaffer, J.; Gallay, O.; Huber, R. *EMBO J.* **1991**, *10*, 1997−2005.

(9) (a) Ji, X.; Zhang, P.; Armstrong, R. N.; Gilliland, G. L. *Biochemistry* **1992**, *31*, 10169−10184. (b) Xiao, G.; Liu, S.; Ji, X.; Johnson, W. W.; Chen, J.; Parsons, J. F.; Stevens, W. J.; Gilliland, G. L.; Armstrong, R. N. *Biochemistry* **1996**, *35*, 4753−4765.

(10) Ji, X.; Armstrong, R. N.; Gilliland, G. L. *Biochemistry* **1993,** *32*, 12949−12954.

(11) Sinning, I.; Gilliland, G. L.; Armstrong, R. N.; Ji, X.; Board, P. G.; Olin, B.; Mannervik, B.; Jones, T. A. *J. Mol. Biol.* **1993**, *232*, 192−212.

(12) (a) Ji, X.; von Rosenvinge, E. C.; Johnson, W. W.; Tomarey, S. I.; Piatigorsky, J.; Armstrong, R. N.; Gilliland, G. L. *Biochemistry* **1995**, *34*, 5317−5328. (b) Lim, K.; Ho, J. X.; Keeling, K.; Gilliland, G. L.; Ji, X.; Ruker, F.; Carter, D. C. *Protein Sci.* **1994**, *3*, 2233−2244. (c) McTigue, M.; Williams, D. R.; Tainer, J. A. *J. Mol. Biol.* **1995**, *246*, 21−27. (d) Wilce, M. C. J.; Board, P. G.; Feil, S. C.; Parker, M. W. *EMBO J.* **1995**, *14*, 2133−2143. (e) Ji, X.; von Rosenvinge, E. C.; Johnson, W. W.; Armstrong, R. N.; Gilliland, G. L. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 8208−8213.

(13) Garcia-Saez, I.; Parraga, A.; Phillips, M. F.; Mantle, T. J.; Coll, M. *J. Mol. Biol.* **1994**, *237*, 298−314.

(14) Dirr, H. W.; Reinemer, P.; Huber, R. *J. Mol. Biol.* **1994**, *243*, 72−92.

(15) Raghunathan, S.; Chandross, R. J.; Kretsinger, R. H.; Allison, T. J.; Penington, C. J.; Rule, G. S. *J. Mol. Biol.* **1994**, *238*, 815−832.

(16) Reinemer, P.; Prade, L.; Hof, P.; Neuefeind, T.; Huber, R.; Zettl, R.; Palme, K.; Schell, J.; Koelln, I.; Bartunik, H. D.; Bieseler, B. *J. Mol. Biol.* **1996**, *255*, 289−309.

(17) Novak, M.; Lin, J. *J. Am. Chem. Soc.* **1996**, *118*, 1302−1308. Rabenstein, D. L. *J. Am. Chem. Soc.* **1973**, *95*, 2797−2803. Cheesman, B. V.; Arnold, A. P.; Rabenstein, D. L. *J. Am. Chem. Soc.* **1988**, *110*, 6359−6364.

(18) Liu, S.; Zhang, P.; Ji, X.; Johnson, W. W.; Gilliland, G. L.; Armstrong, R. N. *J. Biol. Chem.* **1992**, *267*, 4296−4299.

(19) Atkins, W. M.; Wang, R. W.; Bird, A. W.; Newton, D. J.; Lu, A. Y. H. *J. Biol. Chem.* **1993**, *268*, 19188−19191.

(20) Karshikoff, A.; Reinemer, P.; Huber, R.; Ladenstein, R. *Eur. J. Biochem.* **1993**, *215*, 663−670.

(21) Meyer, D. J.; Xia, C.; Coles, B.; Chen, H.; Reinemer, P.; Huber, R.; Ketterer, B. *Biochem. J.* **1993**, *293*, 351−356.

S0002-7863(96)01667-8 CCC: $14.00

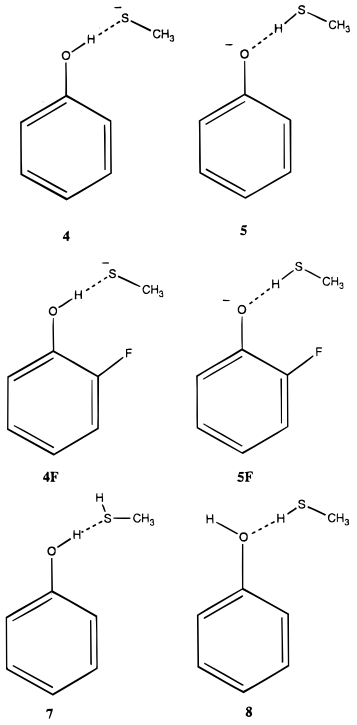
modification of human π1-1 GST by diethyl pyrocarbonate[21] and electrostatic potential analysis of the active site of rat α1-1 GST [20] also indicate a lower pK_a for the active site tyrosine. On the basis of the latter observations, an alternative role for this tyrosine was proposed; under physiological pH, this residue could become a tyrosinate and function as a general base to abstract a proton from the thiol group of GSH. However, in the former case (**1**), the tyrosine acts like a surrogate solvent molecule (e.g., water). Whether or not a "low barrier hydrogen bond" [22−26] plays a role in the GST-catalyzed reaction is not clear. If the proton is centrally located between the oxygen of the tyrosyl residue of GST and the sulfur of the cysteinyl residue of GSH as in **3**, this would be consistent with involvement of a single minimum hydrogen bond in the enzyme−GSH complex.

In the present study, high-level *ab initio* molecular orbital methods are used to investigate the proton location in the deprotonated glutathione−GST binary complex and the strength of the hydrogen bond formed between the active site tyrosine residue and the thiol group of glutathione.

Theoretical Method

All calculations reported here were carried out using either Gaussian 92[27] or Gaussian 94[28] programs. Geometries were fully optimized without any geometrical constraints. In our *ab initio* molecular orbital study, model compounds were used to mimic active site residues; the active site tyrosine is modeled using phenol, and the cysteine side chain of glutathione was modeled by CH_3SH. Chart 1 lists all of the model compounds used in our study. To find the location of the intervening proton, *ab initio* molecular orbital calculations were performed on PhOH- - -$^-$SCH$_3$ (**4**), PhO$^-$- - -HSCH$_3$ (**5**), and the interconversion barrier between them was located. First, structures **4** and **5** are energy minimized and the transition state for the interconversion between them (**6**) is located at several levels of theory (RHF/6-31G*, RHF/6-311+G**, and MP2/6-31G*). Subsequent single-point energies were then calculated at higher levels of theory (MP2/6-31+G*//6-311+G** and MP4/6-31+G*//6-311+G**). We also did calculations at the MP2/6-311+G** level of theory. However, since it gives essentially the same results as the MP2/6-31+G* level of theory, the results from the MP2/6-311+G** level are not reported. Electron correlation effects were included by means of Møller-Plesset perturbation theory up to the fourth order.[29] Only valence shell orbitals were included in the correlation calculations. Diffuse functions were also included in the basis set, since it is well-known that diffuse functions are important for describing the electronic structure of anions.[30] Previous work has shown that the MP2/6-31+G* level of theory is capable of reproducing gas phase experimental interaction enthalpies and free energies between water and thiolate and between water and methoxide.[31] Calculations were also carried out to examine the strength of these hydrogen bonds. The neutral complexes between PhOH and HSCH$_3$ (**7** and **8**) are also investigated.

Chart 1



The interaction free energy was evaluated using enthalpies and entropies determined from RHF/6-31G* harmonic vibrational frequencies. Enthalpies were determined using the following formula:

$$\Delta H_{\mathrm{cal}}{}^{298} = \Delta E_{\mathrm{e}}{}^{0} + \Delta E_{\mathrm{v}}{}^{0} + \Delta(\Delta E_{\mathrm{v}})^{298} + \Delta E_{\mathrm{r}}{}^{298} + \Delta E_{\mathrm{t}}{}^{298} + \Delta PV$$

where the first term is the computed hydrogen bonding energy including correlation, while the second term is the change in zero-point vibrational energy. The third term is the change in vibrational energy on going from 0 to 298 K. The fourth, fifth, and the sixth terms are due to changes in rotation and translation energies,and pressure volume work, respectively. Entropies were evaluated using a standard statistical mechanical approach. All terms except the last one can be calculated with Gaussian 92 and Gaussian 94 programs. The last term in the present case is $-RT$ ($-$0.59 kcal/mol at 298 K).

Results and Discussions

Table 1 lists the calculated electronic energies of each species at the various levels of theory. In the section that follows, the presentation of the results will be organized into two parts: locating the proton and determining the strength of the hydrogen bond.

Location of Proton in the Complex. First, we sought the location of the proton in the complex formed between tyrosine and GSH. The geometries of **4** and **5** are fully optimized at RHF/6-31G* and RHF/6-311+G** levels of theory; vibrational analysis demonstrate that both are minima on the potential energy hypersurface at the Hartree−Fock level. The interconversion transition state (**6**) between **4** and **5** was also located at both RHF/6-31G* and RHF/6-311+G** levels and characterized using vibrational analysis. The calculated structures for **4**−**6** at the RHF/6-311+G** level of theory are shown in Figure 1. Calculated geometrical parameters are summarized in Table

(22) Shan, S.; Loh, S.; Herschlag, D. *Science* **1996**, *272*, 97−101.
(23) Cleland, W. W. *Biochemistry* **1992**, *31*, 317−319.
(24) Cleland, W. W.; Kreevoy, M. M. *Science* **1994**, *264*, 1887−1890.
(25) Gerlt, J. A.; Gassman, P. G. *Biochemistry* **1993**, *32*, 11943−11956. Gerlt, J. A.; Gassman, P. G. *J. Am. Chem. Soc*. **1993**, *115*, 11552−11568.
(26) Frey, P. A.; Whitt, S. A.; Tobin, J. B. *Science* **1994**, *264*, 1927−1930.
(27) Gaussian 92: Frisch; M. J.; Trucks, G. W.; Head-Gordon, M.; Gill, P. M. W.; Wong, M. W.; Foresman, J. B.; Johnson, B. G.; Schlegel, H. B.; Robb, M. A.; Replogle, E. S.; Gomperts, R.; Andres, J. L.; Raghavachari, K.; Binkley, J. S.; Gonzalez, C.; Martin, R. L.; Fox, D. J.; Defrees, D. J.; Baker, J.; Steward, J. J. P.; Pople, J. A., GAUSSIAN, Inc.: Pittsburgh, PA, 1993.
(28) Gaussian 94, Revision B.2: Frisch, M. J.; Trucks, G. W.; Schlegel, H. B.; Gill, P. M. W.; Johnson, B. G.; Robb, M. A.; Cheeseman, J. R.; Keith, T.; Petersson, G. A.; Montgomery, J. A.; Raghavachari, K.; Al-Laham, M. A.; Zakrzewski, V. G.; Ortiz, J. V.; Foresman, J. B.; Cioslowski, J.; Stefanov, B. B.; Nanayakkara, A.; Challacombe, M.; Peng, C. Y.; Ayala, P. Y.; Chen, W.; Wong, M. W.; Andres, J. L.; Replogle, E. S.; Gomperts, R.; Martin, R. L. Fox, D. J.; Binkley, J. S.; Defrees, D. J.; Baker, J.; Stewart, J. P.; Head-Gordon, M.; Gonzalez, C.; Pople, J. A. Gaussian, Inc.: Pittsburgh, PA, 1995.
(29) Møller, C.; Plesset, M. S. *Phys. Rev.* **1934**, *46*, 618−622. Binkley, J. S.; Pople, J. A. *Int. J. Quantum Chem.* **1975**, *9*, 229−236. Pople, J. A.; Binkley, J. S.; Seeger, R. *Int. J. Quantum Chem.* **1976**, *10*, 1−19.
(30) Chandrasekhar, J.; Andrade, J. G.; Schleyer, P. v. R. *J. Am. Chem. Soc*. **1981**, *103*, 5609−5612, 5612−5614.
(31) Zheng, Y.-J.; Merz, K. M., Jr. *J. Comput. Chem*. **1992**, *13*, 1151−1169.

Table 1. Calculated Electronic Energies (in hartrees, 1 hartree = 627.5 kcal/mol) for Each Species Involved in the Present Study

compd	RHF/6-31G*// RHF/6-31G*	RHF/6-311+G**// RHF/6-311+G**	MP2/6-31G*// MP2/6-31G*	MP2/6-31+G*// RHF/6-311+G**	MP4/6-31+G*// RHF/6-311+G**
$HSCH_3$	−437.700 319 7	−437.741 148 7	−437.952 668 4		
$^-SCH_3$	−437.114 863 4	−437.161 348 4	−437.365 953 9	−437.384 471 4	−437.412 156 3
PhOH	−305.558 064 2	−305.639 895 2	−306.490 992 9	−306.508 808 8	−306.542 841 1
PhO^-	−304.968 948 4	−305.060 051 5	−305.911 427 3		
4	−742.706 324 6	−742.830 974 4	−743.903 907 4	−743.935 610 5	−743.995 098
5	−742.687 576 3	−742.816 577 3		−743.929 458 4	−743.988 707
6	−742.683 627 6	−742.810 688 3		−743.931 445 5	−743.987 263 5
4F		−841.710 181 1		−842.962 436 1	
5F		−841.700 772 8		−842.961 211 4	
6F		−841.692 517 1		−842.960 896 9	
7	−743.263 826 2		−744.453 969 0		
8	−743.260 792 2		−744.450 584 8		

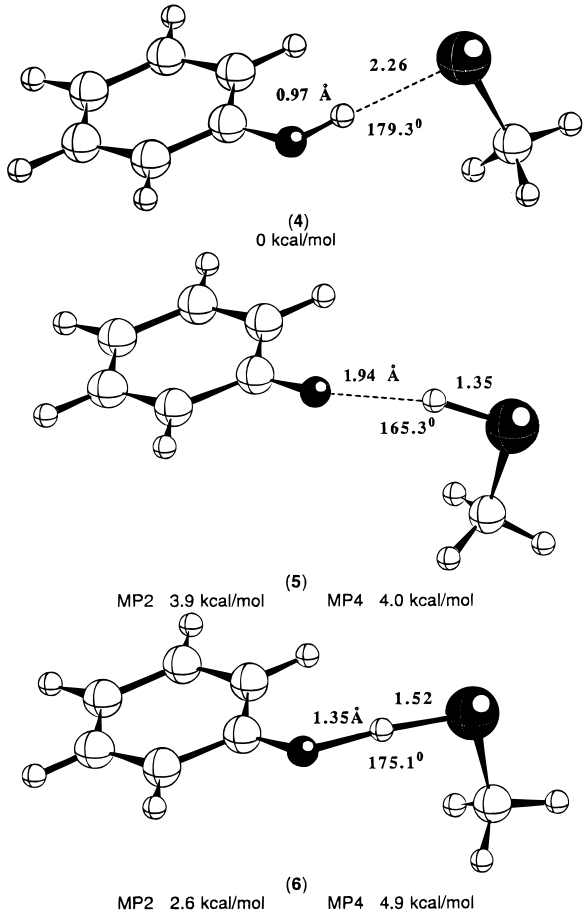


Figure 1. Calculated geometries for **4**−**6** at the RHF/6-311+G** level of theory and relative energies at MP2 and MP4/6-31+G*//RHF/6-311+G** levels.

2. The calculated hydrogen bonding angles at RHF/6-31G* and RHF/6-311+G** levels of theory are very similar, differences being less than 2°. The differences in calculated *r*(O- - -H), *r*(S- - -H), and *r*(O- - -S) distances at both levels of theory are generally less than 0.02 Å except for **5** where the calculated *r*(O- - -H) and *r*(O- - -S) distances differ by 0.09 Å, indicating that the geometry of **5** is sensitive to the level of theory. The calculated distances between the sulfur atom and the phenolic oxygen are consistent with the X-ray crystallographic value for the enzyme−GSH complex (3.23 Å from the 2.2 Å resolution crystal structure).[9]

Complex **4** is predicted to be lower in energy than **5** by 11.8 and 12.7 kcal/mol at the RHF/6-31G* and RHF/6-311+G** levels of theory, respectively. There is a small barrier for the conversion of **5** to **4**; the calculated barrier is 2.5 kcal/mol at the RHF/6-31G* level of theory and 3.7 kcal/mol at the RHF/6-311+G** level. Clearly, both 6-31G* and 6-311+G** give similar results. Even though the geometries may be relatively insensitive to the level of theory and calculated geometries at the HF level are generally very reasonable, electron correlation is very important in order to get reliable energies. We, therefore, carried out single-point energy calculations at MP2/6-31+G* and MP4/6-31+G* levels using the geometries at the 6-311+G** level of theory. Again, at the correlated levels (MP2 and MP4), complex **4** is predicted to be lower in energy than **5**, but the differences are much smaller than those at the Hartree−Fock levels. The calculated energy differences between **4** and **5** are −3.9 and −4.0 kcal/mol at MP2 and MP4 levels, respectively. The interconversion barrier seems to have disappeared at the MP2 level, but at the MP4 level, there is a smaller reverse barrier (Table 2). We also performed geometry optimization at the MP2/6-31G* level; **4** is the only minimum on the potential energy hypersurface at the MP2/6-31G* level. Taken together, the proton is predicted to be located near the phenolic oxygen atom, which is in agreement with previous work by Armstrong and co-workers.[1,18]

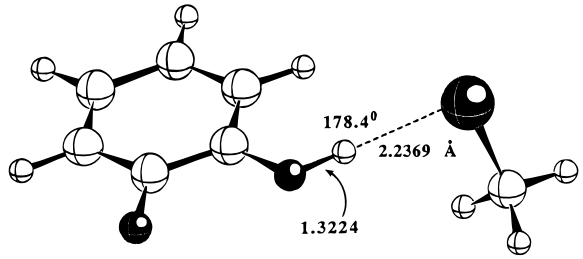
In a recent mutagenesis study, all 14 tyrosyl residues in the M1-1 isoenzyme of GST were replaced by 3-fluorotyrosine and the catalytic activity, UV−visible difference spectra, and solvent deuterium isotope effects were investigated.[32] The introduction of a fluorine atom at the 3-position of the phenolic ring of tyrosine causes a change in the proton location; the spectroscopy results clearly ruled out complex **1**, but both **2** and **3** are consistent with the spectroscopy observation. However, the solvent deuterium isotope results seem to favor **2**. In the GSH bound tetradeca(3-fluorotyrosyl) mutant, the proton now seems to be located near the sulfur atom of the cysteinyl residue of glutathione. Therefore, it would be very interesting to see if theory leads to a consistent result and can provide any new insight. To elucidate the effect of the fluorine atom on the position of the proton, we performed *ab initio* molecular orbital calculations on the complexes formed between 2-fluorophenol (due to the difference in the numbering system between tyrosine and phenol, the 3-fluoro-4-hydroxylphenyl ring of tyrosine is called 2-fluorophenol) and CH_3S^-. The starting geometries are built from the optimized geometries of **4**−**6** at the RHF/6-311+G** level of theory by replacing one of the *ortho* hydrogen atoms with a fluorine atom; geometries were then fully optimized at the RHF/6-311+G** level (Figure 2). Single-point energy calculations were then done at the MP2/6-31+G* level of theory using the RHF/6-311+G** geometries. The calculated energy difference between **4F** and **5F** at the RHF/6-311+G** level is much smaller than that between **4** and **5** (5.9 vs 12.7 kcal/mol). However, at the MP2/6-31+G* level, the energy of these two complexes becomes essentially the same

(32) Parsons, J. F.; Armstrong, R. N. *J. Am. Chem. Soc.* **1996**, *118*, 2295−2296.

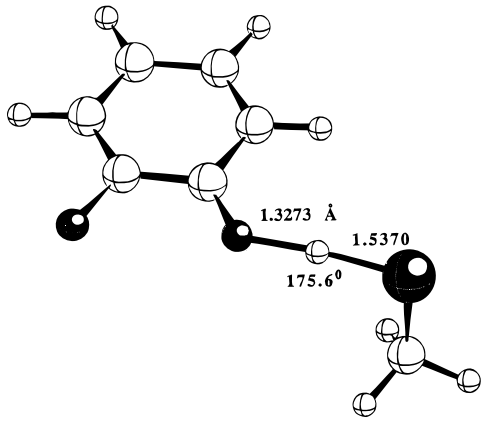
Table 2. Calculated Relative Energies (kcal/mol) and Some Geometrical Parameters (Bond Lengths in Å and Angles in degrees) for **4**−**6**

compd	*r*(O- - -H)	*r*(S- - -H)	*r*(O- - -S)	*θ*(O−H−S)	MP2/6-31+G*	MP4/6-31+G*
4	0.9766[a]	2.2740[a]	3.2506[a]	180.0[a]		
	0.9709[b]	2.2615[b]	3.2323[b]	179.3[b]	0[c]	0[c]
5	1.8520[a]	1.3518[a]	3.1834[a]	166.9[a]		
	1.9445[b]	1.3483[b]	3.2665[b]	165.3[b]	3.9[c]	4.0[c]
6	1.3745[a]	1.5115[a]	2.8821[a]	174.1[a]		
	1.3500[b]	1.5223[b]	2.8697[b]	175.1[b]	2.6[c]	4.9[c]

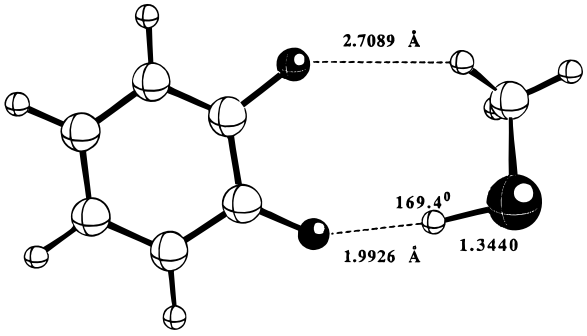
[a] 6-31G* level of theory. [b] 6-311+G** level of theory. [c] Single-point energies using the 6-311+G** geometries.



(**4F**, 0 kcal/mol)



(**6F**, 0.97 kcal/mol)

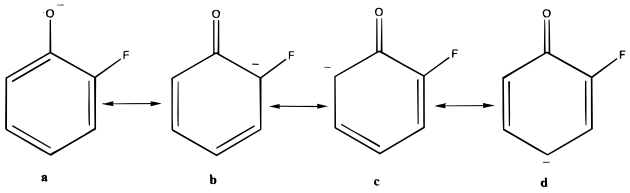


(**5F**, 0.77 kcal/mol)

Figure 2. Calculated geometries for **4F**−**6F** at the RHF/6-31G* level of theory and relative energies at the MP2/6-31+G*//RHF/6-311+G** level.

(the difference in energy being less than 0.8 kcal/mol), indicating that the proton is now probably located between the phenolate and thiolate as would be expected for a low barrier hydrogen bond (or a single minimum hydrogen bond). The reverse barrier (from **5F** to **4F**) is only about 0.2 kcal/mol. This finding is consistent with the fact that 2-fluorophenol is more acidic than phenol. As shown in Figure 2, the structures for **4F** and **5F** are very different, especially the orientation of the thiolate moiety. One may wonder whether this is possible in the enzyme active site. However, it should be pointed out that the energy cost is less than 0.5 kcal/mol for reorienting the thiolate in **5F**, to a similar orientation as in **4F**. As a result, it should not seriously affect the calculated energy difference. In addition,

Scheme 1. Resonance Structures of 2-Fluorophenolate



since the X-ray crystal structure for the fluorotyrosyl mutant is not available, it is unknown what the structure looks like in the active site. Scheme 1 shows the resonance structure for 2-fluorophenolate. Because of its high electronegativity, due to inductive effects, the fluorine atom makes 2-fluorophenolate more stable than phenolate. The introduction of a fluorine atom at the *ortho* position reduces the pK_a of tyrosine by 1.2 pK_a units.[33] The *ab initio* molecular orbital calculations regarding the effect of the fluorine atom on the location of the proton are consistent with the recent mutagenesis study.[32]

Whether the observed differences in UV−visible spectra is solely due to the increased acidity of the tyrosine residue or other steric/electronic factors is not clear; especially, since all 14 tyrosine residues are replaced by 3-fluorotyrosine, the protein may suffer some structural perturbation. However, if acidity is the predominate factor, since 2,3,5,6-tetrafluorotyrosine has a lower pK_a (5.3 compared with 8.8 and 10.0 for 3-fluorotyrosine and tyrosine, respectively),[33] one can predict that replacing the active site tyrosine by 2,3,5,6-tetrafluorotyrosine via site-specific unnatural amino acid mutagenesis,[34] the proton should be located near the sulfur atom of glutathione in the enzyme−GSH complex as in **2**.

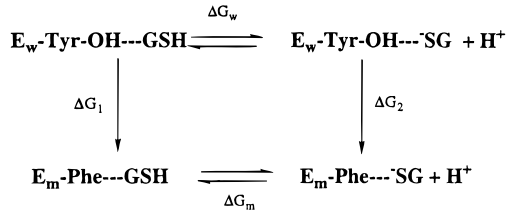
Previous experimental studies gave conflicting results regarding where the proton is located. Our study seems to provide a rationale for this phenomenon. Since the experimental studies were carried out on different subclasses of GSTs and since the pK_a of the active site Tyr is different for different GSTs, the position of the proton could also be different. As discussed before, the pK_a of the active site in α- and π-subclass GSTs is relatively low. For instance, in rat α1-1 GST, the pK_a of the active site Tyr 9 was measured to be in the range 8.3−8.5, which is about 1.8−2.0 pK_a units lower than tyrosine in aqueous solution.[19] On the other hand, the pK_a of the active site Tyr for the μ-subclass is normal (about 10). Especially, in view of the fact that introduction of a fluorine atom at the *ortho* position reduces the pK_a of tyrosine by 1.2 pK_a units, which is enough to shift the location of the proton in the hydrogen bonding complex, one may speculate that, in α- and π-subclass GSTs (and possibly other subclasses of GSTs), the proton is also shifted to the sulfur atom as in the fluorotyrosyl mutant.

Strength of the Hydrogen Bond. In aqueous solution, the thiol group of glutathione has a pK_a of 9,[17] while when it binds

(33) Thorson, J. S.; Chapman, E.; Murphy, E. C.; Schultz, P. G.; Judice, J. K. *J. Am. Chem. Soc.* **1995**, *117*, 1157−1158 and references cited therein.

(34) Noren, C. J.; Anthony-Cahill, S. J.; Griffith, M. C.; Schultz, P. G. *Science* **1989**, *244*, 182−188. Robertson, S. A.; Ellman, J. A.; Schultz, P. G. *J. Am. Chem. Soc.* **1991**, *113*, 2722−2729.

Scheme 2



to GST, the pK_a of the same thiol group is reduced to a value between 6.2 and 6.7.[1,18] More interestingly, when the active site tyrosine is replaced by phenylalanine, the pK_a shifts to a value of 7.8, which is still lower than the aqueous pK_a value of 9. It is clear that GSTs can effectively lower the pK_a of the thiol group of glutathione; the lower pK_a indicates that at physiological pH the thiol group of glutathione, in the active site of GST, is deprotonated. Although other factors such as thiolate stabilization by a helix dipole may contribute, the hydrogen bond between the active site tyrosine and the thiolate is assumed to be the leading factor. Previous *ab initio* molecular orbital calculations indicate that this hydrogen bond is very strong, about 20 kcal/mol;[35] however, the pK_a difference between the wild-type enzyme and the Y6F mutant only indicates a 2.2 kcal/mol free energy difference. Since removal of the hydroxyl of tyrosine (Y6F mutation) causes no noticeable structural change to the protein,[1,9] this free energy difference should reflect the loss of hydrogen bonding between tyrosine and thiolate. The question as to whether this discrepancy is due to the inaccuracy of the previous *ab initio* calculation or some other factors has not been addressed.

Scheme 2 shows a thermodynamic cycle of the titration experiment. ΔG_W and ΔG_m refer to the free energy change associated with the ionization of the thiol moiety of glutathione in the binary complex for the wild-type enzyme and the mutant, respectively. Since in the Y6F mutant phenylalanine cannot form a hydrogen bond with GSH, ΔG_1 refers to the loss of a hydrogen bond in the neutral complex (the left-hand side of the equation), while ΔG_2 refers to the loss of a hydrogen bond in the deprotonated complex (the right-hand side of the equation). Since free energy is a state function, the sum of ΔG_W and ΔG_2 is equal to the sum of ΔG_m and ΔG_1, or $\Delta G_W - \Delta G_m = \Delta G_1 - \Delta G_2$. Therefore, $\Delta G_1 - \Delta G_2$ is equal to $-2.3RT\Delta pK_a$, which is about -2.2 kcal/mol. In the present study, ΔG_1 and ΔG_2 are modeled by the hydrogen bonding free energies in PhOH- - -HSCH$_3$ and PhOH- - -$^-$SCH$_3$, respectively.

For PhOH- - -HSCH$_3$ there are two possible hydrogen-bonded complexes (**7** and **8**), depending on which is the hydrogen bonding acceptor. *Ab initio* molecular orbital calculations at both RHF/6-31G* and MP2/6-31G* levels predict that **7** is lower in energy than **8**. The hydrogen bond is calculated to be -6.5 kcal/mol in **7** at the MP2/6-31G* level. Figure 3 shows the optimized structures for **7** and **8** at the RHF/6-31G* level. As expected, the S- - -O distances are much longer in **7** (3.54 Å) and **8** (3.87 Å) than that in PhOH- - -$^-$SCH$_3$ (**4** in Figure 1); as noted above, the S- - -O distance in **4** (Table 2) is in excellent agreement with the X-ray crystal structural evidence. To calculate the interaction free energy, we followed a standard procedure using the vibrational frequencies calculated at the RHF/6-31G* level of theory. The calculated interaction enthalpy and free energy for **7** are -3.9 and 3.2 kcal/mol, respectively. For PhOH- - -$^-$SCH$_3$, the calculated interaction enthalpy and interaction free energy are -25.2 kcal/mol (-26.6) and -17.3 kcal/mol (-15.9) at the MP4/6-31+G* (MP2/6-31+G*) level, respectively. Therefore, the calculated value for $\Delta G_1 - \Delta G_2$ is -20.5 kcal/mol.

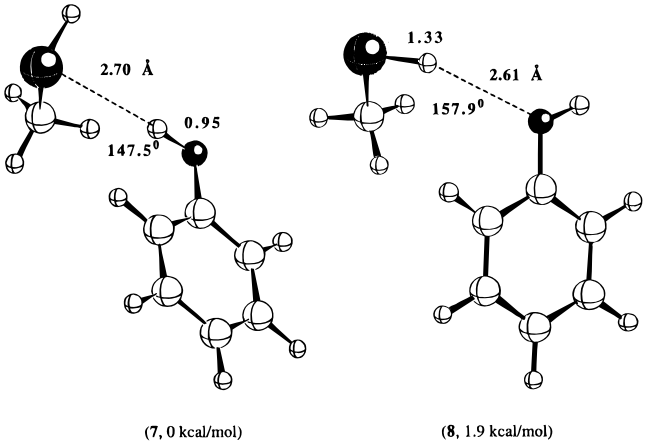


Figure 3. Calculated geometries and relative energies for **7** and **8** at the RHF/6-31G* level of theory.

Why is there such a large discrepancy between the experimental and calculated values? One possible factor is the inaccuracy of the theoretical value, especially the error associated with entropy calculations. However, this disparity is probably too large to be attributed to the inaccuracy of the theoretical value alone. Another possible explanation is that a water molecule in the active site of the mutant protein participates in a hydrogen bond with the thiolate. The X-ray crystallographic structure does indeed show that there are crystallographic waters near the thiol sulfur atom of glutathione. It is conceivable that when the thiol is deprotonated, a crystallographic water moves closer to sulfur to form a stronger hydrogen bond. If this is the case, the term ΔG_2 would represent the free energy difference in hydrogen bonding strength between PhOH- - -$^-$SCH$_3$ (wild-type enzyme) and HOH- - -$^-$SCH$_3$ (mutant enzyme). The calculated interaction enthalpy and free energy for HOH- - -$^-$SCH$_3$ are -13.0 and -8.0 kcal/mol at the MP2(full)/6-31+G* level;[31] the experimental interaction enthalpy and free energy are -14.2 and -8.6 kcal/mol.[36] Now, the calculated ΔG_2 is 8.7 kcal/mol. In the above calculation of entropy, the two hydrogen-bonding partners are treated as independent molecules; although this is reasonable for normal simple hydrogen-bonded complexes formed between two isolated molecules in the gas phase (or in solution), however, this may not be appropriate for hydrogen bond formation within an enzyme−substrate complex. Since in the enzyme−substrate complex the freedom of each hydrogen bonding partner is restricted, the entropic cost of hydrogen bond formation should be smaller than in the gas phase. There is no easy way to estimate the entropy in such a process, which is also dependent on the strength of the hydrogen bond; for a weak hydrogen-bonded complex (loose complex), the entropy of hydrogen bond formation will be small, and for a strong hydrogen-bonded complex (tight complex), it will be larger. The calculated entropy value in this study is probably the upper limit especially for the neutral hydrogen bond between Tyr-OH- - -HSG. By following Guthrie and Kluger,[37] if we take the $T\Delta S$ term as +2.42 kcal/mol (this still may be an overestimation), the calculated ΔG_1 (for PhOH- - -HSCH$_3$) is -1.5 kcal/mol. The calculated $\Delta G_1 - \Delta G_2$ is, therefore, -7.2 kcal/mol, which differs from the experimental value by 5 kcal/mol. This

(35) Liu, S.; Ji, X.; Gilliland, G. L.; Stevens, W. J.; Armstrong, R. N. *J. Am. Chem. Soc.* **1993**, *115*, 7910−7911.

(36) (a) Meot-Ner (Mautner), M. *J. Am. Chem. Soc.* **1988**, *110*, 3854−3858. (b). Gao, J.; Garner, D. S.; Jorgensen, W. L. *J. Am. Cem. Soc.* **1986**, *108*, 4784−4790.

(37) Guthrie, J. P.; Kluger, R. *J. Am. Chem. Soc.* **1993**, *115*, 11569−11572.

remaining difference is probably due to factors such as inaccuracy in the theoretical calculations (especially the uncertainty associated with the entropies) and absence of protein environmental effects in the present study. Indeed, in a previous study of substrate binding to a tRNA synthetase, Lau and Karplus found that the free energy difference in the interaction of the ligand with mutant (Phe) and wild-type (Tyr169) protein is −8.6 kcal/mol,[38] which corresponds most closely to loss of a single interaction between methyl ammonium (substrate) and water (Tyr). In the gas phase, the interaction free energy between methyl ammonium and water is −11.9 kcal/mol;[36a] the calculated value is between −10.6 and −13.6 kcal/mol depending on the level of theory,[31] which is within the uncertainty of experimental value. Clearly, the simulated result (−8.6 kcal/mol) from Lau and Karplus is smaller than the gas phase result (−11.9 kcal/mol), suggesting that the interaction is not optimal in the protein.

The replacement of the hydroxyl group of the tyrosine residue of the wild-type enzyme with a water in the Y6F mutant is reminiscent of the notion of substrate-assisted catalysis.[39] There are several lines of evidence in support of this hypothesis that the role of the active site tyrosine can be replaced by a water or a Ser residue. First, this hypothesis seems to provide an explanation for the observed anomalous Brønsted behavior.[1] It is also in line with a recent crystallographic study on a plant glutathione S-transferase.[16] Although the active site tyrosine seems to be invariant in almost all published sequences of glutathione S-transferases and the involvement of this tyrosine in catalysis has been confirmed by numerous studies,[1−3] surprisingly, in the newly solved crystal structure of a plant glutathione S-transferase, there is no corresponding tyrosine residue; instead, a serine residue seems to have replaced the role of the tyrosine. Recent studies also demonstrated that N-terminal Tyr residues in θ-subclass GSTs are not essential for catalysis; however, in these cases, since the crystal structure is not available, it is not clear whether other residues such as Ser, or even water, are involved in the catalysis as a replacement for tyrosine.[40,41] Protein engineering studies of a dichloromethane dehalogenase/glutathione S-transferase from *Methylophilus* sp. strain DM11 have revealed that Ser 12 but not Tyr 6 is required for enzyme activity.[42] The work by Coleman et al.[43] suggests that Tyr 8 of the rat liver GST (A1-1) contributes to, but is not required for, catalytic activity. Finally, in the X-ray crystal structure of the θ-subclass GST from *Lucilia cuprina*, the N-terminal Tyr residues are too far away from the sulfur atom of the GSH to participate in hydrogen bonding, and the crystal structure also indicated the possible involvement of an N-terminal Ser residue.[12d]

(38) Lau, F. T. K.; Karplus, M. *J. Mol. Biol.* **1994**, *236*, 1049−1066.
(39) Carter, P.; Wells, J. A. *Science* **1987**, *237*, 394−399.
(40) Nishida, M.; Kong, K. H.; Inoue, H.; Takahashi, K. *J. Biol. Chem.* **1994**, *269*, 32536−32541.
(41) Lee, H. C.; Tu, C. P. D. *Biochem. Biophys. Res. Commun.* **1995**, *209*, 327−334.
(42) Vuilleumier, S.; Leisinger, T. *Eur. J. Biochem.* **1996**, *239*, 410−417.
(43) Wang, J. B.; Barycki, J. J.; Coleman, R. F. *Protein Sci.* **1996**, *5*, 1032−1042.

Conclusion

To examine the mechanistic role of the active site tyrosine residue in glutathione S-transferase, *ab initio* molecular orbital calculations were performed on model systems of the active site. The location of the intervening proton in the (Tyr-OH- - -SG)$^-$ complex and the strength of the ensuing hydrogen bond were investigated. According to the present calculations, the proton is attached to the phenolic oxygen in the enzyme−glutathione complex, which is in agreement with results of Armstrong and co-workers.[1,18] However, the proton location can be shifted by changing the pK_a of the active site tyrosine (e.g., by introducing fluorine atoms); this again is consistent with a recent mutagenesis study on the M1-1 isoenzyme of GST where all tyrosines are replaced by 3-fluorotyrosines.[32] It is predicted that, when the active site tyrosine is replaced by 2,3,5,6-tetrafluorotyrosine, the proton will be located near the sulfur atom, which is yet to be confirmed experimentally. Our study also seems to provide a rationale for the previous confusion about where the proton is located. Since changing the protein environment can alter the pK_a of the active site tyrosine residue, for different subclasses of GSTs, the position of the proton could be different.

The strength of the hydrogen bonding interaction between tyrosine and the thiolate has also been investigated in the present study. We propose that, in the Y6F mutant, there is an active site water that hydrogen bonds to the thiolate. Indeed, a recent crystallographic study on the Y96F mutant with bound GSH (neutral form) does indicate that there is a water near the thiol group of GSH.[9b] This hypothesis is consistent with the observed anomalous Brønsted behavior[1] and that serine can functionally replace the active site tyrosine.[16,40−43] The replacement of the hydroxyl group of tyrosine, in the wild-type enzyme, by a water, in the Y6F mutant, is reminiscent of the notion of substrate-assisted catalysis.[39]

Acknowledgment. We would like to thank the reviewers for their insightful comments and suggestions. This work was supported by the Laboratory Directed Research and Development program at Pacific Northwest National Laboratory as part of the Microbial Biotechnology Initiative (R.L.O.). Pacific Northwest National Laboratory is a multiprogram national laboratory operated for U.S. Department of Energy by Battelle Memorial Institute under Contract DE-AC06-76RLO 1830. Y.-J.Z. has an AWU-NW DOE postdoctoral fellowship, which is administered by the Associated Western Universities.

JA961667H