Catalytic Mechanism of the Dihydrofolate Reductase Reaction As Determined by pH Studies†

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ABSTRACT: The variation with pH of the kinetic parameters of the reaction catalyzed by dihydrofolate reductase from *Escherichia coli* has been determined with the aim of elucidating the chemical mechanism of the reaction. The $(V/K)_{DHF}$ and V profiles indicated that protonation enhances the observed rate of interaction of dihydrofolate (DHF) with the enzyme–NADPH complex as well as the maximum velocity of the reaction. The pK_a value of 8.09 observed in the $(V/K)_{DHF}$ profile is similar to that of 7.9 observed in the K_i profile for 2,4-diamino-6,7-dimethylpteridine while the pK_a value of the V profile is displaced to 8.4. From the magnitude of the pH-independent value for $(V/K)_{DHF}$, it is concluded that unprotonated dihydrofolate must react, at neutral pH, with the protonated form of the enzyme. The $^D(V/K)_{DHF}$ value is independent of pH and equal to unity whereas the ^DV value varies as a wave function of pH with limiting values of 1.5 and 1.0 at low and high pH, respectively. It is proposed that dihydrofolate reacts with the unprotonated enzyme–NADPH complex to form a dead-end complex and with the protonated form of the same complex to form a productive complex. Further, it is considered that the protonated carboxyl of Asp-27 at the active site of the enzyme is responsible for the protonation of the N-5 nitrogen of dihydrofolate and that this protonation precedes and facilitates hydride transfer.

Dihydrofolate reductase (5,6,7,8-tetrahydrofolate:$NADP^+$ oxidoreductase, EC 1.5.1.3) catalyzes the NADPH-dependent reduction of dihydrofolate (DHF)[1] to tetrahydrofolate. The inhibition of the enzyme by folate analogues such as methotrexate and trimethoprim has been the subject of intensive investigation (Gready, 1980), and the importance of the interaction of the carboxyl group of Asp-27 with the N-1 nitrogen of 2,4-diaminopteridines has become apparent (Cocco et al., 1981; Matthews et al., 1977; Hood & Roberts, 1978). Less attention has been paid to the function of the aspartate residue in enzymic catalysis, but it has been suggested that, with the enzyme from *Streptococcus faecium*, protonation is important for catalysis and that the hydrogen of the protonated carboxyl group of an aspartate residue acts as an auxiliary catalyst that facilitates hydride transfer from NADPH to DHF (Williams & Morrison, 1981).

A range of kinetic procedures, as outlined by Cleland (1977, 1982) as well as by Tipton & Dixon (1979), has been used to determine the effects of pH on the magnitude of various kinetic parameters and on the deuterium isotope effects of the reaction catalyzed by dihydrofolate reductase from *Escherichia coli*. The results indicate that protonation of the carboxyl group of Asp-27 is important for catalysis and that protonation of DHF precedes hydride transfer from NADPH.

Experimental Procedures

Materials

Dihydrofolate was prepared from folic acid by the method of Blakley (1960) and stored at −20 °C; 2,4-diamino-6,7-dimethylpteridine was a gift from Dr. D. J. Brown. Deuterated NADP, APADPH, and deuterated APADP were synthesized as described previously (Stone & Morrison, 1982). All other chemicals were of the highest purity available commercially. Dihydrofolate reductase was purified from an overproducing mutant of *Escherichia coli* (JFM 228; Smith et al., 1982) by the procedure of Stone & Morrison (1982). The concentration of methotrexate was estimated spectrophotometrically at 302 nm by using a molar extinction coefficient of 22 100 M^{-1} cm^{-1} at pH 13 (Seeger et al., 1949); 2,4-diamino-6,7-dimethylpteridine was estimated in its neutral form by using the extinction coefficient for 2,4-diaminopteridine of 7 200 M^{-1} cm^{-1} at 364 nm (Brown & Jacobsen, 1961), and the concentration of APADPH was determined by using an extinction coefficient of 9 100 M^{-1} cm^{-1} at 363 nm (P-L Biochemicals, 1961). Concentrations of dihydrofolate and NADPH were determined enzymically by using a molar absorbancy change for the dihydrofolate reductase reaction of 11 800 M^{-1} cm^{-1} at 340 nm (Stone & Morrison, 1982).

Methods

Enzyme Assays. Initial velocities for dihydrofolate reductase were determined at 30 °C as described previously (Stone & Morrison, 1982). The buffer used for the assays contained 50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), 25 mM tris(hydroxymethyl)aminomethane (Tris), 25 mM ethanolamine, and 100 mM NaCl. Over the pH range for which initial velocities were measured (5.6–10.4), the ionic strength of this buffer mixture remained essentially constant at $I = 0.15$ (Ellis & Morrison, 1982). The concentration of NADPH or APADPH was maintained constant at 100 μM. At this concentration, the enzyme was saturated with these substrates over the entire pH range tested. The molar absorbance change for the reaction with either coenzyme was 11 800 M^{-1} cm^{-1}. Maximum velocity (V) is expressed in units of reciprocal seconds and the apparent second-order rate constant (V/K) in units of M^{-1} s^{-1}.

The pH of the assay mixture was determined at the temperature of the assay before and after the reaction by using a Radiometer PHM 62 meter standardized with Beckman buffers. For solvent perturbation studies, the pH of the assay mixture was measured in the presence of 20% (v/v) methoxyethanol (Inagami & Sturtevant, 1962; Tipton & Dixon, 1979).

Ultraviolet Difference Spectroscopy. Ultraviolet difference spectra were measured under the same conditions and by use

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[1] Abbreviations: DHF, 7,8-dihydrofolate; APADP(H), 3-acetylpyridine adenine dinucleotide phosphate (reduced).

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Table I: Values for pK_a and pH-Independent Kinetic Parameters Associated with the Reaction Catalyzed by Dihydrofolate Reductase

conditions	parameter determined	pH-independent value of parameter	pK_A	pK_B
dihydrofolate varied, [NADPH] = 100 μM	V (s^{-1})	17.7 ± 0.4		8.38 ± 0.04
	$(V/K)_{DHF}$ (M^{-1} s^{-1} × 10^{-7})	2.00 ± 0.17		8.09 ± 0.06
dihydrofolate varied + 20% (v/v) 2-methoxyethanol, [NADPH] = 100 μM	V (s^{-1})	14.1 ± 0.4		8.68 ± 0.04
	$(V/K)_{DHF}$ (M^{-1} s^{-1} × 10^{-7})	0.89 ± 0.11		7.77 ± 0.03
2,4-diamino-6,7-dimethylpteridine varied	K_i (nM)	12.8 ± 1.2	5.89 ± 0.07	7.90 ± 0.05
2,4-diamino-6,7-dimethylpteridine varied + 20% (v/v) 2-methoxyethanol	K_i (nM)	10.9 ± 1.5		7.18 ± 0.05
dihydrofolate varied, [APADPH] = 100 μM	V (s^{-1})	3.33 ± 0.13		7.87 ± 0.04
	$(V/K)_{DHF}$ (M^{-1} s^{-1} × 10^{-6})	3.72 ± 0.59		7.52 ± 0.06

of the same procedures as those described by Stone & Morrison (1983a).

Data Analysis. Data obtained at each pH value by varying the concentration of DHF (A) were fitted to eq 1 to yield

$$v = \frac{V[A]}{K_a + [A]} \quad (1)$$

values for the maximum velocity (V) and the Michaelis constant (K_a) as well as for V/K_a. In experiments in which the concentration of an inhibitor (I) was varied, the data were fitted to eq 2. Analysis according to eq 2 at each pH value

$$v = \frac{V_{app}}{1 + [I]/K_{i\,app}} \quad (2)$$

yielded an apparent inhibition constant ($K_{i\,app}$) from which a value for the inhibition constant (K_i) at that pH could be calculated by using the relationship given in eq 3 together with

$$K_i = \frac{K_{i\,app}}{1 + [A]/K_a} \quad (3)$$

the known substrate concentration (A) and the previously determined Michaelis constant (K_a). Values obtained for V, V/K_a, and $1/K_i$ were then weighted according to the inverse of their variances and fitted to eq 4 or 5, where y represents

$$y = \frac{C}{1 + K_B/[H]} \quad (4)$$

$$y = \frac{C}{1 + [H]/K_A + K_B/[H]} \quad (5)$$

the value of V, V/K_a, or $1/K_i$ at a particular pH value; C represents the pH-independent value of the parameter; K_A and K_B are acid dissociation constants.

The enthalpy of ionization (ΔH_{ion}) was determined by weighting pK_a values obtained at different temperatures according to the inverse of their variances and fitting to eq 6.

$$pK = \Delta H_{ion}/(2.303RT) \quad (6)$$

Isotope effects [^DV and $^D(V/K)$] were determined by fitting data obtained with the unlabeled or deuterated pyridine nucleotide to eq 1 and then calculating the appropriate ratio.

If the absorbance of a ligand at a particular wavelength is dependent on its state of protonation and if the pK_a of the ligand changes upon binding to the enzyme, the difference spectrum of the enzyme–ligand complex will show a pH dependence that can be described by eq 7 (Hood & Roberts,

$$\Delta\epsilon = -\frac{\epsilon_l + \epsilon_{lh}[H]/K_F}{1 + [H]/K_F} + \frac{\epsilon_{elh} + \epsilon_{el}K_B/[H]}{1 + K_B/[H]} \quad (7)$$

1978; Stone & Morrison, 1983a), where $\Delta\epsilon$ is the change in absorbance per mole of bound ligand; ϵ_l, ϵ_{lh}, ϵ_{el}, and ϵ_{elh} are the extinction coefficients for the free ionized ligand, free protonated ligand, bound ionized ligand, and bound protonated ligand, respectively. K_F and K_B are the acid dissociation constants of the free and bound ligand, respectively. This equation assumes that over the pH range tested there are no changes in the form of the enzyme–ligand complex that will alter the extinction coefficients of the bound ligand species other than ionization of the ligand.

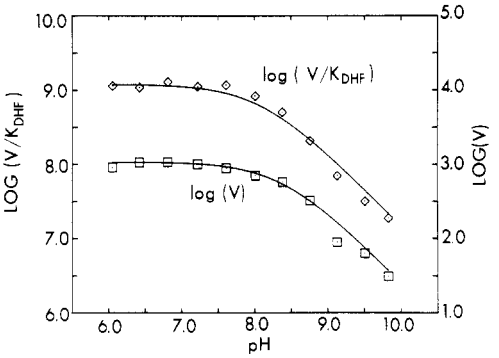


FIGURE 1: Variation with pH of log V and log $(V/K)_{DHF}$ for the reaction catalyzed by dihydrofolate reductase. The units for V and $(V/K)_{DHF}$ are reciprocal seconds and M^{-1} s^{-1}, respectively. The lines represent a best fit to eq 4 with the parameters given in Table I.

Data were fitted to eq 1, 2, 4–6, and a reduced form of eq 7 by using programs written in BASIC to perform weighted, robust regression (Cornish-Bowden & Endrenyi, 1981; Duggleby, 1981). These programs include a procedure for iterative weighting that minimizes the effect of outlying points on the least-squares solution (Mosteller & Tukey, 1977).

Results

pH Dependence of V and V/K with DHF Varied. Values for V and $(V/K)_{DHF}$ were determined over the pH range 6.0–9.9. The pH dependence of these values, which is illustrated in Figure 1, suggests that protonation is important for both the interaction of DHF and the maximum velocity of the reaction. Data at pH values less than 6.0 were difficult to obtain because of the instability of NADPH below this pH. Analysis of the data for the V and $(V/K)_{DHF}$ profiles yielded pK_a values of 8.38 ± 0.02 and 8.09 ± 0.06, respectively (Table I). The effect of 2-methoxyethanol (20%) on the pK_a values is given in Table I. The pK_a value from the V profile is increased by 0.3 pH unit whereas that from the $(V/K)_{DHF}$ profile is decreased by the same amount. For a neutral acid, the pK_a value should increase in the presence of an organic solvent whereas the pK_a value for a cationic acid should not change. A decrease in the pK_a value cannot, however, be explained on the basis of the effect of the organic solvent on an ionizing group exposed to the bulk solvent. The unexpected change in the pK_a value obtained from the $(V/K)_{DHF}$ profile, coupled with the smallness of the change in both values, precludes identification of the group(s) responsible for these pK_a values and suggests that methoxyethanol causes the en-

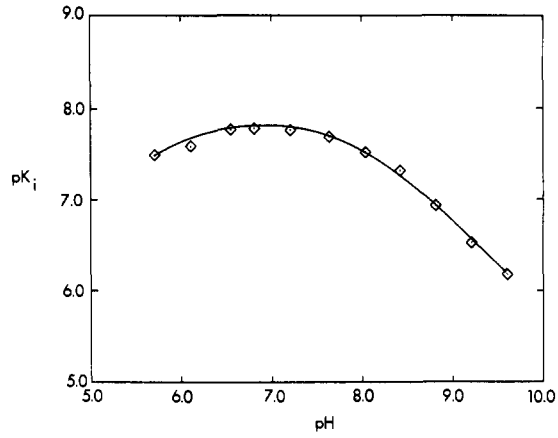


FIGURE 2: Variation with pH of the pK_i (− log K_i) value for 2,4-diamino-6,7-dimethylpteridine. The units for K_i are M^{-1}. The curve represents a best fit to eq 5 with the parameters given in Table I.

zyme to undergo a conformational change [cf. Grace & Dunaway-Mariano (1983)]. The pH dependence of pK_a values was studied at 20, 30, and 39 °C in order to determine the enthalpies of ionization (ΔH_{ion}) for the groups responsible for the pK_a values observed in the profiles. ΔH_{ion} values of 5.7 ± 3.1 and 3.3 ± 1.8 kcal were determined from the V and $(V/K)_{DHF}$ profiles, respectively. The values for ΔH_{ion} lie in between those expected for a carboxyl group and those expected for an imidazole group.

In drawing conclusions from pH studies, it is important to determine if the varied substrate is sticky, that is, if it dissociates from the enzyme more slowly than it reacts to yield products (Cleland, 1977, 1982). The usual check is to determine if an altered pK_a value is obtained with an alternative varied substrate that yields a lower maximum velocity. In the present study, a different approach was used. Thus, it was the nonvaried substrate that was changed to one that gave a lower maximum velocity. Values of 7.87 ± 0.04 and 7.52 ± 0.06 were obtained from the V and $(V/K)_{DHF}$ profiles, respectively, in the presence of APADPH, which gives rise to a 5-fold lower V value than does NADPH.

pH Dependence of Inhibition by a Substrate Analogue. Although the pK_a value determined from a V/K profile may be displaced, the analysis of the pH dependence of the inhibition caused by a substrate analogue will always yield the correct pK_a value. The variation with pH of the inhibition by 2,4-diamino-6,7-dimethylpteridine (Figure 2) yielded a bell-shaped curve with two pK_a values of 5.89 ± 0.07 and 7.90 ± 0.05 (Table I). The lower pK_a value corresponds well with the value of 5.75 ± 0.03 determined for the protonation of the N-1 nitrogen of the pteridine (Stone & Morrison, 1983b). The other pK_a value must represent a group on the enzyme. To examine the nature of this group, its pK_a value was redetermined in the presence of 2-methoxyethanol (20%). The resulting pK_a value was 0.7 pH unit lower, and once again, this result cannot be explained in terms of the effect on the organic solvent on a group exposed to the bulk solvent. The results seem to confirm that the organic solvent causes a conformational change in the enzyme.

Although the bell-shaped curve indicates that protonation is important for the interaction of enzyme with 2,4-diamino-6,7-dimethylpteridine, no conclusions can be reached from such a result as to whether the formation of the enzyme–NADPH–pteridine complex involves protonated and/or unprotonated ligand (Cleland, 1977; Stone & Morrison, 1983b).

Difference Spectroscopy of the Enzyme–Pteridine Complex. In connection with the interpretation of the data of Figure 2,

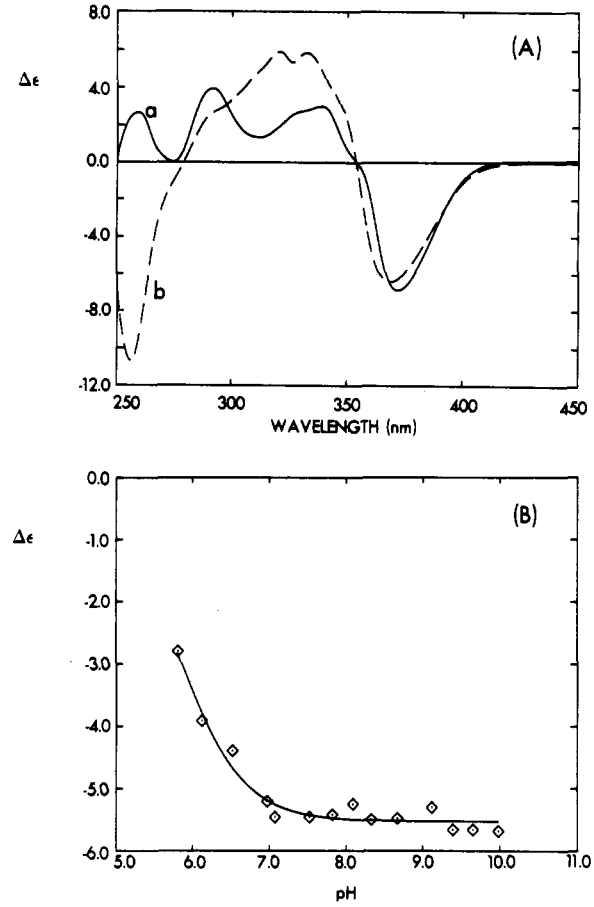


FIGURE 3: (A) Difference spectrum generated on the binding of 2,4-diamino-6,7-dimethylpteridine to the enzyme–NADPH complex at pH 7.6 (a) compared with the protonation spectrum for the pteridine (b). The units for the extinction coefficient (Δε) are M^{-1} cm^{-1} × 10^{-3}. (B) Variation with pH of the difference extinction coefficient (Δε) for the binding of 2,4-diamino-6,7-dimethylpteridine. The units for the extinction coefficient are M^{-1} cm^{-1} × 10^{-3}. The curve represents a best fit to eq 7 with pK_F = 5.77, K_B = 0, ϵ_l = 5690 M^{-1} cm^{-1}, ϵ_{lh} = 60 M^{-1} cm^{-1}, and ϵ_{elh} = 160 M^{-1} cm^{-1}.

investigations were made to determine if 2,4-diamino-6,7-dimethylpteridine is protonated in the ternary complex that it forms with dihydrofolate reductase and NADPH. Figure 3A presents the difference spectrum for the binding of the pteridine in the ternary complex at pH 7.6 and compares this spectrum with the protonation spectrum for the pteridine. The two spectra exhibit similarities that suggest that the pteridine is protonated when bound to the enzyme. Like results have been obtained for the binding of 2,4-diaminopteridines to the enzyme from other sources (Hood & Roberts, 1978; Subramanian & Kaufman, 1978; Poe et al., 1974, 1975; Erickson & Matthews, 1972). The similarity between the two spectra of Figure 3A is most marked between 350 and 400 nm. If the spectral changes between these two wavelengths are due mainly to the increased pK_a of the pteridine in the ternary complex, the amplitude of the spectral band difference at a fixed wavelength will show a pH dependence described by eq 7. This equation predicts that the absolute amplitude of the spectrum will increase in the region of the pK_a of the free ligand (pK_F) and decrease in the region of the bound ligand (pK_B). Figure 3B shows the pH dependence of the amplitude of the binding difference spectrum at 370 nm for the pteridine. The absolute amplitude of the difference spectrum increased in the region of pK_F, but no decrease is observed. Evidently, the pK_F value for the bound pteridine is at least 5 pH units higher than that of the unbound compound. The data were fitted to a modified form of eq 7 in which K_B equaled zero,

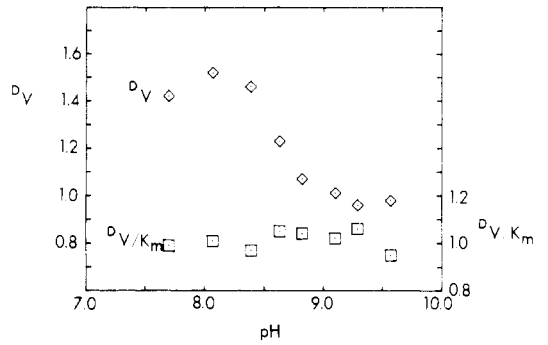


FIGURE 4: Variation with pH of the deuterium isotope effects for the reaction catalyzed by dihydrofolate reductase.

and a value for pK_F of 5.77 ± 0.04 was obtained. This value is in excellent agreement with the independently determined value of 5.75 ± 0.03 for the N-1 nitrogen of the pteridine. Thus, the pteridine is protonated in the ternary complex.

The aforegoing results make it clear not only that the interaction of the enzyme–NADPH complex (E) with 2,4-diamino-6,7-dimethylpteridine results in the formation of a protonated complex (EHL) but also that there is strong association of the proton with the N-1 nitrogen of the pteridine. However, no definitive conclusions can be reached from such thermodynamic results as to the pathway(s) of EHL formation. On the assumption that the EHL complex can be formed by the reactions

$$HE + L \overset{K_1}{\rightleftharpoons} EHL$$

and/or

$$E + LH \overset{K_2}{\rightleftharpoons} EHL$$

values can be obtained for K_1 and K_2. The value for K_1 would be the pH-independent value of 13 nM for K_1 (Figure 2, Table I) while the value for K_2 would be given by the relationship $K_2 = K_{i\,app}K_B/K_A$ (Stone & Morrison, 1983b) and have the lower value of 0.13 nM (Table I).

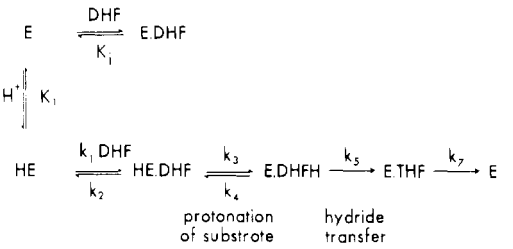
pH Dependence of Isotope Effects. Figure 4 shows the variation with pH of the deuterium isotope effects on V and $(V/K)_{DHF}$ for the dihydrofolate reductase reaction in the presence of saturating NADPH. ^DV decreases from a value of about 1.5 at low pH to a value of 1.0 at high pH. The value of $^D(V/K)_{DHF}$ was constant at 1.0 throughout the pH range tested.

Solvent Isotope Effects. The D_2O solvent isotope effects on V and $(V/K)_{DHF}$ at pH 7.2 were 1.19 ± 0.05 and 1.14 ± 0.06, respectively.

Discussion

From the variation of V and $(V/K)_{DHF}$ with pH (Figure 1), it is apparent that protonation enhances the maximum velocity (V) of the dihydrofolate reductase (E) reaction as well as the rate of interaction of DHF with E–NADPH to yield a productive complex $(V/K)_{DHF}$. In both profiles, only a single ionization is observed, and the similarity of the pK_a values suggests that the same ionizing group at the active center may be responsible for each result. Such a suggestion is supported by the report that the active site of dihydrofolate reductase contains only one ionizing residue, which is Asp-27 for the enzyme from *E. coli* (Bolin et al., 1982). If it had been possible to study the dihydrofolate reductase reaction at lower pH values, it is probable that there would have been observed in the $(V/K)_{DHF}$ profile the pK_a value for the N-5 nitrogen of DHF, which is 3.84 (Poe, 1977). This follows from the fact that when an ionizing enzyme undergoes reaction with an ionizing ligand and the formation of a protonated enzyme–ligand complex is important for reaction, the pH profile will be bell shaped. The two pK_a values will be those for the ligand and the enzyme (Stone & Morrison, 1983b). With such a bell-shaped curve, the pH-independent value of $(V/K)_{DHF}$ could, in theory, represent either a true value for the rate of interaction of protonated enzyme with unprotonated DHF or an apparent value for the rate of reaction of unprotonated enzyme with protonated DHF. Calculation of the true value for the latter interaction with the relationship $(V/K)_{DHF} = K_A \cdot app(V/K_{DHF})/K_B$ [cf. Stone & Morrison (1983b)] and values of 3.84, 8.09, and 2×10^7 M^{-1} s^{-1} (of Table I) for pK_A, pK_B, and $app(V/K)_{DHF}$, respectively, yielded a second-order rate constant of 3.5×10^{11} M^{-1} s^{-1}. This value is considerably greater than 10^9 M^{-1} s^{-1}, which is regarded as the upper limit of the diffusion rate for small molecules. Thus, it can be concluded that the formation of a productive enzyme–NADPH–DHF complex cannot involve interaction between the protonated form of DHF and the ionized form of the enzyme. Instead, at neutral pH, productive binding must occur as a result of reaction between unprotonated DHF and protonated enzyme. Two other points concerning the pH profiles are worthy of note. The first is that the variation in the $(V/K)_{DHF}$ values as a function of pH is due largely to the changes in the V values since K_{DHF} varies only slightly with pH. The second is that neither the $(V/K)_{DHF}$ nor the V profile exhibits any humps or hollows.

Scheme I



Consideration has been given to several schemes that might account for the aforementioned results as well as for the results of the isotope effects. The simplest of these is that which is illustrated in Scheme I. In this scheme, it is assumed that the enzyme–NADPH complex (E) has a single ionizing group and can exist in protonated (HE) and unprotonated (E) forms, that E and HE are in rapid protonic equilibrium, and that both forms of enzyme react with DHF. However, it is considered that only the protonated form of enzyme gives rise to a productive complex and that the presence of DHF on the enzyme prevents dissociation of the proton from HE. The scheme also allows for protonation of DHF by transfer of a proton from HE and for that protonation to precede the hydride transfer, which results in the formation of tetrahydrofolate (THF). The proposed model can be considered as a limiting case of a more general scheme that allows a proton to dissociate from the HE–DHF complex.

The initial velocity equation for the reaction described by Scheme I is given by

$$v = \frac{V'[DHF]/[1 + K_{DHF}'K_1/(K_i[H])]}{K_{DHF}'[(1 + K_1/[H])/[1 + K_{DHF}'K_1/(K_i[H])]] + [DHF]} \quad (8)$$

where V', the pH-independent maximum velocity of the reaction, is equal to $k_3k_5k_7[E_t]/(k_3k_5 + k_3k_7 + k_4k_7 + k_5k_7)$ and K_{DHF}', the pH-independent Michaelis constant, is equal to $k_7(k_2k_4 + k_2k_5 + k_3k_5)/[k_1(k_3k_5 + k_3k_7 + k_4k_7 + k_5k_7)]$.

K_i and K_1 represent dissociation constants for the reactions shown in Scheme I. From eq 8, it follows that the apparent Michaelis constant (K_{DHF}) will vary as a hyperbolic function of [H^+]. Thus, a plot of pK_{DHF} against pH will be wave shaped (Cleland, 1977). But, the prominence of the wave form of the plot will depend on the K_{DHF}'/K_i ratio.

The $(V/K)_{DHF}$ and V relationships for the mechanism given in Scheme I are described by eq 9 and 10. The relationships

$$(V/K)_{DHF} = \frac{k_1k_3k_5[E_t]}{(k_2k_4 + k_2k_5 + k_3k_5)(1 + K_1/[H])} \quad (9)$$

$$V = k_1k_3k_5k_7[E_t]/[k_1(k_3k_5 + k_3k_7 + k_4k_7 + k_5k_7) + (K_1/K_i[H])[k_7(k_2k_4 + k_2k_5 + k_3k_5)]] \quad (10)$$

for ${}^D(V/K)_{DHF}$ and ${}^DV$ have the general forms of eq 11 and

$${}^D(V/K) = \frac{{}^Dk + C_f}{1 + C_f} \quad (11)$$

12, where ${}^Dk$ represents the intrinsic deuterium isotope effect

$${}^DV = \frac{{}^Dk + C_{vf}}{1 + C_{vf}} \quad (12)$$

while C_f and C_{vf} are defined by the relationships given in eq 13 and 14, respectively. Equations 9 and 10 predict that plots

$$C_f = \frac{k_5}{k_4}\left(1 + \frac{k_3}{k_2}\right) \quad (13)$$

$$C_{vf} = \frac{\frac{k_5}{k_4}\left[1 + \frac{k_3}{k_7} + \frac{K_1}{K_i[H]}(k_2 + k_3)\right]}{1 + \frac{k_3}{k_4} + \frac{k_2K_1}{K_i[H]}} \quad (14)$$

of log $(V/K)_{DHF}$ and log V against pH will yield half-bell-shaped curves of the type illustrated in Figure 1. It may be determined that the intersection of the asymptotes with slopes of 0 and −1 for the plot of log $(V/K)_{DHF}$ against pH will occur at the point where pH = pK_1 and thus yield a true value for the ionizing group on the enzyme. By constrast, a true pK_1 value will not be obtained from the log V profile since the intersection will occur at the point where

$$pH = pK_1 - pK_i + pK_{DHF}' \quad (15)$$

The observed pK_1 may be displaced to values that are either above or below the true pK_1 value depending on the values of pK_i and pK_{DHF}'. It is apparent that ${}^D(V/K)_{DHF}$ is independent of pH (eq 11 and 13) while a wave will be obtained for a plot of ${}^DV$ against pH (eq 12 and 14).

The pK_a value of 8.09 ± 0.06 for the ionizing group on the enzyme, as determined from the $(V/K)_{DHF}$ profile, is in good agreement with that of 7.90 ± 0.05 as obtained from the pK_i profile for 2,4-diamino-6,7-dimethylpteridine (Table I). Thus, the data are quantitatively in accord with the model proposed in Scheme I. Further, the pK_a value of 8.38 ± 0.04 from the V profile is displaced as predicted by eq 15. The similarity of the pK_a values from the $(V/K)_{DHF}$ and K_i profiles indicates that the same ionizing group of Asp-27 is involved with the interaction of DHF with the enzyme and with the binding of 2,4-diamino-6,7-dimethylpteridine. Such a finding would usually be interpreted to indicate that protonation of the carboxyl group is of importance for the binding of both substrate and inhibitor. But, it must be recognized that the ionizing group observed in the $(V/K)_{DHF}$ profile could be involved with catalysis and/or release of tetrahydrofolate, rather than with binding. In view of the conclusion that DHF is bound to dihydrofolate reductase in an inverted fashion relative to the binding of an inhibitor such as methotrexate and the postulate that the N-5 nitrogen of DHF is not adjacent to the carboxyl group of Asp-27 (Bolin et al., 1982), it cannot be concluded that this group plays the same role with the substrate and folate analogues, which are 2,4-diaminopteridines. Indeed, the evidence suggests that the ionized carboxyl group of Asp-27 reacts with the N-1 nitrogen of these folate analogues and that the strong interaction is due to the formation of an ion pair in the hydrophobic environment of the active site (Cocco et al., 1981; Hood & Roberts, 1978). On the other hand, it seems clear that the function of the protonated carboxyl group in the enzyme is not with the binding of DHF but with the protonation of its N-5 nitrogen, which would facilitate hydride transfer from NADPH (Huennekens & Scrimgeour, 1964; Lund, 1976). It is for this reason that the ionization of Asp-27 is observed in the $(V/K)_{DHF}$ profile.

From the upward displacement of the true pK_1 value in the V profile by 0.4 log unit (Figure 1, Table I), it may be concluded that the value for K_i is 2–3-fold higher than that for K_{DHF}' (eq 15). The same difference is observed in the limiting values for the wave that is obtained when pK_{DHF} is plotted against pH (cf. eq 8). Upward displacement of the same magnitude is observed when NADPH is replaced with APADPH (Table I). With APADPH, however, lower pK_a values are obtained in the $(V/K)_{DHF}$ and V profiles, and this is probably due to the fact that the two pyridine nucleotides give rise to different active site structures. The similarity of the values for K_i and K_{DHF} is of interest because of earlier results on the binding of DHF to dihydrofolate reductase as a function of pH (Stone & Morrison, 1983a). These showed that, while protonation can markedly affect the binding of folate analogues, it has little effect on the binding of DHF. Thus, the question arises as to whether K_{DHF}' might represent a dissociation constant for the combination of DHF with the protonated form of the enzyme–NADPH complex. Such a conclusion is consistent with studies on the kinetic mechanism of the reaction (Stone & Morrison, 1982). From the relationship given for K_{DHF}, the Michaelis constant would become a dissociation constant when $k_7 > k_5$ and $k_3 < k_2$ and k_5.

The variation with pH of the deuterium isotope effects is as predicted by Scheme I. The value for ${}^D(V/K)_{DHF}$ is invariant with pH (eq 11 and 13) whereas the ${}^DV$ values vary with pH and yield a wave-shaped plot (eq 12 and 14). As ${}^D(V/K)_{DHF}$ is unity at all pH values, the commitment factor (C_f) of $(k_5/k_4)(1 + k_3/k_2)$ must be large (cf. eq 13). This could be due to the fact that $k_5 \gg k_4$ and/or that the product of k_5/k_4 and k_3/k_2 is large. The same conclusion is reached from the ${}^DV$ data at high pH. The relationship for ${}^DV$ under these conditions is identical with that for ${}^D(V/K)_{DHF}$ (eq 11 and 13), and its value is also unity. At low pH, the ${}^DV$ value increases to 1.5, and the relationship for ${}^DV$ is given by eq 12 with C_{vf} expressed as in eq 16. If ${}^Dk$ is assumed to be 6, it

$$C_{vf} = \frac{k_5}{k_3}\left(1 + \frac{k_3}{k_7}\right)\Big/\left(1 + \frac{k_4}{k_3}\right) \quad (16)$$

can be calculated that C_{vf} is 9. On the basis that product release is not rate limiting ($k_7 \gg k_5$) and that k_5/k_4 equals 100, which would allow ${}^D(V/K)_{DHF}$ to be small, it follows that k_3/k_4 would be 10 while k_3/k_5 would equal 0.1. The condition that k_3 is small compared with all other rate constants allows K_{DHF}' to be approximately equal to the dissociation constant, k_2/k_1, and accounts for the small displacement in the V profile.

Thus, the results are in accord with the idea that DHF is not a sticky substrate. Moreover, at high pH, when the enzyme is largely in the unreactive E–NADPH–DHF complex, k_3 would become the sole rate-limiting step, and no isotope effect would be observed because this step is not isotope dependent. It is more likely that k_3 is small because of a conformational change associated with proton transfer rather than because proton transfer itself is slow. Evidence in support of this conclusion comes from the low value observed with the D_2O solvent isotope effect.

On the basis of the kinetic data and with a knowledge of the three-dimensional structure of the active site of the enzyme, a general mechanism can be proposed for the reaction catalyzed by dihydrofolate reductase. It can be considered that, on the binding of NADPH, the active site of the enzyme becomes more hydrophobic so that the pK_a of Asp-27 is elevated from 6.3 in the free enzyme (Stone & Morrison, 1983a) to 8.0 in the binary complex. Thus, at neutral pH the binary complex is present in its protonated form, which can equilibrate rapidly with DHF. However, the proton on Asp-27 in the ternary HE–NADPH–DHF complex appears not to equilibrate rapidly but is transferred to the N-5 nitrogen of DHF in a step that involves a conformational change. Such a transfer is driven by the setting up of a stable hydrogen-bond network (Bolin et al., 1982), which can form with a negatively charged carboxyl group. The formation of such a structure can be expected to cause a fall in the pK_a value for Asp-27 and an increase in that for the N-5 nitrogen of DHF. The latter group should be fully protonated as it is not bound to any other group at the active site of the enzyme. The protonation would facilitate hydride transfer from NADPH to the C-6 carbon of DHF, through formation of a carbonium ion resonance structure, with the resultant production of tetrahydrofolate. The release of this product, together with NADP, would disrupt the hydrogen-bond structure and allow reprotonation of the enzyme. The indications are that the rate-limiting step of the overall sequence is a conformation change associated with the transfer of the proton to DHF.

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