Human Dihydrofolate Reductase: Reduction of Alternative Substrates, pH Effects, and Inhibition by Deazafolates

Elizabeth A. Williams and John F. Morrison*

Division of Biochemistry and Molecular Biology, John Curtin School of Medical Research, Australian National University, GPO Box 334, Canberra, ACT, Australia 2601

Received July 16, 1991; Revised Manuscript Received April 21, 1992

ABSTRACT: The kinetics of the NADPH-dependent reduction of 7,8-dihydrofolate, folate, and 7,8-dihydrobiopterin by human dihydrofolate reductase have been examined over the pH range from 4.0 to 9.5. The V and V/K profiles obtained with the three substrates indicate that a single ionizing residue at the active site of the enzyme must be protonated for catalysis. Both the maximum velocity of the reactions and the rate of interaction of the substrates with the enzyme–NADPH complex decrease in the order dihydrofolate > dihydrobiopterin ≫ folate. From the pK values of the V/K profiles, it can be concluded that, while dihydrofolate behaves as a sticky substrate and dihydrobiopterin exhibits slight stickiness, folate is not a sticky substrate. Further support for this conclusion comes from the results of deuterium isotope effects. The pK values obtained from both the V and V/K_{folate} profiles are similar to the intrinsic pK value of 5.6 for both the free enzyme and the enzyme–NADPH complex. The folate analogue, 5-deazafolate, is not a substrate, but it undergoes strong interaction with the enzyme. This interaction, which is enhanced by the presence of NADPH, is due to protonation of the bound ligand that does not involve the single ionizing group at the active center of the enzyme. Difference spectra yield evidence for the protonation of bound 5-deazafolate and show that, on binding to the enzyme–NADPH complex, the pK of the N-8 atom is raised to about 10 from a value of about 4 in solution. The results are in accord with those of a recent paper on the three-dimensional structure of the enzyme–5-deazafolate complex [Davies, J. F., Delcamp, T. J., Prendergast, N. J., Ashfors, V. A., Freisheim, J. H., & Kraut, J. (1990) *Biochemistry 29,* 9467–9479] which indicate that there is hydrogen bond formation between N-8 of the ligand and the carbonyl group of Ile-7. However, the present findings do not support the idea that bound 5-deazafolate resembles the transition-state complex for folate reduction. Quinazolines also interact strongly with the enzyme but in a pH-independent manner. The dissociation constants for the binary complexes are an order of magnitude lower than that for the binding to the enzyme of unprotonated 5-deazafolate. This difference reflects the hydrophobic nature of the amino acid residues at the active site that are near the N-5 and N-8 nitrogens of bound pterins.

Dihydrofolate reductase (5,6,7,8-tetrahydrofolate:NADP$^+$ oxidoreductase, EC 1.5.1.3, DHFR) is the target enzyme for antibacterial, antimalarial, and antitumor agents. Since a facile isolation procedure became available (Poe et al., 1972), this enzyme from a variety of sources has been the subject of a diverse range of investigations. These include UV–visible spectrocopy (Erickson & Mathews, 1972; Poe et al., 1974; Gupta et al., 1977; Hood & Roberts, 1978; Subramanian & Kaufman, 1978; Stone & Morrison, 1983), NMR spectroscopy (Cocco et al., 1981; Selinsky et al., 1990), and steady-state (Stone & Morrison, 1982, 1984) and rapid reaction kinetics (Fierke et al., 1987; Appleman et al., 1990) as well as X-ray crystallographic studies (Bolin et al., 1982; Filman et al., 1982; Matthews et al., 1985a,b; Oefner et al., 1988; Davies et al., 1990). The result is that dihydrofolate reductase is one of the best understood enzymes with regard to its structure and catalytic function (Morrison, 1991). A point of particular interest has been the mechanism of hydride transfer. The evidence presented by Stone and Morrison (1984) and Morrison and Stone (1988) suggests that, in the ternary enzyme–NADPH–dihydrofolate complex, protonation of the N-5 nitrogen of dihydrofolate by the protonated form of the enzyme precedes and facilitates the hydride transfer. However, protonation of the binary enzyme–dihydrofolate complex is not observed (Stone & Morrison, 1984; Selinsky et al., 1990). By contrast, several inhibitory analogues of dihydrofolate, including methotrexate and trimethoprim, have been shown to be protonated when bound to the free enzyme (Cocco et al., 1981; Stone & Morrison, 1983; Selinsky et al., 1990). In these cases, it is the N-1 nitrogen of the pteridine or pyrimidine ring that is protonated.

Several years ago, it was demonstrated in this laboratory that 5-deazafolate is not reduced by dihydrofolate reductases but is a potent inhibitor of the enzymes from *Escherichia coli* and chicken liver (Stone et al., 1984). Further, it was shown that 5-deazafolate binds to the bacterial enzyme some 4000-fold more tightly than does folate, but the reasons for the enhanced binding of this folate analogue were not pursued. More recently, it has been found that 5-deazafolate is also a strong inhibitor of human dihydrofolate reductase, and investigations were begun to characterize the interaction of the compound with both the free enzyme and the enzyme–NADPH complex. These studies assumed greater significance because of the report on the crystal structure of the binary complex formed between human dihydrofolate reductase and 5-deazafolate (Davies et al., 1990). The X-ray data indicated that the low dissociation constant for the complex is due to the fact that the N-8 of 5-deazafolate is protonated and hydrogen-bonded to a carbonyl group of the enzyme's backbone. Such an interaction does not occur in the binary enzyme–folate complex. It was also suggested by Davies et al. (1990) that this binary complex provides a model for the binding of dihydrofolate to the enzyme and resembles the transition-state complex for the reduction of folate.

The aim of the present investigation has been to characterize kinetically the binding of folate analogues, including 5-dea-

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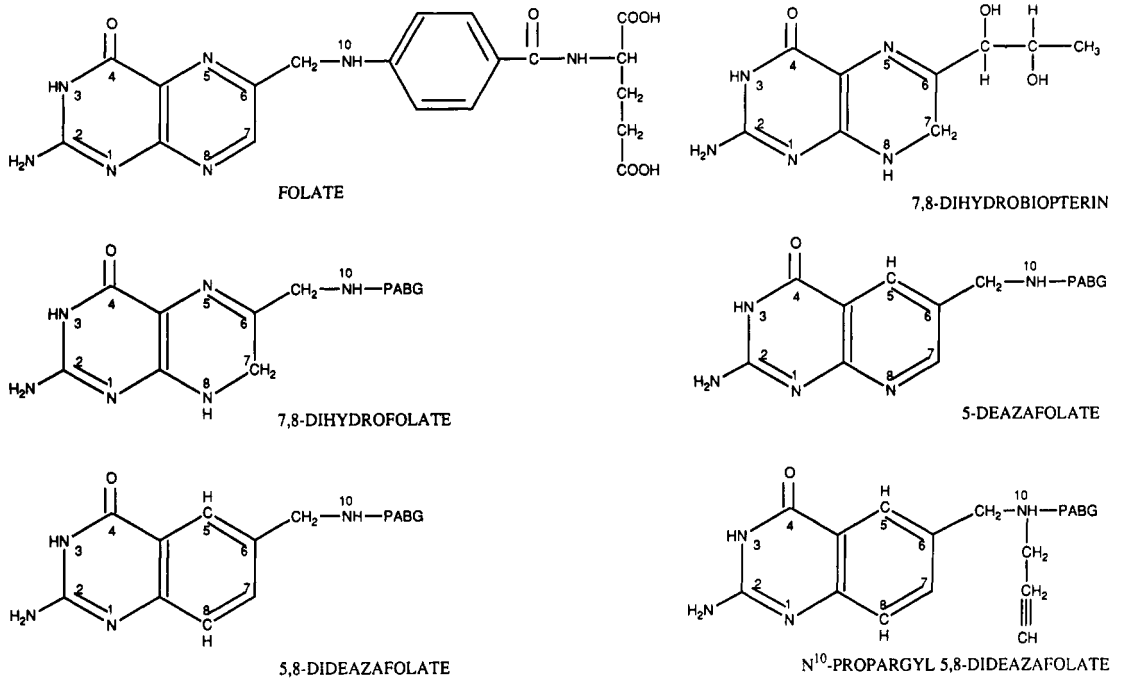


FIGURE 1: Structures of pterins that function as substrates or inhibitors of hDHFR.

zafolate and quinazolines, to both the free form of human dihydrofolate reductase and the enzyme–NADPH complex. As a prelude to this study, the effect of pH on the kinetic parameters of the reaction have been determined by using 7,8-dihydrobiopterin, folate, and 7,8-dihydrofolate (DHF)[1] as alternative substrates. The results indicate that the single ionizing residue at the pterin subsite of hDHFR is involved with catalysis and the binding of trimethoprim and pyrimethamine but not with the binding of 5-deazafolate. They also show that the binding of 5-deazafolate is pH-dependent and that the pK of its N-8 atom is raised about 6 pH units on binding to the hDHFR–NADPH complex.

EXPERIMENTAL PROCEDURES

Materials. Recombinant human dihydrofolate reductase (hDHFR), purified as described by Prendergast et al. (1988), was a gift from Drs. T. Delcamp and J. Freisheim of Ohio Medical College. The concentration of enzyme solutions was determined by fluorescence quenching titrations with the tight-binding inhibitor methotrexate (MTX) as described by Stone and Morrison (1982). Folic acid was supplied by Calbiochem. NADP and NADPH were supplied by Boehringer-Mannheim; [3′,5′,7,9-^{3}H]folic acid, potassium salt, was supplied by Amersham International and had a specific radioactivity of 23.4 Ci/mmol. Dihydrofolate (DHF) was prepared from both labeled and unlabeled folate as described by Blakley (1960). Trimethoprim (TMP), pyrimethamine, and methotrexate (MTX) were obtained from Sigma. Dihydrobiopterin (DHB) was purchased from Schircks Laboratories (Jona, Switzerland) and recrystallized according to the method described by Fukushima and Akino (1968). Solutions were prepared by dissolving the colorless needlelike crystals in 0.5 M NaOH and neutralizing with dilute HCl. 5-Deazafolate was a gift from Dr. R. D. Elliott of the Southern Research Institute (Alabama). 5,8-Dideazafolate was obtained from Dr. J. Hynes of the Medical University of South Carolina, while N^{10}-propargyl-5,8-dideazafolate was a gift from Dr. Ann Jackman of the Institute of Cancer Research (Surrey, U.K.) The structures of pterin substrates and inhibitors are illustrated in Figure 1. Concentrations of substrates and inhibitors were determined spectrophotometrically by using the following extinction coefficients: NADP (pH 7.0), 18 000 cm^{-1} at 259 nm; NADPH (pH 7.0), 6220 cm^{-1} at 340 nm; DHF (pH 7.0), 28 000 cm^{-1} at 282 nm; folate (pH 7.0), 27 000 cm^{-1} at 282 nm; folate (pH 7.0), 7000 cm^{-1} at 350 nm (Dawson et al., 1969); TMP (pH 13.0), 7250 cm^{-1} at 287 nm; pyrimethamine (pH 13.0), 8750 cm^{-1} at 285 nm (Roth & Burchall, 1965); 5-deazafolate (pH 7.0), 24 900 cm^{-1} at 278 nm (Temple et al., 1982); MTX (pH 13.0), 22 100 cm^{-1} at 302 nm (Seeger et al., 1949); and DHB (pH 1.0), 2830 cm^{-1} at 400 nm (Maharaj et al., 1990). The concentration of DHB solutions was also determined enzymatically using DHFR in the presence of an NADPH regenerating system consisting of glucose 6-phosphate and glucose-6-phosphate dehydrogenase from bakers' yeast. The molar extinction change for the reaction under these conditions was 4640 cm^{-1} (see below). The procedure described by Morrison and Stone (1988) was used to prepare (4R)-[4-^{2}H]NADPH (NADPD) that was fully deuterated on the A side.

Methods. (*a*) *Enzyme Assays.* Initial velocities of the reaction were determined spectrophotometrically at 340 nm and 30 °C. The cuvettes had a light path of 1 cm, and NADPH was held constant at 50 μM, which is a saturating concentration at all pH values. DHB was used as an alternative pterin substrate for most kinetic investigations as its Michaelis constant at all pH values falls within a range that is suitable for spectrophotometric assays. By contrast, the Michaelis constants for dihydrofolate and folate are less than 1 μM, which precludes direct determination of their kinetic parameters. For studies with these substrates, initial velocities were determined in the presence of TMP, which acts as a linear competitive inhibitor with respect to the pterin substrate. The concentration of TMP present at each pH was such as to bring the apparent Michaelis constant of the substrate into

[1] Abbreviations: hDHFR, recombinant human dihydrofolate reductase; clDHFR, dihydrofolate reductase from chicken liver; ecDHFR, dihydrofolate reductase from *E. coli*; DHF, dihydrofolate; DHB, dihydrobiopterin; MTX, methotrexate; TMP, trimethoprim.

the region of 10 μM. The true values for the kinetic parameters at any one pH were calculated by using the fixed concentration of, and appropriate K_i value for, TMP. The buffers contained 50 mM MES, 25 mM Tris, and either 25 mM sodium acetate or 25 mM ethanolamine, together with 100 mM NaCl. The ionic strength of the buffers remained essentially constant at 0.15 over the pH range used to determine initial velocities (Ellis & Morrison, 1982) For calculating reaction velocities, the molar absorbance change for the overall reaction was taken to be 10 860 cm^{-1} (cf. Results).

The initial velocity data at each pH value were fitted to eq 1 to obtain values for the maximum velocity (V) and the apparent first-order rate constant (V/K). Second-order rate

$$v = VA/(K + A) \quad (1)$$

constants were calculated from the realtionship $(V/K)/E_t$ by using the knwon concentration of total enzyme (E_t). V and V/K are expressed in units of s^{-1} and M^{-1} s^{-1}, respectively. Data for the variation of V and V/K_{DHB} as a function of pH were fitted to

$$y = C/(1 + (K/H)) \quad (2)$$

where y represents V or V/K, C represents the pH-independent value of V or V/K, and H denotes the hydrogen ion concentration.

(*b*) *Determination of pK Values for Folate Analogues.* Aliquots of solutions of pyrimethamine or 5-deazafolate were diluted into 1.0 mL of buffer at various pH values over the range from 1.0 to 13.0 to give final concentrations of 15.5 and 40 μM, respectively. The spectra were then recorded by using a Cary 2200 spectrophotometer interfaced with a Varian DS 15 computer. The same buffer mixtures, as used for enzyme assays, were used for the pH range from 4.0 to 10.0, while a glycine hydrochloride buffer, adjusted to $I = 0.15$ with NaCl, was used for pH 2.0 and 3.0. For pH 1.0 and 13.0, solutions of 0.1 M HCl and 0.1 M NaOH, respectively, were used. Data for the variation with pH, at a particular wavelength, of the absorbance of each folate analogue were fitted to eq 3 to yield pK values for the compounds.

$$y = \frac{Y_L + Y_H K/H}{1 + (K/H)} \quad (3)$$

(*c*) *Difference Spectroscopy of Enzyme–Ligand Complexes.* UV–visible difference spectra were measured from 450 to 250 nm, with a bandwidth of 1 nm and a scan rate of 1 nm s^{-1}, by using a Cary 2200 spectrophotometer linked to a Varian DS 15 computer. Solutions were placed in quartz split-cuvettes with two compartments, each of which had a light path of 0.5 cm. One compartment of each cuvette contained enzyme, in the presence or absence of a saturating concentration of NADPH, while the other compartment of each cuvette contained ligand. After setting the baseline, the contents of one cuvette were mixed and the difference spectrum was recorded. Spectra were obtained over the pH range from 6.0 to 10.0 by using buffers of the same composition as those described above. The concentration of enzyme–ligand or enzyme–NADPH–ligand complex, present, at each pH, was calculated by using the appropriate dissociation constant. Dissociation contants for enzyme–ligand complexes were determined by fluorescence quenching titration, while those for the release of ligand from the ternary enzyme–NADPH–ligand complexes were obtained from inhibition experiments (see below).

(*d*) *Determination of Dissociation Constants for Enzyme–Ligand Complexes.* (*1*) *Fluorescence Quenching Titration.* Ligand-free hDHFR was diluted to 3 mL with buffer to give a concentration that was not more that 3 times the K_i value of the enzyme–ligand complex. Serial additions of ligand were made, and the fluorescence was measured at 20 °C with excitation and emission wavelengths of 279 and 325 nm, respectively. Corrections were made for dilution and for any inner filter effect before analysis of the data as described by Stone and Morrison (1982). The instability of the free enzyme at lower pH values precluded determination of fluorescence below pH 4.5. Because of the low magnitude of the dissociation constant for the enzyme–5-deazafolate complex under acid conditions, it was not possible to determine such values below pH 6.0. Dissociation constants that varied with pH and gave rise to bell-shaped curves were fitted to

$$y = \frac{C}{1 + (K_1/H) + (H/K_2)} \quad (4)$$

(*2*) *Equilibrium Dialysis.* The dissociation constants for the binding of folate and DHF to the enzyme–NADP complex (K_i) were determined by using equilibrium dialysis with [3′,5′,7,9-^{3}H]folate and DHF. Such experiments were performed in inverted Eppendorf tubes as described by Reinard and Jacobsen (1989). Buffer (260 μL), containing a saturating concentration of NADP (100K_i at all pH values), was present in both the lower (cap) and upper compartments of the Eppendorf tubes. For the determination of the binding of DHF, TMP was also present in both compartments and was added to raise the apparent dissociation constant for this ligand. The concentrations of TMP ranged from 77 μM at pH 6.5 to 1.0 mM at pH 8.5. In addition, the upper compartment contained hDHFR, at a concentration that did not exceed 3 times the K_i value. Ligand concentrations ranged from 0.3 to 5.0 times the K_i value for the dissociation of ligand from the enzyme–NADP–ligand complex. Irrespective of pH, and thus the K_i values for folate and DHF binding, the same number of counts for labeled pterin was used in each tube. Equilibration across the dialysis membrane was allowed to proceed for 24 h, although preliminary experiments showed that equilibrium was achieved by 16 h. Under these conditions, there was no loss of enzyme activity. Samples were removed from each of the compartments, and the distribution of ligand between them was determined by counting in a Packard scintillation counter in 6 mL of Aquasol (Du Pont) scintillation cocktail. The concentrations of the enzyme–NADP complex, the enzyme–NADP–ligand complex, and free ligand were computed, and since it has been established that the stoichiometry of folate and DHF binding to hDHFR is one (Williams & Morrison, 1991), these data were fitted to

$$\frac{[\text{E–NADP}]}{[\text{E–NADP–folate}]} = \frac{K_d}{[\text{folate}]} \quad (5)$$

As the directly determined value for the dissociation constant of DHF is an apparent one (K_d'), the true value (K_d) was calculated by using the relationship $K_d = K_d'/(1 + [\text{TMP}]/K_{iTMP})$.

(*e*) *Kinetic Determination of Dissociation Constants.* Dissociation constants, at pH values above 7.5, for the interaction of 5-deazafolate (I) with the enzyme–NADPH complex were determined kinetically be measuring the initial velocity of the reaction as a function of the inhibitor concentration in the presence of saturating NADPH and a

fixed nonsaturating concentration of DHB.[2] The data were fitted to eq 6

$$v = V_{app}/(1 + (I/K_{iapp})) \quad (6)$$

$$K_i = K_{iapp}/(1 + (A/K)) \quad (7)$$

to obtain values for the apparent inhibition constant (K_{iapp}). True inhibition constants were calculated from the apparent values by using eq 7 together with the fixed concentration of DHB (A) and the Michaelis constant for DHB (K) at the particular pH. With pH values at or below pH 7.5, where tight-binding inhibition by 5-deazafolate is observed, one of two approaches was made to the determination of K_i values. The approach used at pH 7.5 involved the same procedure as described above except that the experiments were performed in the presence of a fixed concentration (44 μM) of TMP (J), which functions as a linear competitive inhibitor with respect to DHB. The introduction of TMP has the effect of raising the apparent K_i value for 5-deazafolate so that tight-binding conditions do not apply. The fitting of the resulting data to eq 6 now yields an apparent K_i value (K_{iapp}) that is dependent on both the A/K_a and the the J/K_j values at each particular pH. True K_i values were determined by using these values together with the relationship given in eq 8.

$$K_i = \frac{K_{iapp}}{1 + (A/K) + (J/K_j)} \quad (8)$$

$$v = \frac{k_{cat}A}{2(K_a + A)}\{[(K_{iapp} + I_t - E_t)^2 + 4K_{iapp}E_t]^{1/2} - (K_{iapp} + I_t - E_t)\} \quad (9)$$

K_i values for 5-deazafolate were also determined in the absence of TMP and the initial velocity data analyzed by fitting to the equation that describes tight-binding inhibition (eqs 7 and 9) according to the procedure of Sculley and Morrison (1986). Comparable K_i values for 5-deazafolate were obtained by the two procedures.

(f) Deuterium Isotope Effects. Initial velocity data were obtained by varying the concentration of each of the pterin substrates in the presence of a fixed, saturating concentration (50 μM) of NADPH or NADPD. When folate and DHF were the variable substrates, TMP was also present to raise their apparent Michaelis constants to levels suitable for steady-state kinetic studies (cf. Table II). The data were fitted to eq 10, where F_i is the fraction of deuterium label in the substrate and $E_{V/K}$ and E_V are the isotope effects minus one for the respective parameters.

$$v = \frac{VA}{K(1 + F_iE_{V/K}) + A(1 + F_iE_V)} \quad (10)$$

RESULTS

Molar Extinction Change for Reduction of DHB by hDHFR. Two methods were used to determine the molar extinction change due to the DHFR-mediated reduction of DHB. For the first, a known concentration of DHB, determined as described under Methods, was reduced enzymatically in the presence of an NADPH regenerating system consisting of glucose 6-phosphate and glucose-6-phosphate dehydrogenase. The observed change in absorbance at 340 nm was 4650 cm^{-1} M^{-1}. Thus, in the absence of the regenerating system, the overall molar extinction change due to the oxidation of NADPH and the reduction of DHB would be 10 870 cm^{-1} M^{-1}. The second method consisted of incubating a known concentration of NADPH, as determined enzymatically, with hDHFR in the presence of excess DHB and measuring the change in absorbance at 340 nm. The mean molar absorbance change from 12 determinations was 10 860 ± 380 cm^{-1} M^{-1}. This value is in very good agreement with that determined by the first procedure. However, it is somewhat lower than the 12 000 cm^{-1} M^{-1} calculated by Smith et al. (1987), which was based on molar extinction coefficients of 6400 at 330 for dihydropterins, 11 920 cm^{-1} M^{-1} reported by Webber and Whiteley (1985), and 12 300 cm^{-1} M^{-1} reported by Kaufman (1967), which was based on experiments utilizing a preparation of DHB with an estimated purity of 90–97%. The reason for the difference may well relate to the fact that when a limiting concentration of DHB is reduced enzymatically in the presence of excess NADPH, the absorbance at 340 nm does not reach a limiting plateau value. Instead, after an initial rapid decrease, the absorbance continues to decrease at a constant slow rate. Thus, it is necessary to extrapolate the linear portion of the curve back to zero time to obtain a true and lower value for the absorbance difference. It is of interest that similar cuves are obtained with folate and DHF. The slow linear rate of decrease in absorbance at 340 nm is directly proportional to the DHFR concentration, irrespective of the source of the enzyme, and is eliminated by the inclusion of an NADPH-recycling system. Thus, it may be that tetrahydrofolate is more susceptible to oxidation when bound to the enzyme than when free in solution. The slow formation of DHF would be observed as a slow decrease in absorbance and result in an apparent consumption of more than stoichiometric amounts of NADPH.

pH Dependence of Kinetic Parameters with DHB as Substrate. In the presence of a fixed saturating concentration of NADPH (50 μM), the initial velocity of the reaction varied hyperbolically as a function of the DHB concentration at all pH values. Analysis of the data yielded values for V and V/K_{DHB} which, when plotted as a function of pH, yielded the results of Figure 2A. These show that only a single ionizing residue is observed in each profile and that the values for V and V/K_{DHB} increase with decreasing pH. Analysis of the data of Figure 2 by fitting to eq 2 gave pH-independent values for V and V/K_{DHB} (Table I) as well as pK values of 7.02 ± 0.2 from the V profile and 6.20 ± 0.06 from the V/K_{DHB} profile. As DHB does not possess an ionizing group with a pK value in the region 6–8 (Maharaj et al., 1990) the latter pK value must be for an ionizing group on the enzyme.

pH Dependence of Kinetic Parameters with DHF and Folate as Substrates. Neither DHF nor folate is a suitable substrate for general kinetic studies on hDHFR because of the relatively low values of their Michaelis constants. But it was of interest to determine the kinetic characteristics of these substrates for comparison with those of DHB. The problems associated with the use of these low K_m substrates were circumvented by measuring initial velocities in the presence of TMP as described under Methods. The data illustrated in Figure 2B,C show that the pH profiles obtained with DHF

[2] Inhibition of the reaction by 5-deazafolate shows some slow-binding characteristics so that at neutral pH the K_i value determined by use of initial velocities is about twice that determined by use of steady-state velocity data. At lower pH values, 5-deazafolate behaves also as a tight-binding inhibitor [cf. Morrison and Walsh (1988)]. It is not practical to study the slow- or slow-tight-binding inhibition by 5-deazafolate of dihydrofolate reductase under acid conditions because of the chemical breakdown of NADPH. Thus, only initial velocity data were used for the determination of inhibition (dissociation) constants for 5-deazafolate.

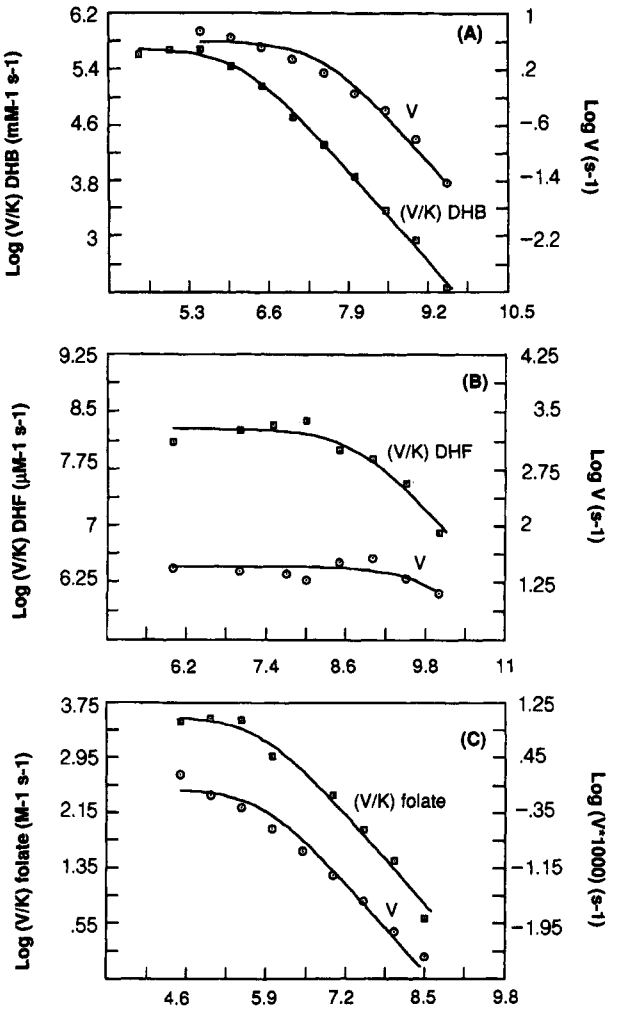


FIGURE 2: Variation with pH of log V and log V/K for alternative substrates of the reaction catalyzed by hDHFR in the presence of 50 μM NADPH. The substrates were (A) dihydrobiopterin (DHB), (B) dihydrofolate (DHF), and (C) folate. The curves represent the best fit of the data to eq 2 and were drawn by using the parameter values that are listed in Table I.

Table I: Rate Constants and pK Values for Interaction of Substrates with hDHFR

substrate	interaction rate[a] (μM^{-1} s^{-1})	max velocity (s^{-1})	pK value: V/K profile	pK value: V profile
folate	0.0030 ± 0.0005	0.0013 ± 0.0003	5.88 ± 0.12	5.73 ± 0.14 5.84 ± 0.13[a]
DHB	0.44 ± 0.01	9.0 ± 2.5	6.20 ± 0.06	7.02 ± 0.20
DHF	190 ± 28	28 ± 3	8.77 ± 0.13	9.94 ± 0.23[b] 9.72 ± 0.16

[a] Value determined from V/KE_t. [b] Determined with both NADPH and pterin substrate present at saturating concentrations of 50 μM.

and folate are qualitatively similar to those obtained with DHB and support the idea that the single ionizing group at the pterin subsite of hDHFR is involved with catalysis. Analysis of the data, by fitting to eq 2, gave the results listed in Table I. Comparison of the data of Table I indicates that DHF is the best substrate as judged by the maximum velocity of the reaction and by the rate at which it interacts with the enzyme–NADPH complex. Indeed, the magnitude of the pH-independent value for $(V/K)_{DHF}$ indicates that with DHF as a substrate the reaction is diffusion limited. While the maximum velocity of the reaction with DHB is reduced only to one-third of that with DHF, its rate of interaction with the

Table II: pH Dependence of Deuterium Isotope Effects with Folate, DHB, and DHF as Variable Substrates

substrate	pH	$^D(V/K)$	^DV
folate[a]	5.0	2.8 ± 0.1	2.8 ± 0.1
	7.8	3.7 ± 0.1	3.7 ± 0.1
DHB	5.0	3.2 ± 0.3	1.7 ± 0.3
	8.0	3.0 ± 0.1	3.0 ± 0.1
DHF[b]	7.2	1.0 ± 0.1	1.0 ± 0.1
	10.1	2.3 ± 0.1	1.4 ± 0.1

[a] Initial velocities were determined in the presence of a saturating concentration of NADPH (50 μM) and TMP at concentrations of 18 (pH 5.0) and 4 μM (pH 7.8). [b] Initial velocities were determined in the presence of a saturating concentration of NADPH (50 μM) and TMP at concentrations of 20 (pH 7.2) and 220 μM (pH 10.1).

enzyme–NADPH complex is 2–3 orders of magnitude lower. Folate is a very poor substrate with respect to both the maximum velocity of the reaction and its rate of interaction to form the ternary complex. The similarity of the pK values from the V and V/K profiles with folate suggests that the pK value of about 5.8 represents the intrinsic pK of the single ionizing residue at the active site of the enzyme. From the increase in the pK values of the V/K profiles, it may be concluded that the stickiness of the substrates decreases in the order DHF > DHB ≫ folate [cf. Cleland (1986)]. The increase in the pK values for the V profiles from 5.8 with folate through 7.0 with DHB to 9.9 with DHF probably reflects the transition from the reaction with folate for which catalysis is rate-limiting to reactions with DHB and DHF where product release becomes, at least partly, rate-limiting (Appleman et al., 1990).

Effect of pH on Deuterium Isotope Effects. The effect of pH on the values for ^DV and $^D(V/K)$, in the presence of a saturating concentration of 50 μM NADPH, was determined to gain information about the rate-limiting step(s) of the reaction with each of the alternative substrates. Folate could not be used as a substrate at pH values less than 5.0 because of enzyme instability, and hence it was not possible to study the reaction under conditions where the enzyme was completely protonated. At pH 5.0, product release is still partly rate-limiting since the values for ^DV and $^D(V/K)_{folate}$ are lower than their limiting values of 3.7 at pH 7.8 (Table II). From the equality of the values for ^DV and $^D(V/K)_{folate}$ it can be concluded that folate does not behave as a sticky substrate (Cleland, 1982). By contrast, at pH 5.0 DHB does exhibit substrate stickiness since the value for $^D(V/K)_{DHB}$ is greater than that for ^DV (Table II). Product release is also partly rate-limiting. Substrate stickiness is eliminated at pH 8.0, although the values for ^DV and $^D(V/K)_{DHB}$ reach a limiting value of only 3.0. This may be a consequence of not being able to use higher pH values, well over the edge of the pH profile, because of the marked increase with pH of the Michaelis constant for DHB. With DHF as substrate, no deuterium isotope effect is observed at neutral pH (Table II), and thus it follows that, under these conditions, catalysis is not rate-limiting. At the higher pH value of 10.1, the increase in the ^DV value from 1.0 to 1.4 indicates that catalysis becomes partly rate-limiting. As the value for $^D(V/K)_{DHF}$ is greater than that for ^DV, it follows that DHF behaves as a sticky substrate.

Determination of Intrinsic pK Value for Ionizing Residue at Active Site of Enzyme. Inhibitory substrate analogues were used to determine the intrinsic pK value of the single ionizing residue in the free enzyme and to confirm that the pK value for the same group in the enzyme–NADPH complex is 5.8 as indicated by the V and V/K_{folate} profiles (Figure 2).

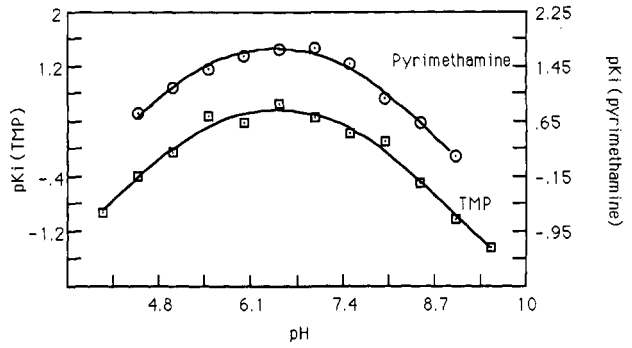


FIGURE 3: Variation with pH of pK_i values for the binding of pyrimethamine to free hDHFR and for the binding of trimethoprim (TMP) to the enzyme–NADPH complex. The theoretical curves were drawn by using values of the parameters determined by fitting the data to eq 4. The data with pyrimethamine were obtained by using fluorescence quenching titration, while those with TMP were obtained kinetically with NADPH at a fixed, saturating concentration of 50 μM. DHB was held constant at each pH at a concentration of about 4 times its Michaelis constant.

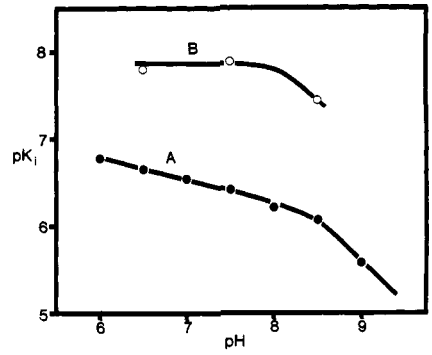


FIGURE 4: Effect of pH on the dissociation constants for the release of folate from the enzyme–folate complex at 20 °C (A) and the enzyme–NADP–folate complex at 4 °C (B). The data were obtained by using the same procedures as described in the legend to Figure 3.

Pyrimethamine was chosen as a suitable analogue for determining the pK value of the free enzyme by means of fluorescence quenching titration over the range from pH 4.5 to 9.5 (see Methods). A plot of pK_i for pyrimethamine[3] as a function of pH (Figure 3) yields a bell-shaped curve with slopes of +1 on the acid side and –1 on the alkaline side. The data fitted well to eq 4 and gave pK values of 5.56 ± 0.09 and 7.34 ± 0.08 for the two ionizing groups. Since the pK of the N-1 nitrogen of pyrimethamine is 7.34 (Roth & Strelitz, 1969), it follows that the value of 5.56 represents the pK value of Glu-30, which is the ionizing residue at the pterin subsite on the enzyme (Oefner et al., 1988). While the data of Figure 3 indicate that the interaction of the enzyme with pyrimethamine is pH-dependent, no conclusions can be reached about whether the complex that forms involves unprotonated enzyme and protonated ligand or protonated enzyme and unprotonated ligand (Stone & Morrison, 1983).

The thermodynamic pK value of the ionizing residue at the active site of the enzyme–NADPH complex was determined kinetically from inhibition studies with DHB as the substrate. The initial velocity of the reaction was measured, in the presence of a saturating concentration of NADPH (50 μM) and over a range of pH values, as a function of the concentration of TMP, which functions as a competitive inhibitor with respect to DHB. The results yielded a bell-shaped curve with slopes of +1 and –1 (Figure 3). The fitting of the data to eq 2 gave pK values of 5.54 ± 0.11 and 7.40 ± 0.10. The latter value can be attributed to the N-1 nitrogen of TMP, which has been reported to be 7.69 (London et al., 1986), while the value of 5.54 must represent the pK value for the glutamate residue at the active site of the enzyme. As the same pK value is observed for Glu-30 with both the free enzyme and the enzyme–NADPH complex, it follows that the presence of NADPH on the enzyme does not influence the behavior of Glu-30 and that the binding of NADPH is pH-independent. The data of Figure 3 also indicate that the interaction of TMP with the enzyme–NADPH complex is pH-dependent, but the same uncertainty exists concerning whether Glu-30 or the inhibitor is protonated in the ternary complex (Stone & Morrison, 1983).

Binding of Folate and DHF to hDHFR. As noted above, hDHFR is capable of utilizing folate as a substrate, although much less effectively than either DHF or DHB. It was, therefore, of interest to study the pH dependence of the interaction of folate with the free enzyme. Values for the dissociation constants of the binary enzyme–folate complex over the pH range from 6.0 to 9.0 were determined by means of fluorescence quenching titrations (see Methods). The resulting data yielded a plot (Figure 4A) which shows that the binding of folate to hDHFR increases with decreasing pH. However, the K_i value does not reach a limiting pH-independent value at low pH. Instead, the pK_i values vary as a linear function of pH up to pH 8.5 with a slope of only –0.3. This apparent low slope value may represent the start of a drawn-out curve that exhibits limiting K_i values at both low and high pH.

Attempts were made to determine the effect of pH on the binding of folate to the enzyme–NADPH complex. Since the maximum velocity of the reaction with folate as a substrate is some 7000-fold lower than with DHB (cf. Table I), the approach involved utilization of folate as a competitive inhibitor of the reaction with respect to DHB. Folate acted as an inhibitor, but at all pH values tested, the linear double-reciprocal plot in the absence of folate became concave-down, curvilinear in its presence. Similar results were obtained with DHF as the pterin substrate. Because of the complexity of the kinetic data, a thermodynamic procedure, with NADP replacing NADPH, was used to determine the effect of bound nucleotide on the binding of folate to hDHFR. NADP binds strongly to the free enzyme with a pH-independent dissociation constant that was determined to be 4 μM. It was present at a saturating concentration of 400 μM, and equilibrium dialysis experiments were performed at pH 6.5, 7.5, and 8.5. The data (Figure 4B) indicate qualitatively that NADP enhances the binding of folate to the enzyme. It is also apparent that the dissociation constant for the interaction of folate with the enzyme–NADP complex reaches a pH-independent value at acid pH and increases at higher pH. The pK values in the region pH 8–9, which are observed in the profiles of Figure 4, undoubtedly represent the pK value for the N3–O4 amide group of folate (Poe, 1977). The binding of DHF to the free enzyme, as determined by fluorescence titration (see Methods), was found to be pH-independent over the range from pH 6.0 to 9.0 and to have a dissociation constant of 0.15 μM (Table III). Equilibrium dialysis experiments at pH 6.5, 7.5, and 8.5 showed that the binding of DHF to the enzyme–NADP complex was also pH-independent. The pH-independent value for the dissociation constant of 0.0054 μM (Table III) indicates that NADP markedly enhances the binding of DHF to hDHFR.

Determination of pK Values for 5-Deazafolate. As a prelude to the study of the effects of pH on the interaction

[3] K_i is used to denote dissociation constants irrespective of whether they were determined kinetically or thermodynamically.

Table III: Dissociation Constants for pH-Independent Interaction of Substrates and Inhibitors with Different Forms of hDHFR

compound	enzyme form[a]	dissociation constant (μM)
5-deazafolate (singly protonated species)	enzyme	0.07
	enzyme–NADPH	0.26
DHF	enzyme	0.15
	enzyme–NADP	0.0054
5,8-dideazafolate	enzyme	0.015
	enzyme–NADPH	0.013
N^{10}-propargyldideazafolate	enzyme–NADPH	0.020

[a] Fixed concentrations of NADPH and NADP were 400 and 50 μM, respectively.

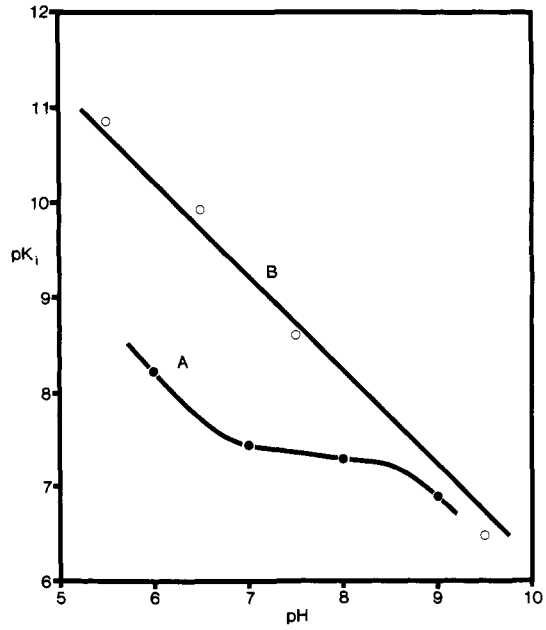


FIGURE 5: Variation with pH of the dissociation constants (K_i) for the interaction of 5-deazafolate with free hDHFR (A) and with the enzyme–NADPH–5-deazafolate complex (B). The dissociation constant for the binary complex was determined fluorometrically, while that for the release of 5-deazafolate from the ternary complex was determined kinetically. For the latter determinations, NADPH was held constant at a saturating concentration of 50 μM while DHB was present at a concentration of about 4 times its Michaelis constant at each pH value.

of 5-deazafolate with hDHFR, spectrophotometric determinations were made of the pK values of the compound (see Methods). Two well-separated ionizing groups were detected. One, with a pK value of 8.90 ± 0.07, was observed at 340 nm and is almost certainly for the N3–O4 amide group of 5-deazafolate. The other, with a pK value of 4.10 ± 0.03, was observed at 360 nm and could be expected to correspond to either the N-1 or the N-8 nitrogen of the compound. However, arguments have been advanced by Dewar et al. (1985) and by Davies et al. (1990) for the N-8 nitrogen being the group with a pK value of about 4.0. The aforementioned values were obtained by fitting separately the two well-spaced sets of data to eq 3. They may be compared with the respective values of 8.38 and 2.35 for the amide group and the N-1 nitrogen of folate (Poe, 1977).

pH Dependence of Binding of 5-Deazafolate to hDHFR. The pH profile for the binding of 5-deazafolate to the free form of hDHFR (Figure 5A) has features in common with that for the binding of folate. An ionizing group with a pK value in the vicinity of 8.5 must be protonated for binding. This value is similar to that of 8.9, as determined for the free ligand, and thus it appears that the protonated form of the amide moiety is important for binding. But binding is enhanced further by protonation of a second ionizing group whose pK value is too low to be determined directly from the data (Figure 5A). On the assumption that it is the protonated form of 5-deazafolate that undergoes interaction with the enzyme, and by taking into account the acid pK value of 4.1 for 5-deazafolate, it may be calculated from the K_i value determined at pH 6.0 that the pH-independent dissociation constant for the binary complex would be 84 pM.

pH Dependence of Binding of 5-Deazafolate to hDHFR–NADPH Complex. The pH profile for the binding of 5-deazafolate to the enzyme–NADPH complex appears to be simply a straight line with a slope of –1.0 (Figure 5B). It is apparent from the data that the binding cannot involve the glutamate residue at the active site of the enzyme, which has a pK value of 5.5, as the pK_i value does not begin to approach a plateau value in the vicinity of pH 5.5. Again it seems reasonable to conclude that protonation of the N-8 nitrogen of 5-deazafolate is of importance for binding. On this basis, and using the pK value of 4.10 for the ionizing group, the pH-independent dissociation constant for the release of 5-deazafolate from the ternary enzyme–NADPH–5-deazafolate complex would be 0.54 pM. A comparison of the K_i profiles for 5-deazafolate in the absence and presence of NADPH suggests that the alkaline limbs of both profiles would have slopes of –1 and that the presence of NADPH on the enzyme enhances the binding of the folate analogue by about 2 orders of magnitude.

Difference Spectra for Binding of 5-Deazafolate in Binary and Ternary Complexes. The application of difference spectroscopy studies, as described under Methods, has demonstrated that when 5-deazafolate is bound to hDHFR, it undergoes protonation–deprotonation reactions. The difference spectrum that is generated on the binding of 5-deazafolate to the free enzyme at pH 7.0 is shown in curve B of Figure 6, while curve A of Figure 6 illustrates the difference spectrum for the free pterin as obtained by subtracting the spectrum for 5-deazafolate at pH 7.0 from that at pH 1.0. The two spectra are similar, with positive bands at 360 and 275 nm as well as

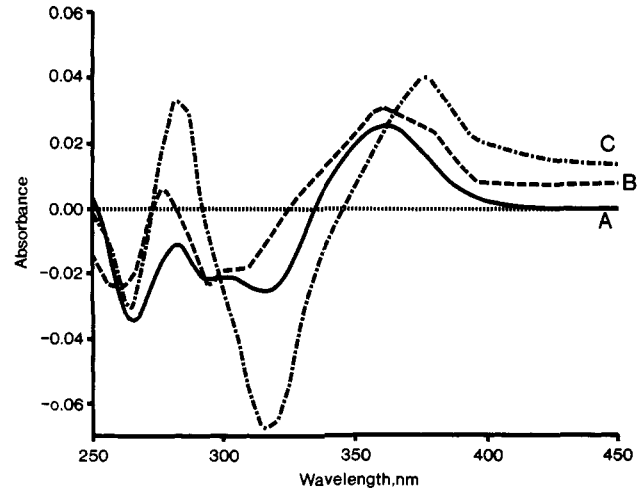


FIGURE 6: Difference spectra for free and bound 5-deazafolate. The difference spectra are for (A) 5-deazafolate (5 μM) at pH 1.0 and pH 7.0, (B) the enzyme–5-deazafolate complex (5 μM) and free 5-deazafolate (5 μM) at pH 7.0, and (C) the enzyme–NADPH–5-deazafolate complex (5 μM) and free 5-deazafolate (5 μM) at pH 7.0. Dissociation constants for the release of 5-deazafolate from the binary and ternary enzyme complexes were used to adjust the concentrations of free and bound ligand.

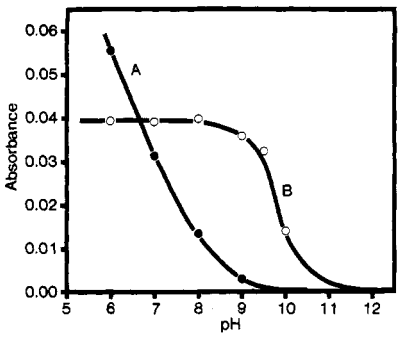


FIGURE 7: Variation with pH of the absorbance of (A) the binary hDHFR–5-deazafolate complex (5 μM) at 360 nm and (B) the ternary hDHFR–NADPH–5-deazafolate complex (5 μM) at 375 nm. The concentrations of the complexes were adjusted as described in the legend to Figure 6.

broad negative bands in the region of 310 nm. These results confirm that 5-deazafolate, with a pK value of 4.1 in free solution, is protonated at pH 7.0 when bound to the enzyme. Similar results were obtained with the ternary enzyme–NADPH–5-deazafolate complex (curve C). The data of Figure 6 also draw attention to the similarity of the difference spectra for the ternary complex (curve C) and the protonated ligand (curve A). The presence of NADPH on the enzyme amplifies both the positive and negative bands of the difference spectrum, particularly those in the UV region, and shifts the positive band in the visible region by about 15 nm.

To determine the pK value of the N-8 atom of 5-deazafolate when bound in binary and ternary complexes, the variation of the amplitudes of the positive bands at 360 and 375 nm, respectively, of the difference spectrum was measured as a function of pH. Prior to analysis of the data, the dissociation constants for the release of 5-deazafolate from the binary and ternary complexes were used to determine the concentration of these complexes (see above). Adjustments were then made to the amplitude values so that the concentration of the complexes at any pH was 5 μM. The data of Figure 7 confirm that, in the ternary complex, the bound 5-deazafolate undergoes a protonation–deprotonation reaction. Analysis of the data of Figure 7 (curve B) showed that the pK of the ligand rises from 4.1 in free solution to 9.8 $\pm$ 0.1 in the ternary complex. It was not possible to obtain from the data of Figure 7 (curve A) a well-determined pK value for the ligand when bound in a binary complex. However, from the results of Figures 5 and 7, it can be expected that the value would be in the vicinity of pH 7. It should be noted that it is the tightness of binding of 5-deazafolate to hDHFR that allows determination of the difference spectrum for the enzyme–5-deazafolate complex at higher pH values.

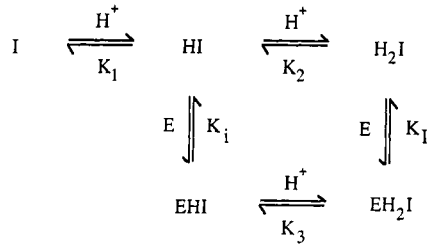
Binding of Quinazolines to hDHFR. Determinations were made of the effect on binding of substituting the N-5 and N-8 atoms of folate by methenyl groups (Figure 1). The dissociation constants for the interaction of 5,8-dideazafolate and its propargyl derivative with hDHFR were found to be pH-independent and unaffected by the presence of NADPH. The values (Table III) are about an order of magnitude lower than the dissociation constant for the binding of DHF to the free enzyme but comparable to that for the interaction of DHF with the enzyme–NADP complex. No evidence was obtained for the protonation of 5,8-dideazafolate when bound to the enzyme in binary or ternary complexes.

DISCUSSION

The present detailed investigation of the interaction of 5-deazafolate with human dihydrofolate reductase (hDHFR) arose from the results of preliminary studies which demonstrated the similarities between the inhibitory behavior of this pterin and the pteridine, methotrexate. Neither compound is a substrate for the enzyme, but both act as potent inhibitors of hDHFR and give rise to slow-binding inhibition. In addition, the inhibitory effects are pH-dependent and enhanced by the presence of NADPH on the enzyme. Thus, it was considered that 5-deazafolate would be bound in an inverted manner relative to the binding of DHF (Bolin et al., 1982). During the course of the present work, the paper by Davies et al. (1990) made it clear that the expectations were wrong and that the strong binding of 5-deazafolate compared with DHF was consistent with the protonation and hydrogen bonding of N-8 of 5-deazafolate to a carbonyl group along the backbone of the enzyme. Nevertheless, it was of importance to characterize, in a quantitative manner, the interaction of 5-deazafolate with hDHFR by the application of a wide range of kinetic and thermodynamic techniques. The results of these investigations prompted additional studies on the interaction of quinazolines with the enzyme.

Kinetic Characteristics of hDHFR Reaction. The successful pursuit of a large part of the present investigations was dependent on the identification of a substrate that is generally suitable for steady-state kinetic studies. The natural substrate, DHF, is difficult to use for these purposes because of its low K_m value. While the reaction has been demonstrated to occur via a random mechanism (Appleman et al., 1990), no steady-state initial velocity patterns have been reported. Dihydrobiopterin (DHB) was found to be a very convenient substrate with K_m values that vary from 80 through 9 to 160 μM over the pH range from 4 through 5.5 to 9.5. Thus, it was possible to obtain directly, for the first time, both V and V/K profiles with hDHFR (Figure 2). The data obtained with DHB indicate that a single ionizing group at the active site of the enzyme must be protonated to obtain the maximum rate of the overall reaction and for the interaction of DHB with the enzyme–NADPH complex. The similarity of the two profiles suggests that the same ionizing residue is being observed in each profile and that this residue is involved with catalysis. As Glu-30 is the only ionizing residue at the pterin subsite of hDHFR (Oefner et al., 1989), it follows that the protonated form of Glu-30 is the source of the proton for reduction of folate and dihydropterins. These conclusions, which are confirmed by the results obtained with DHF and folate (Figure 1), draw attention to the fact that all three substrates of hDHFR must combine with both the protonated and unprotonated forms of the enzyme–NADPH complex. If this were not so, the maximum velocities of the reactions would be pH-independent [cf. Morrison and Stone (1988)]. The pK value of the ionizing group at the active site of the enzyme, as obtained by using folate as the substrate, is similar to that obtained thermodynamically (Table I). These results suggest strongly that the value of 5.6 represents the intrinsic pK of the Glu-30 residue. This value may be compared with those of 6.5 for the Asp-27 residue of the enzyme from *E. coli* (Fierke et al. 1987; Morrison & Stone, 1988; Morrison, 1991) and 5.3 for the Glu-30 residue of the enzyme from chicken liver (Morrison & Stone, 1986). The pH data (Table I) also show clearly that DHF is a good substrate for the enzyme because it is a sticky substrate that can raise artificially the pK of Glu-30 from an intrinsic value of 5.6 to an observed value of about 8.8. By contrast, DHB exhibits only slight stickiness and is a relatively poor substrate. While catalysis is essentially rate-limiting with folate as a substrate, product release limits the rate of reaction with DHF up to pH 10 and with DHB at pH

Scheme I



5. The sickiness of DHB and DHF is confirmed by the data for deuterium isotope effects (Table II).

Binding of 5-Deazafolate to hDHFR. DHFRs from human and bacterial sources exhibit similarities in that neither utilizes 5-deazafolate a substrate but rather both are inhibited strongly by this pterin [cf. Stone and Morrison (1983)]. Indeed, 5-deazafolate is the pterin that is most strongly bound to any DHFR. The binding of 5-deazafolate to hDHFR is enhanced in the presence of NADPH and is pH-dependent for the formation of both the binary and the ternary complexes. It appears that Glu-30 at the active site of the enzyme is not involved with ternary complex formation since there is no evidence to suggest that the binding is beginning to reach a plateau value in the region of pH 5.6 which is the intrinsic pK value of Glu-30 (Figure 5). Such a finding contrasts with the results which indicate that the binding of pyrimethamine and TMP involves protonation of Glu-30 and/or these folate analogues (Figure 3). The difference spectra for the binding of 5-deazafolate in both the binary and ternary complexes are consistent with the proposal that this folate analogue is protonated in the bound state. Quantitative analysis of the difference spectrum data, at a fixed wavelength, as a function of pH reveals that the pK value for N-8 of the molecule is raised about 4 pH units on binding to the free enzyme and about 6 pH units on binding to the enzyme–NADPH complex. Such a shift is consistent with the formation of a hydrogen bond (Davies et al., 1990).

The variation with pH of the binding of 5-deazafolate (I) to hDHFR, as illustrated in Figures 5 and 7, can be accounted for by Scheme I. In this scheme, E represents either free enzyme or the enzyme–NADPH complex, K_I denotes the dissociation constant for the N3–O4 amide group of 5-deazafolate, and K_2 and K_3 represent the respective dissociation constants for the protonation of the N-8 atom of 5-deazafolate in solution and bound to the enzyme. It is assumed that there is neglibible formation of an EI complex. The apparent binding constant (K_I') for the formation of the EHI and EH_2I complexes is described by eq 11. This equation predicts that

$$K_I' = K_I \frac{\left[1 + \frac{K_2}{H}\left(1 + \frac{K_1}{H}\right)\right]}{\left(1 + \frac{K_3}{H}\right)} \qquad (11)$$

with the determined values for the binary EHI complex of K_I = 84 pM, pK_1 = 8.9, and pK_2 = 7.0, a plot of pK_I' against pH would yield a curve of the shape illustrated in Figure 8. These pK values are sufficiently well-separated for the distinct regions of the curve to be observed. The data of Figure 5A for binary complex formation are generally in accord with the predictions except for the fact that it is not possible to observe the plateau region at low pH because of enzyme instability. When 5-deazafolate interacts with the enzyme–NADPH complex, the value of K_I decreases to 0.54 pM while that for pK_3 increases to 9.8 and the predicted curve no longer has distinct regions (Figure 8). The shape of the curve is such

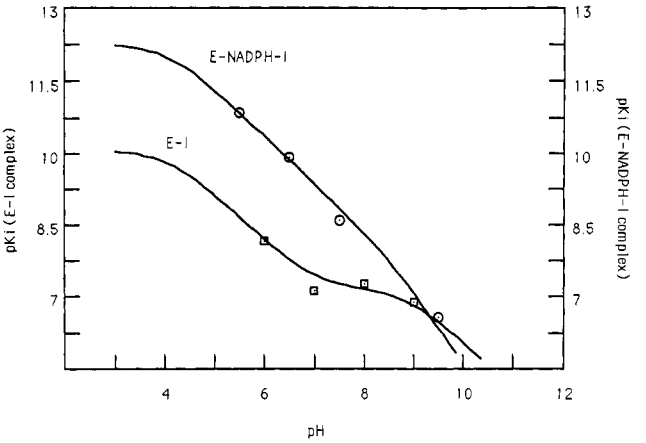


FIGURE 8: Theoretical K_i profiles for the binding of singly and doubly ionized 5-deazafolate (I) to hDHFR in the absence and presence of NADPH. The curves were constructed by using eq 11 together with values of K_I = 84 pM, pK_1 = 8.9, pK_2 = 4.1, and pK_3 = 7.0 (E–I complex) and K_I = 0.54 pM, pK_1 = 8.9, pK_2 = 4.1, and pK_3 = 9.8 (E–NADPH–I complex). The experimental points have been taken from Figure 5.

that experimental data obtained at pH values greater than 5 could appear to yield a straight line with a slope of −1 as observed in Figure 5B. However, the data fit well to the theoretical curve.

It follows from Scheme I that the dissociation constant for the interaction of HI with the free enzyme and with the enzyme–NADPH complex (K_i) can be calculated from the relationship $K_i = K_2K_I/K_3$. Such calculations indicate that the presence of NADPH on the enzyme has little influence on the binding of HI as the values for the release of HI from the E–HI and E–NADPH–HI complexes are about 0.1 and 0.3 μM, respectively (Table III). These values are very much higher than the respective values of 84 and 0.54 pM for the release of H_2I from the E–H_2I and E–NADPH–H_2I complexes but are comparable to the dissociation constants for the interaction of DHF and folate with free hDHFR and to the Michaelis constant for DHF (Table II; Williams & Morrison, 1991). The substrates are not protonated when bound to the enzyme.

Structural Aspects of Binding of 5-Deazafolate to hDHFR. Recent structural studies on hDHFR by Davies et al. (1990) have shown that the binding of 5-deazafolate to the enzyme appears to involve protonation of N-8 which forms a hydrogen bond with the carbonyl backbone of Ile-7. By contrast, the binding of folate does not result in any analogous protonation and hydrogen-bond formation. The conclusion that protonation is of importance for the fomation of the binary enzyme–5-deazafolate is in accord with the present findings that the interaction is pH-dependent (Figure 5) and that the pK for the N-8 atom is considerably elevated on complex formation. Although the structure of the enzyme–folate complex does not indicate any involvement of hydrogen bonding in the interaction (Davies et al., 1990), the present thermodynamic data do show that binding increases with decreasing pH for both the enzyme–folate and enzyme–NADP–folate complexes (Figure 4).

Davies et al. (1990) have also suggested that the bound 5-deazafolate resembles the transition state for folate reduction to DHF. But 5-deazafolate is not a substrate. In addition, the reduction of folate involves Glu-30 and not Ile-7, while the binding of 5-deazafolate involves Ile-7 and not Glu-30. Indeed, it seems likely that it is the very formation of a hydrogen bond between N-8 of 5-deazafolate and the carbonyl of Ile-7

that is responsible for 5-deazafolate not being a substrate. The occurrence of such an interaction is reminiscent of the behavior of antifolates, such as methotrexate, which bind in a different manner to the substrates simply because of fortuitous interactions. Even though, in a hypothetical model, there is an overlap of the nicotinamide ring of NADPH and the pterin ring of 5-deazafolate with the reactive atoms at the optimum distance for hydride transfer, the aforementioned hydrogen bond would cause delocalization of any positive charge on N-8. This, in turn, would reduce the formation of a partial positive charge on the C-7 of the pterin ring, which can be expected to be of importance for the conversion of folate to DHF. But it is the involvement of the protonated form of Glu-30 in the reduction of folate that argues most strongly against the idea that bound 5-deazafolate resembles the transition state for this reaction.

Interactions of Folate with hDHFR. The binding of DHF to both the free enzyme and the enzyme–NADP complex is pH-independent as determined by thermodynamic procedures (Table III). Thus, it might have been expected that the binding of the alternative substrate, folate, would also be pH-independent. This is especially so as the pH profiles obtained with folate as a substrate show that the observed ionizing group is concerned with catalysis (Figure 1). However, this is not the case as the binding of folate to both the free enzyme and the enzyme–NADP complex is pH-dependent. The question must arise as to whether the same complexes are being observed in the kinetic and thermodynamic experiments. If folate were capable of forming both productive and nonproductive complexes and if the latter were more stable, then the major nonproductive complex would be observed in thermodynamic experiments while the minor productive complex would be observed in kinetic experiments. Such a hypothesis would account for the very low pH-independent maximum velocity obtained with folate, relative to that obtained with DHF. If folate were involved with unproductive complex formation, the maximum velocity of the hDHFR reaction with folate would be reduced by the factor $1 + (K_a/K_i)$, where K_a denotes the Michaelis constant for the substrate and K_i represents the dissociation constant for the formation of the nonproductive enzyme–substrate complex. The formation of an unproductive enzyme–NADPH–folate complex would also have the effect of lowering the apparent K_m for folate by the same factor. The pH-independent Michaelis constant for folate of 0.43 μM, as calculated by dividing V by V/K_{folate} (Table I), can be compared with a value of 0.15 μM for DHF. The small difference may argue against the aforementioned proposal, but the Michaelis constant for folate in the productive complex cannot be known.

Structure of Pterin Subsite of hDHFR and Binding of Deazafolates. Apart from the importance of hydrogen bonding for the strong interaction between 5-deazafolate and hDHFR, Davies et al. (1990) have pointed out that the replacement of N-5 of folate by a methenyl group would result in a more favorable nonpolar–nonpolar interaction between C-5 of 5-deazafolate and Leu-22. In this connection, it is of interest to compare the calculated dissociation constant of 0.26 μM for the binding of the unprotonated form of 5-deazafolate to the free form of enzyme with those in the region of 0.015 μM as determined directly for the binding of 5,8-dideazafolate and its N^{10}-propargyl derivative to the same form of hDHFR (Table III). As expected, on the basis of the above argument, the binding of the dideazafolates is an order of magnitude stronger than the unprotonated form of 5-deazafolate. That is, Leu-22 and Ile-7 at the active site of hDHFR prefer to associate with the carbon atoms of the dideazafolates rather than with pterins having a carbon atom at position 5 and an unprotonated nitrogen at position 8. Also as expected, the dideazafolates bind in a pH-independent manner. In connection with the structure of the pterin subsite of hDHFR, Davies et al. (1990) noted that the protonation of N-5 of DHF, as proposed as part of the catalytic mechanism for the reduction of DHF [cf. Morrison (1991)], would result in the formation of an intermediate whose interaction with Leu-22 would be very unfavorable. Nevertheless, the formation of a naked, protonated N-5 atom would facilitate hydride transfer to C-6 for the formation of tetrahydrofolate.

The results of the present studies are also of general significance because they draw attention to the difficulties of reaching conclusions about the mechanism of enzyme action simply on the basis of the structures of the enzyme and its complexes. In the absence of information derived from detailed kinetic investigations of a reaction and/or from the application of site-directed mutagenesis, it is not possible to deduce the role that is played in the catalytic mechanism by amino acid residues at the active site of the enzyme. In addition, conclusions reached from structural data must also take into account those kinetic effects that determine why substrates are good or poor.

ACKNOWLEDGMENT

We thank Dr. W. W. Cleland for his very helpful comments on the manuscript as well as Mrs. Margaret Sneddon and Ms. Beth Schneider for skilled technical assistance.

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