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Determination by Raman Spectroscopy of the pK_a of N5 of Dihydrofolate Bound to Dihydrofolate Reductase: Mechanistic Implications†

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ABSTRACT: Dihydrofolate reductase (DHFR) catalyzes the reduction of dihydrofolate (H_2folate) to tetrahydrofolate by NADPH, and this requires that the pteridine ring be protonated at N5. A long-standing puzzle has been how, at physiological pH, the enzyme can protonate N5 in view of its solution pK_a of 2.6 and the fact that the only proton-donating group in the pterdine binding site, Asp-27, hydrogen bonds not to N5 but to the 2-amino group and N3 of the pterin ring. We have determined the pK_a of N5 of dihydrofolate in the *Escherichia coli* DHFR/NADP$^+$/H_2folate ternary complex by Raman difference spectroscopy and found that the value is 6.5. In contrast, the pK_a of N5 is less than 4.0 in either the binary complex, the ternary complex with an analogue of NADPH (H_2NADPH), or the Asp27 to serine mutant DHFR (D27S) ternary complex with NADP$^+$. Thus, one need not invoke proton donation from Asp-27 to N5 via a series of bound water molecules and/or pteridine-ring substituents. We propose instead that the N5 protonated form of H_2folate is stabilized directly at the active site in the DHFR/NADPH/H_2folate complex by specific interactions that form only in the ternary complex, involving perhaps a bound water molecule, the carboxamide moiety of the coenzyme, and/or the local electrostatic field of the enzyme molecule, to which an important contribution may be made by Asp-27.

Dihydrofolate reductase (5,6,7,8-tetrahydrofolate:NADP oxidoreductase, EC 1.5.1.3, DHFR)[1] catalyzes the reduction of 7,8-dihydrofolate (H_2folate) to 5,6,7,8-tetrahydrofolate (H_4-folate) by facilitating the addition of a proton to N5 of H_2-folate and the transfer of a hydride ion from NADPH to C6. Since H_4folate is essential for the production of purines, thymidylate, and a few amino acids, DHFR is an essential enzyme in a number of biosynthetic pathways. For this reason, it is a target for both antitumor and antimicrobial drugs. Its catalytic mechanism is of obvious interest.

A central issue concerning the catalytic mechanism of DHFR is how the enzyme accomplishes the protonation of N5 of H_2folate's pteridine ring [see, e.g., Kraut and Matthews (1987)]. The solution pK_a of N5 is 2.6 (Maharaj et al., 1990) so that very little H_2folate is protonated at physiological pH. However, the rate of hydride transfer from NADPH to H_2-folate in the presence of *Escherichia coli* DHFR shows approximate Henderson–Hasselbalch dependence with a pK_a of around 6.5 (Fierke et al., 1987; Morrison & Stone, 1988). These authors suggested that a group at the active site of the enzyme with a pK_a of 6.5 is involved, presumably the strictly

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[1] Abbreviations: DHFR, dihydrofolate reductase; H_2folate, 7,8-dihydrofolate; H_4folate, 5,6,7,8-tetrahydrofolate; H_2biopterin, 7,8-dihydrobiopterin; NADPH, reduced β-nicotinamide adenine dinucleotide phosphate; NADP$^+$, oxidized β-nicotinamide adenine dinucleotide phosphate; H_2NADPH, 1,4,5,6-tetrahydronicotinamide adenine dinucleotide phosphate.

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conserved carboxylic acid side chain (Asp-27 in *E. coli*), because it is the only ionizable protein group at the active site. Otherwise, the active site is generally lined with hydrophobic side chains. The importance of Asp-27 in *E. coli* DHFR is shown by studies of Asp-27 to Asn or Ser mutants (D27N, D27S) (Howell et al., 1986). In these mutants, the k_{cat} vs pH profile is shifted dramatically to lower pH. This result was interpreted as showing that the mutant enzymes are unable to facilitate protonation of N5 at neutral pH but can still function at full catalytic activity using a "preprotonated" substrate.

However the exact role of Asp-27 is still a puzzle. X-ray crystallographic results show that its carboxyl group is hydrogen bonded, not to N5, but to the 2-amino group and to N3 of substrate's pteridine ring and is thus on the wrong side of the ring and more than 5 Å from N5. Asp-27 therefore cannot form a direct interaction with N5 nor can it directly donate a proton. Instead, an indirect role has been proposed for Asp-27. In this view, a proton is transferred to N5 via two bound water molecules while the imino–keto tautomer isomerizes to the 4-hydroxyl form with the latter group participating in the proton relay system (Benkovic et al., 1988; Bystroff et al., 1990; Stone & Morrison, 1984; Uchimaru et al., 1989). It has been calculated that, despite the difference in pK_a between the carboxylate of Asp-27 and the (assumed) value of 2.6 for N5, a sufficient degree of N5 protonation occurs to satisfy the observed k_{cat} (Benkovic et al., 1988).

In this communication, we present results from Raman difference spectroscopy, which permits direct observation of the pK_a of N5 in H_2folate bound to DHFR. In this form of vibrational spectroscopy, the Raman bands of bound substrate are obtained by subtracting the Raman spectrum of the protein from that of the protein–ligand complex (Callender & Deng, 1994). In this way the generally sizable interfering protein background spectrum is eliminated. Band assignments may be confirmed by isotopically editing the spectrum using labeled substrates. Here, marker Raman bands are identified for the N5=C6 stretch in both the protonated and unprotonated forms of bound H_2folate. The relative intensities of these bands are proportional to the relative concentrations of the two forms so that a direct measurement of a titration curve is possible by measurement of the marker bands as a function of pH. We find that the pK_a of H_2folate's N5 in the binaryl DHFR/H_2folate complex is less than 4.0. The same is true of H_2folate in the ternary complexes of D27S/$NADP^+$/H_2folate and DHFR/H_2NADPH/H_2folate. In contrast, the pK_a of N5 unexpectedly jumps to 6.5 in the DHFR/$NADP^+$/H_2folate ternary complex.

MATERIALS AND METHODS

$NADP^+$ 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 (Selinsky et al., 1990). 7,8-Dihydrobiopterin (H_2biopterin) and [5-^{15}N]H_2biopterin were obtained from Dr. B. Schircks laboratory (Jona, Switzerland). H_2NADPH was prepared by the following modification of the procedure of Biellmann and Jung (1971). Five milligrams of 5% of palladium on charcoal was added to a solution of 10 mg of NADPH dissolved in 4 mL of H_2O, pH 8, and the resulting mixture was put on ice and hydrogenated by bubbling hydrogen gas. The reaction was stopped just when the absorption at 340 nm is not longer detectable. The mixture was then washed through Centricon-30 centrifuge concentrator (Amicon, Lexington, MA) to remove the solid residue. The filtrate was lyophilized and stored in −80 °C. *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). Mutant DHFR D27S was purified by Dr. Janet Grimsley as described (Villafranca et al., 1983). The apoenzyme concentration was determined spectrophotometrically at 280 nm using a molar extinction coefficients of 31 000 M^{-1} cm^{-1} (Fierke et al., 1987). The concentrations of substrates, coenzyme, and coenzyme analogue were determined at pH 7.0 using the following extinction coefficients: $NADP^+$, 17 500 M^{-1} cm^{-1} at 260 nm; H_2folate, 28 400 M^{-1} cm^{-1} at 282 nm (Blakley, 1969); H_2biopterin, 6170 M^{-1} cm^{-1} at 330 nm (Maharaj et al., 1990); and H_2NADPH, 15 500 M^{-1} cm^{-1} at 288 nm(Biellmann & Jung, 1971), respectively.

Buffer solutions used were as follows: pH 5.4, 100 mM acetate and 0.4 M KCl; pH 6.1, 25 mM Bis-tris and 0.5 M KCl; pH 6.6, 20 mM Bis-tris propane and 0.5 M KCl; pH 7.4 and 8.0, 20 mM Tris and 0.5 M KCl; pH 9.2, 25 mM pyrophosphate and 0.5 M KCl. The pH of the enzyme solution was changed by washing the enzyme in a Centricon-10 centrifuge concentrator (Amicon, Lexington, MA) with the final buffer. Typically, three cycles of diluting and concentrating were needed to reach desired pH. The final enzyme concentration was about 3–5 mM. The binary complex of DHFR/H_2folate was prepared by mixing DHFR and H_2folate in a molar ratio of 1:0.8. The binary complex of DHFR/$NADP^+$ was prepared by mixing DHFR and $NADP^+$ in a molar ratio of 1:1, and the ternary complex of DHFR/$NADP^+$/H_2folate was then prepared by mixing DHFR/$NADP^+$ and H_2folate in a molar ratio of 1:0.8. Under these conditions, essentially all H_2folate was bound to the enzyme (Kraut & Matthews, 1987).

A specially fabricated split-cell cuvette (Hellma Cells) were 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 apoenzyme 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 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. Details concerning the procedures and controls for obtaining Raman difference spectra have been discussed previously (Chen et al., 1987; Deng et al., 1989; Yue et al., 1989).

Data collection and analyses including peak deconvolution and curve fitting were done with the program Igor (WaveMetrics). From the ratio of the intensity of the band characteristic of the neutral or protonated form of N5 (I) versus the intensity of an internal reference band (I_{ref}) which is pH-independent as a function of pH, the pK_a of N5 was determined by fitting the relative intensities to either eq 1 and 2 as appropriate using nonlinear least-squares techniques:

$$I/I_{ref} = \frac{C}{1 + [H]/K_A} \quad (1)$$

$$I/I_{ref} = \frac{C}{1 + K_A/[H]} \quad (2)$$

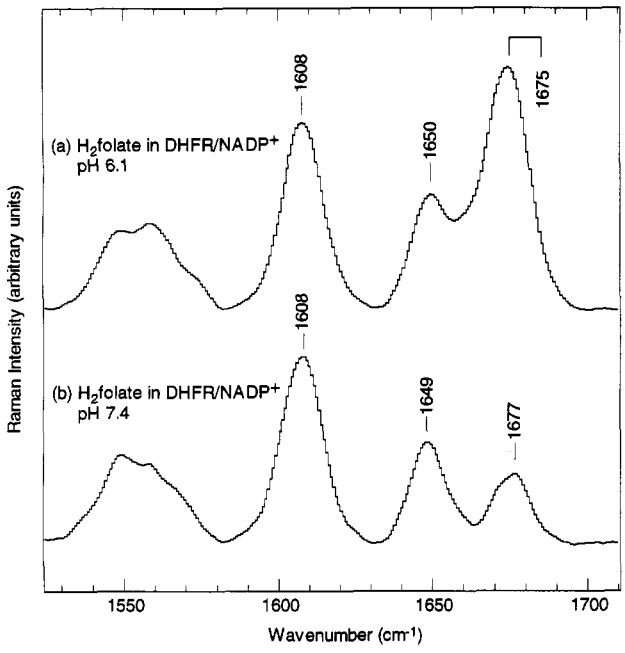

FIGURE 1: Difference Raman spectra of H_2folate bound in the ternary complex of DHFR with NADP+ (DHFR/NADP+:H_2folate = 3.4:2.5) at 4 °C in (a) 25 mM Bis-tris buffer containing 0.5 M KCl, pH 6.1, and (b) 20 mM Tris buffer containing 0.5 M KCl, pH 7.4.

where C represents the maximum relative intensity and K_A the acid dissociation constant.

RESULTS

In Raman difference spectroscopy used here, the nonresonance Raman spectrum of the protein is subtracted from the spectrum of the protein/ligand complex in order to eliminate the generally much stronger protein background spectrum. It is thus possible to determine the vibrational spectrum of ligands bound to proteins for a very wide range of systems since it is not necessary to rely on resonance effects [for review, see Callender and Deng (1994)]. The difference spectrometer used in these studies is capable of detecting ligand signals as small as 0.1% of the protein background (Deng et al., 1993b; Manor et al., 1991; Yue et al., 1989). We do not show any primary protein spectra or that of protein binary and ternary complexes that are used to form the difference spectra in this communication; the difference signals reported here are comparatively large compared to our detection limit of 0.1%.

Figure 1a shows the difference spectrum between H_2folate bound in the ternary complex of DHFR with NADP+ (the DHFR/NADP+/H_2folate ternary complex) and the binary DHFR/NADP+ complex of pH 6.1. Figure 1b is the same but at pH 7.4. The following comments should be kept in mind in order to interpret the spectrum. The relative concentrations of the enzyme, coenzyme, and substrate relative to their dissociation constants are such in this experiment that virtually all the species are bound up as either the ternary or the binary complex (see Materials and Methods). Thus, there are no signals in the difference spectrum arising from unbound substrate. The difference bands shown in Figure 1 are on the order of 10–30% of the strong protein amide-I band (which arises from peptide C=O stretch). The difference bands are quite intense because of preresonance enhancement of H_2-folate's Raman cross sections (H_2folate has a broad absorption of λ_{max} = 282 nm; laser excitation = 568.2 nm). Although it is expected that the vibrational modes of the protein will be affected by the presence of the substrate, it is our general observation that this leads to protein signals which are on the order of one percent of the amide-I band. It is thus almost certain that the bands observed in Figure 1 arise from bound substrate; the isotopic labeling experiments described below confirm this. The characteristic time scale of a vibrational experiment is very fast (faster than picoseconds); therefore, the observed signals are not affected by the slower tumbling motions of the protein or substrate dissociation. Multiple species, such as tautomeric states, show up in the spectrum with their own characteristic bands, and the magnitude of the species marker band is proportional to the concentration of that species.

There are three prominent bands in the spectrum at 1608, 1650, and 1675 cm^{-1}, and the intensity of the 1650 and 1675 cm^{-1} bands is clearly quite sensitive to pH in the range of 5.4–9.2 that we measured. The 1608-cm^{-1} band is assigned to a benzoyl ring mode of H_2folate. A band is found in similar locations of 1,4-disubstituted benzenes while it is absent in the spectra of H_2biopterin (see below), which has the pteridine ring structure of H_2folate but lacks its *p*-amino-benzoyl-L-glutamate group. Moreover, the 1608-cm^{-1} band is present in the spectra of methotrexate and folate (Ozaki et al., 1981; Saperstein et al., 1978); these molecules have substantially different electronic and vibrational spectra of the pterdine ring but contain the *p*-amino-benzoyl-L-glutamate group. As expected from this assignment, we observe little or no change in the intensity of the 1608-cm^{-1} band as a function of pH through 5–9. We therefore use this band as a reference in determining the intensities of the pH-dependent bands.

The 1650- and the 1675-cm^{-1} bands can be assigned to the N5=C6 stretch of the pteridine ring of H_2folate for N5 unprotonated and protonated respectively. The 1650-cm^{-1} band shifts down 31 cm^{-1} to 1619 cm^{-1} for N5=^{13}C6 labeled H_2folate, while the 1675-cm^{-1} band shifts 32 cm^{-1} (data not shown). Moreover, the positions of these bands are close to that of solution models (see below). The mode for either form must be fairly isolated to the N5=C6 group since the observed shifts on ^{13}C labeling are quite close to the predicted shift (36 cm^{-1}) for an isolated diatomic oscillator having the mass of a N=C molecule. In D_2O buffer, the 1675-cm^{-1} band shifts down 16 cm^{-1} to 1661 cm^{-1} (data not shown) while the 1650 cm^{-1} band is unaffected; the 16 cm^{-1} shift is typical of deuteration shifts found in the spectra of compounds containing a protonated –C=NH+ group [see, e.g., Deng et al. (1993a)].

It is of interest to compare the spectra of the protein-bound pteridine ring obtained by Raman difference spectroscopy with the Raman spectra of solution models. H_2biopterin was used as a model for H_2folate because it contains the same pteridine ring as H_2folate and the pK_a of its N5 is the same as that of H_2folate, but it is much more stable and soluble than H_2folate at pH values below 4. The solution pK_a of N5 of the pteridine ring is 2.6 (Selinsky et al., 1990). Figure 2 shows the Raman spectra of H_2biopterin in solution below (panel a; pH 1.6) and above (panel b; pH 5), this pH. The 1635-cm^{-1} band is the unprotonated N5=C6 stretch while the 1671-cm^{-1} band is the protonated N5=C6 stretch. These assignments are confirmed by isotopic labeling studies on H_2-biopterin. The 1671-cm^{-1} band shifts down to 1644 cm^{-1} for [5-^{15}N]H_2biopterin while the 1635-cm^{-1} band shifts to 1618 cm^{-1} (data not shown). It should be noted that the frequency of the unprotonated N5=C6 stretch of H_2biopterin in solution of 1635 cm^{-1} shifts down 6 cm^{-1} upon binding to the enzyme, which is the same for that of H_2folate (shifts down from 1656

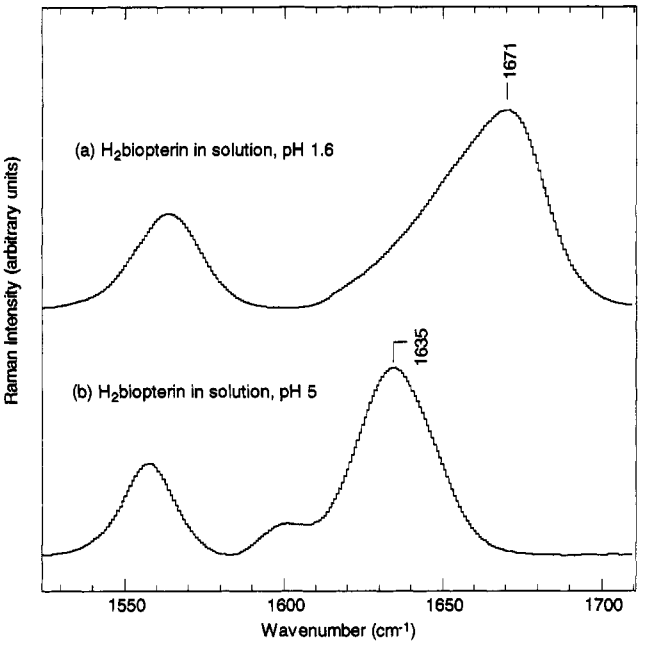

FIGURE 2: Raman spectra of H_2biopterin at 4 °C in solution (7 mM) at (a) pH 1.6 and (b) pH 5.0.

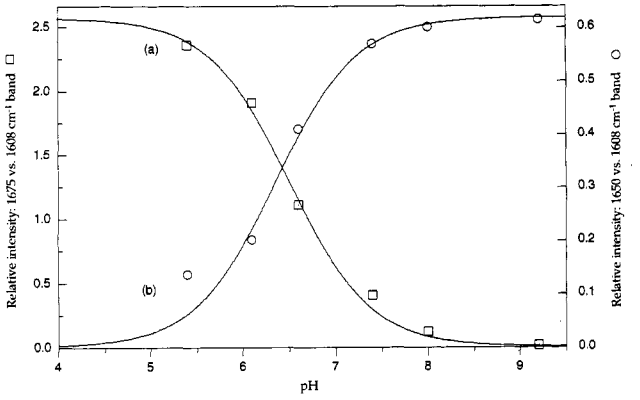

FIGURE 3: pH dependence of the relative intensity (to 1608-cm^{-1} band) of peaks at 1675 cm^{-1} (square) and 1650 cm^{-1} (circle). The solid curves represent the best fits to eq 1 or 2, as appropriate. The values found for the pK_a's were 6.5 (±0.1) using the 1675-cm^{-1} data and 6.4 (±0.1) for 1650 cm^{-1}.

cm^{-1} in solution to 1650 cm^{-1} upon binding), while the shift for the protonated stretch upon binding is not available because H_2biopterin is not protonated in DHFR/NADP$^+$/H_2biopterin ternary complex and the solubility of H_2folate at low pH is too low to allow Raman spectrum of reasonable quality be taken.

From these data, it is clear that the pK_a of H_2folate's N5 nitrogen is very altered in the ternary DHFR/NADP$^+$/H_2-folate complex from its value in solution. Taking the intensities of the 1650- and 1675-cm^{-1} bands as proportional to the concentrations of the neutral and protonated species, respectively, and the 1608-cm^{-1} band as an internal reference, a concentration profile of the neutral and protonated species as a function of pH can be determined. The result of intensity deconvolution for the two bands is plotted in Figure 3. The pK_a's were determined from eq 1 or 2, as appropriate, and a pK_a for N5 was found to be 6.5 (±0.1) from the intensity profile for the 1675-cm^{-1} band and 6.4 (±0.1) from the 1650-cm^{-1} band (reduced chi-square χ_ν^2 = 0.99). Thus, the pK_a of N5 of H_2folate as a ternary complex with DHFR and NADP$^+$ is 6.5, or almost 4 pH units higher than its solution pK_a of 2.6.

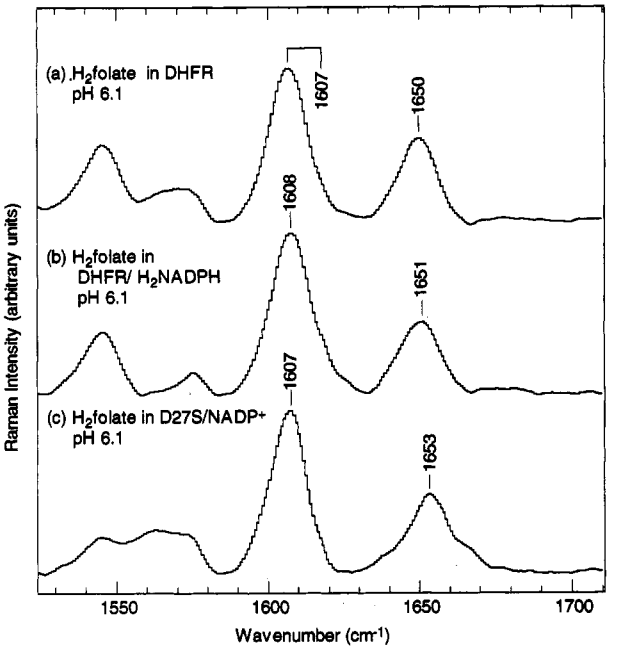

FIGURE 4: Difference Raman spectra of H_2folate bound in (a) the binary complex with DHFR (DHFR:H_2folate = 3.4:2.5), (b) the ternary complex of DHFR with H_2NADPH (DHFR/H_2NADPH: H_2folate = 4.2:2.6), and (c) the ternary complex of mutant D27S with NADP$^+$ (D27S/NADP$^+$:H_2folate = 3.5:2.6) at 4 °C in 25 mM Bis-tris buffer containing 0.5 M KCl, pH 6.1.

It was found that the results described above pertain only to "freshly" prepared samples. While H_2folate bound to *E. coli* DHFR in a binary complex was quite stable in the pH range measured, H_2folate bound in the ternary complex with NADP$^+$ showed substantial changes of its Raman spectrum with time. At pH values below 6.5, the intensity of the 1675-cm^{-1} band began to decrease after about 1 h from preparation; on the other hand, at pH values above 6.5, the intensity of the 1675-cm^{-1} band steadily increased, accompanied by a concomitant intensity decrease of the 1650-cm^{-1} band. The ultraviolet absorption spectrum of dihydrofolate bound to enzyme ternary complex with NADP$^+$ showed a slow progressive change over 2 h at 20 °C and pH 5.8, with an increase in the 350–500-nm region and a decrease in the 280–350-nm region. This seems to be due to a chemical change in the pteridine since it was not reversed by raising the pH to 8.0. It probably reflects slow dismutation of dihydrofolate to folate and tetrahydrofolate and nonenzymatic destruction of the letter (Blakley & Cocco, 1984). The rate of this change was too slow to interfere with spectral measurements on the initial complex.

Figure 4 shows Raman difference experiments on various protein–H_2folate complexes at pH 6.1. Figure 4a shows the difference spectrum of H_2folate bound to DHFR as a binary complex; Figure 4b shows the spectrum of H_2folate bound as a ternary complex with the NADPH analogue H_2NADPH; Figure 4c shows the spectrum of bound H_2folate to the mutant D27S in a ternary complex with NADP$^+$. These spectra did not change with time as did those described above, indicating these species are quite stable. In all of these cases, there is no evidence of the 1675-cm^{-1} band, characteristic of the protonated form of N5 that is found in the ternary DHFR/NADP$^+$/H_2folate complex. From a close examination of the maximum possible value of signal at 1675 cm^{-1} in these spectra and in the spectrum of Figure 1a, it can be determined that the pK_a of N5 of H_2folate bound in these complexes is less than 4.0, assuming simple Henderson–Hasselbalch behavior.

DISCUSSION

The reduction of H_2folate to H_4folate to H_4folate involves the protonation of N5 of the pteridine ring (see the introduction). Indeed, the hydride transfer from NADPH to H_2folate as catalyzed by *E. coli* DHFR is pH-dependent, pK_a = 6.5 (Fierke et al., 1987; Morrison & Stone, 1988). Thus, it has been a puzzle for some time as to how this is accomplished on the enzyme since, under physiological conditions, the amount of protonated N5, assuming a pK_a of 2.6, is very small. Most discussion of this issue has centered on the role of Asp-27 in *E. coli* DHFR, the only ionizable residue in the active site. It has been supposed that this residue donates a proton to N5 via groups of the pteridine ring and/or a series of structural water molecules even though its normal pK_a is around 4.

The results of this investigation suggest that most of this discussion has been wide of the mark. We have determined that the pK_a of N5 of the pteridine ring of H_2folate in the ternary DHFR/$NADP^+$/H_2folate (freshly prepared) complex is 6.5. This is remarkable since, in solution, the pK_a of N5 is 2.6. Thus, the pK_a of N5 is raised about 4 units in this complex. It seems reasonable to suppose that N5 has a pK_a of 6.5 in the biologically relevant DHFR/NADPH/H_2folate ternary complex as well, given all the evidence pointing to a kinetic determinant with this pK_a along the pathway of enzymic catalysis of H_2folate to H_4folate and the evident need for protonation of N5 at physiological pH values. Moreover, there appears to be no significant structure difference in ternary complexes of *E. coli* DHFR associated with the oxidation state of the cofactor, as observed by X-ray crystallography (M. R. Sawaya and J. Kraut, unpublished data). This amounts to assuming that DHFR/$NADP^+$/H_2folate is an appropriate model of the Michaelis complex of the substrates with DHFR. Thus, our results solve a puzzle of how DHFR brings about protonation of N5 along the catalytic pathway. It does this by substantially raising its pK_a in the Michaelis complex. Two questions are raised by our observations. How does the enzyme raise the pK_a value by four units, and how is the proton delivered to N5, and particularly what is the role of Asp-27?

In general, there are many precedents for protein substantially altering the pK_a's of various groups, in particular raising the pK_a values of a –C=N– group. For example, the linkage between the retinal chromophore of visual pigments and bacteriorhodopsin is via this moiety. In these cases, the nitrogen pK_a of solution model lie at about 6.5. However, the pK_a of the –C=N– moiety in bacteriorhodopsin is 13.5 (Druckmann et al., 1982) and in excess of 17 in the bovine visual pigment protein (Steinberg et al., 1993). In these cases, it is believed that the protonated structure is stabilized by the formation of hydrogen bonds between the protein and the $-C=NH^+-$ group. The identity of the protein group that is involved in the hydrogen bond in both protein systems is almost certainly a carboxylate group, probably via a structural water molecule in a hydrogen bond network. It is thus interesting to note that recent X-ray structural studies place a structural water molecule at the active site of DHFR in close proximity to N5 (McTigue et al., 1992; J. Kraut et al., unpublished observations on the *E. coli* enzyme), and this comparison between the retinal containing pigments and DHFR suggests a role for the carbonyl group of Asp-27 as the anion anchor, stabilizing the N5 protonated form of H_2folate by forming hydrogen bond to N5 via structural water molecules (as well as direct hydrogen bonds to NA2 and N3 of the pyrimidine ring, which bears almost a half of positive charge when N5 is protonated; Gready, 1985).

Asp-27 is clearly required to raise the pK_a of N5 to 6.5 since, in the Asp to Ser mutant (D27S), the pK_a of N5 in its ternary complex with H_2folate and $NADP^+$ is substantially less. While Asp-27 is often supposed to be the proton donor to N5, this is not the necessary, or even the simplest conclusion, in view of the present results. More likely the ambient solution acting via a bound water molecule close to N5 is the proton donor, if, as we suppose the pK_a of N5 is raised 4 units upon formation of the DHFR/NADPH/H_2folate ternary complex. In this case the only physical requirements are that a substantial amount of N5 is protonated at physiological pH and that N5 equilibrate with its proton donor at a faster rate than k_{cat}. This is not an unreasonable expectation for a group with pK_a 6.5. Moreover, recent spectroscopic evidence indicates that neither Asp-27 in the *E. coli* enzyme nor Glu-30 in the human enzyme titrate in the pH range 5–9, as would be expected if these were the groups responsible for the observed kinetic pK_a of 6.5. Measurements of the vibrational spectrum of folate bound to *E. coli* DHFR ternary complex with $NADP^+$ show no changes over the pH range 6–9 in bands associated with motions of pteridine ring moieties which are physically close to and interacting with Asp-27. These vibrations would be expected to change if Asp-27 changed its ionization state (Chen, Kraut, and Callender, unpublished data). Additionally, NMR studies (Blakley et al., 1993) show no effect on the NMR spectra of folate bound to human DHFR as a function of pH from 5 to 7. The simplest explanation of these data is that the conserved carboxylic acid side chain at the catalytic sit is ionized throughout the pH range 5–9, since the normal solution pK_a of a carboxylic acid group is around 4.

However, it may be difficult to definitively determine the mechanism by which the pK_a of N5 is raised in DHFR, at least for the *E. coli* enzyme. From our studies, it is clear that the pK_a of N5 is less than 4.0 in the binary complex DHFR/H_2folate and the ternary complexes DHFR/H_2NADPH/H_2-folate and D27S/$NADP^+$/H_2folate, while the (*E. coli*) DHFR/$NADP^+$/H_2folate ternary complex itself is stable for only a couple of hours with regards to the pK_a of N5. Thus, it appears that the presumed elevation of N5's pK_a by DHFR in the catalytically competent DHFR/NADPH/H_2folate ternary complex results from a conjunction of just the right structural features, perhaps involving concerted protein–substrate interactions. Crystallizing and stabilizing the correct DHFR/$NADP^+$/H_2folate complex as a model of the Michaelis complex for X-ray structure determination may be difficult and perhaps impossible because of its limited lifetime.

In conclusion, it appears that we must now replace one puzzle relating to how and from what source a proton is transferred to N5 of a bound substrate molecule with another puzzle relating to how the holoenzyme, i.e., DHFR/cofactor, manages to increase the pK_a of N5 by 4 units, and especially what role Asp-27 plays in this phenomenon. A reasonable conjecture would be that the local electrostatic field of the holoenzyme is responsible. Possible contributions to the formation of a correct set of electrostatics and electrostatic interactions could also include the polar carboxamide moiety of the coenzyme, which is in close proximity to N5, as well as the carboxyl of Asp-27. These conjectures might be amenable to investigation by theoretical calculations like those carried out earlier on substrates bound to DHFR (Bajoreth et al., 1991a–c).

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