pH Dependence of Enzyme Reaction Rates and Deuterium Isotope Effects on the Reduction of a New Mechanism-Based Substrate by Dihydrofolate Reductase (DHFR)†

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ABSTRACT: The enzyme kinetics of the reduction of the substrate 6,8-dimethylpterin by chicken and recombinant human dihydrofolate reductases (DHFRs) have been examined over the pH range 5.0−8.0 in the presence of NADPH or (4*R*)-[^{2}H]NADPH (NADPD). The pH profiles of the catalytic constant ($V_{max}/[E]_o$ or k_{cat}) showed pH independence for chicken DHFR and little pH dependence for human DHFR. For both DHFRs, the pH profiles of the Michaelis constant ($K_{m(substrate)}$) and the apparent second-order rate constant ($V_{max}/K_{m(substrate)}[E]_o$ or $k_{cat}/K_{m(substrate)}$) indicated that two ionizable groups, deduced to be the substrate and the conserved Glu carboxy group in the enzyme active site, should be ionized in their cationic and anionic forms, respectively, for formation of the enzyme−substrate complex and for catalysis. The pK_a values of about 5.3 and 6.5 which were obtained from the pH profiles of $K_{m(substrate)}$ and $k_{cat}/K_{m(substrate)}$ were assigned to the ionizations of the substrate and the enzyme carboxy group, respectively. Deuterium isotope effects on ^DV and $^D(V/K)$ were significant for both enzymes, ~3 for chicken DHFR and ~4 for recombinant human DHFR, and were pH independent. Thus, the rate-limiting step in the enzymic reduction of 6,8-dimethylpterin is hydride-ion transfer at acidic pHs as well as neutral pHs. The results demonstrate that, compared with dihydrofolate, 6,8-dimethylpterin is a superior substrate for mechanistic investigations as it allows direct study of the effects of both enzyme and substrate ionizations involved in the catalysis and also avoids the obscuration of the catalytic rate by product release.

Dihydrofolate reductase (DHFR,[1] EC 1.5.1.3) catalyzes the NADPH-dependent reduction of dihydrofolate (H_2folate) to tetrahydrofolate (H_4folate). At acidic pHs DHFR also catalyzes the reduction of folate to H_4folate using two equivalents of NADPH. As H_4folate derivatives transfer one-carbon units in a wide variety of metabolic reactions including the production of thymidylate, DHFR is essential for the maintenance of the H_4folate pool in the cell. This role has made DHFR a target for development of inhibitors as cytotoxic drugs, and such "antifolates" include the anticancer drug methotrexate and the antibacterial drug trimethoprim (Sirotnak et al., 1984).

Since X-ray crystallographic studies revealed (Bolin et al., 1982) that the DHFR active site contains an ionizable carboxy group, Asp or Glu, the role of the carboxy group in the enzyme catalysis has been a major theme in research on DHFR kinetics. Studies of the pH dependence of complex formation of DHFR with inhibitors indicated a pK_a value for the enzyme carboxy group of ~6.5 (Dunn & King, 1980; Stone & Morrison, 1983; Fierke et al., 1987; Thillet et al., 1990). Previous studies of kinetic and deuterium isotope effects on H_2folate reduction to H_4folate by DHFR have concluded that while the correct ionization state of the carboxy group at physiological pH is required for catalysis, at low to neutral pH product release is the rate-limiting step and only at higher pHs does the hydride-ion transfer step become the rate-limiting step (Beard et al., 1989; Morrison, 1991). This latter fact is reflected in the kinetics as an effective pK_a displacement to ~8 from 6.5 (Fierke et al., 1987; Thillet et al., 1990). However, based on Raman spectroscopy experiments of the ternary complex of DHFR−$NADP^+$−H_2folate, a recent report has criticized the accepted assignment of the pK_a value of ~6.5 to the enzyme carboxy group and argued that it be reassigned to the pK_a for protonation of N^5 of H_2folate (Chen et al., 1994).

Our interests in designing new DHFR inhibitors with different mechanisms of action and pharmacological properties have lead to two new classes of compounds, the 8-alkylpterins and 8-alkyl-N^5-deazapterins (Gready, 1990; Gready et al., 1993), with structures and properties based on the presumed enzymically active form of folate, i.e., protonated on N^8 (Huennekens & Scrimgeour, 1964; Gready, 1985). Preparation, structural studies, and enzymic properties of analogues of these compound classes have been reported (Ivery & Gready, 1992, 1994, 1995; Jeong & Gready, 1992). A significant property of these compounds is their higher basic pK_a, being ~5.5 for the 8-alkylpterin substrates of interest here, compared with a value of ~2.3 for pterin, so that a considerable proportion of cationic form exists at physiological pH.

We have previously reported on the substrate activity and enzymic mechanism for a series of 8-alkylpterins (8-R-Pts)

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[1] Abbreviations: DHFR, dihydrofolate reductase; cl, chicken liver; rh, recombinant human; 8-R-Pt, 8-alkyl-substituted pterin; 6,8-diMe-Pt, 6,8-dimethylpterin; 6,8-diMe-H_2Pt, 6,8-dimethyl-7,8-dihydropterin; 6,8-diMe-H_4Pt, 6,8-dimethyl-5,6,7,8-tetrahydropterin; H_2folate, 7,8-dihydrofolate; H_4folate, 5,6,7,8-tetrahydrofolate; H_2biopterin, 7,8-dihydrobiopterin; NADPD, (4*R*)-[^{2}H]NADPH; ^DV, k_{cat} using NADPH/k_{cat} using NADPD; $^D(V/K)$, $k_{cat}/K_{m(substrate)}$ using NADPH/$k_{cat}/K_{m(substrate)}$ using NADPD.

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with chicken and human DHFRs (Thibault et al., 1989; Gready, 1990; Ivery & Gready, 1993; Ivery & Gready, 1995). These studies indicated that a number of the compounds are good substrates of the enzymes at neutral pH as well as acidic pH, in contrast to the activity of pterins and folate which are at best poor substrates of DHFRs at neutral pH (Blakley, 1984; Kraut & Matthews, 1987), with 6,8-dimethylpterin (6,8-diMe-Pt) being the most efficient substrate on the basis of V_{max} and K_m values (Gready, 1990; Ivery & Gready, 1995). Using UV/vis spectroscopy with recycling of product $NADP^+$, the initial enzymic product was observed to be the 7,8-dihydro form, and this was subsequently reduced further to the 5,6,7,8-tetrahydro form (Thibault et al., 1989; Gready, 1990). These observations have subsequently been confirmed using direct HPLC analysis of enzymic reaction assays (Zhang, 1994). Another feature of the enzymic reduction of the 8-alkylpterins is the dependence on pH of the kinetic parameters with little pH dependence being observed for V_{max} and a bell-shaped pH dependence for $K_{m(substrate)}$ values (Gready, 1990). Also, $K_{m(substrate)}$ values for chicken DHFR were 2−4 times lower than the values for human DHFR (Gready, 1990). From the pH behavior of the $K_{m(substrate)}$ values, the pK_a value of the conserved carboxy group in the enzyme active site was estimated to be about 6.6 (Cummins & Gready, 1993).

As these new mechanism-based compounds were designed to mimic the enzymically active protonated form and bind to the enzyme as the cation, predictions which have now been confirmed directly for the 8-alkyl-N^5-deazpterin inhibitors (Jeong & Gready, 1994a), it is expected that protonation will not be rate-limiting in the enzymic reaction of the 8-alkylpterins. Given the results from pH dependence and deuterium isotope effects studies for H_2folate described above, it was of interest to investigate the rate-limiting step in the enzymic reduction of the 8-alkylpterins. A study of the pH dependence and deuterium isotope effects on the kinetic parameters for reduction of 6,8-dimethylpterin by chicken and human DHFRs is reported.

MATERIALS AND METHODS

Materials. (4*R*)-[^{2}H]NADPH (NADPD) was prepared and purified by an improved procedure recently reported (Jeong & Gready, 1994b). Chicken liver DHFR (clDHFR) was purchased from Sigma and contained some bound cofactor. Cofactor-free recombinant human DHFR (rhDHFR) was a gift from Prof. J. Freisheim. The active concentration of DHFRs was determined to be 41 μM for clDHFR and 30.5 μM for rhDHFR by fluorescence titration with methotrexate (Perkins & Bertino, 1966). NADPH was purchased from Boehringer Mannheim. The concentrations of NADPH and NADPD in water were determined spectrophotometrically using an extinction coefficient of 6200 M^{-1} cm^{-1} at 340 nm (Horecker & Kornberg, 1948; Dawson et al., 1986). The sample of 6,8-dimethylpterin (6,8-diMe-Pt) was purified from an isomeric mixture using a reported method (Ivery & Gready, 1992; Jeong & Gready, 1992) or was a gift from Mr. M. Ivery. The concentration of 6,8-diMe-Pt at pH 2 was determined spectrophotometrically using extinction coefficients of 13 000 and 10 200 M^{-1} cm^{-1} at 262 and 398 nm, respectively (Ivery & Gready, 1995).

Methods. Enzyme assays were carried out at 30 °C in a multicomponent (TMES) buffer containing 25 mM Tris, 50 mM MES, 25 mM ethanolamine, and 100 mM NaCl which maintains a constant ionic strength of $I = 0.15$ in the reaction mixture over the pH range 5−8 (Ellis & Morrison, 1982). The enzyme reaction was started by adding cofactor (6 μL of NADPH or 60 μL of NADPD) and enzyme to preincubated 6,8-diMe-Pt in buffer in a 1 mL plastic cuvette. clDHFR was diluted 1/10 (NADPH assay) or 1/5 (NADPD assay) into pH 6.6 TMES buffer, and 1−4 μL was added to assays. rhDHFR was diluted 1/20 into 0.1 M potassium phosphate buffer (pH 7.5) containing 25 μM NADPH or NADPD, and 2 or 3 μL (NADPH assay) or 4 or 6 μL (NADPD assay) was added to assays. The final concentration of NADPH or NADPD was a saturating concentration of 60 μM (Thibault et al., 1989). Assay 6,8-diMe-Pt concentration ranges used for clDHFR were pH 5.0 and 6.6, 4−20 μM; pH 5.8, 2.5−10 μM; pH 7.4, 10−50 μM; pH 8.0, 40−150 μM, and for rhDHFR were pH 5.0 and 6.6, 10−66 μM; pH 5.8, 5−50 μM; pH 7.4, 33−133 μM; pH 8.0, 80−300 μM. To account for nonenzymic reaction and NADPH decay, assays below pH 7.4 were done with a blank containing all components except DHFR.

The initial rate v of the reduction of 6,8-diMe-Pt was measured using a SHIMADZU UV-160 spectrophotometer. The substrate 6,8-diMe-Pt has a major absorption band at ~400 nm, in which region neither DHFR, NADPH, nor the product 6,8-diMe-H_2Pt absorb. Monitoring of the absorbance change at over 400 nm thus provides a simple means for obtaining the initial rate of disappearance of substrate only, rather than following changes at 340 nm as usual for DHFR assays (Beard et al., 1989; Thibault et al., 1989), and also allows the reaction extinction coefficient to be determined readily. In the present pH studies, the wavelength 407 nm was chosen as it is an isosbestic point for the cation and neutral forms with extinction coefficient 9390 M^{-1} cm^{-1}. As both enzymes were effectively preincubated with cofactor because of small amounts of cofactor present in the chicken DHFR preparation or added to stabilize the diluted human DHFR, no hysteresis in the kinetics from DHFR conformational changes (Appleman et al., 1990) was observed.

Theory and Data Analysis. To investigate the involvement of ionizable groups and their correct ionization states in the formation of enzyme−substrate complex and/or enzymic catalysis, the pH dependence of the kinetic parameters and isotope effects is generally studied (Cleland, 1982). Initial velocity data obtained at each pH by varying the concentration of 6,8-diMe-Pt were fitted to the Michaelis−Menten equation (eq 1) to determine the maximum velocity, V_{max},

$$v = \frac{V_{max}[S]}{K_m + [S]} \quad (1)$$

and the Michaelis constant, K_m, at each pH.

To examine the pH dependence of the parameters, the apparent values of $V_{max}/[E]_o$ (or k_{cat}), K_m and k_{cat}/K_m obtained were fitted to eqs 2−4. These equations were derived (see Appendix) from a suggested scheme (see Scheme 1) for 6,8-diMe-Pt reduction by DHFR (Cummins & Gready, 1993), where $[E]_o$ is the total concentration of enzyme, C_1 and C_2 are pH-independent values of apparent K_m and k_{cat}/K_m, respectively, and K_E and K_S are the acid dissociation constants of enzyme and substrate, respectively.

Data were fitted to the equations by nonlinear regression using the PC program GraFit (Leatherbarrow, 1992). Deu-

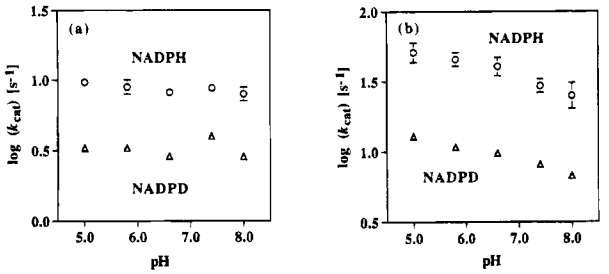


FIGURE 1: Log (k_{cat}) versus pH for 6,8-diMe-Pt reduction by (a) clDHFR with NADPH and NADPD and by (b) rhDHFR with NADPH and NADPD. Standard error bars are shown.

$$\frac{V_{max}^{app}}{[E]_o} = k_{cat}^{app} \text{ (pH independent)} \quad (2)$$

$$K_m^{app} = C_1\left(1 + \frac{[H^+]}{K_E}\right)\left(1 + \frac{K_S}{[H^+]}\right) \quad (3)$$

$$\left(\frac{k_{cat}}{K_m}\right)^{app} = C_2\frac{1}{\left(1 + \frac{[H^+]}{K_E}\right)\left(1 + \frac{K_S}{[H^+]}\right)} \quad (4)$$

terium isotope effects on k_{cat} and k_{cat}/K_m were determined as k_{cat} using NADPH/k_{cat} using NADPD [$=^{D}V$], and ($k_{cat}/K_{m(substrate)}$) using NADPH/($k_{cat}/K_{m(substrate)}$) using NADPD [$=^{D}(V/K)$].

RESULTS

pH Dependence of k_{cat}. The pH profile of the apparent catalytic constant ($V_{max}/[E]_o$ or k_{cat}) for the reduction of 6,8-diMe-Pt showed pH independence over the pH range of 5−8 for clDHFR (Figure 1a) and slight pH dependence for rhDHFR (Figure 1b). The rate of the reduction in the presence of NADPD was about half and one-third of that in the presence of NADPH for clDHFR and rhDHFR, respectively. The catalytic rate of rhDHFR was 3−5 times faster than that of clDHFR in the presence of NADPH and 2−4 times faster in the presence of NADPD.

pH Dependence of $K_{m(substrate)}$. The apparent Michaelis constant ($K_{m(substrate)}$) values with NADPH and with NADPD were in the same range for each enzymic reduction (3.4−94 μM for clDHFR and 13−188 μM for rhDHFR). The pH profiles of the apparent $K_{m(substrate)}$ for both clDHFR and rhDHFR showed a bell-shaped (see Figure 2a,c) curve with the lowest value at pH 5.8 and rapidly rising on either side. The apparent $K_{m(substrate)}$ value for rhDHFR was 2−4 times larger than that for clDHFR in the presence of NADPH and 2−5 times larger in the presence of NADPD. Fitting the data to eq 3 gave the pH-independent value of $K_{m(substrate)}$ (C_1) and values for the two acid dissociation constants (pK_S and pK_E): these are listed in Table 1. For the clDHFR reduction, C_1 was about 0.2 μM, and the pK_a values of the substrate (pK_S) and the enzyme (pK_E) were about 5.3 and 6.3, respectively, in the presence of either NADPH or NADPD. For the rhDHFR reduction, C_1 was about 0.3 μM, and the pK_a values of the substrate (pK_S) and the enzyme (pK_E) were about 5.2 and 6.8, respectively, in the presence of either NADPH or NADPD.

pH Dependence of $k_{cat}/K_{m(substrate)}$. The apparent second-order rate constant ($k_{cat}/K_{m(substrate)}$) values differed between the reactions with NADPH and with NADPD. The pH profiles for both clDHFR and rhDHFR showed a maximum value at pH 5.8, dropping rapidly at higher and lower pHs (Figure 2b,d). Fitting the data to eq 4 gave a pH-independent value of $k_{cat}/K_{m(substrate)}$ (C_2) and values for the two acid dissociation constants (pK_S and pK_E): these are also listed in Table 1. For the clDHFR reduction, C_2 was ~48 and ~25 μM^{-1} s^{-1} in the presence of NADPH and NADPD, respectively. The pK_a values of the substrate (pK_S) and the enzyme (pK_E) were about 5.2 and 6.3, respectively, in the

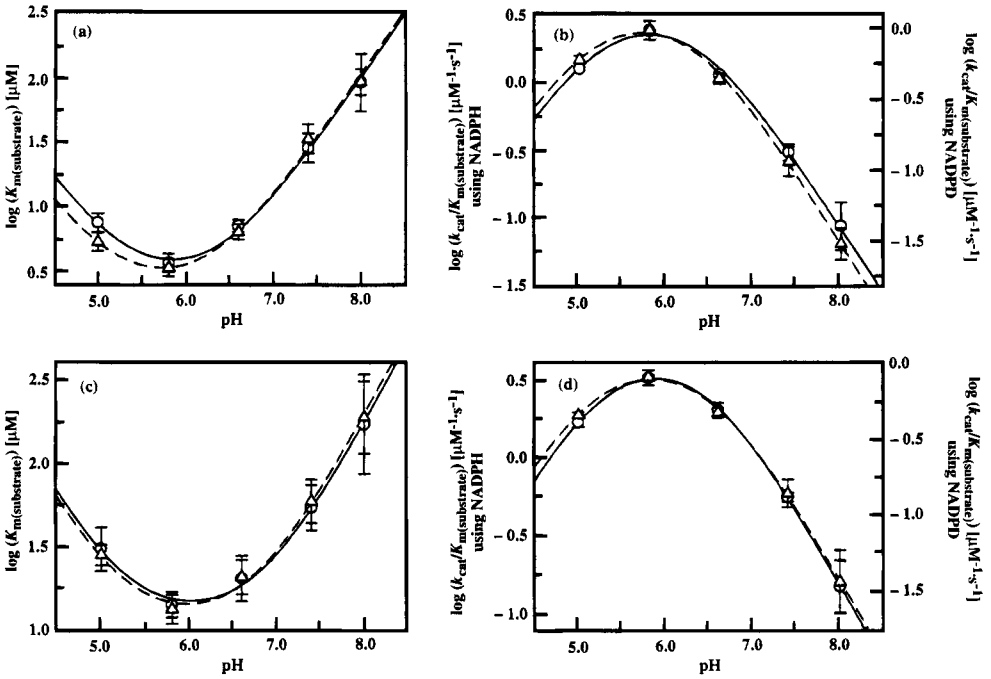


FIGURE 2: pH profiles of (a) log ($K_{m(substrate)}$) and (b) log ($k_{cat}/K_{m(substrate)}$) for 6,8-diMe-Pt reduction by clDHFR, and (c) log ($K_{m(substrate)}$) and (d) log ($k_{cat}/K_{m(substrate)}$) for 6,8-diMe-Pt reduction by rhDHFR. Standard error bars are shown. (○) In the presence of NADPH; (△) in the presence of NADPD.

Table 1: pH-Independent Values and pK_a Values of Ionizable Groups Involved in 6,8-diMe-Pt Reduction by clDHFR and rhDHFR

enzyme reactions	pH-dependent kinetic parameters	pH-independent values (C_1 and C_2) and pK_a values (pK_S and pK_E)	
clDHFR·NADPH	K_m^{app}	C_1 (μM)	0.21 ± 0.09
		pK_S	5.33 ± 0.17
		pK_E	6.37 ± 0.12
	$(k_{cat}/K_m)^{app}$	C_2 (μM^{-1} s^{-1})	48 ± 21
		pK_S	5.24 ± 0.17
		pK_E	6.36 ± 0.11
clDHFR·NADPD	K_m^{app}	C_1 (μM)	0.15 ± 0.11
		pK_S	5.16 ± 0.28
		pK_E	6.30 ± 0.17
	$(k_{cat}/K_m)^{app}$	C_2 (μM^{-1} s^{-1})	25 ± 9
		pK_S	5.09 ± 0.14
		pK_E	6.33 ± 0.08
rhDHFR·NADPH	K_m^{app}	C_1 (μM)	0.27 ± 0.09
		pK_S	5.22 ± 0.12
		pK_E	6.82 ± 0.08
	$(k_{cat}/K_m)^{app}$	C_2 (μM^{-1} s^{-1})	81 ± 9
		pK_S	5.28 ± 0.05
		pK_E	6.48 ± 0.03
rhDHFR·NADPD	K_m^{app}	C_1 (μM)	0.30 ± 0.11
		pK_S	5.20 ± 0.15
		pK_E	6.74 ± 0.09
	$(k_{cat}/K_m)^{app}$	C_2 (μM^{-1} s^{-1})	26 ± 6
		pK_S	5.15 ± 0.09
		pK_E	6.51 ± 0.06

Table 2: Deuterium Isotope Effects on 6,8-diMe-Pt Reduction by clDHFR and rhDHFR

pH	clDHFR		rhDHFR	
	^DV	$^D(V/K)$	^DV	$^D(V/K)$
5.0	2.93 ± 0.23	2.07 ± 0.52	3.95 ± 0.23	3.64 ± 0.72
5.8	2.71 ± 0.29	2.47 ± 0.89	4.23 ± 0.26	3.97 ± 0.81
6.6	2.83 ± 0.16	2.55 ± 0.51	4.14 ± 0.30	4.24 ± 1.08
7.4	2.21 ± 0.17	2.60 ± 0.59	3.64 ± 0.25	3.98 ± 0.88
8.0	2.79 ± 0.22	2.81 ± 0.51	3.73 ± 0.43	4.10 ± 1.41

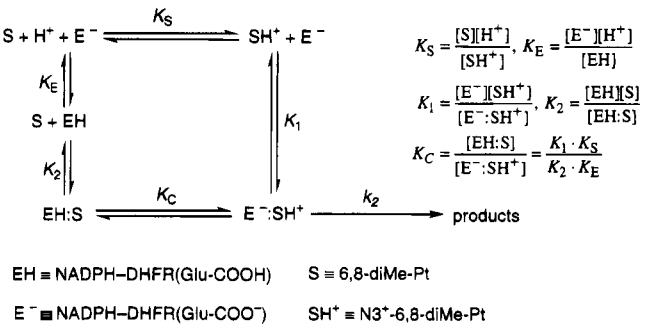
presence of either NADPH or NADPD. For the rhDHFR reduction, C_2 was ~81 and ~26 μM^{-1} s^{-1} in the presence of NADPH and NADPD, respectively, while pK_S and pK_E were about 5.2 and 6.5, respectively, in the presence of either NADPH or NADPD.

Deuterium Isotope Effects. Deuterium isotope effects on ^DV were about 2.2−2.9 for the reduction by clDHFR and about 3.6−4.2 for the reduction by rhDHFR. Deuterium isotope effects on $^D(V/K)$ were about 2.1−2.8 for the reduction by clDHFR and about 3.6−4.2 for the reduction by rhDHFR. These values are listed in Table 2.

DISCUSSION

pH Dependence of k_{cat}. Unlike the natural substrates folate, which has a very low rate, and H_2folate, which has a very low K_m value, 6,8-diMe-Pt is a convenient substrate for steady-state kinetic studies on DHFR catalysis as it has measurable V_{max} and K_m values in the pH range of 5−8. The k_{cat} results for clDHFR reduction showed pH independence (Figure 1a), whereas the k_{cat} results for rhDHFR reduction (Figure 1b) showed slight pH dependence in that the rate decreased as the pH increased. We suggest that this difference in pH dependence between clDHFR and rhDHFR is due to a decrease in the concentration of reactive enzyme−substrate complex (such as E^-:SH^+ in Scheme 1) with

Scheme 1



rhDHFR only at high pH values. pH study of the kinetics of reduction of 8-propylpterin with clDHFR also showed pH independence in the pH range (Ivery & Gready, 1995b), while pH independence or some pH dependence (for 8-methylpterin) were shown in earlier studies (Thibault et al., 1989; Gready, 1990).

pH Dependence of $K_{m(substrate)}$. The concave-up pH profiles of apparent $K_{m(substrate)}$ values for chicken and human DHFRs suggest that two ionizable groups are involved in the formation of enzyme−NADPH−6,8-diMe-Pt complex and that formation of the active complex requires the correct ionization states of the groups (see Figure 2a,c). Data fitting to eq 3 gave the pH-independent value C_1 and two acid dissociation constants for both enzymic reductions. For chicken DHFR reduction, the C_1 values ($K_{m(substrate)}$ values) with NADPH and NADPD are similar [0.21 μM and 0.15 μM, respectively (Table 1)] indicating an insignificant contribution of the catalytic rate constant (k_2 in Scheme 1) to the Michaelis constant. Therefore, the apparent $K_{m(substrate)}$ value at a given pH in the steady-state kinetics can be assumed to be similar to the apparent dissociation constant (K_d) for the enzyme−NADPH−6,8-diMe-Pt complex. The dissociation constant for the binary complex of clDHFR−6,8-diMe-Pt was determined to be 65 μM at pH 5.8 (Ivery & Gready, 1995), while our results indicate a $K_{m(substrate)}$ value for the clDHFR−NADPH−6,8-diMe-Pt complex at pH 5.8 of about 3.5 μM. Therefore, the cofactor NADPH exerts a strong cooperative effect for the binding of 6,8-diMe-Pt to the enzyme with a factor of 18. The values of the two acid dissociation constants obtained from the pH profiles are 5.3 and 6.3. The pK_a of 6,8-diMe-Pt in solution was previously determined to be 5.6 by spectrofluorimetric (Jeong et al., 1993) and spectrophotometric (Ivery & Gready, 1995) methods. Based on this pK_a value, the value of 5.3 can be assigned to the pK_a of N^3 of the substrate (pK_S) and that of 6.3 to the conserved carboxy group (Glu 30 in this case) in chicken DHFR (pK_E). This value of pK_E is comparable with literature values obtained from pH studies on MTX binding to DHFR (Dunn & King, 1980; Stone & Morrison, 1983; Fierke et al., 1987; Thillet et al., 1990).

For human DHFR reduction, C_1 values ($K_{m(substrate)}$ values) with NADPH and NADPD are again similar [0.27 and 0.30 μM, respectively (Table 1)]. The dissociation constant for the binary complex of rhDHFR−6,8-diMe-Pt was determined to be 128 μM at pH 5.8 (Ivery & Gready, 1995), while our $K_{m(substrate)}$ value for the clDHFR−NADPH−6,8-diMe-Pt ternary complex at pH 5.8 is about 14 μM. Again, NADPH exerts a strong cooperativity for the binding of 6,8-diMe-Pt to the enzyme of a factor of 9, which is half that for clDHFR. The values of the two acid dissociation constants obtained

from the pH profiles of rhDHFR reduction are 5.2 and 6.8, which are very similar to those for clDHFR. Overall the bell shape of the pH profile of $K_{m(substrate)}$ values was comparable with the pH profile of the dissociation constants (K_d) of 6,8-dimethyl-N^5-deazapterin, a competitive inhibitor of 6,8-diMe-Pt (Ivery & Gready, 1994), to rhDHFR in the presence of NADPH (Jeong & Gready, 1994a). At pHs lower than 5.8 (K_m) or 6.5 (K_d) both ionizable groups are more protonated inhibiting binding, while at higher pH values both ionizable groups are more deprotonated also inhibiting binding.

pH Dependence of $k_{cat}/K_{m(substrate)}$. The concave-down pH profiles of apparent $k_{cat}/K_{m(substrate)}$ values for chicken and human DHFRs indicate that two ionizable groups are involved not only in the formation of enzyme−NADPH−6,8-diMe-Pt complex but also in the catalysis (Figure 2b,d). Again, formation of the complex and the catalysis require the correct ionization states of the groups. Data fitting to eq 4 gave the pH-independent value C_2 and two acid dissociation constants for both enzymic reductions. For clDHFR reduction, C_2 values with NADPH and NADPD vary by a factor of about 2 [48 and 25 μM^{-1} s^{-1}, respectively (Table 1)]. As the apparent $K_{m(substrate)}$ values do not differ significantly between the reactions with NADPH and with NADPD as discussed above, the main difference in the C_2 values arises from the different catalytic rates with the two cofactors. Therefore, the ratio of the C_2 values (C_2 with NADPH/C_2 with NADPD) of about 2 can be attributed to the intrinsic isotope effect on the catalytic rate for the reduction of 6,8-diMe-Pt by clDHFR. The values of the two acid dissociation constants obtained from the pH profiles are 5.2 and 6.3, similar to those from the $K_{m(substrate)}$ profiles, and may similarly be attributed to pK_S and pK_E, respectively. For rhDHFR reduction, the C_2 values with NADPH and NADPD differ by a factor of 3 [81 and 26 μM^{-1} s^{-1}, respectively (Table 1)], and the values of the two dissociation constants are 5.2 and 6.5.

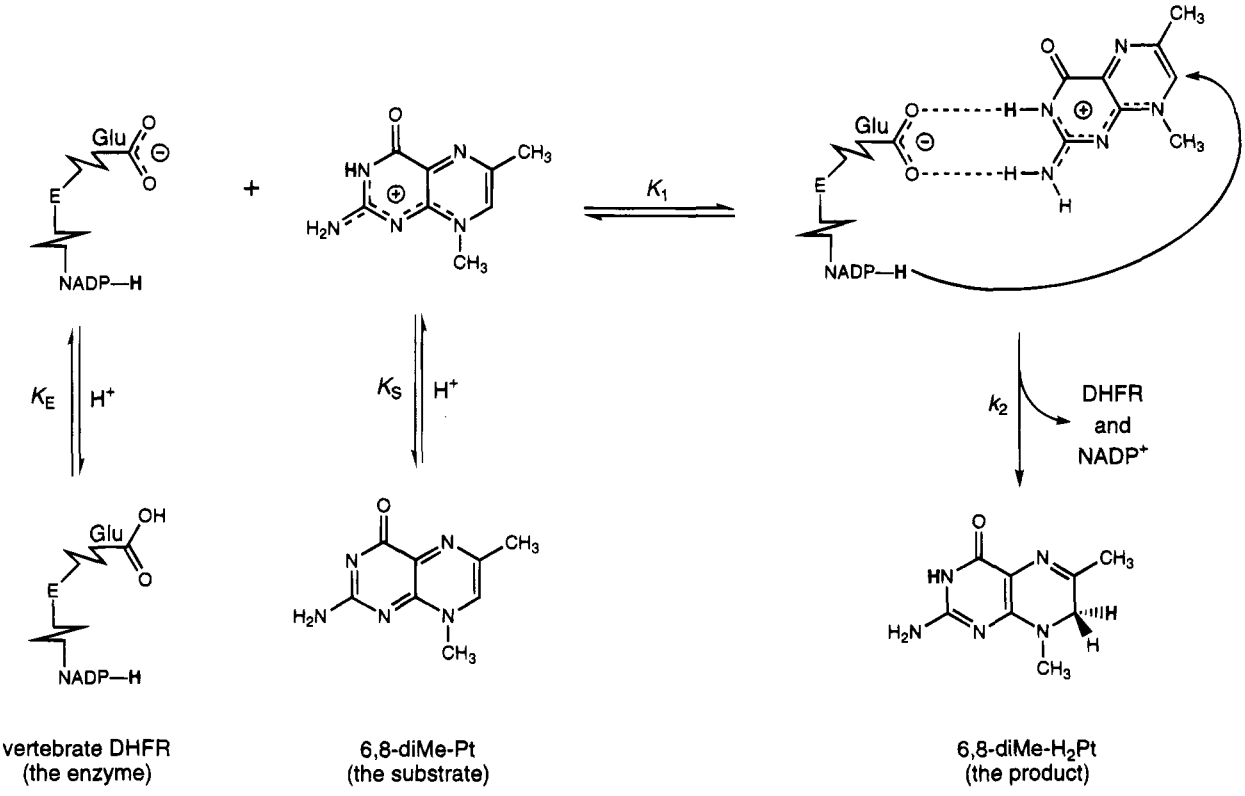
Deuterium Isotope Effects on Kinetic Parameters. Deuterium isotope effects on ^DV and $^D(V/K)$ were both very significant and pH independent over the pH range of 5−8 for the 6,8-diMe-Pt reduction by the two DHFRs. Overall the results indicate that hydride-ion transfer must be the rate-limiting step in the 6,8-diMe-Pt reduction by DHFRs at all pHs, i.e., there is no evidence of product release being rate limiting as found for H_2folate reduction at some pHs as discussed below.

A Proposed Chemical Mechanism. At a given pH, both 6,8-diMe-Pt and the conserved Glu residue in the enzyme active site have two potential forms each: cationic and neutral forms for the substrate and protonated (neutral) and deprotonated (anionic) forms for the enzyme. The pH profiles of apparent $K_{m(substrate)}$ and $k_{cat}/K_{m(substrate)}$ could be interpreted to mean that the formation of the enzyme−NADPH−6,8-diMe-Pt complex and the catalysis require either protonated enzyme−neutral substrate or deprotonated enzyme−cationic substrate. The fact that deuterium isotope effects of ^DV and $^D(V/K)$ were found indicates that hydride-ion transfer not protonation is the rate-limiting step. As the catalytic reduction of the substrate 6,8-diMe-Pt to the product 6,8-diMe-H_2Pt requires a protonation which is generally accepted to precede hydride-ion transfer (Huennekens & Scrimgeour, 1964; Gready, 1985), the deprotonated enzyme−cationic substrate [DHFR(COO^-)−NADPH−6,8-diMe-PtH^+] complex is most likely to be the active complex. While there has been much discussion in the literature of possible mechanisms for a proton transfer from protonated enzyme to N^5 of H_2folate from an initial complex of protonated enzyme−neutral substrate (Stone & Morrison, 1984; Howell et al., 1986; Morrison & Stone, 1988), this is both easier to imagine but also less likely for 6,8-diMe-Pt as substrate. On the one hand the proton shift would only need to occur across a hydrogen bond (Glu-COOH to N^3 of 6,8-diMe-Pt), but on the other hand the spectroscopic evidence for the structurally analogous inhibitor 6,8-dimethyl-N^5-deazapterin (Jeong & Gready, 1994a) and for the related chromophore in methotrexate (Hood & Roberts, 1978; Cocco et al., 1981) suggests that protonated ligand is bound in ternary complexes with NADPH. Consequently, the chemical mechanism proposed in Scheme 2 for the enzymic reduction of 6,8-diMe-Pt in the presence of NADPH is that most consistent with the present and other results. In this mechanism the cationic form of 6,8-diMe-Pt binds to the deprotonated form of DHFR in a similar orientation to that of the natural substrates (folate and biopterin) (Oefner et al., 1988; Bystroff et al., 1990; Davies et al., 1990; McTigue et al., 1992, 1993) to form the DHFR(COO^-)−NADPH−6,8-diMe-PtH^+ complex. The hydride ion is then transferred from NADPH to C7 of 6,8-diMe-PtH^+ to produce 6,8-diMe-H_2Pt.

Comparison with Previous Studies on H_2Folate Reduction. In a number of previous studies on H_2folate reduction by DHFR which were mostly done with *Escherichia coli* enzyme, pH dependence of k_{cat} was found and one pK_a value of ~8 was determined (Howell et al., 1986, 1987, 1990; Beard et al., 1989; Morrison, 1991). One study reported a second pK_a value of ~5, but this finding was not further studied (Howell et al., 1986). Similar observations were made for the pH profile of k_{cat}/K_m (Hermes et al., 1984; Howell et al., 1986, 1987, 1990; Morrison, 1991). The pK_a value of ~8 was assigned to ionization of the enzyme carboxy group in substrate complexes based on a pK_a of ~6.5 of the enzyme assigned from pH studies on methotrexate binding to DHFR (Dunn & King, 1980; Stone & Morrison, 1983; Fierke et al., 1987; Thillet et al., 1990). One interpretation of the increase of the pK_a to ~8 from ~6.5 was that H_2folate is a "sticky substrate" (Cleland, 1982; Morrison, 1991). On the other hand, the increment was argued to be due to a "perturbation" of the real catalytic rate from the slow release of product at low pHs (Cleland, 1982; Fierke et al., 1987; Thillet et al., 1990). This was deduced from deuterium isotope effects on ^DV and $^D(V/K)$ indicating that below pH 7 product release is rate-limiting while at more alkaline pHs hydride transfer is rate-limiting (Stone & Morrison, 1984; Morrison & Stone, 1988; Beard et al., 1989). The story has recently become even more complicated with a report by Chen et al. (1994) based on results from Raman spectroscopy experiments arguing for a reassignment of the pK_a value of 6.5 to protonation of H_2folate on N^5 in the DHFR−$NADP^+$−H_2folate.

From comparison of our results with these studies on H_2-folate reduction, we can make the following observations. First, as will be apparent, H_2folate reduction is unsuitable to probe the DHFR catalytic mechanism because product release obscures the real catalytic rate, especially at low pHs. Consequently, the observed pH dependence is an artifact composed of two processes, product release and hydride-ion transfer, with the balance varying with enzyme source.

Scheme 2



As the catalytic rate of reduction of 6,8-diMe-Pt to 6,8-diMe-H_2Pt is not obscured by product release, our substrate is a good candidate for pH studies. Also, the basic pK_a for N^5 of H_2folate, reported to be ~3.8 (Poe, 1977) or 2.6 (Maharej et al., 1990), is too low to check its effect directly in the pH region where DHFR and NADPH are stable (~5–9). Therefore, although all treatments assumed that correct substrate protonation was required in the catalytic mechanism, no experimental evidence was found for this protonation (Stone & Morrison, 1984; Howell et al., 1986, 1987; Morrison & Stone, 1988). Even if correct, the pK_a reassignment in the recent paper of Chen et al. (1994) does not resolve this problem but merely shifts it to one of evidence for correct enzyme ionization. However, the high pK_a value of ~5.6 for 6,8-diMe-Pt has allowed direct study of the effects of both substrate and enzyme ionizations on the formation of enzyme–substrate complex and on catalysis.

CONCLUSION

The active complex in the reduction of the mechanism-based substrate 6,8-diMe-Pt by DHFRs with NADPH is characterized by a deprotonated enzyme carboxy group which binds protonated 6,8-diMe-Pt. The rate-limiting step for the reaction is hydride-ion transfer at acidic pHs as well as neutral pHs, with no perturbation on the catalytic rate by product release. The pH dependence of the kinetic constants is characterized by two pK_a values representing ionizations of enzyme and substrate. It is suggested that the "well-behaved" kinetics exhibited by 6,8-diMe-Pt make it an attractive substrate for further study of the DHFR catalytic mechanism.

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APPENDIX

$$[S]_o \approx [S] + [SH^+], \text{ where } [S]_o \gg [E]_o$$

$$[E]_o = [E^-] + [EH] + [EH{:}S] + [E^-{:}SH^+]$$

$$[E]_o = [E^-] + \frac{[H^+]}{K_E}[E^-] + \frac{[S][H^+]}{K_2K_E}[E^-] + \frac{[S][H^+]}{K_1K_S}[E^-]$$

$$[S]_o \approx [S] + [SH^+] = [S] + \frac{[S][H^+]}{K_S} = [S]\left(1 + \frac{[H^+]}{K_S}\right), \therefore [S] = \frac{[S]_o}{\left(1 + \frac{[H^+]}{K_S}\right)}$$

$$\frac{v}{[E]_o} = \frac{k_2[E^-{:}SH^+]}{[E]_o}$$

$$= \frac{\frac{k_2}{K_1K_S}[S][H^+][E^-]}{[E^-] + \frac{[H^+]}{K_E}[E^-] + \frac{[S][H^+]}{K_2K_E}[E^-] + \frac{[S][H^+]}{K_1K_S}[E^-]}$$

$$= \frac{\frac{k_2}{K_1K_S}[S]}{\frac{1}{[H^+]} + \frac{1}{K_E} + \frac{[S]}{K_2K_E} + \frac{[S]}{K_1K_S}}$$

$$= \frac{k_2[S]}{K_1K_S\left(\frac{1}{[H^+]} + \frac{1}{K_E}\right) + \left(\frac{K_1K_S}{K_2K_E} + 1\right)[S]}$$

$$= \frac{k_2[S]}{K_1K_S\left(\frac{1}{[H^+]} + \frac{1}{K_E}\right) + (K_C + 1)[S]}$$

$$= \frac{\frac{k_2}{(1 + K_C)}[S]}{\frac{K_1K_S\left(\frac{1}{[H^+]} + \frac{1}{K_E}\right)}{(1 + K_C)} + [S]}$$

$$= \frac{\frac{k_2}{(1 + K_C)}[S]_o}{\frac{K_1K_S\left(\frac{1}{[H^+]} + \frac{1}{K_E}\right)\left(1 + \frac{[H^+]}{K_S}\right)}{(1 + K_C)} + [S]_o}$$

$$\therefore v = \frac{\frac{k_2}{(1 + K_C)}[S]_o[E]_o}{\frac{K_1K_S}{(1 + K_C)}\left(\frac{1}{[H^+]} + \frac{1}{K_E}\right)\left(1 + \frac{[H^+]}{K_S}\right) + [S]_o}$$

$$= \frac{\frac{k_2}{(1 + K_C)}[S]_o[E]_o}{\frac{K_1}{(1 + K_C)}\left(\frac{1}{[H^+]} + \frac{1}{K_E}\right)(K_S + [H^+]) + [S]_o}$$

$$= \frac{\frac{k_2}{(1 + K_C)}[S]_o[E]_o}{\frac{K_1[H^+]}{(1 + K_C)}\left(\frac{1}{[H^+]} + \frac{1}{K_E}\right)\left(1 + \frac{K_S}{[H^+]}\right) + [S]_o}$$

$$= \frac{\frac{k_2}{(1 + K_C)}[S]_o[E]_o}{\frac{K_1}{(1 + K_C)}\left(1 + \frac{[H^+]}{K_E}\right)\left(1 + \frac{K_S}{[H^+]}\right) + [S]_o}$$

$$\left(\text{cf. } v = \frac{V_{max}[S]_o}{K_m + [S]_o}\right)$$

$$V_{max}^{app} = \frac{k_2[E]_o}{(1 + K_C)}$$

$$K_m^{app} = \frac{K_1}{(1 + K_C)}\left(1 + \frac{[H^+]}{K_E}\right)\left(1 + \frac{K_S}{[H^+]}\right)$$

$$\left(\frac{V_{max}}{K_m}\right)^{app} = \frac{k_2}{K_1}\frac{[E]_o}{\left(1 + \frac{[H^+]}{K_E}\right)\left(1 + \frac{K_S}{[H^+]}\right)}$$

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