Structure of Dihydrofolate When Bound to Dihydrofolate Reductase

Hua Deng and Robert Callender*

Contribution from the Department of Biochemistry, Albert Einstein College of Medicine, Bronx, New York 10461

Received April 30, 1998

Abstract: The Raman spectrum of dihydrofolate (H_2folate) complexed with dihydrofolate reductase (DHFR) and $NADP^+$, believed to be an accurate mimic of the productive DHFR/NADPH/H_2folate complex involved in the reaction catalyzed by DHFR, contains bands associated with stretch motions of N5═C6 of bound substrate. However, the assignments of these bands, which are of considerable importance to understanding enzymic mechanism and substrate binding, are in doubt. The vibrational spectra of dihydrofolate, alone and complexed with water and with acetate, have been calculated using quantum mechanical ab initio procedures in order to assign the observed bands. Several structural conclusions follow from these calculations. N5 of H_2folate when bound to DHFR/$NADP^+$ has a pK_a of 6.5. From an examination of deuteration shifts, the immediate environment of N5 of substrate is quite hydrophobic; there does not appears to be an immediate water molecule near enough to form a hydrogen bond with a protonated N5−H. It is suggested that the carboxyl group of Asp27, the only ionizable group in the DHFR binding site, is ionized at physiological pH values and does not donate a proton to substrate during enzymic catalysis. Overall, the results suggest that a major structural attribute of DHFR is to raise the pK_a of N5 4 units when H_2folate binds in the productive ground-state ternary complex. Such a strategy would appear to be responsible for a substantial portion of the rate enhancement in the reaction catalyzed by DHFR.

Introduction

Dihydrofolate reductase (5,6,7,8-tetrahydrofolate:NADP oxidoreductase, EC 1.5.1.3, DHFR) catalyzes the reduction of 7,8-dihydrofolate (H_2folate) to 5,6,7,8-tetrahydrofolate (H_4folate) by facilitating the addition of a proton to N5 of H_2folate and the transfer of a hydride ion from NADPH to C6 (see Figure 1). DHFR is an important enzyme as it is required for the production of purines, thymidylate, and a few amino acids, and it is the target for both antitumor and antimicrobial drugs. Hence, its catalytic mechanism has been under intense scrutiny for some time.

However, despite extensive kinetic, site-directed mutagenesis, X-ray crystallographic, and theoretical molecular modeling studies that have been performed on this enzyme, the reaction mechanism of DHFR is still under debate.[1−3] In fact, the electronic nature of the ground state within the active site in the productive DHFR/NADPH/H_2folate complex is unclear, and this is key to an understanding of the reaction mechanism of DHFR. For example, X-ray crystallographic studies have revealed that the only ionizable group near the pteridine ring is a carboxylic acid, equivalent to Asp27 in *Escherichia coli* DHFR.[4−6] The oxygens of Asp27 are within the hydrogen-bonding distance from N3 and the 2-amino group of the pterin ring but at least 5 Å away from N5. Thus, this group is unable to donate directly a proton to N5 during the reaction. It is possible that there are one or more binding site water molecules interacting with the pterin ring and that the proton that ends up on N5 arrives from the binding site carboxyl group via these water molecules. In this regard, the rate of hydride transfer from NADPH to H_2folate has a pK_a of around 6.5,[7,8] and it has

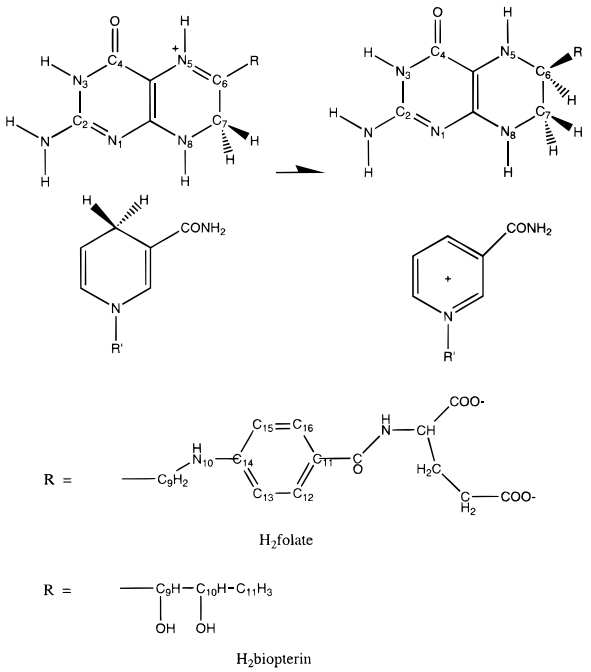

Figure 1. Reaction that is catalyzed by dihydrofolate reductase and structures of the molecules used in the analysis.

(1) Kraut, J.; Matthews, D. A. In *Biological Macromolecules and Assemblies: Active Sites of Enzymes*; Jurnak, F. A., McPherson, A., Eds.; John Wiley: New York, 1987; Vol. 3, pp 1−72.
(2) Blakley, R. L. *Adv. Enzymol.* **1995**, *70*, 23−102.
(3) Cannon, W. R.; Garrison, B. J.; Benkovic, S. J. *J. Am. Chem. Soc.* **1997**, *119*, 2386−2395.
(4) Bolin, J. T.; Filman, D. J.; Matthews, D. A.; Hamlin, R. C.; Kraut, J. *J. Biol. Chem.* **1982**, *257*, 13650−13662.
(5) Filman, D. J.; Bolin, J. T.; Matthews, D. A.; Kraut, J. *J. Biol. Chem.* **1982**, *257*, 13633−13672.
(6) Matthews, D. A.; Alden, R. A.; Bolin, J. T.; Freer, S. T.; Hamlin, R.; Xuong, N.; Kraut, J.; Poe, M.; Williams, M.; Hoogsteen, K. *Science* **1977**, *197*, 425−455.

S0002-7863(98)01497-8 CCC: $15.00
Published on Web 07/23/1998

been supposed that this has to do with an elevated pK_a of the active site carboxyl group. However, a recent Raman study concluded that the pK_a of Asp27 in *E. coli* DHFR is less than 5.0.[9]

Another key issue has to do with whether N5 is protonated in the ground state of the DHFR/NADPH/H$_2$folate complex and, hence, precedes hydride transfer or occurs later in the reaction pathway. A recent study using difference Raman spectroscopy of the DHFR/NADP$^+$/H$_2$folate complex,[10] believed to be an accurate mimic of the productive DHFR/NADPH/H$_2$folate complex, identified two N5═C6 stretch "marker" bands indicating unprotonated (1650 cm^{-1}) or protonated (1675 cm^{-1}) N5. The assignments were based on isotope labeling, comparisons to the spectra of solution models, and the positioning of the bands. On the basis of these assignments, N5 in the binary DHFR/H$_2$folate complex was found to be unprotonated at near neutral pH values. In the ternary DHFR/NADP$^+$/H$_2$folate complex, however, another band at 1675 cm^{-1} was observed. A titration study, using the 1650 and protonated 1675 cm^{-1} marker bands as indicators for unprotonated and protonated species, respectively, showed that the pK_a of N5 is raised from 2.6 in solution[11] to 6.5 in this complex.[10]

However, an alternative interpretation of the Raman results was published recently by Cannon et al.,[3] based on quantum chemical vibrational frequency calculations. Their conclusion is that the N5 of H$_2$pterin is likely to be unprotonated inside DHFR because the N5 protonated form of H$_2$folate is far less favorable energetically compared to other tautomeric forms, especially the 4-hydroxy, N5 unprotonated form (see Figure 2, structure **II**). Furthermore, their calculations show that the N5═C6 stretch frequency of the 4-hydroxy H$_2$pterin is higher than 4-oxy, N5 unprotonated H$_2$pterin, which provided a rationale for the 1675 cm^{-1} band found in the Raman study. On this basis, the two N5═C6 stretch peaks observed in the Raman spectrum of H$_2$folate in the DHFR/NADP$^+$/H$_2$folate complex were assigned to arise from the 4-hydroxy and 4-oxy, N5 unprotonated H$_2$pterin rings, rather than from the 4-oxy, N5 unprotonated and protonated H$_2$pterins. This assignment suggests that the H$_2$pterin ring of H$_2$folate is 4-hydroxy, N5 unprotonated in the Michaelis complex, and the reaction mechanism involves a concerted hydride transfer from C4 of NADPH to C6 of H$_2$pterin and a number of proton transfers, from protonated Asp27 to N3 or the pteridine ring, from the 4-hydroxy to an active site water molecule, and from this water molecule to N5 of H$_2$pterin.[3]

However, in this interpretation of the N5═C6 stretch bands in the Raman spectrum of the ternary complex, a critical observation of the N5═C6 stretch mode was not discussed. When the DHFR ternary complex is suspended in D$_2$O, the 1675 cm^{-1} N5═C6 stretch band shifts down by 14 cm^{-1} while the 1650 cm^{-1} N5═C6 stretch band is unchanged.[10] It is well-known that a C═N stretch mode generally does not shift when the sample is suspended in D$_2$O, unless the nitrogen of the C═N bond is protonated. However, the unprotonated C═N stretch motion in a ring molecule can be coupled with a remote N−H bending motion, so that the deuterium exchange of the hydrogen on the remote nitrogen could cause a frequency shift of the unprotonated C═N stretch mode. For example, it has been found that the unprotonated C═N stretch mode has considerable contribution from a remote N−H bending motion in the adenosine deaminase bound inhibitor, 1-hydroxyl-1,6-dihydropurine ribonucleoside, so that a 8 cm^{-1} shift of the unprotonated C═N stretch mode upon sample deuteration is observed.[12] Cannon et al. showed that the C6═N5 stretch vibrational mode of the 4-hydroxy, N5 unprotonated H$_2$pterin model compound does contain a contribution from N8−H bending.[3] However, whether this coupling between C6═N5 stretch and the remote N8−H bending results in a significant shift of the C6═N5 stretch mode upon H$_2$pterin deuterium exchange has not been reported in their study.

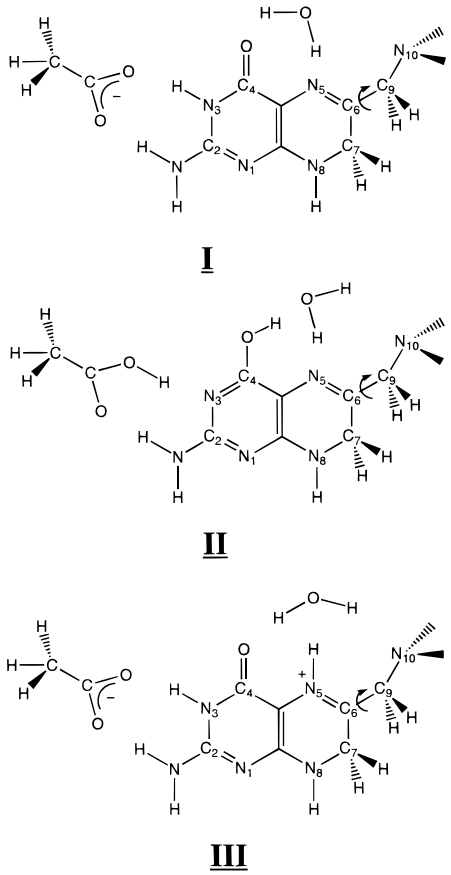

Figure 2. Molecular structures of those molecules for which ab initio calculations were performed showing the approximate locations of the acetate and water molecules relative to the model pterin ring: **I**, 4-oxy, N5 unprotonated; **II**, 4-hydroxy, N5 unprotonated; **III**, 4-oxy, N5 protonated. Syn and anti conformations are achieved by a rotation around the C6−C9 bond, as indicated by the arrow.

Since the Raman results are used as the experimental support for both proposed reaction mechanisms, which are mutually exclusive, correct interpretation of the Raman data is important for elucidating mechanism. Thus, we have conducted extensive ab initio vibrational frequencies calculations on various model systems of H$_2$pterin in order to find out which of the interpretations of the Raman data is correct. In these calculations, the N5═C6 stretch frequency and its shift upon isotopic labeling are determined to study their responses due to the changes in the conformation/tautomerization of H$_2$pterin and the interactions with external carboxyl group and/or water molecule. The calculations take into account the observed deuteration shifts and probe what interactions at the DHFR binding site might be

(7) Fierke, C. A.; Johnson, K. A.; Benkovic, S. J. *Biochemistry* **1987**, *26*, 4085−4092.
(8) Morrison, J. F.; Stone, S. R. *Biochemistry* **1988**, *27*, 5499−5506.
(9) Chen, Y.-Q.; Kraut, J.; Callender, R. *Biophys. J.* **1997**, *72*, 936−941.
(10) Chen, Y.-Q.; Kraut, J.; Blakley, R. L.; Callender, R. *Biochemistry* **1994**, *33*, 7021−7026.
(11) Maharaj, G.; Selinsky, B. S.; Appleman, J. R.; Perlman, M.; London, R. E.; Blakley, R. L. *Biochemistry* **1990**, *29*, 4554−4560.
(12) Deng, H.; Chan, A. Y.; Bagdassarian, C. K.; Estupinan, B.; Ganem, B.; Callender, R. H.; Schramm, V. L. *Biochemistry* **1996**, *35*, 6037−6047.

Table 1. Observed Raman Bands of the C6═N5 Stretch Mode of H_2folate in Solution and in the DHFR/$NADP^+$/H_2folate Ternary Complex and Its Model Compound H_2biopterin in Solution at pH 1.6[a]

	H_2biopterin in soln, N5 protonated	H_2folate in soln, N5 unprotonated	H_2folate in T-complex, N5 protonated	H_2folate in T-complex, N5 unprotonated
native	1671	1655	1675	1650
^{13}C6		−35	−32	−31
^{15}N5	−27			
ND	−25	−1	−14	0

[a] The frequencies are in cm^{-1}. The numbers for the labeled molecules are expressed as frequency shifts relative to that of the unlabeled molecule. The T-complex is the ternary DHFR/$NADP^+$/H_2folate complex, as reported in Chen et al.[10]

important for affecting the vibrational spectra of the bound substrate. Several structural conclusions about the binding site of DHFR and its interaction with bound substrate follow from the results.

Methods

Raman Spectroscopy. The materials and methods for the obtaining the Raman spectra of H_2folate in the DHFR/$NADP^+$/H_2folate complex have been previously reported in detail.[10] $NADP^+$ was obtained from Boehringer Mannheim Co., and 7,8-dihydrofolate (H_2folate) was purchased from Sigma Chemical Co. [6-^{13}C]H_2folate was prepared from labeled folate.[13] *E. coli* DHFR was purified from *E. coli* strain CV634 containing the plasmid pCV29 (wt form I) by using a methotrexate affinity resin (purchased from Pierce). A specially fabricated split-cell cuvette (Hellma Cells) was used to hold the sample. About 25 μL of the binary complex was loaded into one side of the cuvette, while the same amount of apo-enzyme was loaded into the other side, or the ternary complex was loaded into one side and the binary into the other. The cuvette was transferred to a cuvette holder for measurement, maintained at 4 °C. About 120 mW of the 568.2 nm line from a Coherent 2000-CR krypton ion laser (Coherent Radiation Inc., Palo Alto, CA) was used to excite Raman scattering. Data were collected by a Mac IIfx computer (Apple, Cupertino, CA) interfaced with a CCD detector (Princeton Instruments model LN/CCD-1152UV with a ST-135 CCD controller) which is coupled to a Triplemate spectrometer (Spex Industries, Metuchen, NJ). The spectrum of the bound substrate was then obtained by subtraction of one data set from another.

Computational Procedures. Since we are only interested in the vibrational modes of H_2pterin, especially the C═N stretch mode, the folate molecule is truncated at N10 in our model compounds to save computer time. N10 is retained to study the possible interaction between N10 and N5. Three forms of H_2pterin were studied in detail (Figure 2): 4-oxy, N5 unprotonated, 4-hydroxy, N5 unprotonated, and 4-oxy, N5 protonated. The interactions between the pteridine ring and Asp27 were modeled by placing an acetic acid (or acetate) in the vicinity of N3 and the 2-amino group. The hydrogen bonding between N5 and a water molecule is also modeled as show in Figure 2.

The ab initio calculations were carried out on the models by Hartree−Fock method with the 6-31g** basis set, as implemented in Gaussian 94.[14] The geometry of the model compound complexes were first optimized, and then the vibrational normal modes were calculated using the same basis set. True local minima on the potential surface of the complexes for the geometry optimized complexes were verified from the vibrational frequency calculations in which no imaginary frequency was found. In all cases, a stable structure of the complex is achieved without any geometry constraint. To study the effect of the hydrogen bonding between a water molecule and N5, vibrational mode calculations on a series of H_2pterin−water complexes with fixed water−N5 distances were performed. In these calculations, the geometries of the complexes were optimized constraining the fixed water−N5 distance, and the vibrational frequencies were then calculated on these partially optimized complexes.

Results

The C6═N5 stretch mode and its shifts upon isotopic incorporation observed in the Raman spectra[10] of H_2folate at pH 7 (N5 unprotonated) in solution, H_2biopterin at pH 1.6 (N5 protonated) in solution, and the low- and high-pH forms (pK_a = 6.5) of H_2folate in the ternary DHFR/$NADP^+$/H_2folate complex are summarized in Table 1. The solubility of H_2folate at low pH is not sufficient to obtain a decent Raman spectrum of its N5 protonated form. A solution model, H_2biopterin, is used here since it contains the same pteridine ring as H_2folate and the pK_a (=2.6) of its N5 in solution is the same as that of H_2folate,[10] but it is much more stable and soluble at low pH. The C6═N5 stretch mode can be positively identified by its shift upon either ^{13}C or ^{15}N labeling. For a truly localized C═N stretch with a frequency of 1650 cm^{-1}, its ^{15}N and ^{13}C shift should be 25 and 35 cm^{-1}, respectively. The observed shifts of the C6═N5 stretch modes listed in Table 1 (those for ^{13}C6 for the DHFR/$NADP^+$/H_2folate complex are evident in Figure 3c) indicate that all of them are reasonably localized. The deuterations listed in Table 1 were brought about by suspending the samples in D_2O so that all exchangeable protons have been deuterated, including that on N5.

Figure 3a shows the Raman difference spectrum of the DHFR/$NADP^+$/H_2folate complex minus that of the DHFR/$NADP^+$ binary complex (hence the bands in Figure 3a show the spectrum of bound H_2folate). Panel b shows the similar spectrum of deuterated sample while panel c shows the ^{13}C6 isotope edited spectrum of the DHFR/$NADP^+$/H_2folate complex. These spectra were mentioned in Chen et al.[10] but not shown. We graph them here because they are crucial to showing that (1) the 1650 cm^{-1} band is unaffected by deuteration while the 1675 cm^{-1} band shifts down 14 cm^{-1} and (2) both bands involve the C6═N5 stretch since both are shifted by ^{13}C6. The 1608 cm^{-1} band is not due to pterin ring motions;[10] hence, it is unaffected by isotopic incorporation into the ring.

Protonated and unprotonated C═N stretch modes can be distinguished normally on the basis of the frequency shift upon formation of the deuterated moiety, −C═ND−. The N−H bending motion usually couples with the C═N stretch motion because the stretch and bend frequencies are often quite close. Such mode−mode coupling "pushes" apart the frequencies of the two modes from their intrinsic values. For C═ND, the N−D bend frequency downshifts substantially and is no longer significantly coupled to the C═N stretch. As a result, the C═N stretch frequency shifts to its intrinsic value. For example, deuterium exchange of various −C═NH− compounds under different conditions has been observed to result in 12−35 cm^{-1} downward shifts of the C═N stretch.[15−17] For N5 protonated

(13) Selinsky, B. S.; Perlman, M. E.; London, R. E.; Unkefer, C. J.; Mitchell, J.; Blakley, R. L. *Biochemistry* **1990**, *29*, 1290−1296.

(14) Frisch, M. J.; Trucks, G. W.; Head-Gordon, M.; Gill, P. M. W.; Wong, M. W.; J. B. Foresman; Johnson, B. G.; Schlegel, H. B.; Robb, M. A.; Replogle, E. S.; Gomperts, R.; Andres, J. L.; Raghavachari, K.; J. S. Binkley; Gonzalez, C.; Martin, R. L.; Fox, D. J.; Defrees, D. J.; Baker, J.; Stewart, J. J. P.; Pople, J. A. *Gaussian 94, Revision D*; Gaussian, Inc.: Pittsburgh, PA, 1994.

(15) Aton, B.; Doukas, A.; Narva, D.; Callender, R.; Dinur, U.; Honig, B. *Biophys. J.* **1980**, *29*, 79−94.

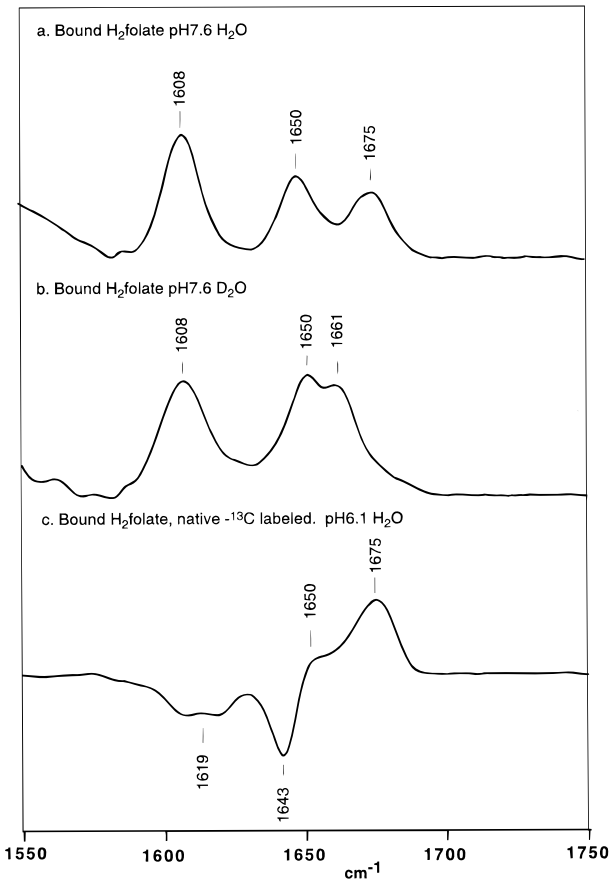

Figure 3. (a) Difference Raman spectra of the ternary complex of DHFR with NADP$^+$ and H$_2$folate ([DHFR/NADP$^+$]/[H$_2$folate] = 3.4/2.5 mM) minus the spectrum of DHFR/NADP$^+$ at 4 °C in 20 mM Tris buffer containing 0.5 M KCl, pH 7.6. The difference bands are about 10−30% of the protein amide-I band and have been assigned to bound H$_2$folate.[10] (b) The same as (a) except the samples have been suspended in D$_2$O so that all the exchangeable protons of the sample have been deuterated. (c) ^{13}C6 isotope edited Raman difference spectrum of DHFR/NADP$^+$/H$_2$folate at pH 6.1 that is the difference spectrum formed by subtracting the spectrum of DHFR/NADP$^+$/[^{13}C6]H$_2$folate from that of DHFR/NADP$^+$/[^{12}C6]H$_2$folate; only modes whose motions involves C6 show up in the ^{13}C6 isotope edited difference spectrum.

H$_2$biopterin in solution, the deuterium shift of the C═N stretch is 25 cm^{-1} (Table 1), but the deuterium shift of the C═N stretch mode of H$_2$folate in the DHFR ternary complex at 1675 cm^{-1} is smaller (14 cm^{-1}; Figure 3b and Table 1). The following questions are in order. If the observed 1675 cm^{-1} band observed for the DHFR/NADP$^+$/H$_2$folate ternary complex is due to the unprotonated C6═N5 stretch of H$_2$pteridine ring, what is the cause that results in a 14 cm^{-1} shift upon sample deuteration? If this band is due to the N5 protonated C6═N5 stretch, why is its deuterium shift significantly smaller than its solution model compound?

In an attempt to provide answers for these questions and other issues related to the vibrational frequency shifts that occur when H$_2$folate binds to DHFR, we have conducted ab initio vibrational frequency calculations on a series model compounds. Before quantitative results from such calculations are presented, we need to point out that the chemical bond lengths, especially the double bonds which contain oxygen or nitrogen, are underestimated by ab initio methods at the Hartree−Fock level compared with those observed values. This is due to the neglect of electron correlation forces in the calculations, the limited basis set, and also that the calculations are "gas phase" which do not treat the interactions with solvent. Consequently, the calculated stretch frequencies of these bonds are overestimated (15−20%) compared to the observed values. Nevertheless, the overestimation of the calculated frequency differences compared to experimentally determined values between different configurations/conformations of a given molecule tends to remain constant. Hence, it is possible to assess the affects of molecule−molecule interactions by employing uniform scaling to the calculated frequencies.

Table 2. Calculated Raman Bands of the C6═N5 Stretch Mode of 4-Oxy, N5 Unprotonated H$_2$pterin in Various Model Complexes Shown in Structure **I** (Figure 2)[a]

molecule complex	isolated	complexed with		
		acetate	water	water and acetate
		A. C6−C9 Anti		
native	1928	1908	1930	1912
^{13}C6	−42	−42	−42	−42
^{15}N5	−25	−25	−25	−25
ND	−1	−1	−1	+2
		B. C6−C9 Syn		
native	1939	1925	1936	1926
^{13}C6	−42	−42	−41	−43
^{15}N5	−25	−25	−25	−25
ND	−1	−2	0	0

[a] The frequencies are in cm^{-1}. The numbers for the labeled molecules are expressed as frequency shifts relative to that of the unlabeled molecule. All frequencies are obtained at the ab initio HF/6-31g** level on fully optimized geometries of the model complexes.

It is known that the dominant species in aqueous solution of H$_2$folate at neutral pH is in the 4-oxy, N5 unprotonated form,[18] which is modeled in structure **I** (Figure 2). The species responsible for the 1650 cm^{-1} band of H$_2$folate in the DHFR/NADP$^+$/H$_2$folate ternary complex is also of this form. Thus, the calculated frequencies of its C6═N5 stretch mode and its isotopic shifts can be compared with experimentally observed values to determine the extent of the overestimation in the calculations. Two conformers, with N10 in either syn or anti to N5 with respect to the C6−C9 bond, are considered in our modeling. Table 2A lists the calculated C6═N5 stretch frequencies and its isotopic shifts of the C6−C9 anti conformer, either from the isolated H$_2$pterin or from one of its three complexes: with an acetate, a water molecule, or with both a water and an acetate, as shown in **I**. In Table 2B, the results from the C6−C9 syn conformer are listed.

The calculated C6═N5 stretch frequencies listed in Table 2 are about 17% higher than the observed values. The calculated shift upon ^{13}C6 labeling is also about 17% larger than the observed shift of the solution H$_2$folate (Table 1). This means that, after a uniform 17% reduction of the calculated frequencies, the ^{13}C isotopic shift of the C═N5 stretch mode is in good agreement with the experimentally observed values. The results in Table 2 show that the C6═N5 stretch frequency is sensitive to at least two factors: conformational change due to a rotation around the C6−C9 bond, which may change the C6═N5 stretch frequency by up to 15 cm^{-1}, and the interaction of N2 and N3 with an external carboxylate group, which may reduce the C6═N5 stretch frequency by up to 20 cm^{-1}. The interaction with a water molecule near N5 (the calculated N5−Hw distance is

(16) Benecky, M. J.; Copeland, R. A.; Hays, T. R.; Lobenstine, E. W.; Rava, R. P.; Pascal, R. A. J.; Spiro, T. G. *J. Biol. Chem.* **1985**, *260*, 11663−11670.

(17) Baasov, T.; Friedman, N.; Sheves, N. *Biochemistry* **1987**, *26*, 3210−3217.

(18) Benkovic, S. J.; Sammons, D.; Armerego, W. L. F.; Waring, P.; Inner, R. *J. Am. Chem. Soc.* **1985**, *107*, 3706−3712.

Table 3. Calculated Raman Bands of the C6═N5 Stretch Mode of 4-Hydroxy, N5 Unprotonated H_2pterin in Various Model Complexes Shown in Structure **II** (Figure 2)[a]

molecule complex	isolated	complexed with acetate	complexed with water	complexed with water and acetate
		A. C6−C9 Anti		
native	1923	1925	1915	1916
^{13}C6	−42	−42	−42	−42
^{15}N5	−25	−25	−25	−25
ND	−1	−1	−1	−1
		B. C6−C9 Syn		
native	1929	1934	1919	1919
^{13}C6	−42	−42	−43	−43
^{15}N5	−25	−25	−25	−25
ND	−1	−1	−1	−1

[a] The frequencies are in cm^{-1}. The numbers for the labeled molecules are expressed as frequency shifts relative to that of the unlabeled molecule. All frequencies are obtained at the ab initio HF/6-31g** level on fully optimized geometries of the model complexes.

Table 4. Calculated Raman Bands of the C6═N5 Stretch Mode of 4-Oxy, N5 Protonated H_2pterin in Various Model Complexes Shown in Structure **III** (Figure 2)[a]

molecule complex	isolated	complexed with acetate	complexed with water	complexed with water and acetate
		A. C6−C9 Anti		
native	1915	1917	1929	1932
^{13}C6	−34	−36	−28	−32
^{15}N5	−27	−27	−24	−27
ND	−20	−20	−30	−27
		B. C6−C9 Syn		
native	1946	1935	1931	1932
^{13}C6	35	−40	−31	−40
^{15}N5	−26	−26	−24	−28
ND	−16	−20	−27	−22

[a] The frequencies are in cm^{-1}. The numbers for the labeled molecules are in the frequency shifts relative to that of the unlabeled molecule. All frequencies are obtained at the ab initio HF/6-31g** level on fully optimized geometries of the model complexes.

2.2 Å for anti conformer and 2.5 Å for syn conformer) has only a relatively small effect (<5 cm^{-1}) on the C6═N5 stretch frequency, as expected, since N5 is unprotonated. Table 2 also shows that the C6═N5 stretch mode is insensitive (shifts <2 cm^{-1}) to the deuterium exchange of the sample in all modeled situations.

Since the energies of the two conformers complexed with a water molecule differ by only about 1 kcal/mol, both may be present in solution. Furthermore, since it is known that the conformation of folate bound in the DHFR ternary complex is closer to C6−C9 syn than to anti,[19] on the basis of the results in Table 2, we can suggest that the observed 5 cm^{-1} downshift of the C6═N5 stretch mode of N5 unprotonated H_2folate upon binding to the DHFR ternary complex (see Table 1) is not due to syn/anti structures but may be the result of the interaction with the ionized carboxylate group of Asp27. Indeed, there is a 3 cm^{-1} upward shift in frequency in this mode when the active site Asp group is mutated to serine.[10]

Tables 3 and 4 show the results of the ab initio calculations on the 4-hydroxy, N5 unprotonated form of H_2pterin (structure **II**; Figure 2) and the 4-oxy, N5 protonated H_2pterin (structure **III**; Figure 2), respectively. The C6═N5 stretch frequencies calculated at the HF/6-31g** level for the anti and syn conformers, as well as for the complexes, are listed in A and B, respectively. It can be seen that the C6═N5 stretch frequency in the 4-hydroxy, N5 unprotonated form of H_2pterin complex with acetate is 9−17 cm^{-1} higher than the corresponding frequency of the 4-oxy, N5 unprotonated H_2pterin (compare column 3 in Tables 2 and 3). This result is consistent with the calculations at HF/3-21g level performed by Cannon et al., which were used to argue that the higher frequency C═N stretch mode observed at 1675 cm^{-1} for H_2folate in the DHFR ternary complex compared to solution may be the result of forming the 4-hydroxy, N5 unprotonated ring from 4-oxy, N5 unprotonated. However, as can be seen from Tables 2 and 3, the N5═C6 stretch frequency of the 4-hydroxy form is only 4 cm^{-1} higher than the 4-oxy form in the anti conformer and 6 cm^{-1} higher in the syn conformer, when a water molecule is added to the model complex near N5. Thus, it is clear that the position of the C6═N5 stretch frequency alone is not a reliable criterion to determine the protonation state of N5 of H_2pterin.

Unlike the 4-oxy, N5 unprotonated H_2pterin, where the interaction with water has little affect, the interaction with a water molecule near N5 of the 4-hydroxy, N5 unprotonated H_2-pterin induces about a 10 cm^{-1} downward shift on the C6═N5 stretch frequency but the addition of the acetic acid does not have a significant effect on the C6═N5 stretch. Our results also show that in all 4-hydroxy, N5 unprotonated H_2pterin model compound complexes, deuterium exchange of the sample does not result in any significant shift of the C6═N6 stretch (Table 3). Since the N8−H bending does have a limited contribution to the C6═N5 stretch mode, further calculations on additional models, in which a water molecule is hydrogen bonded to the N8−H bond, were conducted in order to find out if such hydrogen bonding can increase the coupling between C6═N5 stretching and N8−H bending motions and, hence, increase the deuterium shift of the C6═N5 stretch mode. Our results at HF/6-31g** level suggest that a water molecule hydrogen bonded to N8−H does change the absolute C6═N5 stretch frequency by a small amount (<5 cm^{-1}). However, the deuterium shift of the C6═N5 stretch remains very small (<1 cm^{-1}) no matter whether the water molecule is normally hydrogen bonded (Ow−N8 distance ∼ 3.1 Å) or strongly hydrogen bonded to N8 (Ow−N8 distance ∼ 2.6 Å). Similarly, our calculations also show that hydrogen bonding on the O7−H bond has no effect on the deuterium shift of the unprotonated C6═N5 stretch. Thus, on the basis of the observed 14 cm^{-1} shift upon deuterium exchange (Table 1) of the 1675 cm^{-1} band, our calculations show that this H_2folate band in the DHFR/NADP$^+$/H_2folate ternary complex is not from 4-hydroxy, N5 unprotonated H_2pterin.

On the other hand, the calculations show that deuteration of the 4-oxy, N5 protonated form (Table 4) has a major affect on the frequency of the C6═N5 stretch. It is clear that, while the protonated C6═N5H stretch frequency varies significantly under different conditions, its shift upon deuterium exchange should be larger than 10 cm^{-1} (after 17% reduction of the calculated frequencies) in all cases. Since the 1675 cm^{-1} band of H_2-folate in the DHFR ternary complex shifts 14 cm^{-1} upon deuterium exchange of the sample, it is reasonable to conclude that the observed Raman 1675 cm^{-1} band from the ternary complex is from the N5 protonated form of H_2pterin.

One of the reasons Cannon et al. favored the 4-hydroxy, N5 unprotonated form of H_2pterin over 4-oxy, N5 protonated in the DHFR complex is that the calculated energy of the 4-hydroxy, N5 unprotonated H_2pterin/acetic acid complex is significantly lower than the 4-oxy, N5 protonated H_2pterin/acetate complex, about 20 kcal/mol lower at the HF/3-21g level. Our calculations at HF/6-31g** level on these complexes results in an even larger energy difference; the 4-hydroxy complex is

(19) Bystroff, C.; Oatley, S. J.; Kraut, J. *Biochemistry* **1990**, *29*, 3263−3277.

favored by about 30 kcal/mol. However, we expect that much of this energy difference will disappear when hydrogen bonding and electrostatic interactions with the surrounding environment are considered. H_2pterin and acetate are positively and negatively charged, respectively, in the 4-oxy, N5 protonated H2pterin/acetate complex while H_2pterin and acidic acid in the 4-hydroxy, N5 unprotonated H2pterin/acetic acid complex are not. Thus, their interactions with external polar group or water molecules are expected to be much stronger for the 4-oxy, N5 protonated H_2pterin/acetate complex, and the energy difference between the two complexes will be reduced substantially, if not reversed, when hydrogen-bonded polar groups are included in the complexes. For example, our calculations at HF/6-31g** level on these two systems that includes one additional water molecule near N5 show that the energy difference of the two systems may be reduced by up to 10 kcal/mol. Calculations at HF/3-21g level predict an even larger relative energy reduction in favor of the 4-oxy, N5 protonated H2pterin/acetate complex, by about 14 kcal/mol. Furthermore, calculations at HF/6-31g** level on complexes with a water molecule hydrogen bonded to O7 and to one of the acetate oxygen show that the energy difference between the two complexes is also reduced by about 7 kcal/mol. Thus, it seems reasonable to expect that, with 3−4 polar groups nearby, the 4-oxy, N5 protonated form of H_2pterin may become energetically favorable. In fact, several water molecules and polar groups of protein residues hydrogen bonded to H_2pterin/Asp27 have been reported in the X-ray crystallographic studies of various DHFR/inhibitor complexes. This includes one water molecule hydrogen bonded to δO2 of Asp27 and O7 of pterin, another hydrogen bonded to N5 and O7 of pterin, others hydrogen bonded to N10 and the 2-amino group of pteridine ring.[4−6,19−21] On the basis of these observations, we believe the calculated relative energies of various tautomeric forms of H_2pterin in simplistic models of the binding site are not reliable enough to come to conclusions about the structure of H_2pterin in DHFR.

A detailed examination of the calculated structures and the frequencies listed in Table 4 suggests that the deuterium shift of the of the C6═N5 stretch frequency when N5 is protonated may be correlated with hydrogen bonding to the N5−H bond. For example, in the absence of a water molecule, the deuterium shifts of the C6═N5 stretch in the anti conformer are 20 cm^{-1}, with or without the acetate (Table 4). When a water is added to the complex, the deuterium shifts increase to 27 and 30 cm^{-1}, respectively. For the syn conformer, the situation is more complex because there are two hydrogen bonds that can be formed with N5−H: one from the oxygen of the water and the other from N10 of pterin. The presence of the acetate ion changes the charge distribution in the H_2pterin ring, which in turn changes the N10 position relative to N5. Thus, the deuterium shift in the acetate/H_2pterin complex becomes slightly larger. Addition of a water near N5 of the acetate/H_2pterin complex pushes N10 away from N5; thus, the total hydrogen bonding strength on the N5−H does not increase much and the deuterium shift of the C6═N5 stretch only increases by about 3 cm^{-1} compared to the complex without the water molecule. In any case, this notion that hydrogen-bonding strength to a −C═NH− group affects its shift in frequency upon deuteration is well established in other systems.[17,22−26]

Table 5. Calculated C6═N5 Stretch Frequency and Its Deuterium Shift of the H_2pterin as a Function of Distance between Water and N5

Ow−N5 dist	C6═N5 freq	deuterium shift	rel energy
infinity	1915.4	20.2	15
4	1915.1	20.5	7.68
3.5	1924.7	23.2	4.97
3.2	1927	25.2	2.43
3.05	1928.2	26.7	1.29
2.9	1929.7	28.8	0.49
2.8	1929.3	30.3	0
2.7	1930	32.4	0.31
2.6	1938	43.1	1.32
2.5	1938	47.2	3.36
2.475	1938.1	48.8	4.09
2.45	1938	50.2	4.91
2.4	1937.7	53.2	6.9

[a] The distances are in Å. The frequencies and shifts are in cm^{-1}. The relative energies are in kcal/mol, relative to the fully optimized geometry of the complex. All frequencies are obtained at the ab initio HF/6-31g** level on optimized geometries of the H_2pterin−water complexes with only the distance between the oxygen of the water and N5 of H_2pterin fixed.

To determine whether the C6═N5H stretch frequency and its deuterium shift can be related with the hydrogen-bonding strength on the C═NH moiety, we have conducted systematic calculations mostly on the C6−C9 anti, 4-oxy, N5 protonated H_2pterin/water complex with water oxygen fixed at varying distances from N5. This model has been chosen in our calculations because no counterion is expected in the immediate vicinity of N5 in either aqueous solution or in the DHFR ternary complex, and the complication due to the interaction between N10 and N5 can be separated. In these calculations, the geometries of the complexes are fully optimized except the distance between the water oxygen and N5. The vibrational frequencies were then calculated. All calculations are conducted at the HF level with 6-31g** basis set. The results are shown in Table 5. A couple of calculations were also performed for the C6−C9 syn conformer for comparison. At 2.85 and 2.716 Å, the C6═N5 frequencies (deuterium shifts) are 1930.7 (−26.7) and 1933.5 (−33.0) cm^{-1}, respectively, for the syn conformation.

The results shown in Table 5 suggest that there is a strong correlation between the deuterium shift and hydrogen-bonding strength. The calculated optimal distance between the oxygen of the water and N5 of H_2pterin is 2.8 Å, at which the complex is at a minimum on the potential surface. Moving the water molecule either closer to or further away from N5 will raise the energy of the complex as shown in Table 5. However, the general trend is that the shorter distance between water and N5, the higher the C6═N5H stretch frequency and the larger the deuterium shift of the C6═N5H stretch mode, even when this distance is shorter than the optimal distance. The numbers above for the C6−C9 syn conformation suggest that the C═NH stretch frequency and its deuterium shift are also correlated with the hydrogen-bonding strength for this conformation as well. However, there is an offset of about 3 cm^{-1} compared with the results obtained for the C6−C9 anti conformer.

The calculations, taken in total, show that the C6═N5H stretch frequency of H_2pterin is sensitive to many factors in addition to hydrogen bonding to the N5−H bond. These factors

(20) Bystroff, C.; Kraut, J. *Biochemistry* **1991**, *30*, 2227−2239.
(21) McTigue, M. A.; Davies, J. F., II; Kaufman, B. T.; Kraut, J. *Biochemistry* **1993**, *32*, 6855−6862.
(22) Gilson, H. S. R.; Honig, B. H.; Croteau, A.; Zarrilli, G.; Nakanishi, K. *Biophys. J.* **1988**, *53*, 261−269.
(23) Deng, H.; Huang, L.; Groesbeek, M.; Lugtenburg, J.; Callender, R. H. *J. Phys. Chem.* **1994**, *98*, 4776−4779.
(24) Eyring, G.; Curry, B.; Mathies, R.; Broek, A.; Lugtenburg, J. *J. Am. Chem. Soc.* **1980**, *102*, 5390−5392.
(25) Deng, H.; Callender, R. H. *Biochemistry* **1987**, *26*, 7418−7426.
(26) Lin, S. W.; Sakmar, T. P.; Frank, R. R.; Khorana, H. G.; Mathies, R. A. *Biochemistry* **1992**, *31*, 5105−5111.

include conformational changes of H_2pterin and its interaction with a protein factors remote from N5, as well as local interactions. In contrast, the deuterium shift of the C6═N5H stretch mode is mostly sensitive to its immediate environments and is thus a much better indicator of the hydrogen-bonding strength to the N5−H bond.

Discussion

There are several structural conclusions concerning the electronic nature of H_2folate in DHFR/$NADP^+$/H_2folate that can be made on the basis of comparing the quantum mechanical calculations of the vibrational spectra of H_2folate to the experimental Raman observations (Table 1). It is clear that the key observed 1675 cm^{-1} marker band observed in the ternary complex arises from a tautomer of H_2folate where N5 is protonated. Neither the 4-hydroxy or 4-oxy forms of H_2folate can give rise to the substantial 14 cm^{-1} downward shift observed for the 1675 cm^{-1} marker band upon deuteration of bound H_2-folate unless N5 is protonated. The couplings between remote N−H/O−H bending motions and the unprotonated C6═N5 stretch motion are just not strong enough to account for the observed 14 cm^{-1} shift. It is to be stressed that the current Raman results are not sufficient to determine the tautomeric form of the 4C═O group or if this group is significantly polarized for bound H_2folate in the ternary complex. Such information is important to how the substrate binds and perhaps to enzymic mechanism. Raman studies are now underway to get at this specific issue. However, we believe it unequivocal that N5 of H_2folate bound to DHFR/$NADP^+$ undergoes protonation with a pK_a of 6.5, and this is 4 pH units higher than found for dihydrofolate models in aqueous solution.

The calculations also suggest that there is no water molecule hydrogen bonded with protonated N5 in the ternary DHFR/$NADP^+$/H_2folate complex. The 14 cm^{-1} deuteration shift of the C6═N5 stretch mode of H_2pterin observed in the DHFR/$NADP^+$/H_2folate complex is substantially lower than that found for protonated H_2folate model compound in aqueous solution (−25 cm^{-1}, Table 1). The calculated deuteration shift of the C6═N5 stretch mode in the water complex under optimal hydrogen bonding condition (−25 cm^{-1} after 17% correction) reproduced the deuterium shift in solution, but even the deuteration shifts calculated for "bare" H_2folate/acetate in vacuo are a bit larger than that observed (about 17 cm^{-1} after applying the 17% frequency correction). Further calculations whereby a methyl group was placed close to protonated N5 yielded smaller deuteration shifts (15 cm^{-1}), which is more in line with the Raman observations. It appears that the environment of protonated N5 in the DHFR/$NADP^+$/H_2folate complex is quite hydrophobic. This conclusion is consistent with the results obtained by X-ray spectroscopic studies of the DHFR/$NADP^+$/H_2folate complex. The X-ray structure of this complex indicates that the water molecule near N5 observed in other complexes, such as in DHFR/$NADP^+$/H_2biopterin,[27] is replaced by a hydrophobic group, the side chain of Met20 (Chen and Kraut, private communication). Hence, the structural factor(s) that are responsible for raising the pK_a of N5 by 4 units in the protein complex do not appear to involve a stabilization of the N5−H moiety by hydrogen bonding to a structural water molecule. However, it needs to be pointed out that water molecule can reach N5 of folate in the ternary complex dynamically since the hydrogen on N5 is exchanged to deuterium instantly when the sample is suspended in D_2O.

Finally, the calculations suggest that the 3 cm^{-1} shift observed in the marker band for the N5 unprotonated species when the active site Asp27 group is mutated to serine[10] can be brought about by an unprotonated (i.e., charged $-COO^-$) carboxyl of the aspartate. Hence, an unprotonated N5 is associated with a charged Asp27 in the ternary DHFR/$NADP^+$/H_2folate complex. Therefore, the carboxyl of Asp27 does not serve as the protein acceptor group for a proton transfer from N5 of bound protonated H_2folate. This important structural interpretation of the Raman results is likely correct but tentative because a shift of just a few cm^{-1} could come conceivably from an unknown perturbation of the binding site, other than the change in charge when the Asp residue is replaced by serine. However, this conclusion is consistent with our previous Raman study of the *E. coli* enzyme which examined the vibrational modes of Asp27 directly that suggested the pK_a of Asp27 is less than 5.[10]

Assuming the DHFR/$NADP^+$/H_2folate complex is a structural mimic of the productive DHFR/NADPH/H_2folate complex, we conclude that the enzymic pathway of converting H_2folate to H_4folate involves first the protonation of N5 and then, in a subsequent step, hydride transfer from NADPH to C6 on the basis of the following observations. The pK_a of N5 of bound H_2folate is raise to 6.5 in the ternary complex so that there is substantial protonated H_2folate at physiological pH values. The pK_a of the hydride transfer for *E. coli* DHFR is also 6.5.[7] From kinetic studies of DHFR mutants (particularly the Asp27Ser mutant), hydride transfer rate is greatly diminished. The steady-state kinetic parameter (k_{cat}/K_m) of the Ser-27 mutant was found to be reduced by almost 4 powers of 10 at pH 7, whereas activity−pH profiles indicated that the mutant DHFR rapidly turns over preprotonated substrate but not unprotonated substrate.[28] This behavior would appear to be clearly coupled to the fact that the pK_a of N5 of H_2folate in the Asp27Ser mutant ternary complex has been found to be decreased substantially (below 4).[10]

From a mechanistic point of view, that the protein environment raises the pK_a of N5 four units to 6.5 in the productive ternary complex is reasonable and has been previously proposed.[29] Raising the pK_a of N5 by 4 units means that there is 4 orders of magnitude more productive complex in the physiological pH range provided the transition state energy barrier of hydride transfer is lower in protonated H_2folate compared to unprotonated H_2folate. In fact, the evidence that the transition state barrier to hydride transfer is lower in N5 protonated H_2-folate is strong. The positive charge on C6 is increased significantly when H_2folate becomes protonated at N5. Previous calculations at the HF/3-21g level have shown that it increases about 0.17 units[30] from about 0.3; our present calculations show that the charge on C6 increases 0.12−0.15 units from about 0.25 when N5 becomes protonated in various models. A more positive C6 almost certainly results in a lower barrier to transfer the negatively charged hydride ion from C4 of NADPH to C6 of H_2folate. For example, the hydride transfer rate from C4 of NADH to the >C═O group of pyruvate as catalyzed by lactate dehydrogenase is greatly accelerated as the carbonyl moiety is polarized. A downshift in frequency of the C═O stretch of 35 cm^{-1}, which indicates that the C atom has become more positive and the O atom more negative, is correlated with a $10^{5.5}$-fold

(27) McTigue, M. A.; Davies, J. F., II.; Kaufman, B. T.; Kraut, J. *Biochemistry* **1992**, *31*, 7264−7273.

(28) Howell, E. E.; Villafranca, J. E.; Warren, M. S.; Oatley, S. J.; Kraut, J. *Science* **1986**, *231*, 1123−1128.

(29) Huennekens, F. M.; Scrimgeour, K. G. In *Pteridine Chemistry*; Pfliederer, W., Taylor, E. C., Eds.; MacMillan: Oxford, England, 1964; pp 355−376.

(30) Gready, J. E. *Biochemistry* **1985**, *24*, 4761−4766.

rate enhancement;[31] ab initio calculations estimate that the 30 cm^{-1} frequency shift is equivalent to a buildup of only about 0.03 extra positive charge on the carbon atom (unpublished results). Assuming that the transition state barrier for protonated H$_2$folate is lower than that for unprotonated substrate, other factors that make up the rate acceleration brought about by DHFR would have to with the rate at which protons arrive to N5 in the DHFR/NADPH/H$_2$folate ternary complex and by the rate, which may be dynamically controlled at least partly, at which the hydrogen on C4 of NADPH comes close enough to C6 of bound H$_2$folate to permit hydride transfer.

Although much is known about the structure of DHFR and its complexes with cofactor and substrates, it is not clear just what structural factors are responsible for raising the pK_a of N5 4 units. These is no obvious proton acceptor at the active site that is close enough to stabilized protonated N5 by forming a hydrogen bond. The present results even seem to rule out a hydrogen-bonded network which places a structural water molecule close enough to hydrogen bond to N5−H. In fact, it appears that the immediate environment of N5 is quite hydrophobic. One possible explanation is that the negative charge of the carboxylate of the active site Asp27 would stabilize a protonated substrate even if this group is on the other side of bound substrate relative to N5. It is known that Asp27 is crucial since its mutants result in a drastically decreased pK_a of N5. We also believe that long range electrostatic interactions are likely playing a role. It has been observed in the *E. coli* enzyme that the conformation of DHFR undergoes changes with a pK_a of about 6.5, which involve loop movements (particularly the Met-20 loop) and likely ionization changes of histidine residue-(s) far from the binding site. In addition, perturbation of the indole ring of Trp-22[9] has also been observed with a pK_a of 6.5, and this residue is close to substrate in the binding site. Hence, it appears that long-range motions are affecting the substrate binding site. However, how such changes, which have been observed in the apoprotein, do not affect the pK_a of N5 in the binary DHFR/H$_2$folate complex but do affect N5 in the ternary DHFR/NADP$^+$/H$_2$folate complex is a puzzle.

(31) Deng, H.; Zheng, J.; Clarke, A.; Holbrook, J. J.; Callender, R.; Burgner, J. W. *Biochemistry* **1994**, *33*, 2297−2305.

Acknowledgment. This work was supported in part by a grant (GM35183) from the Institute of General Medicine, from the National Institutes of Health.

JA9814974