Dihydrofolate Reductase from *Escherichia coli*: The Kinetic Mechanism with NADPH and Reduced Acetylpyridine Adenine Dinucleotide Phosphate as Substrates

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ABSTRACT: Kinetic studies on the reaction catalyzed by dihydrofolate reductase from *Escherichia coli* have been undertaken with the aim of characterizing further the kinetic mechanism of the reaction. For this purpose, the kinetic properties of substrates were determined by measurement of (a) initial velocities over a wide range of substrate concentrations and (b) the stickiness of substrates in ternary enzyme complexes. Stickiness is defined as the rate at which a substrate reacts to give products relative to the rate at which that substrate dissociates. Stickiness was determined by varying the viscosity of reaction mixtures and the concentration of one substrate in the presence of a saturating concentration of the other substrate. The results indicate that NADPH is sticky in the enzyme–NADPH–dihydrofolate complex, while dihydrofolate is much less sticky in this complex. At higher concentrations, NADPH functions as an activator through the formation of an enzyme–NADPH–tetrahydrofolate from which tetrahydrofolate is released more rapidly than from an enzyme–tetrahydrofolate complex. Higher concentrations of dihydrofolate also cause enzyme activation, and it appears that this effect is due to the ability of dihydrofolate to displace tetrahydrofolate from a binary enzyme complex through the formation of a transitory enzyme–tetrahydrofolate–dihydrofolate complex. As NADPH and dihydrofolate function as activators and as NADPH behaves as a sticky substrate, the kinetic mechanism of the dihydrofolate reductase reaction with the natural substrates is steady-state random. By contrast with NADPH, reduced 3-acetylpyridine adenine dinucleotide phosphate exhibits only slight stickiness and does not function as an activator. The kinetic data indicate that the slower reaction with the alternate pyridine nucleotide substrate conforms to a rapid equilibrium, random mechanism. However, catalysis is not rate-limiting. Deuterium isotope effect data show that the slow step along the reaction sequence is an isomerization reaction that precedes and/or follows the catalytic step.

Dihydrofolate reductase catalyzes the NADPH-dependent reduction of dihydrofolate (DHF)[1] to tetrahydrofolate, which is subsequently used as a cofactor in nucleotide biosynthesis. Inhibition of dihydrofolate reductase by antifolate drugs is important in the chemotherapeutic treatment of several diseases (McCormack, 1981; Roth & Cheng, 1982). Consequently, the mechanism of this inhibition has been studied extensively (McCormack, 1981; Roth & Cheng, 1982; Gready, 1980; Hitchings & Smith, 1980). Fewer investigations have dealt with the kinetic mechanism of the enzyme-catalyzed reaction. Earlier reports suggested that the mechanism could be either random (Burchall & Chan, 1969; McCullough et al., 1971) or ordered (Blakley et al., 1971). The results of a recent study from this laboratory were consistent with a random kinetic mechanism for the enzyme from *Escherichia coli* (Stone & Morrison, 1982). With the enzyme from this source, a discrepancy was observed between the kinetically and thermodynamically determined values for the dissociation constant of the enzyme–NADPH complex. To explain this discrepancy, it was proposed that the mechanism was partial steady-state random with DHF being the sticky substrate. However, the results of recent pH studies indicate that DHF is not a very sticky substrate (Morrison & Stone, 1988).

The present study was initiated to determine if either of the two substrates is sticky. The stickiness of a substrate can be determined by the method of isotope trapping (Rose, 1980) or by a relatively simple procedure that involves measurement of the variation of the initial velocity of the reaction as a function of viscosity (Brouwer & Kirsch, 1982). The latter method has been used in the present study to show that NADPH is a much stickier substrate than is DHF. The results of kinetic investigations indicate that the dihydrofolate reductase reaction exhibits a random kinetic mechanism with both NADPH and reduced acetylpyridine adenine dinucleotide (APADPH) as substrates. But it is only with APADPH that the reaction can be described as being rapid equilibrium, random. The conclusions about the mechanism with NADPH and DHF as substrates are in agreement with those reached recently by Fierke et al. (1987) from the results of pre-steady-state kinetic studies.

Theory and Data Analysis

Viscosity Effects. On the basis of the conclusions reached in the accompanying paper, it will be considered that the dihydrofolate reductase reaction proceeds, at neutral pH, via

$$\mathrm{HE} \underset{k_2}{\overset{k_{1A}}{\rightleftharpoons}} \mathrm{HEA} \underset{k_4}{\overset{k_3}{\rightleftharpoons}} \mathrm{EAH} \xrightarrow{k_5} \mathrm{EP} \xrightarrow{k_7} \mathrm{E} \qquad (1)$$

HE represents the protonated form of the enzyme–NADPH-(APADPH) complex. k_3 and k_5 denote, respectively, the rates of protonation of the N-5 nitrogen of DHF(A) by protonated

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[1] Abbreviations: DHF, dihydrofolate; THF, tetrahydrofolate; APADPH, reduced 3-acetylpyridine adenine dinucleotide phosphate; Tris, tris(hydroxymethyl)aminomethane; MES, 2-(*N*-morpholino)-ethanesulfonic acid.

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enzyme and the subsequent hydride transfer from NADPH-(APADPH) to the C-6 position of the substrate. k_7 represents the rate of release of NADP(APADP) and THF(P) from the enzyme. In the presence of a saturating concentration of DHF and with NADPH(APADPH) as the variable substrate, the same scheme would apply. However, HE would now represent the protonated form of the enzyme–DHF complex, while A would denote NADPH(APADPH).

The observed rate constant for the association of A with the binary form of enzyme (V/K_aE_t) would be given by

$$\frac{V}{KE_t} = \frac{k_1k_3k_5}{k_2(k_4 + k_5) + k_3k_5} \tag{2}$$

where V, K, and E_t represent the maximum velocity of the reaction, the Michaelis constant for the variable substrate with the other substrate saturating, and the total enzyme concentration, respectively. The magnitude of the rate constants k_1 and k_2 should be dependent on viscosity (Brouwer & Kirsch, 1982; Nakatani & Dunford, 1979). The dependence is given by

$$k_1{}^0\eta^0 = k_1\eta \qquad k_2{}^0\eta^0 = k_2\eta \tag{3}$$

where k_1 and k_2 are the rate constants in a medium of viscosity, η, and $k_1{}^0$ and $k_2{}^0$ are the rate constants in the absence of added viscogenic reagent when the viscosity is η^0. Substitution into eq 2 of the relationships for k_1 and k_2 yields

$$\frac{V}{KE_t} = \frac{k_1\left(\frac{\eta^0}{\eta}\right)}{1 + \left(\frac{1 + k_4/k_5}{k_3/k_2}\right)\left(\frac{\eta^0}{\eta}\right)} \tag{4}$$

When this equation is written in double-reciprocal form as eq 5, it is apparent that a plot of KE_t/V against relative viscosity

$$\frac{KE_t}{V} = \frac{1}{k_1}\left(\frac{\eta}{\eta^0}\right) + \frac{1}{k_1}\left(\frac{1 + k_4/k_5}{k_3/k_2}\right) \tag{5}$$

(η/η^0) will be linear. The slope of the plot will yield a value for k_1, which is the bimolecular rate constant for the interaction of A with HE. The value will be in units of M^{-1} s^{-1} if E_t and K/V are expressed as M and s^{-1}, respectively. The horizontal intercept of the replot gives a reciprocal value for the stickiness factor, $(k_3/k_2)/(1 + k_4/k_5)$, which is the ratio of the net rate constant for the reaction of A with HE, through the irreversible hydride transfer step (k_5), to the true dissociation constant (k_2) for the release of A from HEA (Cleland, 1982).

The maximum velocity for the reaction scheme of eq 1 is given by

$$\frac{V}{E_t} = \frac{k_3k_5k_7}{k_3k_5 + k_3k_7 + k_4k_7 + k_5k_7} \tag{6}$$

When the product release step (k_7) is affected by viscosity, the equation becomes

$$\frac{V}{E_t} = \frac{k_7\left(\frac{\eta^0}{\eta}\right)}{1 + \left[\frac{k_7(k_3 + k_4 + k_5)}{k_3k_5}\right]\left(\frac{\eta^0}{\eta}\right)} \tag{7}$$

which can be expressed in double-reciprocal form as

$$\frac{E_t}{V} = \frac{1}{k_7}\left(\frac{\eta}{\eta^0}\right) + \frac{k_3 + k_4 + k_5}{k_3k_5} \tag{8}$$

Thus, the intercepts of double-reciprocal plots of initial velocity as a function of A (i.e., $1/V$ values) will vary linearly with the relative velocity. The slope and intercept of the secondary plot of E_t/V against η/η^0 (eq 8) will yield values for k_7 and $k_3k_5/(k_3 + k_4 + k_5)$, respectively. The latter relationship represents the net rate constant for the two catalytic steps, viz., protonation and hydride transfer, of eq 1. When E_t is in M and V in M/s, units of k_7 will be s^{-1}.

The observation of an effect of viscosity on the intercepts of reciprocal plots of velocity against substrate concentration could also be due to the formation of an unproductive enzyme–substrate complex. If it is considered that the HE form of enzyme in eq 1 undergoes the reaction

$$\mathrm{HE} + \mathrm{A} \rightleftharpoons[K_i] \mathrm{AHE} \tag{9}$$

where AHE represents a dead-end or unproductive complex, eq 6 changes to

$$V/E_t = k_1k_3k_5k_7/[(k_7/K_i) \times (k_2k_4 + k_2k_5 + k_3k_5) + k_1(k_3k_5 + k_3k_7 + k_4k_7 + k_5k_7)] \tag{10}$$

When it is assumed that viscosity has no effect on k_7 but affects only k_1 and k_2 of eq 1, then

$$V/E_t = k_3k_5k_7(\eta^0/\eta)/[(k_7K_{ia}/K_i)(k_4 + k_5)(\eta^0/\eta) + (k_3k_5k_7/k_1K_i) + (k_3k_5 + k_3k_7 + k_4k_7 + k_5k_7)(\eta^0/\eta)] \tag{11}$$

and the reciprocal form of the equation becomes

$$\frac{E_t}{V} = \frac{1}{k_1K_i}\left(\frac{\eta}{\eta^0}\right) + \frac{1}{k_3}\left(1 + \frac{k_4}{k_5}\right)\left[1 + \frac{K_{ia}}{K_i} + \frac{k_3(1 + k_5/k_7)}{k_4 + k_5}\right] \tag{12}$$

In eq 11 and 12, K_{ia} is the dissociation constant of the HEA complex and equal to k_2/k_1. While the relationships for the horizontal and vertical intercepts from eq 12 are complex, a value for k_1K_i can be obtained from the slope of a plot of E_t/V against (η/η^0). Since the slope of a plot of KE_t/V against viscosity yields a value for k_1 (eq 5), the dissociation constant for the formation of an unproductive enzyme–substrate complex (K_i) could be determined.

Analysis of Viscosity Data. Initial velocity data obtained by varying one substrate in the presence of different viscosities were fitted to eq 13 by using weighted, robust linear regression

$$v = \frac{VA}{K + A} \tag{13}$$

(Cornish-Bowden & Endrenyi, 1981). The resulting values for V/K and V were weighted according to their variances and fitted to eq 4, 7, or 12 by using weighted, robust nonlinear regression (Duggleby, 1981).

Nonlinear Double-Reciprocal Plots. In the absence of products and with one substrate concentration held constant at a nonsaturating concentration, initial velocity data for a steady-state random bireactant mechanism can be described by the general equation

$$v = \frac{V(A^2 + dA)}{A^2 + bA + c} \tag{14}$$

where A denotes the variable substrate and V, b, c, and d are parameters composed of terms that are the products of various rate constants and the concentration of the fixed substrate (Morrison, 1969). In double-reciprocal form eq 14 describes

a 2/1 function (Cleland, 1963). Data yielding double-reciprocal plots that could be described by such a function were fitted to eq 14. For this purpose, weighted nonlinear regression that assumed proportional standard errors was used. The algorithm used was the Levenberg–Morrison–Marquardt algorithm as implemented by Osborne (1976).

Deuterium Isotope Effects. Initial velocities were obtained by (a) varying the concentration of DHF at a fixed, saturating concentration of either APADPH or APADPD or (b) varying the concentration of APADPH or APADPD at a saturating concentration of DHF. The resulting data were fitted to eq 15, where $E_{V/K}$ and E_V are the isotope effects minus one and

$$v = \frac{VA}{K(1 + F_i E_{V/K}) + A(1 + F_i E_V)} \quad (15)$$

F_i represents the fraction of deuterium label in the substrate.

Experimental Procedures

Materials. DHF was prepared from folic acid by using the method of Blakley (1960). Sucrose was obtained from BDH Chemicals, Australia, and purified by treatment with activated charcoal and recrystallization from ethanol. Reduced 3-acetylpyridine adenine dinucleotide phosphate (APADPH) was synthesized and purified as described previously (Stone & Morrison, 1984). Reduced APADP, containing deuterium on the A-side at position 4 of the nicotinamide ring, was prepared and analyzed by the same procedures as those used to prepare and analyze NADPD (accompanying paper). The product was fully deuteriated on the A-side. All other chemicals were of the highest quality available commercially. Dihydrofolate reductase was purified from an overproducing mutant of *E. coli* (Smith et al., 1982) by using the procedure of Stone and Morrison (1982). The concentration of APADPH was estimated spectrophotometrically by using an extinction coefficient of 9100 M^{-1} cm^{-1} at 363 nm (P-L Biochemicals, 1981) Concentrations of DHF and NADPH were determined enzymically by using a molar absorbancy change for the dihydrolfate reductase reaction of 11 800 cm^{-1} (Stone & Morrison, 1982).

Methods. (*a*) *Enzyme Assays.* Initial velocities were determined spectrophotometrically by following the decrease in absorbance at 340 nm and 30 °C (Stone & Morrison, 1982). The assay buffer at pH 7.4 contained 50 mM tris(hydroxymethyl)aminomethane (Tris), 25 mM sodium acetate, 25 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), and 100 mM NaCl. For viscosity experiments sucrose buffer solutions were made up in a manner such that the concentrations of the buffer components and the pH were identical with those of the reference buffer. The molar absorbancy change of the reaction with either NADPH or APADPH as the hydride donor was 11 800 cm^{-1} at 340 nm.

(*b*) *Viscosity Measurements.* The relative viscosities (η/η^0) of buffer solution containing various concentrations of sucrose were measured at 30 °C by using a Ubbelohde viscometer. The assay buffer was used as the reference solution.

Results

Effect of Viscosity on Reaction. The effect of viscosity on the initial velocity of the dihydrofolate reductase reaction has been determined at pH 7.4 with DHF, NADPH, and APADPH as variable substrates. The nonvaried substrate was held constant at a saturating concentration. The results indicate that the value of V/K_{DHF} is not dependent on viscosity in the presence of APADPH (100 μM), although the value of V decreases with viscosity (Figure 1A). But in the presence

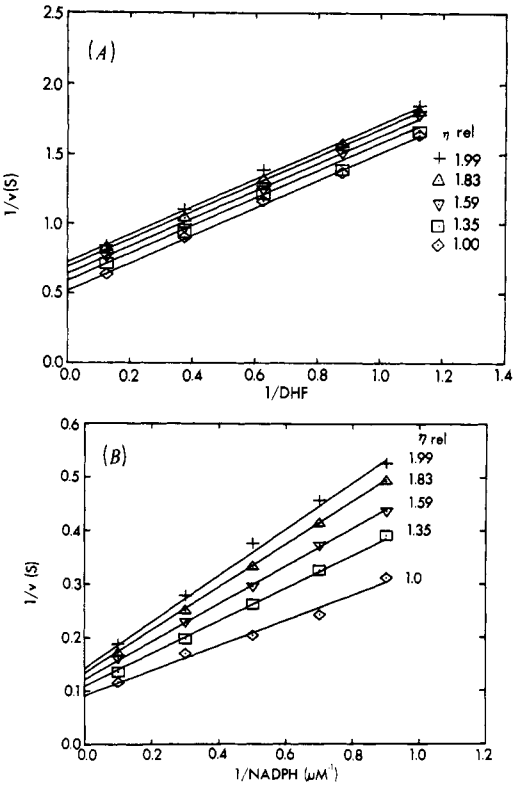


FIGURE 1: Effect of viscosity on the initial velocity of the reaction. (A) Dihydrofolate (DHF) was the variable substrate with reduced acetylpyridine adenine dinucleotide (APADPH) held constant at 100 μM. (B) NADPH was the variable substrate with DHF at a fixed concentration of 40 μM. The concentration of enzyme was 0.38 nM. The units of velocity (v) are s^{-1}, while the units of viscosity are given as relative to the assay buffer. The relative viscosity ($\eta_{rel} = \eta/\eta^0$) was increased by using sucrose.

of NADPH (100 μM), both V/K_{DHF} and V increase with viscosity (data not shown). In the presence of DHF (40 μM), the V/K values for both pyridine nucleotides, as well as the V values, are dependent on viscosity. The results obtained with NADPH as the variable substrate are shown in Figure 1B. Analyses of the viscosity data, as outlined in eq 5 and 8, yielded values (Table I) for the association rate constants (k_1), the stickiness ratios $[(k_3/k_2)/(1 + k_4/k_5)]$, the product release ($k_7$), and the net rate constants for the catalytic steps $[k_3k_5/(k_3 + k_4 + k_5)]$. Alternatively, the values obtained for k_7 could represent those for k_1K_i (cf. eq 12).

Effect of Higher Substrate Concentration on the Initial Velocity of Reaction. The dependence of the velocity of the reaction on the concentration of APADPH and NADPH was studied over an extended range of concentrations, and the results are presented in Figure 2. When APADPH was varied, the dihydrofolate reductase reaction follows Michaelis–Menten kinetics over the concentration range 1–100 μM (Figure 2B) at both low and high concentrations of DHF. With NADPH, however, substantial deviations from Michaelis–Menten kinetics were found at nucleotide concentrations above 10 μM (Figure 2A). The double-reciprocal plots, which exhibit downward curvature that is independent of the DHF concentration, could be fitted to the equation that describes a 2/1 function (eq 10). The resulting values are given in the legend to Figure 2. This downward curvature was not observed by Fierke et al. (1987), although it is predicted by their data. Higher concentrations of DHF also gave rise to double-reciprocal plots that were concave down with concentrations of NADPH ranging from 2.5 to 100 μM (Figure 3A). By contrast, there was little or no downward curvature when

Table I: Determination from Viscosity Effects of the Magnitude of Rate Constants for the Interaction of DHF and Pyridine Nucleotides with Binary Complexes of Dihydrofolate Reductase[a]

variable substrate	fixed substrate	slope replot data		intercept replot data		max velocity[d] (s^{-1})	K_i nonproductive complex, k_7/k_1 (μM)
		k_1 (μM^{-1} s^{-1})	$(k_3/k_2)/(1 + k_4/k_5)$[b]	k_7 (s^{-1})	$k_3k_5/(k_3 + k_4 + k_5)$[c] (s^{-1})		
DHF	NADPH (100 μM)	44 ± 9	0.18 ± 0.05	50 ± 6	28 ± 3	18	1.1 ± 0.3
NADPH	DHF (40 μM)	4.6 ± 0.2	11.0 ± 1.3	20 ± 2	25 ± 5	11	4.3 ± 0.5
DHF	APADPH (100 μM)			3.3 ± 0.1	4.8 ± 0.2	2	
APADPH	DHF (40 μM)	0.81 ± 0.07	0.57 ± 0.09	8.7 ± 2.4	2.4 ± 0.3	2	10.7 ± 3.1

[a] The slopes and intercepts for primary plots of $1/v$ against 1/variable substrate were analyzed according to eq 5 and 8. [b] Stickiness ratio for substrate in the given mechanism (eq 1) as described by Cleland (1982). [c] Net rate constant for the catalytic steps of eq 1 and equal to $1/[(1/k_5) + (k_4 + k_5)/k_3k_5]$. [d] Calculated by using the values for k_7 and the net rate constant for the catalytic steps as well as the relationship $V/E_t = 1/[1/k_7 + 1/k_5 + (k_4 + k_5)/k_3k_5]$.

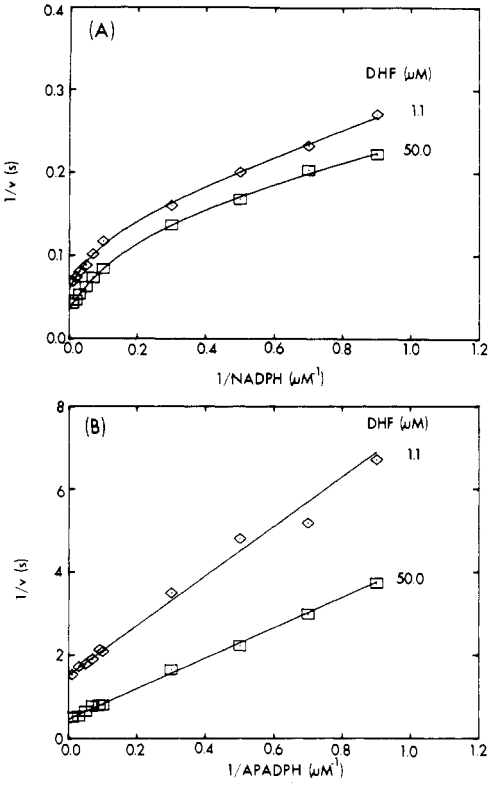


FIGURE 2: Variation of the initial velocity of the reaction with (A) NADPH and (B) APADPH as the variable substrates in the presence of fixed concentrations of DHF. Initial velocity assays were performed as described under Methods at the indicated concentrations of DHF and with (A) 0.42 or (B) 1.9 nM enzyme. (A) Lines represent the best fit of the data to eq 14. With 1.1 μM DHF, the values of the parameters were $V = 16.8$ s^{-1}, $b = 22.8$ μM, $c = 21.6$ μM^2, and $d = 8.5$ μM. With 50 μM DHF, the values were $V = 28.4$ s^{-1}, $b = 22.8$ μM, $c = 11.8$ μM^2, and $d = 4.3$ μM. (B) Lines represent the best fit of the data to eq 13. With 1.1 μM DHF, the values of V and K were 0.66 s^{-1} and 3.9 μM, respectively. With 50 μM DHF, the values for V and K were 2.1 s^{-1} and 7.9 μM, respectively.

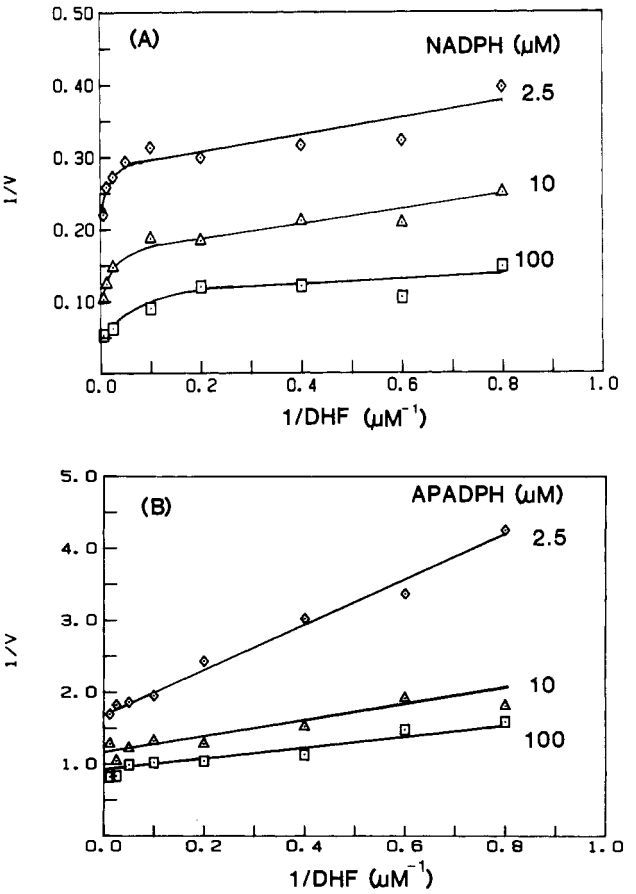


FIGURE 3: Variation of the initial velocity of the reaction with DHF varied over an extended range of concentrations and either (A) NADPH or (B) APADPH held constant at different fixed concentrations.

DHF was varied in the presence of APADPH (Figure 3B).

Deuterium Isotope Effects with APADPD. The pH dependence of the deuterium isotope effects has been determined with both DHF and APADPH as variable substrates. The determinations of the initial velocities with DHF as the varied substrate in the presence of either APADPH or APADPD were performed by having 2,4-diamino-6,7-dimethylpteridine (DADMP) present at a fixed concentration of 6 μM. This compound, which acts as an inhibitory analogue of DHF (Stone & Morrison, 1986), was added to facilitate the measurement of velocities by raising the Michaelis constant for DHF at each pH. The effect of the inhibitor was greatest at pH 6.6 with an increase in the value for K_{DHF} from 1 to about 30 μM. The increase was less at higher pH values because

Table II: Effect of pH on the Deuterium Isotope Effect with DHF and APADPH as Variable Substrates[a]

pH	DHF		APADPH	
	$^D(V/K)$	^DV	$^D(V/K)$	^DV
6.6	1.6 ± 0.1	1.4 ± 0.1	2.0 ± 0.1	1.3 ± 0.1
8.2	2.1 ± 0.1	1.9 ± 0.1		2.1 ± 0.1
9.6	3.3 ± 0.2	2.4 ± 0.2	2.6 ± 0.1	2.9 ± 0.2

[a] Values for $^D(V/K)$ and ^DV were determined in the presence of a fixed concentration (6 μM) of 2,4-diamino-6,7-dimethylpteridine. The concentration of APADPH was held constant at 50 μM with DHF as the variable substrate, while the concentration of DHF was fixed at 40 μM when APADPH was the variable substrate.

the inhibition by DADMP decreases with increasing pH, while the value for K_{DHF} is largely pH independent. As DADMP functions as a classical competitive inhibitor with respect to DHF, maximum velocities will not be affected. The observed

values for V/K at any pH with APADPH or APADPD will be reduced by the factor $1 + I/K_i$. But true $^D(V/K)$ values will be obtained as they are ratios of the V/K values observed with the protio- and deuteriopyridine nucleotides. The results (Table II) indicate that the values for ^DV, $^D(V/K_{DHF})$, and $^D(V/K_{APADPH})$ increase to a limiting value in the region of 3 as the pH is increased from 6.6 to 9.6.

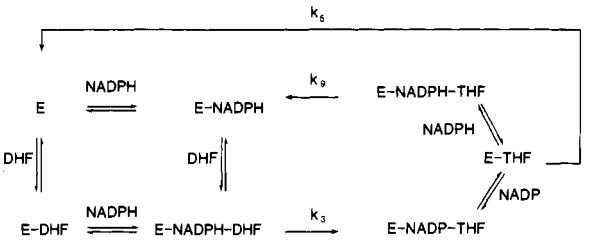
Discussion

Viscosity Effects. The effect of viscosity on an enzyme-catalyzed reaction could be due to either a general effect on the structure of the enzyme or a specific effect on diffusion-controlled steps. The existence of a substrate whose V/K value is independent of viscosity provides an assurance that the effects observed with other substrates are not due to a general perturbation of enzyme structure (Brouwer & Kirsch, 1982). Thus, the lack of dependence on viscosity of the V/K_{DHF} value in the presence of APADPH indicates that it is possible to interpret in terms of eq 5 the data obtained with substrates whose V/K values do vary with viscosity. The bimolecular rate constant of 44 μM^{-1} s^{-1} for the reaction of DHF with the enzyme–NADPH complex and of 4.6 μM^{-1} s^{-1} for the reaction of NADPH with the enzyme–DHF complex (Table I) may be compared with values of 40 and 5 μM^{-1} s^{-1} as reported by Fierke et al. (1987) with dihydrofolate reductase from *E. coli*. APADPH interacts with the enzyme–DHF complex more slowly than does NADPH (Table I).

The fitting of viscosity data to eq 5 also yields values for the stickiness ratio (S_r) associated with substrates (Table I; Cleland, 1982), and it should be noted that this ratio is the reciprocal of the partition ratio as defined by Brouwer and Kirsch (1982). The S_r value of 11.0 for the reaction of NADPH with the enzyme–DHF complex (Table I) indicates that NADPH behaves as a sticky substrate. By contrast, DHF and APADPH exhibit only a small degree of stickiness, as the S_r values are less than 1. The present findings are generally in accord with those reached from studies on deuterium isotope effects (accompanying paper), although the magnitude of the S_r values is lower.

On the assumption that product release is affected by viscosity, the variation of V/E_t as a function of viscosity in the presence of a saturating concentration of the other substrate will yield values for the maximum rate of product release (k_7) and the net rate constant for the catalytic steps (cf. Theory and Data Analysis eq 7, 8). The results (Table I) show that, as expected, the net rate constant for the catalytic steps is the same irrespective of whether DHF or NADPH is the variable substrate. However, the maximum rate of product release, as well as the maximum velocity of the reaction, is significantly greater when DHF is the variable substrate and NADPH is held constant at a relatively high concentration than when the reverse situation applies. This is because the highest variable concentration of NADPH was 10 μM with a fixed concentration of DHF (Figure 1B), and concentrations of NADPH from 10 to 100 μM are required for activation (Figure 2A). The magnitude of the values for k_7 and the net rate constant for the catalytic steps suggests that both product release and the catalytic steps determine the maximum velocity of the reaction (Table I). Such a result is at variance with the conclusions of Fierke et al. (1987), who conclude that product release at neutral pH is solely rate-limiting. The values for the net rate constants of the catalytic steps are not in such good agreement with DHF and APADPH as variable substrates (Table I). This limits the conclusions that can be drawn about the effects of viscosity on the reaction with the alternate pyridine nucleotide substrate. But the data indicate that there is no activation by higher concentrations of APADPH and that the maximum velocity of the reaction with this nucleotide substrate is lower than with NADPH [cf. Stone et al. (1984)].

Scheme I



The effect of viscosity on the maximum velocity of the reaction could also be due to the formation of nonproductive ternary complexes. The kinetic theory, which is outlined under Theory and Data Analysis, shows that, under these circumstances, the slope of a secondary plot of the apparent maximum velocity as a function of viscosity yields a value for k_1K_i rather than for k_7 (Table I). As the value for the k_1, the second-order rate constant for the reaction of the variable substrate with a binary enzyme–substrate complex, can be obtained from a replot of primary viscosity data, a value can be calculated for K_i (cf. Theory and Data Analysis, Table I). The resulting values for K_i, which represents the dissociation constant for the nonproductive ternary enzyme–substrate complex, suggest that nonproductive complex formation would be greater with NADPH than with APADPH. Such a conclusion is not in accord with the finding that the maximum velocity is greater in the presence of NADPH (Table I; Stone et al., 1984), and thus this model can be discarded.

Effect on Initial Velocity of Higher Concentrations of NADPH and DHF. It has been considered previously that the kinetic mechanism of the reaction catalyzed by dihydrofolate reductase from *E. coli* is essentially rapid equilibrium, random. However, a 7-fold difference was found in the values for the dissociation constant of the enzyme–NADPH complex when determined by kinetic and thermodynamic procedures (Stone & Morrison, 1982). Such a result indicates that the kinetic mechanism is not truly rapid equilibrium, random. This conclusion is supported by the findings that NADPH behaves as a sticky substrate (Table I) and that double-reciprocal plots of velocity as a function of a wide range of NADPH concentrations are concave down (Figure 2A). Such downward curvature in the presence of a nonsaturating concentration of DHF is in accord with the reaction conforming to a steady-state random mechanism. However, as curvature persists in the presence of a saturating concentration of DHF, the data cannot be accounted for simply on the basis of the steady-state random addition of the two substrates to the enzyme. The substrate activation of the enzyme with NADPH concentrations greater than 10 μM can be accounted for by Scheme I. This scheme allows the dihydrofolate reductase reaction to occur via a random mechanism that involves the presence, under steady-state conditions, of a kinetically significant concentration of enzyme–THF. Evidence for the formation of such a complex has been obtained previously from inhibition studies with thionicotinamide nucleotide diphosphate (Stone et al., 1984). Further, the scheme proposes that, at higher concentrations, NADPH can combine with this complex to enhance the rate of THF release from the ternary enzyme–THF–NADPH complex relative to that from the binary enzyme–THF complex ($k_9 > k_5$). It is also consistent with the earlier report that there is no formation of a dead-end enzyme–NADPH–THF complex (Stone & Morrison, 1982). The full steady-state rate equation for this kinetic mechanism

has been derived by Dalziel and Dickinson (1966). The equation predicts that, with NADPH as the variable substrate in the presence of both low and high concentrations, double-reciprocal plots will be described by a 2/1 function and hence can have the shape of those illustrated in Figure 2A. Fierke et al. (1987) have also concluded that NADPH combines with the enzyme–THF complex and that the interaction is essential for the release of the product at a rate commensurate with the maximum velocity of the reaction. They determined the rates of dissociation of THF from the enzyme–THF and enzyme–THF–NADPH complexes to be 1.4 and 12 s^{-1}, respectively. The values are lower than the 20 and 50 s^{-1} reported in Table I. In this connection the question must arise as to whether the binding of THF in the complex formed by mixing enzyme and THF is the same as that in the complex produced on the release of NADP from the ternary enzyme–NADP–THF complex. It is conceivable that, on mixing with the enzyme, THF could be bound to some extent in a nonproductive manner to form a binary complex with thermodynamic properties different from those of the complex formed enzymically.

The activation of the enzyme by concentrations of DHF that are considerably higher than the Michaelis constant for this substrate (Figure 3) cannot be due to the interaction of DHF with an enzyme–NADP complex. Product inhibition patterns indicate clearly that the ternary enzyme–NADP–DHF complex is a dead-end complex (Stone & Morrison, 1982). Therefore, it seems that the activation by DHF may well be due to its ability to displace THF by a mechanism that involves a bimolecular collision between an enzyme–THF complex and DHF to form a transitory enzyme–THF–DHF complex. Because THF and DHF are large molecules with common structural features, it is conceivable that, in forming a short-lived ternary complex, they bind to dihydrofolate reductase through different complementary moieties. For example, THF might bind through the (*p*-aminobenzoyl)-glutamate moiety, while DHF binds through the pterin moiety. The binding of DHF would commence before THF has fully dissociated from the binary enzyme–THF complex.

The results obtained with APADPH differ from those observed with NADPH. Double-reciprocal plots indicate that there is no substrate activation by this nucleotide (Figure 1B), while the viscosity data show that APADPH is not a very sticky substrate (Table I). In addition, the maximum velocity of the reaction with APADPH is some 7 times slower than with NADPH, and similar values are obtained for the dissociation constant of the binary enzyme–APADPH complex when determined by kinetic and thermodynamic procedures (Stone & Morrison, 1984). The findings provide strong evidence for the idea that, with APADPH as the hydride donor, the reaction mechanism is essentially rapid equilibrium, random. That is, the reaction occurs under conditions where catalysis is rate-limiting and there is no kinetically significant steady-state concentration of an enzyme–THF complex on the release side of the reaction sequence. This conclusion is also consistent with the finding that there is no substrate activation by higher concentrations of DHF in the presence of APADPH (Figure 3B).

Deuterium Isotope Effects with APADPH. The occurrence of deuterium isotope effects with both APADPH and DHF as variable substrates confirms that the kinetic mechanism of the reaction with these substrates is of the random type. On the basis of the aforementioned conclusion that catalysis is rate-limiting with APADPH as the hydride donor, it follows that intrinsic isotope effects should be observed for $^{D}(V/K_{DHF})$, $^{D}(V/K_{APADPH})$, and ^{D}V at all pH values.[2] However, this is not the case (Table II). Both the ^{D}V and $^{D}(V/K)$ values are relatively low at pH 6.6 and increase, at higher pH, toward a limiting value of about 3 as found with NADPH (accompanying paper). This finding suggests that the conversion of the enzyme–APADPH–DHF complex to an enzyme–APADP–THF complex is preceded and/or followed by a slower, pH-independent isomerization step that is responsible for the lower maximum velocity observed with APADPH as compared with NADPH (Table I). It is not until catalysis becomes rate-limiting at higher pH that the isotope effects for ^{D}V and $^{D}(V/K)$ approach an intrinsic value. With a slow isomerization step preceding and/or following the catalytic step, the internal commitments of the relationships for $^{D}(V/K_{DHF})$ and $^{D}(V/K_{APADPH})$ would be high and be reflected in the relatively low values for these parameters in the pH-independent region (Table II; Cleland, 1982). A slow isomerization step would also bring to equilibrium all the steps preceding the isomerization. Thus, the reaction with APADPH would conform to a rapid equilibrium, random kinetic mechanism. It remains to be determined why viscosity affects the maximum velocity of the reaction with APADPH and DHF.

Conclusion. The present results draw attention to the need for the application of a wide range of techniques in connection with the elucidation of an enzyme reaction mechanism. The kinetic and thermodynamic data for the dihydrofolate reductase reaction with APADPH as the nucleotide substrate suggest that the catalytic step is rate-limiting. However, the isotope effect data indicate that it is not this step which is rate-limiting but rather one that precedes and/or follows the conversion of substrates to products. Viscosity studies provide a useful means of determining second-order rate constants for the reaction of the second substrate of a Bi-Bi mechanism with a binary enzyme-first substrate complex. They are also useful as a means of gaining information about substrate stickiness.

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Registry No. NADPH, 53-57-6; DHF, 4033-27-6; APADPH, 2737-69-1; D_2, 7782-39-0; DHF reductase, 9002-03-3.

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[2] Articles by Cleland (1982, 1986) should be consulted for a detailed discussion of the interpretation of deuterium isotope effect data.

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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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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.

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