# Second-Sphere Electrostatic Effects in the Active Site of Glutathione S-Transferase. Observation of an On-Face Hydrogen Bond between the Side Chain of Threonine 13 and the π-Cloud of Tyrosine 6 and Its Influence on Catalysis

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*Received April 1, 1993*

Weak electrostatic interactions between electropositive groups and the electron-rich π-clouds of aromatic rings are commonly observed and are thought to contribute significantly to the stability of folded protein structures.[1] However, only in rare instances have such interactions been implicated in the catalytic function of an enzyme.[2] In this paper we describe the observation of an on-face hydrogen bond between the side-chain hydroxyl group of threonine 13 and the aromatic ring of tyrosine 6 in the active site of isoenzyme 3–3 of glutathione (GSH) transferase (EC 2.5.1.18). This type of hydrogen bond appears to have received little attention in protein structures[3] and more importantly seems, in this instance, to affect catalysis by contributing a second-sphere interaction which influences the hydrogen bond between the hydroxyl group of tyrosine 6 and the sulfur of bound glutathione anion ($GS^-$) (e.g., Thr13-OH···π-Tyr6-OH···$^-$SG). *Ab initio* calculations, which model this interaction with a geometrically constrained methanol–*p*-cresol–methanethiol trimer, suggest that the on-face hydrogen bond could have a significant effect (ca. 6.3 kJ/mol in the gas phase) on the proton affinity of the thiol. Kinetic measurements of the $pK_a$ of GSH bound in the native and two mutant enzymes, T13V and T13A, in which the on-face hydrogen bond is eliminated, indicate that this interaction does contribute measurably (ca. −4 kJ/mol) to the ability of the hydroxyl group of tyrosine 6 to stabilize the thiolate anion.

The GSH transferases catalyze the general reaction GSH + R–X → GSR + XH. Three-dimensional structures of GSH transferase in complex with the physiological substrate GSH as well as products of reaction of GSH with phenanthrene 9,10-oxide have been solved at resolutions of 2.2 and 1.8 Å, respectively.[4] These structures and the structures of other isoenzymes,[5] along with kinetic properties of site-specific mutants,[6] have suggested that the catalytic role of the conserved, active-site tyrosine (tyrosine 6) is to donate a hydrogen bond to and stabilize the thiolate of the bound $GS^-$ (e.g., Tyr6-OH···$^-$SG). Spectroscopic evidence and the dependence of $k_{cat}/K_m^{CDNB}$ on pH (at saturating GSH) in the addition of GSH to 1-chloro-2,4-dinitrobenzene (CDNB) indicate that the electrostatic environment of the active site lowers the $pK_a$ of GSH from about 9 in aqueous solution to 6.2 on the enzyme surface.[6a] Close examination of the crystal structures reveals well-defined electron density for the side chain of threonine 13. The *R*-configuration of the β-carbon places the oxygen atom (as opposed to the methyl group) of the side chain directly above and about 3.4–3.5 Å from the centroid of the aromatic ring of tyrosine 6 (Figure 1). This distance is consistent with the hydroxyl group donating a hydrogen bond to the π-electron cloud of the aromatic ring. That this on-face hydrogen bond involves a residue in the active site that directly participates in catalysis compelled us to examine its possible influence on the catalytic properties of the enzyme.

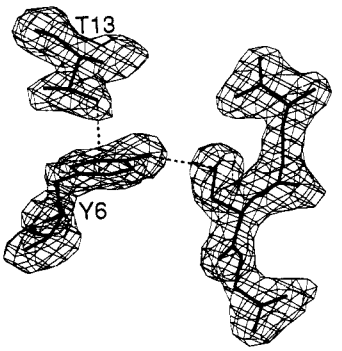


**Figure 1.** View of the hydrogen bond network in the active site of isoenzyme 3-3 of glutathione S-transferase. A portion of the $2F_o - F_c$ electron density map contoured at 1.0σ shows both the on-face hydrogen bond between the hydroxyl group of threonine 13 and the π-electron cloud of tyrosine 6 and the hydrogen bond between the hydroxyl group of tyrosine 6 and the sulfur of $GS^-$. Hydrogen bonding distances are given in Table I.

In order to get an estimate of the strength of the hydrogen bond to the π-cloud and the indirect effect of this interaction on the stabilization of the anionic sulfur in $GS^-$, *ab initio* calculations were performed with methanol, *p*-cresol, and methanethiol as models for threonine, tyrosine, and GSH, respectively. The initial geometry of the complex was constructed using the non-hydrogen coordinates of the refined crystal structure. With these coordinates frozen, optimizations of the proton positions and the hydrogen bond distances were carried out at the SCF level of theory with a 3-21G* Gaussian basis set. The geometries were optimized for both neutral and deprotonated methanethiol, with and without the methanol binding to the face of the *p*-cresol ring. Interaction energies were calculated in a second step at the SCF/MP2 level using a 6-31+G* basis set at the 3-21G* optimum geometry.[7] The MP2 level of theory incorporates electron correlation effects including dispersion, and the large basis set provides a good description of the electrostatic properties of the molecules in the complex, including the methanethiol anion. The

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**Table I.** Sulfur Proton Affinities and Optimum Hydrogen Bond Distances and Energies from *ab Initio* MP2/6-31+G*/3-21G* Calculations

| | | S⁻···O | | ring···O | |
|---|---|---|---|---|---|
| species | proton affinity[a] (kJ/mol) | distance (Å) | energy (kJ/mol) | distance (Å) | energy (kJ/mol) |
| $CH_3S^-$ | 1488.0 | | | | |
| $CH_3S^-$/*p*-cresol | 1410.0 | 3.243[b] | −75.0 | | |
| $CH_3S^-$/*p*-cresol/$CH_3OH$ | 1403.7 | 3.225 | −82.7 | 3.508 | −19.6 |
| $GS^-$/Tyr/Thr-OH[c] | | 3.23 | | 3.4 | |

[a] Energy required to form the anion from the neutral species. [b] The optimized distance (S···O) to neutral sulfur of $CH_3SH$ is 3.54 Å. [c] Experimental distances from 2.2-Å resolution crystal structure.[4a] The hydrogen bond distance to the aromatic ring was measured from the hydroxyl oxygen of threonine 13 to the center of the ring of tyrosine 6, as indicated in Figure 1.

results are presented in Table I. The optimized and experimental hydrogen bond distances are in excellent agreement and support the interpretation of the sulfur anion in the crystal structure. However, the closeness of the agreement must be considered fortuitous in light of the simplicity of the model and the uncertainties in the crystallographic coordinates. The direct hydrogen bond between sulfur and the hydroxyl group of *p*-cresol lowers the methanethiol deprotonation energy by nearly 80 kJ/mol, which is more than enough to account for the observed lowering of the p$K_a$ (2.8 p$K$ units, 15.9 kJ/mol) of GSH when it is complexed with the enzyme. The calculated number is probably an upper bound, since the model does not take into account solvation effects or the influence of the other protein residues. The methanol hydrogen bond to the face of the *p*-cresol ring, which, in complex with $CH_3S^-$, has an energy of −19.6 kJ/mol, results in a decreased proton affinity of $CH_3S^-$ by an additional 6.3 kJ/mol. A separate calculation with the *p*-cresol deleted yields only a 1.7 kJ/mol decrease for the direct effect of the second-sphere methanol on the sulfur proton affinity, a result which suggests that the bulk of the effect on sulfur is relayed through the aromatic ring. In addition, rotation of the hydroxyl group of methanol 180° away from the aromatic ring to reverse the polarity of the interaction results in a 19 kJ/mol increase in the thiolate proton affinity rather that the decrease found for the optimum geometry. Thus, the hydrogen bond to the face of the aromatic ring makes the *p*-cresol a better hydrogen bond donor and strengthens the interaction between *p*-cresol and $CH_3S^-$. The absence of this on-face hydrogen bond should increase the p$K_a$ of GSH in the protein complex by a measurable amount.

In principle, the magnitude of the influence of the on-face hydrogen bond on the p$K_a$ of bound GSH can be assessed by mutagenic removal of the side-chain hydroxyl group of threonine 13. Inasmuch as small geometry changes introduced by even the most conservative mutations might cause measurable perturbation of the p$K_a$ of bound GSH, two mutants, T13V and T13A, were prepared and analyzed. The pH dependencies of $\log(k_{cat}/K_m^{CDNB})$ of the native enzyme and the two mutants are illustrated in Figure 2. Both mutations cause an increase in the apparent p$K_a$ of enzyme-bound GSH of about 0.7 units, well outside the experimental error of the measurements. This result is consistent with an increased proton affinity of the hydroxyl group of tyrosine 6, a decreased hydrogen bond strength to sulfur, and a decreased stability of the thiolate upon removal of the on-face hydrogen bond. It is encouraging that the same magnitude of change is observed in two mutants that have different steric requirements.[9] This fact mitigates against a significant perturbation of the observed p$K_a$ by small geometric differences that might be introduced in the active site. Furthermore, a $\Delta pK_a = 0.7$ corresponds to an increase in proton affinity of 4 kJ/mol, which

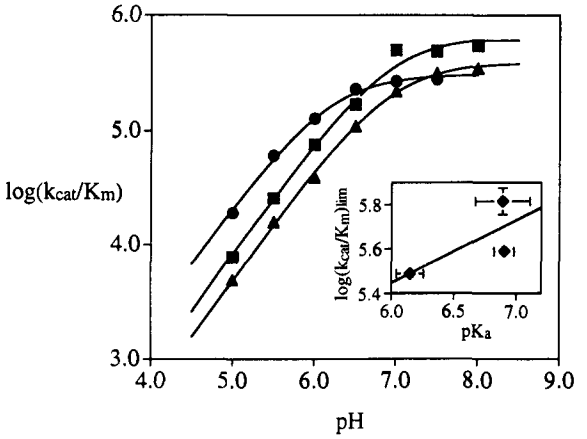


**Figure 2.** Dependence of $k_{cat}/K_m^{CDNB}$ on pH under conditions of saturating (2 mM) GSH for isoenzyme 3-3 (●), the T13A (▲), and T13V (■) mutants. Experimental conditions are the same as previously described.[6a] The solid lines are fits of the experimental data to the equation $\log(k_{cat}/K_m) = \log(C/1 + [H^+]/K_a)$ using the program HABELL,[8] where $C = k_{cat}/K_m^{lim}$, the limiting value of $k_{cat}/K_m^{CDNB}$ at high pH, $K_a$ is the apparent acid dissociation constant for (E·GSH ⇌ E·GS⁻ + H⁺), and [H⁺] is the hydronium ion concentration. Values of the constants obtained from regression analyses are isoenzyme 3-3, p$K_a$ = 6.15 ± 0.11, $k_{cat}/K_m^{lim} = (3.08 \pm 0.15) \times 10^5\ M^{-1}\ s^{-1}$; T13A, p$K_a$ = 6.90 ± 0.08, $k_{cat}/K_m^{lim} = (3.88 \pm 0.18) \times 10^5\ M^{-1}s^{-1}$; and T13V, p$K_a$ = 6.89 ± 0.23, $k_{cat}/K_m^{lim} = (6.55 \pm 0.79) \times 10^5\ M^{-1}s^{-1}$. The inset shows a Brønsted-type plot of $\log(k_{cat}/K_m)^{lim}$ vs the apparent p$K_a$ of E·GSH.

is of the same order of magnitude as that calculated from the model (Table I).

It should also be noted that an increase in the reactivity of enzyme-bound $GS^-$ would be anticipated to accompany an increased basicity of a nucleophile for any reaction in which the Brønsted $\beta_{nuc} > 0$. This appears to be the case with the mutants. Although the data are quite limited and cover a narrow range of p$K_a$, a Brønsted-type plot (Figure 2, inset) of $\log(k_{cat}/K_m)^{lim}$ vs the p$K_a$ of bound GSH provides an estimate of $\beta_{nuc}$ for $GS^-$ in the active site of 0.3 ± 0.2, which is within experimental error of that found for reaction of thiolates with CDNB in aqueous solution ($\beta_{nuc} = 0.16 \pm 0.02$).[10] This result is consistent with an early transition state with little bond formation between sulfur and the electrophilic carbon in the reactions of both E·$GS^-$ and $GS^-_{(aq)}$ with CDNB. Additional perturbations of second-sphere electrostatic effects should permit further refinement of this analysis.

Finally, we suggest that the on-face hydrogen bond and perhaps cation–$\pi$ interactions can act as electronic substituents on aromatic systems that are analogous to more classical covalent ortho, meta, and para substituents. We propose that this type of electronic effect be termed a "nephotic" substituent effect, from the Greek *nephos* (cloud), to reflect the geometry of its origin and the fact that the effect appears to be transmitted via the $\pi$-system of aromatic ring.

**Acknowledgment.** This work was supported by a grant from the National Institute of General Medical Sciences (GM 30910). We thank Mr. Tat Chi Ho for his help in preparing the mutant enzymes.

(9) Replacement of the hydroxyl group with a methyl group as in T13V is expected to increase steric crowding slightly by substituting a repulsive van der Waals contact for the attractive electrostatic contact of the on-face hydrogen bond. Just the opposite problem could be anticipated in the T13A mutant, where replacement of both the hydroxyl group and the γ-methyl group with hydrogen would tend to reduce steric crowding. The 1.9-Å crystal structure of T13A indicates that the relationship of tyrosine 6 to the sulfur of $GS^-$ does not change in that mutant.

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