pH-Dependent Conformational Changes in *Escherichia coli* Dihydrofolate Reductase Revealed by Raman Difference Spectroscopy

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ABSTRACT The catalytic site of all dihydrofolate reductases contains an invariant carboxylic acid, equivalent to Asp-27 in *Escherichia coli* dihydrofolate reductase (ecDHFR). It has been found that various kinetic and ligand binding properties of ecDHFR show a pH profile with a pK_a of about 6.5. The group responsible for this pK_a is often assumed to be the carboxyl group of Asp-27. To determine the ionization state of this carboxyl and its pK_a, we have employed a novel method, based on Raman difference spectroscopy, to obtain its vibrational spectrum in situ. The method is general for the study of protein carboxyl groups, which are often significantly implicated in protein function and structure; this study establishes the method's limits and problems. The Raman difference spectrum between wild-type ecDHFR and the Asp-27 to serine mutant (D27S) in the pH range 5.6–9.0 has been taken. No protonation of the carboxyl group was detected, implying that its pK_a is probably less than 5.0. We did, however, detect a pH dependence in the intensity of Raman bands in the difference spectrum with a pK_a of 6.3, indicating that the apo enzyme undergoes a pH-dependent conformational change. Because the carboxyl group of Asp-27 at the active site is the only ionizable group in the binding site, other groups, away from the catalytic site, must be responsible for the pH behavior of ecDHFR.

INTRODUCTION

Dihydrofolate reductase (DHFR) catalyzes the reduction of 7,8-dihydrofolate (DHF) by NADPH to form 5,6,7,8-tetrahydrofolate and $NADP^+$. The active site of all DHFRs contains an invariant carboxylic acid residue, aspartic acid in bacteria and glutamic acid in vertebrates, which is the only ionizable residue in the active-site cavity. X-ray crystallographic data show that this carboxylic side chain interacts directly with bound folates but not with NADPH or $NADP^+$ (Bolin et al., 1982; Bystroff et al., 1990). It has been proposed that DHFR promotes the hydride transfer from NADPH to substrates by facilitating protonation of N5 of DHF or N8 of folate (Huennekens and Scrimgeour, 1964). That the active-site carboxylic acid residue is implicated in this function was demonstrated by site-direct mutagenesis experiments in which Asp-27 of ecDHFR was changed to asparagine (D27N) or serine (D27S) (Howell et al., 1986). The steady-state kinetic parameter (k_{cat}/K_m) of the Ser-27 mutant was found to be lower than that of the wild-type enzyme by a factor of 8000 at pH 7.0, whereas activity-pH profiles indicated that the mutant DHFR rapidly turns over preprotonated substrate but not unprotonated substrate. We have found by Raman difference spectroscopy that the pK_a of N5 of bound DHF is raised by four units to 6.5 from its solution value (Chen et al., 1994). An increase of four orders of magnitude in concentration of bound protonated substrate at physiological pH agrees very well with the kinetic studies of the mutants.

X-ray crystallographic studies have shown that the Asp-27 side chain forms hydrogen bonds to the 2-amino group and to N3 of DHF's pteridine ring rather than N5 or N8 (Bolin et al., 1982; Bystroff et al., 1990). Thus Asp-27 cannot be the ultimate proton donor to the key nitrogen of substrate. Instead, an indirect role for the carboxyl group has been proposed (Bystroff et al., 1990; Morrison and Stone, 1988; Uchimaru et al., 1989). An essential step in most versions of the protonation mechanism is that bound pterin substrates undergo enolization from the 4-keto to the 4-hydroxyl tautomer and that the 4-hydroxyl group then becomes part of a proton relay system for proton donation to the substrate. The assumption here is that the active-site carboxyl group is protonated below pH 6.5, which was prompted by the observation that the hydride transfer rate exhibits a kinetic pK_a of 6.5 (Fierke et al., 1987). Earlier studies on the binding of 2,4-diaminopteridine inhibitors to DHFR have also shown that the binding constant is pH dependent, with a pK_a of about 6.3 (Stone and Morrison, 1983). Thus it has often been supposed that the active-site carboxyl group has a highly elevated pK_a.

However, a recent study of human DHFR as the enzyme-folate binary complex showed no changes in substrate NMR signals throughout the pH range 6–9 (Blakley et al., 1993). This was taken to indicate that there is no ionization change in Glu-30, the equivalent active-site carboxyl group, because protonation/deprotonation of the Glu-30 side chain should have resulted in detectable chemical shift changes on the interacting substrate groups. Furthermore, our previous Raman studies of the pK_a of N5 of DHF, performed on the ternary complex of ecDHFR · DHF · $NADP^+$ (Chen et al.,

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1994), found a value of 6.5 and that the side chain of Asp-27 is probably in its ionized form, stabilizing the N5-protonated DHF through electrostatic interactions. It is therefore evident that a direct measurement of the ionization state of Asp-27 would be very useful.

The goal of this study is to measure the pH-dependent changes in the structure of the apo enzyme of *Escherichia coli* DHFR (ecDHFR). It is particularly interesting to determine directly the pK_a of the active-site Asp-27. In general, it is often difficult to determine the ionization state of specific protein groups. Here we have employed Raman difference spectroscopy, in which the protein group is somehow tagged, the spectra of the tagged and the native protein are compared, and the difference spectrum is calculated. The difference spectrum then contains only the spectrum of the tagged group in the ideal case, all other bands canceling out (Callender and Deng, 1994). Because the vibrational spectra of ionizable molecular groups, like carboxyl moieties, differ substantially depending on ionization state, this method is widely applicable in principle to determining the pK_a's of key groups. The approach taken here is to determine the difference spectrum between ecDHFR and its D27S mutant, where the active-site carboxyl group has been replaced by serine (Howell et al., 1986). This difference spectrum will contain positive bands of the Asp-27 carboxyl group of the native protein, which are diagnostic of its ionization state, as well as negative bands due to Ser-27 of the D27S mutant. As will be seen below, the D27S substitution additionally affects the structure of the protein in many subtle ways, and these differences are present up the Raman difference spectrum, many as uninterpretable weak bands. Nevertheless, it was possible to determine the ionization state of the active-site carboxyl group. We have found that the carboxyl group is ionized from pH ~ 5.6 and above, demonstrating that its pK_a is less than about 5.0. Interestingly, many features in the Raman difference spectra that are not associated with the carboxyl groups of Asp residues have a pH profile with a pK_a of 6.3. These results show that ecDHFR undergoes a pH-dependent conformation change.

MATERIALS AND METHODS

Tryptophol was purchased from Sigma Chemical Co. All other chemicals were of the highest purity available commercially. Wild-type ecDHFR was purified from *E. coli* strain CV634 containing the plasmid pCV29 by using a methotrexate affinity resin (purchased from Pierce). The D27S mutant of ecDHFR was purified by Dr. Janet Grimsley as described elsewhere (Villafranca et al., 1983). The apo-enzyme concentration was determined spectrophotometrically at 280 nm, by using a molar extinction coefficient of 31,000 M^{-1} cm^{-1} (Fierke et al., 1987).

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 dilution and concentration were needed to reach the desired pH. The final enzyme concentration was about 3–5 mM.

A specially fabricated split-cell cuvette (Hellma Cells) was used to hold the sample. About 25 μl of the wild-type ecDHFR was loaded into one side of the cuvette, and the same amount of the D27S mutant at a similar concentration was loaded into the other side. The cuvette was transferred to a cuvette holder for measurement, maintained at 4°C. About 120 mW of a 568.2-nm line from a Coherent 2000-CR krypton ion laser (Coherent Radiation, Palo Alto, CA) was used to excite Raman scattering. Data were collected with a Macintosh IIfx computer (Apple, Cupertino, CA) interfaced with a CCD detector (Princeton Instruments model LN/CCD-1152UV with a ST-135 CCD controller) coupled to a Triplemate spectrometer (Spex Industries, Metuchen, NJ). Spectra were taken alternatively from each cuvette to reduce systematic errors and to avoid sample damage. We found that the enzyme specific activities were essentially identical before and after data collection under these conditions (38 mM min^{-1} mg^{-1}), implying that damage to the enzyme due to laser irradiation was minimal. The spectra from each sample were then added together, and the difference spectrum was then obtained by subtraction of one data set from another. It contains the differences between the side chain of aspartate and serine as well as the differences due to perturbations of the mutation. Details concerning the procedures and controls for obtaining Raman difference spectra can be found in a review of the technique (Callender and Deng, 1994).

Data collection and analysis were done with the program Igor (WaveMetrics). The ratio of the relative amplitude between the positive band at 1542 cm^{-1} and the negative one at 1553 cm^{-1} in the difference spectrum (A_{diff}) to the amplitude of the protein band at 1450 cm^{-1} in the original wild-type ecDHFR spectrum (A_{1450}) was calculated at each pH, and the result was fitted to the Henderson-Hasselbalch equation using nonlinear least-squares techniques:

$$A_{diff}/A_{1450} = \frac{C}{1 + K_A/[H]}, \quad (1)$$

where C represents the maximum amplitude and K_A is the acid dissociation constant.

RESULTS

The side-chain carboxylic acid group of aspartate, when protonated, has a characteristic C=O stretch band at 1720–1770 cm^{-1}, depending on the degree of hydrogen bonding (toward the lower end for very strong H bonding) and effective dielectric constant (Dioumaev and Braiman, 1995), and a symmetrical CO_2^- stretch band at 1360–1450 cm^{-1} when ionized (Daimay et al., 1991). By comparing the intensities from ecDHFR and model spectra of carboxyls at different pH values, we found that the Raman intensity of C=O stretch at 1738 cm^{-1} for the protonated carboxyl group of aspartate at pH 2 is 0.7% of the intensity of the protonated C=O stretch band at the peak of ecDHFR's amide I band (at 1667 cm^{-1}), whereas the intensity of the unprotonated CO_2^- stretch is about 2%. If the carboxyl group of Asp-27 has a pK_a of 6.5, it would be mostly protonated at pH 5.6, whereas all the other carboxyl groups are mostly ionized. In this case, the difference Raman spectrum between the wild-type ecDHFR and the mutant D27S at pH 5.6 would contain a C=O stretch band at 1720–1770 cm^{-1}, with an intensity about 0.7% of that of protein amide I band. If Asp-27 has a normal pK_a of about 4, a CO_2^- stretch band at 1360–1450 cm^{-1} with an intensity about 2.0% of that of protein amide I band would be expected. The difference spectrometer used in our studies is capable of detecting signal changes as small as 0.1% of the protein amide I band. Therefore, in principle, it is possible to distinguish whether the side chain of Asp-27 is proto-

nated or ionized by simply detecting the presence or absence of these marker bands. The protonated carboxyl C=O stretch is particularly convenient, because its frequency at 1720–1770 cm^{-1} overlaps no other vibrational frequencies of any amino acid. This band therefore lies in an otherwise vibrationally silent spectral region of proteins, like DHFR, that do not contain prosthetic groups.

Fig. 1 *a* shows the difference Raman spectrum between wild-type DHFR and mutant D27S at pH 5.6, scaled to the percentage of the protein amide I band. Most of the difference bands are quite weak, but almost all of the features are reproducible. If the carboxyl group of Asp-27 has a pK_a of above 6, it will be mostly protonated at pH 5.6, and one would expect a band at the protonated marker band position. However, there is no band in the 1720–1770 cm^{-1} region in Fig. 1 *a*. In fact, no band is observed down to 1685 cm^{-1} with a signal-to-noise of 0.2% of the amide I intensity. From an expected intensity of 0.7% of a fully protonated Asp group to the signal to noise, it can be concluded that the Asp-27 group is at least 70% ionized. Consistent with this assignment, a positive band at 1436 cm^{-1} is observed that has an intensity of about 2% of that of the protein amide I band. Because this band is near the correct position and is of the predicted intensity, it can be assigned to the symmetrical CO_2^- stretch. This assignment must remain somewhat tentative because other bands, due to protein changes arising from the D27S mutation, can show up in this region, as will be shown below. It is not feasible to perform measurements at lower values of pH, to protonate the group, because ecDHFR is unstable under more acidic conditions.

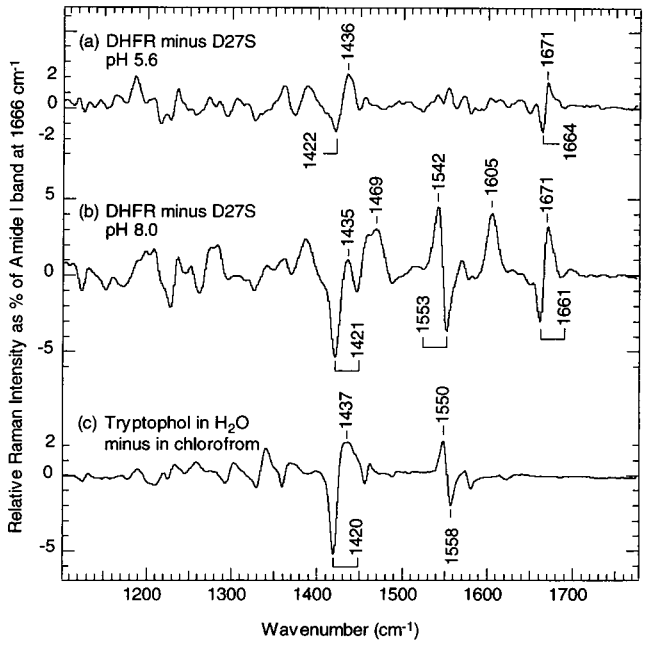

FIGURE 1 Raman difference spectra between wt DHFR and mutant D27S in (*a*) 25 mM MES (4-morpholineethanesulfonic acid) and 0.3 M KCl, pH 5.6; (*b*) 10 mM Tris and 0.5 M KCl, pH 8.0; (*c*) Raman spectrum of tryptophol in H_2O minus that in chloroform (solvent spectra were subtracted out).

The difference Raman spectrum between wild-type ecDHFR and the mutant D27S at higher pH is much more complicated. For example, Fig. 1 *b* shows the difference spectrum at pH 8. Several bands are observed in the difference spectrum that have an intensity of 3–5% of the protein amide I band. These difference bands arise from substantial structural changes between the wild-type and D27S mutant protein under alkaline conditions. Several residues apparently find themselves in altered environments, giving rise to numerous differences in the Raman spectra. In general, it is very difficult to assign these features because they may arise from anywhere in the protein. However, the negative/positive set of bands at 1661/1671 cm^{-1} in Fig. 1, *a* and *b*, can be assigned to perturbation to the protein backbone carbonyl, which shifts the frequency of the amide I C=O stretch from 1671 cm^{-1} to 1661 cm^{-1}. The position of the amide I band is known to be sensitive to protein secondary structure. From the relative intensity of these bands compared to the intensity of the amide I band, it can be estimated that three to four carbonyls, net, are affected by the mutation at pH 8, whereas only one or two are affected at pH 5.6.

In addition, two pairs of positive/negative bands at 1435/1421 and 1542/1553 cm^{-1} (Fig. 1 *b*) can be assigned to perturbation of one or more tryptophan residues. It has been shown that the N_ε-H bending frequency of tryptophan's indole ring (W6 mode) is sensitive to hydrogen bonding, ranging from 1422 cm^{-1} in the nonbonded state to 1441 cm^{-1} in a strongly hydrogen bonded state (Miura et al., 1989). The presence of a positive band at 1435 cm^{-1} and a negative band at 1421 cm^{-1} suggests that one or more tryptophan residues in the wild-type apo enzyme are more exposed to solvent (strongly hydrogen bonded) than that in the mutant D27S (weakly or not hydrogen bonded). This assignment is also confirmed by the positive/negative pair at 1437/1420 cm^{-1} in Fig. 1 *c*, which graphs the difference spectrum between tryptophol in water and tryptophol in chloroform (tryptophol has the same indole ring as tryptophan, shown below).

Another indole ring mode of tryptophan (W3) has an intense band at 1542–1560 cm^{-1}, which has a major contribution from the C_γ-$C_{\delta 1}$ stretch (Takeuchi and Harada, 1986). Besides being affected by the polarity of the solvent environment, as shown by the 1550/1558 cm^{-1} pair in Fig. 1 *c*, it is also sensitive to ring conformation. Studies of a series of crystalline tryptophan derivatives have shown that the W3 frequency increases sigmoidally with $\chi^{2,1}$, the dihedral angle about the bond connecting the indole ring with the C_β atom of the tryptophan side chain (Miura et al., 1989; Maruyama and Takeuchi, 1995), and an empirical equation was found as follows:

$$\nu(\mathrm{W3}) = 1542 + 6.7*[\cos(3*|\chi^{2,1}|) + 1]^{1,2},$$

where ν(W3) is the W3 frequency in cm^{-1} and $\chi^{2,1}$ is expressed in degrees.

There are five tryptophan residues, Trp-22, 30, 47, 74, and 133, in ecDHFR, and their dihedral angles $\chi^{2,1}$ in the

x-ray structure of wild-type apo enzyme (Bystroff and Kraut, 1991) are 50°, −90°, −109°, 82°, and 83°, corresponding to vibrational frequencies of 1543, 1550, 1556, 1547, and 1547 cm^{-1}, respectively, as calculated from the empirical equation. Only Trp-22 has a dihedral angle that gives a frequency close to the 1542 cm^{-1} positive band found in Fig. 1 *b*. It thus seems reasonable to assign the 1542 cm^{-1} band in Fig. 1 *b* to Trp-22 in the wild-type protein at pH 8.0 and the 1553 cm^{-1} negative band to Trp-22 in the D27S mutant. Trp-22 is located in the Met-20 loop (residues 9–23), which forms part of the active site. Both x-ray crystallography (Bystroff et al., 1990; Bystroff and Kraut, 1991) and NMR (Falzone et al., 1994) studies have revealed that this loop is very flexible and easily perturbed, so it is not surprising that mutation of Asp-27, the only charged residue at the active site, to a serine would have a substantial effect on the conformation of Trp-22.

The amplitude of the positive/negative pair of bands at 1542/1553 cm^{-1} relative to the protein band at 1450 cm^{-1} (A_{diff}/A_{1450}) did have a pH-dependent profile, shown in Fig. 2. This pH-dependent relative amplitude was fitted to the Henderson-Hasselbalch equation (Eq. 1), and a pK_a of 6.3 (± 0.1) was obtained. This shows that the solution conformation of the apo enzyme is pH dependent. As argued above, the carboxyl group of Asp-27 is fully ionized above pH 5.6, so some other ionizable group or groups away from the active site must be able to trigger pH-dependent conformational changes that are seen in the Raman difference spectrum. Indeed, recent x-ray crystallographic results on the ecDHFR · DHF binary complex at different pH's (Chen et al., manuscript in preparation) suggest pH-dependent changes in protein structure, particularly within the Met-20 loop, and its interactions with the rest of the protein.

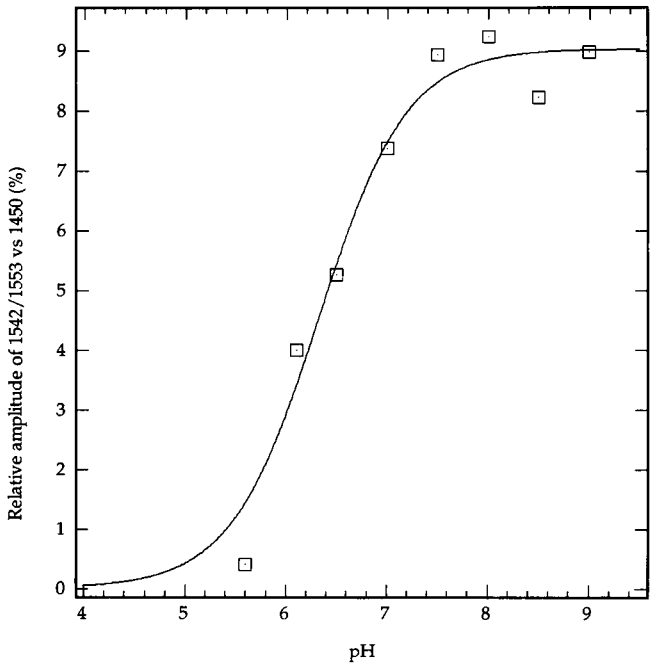

FIGURE 2 The pH dependence of the relative amplitude of the derivative bands at 1542 and 1553 cm^{-1} (to protein band at 1450 cm^{-1}). The solid line represents the best fit of data points to a titration curve with amplitude and pK_a as the only parameters. The value found for pK_a was 6.3 (± 0.1).

DISCUSSION

The pK_a of the active-site carboxyl group is not elevated

Enzyme activities are often found to be pH dependent because they rely on a specific state of ionization of certain ionizable residues. The effect of pH on ecDHFR was first investigated systematically by Stone and Morrison (1983, 1984). A group on the enzyme with a pK_a value of about 6.3 was found to be involved in the formation of the binary complex with 2,4-diamino-6,7-dimethypteridine (Stone and Morrison, 1983). Kinetic parameters of the ecDHFR-catalyzed reaction were also found to have distinct pH profiles (Stone and Morrison, 1984), with a pK_a of 8.4 for the maximum velocity (*V*), a pK_a of 8.1 for the apparent second-order rate constant (*V/K*), and a pK_a of 7.9 for the inhibition by 2,4-diamino-6,7-dimethypteridine. It was later found by Fierke et al. (1987) that the pK_a of about 8.4 observed in the steady-state kinetics of the enzyme reaction was an "apparent" pK_a, resulting from a change in the rate-determining step from product release at low pH to hydride transfer above pH 8.4. Using the stopped-flow technique, they found that the rate of hydride transfer could be described by a single pH-dependent step with a pK_a of 6.5. Since then, these methods have been applied to various mutants of DHFR, and pK_a's for binding and kinetic parameters have been similarly observed, with values ranging from 5.6 to 8.6 (see a summary by Blakley et al., 1993). In the absence of evidence to the contrary, the group responsible for this widely varying pK_a is almost always assumed to be the ionizable group at the active site (Asp-27 in the wild-type ecDHFR).

However, there is no firm basis for believing the carboxyl group of Asp-27 has an elevated pK_a from the normal value of about 4, other than the fact that this group is the only ionizable residue at the catalytic site. The current results from Raman difference spectra between the wild-type ecDHFR and the mutant D27S show that the carboxyl group of Asp-27 is, however, fully ionized throughout the pH range 5.6–9. This is in agreement with an inference from NMR studies of human DHFR that the corresponding active-site Glu-30 carboxyl group in that enzyme is also ionized in the pH range 6–9 (Blakley et al., 1993). Furthermore, if the carboxyl group had a highly elevated pK_a, mutation of Asp-27 to serine should have no effect on the isoelectric point (pI) of ecDHFR, which is 4.72 in the wild type. However, the pI for the D27S mutant is raised to 4.88 (Howell et al., 1987). Similarly, the pI for *Lactobacillus casei* DHFR increases from 6.25 in the wild type to 6.9 in the Asp-26 to asparagine mutant (Basran et al., 1995). These results are inconsistent with the assumption that the car-

boxyl group of this residue has a highly elevated pK_a. Other evidence as pointed out by Blakley et al. (1993) includes the following: (1) The active site of DHFR contains structural water molecules, which is inconsistent with the assumption that the pK_a is elevated by a hydrophobic microenvironment. (2) When Asp-27 is replaced by a cysteine residue, the Cys-27 sulfhydryl exhibits its normal pK_a of 8.56. (3) The dissociation rate, k_{off}, for folate from complexes of ecDHFR exhibits little or no pH dependence. Taking all of the evidence together, we conclude that the active-site carboxyl group has a normal pK_a of less than 5.

We have shown in a previous paper (Chen et al., 1994) that the pK_a of N5 of DHF is elevated by four units from its solution value of 2.6 to a bound value of 6.5 in the ternary complex of ecDHFR · DHF · $NADP^+$, suggesting that protonation of N5 of DHF is responsible for the "intrinsic" pK_a measured from the rate of hydride transfer (Fierke et al., 1987). However, because the pK_a of DHF is not measurably elevated in the ecDHFR · DHF binary complex, pH dependence is observed for inhibitor binding where DHF is not present, and the apo enzyme also shows pH-dependent conformational changes (see discussion below), some other group or groups on the enzyme must contribute to the observed pH dependence of DHFR's binding and kinetic properties.

Function of the active-site carboxyl group

What, then, is the role of Asp-27 in catalysis if its carboxyl side chain remains ionized? Because mutation of Asp-27 to asparagine or serine reduces the activity of the enzyme by several orders of magnitude (Howell et al., 1986), this carboxyl group must play an important role in catalysis. The most obvious role is substrate binding. X-ray crystallographic studies show that the Asp-27 side chain forms hydrogen bonds to the 2-amino group and to N3 of the pteridine ring (Bolin et al., 1982; Bystroff et al., 1990).

The most important role for a negatively charged carboxyl group of Asp-27 is probably to stabilize the protonated substrate. This was demonstrated by our finding that the pK_a of DHF's N5 is raised four units from its value of 2.6 in solution to 6.5 in the wild-type ecDHFR · DHF · $NADP^+$ ternary complex, but no elevation was observed in the mutant D27S · DHF · $NADP^+$ (Chen et al., 1994). Further support comes from the observation that when Thr-113 is changed to a glutamate in addition to the replacement of Asp-27 by serine, the resulting double-mutant enzyme has a three-fold greater k_{cat} than the D27S mutant (Howell et al., 1987), implying that the displaced carboxyl group of Glu-113 could also stabilize the protonated DHF, but with greatly decreased efficiency.

pH-dependent structural changes take place in the apo enzyme of ecDHFR

Our Raman data show a pH dependence with a pK_a of 6.3 in the intensity of two pairs of positive/negative bands in the difference spectra between the wild-type ecDHFR and the mutant D27S, tentatively assigned to perturbation of the indole ring of Trp-22 (Fig. 2 shows the titration of the 1542/1553 cm^{-1} pair). In addition, the same pH dependence was found for bands in the Raman difference spectrum that arise from the structure sensitive protein amide I bands. These features in the difference spectrum show, definitively, that the solution structure of ecDHFR apo enzyme undergoes pH-dependent conformational changes, so that ecDHFR is in equilibrium between high- and low-pH forms. This pH dependence in structure is likely to be associated with the essentially same pK_a found for the binding of certain inhibitors (Stone and Morrison, 1983). For example, only the low-pH form of DHFR may bind them with significant affinity. It is also possible that the low-pH conformer binds protonated substrate preferentially in competent ternary complexes, so that the pK_a of N5 is 6.5 when on the enzyme. Further study is required on this latter point, because the pK_a of N5 is not raised in DHFR/DHF binary complexes (Chen et al., 1994).

Recent x-ray crystallographic studies, in agreement with the present Raman results that pertain to solution structures, have also found changes in the crystal structures of ecDHFR · DHF binary complexes when the pH is changed from 7.2 to 5.1, particularly in the Met-20 loop (residues 9–23) (Chen et al., manuscript in preparation). These changes seem to be associated with changes of protonation states of histidine residues, particularly His-45, His-124, and His-149. Indeed, mutations at distal sites (away from the active site) have been found to have substantial effects on the pK_a determined from the rate of the hydride transfer, which is elevated from 6.5 in the wild-type ecDHFR to 7.5 in the H45Q mutant, and 8.4 in the R44L mutants (Adams et al., 1989), and 7.3 in the H45R/W47Y/I50F triple mutant (Li and Benkovic, 1991). Thus, protonation of groups away from the active site must contribute to the pH dependence of the enzyme's binding and kinetic properties.

CONCLUSION

Raman difference spectra between wild-type ecDHFR and the D27S mutant show that the carboxyl side chain of Asp-27 at the active site is ionized in the pH range 5.6–9.0. Thus it appears that the most important role for the invariant carboxyl group is to stabilize the N5-protonated DHF substrate in the enzyme ternary complex with cofactor.

We did, however, detect pH-dependent conformational changes in the apo enzyme, as reflected by the pH dependence of Raman bands from the indole ring of a tryptophan residue(s), presumably Trp-22. These conformational changes at the active site could result from protonation/deprotonation of groups away from the catalytic site. These distal groups are likely to be responsible for the pH dependence of DHFR's binding properties and perhaps, ultimately, the pH dependence of some of its catalytic kinetic parameters.

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REFERENCES

Adams, J., K. Johnson, R. Matthews, and S. J. Benkovic. 1989. Effects of distal point-site mutations on the binding and catalysis of dihydrofolate reductase from *Escherichia coli*. *Biochemistry*. 28:6611–6618.

Appleman, J. R., E. E. Howell, J. Kraut, and R. L. Blakley. 1990. Role of aspartate-27 of dihydrofolate reductase from *Escherichia coli* in interconversion of active and inactive enzyme conformers and binding of NADPH. *J. Biol. Chem.* 265:5579–5584.

Basran, J., M. G. Casarotto, I. L. Barsukov, and G. C. K. Roberts. 1995. Role of the active-site carboxylate in dihydrofolate reductase—kinetic and spectroscopic studies of the aspartate 26 to asparagine mutant of the *Lactobacillus casei* enzyme. *Biochemistry*. 34:2872–2882.

Blakley, R. L., J. R. Appleman, J. H. Freisheim, and M. J. Jablonsky. 1993. ^{13}C and ^{15}N nuclear magnetic resonance evidence that the active site carboxyl group of dihydrofolate reductase is not involved in the relay of a proton to substrate. *Arch. Biochem. Biophys.* 306:501–509.

Bolin, J. T., D. J. Filman, D. A. Matthews, R. C. Hamlin, and J. Kraut. 1982. Crystal structures of *Escherichia coli* and *Lactobacillus casei* dihydrofolate reductase refined at 1.7 ≈ resolution I. General features and binding of methotrexate. *J. Biol. Chem.* 257:13650–13662.

Bystroff, C., and J. Kraut. 1991. Crystal structure of unliganded *Escherichia coli* dihydrofolate reductase. Ligand-induced conformational changes and cooperativity in binding. *Biochemistry*. 30:2227–2239.

Bystroff, C., S. J. Oatley, and J. Kraut. 1990. Crystal structure of *Escherichia coli* dihydrofolate reductase: the binding of $NADP^+$ holoenzyme and the folate-$NADP^+$ ternary complex. Substrate binding and a model for the transition state. *Biochemistry*. 29:3263–3277.

Callender, R., and H. Deng. 1994. Nonresonance raman difference spectroscopy—a general probe of protein structure, ligand binding, enzymatic catalysis, and the structures of other biomacromolecules. *Annu. Rev. Biophys. Biomol. Struct.* 23:215–245.

Chen, Y.-Q., J. Kraut, R. L. Blakley, and R. Callender. 1994. Determination by Raman spectroscopy of the pKa of N5 of dihydrofolate bound to *Escherichia coli* dihydrofolate reductase—mechanistic implications. *Biochemistry*. 33:7021–7026.

Daimay, L.-V., N. B. Colthup, W. G. Fateley, and J. G. Grasselli. 1991. The Handbook of Infrared and Raman Characteristic Frequencies of Organic Molecules. Academic Press, San Diego, CA.

Dioumaev, A. K., and M. S. Braiman. 1995. Modeling vibrational spectra of amino acid side chains in proteins—the carbonyl stretch frequency of buried carboxylic residues. *J. Am. Chem. Soc.* 117:10572–10574.

Falzone, C. J., P. E. Wright, and S. J. Benkovic. 1994. Dynamics of a flexible loop in dihydrofolate reductase from *Escherichia coli* and its implications for catalysis. *Biochemistry*. 33:439–442.

Fierke, C. A., K. A. Johnson, and S. J. Benkovic. 1987. Construction and evaluation of the kinetic scheme associated with dihydrofolate reductase from *Escherichia coli*. *Biochemistry*. 26:4085–4092.

Howell, E. E., J. E. Villafranca, M. S. Warren, S. J. Oatley, and J. Kraut. 1986. Functional role of aspartic acid-27 in dihydrofolate reductase revealed by mutagenesis. *Science*. 231:1123–1128.

Howell, E. E., M. S. Warren, C. L. J. Booth, J. E. Villafranca, and J. Kraut. 1987. Construction of an altered proton donation mechanism in *Escherichia coli* dihydrofolate reductase. *Biochemistry*. 26:8591–8598.

Huennekens, F. M., and K. G. Scrimgeour. 1964. Tetrahydrofolic acid–the coenzyme of one-carbon metabolism. *In* Pteridine Chemistry (W. Pfliederer and E. C. Taylor, editors. MacMillan, Oxford, England. 355–376.

Li, L., and S. J. Benkovic. 1991. Impact on catalysis of secondary structural manipulation of the αC-helix of *Escherichia coli* dihydrofolate reductase. *Biochemistry*. 30:1470–1478.

Maruyama, T., and H. Takeuchi. 1995. Effects of hydrogen bonding and side-chain conformation on the Raman bands of tryptophan-2,4,5,6,7-d5. *J. Raman Spectrosc.* 26:319–324.

Miura, T., H. Takeuchi, and I. Harada. 1989. Tryptophan Raman bands sensitive to hydrogen bonding and side-chain conformation. *J. Raman Spectrosc.* 20:667–671.

Morrison, J. F., and S. R. Stone. 1988. Mechanism of the reaction catalysed by dihydrofolate reductase from *Escherichia coli:* pH and deuterium isotope effects with NADPH as the variable substrate. *Biochemistry*. 27:5499–5506.

Stone, S. R., and J. F. Morrison. 1983. The pH-dependence of the binding of dihydrofolate and substrate analogues to dihydrofolate reductase from *Escherichia coli*. *Biochim. Biophys. Acta*. 745:247–258.

Stone, S. R., and J. F. Morrison. 1984. Catalytic mechanism of the dihydrofolate reductase reaction as determined by pH studies. *Biochemistry*. 23:2753–2758.

Takeuchi, H., and I. Harada. 1986. Normal coordinate analysis of indole ring. *Spectrochim Acta A*. 42:1069–1078.

Uchimaru, T., S. Tsuzuki, K. Tanabe, S. J. Benkovic, K. Furukawa, and K. Taira. 1989. Computational studies on pterins and speculations on the mechanism of action of dihydrofolate reductase. *Biochim. Biophys. Res. Commun.* 161:64–68.

Villafranca, J. E., E. E. Howell, D. H. Voet, M. S. Strobel, R. C. Ogden, J. N. Abelson, and J. Kraut. 1983. Directed mutagenesis of dihydrofolate reductase. *Science*. 222:782–788.