Combined Quantum and Molecular Mechanics (QM/MM) Study of the Ionization State of 8-Methylpterin Substrate Bound to Dihydrofolate Reductase

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The prediction of stabilities of ion pairs relative to the corresponding neutral pairs is a challenging computational problem but very important in biological systems where the occurrence of such pairs is often mechanistically significant. Here we have computed the relative free energy for the protonated and neutral forms of the mechanism-based substrate 8-methylpterin bound to dihydrofolate reductase (DHFR) in a ternary complex with cofactor nicotinamide adenine dinucleotide phosphate (NADPH). The free energy components required to calculate relative affinities of the protonated substrate hydrogen (H) bonded to the unprotonated form of the conserved active-site carboxylate residue (Glu30 for chicken DHFR) and neutral substrate H bonded to the neutral (carboxylic acid) form of Glu30 were obtained using both ab initio QM methods and combined semiempirical (AM1) QM and MM (QM/MM) methods. The energy difference for the H-bonded systems was first calculated in a vacuum using ab initio QM methods for a model system consisting of the Glu30 side chain and the substrate. The free energy of interaction of this system with the protein/solvent surroundings was then computed using a coordinate-coupled free energy perturbation (FEP) method implemented within the molecular dynamics (MD) simulation scheme. The ab initio QM results show the neutral form is more stable in a vacuum, but that this relative stability is reduced in the enzyme due to the more favorable electrostatic interactions made by the ion pair. The neutral pair is calculated to be approximately 5 ± 2 kcal/mol ($\Delta\Delta G_{bind}$) more stable than the ion pair, but the results are suggestive of a small free energy gap and the possibility of a low energy barrier for the proton transfer. The results are in qualitative agreement with other theoretical studies on similar types of H-bonded systems but appear to contradict our own experimental data for these DHFR ligands. Reasons for the apparent discrepancy are discussed.

Introduction

Dihydrofolate reductase (DHFR) catalyzes the reduction of folate to dihydrofolate and tetrahydrofolate (THF). These DHFR catalyzed reductions take place via a hydride ion transfer between nicotinamide adenine dinucleotide phosphate (NADPH) and the substrates. The THF reduction product belongs to a cofactor class essential for DNA replication in cell division, making DHFR a prime target for antibacterial and anticancer drugs. As part of a broader investigation into the catalytic mechanism of DHFR, we have previously used molecular dynamics and free energy perturbation (MD/FEP) and combined quantum mechanical and molecular mechanical (QM/MM) methods in a study of the hydride ion transfer between NADPH and 8-methylpterin catalyzed by DHFR.[1,2] 8-Methylpterin is the lead member of a class of novel mechanism based substrates and inhibitors of DHFR developed by us.[3,4] This substrate activity and other experimental evidence suggest that 8-substituted pterins bind the active site of DHFR in a similar orientation to the natural substrates, that is, they are assumed hydrogen (H) bonded to a conserved acidic residue (Asp/Glu) in the active site of DHFR. Figure 1 shows 8-methylpterin H bonded to the Glu30 residue of avian DHFR. Clearly, the H bonded complex can exist in either the N3 protonated (ion pair) or Glu30 (OE2) protonated (neutral pair) forms. The binding of the N3 protonated form of 8-methylpterin substrate (SH^+) to DHFR is important mechanistically as this is the form believed to be activated toward hydride ion transfer from NADPH,[5] i.e.

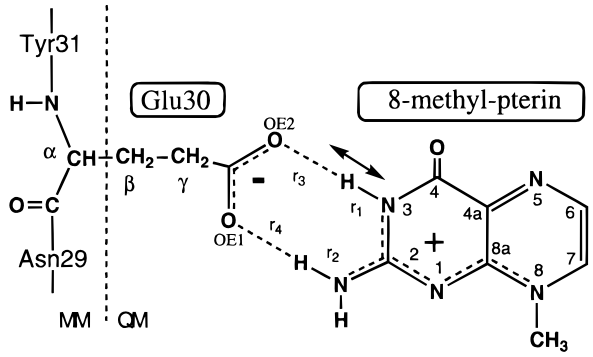

Figure 1. H-bonding between the protonated 8-methylpterin substrate and side chain of Glu30 in avian DHFR.

$$SH^+ + NADPH \rightarrow SH_2 + NADP^+ \qquad (1)$$

From experimentally determined K_m values it is not possible to determine the relative stabilities of the ion-pair and neutral-pair forms as both give rise to the same pH dependence,[6,7] i.e.

$$K_m = K\left[1 + \frac{K_S}{[H^+]}\right]\left[1 + \frac{[H^+]}{K_E}\right] \qquad (2)$$

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$$K = K_d\left[1 + \frac{K_d K_S}{K_{d'} K_E}\right]^{-1} \quad (3)$$

where K_d and $K_{d'}$ are the dissociation constants for the protonated and unprotonated substrates, respectively. The remaining molecular constants are the acid dissociation constants of the protonated substrate (K_S) and the carboxylic acid side chain of Asp or Glu in the enzyme active site (K_E). It is not feasible to perform a direct experimental determination of both K_d and $K_{d'}$. However, while it is also not feasible to compute K_d and $K_{d'}$, in the present work we show that by using experimental K_S and K_E values together with the results of computational studies, we can evaluate the relative free energy of binding

$$\Delta\Delta G_{bind} = -RT\ln(K_d/K_{d'}) \quad (4)$$

It is known from other theoretical studies on the comparable formate-guanidinium[8] and acetate-methylguanidinium[9] complexes that the electrostatic environment influences the relative free energy of the two forms. In media with high dielectric constants, such as water ($\epsilon = 80$), the ion pair is more stable, whereas in a vacuum the neutral form is favored. In low dielectric media ($\epsilon < 10$), either form may be possible. Thus, for example, in nonpolar interior regions of a protein or in lipid membranes where the dielectric constant is sufficiently small, the neutral forms of H-bonded complexes between acidic and basic amino acid residues may be observed. On the protein surface, however, solvent stabilization will more likely favor the ionic form. For the binding of ionizable ligands, such as 8-methylpterin, there exists an equilibrium between the protonated and unprotonated substrates involving enzyme-bound and solution phases. The thermodynamic stability of the ion-pair form over the neutral H-bonded complex in the enzyme also depends on the interactions of solvent water with the unbound substrate and unbound enzyme. This information on the relative stability of the protonation/ionization states of the unbound forms is already contained in the K_S and K_E values. For the bound species, the H-bond interaction can be accurately modeled using ab initio QM methods, while the effects of the surrounding protein/solvent environment can be simulated using molecular dynamics with semiempirical QM/MM force fields. Thus, in the present work we focus on using QM and combined QM/MM methods in an effort to understand the differences in binding of the ion-pair and neutral-pair species for 8-methylpterin bound to DHFR.

Theory and Methods

Free Energy of Binding. The thermodynamic cycle for the binding of the N3-protonated (SH^+) and neutral (S) substrates to the unprotonated Glu30 residue in the enzyme (E^-) and protonated Glu30 residue in the enzyme (EH) is illustrated in Figure 2. The relative free energy of binding for the ion-pair (E^-:SH^+) and neutral-pair (EH:S) forms of the substrate−enzyme complex is given by

$$\Delta\Delta G_{bind} = \Delta G_{bind}(E^-{:}SH^+) - \Delta G_{bind}(EH{:}S) = \Delta G_2 - \Delta G_1 \quad (5)$$

The binding energy difference is calculated in terms of the free energies (ΔG_2 and ΔG_1) of the ionization processes involved in the formation of the complexes, rather than in terms of the direct binding free energy. Experimentally, the free energy of

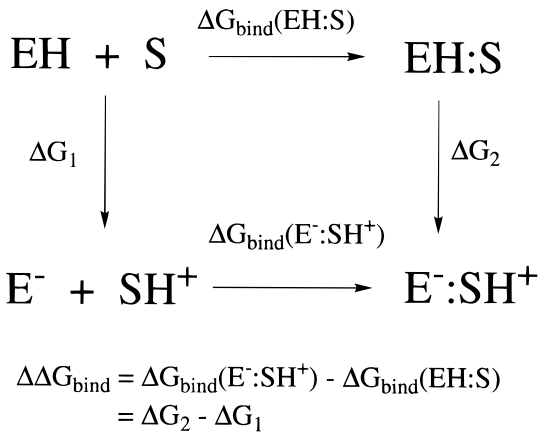

Figure 2. Thermodynamic cycle for binding the ion-pair (E^-:SH^+) and neutral-pair (EH:S) forms by an H-bond interaction between substrate S and enzyme E.

ionization (ΔG_1) may be readily obtained from K_S and K_E, i.e., the experimental pK_a's of SH^+ and EH, respectively,

$$\Delta G_1 = 2.3RT\,[pK_E - pK_S] \quad (6)$$

Using the experimental value[4] of $pK_S = 5.2 \pm 0.1$ and experimental estimate of $pK_E = 4.0 \pm 1.0$, we obtain

$$\Delta\Delta G_{bind} = \Delta G_2 + 1.7 \pm 1.4 \text{ kcal/mol} \quad (7)$$

The uncertainty in ΔG_1 arises from the lack of a precise value for pK_E. The pK_E of 4.0 used to obtain eq 7 was chosen as it is close to the value of 4.4 for the side chain of glutamic acid in solution and is consistent with the most recent experimental and theoretical evidence for Glu30 in the enzyme. A fit of the pH dependence data for the apparent dissociation constants of a series of 8-substituted pterin analogue (8-R-N5-deazapterin) inhibitors yields pK_E values ranging from 3.6 to 6.4,[10] although spectroscopic studies have placed an upper limit of approximately 5.[11,12] Another theoretical estimate suggests a lower limit of approximately 3.[13] Thus, we estimate the uncertainty in pK_E to be about one pK unit (1.4 kcal/mol).

To compute ΔG_2 we define a model system partitioned into a smaller part that can be treated using high level ab initio QM and the much larger remainder of the protein/solvent system which is described using MM force fields (Figure 1). The QM part consists of the substrate molecule SH^+ and S, and the Glu30 side chain L−R−COO^- and L−R−COOH where R = $(CH_2)_2$ and L is an additional "link" atom required to satisfy valency. The free energy difference ΔG_2 between the two complexes E^-:SH^+ and EH:S can then be expressed in the form[14−16]

$$\Delta G_2 = \Delta E_2 + \Delta G_2^{int} \quad (8)$$

where ΔE_2 is the ab initio QM energy difference between the two H-bonded complexes L−R−COO^-:SH^+ and L−R−COOH:S, computed using the *Gaussian 98* program,[17] and ΔG_2^{int} is the difference in free energy for the interaction of the (semiempirical) QM system with the MM protein/solvent environment. The validity of eq 8 partly depends on whether expressing the total free energy as the sum of the two components gives an accurate representation of the real system. If so, each component can be calculated using a different method. We demonstrated previously[1] that expressing a total free energy as the sum of component free energies is valid, at least for the types of reacting systems being considered here. However, it needs to be emphasized, that eq 8 remains an approximation and that it does

not properly account for polarization of the QM solute by the surrounding medium at the ab initio QM level. Problems can occur when describing charge separation between QM fragments over large distances in highly polar environments.[18] In the present system, however, the donor and acceptor in the proton transfer are always kept at a close H-bonding distance (2.5 to 3 Å) in a relatively low dielectric (protein) medium.

The free energy difference of interaction is computed using a coordinate-coupled free energy perturbation method,[1,19]

$$\Delta G_2^{\text{int}}(\mathbf{r},\mathbf{r}\pm\Delta\mathbf{r}) = -(RT)^{-1}\ln\langle\exp[-(E^{\text{int}}(\mathbf{r}\pm\Delta\mathbf{r}) - E^{\text{int}}(\mathbf{r}))/RT]\rangle_{\text{r}} \quad (9)$$

where **r** defines the reaction coordinate for the transfer of the acidic proton between N3 and OE2 through increments of $\Delta\mathbf{r}$. Note that although eq 9 depends only on the interaction energy between the QM and MM systems and not the MM energy itself, for the approximate model we are using (eq 8) it is exact since the coordinates **r** depend only on the QM system. A rigorous treatment of this system at the ab initio QM level represents a challenge as it would require that all QM-system coordinates be included in the MD except those orthogonal to the reaction coordinate.[20]

Although it is not computationally feasible to carry out MD using the ab initio QM surface for the present system, we are able to implement a proper QM/MM treatment at the semiempirical QM level. To obtain the ensemble averages in eq 9, we perform MD simulations with the total energy of the QM/MM system given by

$$E_{\text{T}} = E_{\text{QM}} + E_{\text{MM}} + E_{\text{QM/MM}} \quad (10)$$

where E_{QM} is the energy of the quantum system, E_{MM} is the energy of the molecular mechanics part of the system, and $E_{\text{QM/MM}}$ is the interaction energy between the quantum and molecular mechanics parts of the system. In the present QM/MM MD simulation model, E_{QM} may be computed using any of the semiempirical AM1,[21] MNDO,[22] and PM3[23] parameterizations. The molecular mechanics systems are described using the united-atom and all-atom force fields of Weiner et al.[24,25] and Cornell et al.[26] for the protein and the TIP3P force field[27] for water. The QM/MM interaction energy is the sum of electrostatic/polarization and van der Waals terms,

$$E^{\text{int}} = E_{\text{QM/MM}} = E_{\text{ele}} + E_{\text{vdW}} \quad (11)$$

The method of calculating E_{ele} is described elsewhere.[28,29]

Although an empirical estimate of ΔG_1 can be readily obtained via eq 6, a theoretical value can also be obtained for comparison and serves as a useful test of the methodology. The free energy (ΔG_1) required to ionize substrate and enzyme in aqueous solution (see Figure 2)

$$\text{EH} + \text{S} \rightarrow \text{E}^- + \text{SH}^+ \quad (12)$$

can be written in terms of ab initio QM and QM/MM components analogous to eq 8

$$\Delta G_1 = \Delta E_1 + \Delta G_1^{\text{int}} \quad (13)$$

where the ab initio QM energy difference is given by

$$\Delta E_1 = \text{E}(\text{SH}^+) + \text{E}(\text{L}-\text{R}-\text{COO}^-) - \text{E}(\text{S}) - \text{E}(\text{L}-\text{R}-\text{COOH}) \quad (14)$$

and the QM/MM interaction term is

$$\Delta G_1^{\text{int}} = \Delta G_{\text{ele}}(\text{SH}^+) + \Delta G_{\text{ele}}(\text{L}-\text{R}-\text{CO}_2^-) - \Delta G_{\text{ele}}(\text{S}) - \Delta G_{\text{ele}}(\text{L}-\text{R}-\text{CO}_2\text{H}) \quad (15)$$

where the free energy (ΔG_{ele}) due to electrostatic/polarization interactions between the QM and MM systems can be expressed as[28,29]

$$\Delta G_{\text{ele}} = N^{-1}\sum_{i=1}^{N}[E_{\text{ele}}]_i + \frac{1-\epsilon}{2\epsilon r_{\text{cut}}}\text{Q}^2 \quad (16)$$

The first term in eq 16 is calculated in a molecular dynamics (MD) simulation by the creation or annihilation of the solute−solvent interaction terms via the λ coupling parameter $E_{\text{ele}} \rightarrow (1-\lambda)E_{\text{ele}}$ in eq 11. N is the number of integration time steps and $[E_{\text{ele}}]_i$ is the electrostatic/polarization part of the QM/MM interaction evaluated using the configuration obtained at the ith time step in the MD simulation. The second term is a Born correction in which ϵ is the dielectric constant of the solvent and Q is the total charge on the QM region. There are remaining nonpolar cavity and van der Waals free energy terms ($\Delta G_{\text{c/vdw}}$) we have neglected as these have been shown to be relatively small.[30] Note also that the difference in ΔG_{ele} computed for the H-bonded complexes gives another estimate of ΔG_2^{int}. Another method of estimating the free energy for protein−ligand binding is by use of the linear response assumption.[31] If the free energy change ΔG_{ele} is linear in λ, the ratio of the free energy to the average electrostatic energy is exactly one-half,[31,32]

$$\Delta G_{\text{ele}}/\langle E_{\text{ele}}\rangle = 1/2 \quad (17)$$

As this has been shown to be only approximately true for QM/MM methods,[29] we obtain a more accurate estimate of the free energies by

$$\Delta G_{\text{ele}} \cong \alpha\langle E_{\text{ele}}\rangle \quad (18)$$

where α is a parameter determined by free energy simulations on similar systems.

Link Atoms. It should be emphasized that there is no unique way of defining the Hamiltonian of the system at the QM/MM boundary.[33−35] The treatment of link (L) atoms used in this work follows from our previous study of hydride ion transfer, as it is relatively simple to implement and appears well suited to polar and π-electron delocalized systems where electronic charge reorganization is important. Terms in the MM force field describing bonds, angles, and dihedrals that include both QM and MM atoms are retained in the model Hamiltonian, and thus the geometry at the QM/MM interface is largely maintained using purely MM potentials. Clearly, however, the choice of L atom affects the electronic structure of the QM part of the system and, hence, its properties. In a previous study,[1] we found that the choice of L atoms can have a profound effect on the free energy of hydride ion transfer but that there was a correlation of the free energy with the net charge on the QM system which is also dependent on the type of L atom. For the present study we have used and evaluated both an electron donating atom (L = H) and an electron withdrawing atom (L = F). The degree of electronegativity can be changed for each of these by simply scaling the core charge of the link atom, thereby modifying the charge on the QM system. The scaled core-charge L atoms are denoted by H* and F*. To determine the appropriate charge polarizations and, hence, L atoms, it is necessary to model

accurately the covalent bonds between the QM and MM regions. To achieve this we define a separate reference QM system, L-Glu, that includes all of the Glu30 residue atoms beyond the QM/MM boundary. The system is terminated by L atoms at each of the peptide linkages with Tyr31 and Asn29 (Figure 1). Note, however, that certain interactions within the reference QM system would correspond to terms in $E_{QM/MM}$ (eq 10) in the MD simulations. As there is in general no rigorous way of correcting for the effects of this double counting of interactions, the reference system is kept as small as possible.

Molecular Dynamics Simulations. The starting coordinates for the ternary complex consisting of DHFR, NADPH cofactor, and 8-methylpterin were based on the chicken liver DHFR·$NADP^+$·biopterin X-ray structure[36] as described in previous work.[37] The ternary complex including crystallographic water was solvated using a 34 Å shell of TIP3P water[27] containing approximately 3000 water molecules (approximately 12000 atoms in total). For simulations with the Weiner et al. force fields, the all-atom force field[25] was used for protein residues within 8 Å of the substrate, with the remainder of the protein represented by the united-atom force field.[24] For solvation of the unbound substrate, S and SH^+ were placed in a periodic box of approximately 1000 TIP3P waters. The solvated systems were initially energy minimized using conjugate gradients, followed by the MD simulations. We used the constant temperature algorithm of Berendsen et al.[38] to perform the MD simulations with a time step of 0.001 ps and hydrogen masses set to 3 amu. The temperature was set at 300 K with a relaxation time of 0.1 ps. For the periodic box simulations, the pressure was set at 1 atm. As in our previous QM/MM studies on aqueous solvation,[28,29] a 12 Å cutoff was used for the QM/MM interactions in SH^+ and a 9 Å cutoff for S. This is to ensure that proper calibration with experimental solvation free-energy data is obtained. Due to the presence of charged amino acid groups in simulations of the enzyme, no cutoff was used for the QM/MM interactions. To obtain an improved description of the long-range electrostatic interactions for the MM parts of the systems, a 9 Å cutoff was imposed for all polar−polar residue and polar−charge residue interactions, while all interactions between charged residues were retained. It is well known that truncation of polar−polar residue interactions can lead to an overpolarization of charged solute species and, hence, an overestimation of free energies.[29,39] This overpolarization effect, although very much weaker for the neutral "solute" QM system used in the present study, may still persist due to the presence of a highly polar salt bridge within the QM region. However, the charged protein residues will be subject to the full extent of the overpolarization by the solvent shell, and this may have an indirect effect on the interaction free energy.

The enzyme simulations generally required a minimum of 100 ps equilibration with a further minimum of 100 ps for obtaining averages of the energies (eq 18). The free energies ΔG_{ele} were obtained by thermodynamic integration over a minimum of 50 ps simulation time using a continuously coupled method.[28] The free energy change was then recalculated by performing the reverse integration to obtain a mean and standard error. In the coordinate-coupled FEP calculations, the distances $\{\mathbf{r}\}$ in Figure 1 were fixed using SHAKE[40] and the free energy change calculated at $\{\mathbf{r} \pm \Delta\mathbf{r}\}$ as the mean of plus and minus perturbations[1] over 20 intervals (windows) along the reaction coordinate with 3 ps each of equilibration and data collection per window, i.e., a total of 60 ps after the initial equilibration. These sampling times of 50 to 100 ps have been shown to be adequate for computing electrostatic free energy changes.[29] The MD simulation and free energy QM/MM calculations were performed using Molecular Orbital Programs for Simulations (MOPS).[41]

TABLE 1: Bonding and H-bonding Distances (Å) in Figure 1 Computed for the Neutral-Pair and Ion-Pair Complexes at Semiempirical and Ab Initio QM Levels in Vacuum

QM part[a]	dist	L[a]	AM1	PM3	HF/6-31G	HF/6-31G*	MP2/6-31G*
neutral pair	r_1	H	2.479	1.779	1.767	1.912	1.767
		F	2.434	1.773	1.727	1.889	1.743
	r_2	H	0.996	1.009	1.004	1.003	1.025
		F	0.996	1.009	1.003	1.002	1.024
	r_3	H	0.976	0.979	0.989	0.972	1.014
		F	0.976	0.979	0.994	0.974	1.018
	r_4	H	2.089	1.802	1.882	1.963	1.864
		F	2.092	1.803	1.899	1.975	1.872
ionized pair	r_1	H	1.029	1.051	1.056	1.050	1.100
		F	1.028	1.049	1.055	1.049	1.108
	r_2	H	1.045	1.040	1.049	1.051	1.104
		F	1.039	1.039	1.041	1.045	1.083
	r_3	H	1.912	1.687	1.571	1.622	1.522
		F	1.919	1.691	1.573	1.626	1.498
	r_4	H	1.776	1.657	1.580	1.592	1.487
		F	1.834	1.662	1.615	1.619	1.548

[a] QM part used to model the side chain of Glu30 complexed with 8-methylpterin substrate (S) to form the neutral-pair (L−R−COOH:S) and ion-pair (L−R−COO^-:SH^+) complexes. R = CH_2−CH_2, L = link atom.

Results and Discussion

QM Calculations. As we require a reasonably accurate description of the H-bond geometry at the semiempirical level, we have compared AM1 and PM3 with ab initio methods. We did not consider the MNDO method as its inability to reproduce H bonding is well known. All methods predict stable structures for both the neutral-pair and ion-pair forms with well-defined potential energy minima. The H bond distances in Table 1 show that PM3 is the model of choice for the subsequent QM/MM calculations. The AM1 method, while predicting stable neutral-pair and ion-pair complexes, overestimates the H-bond distances r_1 and r_4 for the neutral pair and r_3 and r_4 for the ion pair. Considering all methods, the maximum differences between results with link atoms H and F are not more than 0.06 Å. A significant proportion of this difference for the ab initio methods is most likely attributable to basis set superposition error (BSSE), i.e., arising from incompleteness of the basis set in calculations on the H-bonded complexes.[42] For PM3 this difference is only 0.006 Å. Thus, the choice of link atom has a relatively small effect on the description of the H-bond geometry, particularly at the PM3 level.

The energy differences are given in Table 2 for various choices of basis sets, optimized geometries, and link atoms H and F. Compared with the ab initio HF and MP2 methods, the AM1 and PM3 methods overestimate the stability of the neutral form over the ion pair (ΔE_2). At the HF/6-31G level, the energy differences ΔE_2 are only 3.1 kcal/mol (L=H) and 1.4 kcal/mol (L=F). However, the results obtained using larger basis sets containing polarization functions (6-31G*, 6-311+G**) range from 8.7 to 11.8 kcal/mol. The effects of electron correlation (MP2) reduce the range from 5.7 to 7.8 kcal/mol. The H link atom gives values 1 to 2 kcal/mol larger than the corresponding calculations using the F link atom. If we extend the QM system to include all atoms of residue Glu30 (H-Glu and F-Glu) we observe a modest reduction in the PM3 energy difference ΔE_2. Note, however, that at least some of this energy difference must be regarded as artificial as it includes terms that correspond to nonbonded interactions that would be accounted for within the

TABLE 2: QM Energy Differences ΔE_1, ΔE_2, and $\Delta E_2 - \Delta E_1$ (kcal/mol) Computed at Semiempirical and Ab Initio Levels in Vacuum

QM method	link	ΔE_1[a]	ΔE_2[b]	$\Delta E_2 - \Delta E_1$[c]
AM1	H	114.1	20.7	−93.4
	F	106.7	18.6	−88.1
PM3	H	118.8	19.3	−99.5
	F	114.4	17.9	−96.5
	H-Glu[d]	101.6	17.2	−84.4
	F-Glu[d]	105.1	16.6	−88.5
HF/6-31G	H	101.3	3.1	−98.2
	F	93.7	1.4	−92.3
HF/6-31G*	H	109.1	11.4	−97.7
	F	104.2	9.7	−94.5
HF/6-311+G**//HF/6-31G	H	106.0	10.6	−95.4
	F	99.7	8.7	−91.0
//HF/6-31G*	H	105.8	11.8	−94.0
	F	100.3	10.0	−90.3
MP2/6-31G*	H	110.2	6.9	−103.3
	F	105.3	5.8	−99.5
MP2/6-311+G**//HF/6-31G	H	105.1	7.4	−97.7
	F	99.5	6.1	−93.4
//HF/6-31G*	H	105.0	7.8	−97.2
	F	99.8	6.4	−93.4
//MP2/6-31G*	H	106.0	7.1	−98.9
	F	100.8	5.7	−95.1

[a] $\Delta E_1 = E(SH^+) + E(L-R-COO^-) - E(S) - E(L-R-COOH)$. [b] $\Delta E_2 = E(L-R-COO^-:SH^+) - E(L-R-COOH:S)$. [c] $\Delta E_2 - \Delta E_1$ is the change in H-bond energy on going from the neutral-pair to ion-pair complex. [d] Reference system consisting of all atoms in the Glu30 residue plus H or F groups instead of the peptide linkages at Tyr31 and Asn29 (see text and Figure 1).

QM/MM model. This is apparent in the decrease in the H-bond energy ($\Delta E_2 - \Delta E_1$) for the reference H-Glu and F-Glu models relative to the smaller H and F models. Due to the pairwise nature of the interactions in the NDDO approximation (i.e., PM3, AM1, and MNDO), it is possible to largely correct for the effects of these terms by simply subtracting them from the total semiempirical energy. The corrected ΔE_2 values are 18.6 and 18.0 which are closer to 19.3 and 17.9 for L=H and L=F, respectively.

The difference in the H-bond energy ($\Delta E_2 - \Delta E_1$) is 90−100 kcal/mol in favor of the ion pair. However, this H-bond energy is not sufficient to offset the cost of ionizing the molecular fragments (ΔE_1). Consequently, the neutral-pair form is the more stable species in the gas phase. Note that the energies given in Table 2 have not been corrected for thermal effects or BSSE. For the 6-31G* basis, the BSSE in ΔE_2 is computed using the standard counterpoise method[43] to be −2.0 kcal/mol. However, the BSSE is reduced to only 0.1 kcal/mol for the larger 6-311+G** basis. Thermal effects based on a normal-mode analysis are calculated to be similarly small: with zero point vibrational and thermal free energy corrections, ΔE_2 at the HF/6-31G level increases from 1.4 to 1.6 kcal/mol. The choice of optimization level for the geometry has a relatively minor effect on the energy differences. Thus, an estimate of the free energy differences should be obtainable from ΔE_1 and ΔE_2 by taking the mean of energies for L=H and L=F given in Table 2. From the MP2/6-311+G** energies obtained at the MP2/6-31G* geometry we estimate $\Delta E_1 = 103.4 \pm 2.6$ kcal/mol and $\Delta E_2 = 6.4 \pm 0.7$ kcal/mol from the mean of the L=H and L=F energies.

QM/MM Calculations. The electronic components of the interaction free energies (ΔG_{ele}) of the neutral and protonated substrate molecules in aqueous solution are given in Table 3. We also include results for the nonpolarization model,[29] in which the QM electron density is not polarized by the MM atomic charges. As the QM/MM interaction terms have been separately calibrated for the AM1, PM3, and MNDO methods to reproduce experimental free energies,[28,29] we use the mean value of the AM1, PM3, and MNDO results for the theoretical estimate of aqueous solvation free energy. For the neutral and protonated substrate we obtain (polarization model) $\Delta G_{ele}(S) = -30.0 \pm 3.0$ kcal/mol and $\Delta G_{ele}(SH^+) = -68.4 \pm 0.8$ kcal/mol, respectively. The value for the cation is comparable with values of −66.6 and −68.5 kcal/mol obtained by Sitkoff et al.[30] for the methylimidazolium and *N*-propylguanidinium cations, respectively. The relative polarization contribution to the free energy for the cation is small (∼10%), consistent with the previous QM/MM study of ion hydration,[29] but it is relatively greater (∼30%) for the neutral species.[28]

In the calculation of ΔG_{ele} for the carboxyl side chain, the MM system consists of protein in addition to water molecules. Link atoms are required for this part of the calculation as the side chain is covalently bonded to the MM system. The net charges on −R−COO$^-$ and −R−COOH for various link (L) atoms are given in Table 4. To determine appropriate scalings for the core charges, we compare with the charges obtained from the extended (reference) QM system consisting of all atoms of the Glu30 residue with different terminating atom groups, H, F, or CH_3. Without scaling of the core charge, we can see from the net charges that the electronegativity of F is too high, whereas for H it is too low. However, by scaling the core charges by 0.92 and 1.03 for H and F, respectively, we obtain reasonable agreement with the net charge for the reference QM system.

Values for ΔG_{ele} for the neutral (−R−COOH) and ionized (−R−COO$^-$) carboxyl side chain are given in Table 5 for different L atoms, with and without electronic polarization. For

TABLE 3: Electrostatic/Polarization Components ΔG_{ele} (kcal/mol) (eq 16) of the Aqueous Solvation Energies of Neutral Substrate (S) and Protonated Substrate (SH^+) Computed Using the Semiempirical QM/MM Models

QM species[a]	model[b]	$\langle E_{ele} \rangle$	$\langle E_{vdW} \rangle$	ΔG_{ele}[c]	$\Delta G_{ele}/\langle E_{ele} \rangle$
neutral	AM1	−86.7 ± 0.4	−19.5 ± 0.2	−27.2 ± 0.7	0.31
	PM3	−106.1 ± 0.5	−16.6 ± 0.1	−27.2 ± 0.5	0.26
	MNDO	−98.8 ± 0.8	−16.9 ± 0.2	−32.5 ± 0.3	0.33
	AM1(np)	−35.7 ± 0.6	−21.9 ± 0.2	−17.2 ± 0.3	0.48
	PM3(np)	−46.0 ± 0.4	−20.1 ± 0.1	−21.7 ± 0.2	0.47
	MNDO(np)	−45.8 ± 0.4	−19.9 ± 0.2	−21.9 ± 0.1	0.48
ionized	AM1	−128.3 ± 0.7	−18.3 ± 0.3	−67.8 ± 0.2	0.42
	PM3	−128.8 ± 1.2	−16.6 ± 0.2	−69.2 ± 0.6	0.43
	MNDO	−129.2 ± 1.0	−15.6 ± 0.3	−68.2 ± 0.1	0.42
	AM1(np)	−100.0 ± 0.8	−20.8 ± 0.2	−62.2 ± 0.1	0.48
	PM3(np)	−99.3 ± 0.9	−19.0 ± 0.3	−62.9 ± 1.1	0.49
	MNDO(np)	−102.5 ± 0.8	−18.4 ± 0.4	−63.3 ± 0.7	0.48

[a] Neutral substrate (S) and ionized, i.e., protonated, substrate (SH^+). [b] (np) = nonpolarization model. [c] Includes a Born correction in eq 16 of −13.8 kcal/mol (r_{cut} = 12 Å, ϵ = 79) for ionized substrate.

TABLE 4: Net PM3 Charge on the Neutral and Ionized Side Chain of Glu30 [L−R−COOH and L−R−COO$^-$), R = $(CH_2)_2$] in Unbound and Substrate-Bound Forms Computed for the Various Choices of Link (L) Atoms

L	L−R−COOH	L−R−COO$^-$	L−R−COOH:S	L−R−COO$^-$:SH$^+$
H	−0.052	−1.028	−0.360	−0.815
F	0.145	−0.816	−0.173	−0.628
H*[a]	0.044	−0.929	−0.136	−0.709
F*[b]	0.044	−0.926	−0.280	−0.738
H-Glu[c]	0.027	−0.930	−0.291	−0.740
F-Glu[c]	0.043	−0.900	−0.257	−0.710
CH_3-Glu[c]	0.011	−0.928	−0.183	−0.743

[a] H* = hydrogen atom with core charge scaled by 0.92. [b] F* = fluorine atom with core charge scaled by 1.03. [c] Reference system. All atoms in the Glu30 residue plus H, F, or CH_3 groups in place of the peptide linkages at Tyr31 and Asn29 (see text and Figure 1).

TABLE 5: PM3 Electrostatic/Polarization Components ΔG_{ele} (kcal/mol) (eq 16) for the Interaction between Neutral and Ionized Forms of the Glu30 Side Chain with the MM Part of the Solvated DHFR System with No Substrate in the Active Site (EH and E$^-$ Models, See Figure 2)

QM species[a]	L[b]	$\langle E_{ele}\rangle$	$\langle E_{vdW}\rangle$	ΔG_{ele}[c]	$\Delta G_{ele}/\langle E_{ele}\rangle$
neutral	H	−46.5 ± 1.4	−5.6 ± 0.3	−17.7 ± 0.2	0.38
	F	−43.2 ± 1.6	−6.9 ± 0.6	−16.0 ± 1.8	0.37
neutral[d]	F*	−30.1 ± 2.2	−6.8 ± 0.8	−11.3 ± 0.9	0.37
neutral (np)	H	−11.0 ± 0.9	−8.3 ± 0.1	−4.6 ± 0.5	0.42
	F	−13.8 ± 1.5	−9.8 ± 0.8	−5.5 ± 0.1	0.40
ionized	H	−528.5 ± 3.4	5.8 ± 0.1	−165.8 ± 5.3	0.30
	F	−179.6 ± 2.5	−4.1 ± 0.2	−73.6 ± 4.2	0.38
	H*	−508.1 ± 4.5	4.9 ± 0.1	−154.2 ± 6.7	0.29
	F*	−218.9 ± 2.6	−3.8 ± 0.2	−84.5 ± 2.5	0.36
ionized[d]	F*	−193.3 ± 1.6	−5.2 ± 0.2	−74.7 ± 2.5	0.36
ionized (np)	H	−140.0 ± 1.9	−5.5 ± 0.4	−63.8 ± 3.9	0.42
	F	−108.9 ± 1.1	−5.4 ± 0.1	−51.2 ± 4.2	0.43
	H*	−146.9 ± 1.3	−4.9 ± 0.3	−77.1 ± 0.4	0.49
	F*	−130.3 ± 1.5	−5.5 ± 0.2	−59.2 ± 2.6	0.41

[a] Neutral (L−R−COOH) and ion (L−R−COO$^-$) forms of Glu30 side chain. (np) = nonpolarization model. [b] Link atom: core charges scaled by 0.92 and 1.03 for H* and F*, respectively (Table 4). [c] Includes Born correction in eq 16 of −4.9 kcal/mol (r_{cut} = 34 Å, ϵ = 79). [d] Using Cornell et al.[26] force field for protein.

the neutral species, the L atom characteristics have an insignificant effect on the free energy. For the anion, however, the variations are very much larger. In particular, the use of H and H* atoms for L leads to a significant overpolarization that would clearly predict a value for ΔG_1 far in excess (∼ −80 kcal/mol) of the experimental estimate of −1.7 kcal. In contrast, the free energies for L=F and F* obtained using the polarization model, and results for L=H, H*, F, and F* obtained using the nonpolarization model behave more predictably. We suspect, therefore, that, at least for this particular system, L atoms based on H will also not produce reliable predictions of ΔG_2 for the polarization model. As electronic polarization has a significant effect on both ΔG_{ele} and $\langle E_{ele}\rangle$, it consistently gives lower $\Delta G_{ele}/\langle E_{ele}\rangle$ values than the nonpolarization model in agreement with previous QM/MM studies on ion solvation.[29] It is apparent that the force fields of Weiner et al.[24,25] and Cornell et al.[26] produce significantly different interaction energies E_{ele}. This is not surprising due to the differences in atomic charges, and such differences between MM force-field in general have been noted in electrostatic model calculations on the stability of protein salt bridges.[44]

In Table 6 we report the average QM and QM/MM energy components (eqs 10 and 11) obtained from the MD simulations on the substrate−DHFR complexes. Note that the differences between neutral and ionized pairs for $\langle E_{vdW}\rangle$ are minimal, justifying the neglect of these terms in the free energy calculations. Also, the corresponding differences for $\langle E_{QM}\rangle$ are consistent with the energy change, ΔE_2 (PM3), for the noninteracting QM system (Table 2), i.e., the neutral form is lower in energy. For a given model, the average electrostatic interaction energies $\langle E_{ele}\rangle$ together with the $\Delta G_{ele}/\langle E_{ele}\rangle$ values (Table 5) can be used to estimate, via eq 18,[29,31,32] the free energy difference

$$\Delta G_2^{int} = \alpha[\langle E_{ele}\rangle(\text{ionized}) - \langle E_{ele}\rangle(\text{neutral})] \qquad (19)$$

between neutral and ionized pairs. Excluding the polarization model calculations using H link atoms, we obtain ΔG_2^{int} values ranging from −4.9 kcal/mol for polarization (α = 0.37) to −2.1 kcal/mol for nonpolarization (α = 0.42) models. As for the simulations on E^- (Table 5), we obtain a significantly larger polarization energy for H and H* link atoms, compared with F and F*, which leads (L=H) to ΔG_2^{int} = −11.7 kcal/mol.

The QM/MM contribution ΔG_2^{int} to the relative free energy of binding can also be computed using the coordinate-coupled FEP method (eq 9). For these calculations we used L=F* for the two protein force fields, except L=F was used for the neutral-pair simulation with the Weiner et al. force fields, based on the results in Tables 4, 5, and 6. As discussed above, we can also use results in Tables 3 and 5 to estimate ΔG_1 for comparison with the experimental estimate. The results are summarized in Table 7. As a further test of our predictions, we performed simulations for the H-bonded QM system only in a purely aqueous environment (i.e., in the absence of protein). The aqueous solvent effects on the relative stability of the two forms were computed by placing the H-bonded substrate and Glu30 side chain at the center of a 20 Å radius sphere of TIP3P water molecules and performing the FEP simulations. The resulting ΔG_2^{int} can be compared with ab initio self-consistent reaction field (SCRF) calculations on the similar formyl/guanidine system in water.

For the Weiner et al.[24,25] force field, we obtain ΔG_2^{int} = −2.5 ± 0.2 kcal/mol, and for the Cornell et al.[26] force field ΔG_2^{int} = −3.6 ± 0.2, which are comparable with the estimates derived from $\langle E_{ele}\rangle$ values in Table 6 noted above. Adding these to ΔE_2 = 6.4 ± 0.7 kcal/mol obtained above (mean of MP2/6-311+G**//MP2/6-31G* results in Table 2) and correcting for ΔG_1 = 1.7± 1.4 gives $\Delta\Delta G_{bind}$ of 5.6 ± 2.1 and 4.5 ± 2.1 kcal/mol, respectively. Thus, as for the nonenzyme-bound system, the neutral-pair complex is predicted to be the more thermodynamically stable. For the Weiner et al.[24,25] and Cornell et al.[26] force fields, we obtain ΔG_1 = −3.5 ± 2.5 and 1.6 ± 2.4 kcal/mol, respectively, compared with the experimental estimate of −1.7 ± 1.4 kcal/mol. Thus, the predicted free energies agree with the experimental estimate within the error limits, which are, however, relatively large. The value of ΔG_2^{int} = −8.6 kcal/mol in aqueous solution is in good agreement with the value of −11.1 kcal/mol obtained by Zheng and Ornstein[8] for the smaller formyl/guanidine complex. Moreover, the −3.8 kcal/mol value obtained by Zheng and Ornstein[8] for a low dielectric constant medium ($\epsilon \cong 2$) is close to our FEP calculated values of −2.5 and −3.6 kcal/mol. It is also interesting to compare our results with the theoretical study of Cannon et al.[13] for binding of the carboxyl (Asp27 in *E. coli*) group with methotrexate inhibitor. Their H-bond model is also based on ab initio QM methods for the H-bonded system but with Poisson−Boltzmann calculations for the electrostatic effects of the protein/solvent environment. For low dielectric constants (ϵ < 10), they find that the neutral pair is the more stable complex. However, it is well known that the stabilities of ion pairs depend critically on the different microenvironments within

TABLE 6: QM/MM ($\langle E_{ele}\rangle + \langle E_{vdW}\rangle$) and QM ($\langle E_{QM}\rangle$) and Total Averaged Energies ($\langle E_{QM}\rangle + \langle E_{ele}\rangle + \langle E_{vdW}\rangle$) (kcal/mol) for Neutral-Pair and Ionized-Pair Complexes (EH:S and E^-:SH^+ Complexes, See Figure 2)

QM species[a]	L[b]	$\langle E_{ele}\rangle$	$\langle E_{vdW}\rangle$	$\langle E_{QM}\rangle$	$\langle E_{total}\rangle$
neutral pair	H	-89.0 ± 1.8	-29.9 ± 0.9	-37.5 ± 1.9	-156.4 ± 2.0
	F	-72.5 ± 2.6	-31.3 ± 0.7	-81.8 ± 2.5	-185.6 ± 2.6
neutral pair[c]	F*	-57.4 ± 1.1	-40.0 ± 0.7	-82.3 ± 0.6	-179.8 ± 1.5
neutral pair (np)	H	-26.7 ± 1.4	-33.2 ± 1.1	-63.0 ± 1.3	-122.9 ± 1.6
	F	-27.5 ± 1.2	-33.4 ± 0.7	-103.3 ± 1.3	-164.2 ± 1.3
ionized pair	H	-120.6 ± 2.5	-29.7 ± 0.1	-8.7 ± 1.4	-159.0 ± 2.0
	F	-85.8 ± 1.9	-31.4 ± 0.8	-56.4 ± 0.5	-173.6 ± 1.5
	H*	-148.4 ± 2.0	-28.5 ± 1.2	-5.2 ± 0.6	-182.1 ± 1.2
	F*	-86.6 ± 2.0	-32.3 ± 1.8	-55.8 ± 1.2	-174.7 ± 2.1
ionized pair [c]	F*	-68.6 ± 0.7	-38.5 ± 1.1	-67.9 ± 0.7	-174.9 ± 1.0
ionized pair (np)	H	-31.7 ± 1.7	-33.7 ± 0.2	-41.6 ± 1.5	-107.0 ± 1.6
	F	-34.1 ± 1.3	-32.6 ± 0.5	-83.7 ± 1.9	-150.4 ± 1.7
	H*	-37.5 ± 1.8	-31.5 ± 0.6	-49.5 ± 1.6	-118.5 ± 1.8
	F*	-33.5 ± 0.8	-33.8 ± 0.6	-76.8 ± 0.5	-144.1 ± 0.8

[a] Neutral-pair (L−R−COOH:S) and ion-pair ($L-R-COO^-:SH^+$) complexes. (np) = non polarization model. [b] Link atom: H*, F* have core charges scaled by 0.92 and 1.03, respectively (Table 4). [c] Using Cornell et al.[26] force field for protein.

TABLE 7: Electrostatic Components for the Relative Free Energies (kcal/mol) of the Neutral-Pair and Ion-Pair Enzyme−Substrate Complexes ΔG_2^{int}, the Electrostatic Free Energy Change for Ionization of the Unbound Forms ΔG_1^{int}, and the Corresponding Totals Given by $\Delta\Delta G_{bind}$ and ΔG_1, Respectively

	ΔG_2^{int} [a]	$\Delta\Delta G_{bind}$ [b]	ΔG_1^{int} [c]	ΔG_1 [d]
protein/solvent[e]	-2.5 ± 0.2	5.6 ± 2.1	-106.9 ± 2.5	-3.5 ± 2.5
protein/solvent[f]	-3.6 ± 0.2	4.5 ± 2.1	-101.8 ± 2.4	1.6 ± 2.4
solvent[g]	-8.6 ± 0.5	-0.5 ± 2.1	N/A	N/A
experiment (see text)	N/A	N/A	N/A	-1.7 ± 1.4

[a] ΔG_2^{int} calculated using eq 9 (polarization model with L=F*, except with L=F for neutral-pair simulation with Weiner et al.[24,25] force field). [b] $\Delta\Delta G_{bind} = \Delta G_2 + 1.7$ kcal/mol (eq 7), where $\Delta G_2 = \Delta E_2 + \Delta G_2^{int}$, and with $\Delta E_2 = 6.4 \pm 0.7$ kcal/mol (Table 2). [c] ΔG_1^{int} calculated using eq 15 (polarization model with L=F*, except with L=F for EH simulation with Weiner et al.[24,25] force field, from results in Tables 3 and 5). [d] $\Delta G_1 = \Delta E_1 + \Delta G_1^{int}$, where $\Delta E_1 = 103.4 \pm 2.6$ kcal/mol (Table 2). [e] Weiner et al.[24,25] force field for DHFR. [f] Cornell et al.[26] force field for DHFR. [g] Hypothetical solvent-only system (see text).

proteins.[45,46] A recent theoretical study[9] on acetate−methylguanidinium ions as a model for Asp/Glu−Arg salt bridges in a wide selection of proteins suggests that the ion-pair forms may predominate.

Correlation with Experimental Data. Spectrofluorimetric studies[47] at neutral pH of the inhibitor analogue 6,8-dimethyl-N5-deazapterin bound to the recombinant human DHFR·NADPH complex show spectra characteristic of the protonated form of the ligand, i.e., suggestive of an ion-pair complex. Our recent UV−vis spectral difference data for binding of a tighter-binding analogue of this inhibitor series show more clearly that the ligand is bound as the cation (Fan et al., unpublished results). However, note that 6,8-dimethyl-N5-deazapterin has a pK_a of 6.6[10] compared with 5.2 for 8-methylpterin[4] and ~5.5 for the inhibitor methotrexate. While the higher pK_a of 6.6 would clearly increase ΔG_1 to favor the neutral pair (eq 6), the finding of bound cation suggests a proportionately greater contribution from ΔG_2 in order to stabilize the ion-pair form. While these spectral results alone cannot rule out the EH:SH^+ complex, such a complex is inconsistent with literature data on the pK_a of the Glu30 group reviewed previously. The finding of Cannon et al.[13] for methotrexate binding also apparently contradicts NMR experiments[48−50] and pH-dependent competitive binding studies[51] which indicate the ion pair and not the neutral pair is the more stable. Cannon et al.[13] suggest the existence of a single potential well or the effects of aromatic rings in contact with the pteridine ring as possible reasons for the discrepancy. These possibilities merit further study for our system. The latter effects associated with charge transfer and mutual polarization of the ring systems may well reduce the free energy gap between neutral-pair and ion-pair forms, but our current model cannot represent these subtle effects as it does not allow for polarization of the MM system or charge transfer between QM and MM systems. However, our recent feasibility studies on the use of the localized MO linear-scaling semiempirical method,[52] implemented in MOPAC2000,[53] for calculations on DHFR ligand complexes suggest such studies incorporating charge transfer and mutual polarization will be possible (Titmuss et al., unpublished results). The possibility of a single potential well in the enzyme seems less likely due to the difference between ΔE_2 and ΔG_2^{int}. However, for the type of H-bonded systems considered in the present work, it is clear that polar environments can reduce the free energy of activation for the transfer of the proton to form the ion-pair complex.[8,9,13,45]

Conclusions

The semiempirical PM3 method yields geometries for the H-bonded complexes in reasonable agreement with ab initio QM methods and is sufficient for use in simulation protocols for the purpose of free energy calculation. However, the results show that semiempirical methods do not provide a quantitative prediction of the energy difference between the neutral-pair and ion-pair complexes in a vacuum. The free energy difference in a vacuum was estimated using high level ab initio QM methods, while the effects of the protein/solvent systems were modeled using QM/MM methods based on semiempirical QM. The results of the present QM and QM/MM calculations are consistent with other theoretical studies on similar systems in that the neutral pair is the preferred complex in a vacuum, but polar interactions favor the larger dipole of the ion pair, reducing this neutral-pair stability. In a vacuum the energy cost of ionizing the monomers exceeds the stabilizing effect of the increased H-bond interaction in the ion pair. The protein can provide the stabilization required to lower the free energy of the ion pair relative to the neutral pair. The QM/MM calculations predict such a lowering of the relative free energy in the enzyme but not to the extent that the ion pair becomes thermodynamically more stable. However, based on the level of uncertainty in the computations and experimental estimate of pK_E, a small free energy difference is quite possible, which may indicate a small energy barrier for the interconversion of the neutral-pair and ion-pair complexes. As the free energy difference for the

enzyme-bound pair forms appears small, further detailed ab initio level QM studies of the proton transfer are being undertaken. Also, a more accurate treatment of long-range electrostatic effects and enzyme environmental effects allowing charge transfer and mutual polarization between ligand and enzyme is required to define how large these additional perturbations are and whether they might modulate the relative stabilities of the pair forms.

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