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Mechanism of the Reaction Catalyzed by Dihydrofolate Reductase from *Escherichia coli*: pH and Deuterium Isotope Effects with NADPH as the Variable Substrate

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Received September 29, 1987; Revised Manuscript Received January 26, 1988

ABSTRACT: The variations with pH of the kinetic parameters and primary deuterium isotope effects for the reaction of NADPH with dihydrofolate reductase from *Escherichia coli* have been determined. The aims of the investigations were to elucidate the chemical mechanism of the reaction and to obtain information about the location of the rate-limiting steps. The V and V/K_{NADPH} profiles indicate that a single ionizing group at the active center of the enzyme must be protonated for catalysis, whereas the K_i profiles show that the binding of NADPH to the free enzyme and of ATP-ribose to the enzyme–dihydrofolate complex is pH independent. From the results of deuterium isotope effects on V/K_{NADPH}, it is concluded that NADPH behaves as a sticky substrate. It is this stickiness that raises artificially the intrinsic pK value of 6.4 for the Asp-27 residue of the enzyme–dihydrofolate complex [Howell, E. E., Villafranca, J. E., Warren, M. S., Oatley, S. J., & Kraut, J. (1986) *Science (Washington, D.C.) 231*, 1123] to an observed value of 8.9. Thus, the binary enzyme complex is largely protonated at neutral pH. The elevation of the intrinsic pK value of 6.4 for the ternary enzyme–NADPH–dihydrofolate complex to 8.5 is not due to the kinetic effects of substrates. Rather, it is the consequence of the lower, pH-independent rate of product release and the faster pH-dependent catalytic step. At neutral pH, the proportion of enzyme present as a protonated ternary enzyme–substrate complex is sufficient to keep catalysis faster than product release. The data for deuterium isotope and deuterium solvent isotope effects are consistent with the postulate that, for the reduction of dihydrofolate to tetrahydrofolate, protonation precedes hydride transfer. A scheme is proposed for the indirect transfer of a proton from the enzyme to dihydrofolate. Dihydrofolate reductase is another enzyme whose catalytic efficiency is limited by product release rather than by the chemistry of the reaction.

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 enzyme from *Escherichia coli* possesses distinct subsites for the pyridine nucleotide and pterin substrates (Stone & Morrison, 1982), and there exists at the active site only a single ionizing Asp-27 residue, which is located within the pterin subsite (Bolin et al., 1982; Filman et al., 1982). pH and deuterium isotope effect studies with DHF as the variable substrate have shown that this residue is not involved with the binding of DHF, but with catalysis. It acts as a source of protons for the protonation of the N-5 nitrogen of DHF, which facilitates hydride transfer from NADPH to the C-6 carbon

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[1] Abbreviations: DHF, 7,8-dihydrofolate; THF, 5,6,7,8-tetrahydrofolate; APADP(H), 3-acetylpyridine adenine dinucleotide phosphate (reduced); ATP-ribose, 2′-monophosphoadenosine-5′-diphosphoribose; DADMP, 2,4-diamino-6,7-dimethylpteridine; ^{D}V and $^{D}(V/K)$, deuterium isotope effects on V and V/K, respectively; ^{D}k, intrinsic deuterium isotope effect; MES, 2-(N-morpholino)ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; HEPES, N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid.

0006-2960/88/0427-5499$01.50/0

(Stone & Morrison, 1984; Howell et al., 1986).

The present paper reports the results of pH and deuterium isotope effect studies with dihydrofolate reductase from *E. coli* when NADPH is used as the variable substrate in the presence of a saturating concentration of DHF. The data confirm the earlier conclusions of Stone and Morrison (1984) and Howell et al. (1986) about the role of Asp-27 in the catalytic mechanism. They also draw attention to the way in which sticky substrates[2] for a dehydrogenase can raise artificially the pK value of the ionizing group at the active site of the enzyme and thereby facilitate the protonation that accompanies hydride transfer. These results, together with the stopped-flow data of Fierke et al. (1987), establish the basic kinetic and chemical mechanisms of the dihydrofolate, reductase reaction. A preliminary report of the present work has been given (Morrison & Stone, 1986).

Experimental Procedures

Materials

Dihydrofolate was prepared from folic acid by the method of Blakley (1960). NADPD with deuterium on the A-side was prepared by reducing NADP with alcohol dehydrogenase from *Leuconostoc mesenteroides* (Research Plus Inc.) in the presence of deuteriated ethanol (Merck) at pH 10.2. The product was purified by the method of Viola et al. (1979). To check that the final product was fully deuteriated on the A-side, a solution (0.16 mM) was completely oxidized by using glutamate dehydrogenase in the presence of α-ketoglutarate (0.5 mM) and NH_4Cl (1.0 mM). After filtering through a YM5 membrane (Amicon), the solution was freeze-dried and the NMR spectrum determined (Arnold & You, 1978). The absence of a resonance peak at δ 8.95 in the spectrum of NADP indicated that the original reduced pyridine nucleotide (NADPD) was fully deuteriated on the A-side. Reduced acetylpyridine adenine dinucleotide phosphate (APADPH) was prepared and purified as described by Stone and Morrison (1982). All other chemicals were of the highest quality available commercially. Dihydrofolate reductase was purified from an overproducing mutant of *E. coli* K12 (JFM 228; Smith et al., 1982) by using the procedure of Stone and Morrison (1982). Fluorescence titration with methotrexate was used to determine the concentration of the enzyme (Perkins & Bertino, 1966). The concentration of ATP-ribose was estimated spectrophotometrically by using an extinction coefficient of 15 300 M^{-1} cm^{-1} at 259 nm (Dawson et al., 1969). Concentrations of DHF and NADPH were determined enzymically by using a molar absorbancy change for the dihydrofolate reductase reaction of 11 800 cm^{-1} at 340 nm (Stone & Morrison, 1982).

Methods

Enzyme Assays. Initial velocities of the reaction were determined spectrophotometrically at 340 nm and 30 °C. The cuvettes had a 1-cm light path, and DHF was present at a saturating concentration of 40 μM (Stone & Morrison, 1982, 1984). The buffer 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 used to determine initial velocities, the ionic strength of the reaction mixture remained essentially constant at I = 0.15 (Ellis & Morrison, 1982). The molar absorbancy change for the reaction was 11 800 cm^{-1}. Maximum velocity (V) is expressed in units of s^{-1} and the apparent second-order rate constant (V/K) in units of M^{-1} s^{-1}. Solvent perturbation studies were conducted as previously described (Stone & Morrison, 1984) by using the method of Inagami and Sturtevant (1960). Deuterium solvent isotope effects were determined by using single nonvolatile buffers. N-(2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) and 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) were used at a concentration of 50 mM for experiments at pH 7.4 and pH 10.0, respectively. For experiments in D_2O, each buffer was dissolved in D_2O, brought to the appropriate pH with NaOD, and lyophilized. The solid was taken up in D_2O, lyophilized again, and redissolved in D_2O. Substrates were also dissolved in D_2O and treated in a similar manner.

Fluorescence Titration. This method was used to determine the dissociation constant for the binary enzyme–NADPH complex. Titrations were performed as described by Stone and Morrison (1983). Corrections were made for the absorption of light by the ligand as described by Birdsall et al. (1980), and the corrected data were analyzed by using nonlinear regression to yield a value for the dissociation constant.

Data Analysis. Initial velocity data obtained at each pH value by varying the concentration of the pyridine nucleotide (A) were fitted to eq 1 to obtain values for the maximum

$$v = \frac{VA}{K + A} \tag{1}$$

velocity (V) and the apparent first-order rate constant (V/K). The second-order rate constant $[(V/K)/E_t]$ was calculated by using the known concentration of enzyme (E_t). Initial velocities obtained at each pH value by varying the concentration of ATP-ribose (I) in the presence of fixed concentrations of DHF (40 μM) and NADPH (10 μM) were fitted to eq 2 to

$$v = \frac{V_{app}}{1 + I/K_{iapp}} \tag{2}$$

obtain values for the apparent inhibition constants (K_{iapp}). True inhibition constants (K_i) were calculated from the apparent values by using eq 3 together with the fixed concentration of NADPH (A) and the Michaelis constant for NADPH at the particular pH. The Michaelis constant for NADPH (K_a), at any particular pH, was calculated by using

$$K_i = \frac{K_{iapp}}{1 + A/K} \tag{3}$$

$$K_{NADPH} = \left[\frac{\text{pH-independent } V}{\text{pH-independent } V/K}\right]\left[\frac{1 + K_2/H}{1 + K_1/H}\right] \tag{4}$$

where K_1 and K_2 are ionization constants determined from the V and V/K profiles, respectively. The same procedure was used for calculation of the Michaelis constants for DHF at various pH values. The pH-independent values were taken from Stone and Morrison (1984). Values for V and V/K were weighted according to the inverse of their variances and fitted to eq 5, where y represents V or V/K, C represents the pH-independent value of the parameter, H denotes the hydrogen ion concentration, and K is an acid dissociation constant.

$$y = \frac{C}{1 + K/H} \tag{5}$$

Initial velocity data obtained by varying the concentration of NADPH or NADPD in the presence of a fixed concen-

[2] The stickiness ratio for a substrate is defined as the ratio of the net rate constant for reaction of the first collision complex through the first irreversible step to the rate constant for the dissociation of the collision complex (Cleland, 1982b).

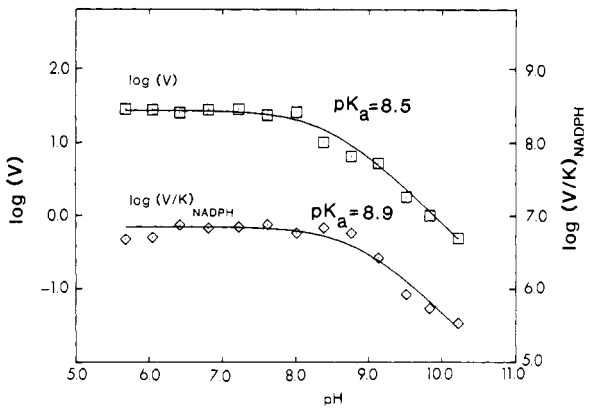

FIGURE 1: Variation with pH of log (V) and log (V/K_{NADPH}) for the reaction catalyzed by dihydrofolate reductase in the presence of 40 μM DHF. The units of V and V/K_{NADPH} are s^{-1} and M^{-1} s^{-1}, respectively. The curves represent the best fit of the data to eq 5 and were drawn by using the parameter values of Table I.

tration of DHF or by varying the concentration of DHF at a fixed concentration of NADPH or NADPD were fitted to eq 6. In this equation, F_i is the fraction of deuterium label

$$v = \frac{VA}{K(1 + F_iE_{V/K}) + A(1 + F_iE_V)} \quad (6)$$

in the substrate, while $E_{V/K}$ and E_V are the isotope effects minus one for the respective parameters. Kinetic data were analyzed by using the computer programs of Cleland (1979).

Calculation of pK Displacement in the Presence of D_2O. The observed value of a kinetic parameter such as V or V/K that varies with pH and which is altered in the presence of D_2O can be described by eq 7 and 8. In these equations, P_H

$$P_{H_2O} = \frac{P_H}{1 + K_1/H} \quad (7)$$

$$P_{D_2O} = \frac{P_D}{1 + K_1/\alpha H} \quad (8)$$

and P_D represent pH-independent values of the parameters in H_2O or D_2O; P_{H_2O} and P_{D_2O} represent oberved values for the parameters in the presence of H_2O and D_2O, respectively. K_1 denotes the dissociation constant of the ionizing group on the enzyme that must be protonated for maximum activity. α represents the relative change in the value of K_1 in the presence of D_2O. H denotes the H ion concentration. At low and high pH values, P_{H_2O}/P_{D_2O} is equal to P_H/P_D and $P_H/P_D\alpha$, respectively. Thus, the ratio of the latter values yields a value for α, and it follows that

$$\text{displacement in pH profile } (\Delta pK) = \log \alpha = \log\left[\frac{(P_H/P_D)_{\text{low pH}}}{(P_H/P_D)_{\text{high pH}}}\right] \quad (9)$$

Equation 9 was used for calculations of the ΔpK_a values of Table III.

RESULTS

pH Dependence of V and V/K_{NADPH}. With DHF present at a saturating concentration of 40 μM at all pH values, both V and V/K_{NADPH} decrease with increasing pH (Figure 1). The data fitted well to eq 5 and yielded pK values of 8.47 ± 0.03 and 8.85 ± 0.06 from the V and V/K_{NADPH} profiles, respectively. NADPH does not have an ionizing group in the region of pH 8–9, and thus the pK values must be for a group

Table I: pK Values and pH-Independent Parameters for the Reaction of Pyridine Nucleotides with the Enzyme–DHF Complex[a]

variable substrate	parameter	value of pH-independent parameter	pK
NADPH	V (s^{-1})	13.2 ± 0.6	8.47 ± 0.03
	V/K_{NADPH} (μM^{-1} s^{-1})	3.4 ± 0.2	8.85 ± 0.06
NADPH + 20% (v/v) 2-methoxyethanol	V (s^{-1})	9.4 ± 0.5	8.73 ± 0.05
	V/K_{NADPH} (μM^{-1} s^{-1})	4.2 ± 0.2	8.95 ± 0.06
APADPH	V (s^{-1})	1.6 ± 0.1	7.95 ± 0.10
	V/K_{APADPH} (μM^{-1} s^{-1})	0.49 ± 0.12	7.37 ± 0.10

[a] DHF was held constant at 40 μM.

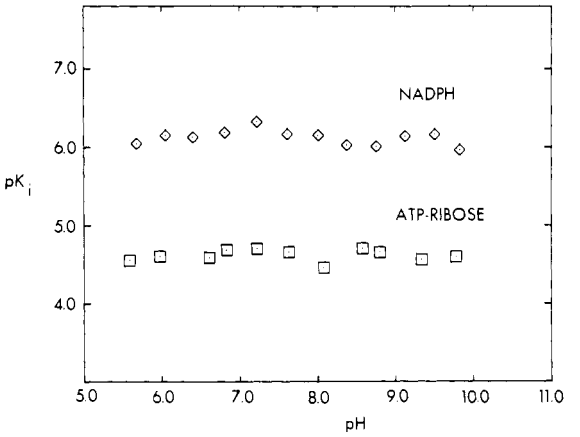

FIGURE 2: pK_i profiles for the interaction of NADPH with the free form of enzyme and of ATP-ribose with the enzyme–DHF complex. The K_i value for the enzyme–NADPH complex was determined by fluorescence titration (see Methods). The K_i values for the release of ATP-ribose from the enzyme–DHF–ATP-ribose complex were determined kinetically by varying the concentration of ATP-ribose in the presence of fixed concentrations of DHF (40 μM) and NADPH (10 μM). Apparent dissociation constants were determined by fitting the data to eq 2. The resulting values were then used to calculate the true inhibition constants by means of eq 3.

or groups on the enzyme. As APADPH is a slow substrate with a maximum velocity at pH 7.4 about 7-fold lower than with NADPH (Stone et al., 1984), pH profiles were also obtained with this alternative substrate. The shapes of the profiles were similar to those of Figure 1. Analysis of the data yielded pK values of 7.95 ± 0.10 and 7.37 ± 0.10 from the V and V/K_{APADPH} profiles, respectively. The pK values as well as the pH-independent values for V and V/K from each pH profile are listed in Table I.

Effect of Solvent and Temperature on pK Values. In the presence of 20% (v/v) methoxyethanol, the pK value obtained from the V profile was increased 0.26 pH unit, while that from the V/K_{NADPH} profile was increased by 0.10 pH unit (Table I). Although the increases in pK values are small, they are consistent with each of the observed ionizing groups (Figure 1) being a neutral acid (Inagami & Sturtevant, 1960; Cleland, 1977; Tipton & Dixon, 1979). There was no significant variation of the pK values from the V and V/K_{NADPH} profiles when the experiments were performed at 21, 30, and 39 °C. Such a result is consistent with the ionizing group(s) having the property of a carboxylic acid.

pH Dependence of the Binding of NADPH and ATP-ribose. The interaction of NADPH with the free enzyme as a function of pH was determined by fluorometric titration (cf. Methods) and found to be independent of pH (Figure 2). The binding of ATP-ribose to the enzyme–DHF complex was determined

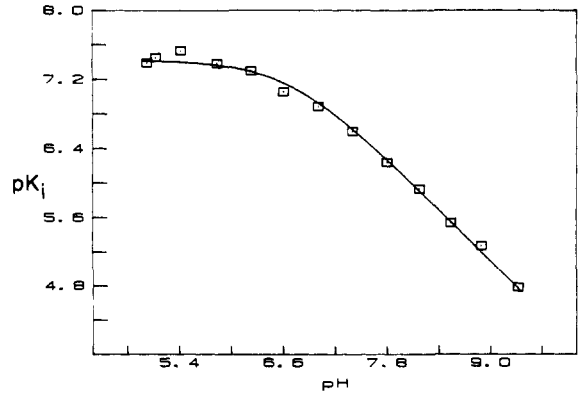

FIGURE 3: Variation with pH of the pK_i value for the binding of 2,4-diamino-6,7-dimethylpteridine (DADMP) to the enzyme–NADPH complex. The theoretical curve was drawn by using parameter values obtained by fitting the data to eq 5. The inhibition experiments were performed in the presence of fixed concentrations of NADPH (100 μM) and DHF (20 μM).

Table II: pH Dependence of Deuterium Isotope Effects[a]

	NADPH[b]		DHF[c]	
pH	^DV	$^D(V/K)$	^DV	$^D(V/K)$
7.0	1.3 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	1.8 ± 0.1
7.8	2.1 ± 0.1	1.1 ± 0.1	1.7 ± 0.1	2.8 ± 0.2
8.6	2.2 ± 0.2	1.9 ± 0.1	2.5 ± 0.1	2.8 ± 0.2
9.3	3.3 ± 0.1	2.1 ± 0.1	3.0 ± 0.2	2.9 ± 0.2
10.2	2.9 ± 0.2	3.0 ± 0.2	2.8 ± 0.2	3.0 ± 0.2

[a] Weighted mean values of two or three determinations. [b] DHF was held constant at 40 μM. [c] NADPH was held constant at 50 μM.

kinetically by measuring the inhibition of the reaction by increasing concentrations of ATP-ribose in the presence of fixed concentrations of DHF and NADPH. The true dissociation constant for the release of ATP-ribose from the enzyme–DHF–ATP-ribose complex is also pH independent (Figure 2).

pH Dependence of Binding of DADMP to Enzyme–NADPH Complex. As the binding of NADPH to the free enzyme was pH independent (Figure 2), it follows that the free enzyme and the enzyme–NADPH complex should exhibit the same pK value. However, it has been reported that the value for the enzyme–NADPH complex is 7.9 (Stone & Morrison, 1984), while that for the free enzyme is 6.3 (Stone & Morrison, 1983). This discrepancy prompted a reinvestigation of the pK value of the enzyme–NADPH complex by determining the effect of pH on the inhibition of the reaction by the folate analogue 2,4-diamino-6,7-dimethylpteridine (DADMP). The results illustrated in Figure 3 yielded a pK value of 6.39 ± 0.07 when a weighted fit was made to eq 5. The weighting factor for each K_i value was equal to the reciprocal of the variance of the value. The pH-independent K_i value was 29 μM.

pH Dependence of Deuterium Isotope Effects. The effect of pH on the values for ^DV and $^D(V/K_{NADPH})$, in the presence of a saturating concentration of DHF, was determined to gain information about the rate limiting step(s) of the reaction and to elucidate the kinetic characteristics of NADPH. The results (Table II) show that there is an increase in the value of ^DV from 1.1–1.3 at pH 7.0 to a limiting value of about 3.0 at pH 10.2. Thus, the rate of product release must be, at least partly, rate-limiting at neutral pH. Product release cannot be solely rate-limiting for then the value of ^DV at this pH would be unity. The increase with pH of the value for $^D(V/K_{NADPH})$ indicates that NADPH behaves as a sticky substrate at neutral pH. It will be noted that the limiting value at high pH for $^D(V/K_{NADPH})$ is also about 3. Fierke et al. (1987) have reported a value of 3 from direct measurement of the deuterium isotope effect on the catalytic step.

At higher pH values, the magnitude of the ^DV values is considerably greater than those previously obtained by varying DHF with NADPH present at a saturating concentration of 100 μM (Stone & Morrison, 1984). As a result, a reinvestigation has been made of the isotope effects with DHF as the variable substrate, and the results are included in Table II. It will be noted that, as expected, comparable values are obtained for ^DV with the two substrates. The values for V/K_{DHF} are higher than previously reported (Stone & Morrison, 1984) and increase from 1.8 at pH 7.0 to a limiting value of 3.0 at pH 10.2. The earlier results must be in error, presumably due to not having used fully deuteriated NADPD. The product prepared for the earlier experiments was not checked by NMR as in the present study (see Materials).

Deuterium Isotope Effects Varying NADPH at pH 7.0. Because of the activation of the enzyme by higher concentrations of NADPH (Stone & Morrison, 1988), the effect of the NADPH concentration on $^D(V/K_{DHF})$ and ^DV values was also determined. These experiments were performed in the presence of 2,4-diamino-6,7-dimethylpteridine (DADMP), which acts as a classical competitive inhibitor of the enzyme with respect to DHF (Stone & Morrison, 1986, 1988). This pteridine was added to reaction mixtures at a concentration of 6.0 μM so as to raise the apparent Michaelis constant at pH 7.0 from about 1 to 30 μM and thereby increase the accuracy with which the ^DV and $^D(V/K_{DHF})$ values could be determined with a cuvette of 1-cm light path. The results showed that, with increasing concentrations of NADPH from 10 to 50 μM, the $^D(V/K_{DHF})$ value does not vary. Thus, the external forward commitment for the random dihydrofolate reductase reaction (Cleland, 1982b) is at a maximum value with NADPH at 10 μM. On the other hand, the ^DV value appears to increase slightly from 1.2 ± 0.2 to 1.6 ± 0.2 as the NADPH concentration increases from 10 to 50 μM. This result is to be expected as higher concentrations of NADPH facilitate the release of THF from the enzyme–THF complex, through the formation of an enzyme–THF–NADPH complex, which makes product release less rate-limiting and hydride transfer more so (Stone & Morrison, 1988; Fierke et al., 1987).

Deuterium Solvent Isotope Effects. Experiments on the effect of D_2O on the kinetic parameters of the reaction and on the deuterium isotope effects were undertaken to determine if D_2O could slow down the protonation step of catalysis with either NADPH or NADPD as the nucleotide substrate. They were performed in the presence of single, nonvolatile buffers at pH 7.4 and pH 10.0. The change in buffer composition had only a small effect on the magnitudes of the values for ^DV and $^D(V/K_{DHF})$ as judged by the data of Tables II and III. In the presence of D_2O, there is a significant reduction of the isotope effects and a deuterium solvent isotope effect is observed at both pH values (Table III). The effect is normal at pH 7.4 but inverse at pH 10.0. The latter result is due to the upward displacement of the observed pK values of the V and V/K profiles in the presence of D_2O. Calculations using the relationships given under Data Analysis and the results of Table III show that there is, in the presence of D_2O, an average shift of about 0.2 pH unit in the V profile and of 0.6 pH unit in the V/K profile.

Discussion

Pathways of Ternary Complex Formation. The variation of V and V/K_{NADPH} with pH (Figure 1) indicates that a single ionizing group at the active site of dihydrofolate reductase must

Table III: Deuterium and Deuterium Solvent Isotope Effects with DHF as the Variable Substrate[a]

parameter[b]	pH 7.4	pH 10.0	ΔpK[c]
$^{D}V_{H_2O}$	1.5 ± 0.1	3.3 ± 0.2	
$^{D}V_{D_2O}$	1.5 ± 0.1	2.6 ± 0.2	
$^{D}(V/K)_{H_2O}$	1.7 ± 0.1	3.3 ± 0.2	
$^{D}(V/K)_{D_2O}$	1.4 ± 0.1	2.8 ± 0.1	
$^{D_2O}V_H$	1.3 ± 0.1	0.79 ± 0.06	0.20
$^{D_2O}V_D$	1.1 ± 0.1	0.64 ± 0.06	0.23
$^{D_2O}(V/K)_H$	1.8 ± 0.1	0.49 ● 0.03	0.57
$^{D_2O}(V/K)_D$	1.5 ± 0.1	0.34 ± 0.02	0.63

[a] Experiments were performed in the presence of 50 mM HEPES (pH 7.4) or 50 mM CAPS (pH 10.0). The buffers were prepared as described under Methods. [b] The superscript associated with the parameters refers to the deuterium (D) isotope effect or to the deuterium solvent (D_2O) isotope effect. The subscripts indicate the solvent in which the results were obtained (H_2O, D_2O) or the form of reduced NADP (NADPH, NADPD) held constant at 50 μM. [c] These values represent the shift in the observed pK_a values of V and V/K_{DHF} profiles that occurs in the presence of D_2O. They were calculated as described under Data Analysis.

be protonated to obtain the maximum rate for the overall reaction and for the interaction of NADPH with the enzyme–DHF complex to form a productive ternary complex. The similar shapes of the V and V/K_{NADPH} profiles suggest that the same ionizing group, which is involved with catalysis, is being observed in each profile. This conclusion is supported by the finding (Figure 2) that the binding of NADPH to the free enzyme and of ATP-ribose to the enzyme–DHF–complex is independent of pH. The results of the solvent perturbation studies (Table I) are consistent with the ionizing group being a neutral acid, while the nonsignificant value obtained for the heat of ionization is consistent with the group being a carboxylic acid (Tipton & Dixon, 1979; Cleland, 1977). The X-ray crystal structure of dihydrofolate reductase (Bolin et al., 1982); Filman et al., 1982; Matthews et al., 1985) shows that Asp-27 is the only ionizing group within the active site of the enzyme. Thus, it follows that this amino acid residue must be responsible for the pK values observed in the pH profiles.

From the decrease with pH of both V and V/K_{NADPH}, it can be concluded that NADPH must react with both the protonated and unprotonated forms of the enzyme–DHF complex and that only the protonated (HE–DHF–NADPH) complex is productive. Further, as NADPH behaves as a sticky substrate (Table II; Stone & Morrison, 1988), allowance must be made for the reversible protonation of the nonproductive (E–DHF–NADPH) complex to form the productive ternary complex. The reactions are shown in Figure 4, which also illustrates the possible sequences for the reduction of DHF to THF. Irrespective of the order of protonation and hydride transfer, the scheme proposes that it is the protonated form of the carboxyl group of Asp-27 of the dihydrofolate reductase that is the source of the proton for the reduction. The same conclusion has been reached previously from experiments with DHF as the variable substrate (Stone & Morrison, 1984). Since the observed pK values for the enzyme–DHF and enzyme–DHF–NADPH complexes are 8.9 and 8.5, respectively, it appears that both complexes would be largely protonated at neutral pH. The kinetic behavior of APADPH is similar to that of NADPH, although lower pK values are observed in the V and V/K profiles (Table I). Thus, the kinetic mechanism of Figure 4 would apply with this alternative nucleotide substrate, but the degree of protonation of the enzyme–DHF and enzyme–DHF–APADPH complexes at neutral pH would be less than with NADPH.

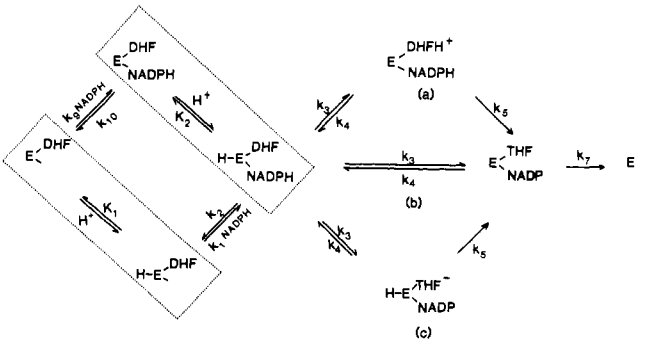

FIGURE 4: Kinetic and chemical mechanisms for the formation of tetrahydrofolate from dihydrofolate as catalyzed by dihydrofolate reductase. The dotted lines represent rapid equilibrium segments for the protonic equilibria. In the right-hand section, pathway a represents protonation before hydride transfer, pathway b denotes concerted protonation and hydride transfer, while pathway c represents hydride transfer before protonation.

Order of Protonation and Hydride Transfer. The values for $^{D}(V/K_{NADPH})$ and ^{D}V increase with pH to the same limiting value of 3 (Table II), and they can be analyzed in terms of the quantitative relationships that apply to the three mechanisms for protonation and hydride transfer that are illustrated in Table IV.[3]

Table IV: Relationships for the Parameters Associated with the Mechanisms of Figure 4

parameter	mechanism (a) protonation before hydride transfer	(b) concerted protonation and hydride transfer	(c) hydride transfer before protonation
$\frac{V}{KE_t}$	$[k_1k_3k_5/(1 + K_1/H)]/[k_2(k_4 + k_5) + k_3k_5/(1 + K_1k_9/Hk_1)]$	$[k_1k_3k_5/(1 + K_1/H)]/[k_2k_7 + k_3k_7 (1 + K_1k_9/Hk_1)]$	relationships are the same as those given under (a)
crossover point for V/K profile	$pH = pK_1 + \log [1 + (k_3/k_2)/(1 + k_4/k_5)]$	$pH = pK_1 + \log (1 + k_3/k_2)$	
$\frac{V}{E_t}$	$[k_5k_7/(k_5 + k_7)]/[1 + [k_7(k_4 + k_5)/k_3(k_5 + k_7) (1 + K_2/H)]$	$k_3k_7/[k_3 + k_7 (1 + K_2/H)]$	
crossover point for V profile	$pH = pK_2 + \log [1 + k_3(k_5 + k_7)/k_7(k_4 + k_5)]$ or $pH = pK_1 + \log [1 + [k_1k_{10}(k_3 + k_4 + k_5 + k_3k_5/k_7)]/[k_2k_9(k_4 + k_5)]]$	$pH = pK_2 + \log (1 + k_3/k_7)$	
$^{D}(V/K)$			
C_f, low pH	$k_5/k_4 (1 + k_3/k_2)$	k_3/k_2	$k_3/k_2 + {}^{D}K_{eq}k_4/k_5$
C_f, high pH	k_5/k_4	0	$^{D}K_{eq}k_4/k_5$
^{D}V			
C_{Vf}, low pH	$[k_5/k_4 (1 + k_3/k_7)]/(1 + k_3/k_4)$	k_3/k_7	$^{D}K_{eq}k_4/[k_5 + k_3/k_5(1 + k_5/k_7)]$
C_{Vf}, high pH	k_5/k_4	0	$^{D}K_{eq}k_4/k_5$

Table V: Ratio of Rate Constants As Calculated from Deuterium Isotope Effects[a]

assumed value of ^Dk[b]	ratio of rate constants: k_3/k_2 (a)[c]	k_3/k_2 (b)	k_3/k_2 (c)	k_5/k_4 (a)	$^DK_{eq}k_4/k_5$ (b)	$(1 + k_3/k_7)/(1 + k_3/k_4)$ (a)	$(k_3/k_5)(1 + k_5/k_7)$ (b)	k_3/k_7 (c)
3	∞	19	19	0	0	∞	6	6
4	57	29	29	0.5	0.5	18	9	9
5	38	38	39	1.0	1.0	12	11	12
6	32	48	49	1.5	1.5	10	14	16

[a] Values for $^D(V/K_{NADPH})$ at low and high pH were taken to be 1.1 and 3.0, respectively. Values for ^DV at low and high pH were taken to be 1.3 and 3.0, respectively. Calculations were made by using the relationships given in Table IV and the data of Table II. [b] ^Dk represents the intrinsic deuterium isotope effect. [c] (a), (b), and (c) denote the mechanisms illustrated in Figure 4.

If the value of ^Dk were 3, then it must be concluded that the protonation and hydride-transfer reactions are concerted, since for such a mechanism (mechanism b of Figure 4) the intrinsic isotope effect is observed at alkaline pH; i.e., the commitment factors, C_f and C_{Vf}, are zero (Table IV). However, such a sequence is not essential, as it has been demonstrated that chemically protonated DHF functions as a substrate with a mutant form of dihydrofolate reductase that does not contain an ionizing amino acid residue at its active site (Howell et al., 1986). If the value of ^Dk is greater than 3, the deuterium isotope effect data could also be consistent with the transfer of a hydride ion to DHF that has been protonated by migration of a proton from Asp-27. In this case, a finite internal commitment would remain at high pH.

In the absence of data for the intrinsic isotope effect value (^Dk), values of the commitments, C_f and C_{Vf} (Table IV), for each of mechanisms a–c of Figure 4 have been calculated by using the relationships (Cleland, 1982b)

$$^D(V/K) = \frac{^Dk + C_f}{1 + C_f} \qquad ^DV = \frac{^Dk + C_{Vf}}{1 + C_{Vf}} \tag{9a}$$

and assuming a range of values for ^Dk (Table V). Irrespective of the ^Dk value, the ratio k_3/k_2 is of a magnitude expected for a sticky substrate, while the values of k_4 and k_5 are comparable. The latter result tends to eliminate the mechanism involving hydride transfer before protonation. The transfer of a hydride ion to DHF would yield a negatively charged N-5 nitrogen that would undergo very rapid protonation. Consequently, k_5 would be expected to be very much greater than k_4 if mechanism c were to apply. As mechanism c is inconsistent with the experimental data and unlikely on chemical grounds, it must be discarded. The conclusion that the reduction of DHF by dihydrofolate reductase proceeds by protonation of the substrate., followed by hydride transfer, or by an essentially concerted reaction is in accord with the demands of chemistry (Lund, 1975) and the original proposal of Huennekens and Scrimgeour (1964). The finding that the values for ^DV and $^D(V/K)_{DHF}$ are lower in D_2O than in H_2O (Table III) supports the conclusion that the reduction of the imine double bond of DHF occurs in a stepwise manner with protonation preceding hydride transfer. D_2O would slow down the protonation step and thereby reduce the deuterium isotope effect on hydride transfer by increasing the value of k_5/k_4.

Rate-Limiting Step(s) of the Reaction Sequence. From the increase with pH of the ^DV values, it can be concluded that product release is at least partly rate-limiting at neutral pH. As catalysis slows down with increasing pH, product release becomes less rate-limiting, and a relatively large deuterium isotope effect is observed. This conclusion is in accord with the results obtained from stopped-flow experiments by Fierke et al. (1987). They found that catalysis is dependent on the protonation of an ionizing amino acid residue with a pK value of 6.5, while the rate of product release is independent of pH. Further, they showed that the pH-independent rate of catalysis is about 80-fold higher than the rate of product release. As the pK value from the V profile is 8.5 (Figure 1, Table I), this is the pH at which catalysis and product release have equal rates and above which the pH profiles become limited by catalysis.

Intrinsic pK Values for Enzyme–DHF and Enzyme–NADPH Complexes. As the binding of NADPH to the free enzyme is pH independent (Figure 2), the pK value of the binary HE–NADPH complex must be the same as that for the HE form of enzyme. The latter value has been determined by Stone and Morrison (1983) to be 6.3. The above results, together with the finding that the interaction of DHF with the free enzyme is pH independent (Stone & Morrison, 1983; Fierke et al., 1987), lead to the conclusion that the binary enzyme–DHF complex and the ternary enzyme–NADPH–DHF complex must also have pK values of 6.3. The conclusion is in accord with the pH-independent binding of ATP-ribose to the enzyme–DHF complex (Figure 2). More importantly, it is in agreement with the finding by Fierke et al. (1987) that the directly determined pK value for the HE–NADPH–DHF complex is 6.5.

The report by Stone and Morrison (1984) that the pK value of the HE–NADPH complex is 7.9 is at variance with the conclusion that bound substrate does not affect the pK value of the single ionizing group at the active site of the enzyme. A reinvestigation of the effect of pH on the binding of 2,4-diamino-6,7-dimethylpteridine (DADMP) to the enzyme–NADPH complex (Figure 3) shows that the earlier value was in error. The present studies yield a value of 6.4. The pH data indicate that the pK value of the carboxyl group of Asp-27 at the active site of dihydrofolate reductase is higher than that of the α-carboxyl of free aspartate. This would be due to the hydrophobic environment of the active site (Bolin et al., 1982).

The observed pK value of 8.9 for the enzyme–DHF complex from the V/K_{NADPH} profile (Figure 1) is considerably greater than the intrinsic value of 6.4. Such a result is obtained

[3] It should be mentioned that, in deriving the relationships of Table V, no assumptions have been made about the equality of the rate constants for the interaction of NADPH with the protonated and unprotonated forms of enzyme in Figure 4. However, as the pH profiles are smooth curves without humps or hollows [cf. Cleland (1986)], it is likely that $k_1 = k_9$ and $k_2 = k_{10}$. It should also be noted that the relationship, given in terms of pK_1, for the intersection point of the asymptotes of the V profiles for mechanisms a and c also applies when the ternary enzyme–substrate complexes do not undergo protonation–deprotonation reactions and the variable substrate is not sticky [cf. Scheme I of Stone and Morrison (1984)]. The same limiting value of 3 is obtained for ^DV and $^D(V/K)_{DHF}$ with DHF as the variable substrate (Table II). This is as expected since the steps after the formation of the ternary complexes are identical, irrespective of which substrate is varied. Conclusions about the order of protonation and hydride transfer would be facilitated by a knowledge of the intrinsic deuterium isotope effect (^Dk) associated with the dihydrofolate reductase reaction. However, such information is not available and is likely to be difficult to obtain because of the inherent instability of THF.

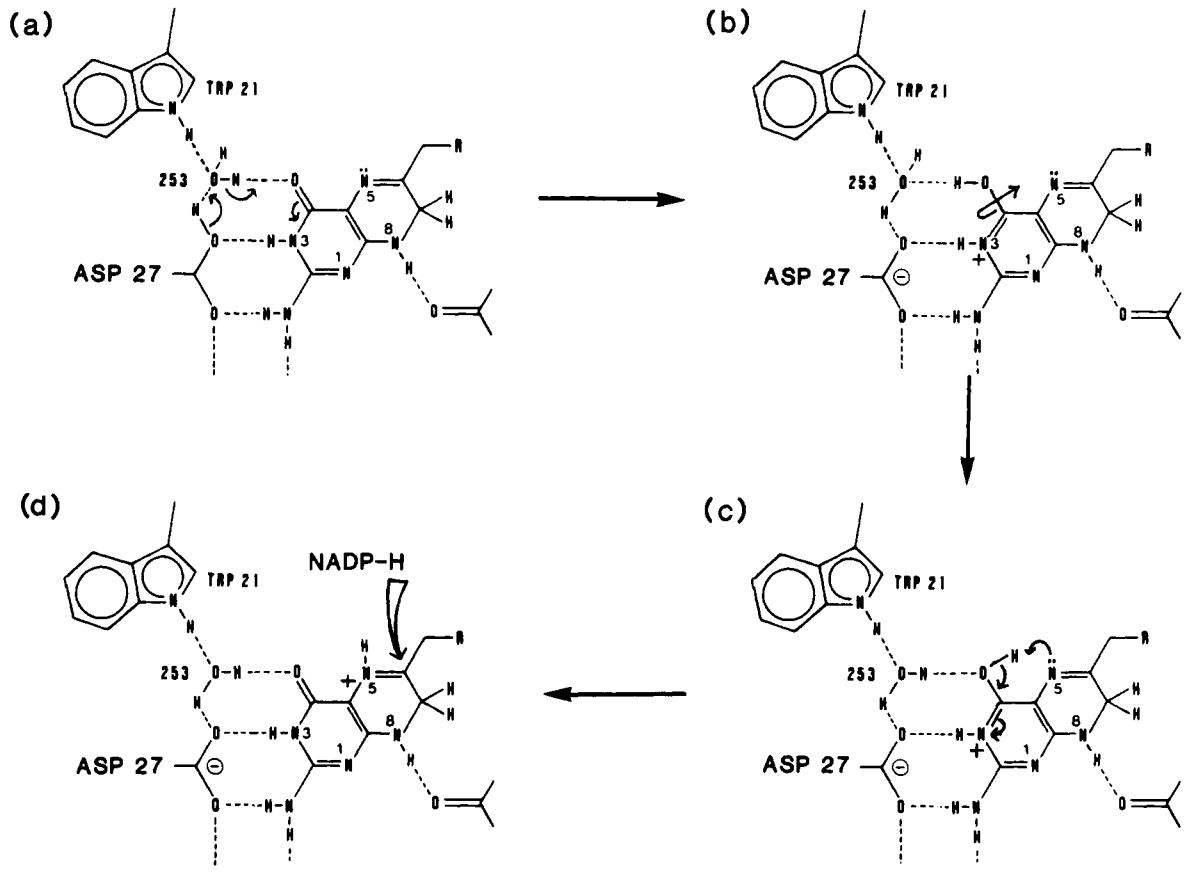

FIGURE 5: Proposed chemical mechanism of the transfer of a proton from the protonated carboxyl group of Asp-27 of dihydrofolate reductase to the N-5 nitrogen of dihydrofolate.

because NADPH behaves as a stick substrate, and the stickiness displaces outward the true pK value (Cleland, 1982b, 1986). In theory, it is possible to calculate an intrinsic pK by subtracting from the observed pK value of a V/K profile the log of the value of the displacement or stickiness term. The relationships that describe the stickiness terms for each of the three possible reduction mechanisms are given as log terms in the expressions for the crossover point of V/K profiles in Table IV. It may be shown that for mechanism a, as well as for mechanism c, the stickiness term is given by the relationship

$$[^{D}(V/K)_{\text{high pH}} - 1]/[^{D}(V/K)_{\text{low pH}} - 1] \qquad (10)$$

which is independent of the magnitude of the value for the intrinsic isotope effect. The calculated values for the stickiness term associated with the reactions involving both NADPH and DHF are given in Table VI, which also lists the calculated intrinsic pK values for binary enzyme–substrate complexes as well as the directly determined pK value for the free enzyme. It is apparent that the calculated values for the binary enzyme–substrate complexes are about 1 pH unit higher than the true pK value. The error is undoubtedly due to the difficulty of determining precisely the value for $^{D}(V/K)_{\text{low pH}}$ when that value approaches 1.

The results of viscosity experiments (Stone & Morrison, 1988) suggest that APADPH and DHF are not very sticky substrates. In this event the pK values observed in the V/K profiles for these substrates would represent true pK values. However, this is not the case (Table VI). Thus, it appears that while viscosity studies allow the detection of very sticky substrates, they may well not allow the identification of those that are only moderately sticky. It should be mentioned that with a nonsticky variable substrate it would not be necessary to allow for the protonic equilibrium between the two ternary

Table VI: Observed and True Calculated pK Values of Asp-27 in Complexes of Dihydrofolate Reductase[a]

enzyme complex	obsd pK value	stickiness term	calcd pK value
free enzyme	6.3[b]		6.3[a]
enzyme–DHF	8.9	20	7.6
	7.4	1.6[c]	7.2
enzyme–NADPH	8.1[d]	2.5	7.7
enzyme–APADPH	7.5[c]	1.6	7.0

[a] Stickiness terms were calculated by using the relationship $[^{D}(V/K)_{\text{high pH}} - 1]/[^{D}(V/K)_{\text{low pH}} - 1]$ and the data of Table II. Calculated pK values were determined by substracting log (stickiness term) from the observed pK value (cf. Table V). [b] Value taken from Stone and Morrison (1983). [c] Values for $^{D}(V/K_{\text{APADPH}})$ were taken from Stone and Morrison (1988). [d] pK value from Stone and Morrison (1984).

complexes of Figure 4 and that the intrinsic pK value for the binary enzyme–substrate complex would be obtained from the V/K profile [cf. Stone and Morrison (1984)].

Effect of D_2O on Observed pK Values. In the presence of D_2O, the observed pK value from the V/K_{DHF} profile is shifted by an average of 0.6 pH unit (Table III). This shift is greater by 0.2 pH unit than the average shift of 0.4 pH unit. A full displacement of 0.6 pH unit is not observed in the V profile (Table IV). This is because the intersection point of the asymptotes of the V profile (Figure 1) does not represent a true pK value. Rather, as outlined above, it is the intersection point of the profiles for pH-dependent catalysis and pH-independent product release. The observed displacement of 0.2 pH unit suggests that, in D_2O, catalysis is slowed by a greater factor than product release.

Chemical Mechanism for the Reduction of DHF. The ability of the substrates, NADPH and DHF, to raise artificially the pK value of the single ionizing group at the active

site of dihydrofolate reductase (Asp-27) ensures that both binary enzyme complexes exist in their protonated forms at neutral pH (Table VI). The pK value of 8.5 for the ternary enzyme complex ensures that a sufficiently high proportion of this complex is present at neutral pH in protonated form so as to keep catalysis faster than product release. Thus, the role of Asp-27 is as the source of a proton for the protonation of DHF that, as indicated above, occurs prior to hydride transfer. The latter reaction can still occur even with a mutant form of enzyme that cannot donate a proton to DHF provided that the substrate is present in protonated form (Howell et al., 1986).

On the basis of the above conclusions it might be anticipated that DHF would be bound to dihydrofolate reductase so that its N-5 nitrogen was in close proximity to the protonated carboxyl group of Asp-27. Although suitable crystals have not been obtained for the determination of the structure of the enzyme–DHF complex, modeling studies on the basis of the structure of the enzyme–methotrexate complex indicate that the proton transfer from Asp-27 to DHF cannot be direct (Bolin et al., 1982). However, on the basis of the structure proposed for the enzyme–DHF complex, it is possible to postulate a reasonable mechanism for the proton transfer. This mechanism is illustrated in Figure 5. The figure shows that the water molecule (Wat-253) at the active site, which is bound to Trp-21 and methotrexate in the enzyme–methotrexate complex (Bolin et al., 1982), is hydrogen-bonded to Trp-21, protonated Asp-27, and the carboxyl group of DHF in the ternary enzyme–substrate complex (panel a). As a consequence of the indicated proton transfer, Asp-27 becomes negatively charged, the N-3 nitrogen of DHF assumes a positive charge, and there is formation of the enolic form of DHF (panel b). The enolic group can rotate about its single bond so as to bring the hydrogen atom close to the N-5 nitrogen of DHF (panel c). The lone pair of electrons on the nitrogen atom can now make a nucleophilic attack on the enolic hydrogen, which would be facilitated by the subsequent movement of electrons toward the positively charged N-3 nitrogen. The result (panel d) is the formation of a positive charge on the N-5 nitrogen, which can be regarded as a naked atom since there are no other groups within its vicinity (Bolin et al., 1982). The development of carbonium ion character at the C-6 carbon would facilitate hydride transfer from NADPH and the formation of THF. The hydrogen-bonding network of the enzyme–substrate complex can be considered to play an important role in the catalytic mechanism by decreasing the basicity of Asp-27 while, at the same time, increasing the basicity of the N-5 nitrogen. Such a reversal is essential for transfer of a proton from Asp-27, with a pK value of 6.4 in the ternary enzyme–substrate complex, to the N-5 nitrogen of DHF with a pK value of 3.8 (Poe, 1977). Certainly, the hydrogen-bonding system associated with the ionized form of Asp-27 would make more difficult the protonation of Asp-27 so that the pK value of this group would be decreased. It is possible that a water molecule, hydrogen-bonded to both the enolic hydrogen and the N-5 nitrogen (panel c), could be involved with protonation of this nitrogen. But there is no evidence for the presence of a water molecule in this position.

ACKNOWLEDGMENTS

We are especially grateful to Margaret K. Sneddon for highly skilled technical assistance. We are also most appreciative of the time given by Drs. Armarego and Cleland to discussions of this work. We thank Dr. Benkovic for making available a preprint of the paper by Fierke et al. (1987).

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