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Protein Engineering of Dihydrofolate Reductase. pH Dependency of Phe-31 Mutants

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Site-specific mutations on dihydrofolate reductase from *Escherichia coli* at the Phe-31 site have generated (Try-31)-DHFR and (Val-31)-DHFR mutant enzymes. The pH dependence of log V and log V/K_{DHF} for these enzymes suggests that protonation is important for both the interaction of dihydrofolate and the maximum velocity of the reaction. More importantly, a "hollow" is observed for the Tyr-31 mutant in a log V/K-pH profile, necessitating a modification of the wild-type kinetic scheme. The intrinsic pK_a of 5.8, obtained based on the modified more general kinetic scheme, for the Tyr-31 mutant agrees well with that obtained from inhibition studies by 2,4-diamino-6,7-dimethylpteridine.

During the last two decades the interest and research in enzyme chemistry has expanded so rapidly with applications of physical organic techniques[1] that it now occupies an important position in chemistry as a whole. Moreover, recent recombinant DNA technology has revolutionized our ability to tailor the structure and activity of enzymes by manipulation of their genes.[2-8]

As described in the preceding paper,[8] our interest in the mechanism of dihydrofolate reductase (DHFR), which catalyzes the reduction of dihydrofolate (H_2-folate) to tetrahydrofolate (H_4-folate) with NADPH as the cofactor,[9] has led us to carry out site-specific mutations on this enzyme at the Phe-31 site. From the crystallographic structure[10,11] the Phe-31 is located at the dihydrofolate binding site and interacts with both the pteridine ring and the *p*-aminobenzoyl moiety of the substrate.

The first mutation (Phe-31 → Tyr-31) has resulted in an only five-fold increase in K_{DHF} (K_M with saturating NADPH and varying dihydrofolate), despite the fact that the mutation has introduced a polar Tyr-group into a hydrophobic binding site, and a 2.5-fold increase in the maximum velocity. Similarly, the second mutation (Phe-31 → Val-31) resulted in a 25-fold increase in K_{DHF} with a 2.2-fold increase in the maximum velocity. Thus, in both mutant enzymes the decrease in binding has not been translated into a loss of catalytic efficiency.

We report here the pH dependency of log V and log V/K_{DHF} for these mutant enzymes. Although the pH dependencies of these values for the Val-31 mutant DHFR are clearly half-bell-shaped as they are for the wild-type DHFR,[12] a "hollow"[13] is observed for the Tyr-31 DHFR. The kinetic scheme proposed for the wild-type DHFR[12] is inadequate to explain this "hollow" phenomenon, necessitating a modification of the wild-type kinetic scheme. The modified more general kinetic scheme may also be applicable to explain the wild-type steady-state kinetic data.

Experimental

Mutant Enzymes. Preparation of the mutant enzymes (Tyr-31 and Val-31) has been described.[8]

Kinetics and Data Analysis. Initial velocities for dihydrofolate reductase were determined at 25 °C following the disappearance of NADPH and DHF at 340 nm (ε=11800 M^{-1} cm^{-1} (1 M=1 mol dm^{-3})). The buffer used for the assays contained 50 mM 2-morpholinoethanesulfonic acid (MES), 25 mM tris(hydroxymethyl)aminomethane (Tris), 25 mM 2-aminoethanol, and 100 mM NaCl (MTEN buffer). The concentration of NADPH was maintained constant at 60 μM. The concentrations of DHF and enzymes are listed in Table 1.

Data obtained at each pH value by varying the concentration of DHF were fitted to Eq. 1 by a nonlinear computer fitting program to yield values for the maximum velocity (V) and the Michaelis constant (K_a) as well as for V/K_a.

$$v = \frac{V[\mathrm{DHF}]}{K_a + [\mathrm{DHF}]}. \quad (1)$$

The pH-rate profiles were fitted to Eq. 2, and for the special case with a "hollow" phenomenon (Fig. 2(c)) Eq. 3 was used.

Table 1. Summary of pH-Independent Values of V, V/K_{DHF}, K_{DHF}, Apparent p$K_a(V)$, and p$K_a(V/K)$ Values for Tyr-31 and Val-31[a]

	V/s^{-1}	V/K / 10^{-6} M^{-1} s^{-1}	K_{DHF}/μM	p$K_a[V]$	p$K_a[V/K]$
Wild-type	12	11	1.1[b]	8.4[b]	8.1[b]
Tyr-31	30	5.3	5.7	7.9	6.8
Val-31	26	0.97	27	7.4	6.9

a) Conditions: 60 μM NADPH, 3.25—120 μM H_2-folate, 1.1—500 nM (Tyr-31)-DHFR, 6.1—730 nM (Val-31)-DHFR, MTEN buffer, 25 °C, 340 nm. b) Taken from Ref. 12.

$$V = \frac{V_{max}}{\left\{1+\frac{K_a}{(H)}\right\}} \quad \text{or} \quad V/K = \frac{(V/K)_{max}}{\left\{1+\frac{K_a}{(H)}\right\}} \tag{2}$$

$$V/K = \frac{C_1\left\{1+\frac{C_2K_1}{(H)}\right\}}{\left\{1+\frac{K_1}{(H)}\right\}\left\{1+\frac{C_3K_1}{(H)}\right\}} \tag{3}$$

Results

Table 1 summarizes the V, V/K, K_M, and pK_a values observed in pH-rate profiles determined for the purified mutant enzymes, Tyr-31 and Val-31. The reported V and V/K terms are pH-independent. The kinetic experiments were performed under conditions similar to those employed by Stone and Morrison.[12] Both of these mutants have V values significantly larger than that of wild-type, while the V/K values are smaller. Also, each mutation results in a pK_a shift to lower pH in both the V and V/K profiles.

The Tyr-31 mutant has a 2.5-times higher V value than that of wild-type in the pH independent region, indicating that, under saturating DHF conditions, the Tyr-31 mutant is a better catalyst than the wild-type enzyme. The increase in V may in one interpretation result from the accelerated product release step which may constitute a significant part of the rate determining step. The Tyr-31 mutant has only a two-fold smaller value for V/K_{DHF} owing to a 5-fold increase in K_{DHF} at low pH. At high pH values, however, the K_{DHF} value increases to 300 μM (pH 9.5), possibly due to the ionization of Tyr, drastically decreasing the hydrophobicity of the side chain.

The effect of the Val-31 mutation is similar; a 25-fold increase in K_{DHF} and a 2.2-fold increase in V. This increase in K_{DHF} is probably a reflection of a higher K_D value for H_2-folate, by analogy with a parallel increase in the dissociation constant for methotrexate resin. The decrease in the apparent pK_a value could be due to the introduction of water in the active site to fill the volume originally occupied by the Phe-31. However, the pK_a value of 8.1 of wild-type in the V/K profile may not reflect the intrinsic pK_a as originally thought.[12] This point will be discussed in more detail in the next section.

In Fig. 1 are shown the pH dependence of log V and log V/K_{DHF} for the reaction catalyzed by the Val-31 mutant DHFR. Values for V and V/K_{DHF} were determined over the pH range 5.0—9.8. The pH dependence of these values suggests that protonation is important for both the interaction of DHF and the maximum velocity of the reaction. The initial velocities, especially at pH values less than 6.0, were corrected for the decomposition of NADPH. Both profiles fit to the theoretical lines calculated from Eq. 2 with the parameters given in Table 1.

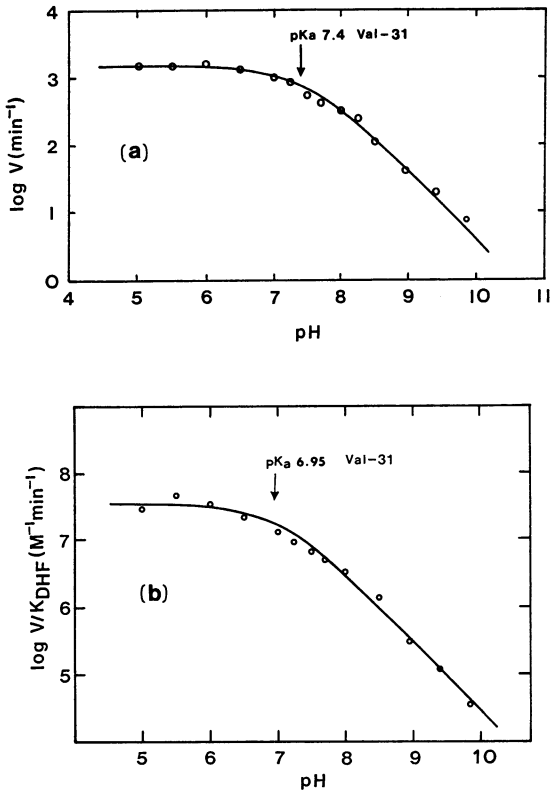
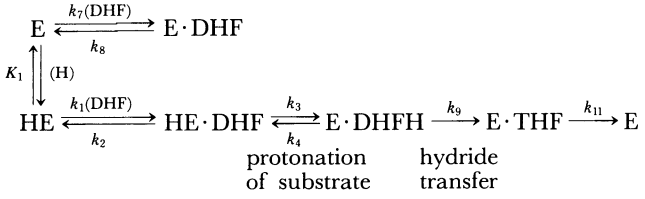


Fig. 1. (a) The pH dependence of log V for the reaction catalyzed by the Val-31 mutant DHFR. (b) The pH dependence of log V/K_{DHF} for the Val-31. The lines represent a best fit to Eq. 2 with the parameters given in Table 1.

Figure 2 illustrates the analogous pH dependence of log V and log V/K_{DHF} for the Tyr-31 mutant. Again, the overall pH dependence of these values indicates that the protonation is important for both the interaction of DHF and the maximum velocity of the reaction. The theoretical lines for Fig. 2(a) and (b) were drawn according to Eq. 2 with the apparent pK_a's shown in the profiles also given in Table 1. Although the theoretical curve in Fig. 2(a) fits the experimental points satisfactorily,that of Fig. 2(b) is inadequate because of the observed "hollow". The use of Eq. 2 for describing the data in Fig. 2(b), was based on the assumption that the kinetic scheme for Tyr-31 is same as that of the wild-type DHFR proposed by Stone and Morrison:[12]



Scheme 1.

where E denotes the enzyme-NADPH binary complex since the kinetic studies were carried out under the saturation with NADPH. The V/K_{DHF} equation for

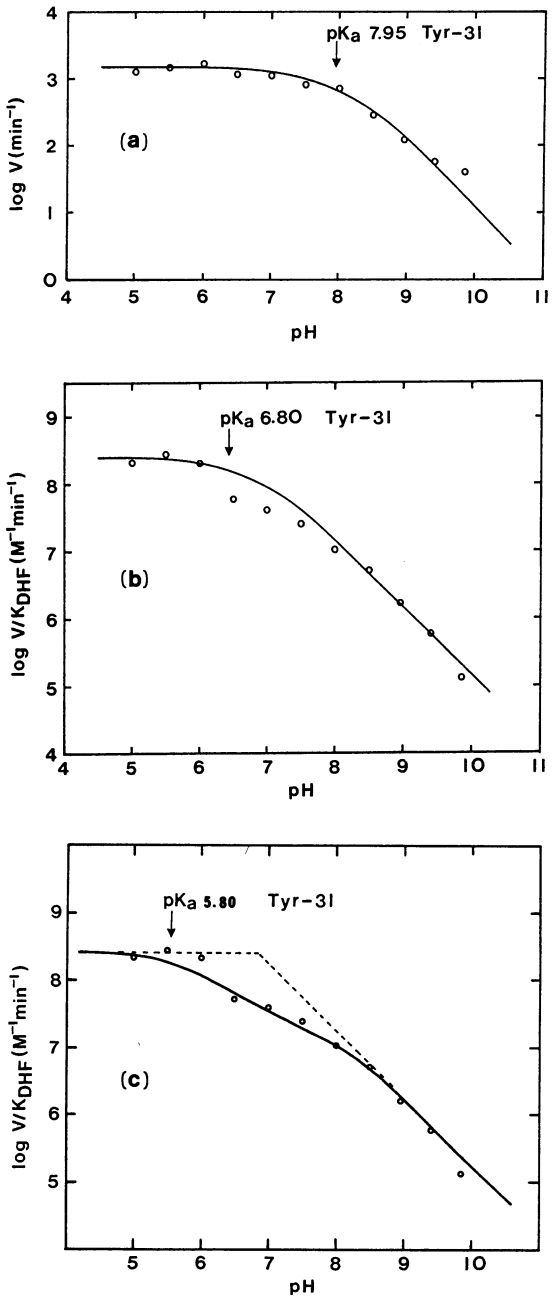


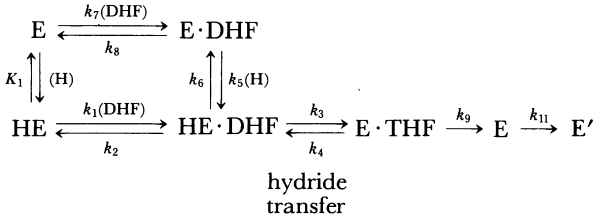
Fig. 2. (a) The pH dependence of $\log V$ for the Tyr-31, where the solid line is the theoretical curve based on Eq. 2. (b) The pH dependence of $\log V/K_{DHF}$ for the Tyr-31, where the theoretical line is based on Eq. 2. (c) The pH dependence of $\log V/K_{DHF}$, same as in (b), for the Tyr-31, where the theoretical line is drawn according to Eq. 7 with the intrinsic pK_a of 5.8 and the conditions of $k_1=10\,k_7$, $k_3=10\,k_6$, $k_6=10\,k_2$, and $k_9=10\,k_4$. The intercept of the dashed line corresponds to the apparent pK_a of 6.8.

the reaction described by Scheme 1 is given by

$$\frac{V}{K}=\frac{k_1k_3k_9\ (E_T)}{(k_2k_4+k_2k_9+k_3k_9)\left\{1+\dfrac{K_1}{(H)}\right\}} \tag{4}$$

which contains only one pH-dependent term yielding a half-bell-shaped curve of the type illustrated in Fig. 1(b).

Since the Eq. 4 derived on the basis of Scheme 1 cannot adequately describe the "hollow" phenomenon[13] of the Tyr-31 mutant shown in Fig. 2(b), the kinetic scheme proposed by Stone and Morrison was expanded to the following more general case:



Scheme 2.

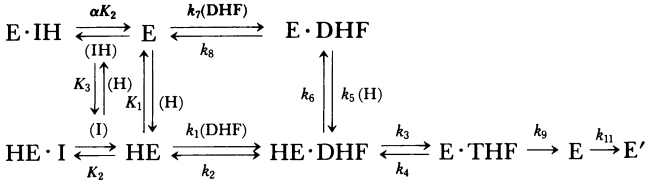
where E and E′ denote the enzyme-NADPH complex and free enzyme, respectively. The hydride transfer and protonation of DHF were combined into a single, reversible step (k_3, k_4) with k_9 and k_{11} representing rate coefficients associated with product release. The initial velocity equation for the reaction described by Scheme 2 is given by:

$$\nu=\frac{k_1k_3k_9(k_8+k_5H+\dfrac{k_5k_7K_1}{k_1})(E_T)(DHF)}{\begin{aligned}&(k_3k_7k_9+k_6k_7k_9+k_4k_6k_7+k_2k_4k_7+k_2k_7k_9+k_5Hk_7k_9+\\&k_4k_5Hk_7+k_3k_5Hk_7+k_3k_5Hk_7k_9/k_{11})\cdot\frac{K_1}{(H)}\\&+k_1(k_4k_6+k_6k_9+k_4k_5H+k_5Hk_9+k_4k_8+k_8k_9+\\&k_3k_5H+k_3k_8+k_3k_5Hk_9/k_{11}+k_3k_8k_9/k_{11})\\(DHF)+&\frac{}{(k_3k_8k_9+k_6k_8k_9+k_4k_6k_8+k_3k_5Hk_9+k_2k_4k_5H\\&+k_2k_5Hk_9+k_2k_4k_8+k_2k_8k_9)\cdot\left\{1+\frac{K_1}{(H)}\right\}\\&\frac{}{(k_3k_7k_9+k_6k_7k_9+k_4k_6k_7+k_2k_4k_7+k_2k_7k_9\\&+k_5Hk_7k_9+k_4k_5Hk_7+k_3k_5Hk_7+\\&k_3k_5Hk_7k_9/k_{11})\cdot\frac{K_1}{(H)}+k_1(k_4k_6+k_6k_9+k_4k_5H+\\&k_5Hk_9+k_4k_8+k_8k_9+k_3k_5H+k_3k_8+\\&k_3k_5Hk_9/k_{11}+k_3k_8k_9/k_{11})\end{aligned}} \tag{5}$$

The V and V/K_{DHF} relationships for the mechanism given in Scheme 2 are described by Eqs. 6 and 7.

$$V=\frac{\dfrac{k_3k_9k_{11}(E_T)}{k_3k_9+k_3k_{11}+k_4k_{11}+k_9k_{11}}\left\{1+(1+\dfrac{k_2}{k_6})\dfrac{k_7K_1}{k_1(H)}\right\}}{\begin{aligned}&1+\left\{\frac{k_7}{k_1}+(\frac{k_8}{k_6}+\frac{k_4k_{11}+k_9k_{11}}{k_3k_9+k_3k_{11}+k_4k_{11}+k_9k_{11}})\frac{k_2k_7}{k_1k_8}\right\}\frac{K_1}{(H)}\\&+\left\{\frac{k_7k_{11}(k_2k_4+k_2k_9+k_3k_9+k_4k_6+k_6k_9)}{k_1k_5(k_3k_9+k_3k_{11}+k_4k_{11}+k_9k_{11})}\right\}\frac{K_1}{(H)^2}\end{aligned}} \tag{6}$$

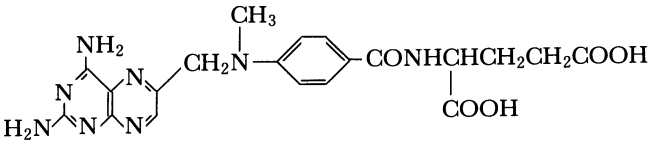
$$\frac{V}{K}=\frac{\dfrac{k_1k_3k_9(E_T)}{k_2k_4+k_9(k_2+k_3)}\left\{1+(1+\dfrac{k_2}{k_6})\dfrac{k_7K_1}{k_1(H)}\right\}}{\left\{1+\dfrac{K_1}{(H)}\right\}\left\{1+(1+\dfrac{k_6}{k_2+\dfrac{k_3}{1+\dfrac{k_4}{k_9}}})\cdot\dfrac{k_2k_7K_1}{k_6k_1(H)}\right\}} \quad (7)$$



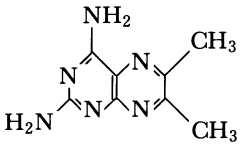
Scheme 3.

The Eq. 7, from which the individual rate constants can be combined to form constants C_1, C_2, and C_3 of Eq. 3, has three pH-dependent terms. Thus, depending on the magnitude of C_2 and C_3 in Eq. 3, a fit for the "hollow" in the pH-log V/K profile can be generated. The theoretical curve for the pH-log V/K_{DHF} profile of the Tyr-31 mutant is drawn in Fig. 2(c) according to Eq. 3 with the following parameters: $C_1=5.3\times10^6$ M^{-1} s^{-1} (pH-independent value of V/K_{DHF} in Table 1); $C_2=0.1$, $C_3=0.01$, and the intrinsic pK_a of 5.8 (pK_1 =5.8). Although the individual rate constants in Eq. 7 are not known, the condition of $C_2=0.1$ and $C_3=0.01$ may be met, as one of several possibilities, when $k_6/k_2=k_3/k_6=k_1/k_7=k_9/k_4=10$ in Eq. 7.

Shown in Fig. 3 is the pH dependence of inhibition for the Tyr-31 mutant by a substrate analogue, 2,4-diamino-6,7-dimethylpteridine, which differs from methotrexate (MTX) by the absence of the side chain. Although the pK_a value determined from a V/K profile may be displaced, the analysis of the pH dependence of



MTX(methotrexate)



2,4-diamino-6,7-dimethylpteridine

the inhibition caused by a substrate analogue will generally[14] yield the correct[12,13] pK_a value. The variation with pH of the inhibition of the wild-type DHFR by 2,4-diamino-6,7-dimethylpteridine yielded a bell-shaped curve with two pK_a values of 5.9 and 7.9.[12] The lower pK_a value corresponds to the value of 5.75 determined for the protonation of the N-1 nitrogen of the pteridine.[12,15] As can be seen from Fig. 3, however, the mutation Phe-31 → Tyr-31 has shifted the higher pK_a value of the wild-type, Phe-31, to a much lower pH region. The solid line in Fig. 3 is drawn according to Eq. 8 based on Scheme 3.

In Eq. 8, K_i(app) denotes the apparent inhibition constant which can be obtained by plotting $1/v$ vs. the inhibitor concentration (Dixon plot).[16] K_1, K_2, and K_3 are defined in Scheme 3, of which pK_3, the protonation

$$K_i(\text{app})=\frac{K_2\left\{1+\dfrac{(H)}{K_3}+\dfrac{K_1}{(H)}+\dfrac{K_1}{K_3}\right\}}{1+\dfrac{K_1}{\alpha K_3}}, \quad (8)$$

of the N-1 nitrogen of the pteridine inhibitor, is already reported to be 5.75.[15] Since, at low pH, the inhibition of the Tyr-31 mutant by 2,4-diamino-6,7-dimethylpteridine was comparable to that of the wild-type enzyme, the pH independent value of the inhibition constant, K_2, was assumed to be 13 nM which was the reported value for the wild-type enzyme.[12] Assuming that α equals unity, two theoretical lines are drawn in Fig. 3: The intrinsic pK_a of the enzyme, pK_1, is assumed to be 5.8 for the solid line, and 6.8 for the dashed line. Although the experimental points are limited, the solid line with the intrinsic pK_a of 5.8 better fits to the experimental data. This intrinsic pK_a of 5.8, obtained by the inhibition study, agrees well with the intrinsic pK_a of 5.8 obtained from the "hollow" profile of Fig. 2(c).

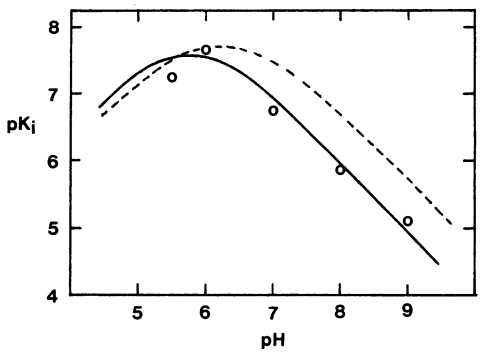


Fig. 3. Variation with pH of the pK_i ($-\log K_i$) value of the Tyr-31 mutant for 2,4-diamino-6,7-dimethylpteridine. The units for K_i are M. The theoretical curves are drawn according to the Eq. 8 with the parameters of pK_3=5.75, K_2=13 nM, α=1, and for the solid curve pK_1=5.80, and for the dashed curve pK_1=6.80.

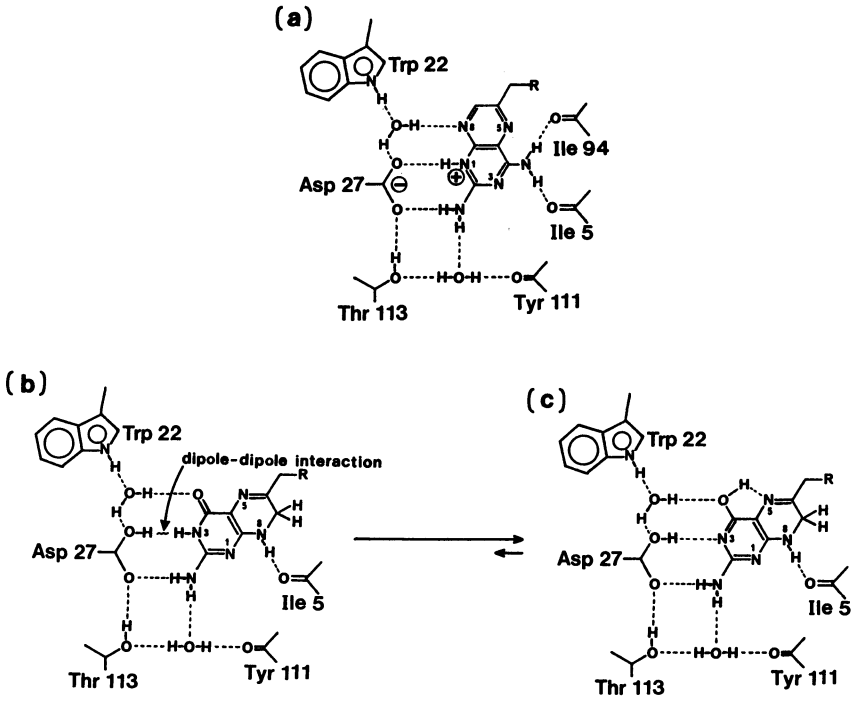


Fig. 4. Schematic representation of hydrogen bonding interaction between dihydrofolate reductase and the pteridine portions of (a) methotrexate and (b), (c) 7,8-dihydrofolate. At neutral pH, the enzyme bound methotrexate is protonated, on the other hand, the enzyme bound dihydrofolate is not protonated.

Discussion

General Kinetic Scheme with Saturating NADPH. From the variation of V and V/K_{DHF} with pH in Figs. 1 and 2, it is apparent that protonation enhances the maximum velocity, V, of the dihydrofolate reductase reaction as well as the flux rate V/K_{DHF}, with the enzyme-NADPH binary complex to yield a productive complex. For the wild-type *E. coli* DHFR, activity is dependent on a group with a pK_{a1} of about 8 that must be protonated, and on another group with a pK_{a2} of about 5 that must be unprotonated.[17] Moreover, it has been suggested that Asp-27 is responsible for the pK_a of 8.[12] In view of the conclusion that DHF is bound to DHFR in an inverted fashion relative to the binding of an inhibitor such as methotrexate and, at neutral pH, productive binding must occur as a result of reaction between unprotonated DHF and protonated enzyme (protonated Asp-27), a modified representation[10] of the hydrogen bonding interaction between DHFR and DHF based on the X-ray crystallographic structure of the methotrexate-DHFR complex is given in Fig. 4(a). One may speculate that tautomerization of the initial complex relieves an unfavorable dipole-dipole interaction and promotes protonation of the N-5 nitrogen, thereby facilitating the following hydride transfer reaction (Figs. 4(b) and (c)).

A general kinetic scheme with saturating NADPH has been proposed[12] as reproduced in Scheme 1. The V/K_{DHF} expression for the reaction described by Scheme 1 is given by Eq. 4. When log V/K_{DHF} is plotted versus pH, the curve described by Eq. 4 is half-bell-shaped with asymptotes given by Eqs. 9 and 10 at low and high pHs, respectively.

$$\log V/K_{DHF} = \log \left(\frac{k_1 k_3 k_9 E_T}{k_2 k_4 + k_2 k_9 + k_3 k_9} \right) \quad (9)$$

$$\log V/K_{DHF} = \log \left(\frac{k_1 k_3 k_9 E_T}{k_2 k_4 + k_2 k_9 + k_3 k_9} \right) - pH + pK_1 \quad (10)$$

It is apparent from these equations that the intersection of the asymptotes with slopes of 0 (Eq. 9) and -1 (Eq. 10) for the plot of log V/K_{DHF} against pH will occur at the point where pH=pK_1 and thus yield a true value for the ionizing group on the enzyme. Namely, the pK_a of 8.1 obtained from the log V/K_{DHF}-pH profile of the wild-type enzyme must be the intrinsic pK_a of DHFR.

Although the pK_a of 6.9 for Val-31 mutant obtained from the log V/K_{DHF}-pH profile (Fig. 1(b)) may be considered to be the intrinsic pK_a of the Val-31 mutant presuming that the general kinetic scheme (Scheme 1) still is applicable for this mutant enzyme, the same interpretation cannot be ascribed to the Tyr-31 mutant. In order to accomodate the "hollow" phenome-

nom observed in the log V/K_{DHF}-pH profile for the Tyr-31 mutant, Scheme 1 has been expanded as shown in Scheme 2, where the proton on Asp-27 in the ternary HE-NADPH-DHF complex can now communicate with the solvent. The V/K_{DHF} relationship for the expanded mechanism is described by Eq. 3 or 7. Since there are three pH-dependent terms in these equations, the "hollow" phenomenon can be rationalized. The minimum requirement to observe a hollow in a log V/K_{DHF}-pH profile is that the varied substrate is sticky, that is, the substrate dissociates from the enzyme more slowly than it reacts to yield products ($k_2<k_3$). Otherwise, if $k_2>k_3$, the V/K_{DHF} expression reduces to Eq. 11, with a single dependency on pH.

$$\frac{V}{K}=\frac{k_1k_3k_9(E_T)}{k_2(k_4+k_9)\left\{1+\frac{K_1}{(H)}\right\}}=\frac{C_1'}{\left\{1+\frac{K_1}{(H)}\right\}} \tag{11}$$

From Eq. 3 the asymptotes of the log V/K_{DHF}-pH profile for the expanded mechanism described by Scheme 2 are calculated to give Eqs. 12 and 13 at low and high pHs, respectively.

$$\log V/K=\log C_1 \tag{12}$$

$$\log V/K=\log C_1+\log C_2-\log C_3-\mathrm{pH}+\mathrm{p}K_1 \tag{13}$$

Thus, a true pK_1 value will not be obtained from the log V/K-pH profile of the expanded mechanism since the intersection will occur at the point where:

$$\mathrm{pH}=\mathrm{p}K_1+\log C_2-\log C_3. \tag{14}$$

A theoretical line for the hollow of the Tyr-31 mutant is drawn in Fig. 2(c) according to Eq. 3 with the conditions of pK_1=5.8, C_1=5.3×10^6 (M^{-1} s^{-1}), C_2=0.1, and C_3=0.01. This fitting indicates that the apparent pK_a of Tyr-31 is shifted up by one pH unit from its intrinsic pK_1 of 5.8.

In order to confirm this intrinsic pK_a value of 5.8, inhibition studies by 2,4-diamino-6,7-dimethylpteridine were carried out according to Scheme 3. Judging from the two theorectical curves given in Fig. 3, the solid curve with pK_1=5.8 better fits to the experimental points than the dashed curve with pK_1=6.8 which were expected to be the intrinsic pK_a if the original mechanism of Scheme 1 were applicable to the Tyr-31 mutant enzyme. Thus, both the hollow and the inhibition studies are in accord with the intrinsic pK_a of 5.8 for the Tyr-31 mutant.

Conclusion

Examination of the engineered proteins (Tyr-31 DHFR and Val-31 DHFR) has clarified the kinetic sequence for wild-type DHFR. Specifically, the observation of a hollow in the pH-log V/K profile for the Tyr-31 mutant DHFR has necessitated a modification of the wild-type kinetic scheme (Scheme 1) to a more general case (Scheme 2), where the proton on Asp-27 in the ternary complex can now communicate with the solvent. This mechanism can adequately rationalize the kinetic data for both the wild-type and the mutant enzymes. In order to support further our mechanism, more detailed kinetic studies such as deuterium isotope effects and pre-steady state kinetics have been under taken.

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