Effect of Enzyme and Ligand Protonation on the Binding of Folates to Recombinant Human Dihydrofolate Reductase: Implications for the Evolution of Eukaryotic Enzyme Efficiency†

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ABSTRACT: There is marked pH dependence of the rate constant (k_{off}) for tetrahydrofolate (H_4folate) dissociation from its ternary complex with human dihydrofolate reductase (hDHFR) and NADPH. Similar pH dependence of H_4folate dissociation from the ternary complex of a variant of hDHFR with the substitution $Phe^{31} \rightarrow$ Leu (F31L hDHFR) causes this dissociation to become rate limiting in the enzyme mechanism at pH $\approx$ 5, and this accounts for the marked decrease in k_{cat} for this variant as the pH is decreased from 7 to 5. This decreased k_{cat} at low pH is not seen for most DHFRs. k_{off} for dissociation of folate, dihydrofolate (H_2folate), and H_4folate from their binary complexes with hDHFR is similarly pH dependent. For all the complexes examined, the pH dependence of k_{off} in the range pH 5–7 is well described by a pK_a of about 6.2 and must be due to ionization of a group on the enzyme. In the higher pH range (7–10), k_{off} increases further as the pH is raised, and this relation is governed by a second pK_a which is close to the pK_a for ionization of the amide group (HN^3–C^4O) of the respective ligands. Thus, ionization of the ligand amide group also increases k_{off}. Evidence is presented that the dependence of pH on k_{off} for hDHFR accounts for the shape of the k_{cat} versus pH curve for both hDHFR as well as its F31L variant and contributes to the higher efficiency of hDHFR compared with bacterial DHFR.

It might be expected that the binding of the substrates folate and dihydrofolate (H_2folate)[1] to the active site of dihydrofolate reductase (DHFR) would be strongly influenced by pH. These substrates, as well as the product tetrahydrofolate (H_4folate), have an amide group (HN^3–C^4O) that ionizes not far above physiological pH, and this ionization might be expected to affect binding. In addition, the crystal structure of the folate complex of human DHFR (hDHFR) indicates that folate bound in the active site has one side of the pteridine ring in close proximity to the side-chain carboxyl group of Glu^{30} (Oefner et al., 1988; Davies et al., 1990). Glu^{30} is the only ionizable group in the active site and is strictly conserved in DHFR from eukaryotic organisms. Bacterial DHFRs have a corresponding Asp residue (Asp^{27} in ecDHFR), and in the crystal structure of the ecDHFR·NADP·folate complex the interaction of the pteridine ring of bound folate with the Asp^{27} carboxyl closely resembles the interaction with Glu^{30} in the hDHFR complex (Bystroff et al., 1990).

There have, however, been relatively few studies of the effect of pH on the binding of these ligands to DHFR. Those that have appeared were made with ecDHFR and showed only small pH effects on binding. Stone and Morrison (1983) reported that K_d for the binary complex of H_2folate increased about 4-fold as the pH was lowered from 7, with a pK_a value of 5.73 ± 0.21. A similar small increase in K_d occurred as the pH was raised above 7, with a pK_a of 9.64 ± 0.08. The latter pK_a was assigned to the HN^3–C^4O amide group of H_2folate (though this is far from the actual pK_a value; Maharaj et al., 1990) and the former to a group on the enzyme, which was assumed to be the active-site carboxyl (Asp^{27}). Fierke et al. (1987) reported that the association and dissociation rate constants, k_{on} and k_{off}, were essentially the same at pH 6.0 and pH 9.0 for H_2folate in its binary complex and in its ternary complex with NADP also bound. k_{on} for H_4folate was also the same at pH 6.0 and pH 9.0 for its binary complex, although k_{off} was at least 4 times higher at the higher pH than at the lower. For the ternary complex E·H_4folate·NADPH, k_{on} and k_{off} for H_4folate were the same at both pH values.

As part of our studies on the binding of ligands in the active site of hDHFR, we have investigated the effect of pH on the binding of folate, H_2folate, and H_4folate to hDHFR. In contrast to the reported minimal effect of pH on binding of folates to ecDHFR, which we have confirmed, k_{off} values for dissociation of these ligands from hDHFR show considerable decreases as the pH is raised above 5.

Materials and Methods

Materials. NADPH, folic acid, and MTX were from Sigma. 5-Deazafolic acid was a generous gift from Dr. Robert D. Elliott, Southern Research Institute. wt hDHFR was produced as previously described (Prendergast et al., 1988) or by use of the expression vector pDS hDHFR (Stüber et al.,

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[1] Abbreviations: DHFR, dihydrofolate reductase (EC 1.5.1.3); hDHFR, recombinant human DHFR; ecDHFR, dihydrofolate reductase from *Escherichia coli*; H_2folate, 7,8-dihydrofolate; H_4folate, (6*S*)-5,6,7,8-tetrahydrofolate; MATS, 25 mM MES, 25 mM acetate, 50 mM Tris, 100 mM NaCl, and 0.02% sodium azide; MTEN, 50 mM MES, 25 mM Tris, 25 mM ethanolamine, 100 mM NaCl; MTX, methotrexate; MES, 2-(*N*-morpholino)ethanesulfonic acid; Tris, tris(hydroxymethyl)-aminomethane; wt, wild type; F31L, mutant hDHFR with $Phe^{31} \rightarrow$ Leu.

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1986) which was kindly provided by Dr. D. Stüber. Other materials were as previously described (Appleman et al., 1989, 1990; Tsay et al., 1990).

The concentration of H_2folate was determined enzymatically (Maharaj et al., 1990). The concentrations of other ligands were determined spectrophotometrically by using the following molar extinction coefficients (M^{-1} cm^{-1}): folic acid, pH 7, 282 nm (27 000) and 350 nm (7000) (Blair & Saunders, 1970); H_4folate 298 nm (28 000) (Huennekens et al., 1963); MTX, pH 13, 257 nm (23 000), 302 nm (22 000), and 370 nm (7100) (Seeger et al., 1949); 5-deazafolic acid, pH 7, 278 nm (24 900) and 295 nm (23 800) (Temple et al., 1982); NADPH, 340 nm (6220). Enzyme concentration was determined by titrating with MTX (Appleman et al., 1990).

Experimental Conditions. Measurements were made at 20 °C in the buffer mixtures MATS (pH 5–9) or MTEN (pH 9.5–10) unless otherwise indicated. Both buffers were supplemented with 50 mM 2-mercaptoethanol. The pH was adjusted with either HCl or NaOH as appropriate. The ionic strengths of these buffer systems are constant over the pH range used in this study.

Determination of Dissociation Rate Constants by Competition Methods. This was carried out as previously described (Appleman et al., 1990) with MTX or 5-deazafolate as the competing ligand. Change in absorbance at 380 nm or in fluorescence was monitored. The observed rate constants for folate, H_2folate, and H_4folate dissociation from their binary complexes with wt hDHFR were independent of the concentration of the competing ligand (MTX) over the range 10–200 μM at pH values of 5, 7.5, and 8.5. The dissociation rate constant, k_{off}, at each pH between 5.3 and 10 was determined using 50 μM MTX. Because the pK for N^1 of MTX is 5.73 (Cocco et al., 1981), MTX is largely protonated at pH 5 so that at this pH, its binding cannot be readily observed by absorbance change. However, values of k_{off} for H_2folate and H_4folate at pH 5 could be determined by the fluorescence changes occurring when they are displaced by MTX. Displacement of folate did not give a significant fluorescence change, and k_{off} for folate at pH 5 was determined from the absorbance change at 360 nm with 5-deazafolate as competing agent. The pH of each reaction mixture was determined before and after each condition. The pH dependence of k_{off} for dissociation of folate, H_2folate, and H_4folate from their binary complexes with either wt or F31L hDHFR was fitted to

$$k_{off} = \frac{(k_{off,HE}[H^+]/K_1 + k_{off,E})(1 + K_2/[H^+])}{1 + [H^+]/K_1} \quad (1)$$

where $k_{off,HE}$ and $k_{off,E}$ are limiting values of k_{off} at low and neutral pH, respectively, K_1 and K_2 are acid dissociation constants, and $[H^+]$ is the hydrogen ion concentration.

Because there is a strong negative cooperativity for H_4folate binding to the E·NADPH complex, k_{off} for H_4folate dissociation from this abortive ternary complex by competition with MTX was complicated by difficulty in saturating the enzyme with NADPH. The competition experiment at each pH was performed on a stopped-flow spectrophotometer by mixing equal volumes of a solution of the preincubated enzyme·H_4folate complex with a solution of MTX containing NADPH. A series of NADPH concentrations was used, and the true k_{off} was calculated as described previously (Tsay et al., 1990). The pH dependence of k_{off} for H_4folate dissociation from its ternary complex with F3IL rhDHFR·NADPH was fitted to

$$k_{off} = \frac{k_{off,HE}[H^+]/K + k_{off,E}}{1 + [H^+]/K} \quad (2)$$

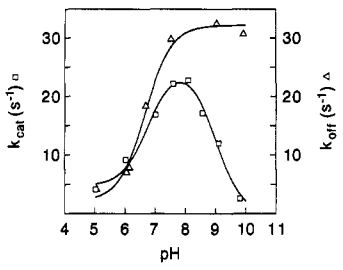

FIGURE 1: pH dependence of k_{cat} (s^{-1}) for F31L hDHFR and of k_{off} (s^{-1}) for H_4folate release from the F31L hDHFR·NADPH·H_4folate ternary complex. The pH vs k_{cat} profile is from Tsay et al. (1990). k_{off} at each pH was determined at a series of NADPH concentrations in competition experiments, and the solid curve is a best fit to eq 2 as described in Materials and Methods.

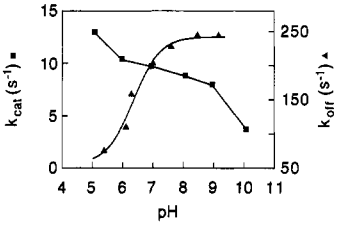

FIGURE 2: pH dependence of k_{cat} (s^{-1}) for wild type and of k_{off} for H_4folate release from wt hDHFR·NADPH·H_4folate ternary complex. The pH vs k_{cat} profile was taken from Beard et al. (1989). k_{off} at each pH was determined as described in Figure 1.

where $k_{off,HE}$ and $k_{off,E}$ are limiting values of k_{off} at low and high pH, respectively, K is the acid dissociation constant, and $[H^+]$ is the hydrogen ion concentration.

Computer Simulation of the k_{cat}/pH Relationship for hDHFR. The following assumptions were made: (1) a single value of p$K_{a,EL}$ and of $k_{off,EH}/k_{off,E}$ describes the pH dependence of the dissociation of both H_2folate and H_4folate from their binary and all their ternary complexes with hDHFR; (2) k_{on} for folates and k_{off} and k_{on} for NADPH and NADP in all complexes have the values determined at pH 7.65 (Appleman et al., 1990) at all pH values; (3) the increase in k_{off} governed by pK_{a2} can be ignored because hydride transfer is primarily rate limiting at pH values in the vicinity of pK_{a2}. Values of $k_{off,E}$ for the folates were those measured at pH 7.65. The value of k_{hyd}, the rate constant for the conversion of enzyme-bound substrates to enzyme-bound products, was assumed to be pH dependent with a maximum value at low pH of 39 700 s^{-1} and governed by a pK_a of 6.2 (J. R. Appleman and R. L. Blakley, unpublished results).[2] Simulation was carried out by methods previously employed (Appleman et al., 1990).

Results and Discussion

Dissociation of H_4folate from Its Complex with F31L hDHFR and NADPH. As we previously reported (Tsay et al., 1990), a major difference in the properties of the F31L variant of hDHFR compared with wild-type (wt) enzyme is the shape of the curve relating k_{cat} to pH (Figure 1). Whereas wt hDHFR has a relatively flat curve showing only a small increase of activity at low pH and a modest decrease at high

[2] The value of the pK_a governing hydride transfer is estimated to be 6.2 from studies with alternate substrates. k_{hyd} is the rate constant governing the rate of hydride transfer and is taken from the limiting value of 39 700 s^{-1} at low pH that is calculated from the observed value of hydride transfer (1360 s^{-1}) at pH 7.65 and a pK_a of 6.2. Other methods for the estimation of the pH dependence for hydride transfer can be made, but they do not significantly alter the results of the simulations reported in this study.

Table I: Parameters Characterizing the Interaction of the Active-Site Residue of hDHFR with the Pteridine Ring of Folate Derivatives[a]

enzyme complex	dissociating ligand	$k_{off,HE}$[b] (s^{-1})	$k_{off,E}$[c] (s^{-1})	$k_{off,E}/k_{off,HE}$	pK_{a1}	pK_{a2}
wt·folate	folate	<3[d]	29.9 ± 1.6	>10	5.53 ± 1.6	8.36 ± 1.6
wt·H_2folate	H_2folate	2.0 ± 0.6	18.5 ± 0.5	9.3 ± 2.8	6.37 ± 0.09	11.2 ± 0.3
wt·H_4folate	H_4folate	0.80 ± 0.19	5.85 ± 0.14	7.3 ± 1.8	6.17 ± 0.08	10.30 ± 0.05
F31L·H_4folate	H_4folate	0.021 ± 0.008	0.149 ● 0.004	7.1 ± 2.7	6.25 ± 0.11	10.6 ± 0.1
wt·H_4folate·NADPH	H_4folate	56 ± 16	242 ● 7	4.3 ± 1.2	6.4 ± 0.1	*e*
F31L·H_4folate·NADPH	H_4folate	2.2 ± 1.5	32.2 ± 1.0	14.6 ± 10.0	6.7 ± 0.1	*e*

[a] From dependence of pH of dissociation rate constants (k_{off}) for binary complexes (Figures 3 and 4) and for ternary complexes (Figures 1 and 2). [b] Limiting value of k_{off} at acidic pH. [c] Limiting value of k_{off} at neutral pH. [d] When the best-fit values of the parameters $k_{off,HE}$, $k_{off,E}$, and pK_{a2} were determined by fitting the pH dependence of k_{off} for folate, that for $k_{off,HE}$ was less than zero. Since this is not physically possible, the best-fit values of the other parameters were determined with the value of $k_{off,HE}$ held constant at zero. The value of $k_{off,HE}$ is less than 3 since this value was measured at pH 5. [e] pK_{a2} not accessible due to weak binding of NADPH at high pH.

pH (Figure 2), the mutant enzyme has a bell-shaped curve with a 10-fold variation in activity over the pH range 5–10. The decrease in activity of both enzymes at high pH can be explained by a decrease in the rate of hydride transfer. Evidence for this decrease is found in the increasing isotope effect above pH 8 when NADPD is substituted for NADPH. Below pH 7, however, the catalytic cycling is slower than hydride transfer because the release of product is rate limiting. Determination of all association and dissociation rate constants for all binary and ternary complexes permitted complete definition of the branched mechanism by which F31L hDHFR functions (Tsay et al., 1990). In this mechanism, dissociation of H_4folate from the E·H_4folate·NADPH complex is a major determinant of the steady-state rate of catalysis at pH 7.65. It therefore seemed likely that a decrease in the rate of H_4folate dissociation as the pH decreases below 7 might cause the decrease in steady-state activity. As seen in Figure 1, this is indeed the case. There is an approximately 15-fold decrease in k_{off} as the pH decreases from 9 to 5, and it is clear that at low pH the rate of this dissociation must limit k_{cat}.

Dissociation of H_4folate from the Ternary Complex with NADPH and wt Enzyme. The next question to be addressed was why similar behavior is not reflected in the k_{cat} versus pH curve for wt enzyme. One possibility is that pH dependence of k_{off} for H_4folate is peculiar to the F31L mutant. Accordingly, we investigated wt hDHFR for similar behavior, with the results shown in Figure 2. Although at any selected pH the value of k_{off} for wt is an order of magnitude greater than that for F31L, the shape of the curve describing the decrease in k_{off} with decreasing pH is very similar. The pH dependence of k_{off} for H_4folate from this ternary complex is therefore an intrinsic property of hDHFR. However, this change in k_{off} with pH is not obviously reflected in the k_{cat} versus pH relationship for wt enzyme, because at no pH value is the dissociation of H_4folate from the ternary complex with wt hDHFR and NADPH the sole rate-limiting step in the mechanism. At all pH values measured, k_{off} is much greater than k_{cat} (5-fold at pH 5 increasing to 70-fold at pH 10).

Effect of pH on Dissociation of Folates from Binary Complexes with wt hDHFR. In view of the marked pH dependence of H_4folate dissociation from the ternary complex, the rate constants for dissociation of folate, H_2folate, and H_4folate from their binary complexes with wt hDHFR were determined as a function of pH. In all three cases, k_{off} decreased as the pH was decreased from 7 to 5 (Figure 3). In each case, a single pK_a described this dependence well, as indicated by the curves, and the pK_a values were similar (5.5–6.4) (Table I) for all three binary complexes as well as for H_4folate dissociation (6.4 and 6.7) from the two ternary complexes already described.

In the case of the binary complexes, we were able to extend measurement of k_{off} to higher pH, where it became evident that a second proton dissociation was increasing the rate of

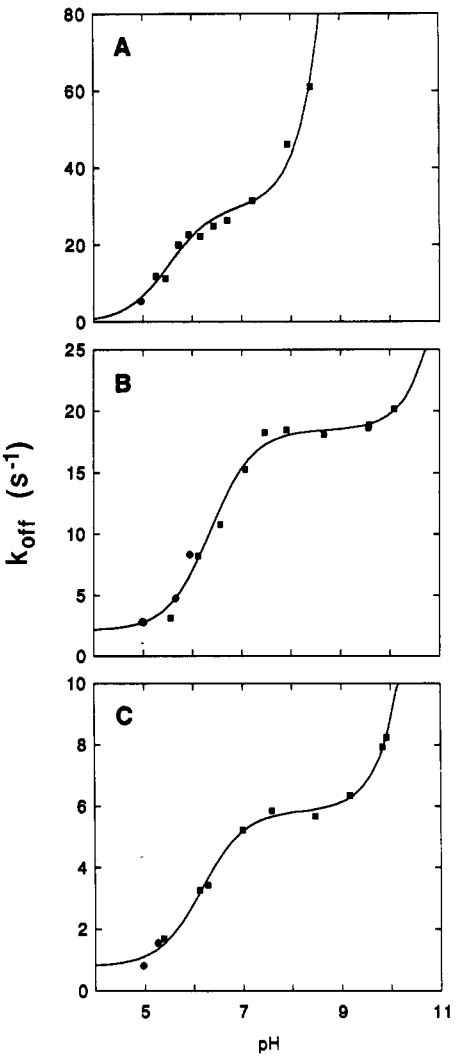

FIGURE 3: pH dependence of k_{off} (s^{-1}) for folate (panel A), H_2folate (panel B), and H_4folate (panel C) dissociation from their binary complexes with wt hDHFR. k_{off} was determined by competition either with MTX (●) or with 5-deazafolic acid (■). Final concentrations: wt hDHFR, 2 μM; folates, 3 μM; MTX, 50 μM; 5-deazafolic acid, 10–40 μM. The solid curves are best fits to eq 1 as described in Materials and Methods.

ligand dissociation (Figure 3 and Table I). However, the pK_a value for this dissociation was significantly different for each ligand, increasing in the order folate < H_4folate < H_2folate. On the other hand, there was little difference between values of this pK_a for H_4folate dissociation from binary complexes with wt and F31L·hDHFR (Table I).

Effect of pH on Dissociation of Folates from Binary Complexes with ecDHFR. It was reported previously that the rate of dissociation of folates from ecDHFR was nearly the same at pH 6 and 9 (Fierke et al., 1987). However, it was

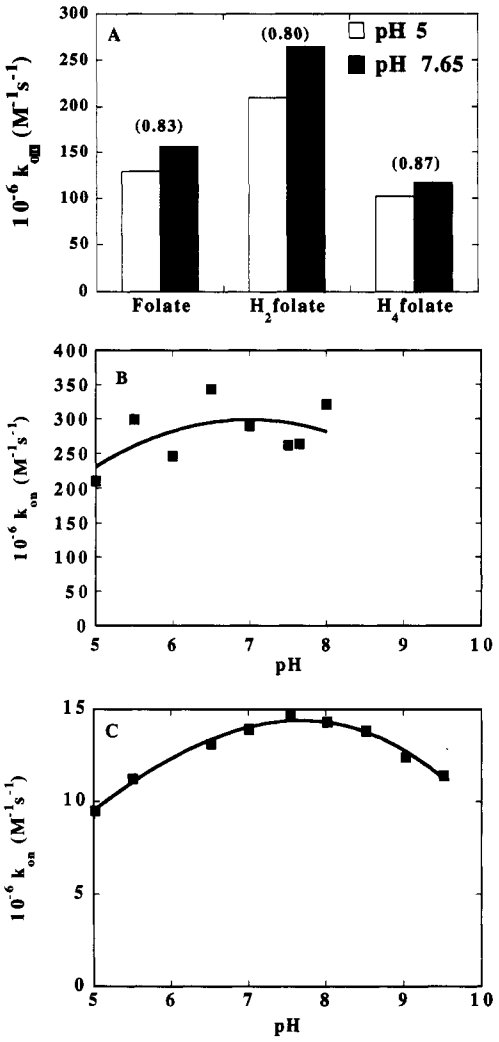

FIGURE 4: (A) Comparison of k_{on} (μM^{-1} s^{-1}) for binding of folates to wt hDHFR at pH 5 and 7.65. (B) pH dependence of k_{on} for binding of H_2folate to wt hDHFR. (C) pH dependence of k_{on} for binding of H_4folate to F31L·hDHFR·NADPH.

possible that some pH dependence might have been missed. We therefore examined k_{off} for folate and H_2folate dissociation from binary complexes with ecDHFR over the pH range 5–8. k_{off} for H_2folate is 10.7 ± 0.3 s^{-1} under the conditions used and is independent of pH over this range. We have not extended these measurements in order to determine whether a pK_a corresponding to pK_{a2} with hDHFR can be observed. With folate, an increase in k_{off} is observed in the pH range 5–10, like that observed with hDHFR. Thus, only one of the two protonations that alter the rate of dissociation of folates from hDHFR is observed with ecDHFR.

Effect of pH on Rate Constants for Association of Folates with hDHFR To Form Enzyme Complexes. We have examined the pH dependence of values of k_{on}, the rate constant governing the rate of binding of folates to hDHFR. Although k_{on} depends upon the exact nature of the folate, these values are not appreciably different at pH 5 and 7.65 (Figure 4A). Furthermore, very little pH dependence was observed in much more complete pH profiles of k_{on} for binding of H_2folate to hDHFR (Figure 4B) and H_4folate to F31L hDHFR·NADPH (Figure 4C). This is not particularly surprising since dissociation rates are often far more sensitive than are association rates to specific interactions between ligands and enzymes (Hammes & Schimmel, 1970).

Dependence of k_{off} for Folates on Protein Protonation. The results presented indicate clearly that the dissociation of folate, H_2folate, and H_4folate from complexes with hDHFR is greatly accelerated by the deprotonation of two groups in the complex with quite different pK_a values. For one of these groups, the pK_a is close to 6.2 regardless of which of the three ligands is involved in the complex or whether the complex involves wt or F31L hDHFR. Since none of the pteridine ligands have a pK_a value close to 6.2 (Temple & Montgomery, 1984), the group must be on the enzyme. Although a logical candidate might seem to be the Glu^{30} side-chain carboxyl, there are a number of difficulties for this assignment which will be discussed in a subsequent publication. It has been reported that replacement of the αC helix of ecDHFR and incorporation of an attached hairpin turn at the C-terminus of his helix corresponding to the sequence from DHFR from *Lactobacillus casei* produces a pH dependence in the rate of dissociation of H_4folate from ecDHFR·NADPH resembling that which we report herein for hDHFR·NADPH (Li & Benkovic, 1991). Thus, elements within the corresponding helix in hDHFR must be considered candidates. However, no residues in this helix in either the altered ecDHFR or in hDHFR seem likely to undergo ionization in the appropriate pH range. It may be that pK_{a1} is not produced by the ionization of any single residue. An alternate hypothesis is that it is instead the result of several ionizations that alter the electrostatic field in the active site of the enzyme, thus changing the rate of ligand dissociation. The exact orientation of charged residues throughout the enzyme would then be critical in determining the electrostatic potential experienced by bound ligand, so that the altered pH dependence of k_{off} for the ecDHFR mutant may be due to structural perturbations rather than directly due to the introduction of an ionizing group.

Table II: Comparison of pK_{a2} for Effect of pH on k_{off} for Binary Complexes of Folates and pK_a for Their HN^3–C^4O Amide Groups

ligand	hDHFR	pK_{a2}[a]	HN^3–C^4O pK_a[b]	reference
folate	wt	8.36 ± 1.6	8.38	Cocco et al., 1981
H_2folate	wt	11.2 ± 0.3	10.81 ± 0.01	Maharaj et al., 1990
H_4folate	wt	10.3 ± 0.05	10.50	Kallen & Jancks, 1966
	F31L	10.6 ± 0.1		

[a] From Table I. [b] From reference shown.

Dependence of k_{off} for Folates on Ligand Protonation. pK_{a2}, the higher pK_a that influences the rate of dissociation of folate ligands, is different for each ligand and in each case is quite close to the value of pK_a for dissociation of a proton from the HN^3–C^4O group of the pteridine ring (Table II). It therefore seems reasonable to conclude that deprotonation of this group on the bound ligand further increases its rate of dissociation from complexes with hDHFR. Loss of the proton from N^3 of the ligand results in loss of one hydrogen bond between bound ligand and enzyme, and in addition, the close proximity of two negative charges further decreases the binding energy. This proximity might be alleviated by inversion of the pteridine ring, although there is no direct evidence that this occurs.

Relation of pH Dependence of k_{off} for Folates to pH Dependence of k_{cat}. k_{cat} for hDHFR declines with increase of pH above 7 (Figure 2) due to the effect of pH on the rate of hydride transfer. This is a characteristic property of DHFR from all sources that we have studied (Beard et al., 1989). However, as the pH is decreased below 7, k_{cat} for hDHFR continues to rise. This behavior is also shown by bovine, murine, and chicken DHFR, but not by ecDHFR (Beard et al., 1989). We have investigated whether this different pH dependence of k_{cat} for eukaryotic DHFR is related to the ligand-binding/enzyme-protonation relationship we have observed for hDHFR.

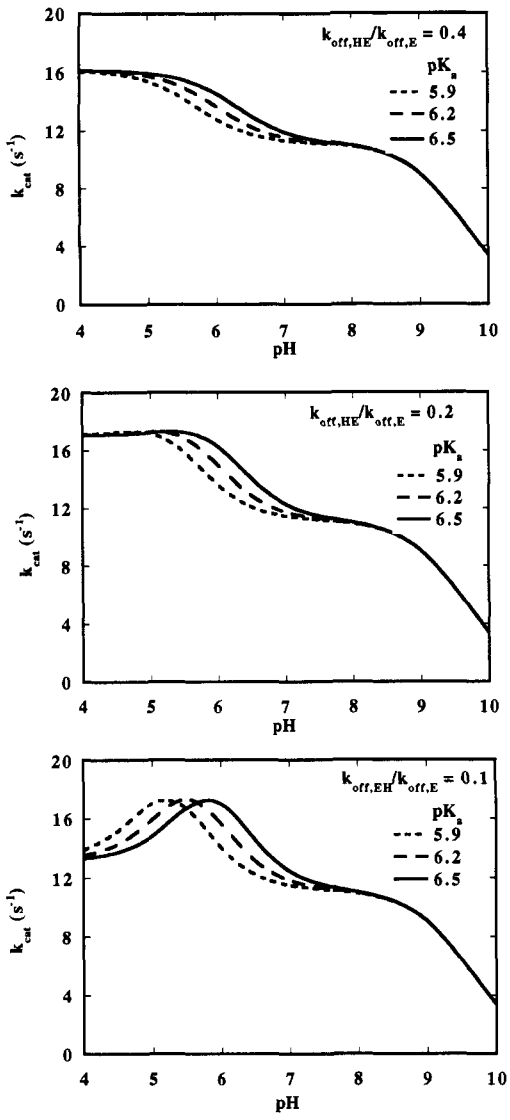

FIGURE 5: Simulated dependence of k_{cat} on pH. Each panel contains three curves corresponding to different values of $pK_{a,EL}$ (given in the figure). The difference in the results between panels is due to the choice of $k_{off,HE}/k_{off,E}$ (given in figure). Simulations were carried out with 100 μM NADPH and 100 μM H_2folate.

The results of computer simulations are shown in Figure 5. A pK_{a1} of 5.9–6.2 and $k_{off,HE}/k_{off,E}$ 0.2–0.4 gave curves that correctly predicted general dependence on pH. The residual discrepancy between these predictions and experimental values is probably due mainly to the simplifying assumption of a single value of $k_{off,HE}/k_{off,E}$ for dissociations of both H_2folate and H_4folate from all their complexes, which is contrary to observation (Table I). However, in the case of H_4folate, further departure from prediction may also be caused by the protonation of N^5 with a pK_a of 4.8 (Kallen & Jencks, 1966).

It is not intuitively obvious that $k_{off,E} > k_{off,HE}$ will cause the pH dependence of k_{cat} at low pH, but this is clearer when one considers the distribution of enzyme among its complexes during approach to steady state (Appleman et al., 1990) at pH 7.65 and at pH 5 (Figure 6). When substrates are saturating, three complexes are present in major amounts at steady state: E·NADP·H_4folate, E·NADPH·H_4folate, and E·NADP·H_2folate.[3] At pH 7.65 (Figure 6A), the last pre-

[3] At 100 μM NADPH, a significant amount of enzyme (13%) is in the hDHFR·H_4folate complex at steady state.

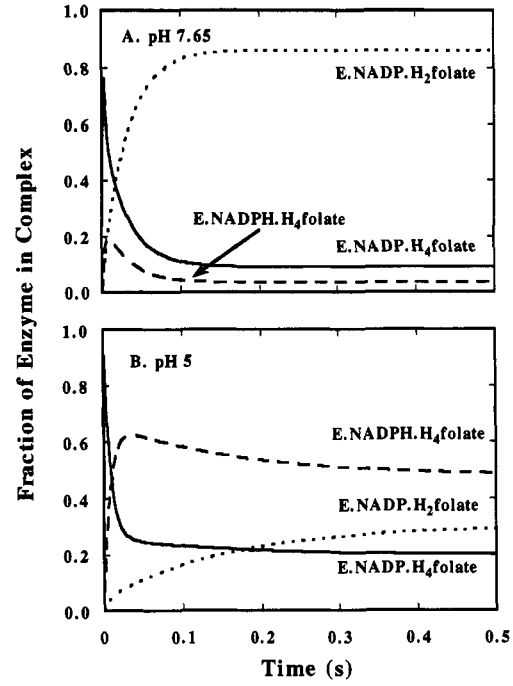

FIGURE 6: Simulated time courses at pH 7.65 (A) and at pH 5 (B) for the distribution of enzyme species as steady state is approached after initiation of catalysis by mixing H_2folate with enzyme preincubated with NADPH. The concentrations of NADPH and H_2folate are 1000 μM and 100 μM, respectively. The distribution has reached steady state by 0.5 s at pH 7.65, and at this time is nearly at steady state at pH 5.

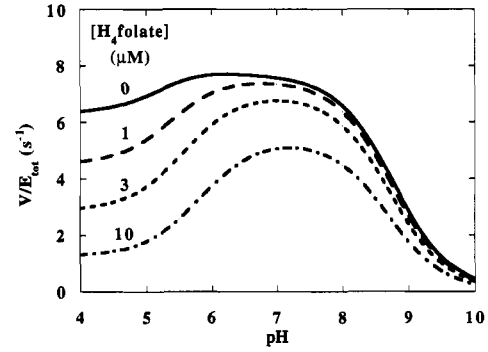

FIGURE 7: Simulated dependence of catalytic rate on pH with concentrations of substrates and products like those anticipated to occur in human tissue (Appleman et al., 1990). These are NADPH, 2000 μM; NADP, 20 μM; and H_2folate, 0.1 μM. The concentration of H_4folate for each curve is shown in the figure.

dominates at steady state because of the slow dissociation of NADP from this complex, and flux through the loop of the pathway involving this complex is therefore relatively slow but accounts for about two-thirds of the product release. At pH 5, substrate binding and hydride transfer are very fast (as at pH 7.65), but dissociation of H_4folate from the ternary product complex is slower, and this has two effects. More of the E·NADP·H_4folate complex is present at steady state, and less flux (8%) is diverted through the "bottleneck" complex, E·NADP·H_2folate, with less accumulation of the latter. However, the increase in k_{cat} is modest because the dissociation of H_4folate from E·NADPH·H_4folate also decreases by a factor of 7 and becomes the rate-limiting step.

Evolutionary Significance of Increased Dissociation of Folates with DHFR Protonation. The efficiency of an enzyme is indicated by the turnover rate at intracellular substrate concentrations, not by k_{cat}, the reaction rate with saturating substrates (Albery & Knowles, 1976). For DHFR this means that it is the rate at low H_2folate concentration (≈0.1 μM)

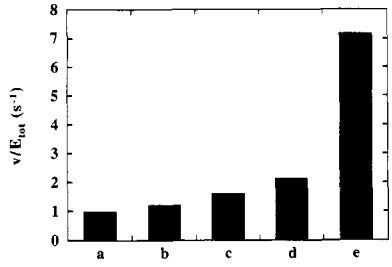

FIGURE 8: Effect of various factors in the evolution of the catalytic efficiency of hDHFR. (a) v/E_{tot} at 20 °C and pH 7.4 for ecDHFR under conditions similar to those occurring in this organism (Benkovic et al., 1988): NADPH, 1000 μM; NADP, 1500 μM; H_2folate, 0.3 μM; H_4folate, 13 μM. Rate constants for this computation were taken from Fierke et al. (1987) and adjusted to 20 °C assuming $Q_{10} = 2$. (b) v/E_{tot} for ecDHFR at 20 °C and pH 7.4, but under conditions more representative of eukaryotic cells (Appleman et al., 1990): NADPH, 2000 μM; NADP, 20 μM; H_2folate, 0.1 μM; and H_4folate, 1 μM. (c) Calculation as for (b) but with pH-independent k_{hyd} increased 70-fold to that for hDHFR under these conditions. The internal equilibrium is maintained by proportionately increasing the value of $k_{r,hyd}$ which governs the rate of conversion of enzyme-bound NADP and H_4folate to enzyme-bound NADPH and H_2folate. (d) As for (c), but with k_{off} values for H_2folate and H_4folate dissociation from their complexes increased 10-fold. (e) As for (c), but with k_{on} and k_{off} values as determined for hDHFR.

that must be considered. Computer simulations of this rate (v/E_{total}) for DHFR as a function of pH are shown in Figure 7. Unlike k_{cat}, v/E_{total} increases as the pH increases from 5 to 7 and is maximized near physiological pH. No such maximization of efficiency at physiological pH occurs in the case of ecDHFR. This maximization is intensified at higher concentrations of H_4folate. H_4folate levels are tissue specific, and are likely to range from about 0.4 μM to as high as 3 μM in human tissue.[4] H_4folate is reported to be especially abundant in liver and kidney, where total folate pools are elevated, and in brain, where an unusual distribution of reduced folate forms occurs.

Comparison of ecDHFR and hDHFR efficiencies under various conditions are shown in Figure 8. The efficiency of ecDHFR at concentrations of substrates present in bacteria (a) is about 16% of the efficiency of hDHFR at substrate concentrations present in eukaryotic cells (e). This difference is not due to substrate concentration differences (b), and even the 70-fold higher rate of hydride transfer contributes only slightly (c). However, the combination of these two factors with a 10-fold increase in k_{off} for folate (like that produced by pH dependence in the case of hDHFR) caused an almost 2-fold improvement in efficiency (d). Other factors, including differences in cooperativity of ligand binding, and in k_{on} values for substrates, produce the additional efficiency advantage of hDHFR over ecDHFR.

Studies of conservative amino acid substitutions in hDHFR at residues invariant in many species (Tsay et al., 1990; Beard et al., 1991) indicate that relatively small decreases in v/E_{total} constitute an apparently significant selective disadvantage. Consequently, the evolution of hDHFR (and probably other vertebrate DHFRs) seems to have required the incorporation of structural features that have produced both the extremely efficient hydride transfer system and also the more favorable k_{off} values for folates at physiological pH. Development of the latter is probably closely related to the increase of these k_{off} values with deprotonation of enzyme.

[4] These values are indirectly derived from reports of H_4folate concentration and distribution of folates in human cell lines and in mammalian tissues from nonhuman sources and from concentrations of total reduced folates in human tissues (Allegra et al., 1986; Cichowicz & Shane, 1987a,b; Cossins, 1984; Green et al., 1988; Houghton et al., 1990; Kashanis & Cooper, 1985).

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Identification of the Phosphoribulokinase Sugar Phosphate Binding Domain†

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ABSTRACT: A recombinant form of *Rhodobacter sphaeroides* phosphoribulokinase (form I; NADH dependent) has been expressed in and purified to homogeneity from *Escherichia coli* that harbor the *prk*A gene in the plasmid pKP1565b. Restriction digestion of the phosphoribulokinase-encoding plasmid produces a tractable 450 bp fragment that encodes amino acid residues 28–179, which include a region (residues 42–54) highly conserved among phosphoribulokinase proteins. Using overlap extension polymerase chain reaction methodology, directed mutagenesis was performed to produce mutant proteins in which basic residues in this conserved region were replaced by neutral amino acids. Lysine-53, implicated by affinity labeling studies, has been replaced by methionine; little effect on substrate binding or catalysis is apparent. In contrast, when histidine-45 is replaced by asparagine, a 40-fold increase in the K_m for ribulose 5-phosphate results; a 200-fold increase results when arginine-49 is replaced by glutamine. Implication of this region as part of the sugar phosphate binding site is compatible with previous results that indicate targeting by an ATP analogue containing a reactive functionality esterified to the γ-phosphoryl group. The phosphoribulokinase reaction involves a single in-line phosphoryl transfer, requiring that the γ-phosphoryl of ATP be closely juxtaposed to the bound cosubstrate. It follows that any reactive group attached to the γ-phosphoryl in a nucleotide analogue that is bound to PRK in the absence of the cosubstrate will be favorably positioned to modify the sugar phosphate binding site.

Phosphoribulokinase (PRK;[1] EC 2.7.1.19) catalyzes a key step in Calvin's reductive pentose phosphate cycle, namely, the synthesis of the CO_2 acceptor ribulose 1,5-bisphosphate. The plant and algal PRKs are active as dimers and typically exhibit turnover numbers (Krieger & Miziorko, 1986; Porter et al., 1986) that are 1 order of magnitude higher than some octameric bacterial PRKs (Tabita, 1988). As might be expected for an enzyme that catalyzes a key metabolic step, PRK activity is subject to regulation. Plant and algal PRKs are interconverted between active and inactive forms by thiol/disulfide exchange (Buchanan, 1980) while bacterial PRKs are subject to allosteric regulation (Abdelal & Schlegel, 1974; Tabita, 1980; Rippel & Bowien, 1984).

The active site of spinach PRK has been investigated in some detail. The N-terminal portion of the protein has been identified as a consensus ATP binding domain and has, in fact, been affinity-labeled by reactive ATP analogues (Kreiger & Miziorko, 1986; Kreiger et al., 1987). Cysteine-16 in this domain is not catalytically essential (Porter & Hartman, 1988) and is not conserved in prokaryotic PRKs but controls activity in eukaryotic PRKs by reversibly forming a disulfide with cysteine-55 (Porter et al., 1988). Affinity labeling with lysine-directed reactive ATP analogues has recently implicated the region around lysine-68 as part of the catalytic domain (Miziorko et al., 1990). Upon mapping lysine-68 within the catalytic site, we suggested that this basic residue might function to stabilize binding of the nucleotide substrate (Miziorko et al., 1990). As initial sequence information on bacterial PRKs became available, the presence of a homologous lysine in the eukaryotic enzyme was suggested (Kossman et al., 1989) as well as discounted (Gibson et al., 1990). Thus, the significance and function of this residue remain to be established.

Systems for heterologous expression of *Rhodobacter sphaeroides* PRK have been developed by Hallenbeck and Kaplan (1987) and by Gibson and Tabita (1987). We have adopted Hallenbeck and Kaplan's expression system for the allosterically controlled PRK A (form I) in order to develop a model system useful for exploring structure/function correlations that account for PRK's regulation and catalysis. This report provides an indication of the value of this experimental

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[1] Abbreviations: PRK, phosphoribulokinase; Ru5P, ribulose 5-phosphate; RuBP, ribulose 1,5-bisphosphate; IPTG, isopropyl thiogalactoside; oePCR, overlap extension polymerase chain reaction.

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