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Role of the Conserved Active Site Residue Tryptophan-24 of Human Dihydrofolate Reductase As Revealed by Mutagenesis†

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ABSTRACT: The active sites of all bacterial and vertebrate dihydrofolate reductases that have been examined have a tryptophan residue near the binding sites for NADPH and dihydrofolate. In cases where the three-dimensional structure has been determined by X-ray crystallography, this conserved tryptophan residue makes hydrophobic and van der Waals interactions with the nicotinamide moiety of bound NADPH, and its indole nitrogen interacts with the C^4 oxygen of bound folate through a bridge provided by a bound water molecule. We have addressed the question of why even the very conservative replacement of this tryptophan by phenylalanine does not seem to occur naturally. Human dihydrofolate reductase with this replacement of tryptophan by phenylalanine has increased rate constants for dissociation of substrates and products and a considerably decreased rate of hydride transfer. These cause some changes in steady-state kinetic behavior, including substantial increases in Michaelis constants for NADPH and dihydrofolate, but there is also a 3-fold increase in k_{cat}. The branched mechanistic pathway for this enzyme has been completely defined and is sufficiently different from that of wild-type enzyme to cause changes in some transient-state kinetics. The most critical changes resulting from the amino acid substitution appear to be a 50% decrease in stability and a decrease in efficiency from 69% to 21% under intracellular conditions.

Among amino acid residues at the active site of dihydrofolate reductase (DHFR)[1] are several that are conserved in all the known sequences of the enzyme from vertebrate species. Although bacterial DHFRs have structures congruent with the vertebrate enzyme, their sequence homology with vertebrate DHFR is quite low. Nevertheless, some of these active site residues are conserved in bacterial DHFR sequences as

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[1] Abbreviations: DHFR, dihydrofolate reductase; ecDHFR, *Escherichia coli* DHFR; lcDHFR, *Lactobacillus casei* DHFR; rhDHFR, recombinant human DHFR; 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; MTX, methotrexate; NADPD, (4*R*)-[^{2}H]NADPH; MES, 2-(*N*-morpholino)ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; wt, wild type; W24F, mutant rhDHFR with $Trp^{24} \rightarrow Phe$. $k_{cat} = V_{max}/[E]$, where V_{max} is the steady-state maximum velocity and [E] is the enzyme concentration.

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well. Among these is a tryptophan,[2] Trp[24], which is near the binding sites for both folate and NADPH (Filman et al., 1982; Volz et al., 1982; Stammers et al., 1987; Oefner et al., 1988). Elucidation of the evolutionary advantage conferred by this residue, compared with another aromatic amino acid, might give insights about the catalytic mechanism. Accordingly, we have investigated a mutant of recombinant human DHFR (rhDHFR) in which Trp[24] is replaced by phenylalanine (W24F rhDHFR) (Huang et al., 1989).

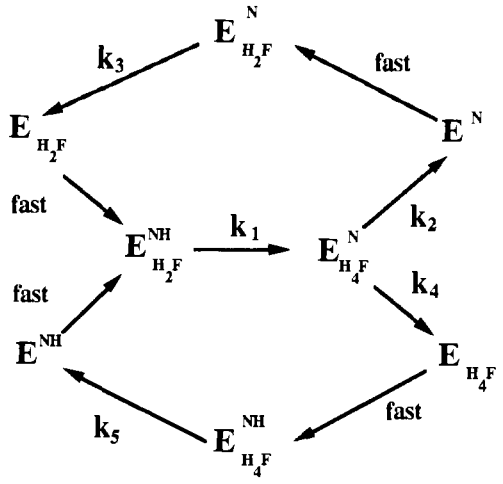
Several explanations for the strict conservation of Trp[24] can be suggested. It might be related to proper binding of one or both substrates. A second possibility is that substitution of another side chain for that of Trp[24] might disturb the proximity and orientation of the nicotinamide and pteridine moieties of the substrates, to which the rate of hydride transfer is very sensitive (Wu & Houk, 1987; Benkovic et al, 1988). Substitution of a less bulky side chain could also change the equilibrium between one or more of the conformational equilibria that have been shown to exist in rhDHFR apoenzyme and its complexes with ligands. These equilibria exert important effects on substrate binding and hydride transfer (Appleman et al., 1990a; Beard et al., 1989). Finally, substitution of a smaller side chain might result in less favorable hydrophobic packing with a consequent decrease in protein stability. We have characterized the behavior or W24F rhDHFR in order to evaluate the relative contribution of each of these factors to the selective advantage of a tryptophan at position 24. While this work was in progress, reports appeared of the substitution of other amino acids for Trp[24] in DHFR from other sources: Trp → Leu in *Lactobacillus casei* DHFR (Andrews et al., 1978) and Trp → Arg in mouse DHFR (Thillet et al., 1988). However, few details of kinetic behavior were reported in the latter study, in which a number of mutants of mouse DHFR were reported. The kinetic study of the *L. casei* DHFR mutant, though more detailed, explored changes only in selected rate constants, so that only a partial comparison with our study is possible. Amino acid substitutions at many other positions in the sequence of *Escherichia coli* DHFR have been reported [see, for example, Chen et al. (1987), Murphy and Benkovic (1989), and Adams et al. (1989)], as well as in rhDHFR (Schweitzer et al., 1989) and mouse DHFR (Thillet et al., 1988), but those studies do not address the same issues as the present investigation.

Materials and Methods

Unless otherwise noted, the materials and experimental methods used in this study are as previously described (Beard et al., 1989; Appleman et al., 1990a). Measurements were made at 20 °C in MATS buffer, pH 7.50. For experiments in which H_4folate was added to the reaction mixture, 50 mM 2-mercaptoethanol was also present.

Enzyme Preparations. Previously described methods were used in the preparation of mutant W24F rhDHFR (Huang et al., 1989). Fractions from the MTX affinity column containing enzyme activity were pooled, rapidly frozen in liquid nitrogen, and stored at −70 °C. To remove enzyme-bound H_2folate, 2.5 mL of enzyme solution was thawed and passed over a 1.5 × 5.1 cm prepackaged Sephadex G-25 M column equilibrated with 10 mM potassium phosphate, pH 7.5, to remove most of the H_2folate and exchange buffers. The remaining H_2folate was removed by loading the pooled enzyme-containing fractions from this column on a hydroxylapatite column (0.5 × 14 cm) equilibrated with 10 mM potassium phosphate, pH 7.5. The column was washed with 10 mL of the same buffer, and enzyme was eluted at a flow rate of 0.25 mL/min with a linear gradient buffer of potassium phosphate, pH 7.5, generated from 35 mL of 10 mM buffer and 35 mL of 200 mM. Two peaks of activity were combined separately, and each was assayed for enzyme-bound H_2folate by measuring the change in absorbance at 340 nm upon dilution of 3 μM enzyme with an equal volume of 80 μM NADPH, pH 7.5, using a stopped-flow spectrophotometer. The rate of the chemical transformation at this pH is slow enough with W24F rhDHFR for most of the conversion of enzyme-bound substrates to enzyme-bound products to be recorded, in contrast to wild-type rhDHFR (wt rhDHFR) (Beard et al., 1989). As reported earlier for H_2folate removal from bovine liver DHFR (Kaufman & Kemerer, 1976), the second peak was found to be substrate-free. Combined fractions were frozen in liquid nitrogen and stored at −70 °C either immediately or after exchanging the buffer with MATS by passing the enzyme preparation over a Sephadex G-25 M column.

Scheme I[a]



[a] NH = NADPH, N = NADP, H_2F = H_2folate, H_4F = H_4folate, k_{2-5} = product dissociation rate constants, and k_1 = forward rate constant for chemical transformation.

Determination of Association and Dissociation Rate Constants. When time courses for binding were used, the data were analyzed by equations for second-order or pseudo-first-order reactions (Appleman et al., 1988b). Dissociation rate constants were also determined by competition methods (Appleman et al., 1988a, 1990).

Calculation of the Rate Constant for NADP Dissociation from the Ternary Product Complex. The change in protein fluorescence associated with formation of this complex was too small for determination of the dissociation rate constant for NADP (k_4 in Scheme I) by direct binding or competition methods with W24F rhDHFR. The steady-state rate equation can be derived from the expressions for the steady-state distribution of ternary product complexes (see below), and the expression for k_4 is given by eq 1. This equation is applicable

$$k_4 = k_5(k_{cat}k_1k_2 + k_{cat}k_1k_3 + k_{cat}k_2k_3 - k_1k_2k_3)/(k_1k_3k_5 - k_{cat}k_1k_3 - k_{cat}k_3k_5) \quad (1)$$

provided substrates are at saturating concentrations, product concentrations are zero, and the rate of the reverse reaction is negligible. We have determined that k_5 is very high so that k_4 was calculated according to eq 2 from measured values of the other rate constants. As a check on this calculated value

$$k_4 = (k_{cat}k_1k_2 + k_{cat}k_1k_3 + k_{cat}k_2k_3 - k_1k_2k_3)/k_3(k_1 - k_{cat}) \quad (2)$$

[2] The sequence numbering used throughout is for rhDHFR.

of k_4, it was used in eq 2 to calculate the value of k_{cat} at pH 6.1. The rate constant k_1 for the chemical transformation was measured at this pH, and it was assumed that k_2, k_3, and k_4 have the values determined at pH 7.5. This calculation gave a value of 69 s^{-1} for k_{cat} at pH 6.1, as compared with an experimental value of 69 ± 3 s^{-1}.

Calculation of the Association Rate Constant for NADP. The competition method was that used previously (Appleman et al., 1988a, 1990a). The relationship between the observed rate constant, k_{obs}, and the concentrations of the dissociating ligand L_1 and the trapping ligand L_2 is

$$k_{obs} = (k_{-1}k_2L_2 + k_{-1}k_{-2} + k_1k_{-2}L_1)/(k_{-1} + k_1 + k_2L_2)$$

where k_1 and k_2 are association rate constants for L_1 and L_2, respectively, and k_{-1} and k_{-2} are corresponding dissociation rate constants. Since in these experiments $k_{-1} \ll k_2L_2 \gg k_{-2}$, this can be simplified without significant error to

$$k_{obs} = (k_{-1}k_2L_2 + k_1k_{-2}L_1)/(k_1L_1 + k_2L_2)$$

As discussed by Appleman et al. (1988a) and Tsay et al. (1990), when $k_2L_2 \gg k_1L_1$, but the condition $k_2L_2/k_{-2} \gg k_1L_1/k_{-1}$ is not met, k_{obs} is linearly related to $1/L_2$.

$$k_{obs} = k_{-1} + (k_1k_{-2}L_1/k_2)(1/L_2)$$

Such behavior was observed for E·NADPH with NADP as the competing ligand. In these experiments, the concentration of NADPH (L_1) was held constant so that the dependence of k_{obs} on $1/L_2$ is linear with ordinate intercept = k_{-1}, the dissociation rate constant for NADPH, and slope = $k_1k_{-2}L_1/k_2$. From the fit slope and from k_{-2} determined by relaxation, and the concentration of NADPH used, the association rate constant, k_2, for the competing ligand, NADP, can be calculated.

Calculation of K_D for Ligands in Ternary Complexes. In some instances where rate constants could not be directly measured, they could be calculated from thermodynamic equilibrium considerations. In the formation of a ternary complex from apoenzyme, two alternative pathways are possible for its formation, and the product of the dissociation constants for each pathway should be equal. From three of the four dissociation constants the remaining one can be calculated. If one of the binding constants (i.e., k_{on} or k_{off}) for this step is known, then the other can be calculated from the calculated dissociation constant (K_D).

Reverse Rate. The reverse rate was measured experimentally at pH 7.5 by preincubating 3 μM W24F rhDHFR with 3 mM NADP and rapidly diluting 2-fold with H_4folate (20–80 μM) at 20 °C in a stopped-flow spectrophotometer and collecting 800-ms time courses of NADPH formation (from absorbance increase at 340 nm). The rate constant for hydride transfer in the reverse reaction ($k_{r,H}$) was estimated to be 4.0 s^{-1} by simulations of these time courses by using a modified CRICF program (Appleman et al., 1990a). This value agrees well with a value that can be calculated from the overall equilibrium constant for the reaction as described by Appleman et al. (1990a), 2.3 s^{-1}.

Determination of the K_D for H_4folate in E·NADPH·H_4folate. Measurement of the dissociation rate constant of NADPH from the E·NADPH·H_4folate complex by competition requires that both ligands be saturating and that the trapping ligand (NADP) be in large excess over the ligand it is displacing (NADPH). If the second ligand (H_4folate) is not saturating, then the value of k_{off} obtained is an apparent one and is hyperbolically dependent on H_4folate concentration (Tsay et al., 1990). From the H_4folate dependence of the apparent k_{off} for NADPH from E·NADPH·H_4folate, an apparent K_D for H_4folate in this complex can be calculated. Competition experiments to determine k_{off} for H_4folate release from the E·NADPH·H_4folate complex revealed that k_{off} for H_4folate ≫ k_{off} for NADPH from this complex. Under these conditions, the K_D determined for H_4folate is a true K_D.

Table I: Steady-State Kinetic Parameters for Mutant and Wild-Type rhDHFR

	W24F[a]	wt[b]
$K_{m(H_2folate)}$ (μM)	2.5 ± 0.1	0.1
$K_{m(NADPH)}$ (μM)	3.4 ± 0.2	0.16, 4.2
k_{cat} (s^{-1})	39.8 ± 0.5	12.5
$K_{i(NADP)}$ (μM)	6.7 ± 0.5[c]	0.21[c]
$K_{i(H_4folate)}$ (μM)	43 ± 3[d]	activates (K_{act} 49 μM)

[a] At pH 7.5. [b] Values at pH 7.65. From Appleman et al. (1990a). [c] Initial velocities obtained in the presence of 40 μM NADPH, 50 μM H_2folate, and up to 200 μM NADP were fitted by a nonlinear least-squares method to the equation for linear competitive inhibition. In the case of wt enzyme $K_{m(NADPH)}$ of 0.16 was used. [d] Apparent value obtained in the presence of 50 μM NADPH, 10 μM H_2folate, and up to 100 μM H_4folate.

Simulation of Catalytic Behavior from Rate Constants. The modified CRICF computer program was used as described previously (Appleman et al., 1990a) to simulate the time courses of catalytic reactions, distribution of enzyme complexes during catalytic cycling, and the dependence of steady-state catalytic rates on concentrations of substrates and products. These latter rates were subsequently analyzed by the same methods employed with corresponding experimental data to obtain predicted values for steady-state parameters including K_ms for the substrates and K_is for the products.

Results

Steady-State Behavior of W24F rhDHFR. Comparison of the kinetic constants for mutant and wt rhDHFR (Table I) indicates that k_{cat} is actually increased more than 3-fold by the amino acid substitution, but that K_m for both substrates is increased more than 20 times. In the case of NADPH the comparison is with the lower of two K_m values seen for wt enzyme. Since the wt enzyme has a mechanism involving branching into several pathways, the two major ones both involving an abortive enzyme–substrate–product complex (Appleman et al., 1990a), the basis for the increases in K_m values is complex and requires determination of rate constants for reactions in both directions for each step in each pathway of the mechanism.

When the pH dependence of k_{cat} for W24F rhDHFR was investigated (Figure 1A), k_{cat} was found to increase 100-fold when the pH was decreased from 9.5 to 6.0. When this dependence was plotted in log form, it was clear that the increase in activity depends on protonation of a group (or, less probably, groups) with a single apparent pK_a of 7.6 ± 0.1 (Figure 1B). The wt enzyme shows no comparable changes of activity over the same pH range.

Part of the explanation for the altered dependence of activity on pH was revealed when the pH dependence of $^Dk_{cat}$ was examined. $^Dk_{cat}$ is the primary deuterium isotope effect and is defined by $k_{cat(NADPH)}/k_{cat(NADPD)}$. The variation of its value for W24F rhDHFR with pH is shown in Figure 1C. At pH ≥ 9 $^Dk_{cat}$ is 3.4, a value indicating that the hydride-transfer step is rate-limiting, but at pH 5–6 $^Dk_{cat} \approx 1$ so that the steady state is now limited entirely by other mechanistic steps, probably product dissociation. The activity/pH relationship and the apparent pK_a of 7.6 reflect this change in rate-limiting step as illustrated in Figure 1B. The apparent pK_a is, in fact, the pH at which the rate of hydride transfer equals the rate of product release (or other rate-limiting step). Such behavior

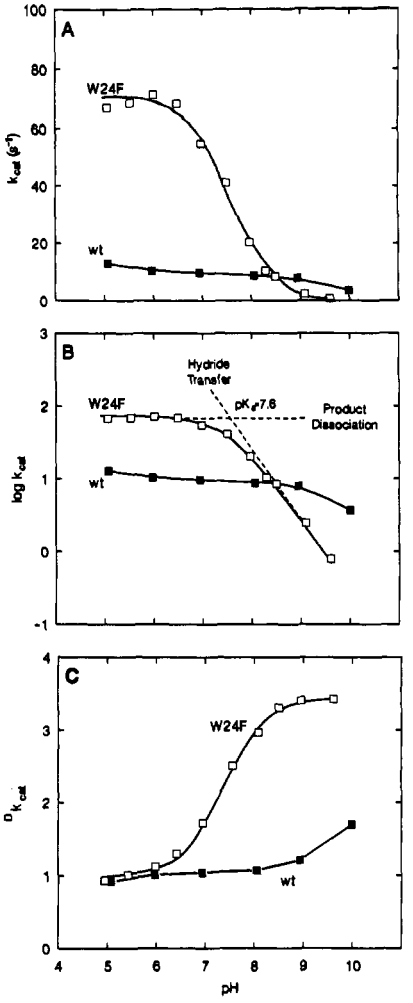


FIGURE 1: Dependence of k_{cat} and $^{D}k_{cat}$ on pH for wt and W24F rhDHFR. Reaction conditions were 100 μM substrates in a MATS buffer mixture at 20 °C. The data for the mutant enzyme were fitted to the equation $k_{cat} = {}^{*}k_{cat}/[1 + 10(pH - pK_a)]$, where $^{*}k_{cat}$ is the pH-independent rate of product release. (A) pH dependence of k_{cat}; (B) pH dependence of log k_{cat}; (C) pH dependence of $^{D}k_{cat}$ [= $k_{cat(NADPH)}/k_{cat(NADPD)}$]. The pH dependence of k_{cat} and $^{D}k_{cat}$ for wt rhDHFR is from Beard et al. (1989). Wild-type rhDHFR (wt, ■); W24F rhDHFR (W24F, □).

and its analysis for DHFR from *E. coli* was originally reported by Fierke et al. (1987). In the case of wt rhDHFR hydride transfer is not the rate-limiting step at any pH (Figure 1C) and only starts to contribute to limitation of the steady-state rate above pH 9.

Hydride Transfer. When product release is the only step that limits the steady-state rate of catalysis by an enzyme, rapid mixing of enzyme and substrates is followed by a burst of rapid product formation. This corresponds to chemical transformation of enzyme-bound substrates to enzyme-bound products. Thereafter, the rate of product formation is the constant steady-state rate governed by product release. The transient-state kinetics for W24F at pH 7.5 are shown in Figure 2. It is clear that the rate of formation of the first equivalent of product is not much faster than subsequent product formation. This indicates that the rate of hydride transfer is not much faster than the steady-state rate, which is consistent with $^{D}k_{cat} = 2.4$ at pH 7.5 (Figure 1C). This makes measurement of the "burst" rate difficult. Moreover, there is a continuing rate change during the formation of several equivalents of product. Such hysteretic behavior has been observed for wt rhDHFR (Appleman et al., 1989) and in that case is due to a change in the relative flux of reactants

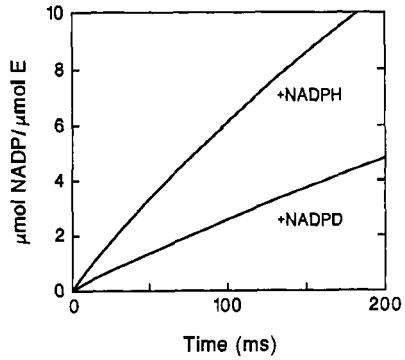


FIGURE 2: Transient-state catalysis: effect of NADPD on the time course of product formation by W24F rhDHFR. Enzyme was preincubated with H_2folate, and the reaction was initiated by rapidly mixing with either NADPH or NADPD. Final concentrations were as follows: W24F rhDHFR, 4.8 μM; substrates 100 μM. The final measured pH was 7.55 (20 °C).

Table II: Observed Transient-State Kinetics and Rate Constants for Isomerization and Hydride Transfer[a]

rhDHFR	pH	k_{burst}	$^{D}k_{burst}$	$k_{hydride}$	$k_{isomerization}$
wt[b]	7.65	1400	2.1	3000	2200
W24F	7.55	*c*	3.5[c]	79[c]	≫79
W24F	6.10	1700[d]	2.4	2600	3000

[a] Rate constants for $k_{hydride}$ and $k_{isomerization}$ were determined from k_{burst} and $^{D}k_{burst}$ as described previously (Beard et al., 1989). Values are for 20 °C. $^{D}k_{burst} = k_{burst(NADPH)}/k_{burst(NADPD)}$. [b] Values for wt enzyme are from Beard et al. (1989). [c] No true burst was observed. $k_{hydride}$ was the rate over 2–7 ms after mixing. See text and Figure 2. [d] Measured at 4 °C and corrected to 20 °C.

through the two pathways of a branched mechanism (Appleman et al., 1990a). After several turnovers under these conditions, product inhibition also begins to decrease the rate. Similar behavior would account for the observed W24F transient-state kinetics.

In view of these complexities the rate constant for hydride transfer was calculated from the initial rate of product formation over the period 2–7 ms after mixing. The initial rate was also measured with NADPD as substrate, and the ratio of these two rates gave the isotope effect $^{D}k_{burst}$ (Table II). Since $^{D}k_{burst}$ was high (3.5), the initial rate is entirely governed by hydride transfer and is identical with the rate of the latter.

At pH 6.1, the first turnover is so rapid at 20 °C it occurs during the dead time of the stopped-flow instrument. At lower temperature, a well-defined burst of product formation occurred and the rate constant and isotope effect could be determined in the usual way (Table II). The $^{D}k_{burst}$ of 2.4 at pH 6.1 indicates that both hydride transfer and another step contribute to limiting the rate of transformation of bound substrates to bound products. A similar result encountered for wt rhDHFR and other vertebrate DHFRs has been interpreted as indicating an obligatory isomerization of the ternary substrate complex (Beard et al., 1989), and the rate constant for the isomerization can be calculated as well as the rate constant for hydride transfer (Table II). At pH 7.55 hydride transfer is presumably much slower than isomerization.

The data in Table II clearly indicate that the rate of hydride transfer is greatly decreased by the Trp[24] → Phe substitution. This is consistent with the data in Figure 1, which indicate that, in contrast to wt enzyme, hydride transfer limits the steady-state rate for this mutant enzyme over much of the accessible pH range.

Association and Dissociation Rate Constants for Complexes of Mutant DHFR. In order to assess the effects of the amino acid substitution on ligand binding, k_{on} and k_{off} were deter-

Table III: Association Rate Constants for Ligand Binding to Wild-Type and W24F rhDHFR[a]

ligand	form of enzyme combining	$k_{on} \times 10^{-6}$ (M^{-1} s^{-1}) W24F	wt[b]	ratio W24F/wt
folate	E	130 ± 13	156	0.8
H_2folate	E	100 ± 6	264	0.4
	E·NADPH	97 ± 6	98	1.0
	E·NADP	58 ± 2	110	0.5
H_4folate	E	44 ± 4	117	0.4
	E·NADP	34 ± 2	24	1.4
NADP	E	19 ± 2[c]	17	1.1
	E·H_2folate	17 ± 1	20	0.9
NADPH	E	28 ± 1	38	0.7

[a] Except where noted, these values were calculated from time courses of fluorescence transients accompanying ligand binding as described previously (Appleman et al., 1990a). [b] Values for wild-type rhDHFR are taken from Appleman et al. (1990a). [c] The association rate constant for NADP binding to W24F rhDHFR was determined as described under Materials and Methods.

Table IV: Dissociation Rate Constants Obtained by the Relaxation Method for Binary Complexes of Wild-Type and W24F rhDHFR[a]

ligand	form of enzyme dissociating	k_{off} (s^{-1}) W24F	wt[b]	ratio W24F/wt
folate	E·folate	82 ± 13	27	3.0
H_2folate	E·H_2folate	16 ± 6	12	1.3
H_4folate	E·H_4folate	51 ± 2	5.4	9.4
	E·NADP·H_4folate	120 ± 8	63	1.9
NADP	E·NADP·H_2folate	50 ± 2	4.6	10.9
NADPH	E·NADPH	2.8 ± 1	1.3	2.2

[a] These values were calculated from time courses of fluorescence transients accompanying ligand binding as described previously (Appleman et al., 1990a). [b] Values for wild-type rhDHFR are taken from Appleman et al. (1990a).

Scheme II: Interconversion of Apoenzyme Conformers and Binding of Ligands to DHFR

$$E' \overset{K_{eq}}{\rightleftharpoons} E + L \overset{k_{on}}{\underset{k_{off}}{\rightleftharpoons}} EL$$

mined for substrates and products. The association rate constants, k_{on}, that were measurable by stopped-flow fluorimetric observation of ligand binding were not much different from those determined for wt rhDHFR (Table III). Dissociation rate constants, k_{off}, determined either by the relaxation method (Table IV) or by competition (Table V) were increased compared with the wt enzyme, with the exception of NADPH dissociation from the E·NADPH·H_4folate complex. However, the increases in dissociation rates compared with wt enzyme were modest, the highest being by a factor of 11.

Apoenzyme Conformer Equilibrium and Effect on Equilibrium Dissociation Constants. In measuring fluorescence transients associated with ligand binding, it was observed that the fluorescence increase was biphasic for NADPH binding (Figure 3) as in the case of wt rhDHFR (Appleman et al., 1990a). This is due to the existence of an equilibrium between at least two conformers of the apoenzyme, only one of which binds ligand to a significant extent, as indicated in Scheme II. A further indication of this equilibrium between apoenzyme conformers was provided by the greater value of equilibrium dissociation constants determined by fluorescence titration and kinetic dissociation constants obtained from k_{off}/k_{on} (Table VI). If Scheme II adequately describes this behavior, then $K_{eq} = [K_D/(k_{off}/k_{on}) - 1]^{-1}$ and estimates of K_{eq} for W24F rhDHFR range from 0.31 to 0.67. From the ratio of the amplitudes in the slow and fast phases in Figure 3 K_{eq} was estimated as 0.31. In the case of wild-type enzyme

Table V: Dissociation Rate Constants Obtained by the Competition Method for Wild-Type and W24F rhDHFR

ligand	form of enzyme	trapping ligand	k_{off} (s^{-1}) W24F	wt[a]	ratio W24F/wt
folate	E·folate[b]	MTX	83 ± 8	25	3.3
H_2folate	E·H_2folate[b]	MTX	22 ± 2	14	1.6
	E·NADPH·H_2folate[b]	MTX	350 ± 4	94	3.7
	E·NADP·H_2folate[b]	MTX	4.9 ± 0.3	1.3	3.8
H_4folate	E·H_4folate[b]	MTX	47 ± 2	5	9.4
	E·NADPH·H_4folate[b]	MTX	≫300[c]	225	≫1.3
	E·NADP·H_4folate[b]	MTX	125 ± 5	46	2.7
NADP	E·NADP[d]	NADPH	250 ± 8	32	7.8
NADPH	E·NADPH[e]	NADP	3.2 ± 0.5	1.7	1.9
	E·NADPH·H_2folate[e]	NADP	60 ± 3	19	3.1
	E·NADPH·H_4folate[e]	NADP	32 ± 1	100	0.3

[a] Values for wild-type rhDHFR are taken from Appleman et al. (1990a). [b] Values was calculated from time courses of decreasing absorbance at 380 nm accompanying protonation of MTX upon binding to W24F rhDHFR. [c] It was not possible to use concentrations of H_4folate and NADPH necessary to saturate enzyme with these ligands. The true dissociation rate constant was much greater than measured with achievable NADPH concentrations. [d] Value was calculated from time courses of increasing fluorescence intensity due to energy transfer from W24F rhDHFR to NADPH as NADPH replaces NADP. [e] Value was calculated from time courses of decreasing fluorescence intensity due to loss of energy transfer from W24F rhDHFR to NADPH as NADP replaces NADPH.

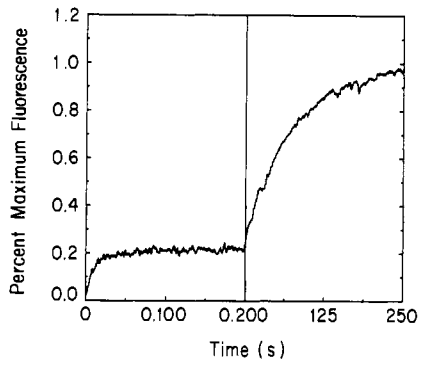


FIGURE 3: Biphasic time course of NADPH binding to W24F rhDHFR. Enzyme was mixed with NADPH in a stopped-flow spectrophotometer. The reaction was monitored by an increase in NADPH fluorescence due to energy transfer from protein excited at 280 nm. Final concentrations of W24F rhDHFR and NADPH were 0.5 and 3 μM, respectively.

the data for H_4folate, NADPH, and NADP give a mean value of 6.9 for K_{eq}, so that there appears to be a significant effect of the mutation on the apoenzyme conformational equilibrium with displacement toward the nonbinding form or forms. Binding of H_2folate, and especially of folate, to wt rhDHFR seems to be more complex than described by Scheme II since the data for these ligands do not fit the relationship for K_{eq}.

Cooperativity of Ligand Binding. Ternary complexes of wt rhDHFR show an almost 2800-fold range of cooperativity from a positive cooperativity of over 7 for the E·NADP·H_2-folate complex to a negative cooperativity of 0.0025 for the E·NADPH·H_4folate complex (Table VII). In the W24F enzyme the negative cooperativity was greatly weakened for the E·NADP·H_4folate and E·NADPH·H_4folate complexes.

Complete Kinetic Scheme. The complete branched kinetic mechanism for W24F rhDHFR is shown in Scheme III, together with rate constants in both directions for each step.

Stability of the Mutant Enzyme. When either wt or W24F rhDHFR is incubated at 37 °C under conditions approximating intracellular conditions in regard to pH, ionic strength, and NADPH, the enzyme shows a biphasic loss of activity (Figure 4). The first, quite rapid phase results in a loss of 20–25% of activity (as measured under standard assay conditions), and the second, slow phase results in the loss of the remaining activity. The rate constants for both phases are

Table VI: Kinetic and Equilibrium Dissociation Constants for Binary Complex Formation for wt and W24F rhDHFR

	k_{off}/k_{on}[a]			K_D[b]			$K_D/(k_{off}/k_{on})$	
ligand	W24F (μM)	wt (μM)	ratio W24F/wt	W24F (μM)	wt (μM)	ratio W24/wt	W24F	wt
folate	0.64 ± 0.09	0.17	3.8	2.7 ± 0.4	0.08	33.7	4.2 ± 0.9	0.5
H_2folate	0.22 ■ 0.02	0.05	7.3	0.93 ± 0.11	0.12	7.8	4.2 ± 0.6	2.3
H_4folate	1.07 ± 0.11	0.04	26.8	2.7 ± 0.2	0.05	48.0	2.5 ± 0.3	1.1
NADP	13.2 ± 1.4	2.3	5.7	40 ± 10	2.3	17.4	3.0 ± 0.8	1.2
NADPH	0.11 ± 0.02	0.05	2.2	0.33 ± 0.03	0.05	6.6	3.0 ± 0.6	1.1

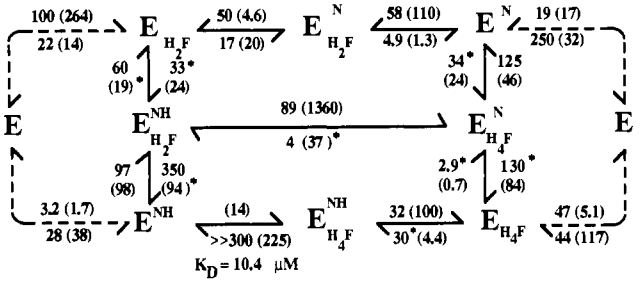
[a] Values of k_{off} are from the competition method (Table V), and values of k_{on} are from the relaxation method (Table III). Values for wild-type rhDHFR are taken from Appleman et al. (1990a). [b] Values for wt rhDHFR were taken from Appleman et al. (1990a). Values for W24F rhDHFR were determined in MATS, pH 7.5, as described by Appleman et al. (1988a).

Table VII: Dissociation Constants and Binding Cooperativity in Formation of Ternary Complexes

		k_{off}/k_{on}[b,c] (μM)		degree of cooperativity[a]	
ternary complex	ligand	W24F	wt	W24F	wt[c]
E·NADP· H_2folate	NADP	2.94 ± 0.21	0.23	3.00 ± 0.27[d]	7.18
	H_2folate	0.08 ± 0.01	0.012		
E·NADP· H_4folate	H_4folate	3.68 ± 0.26	1.92	0.291 ± 0.036	0.023
E·NADPH· H_2folate	H_2folate	3.61 ± 0.23	0.96	0.061 ± 0.007	0.055
E·NADPH· H_4folate	H_4folate	10.4 ± 1.0[e]	16.1	0.103 ± 0.014	0.0025

[a] Degree of cooperativity is the ratio of k_{off}/k_{on} for binary complex from Table VI to k_{off}/k_{on} for ternary complex. It should be noted that this is the inverse of the ratio we used previously (Appleman et al., 1990a). [b] For binding of ligand to form ternary complex. Values of k_{on} are from Table III, and those for k_{off} are from Table V where available or otherwise from Table IV. [c] Values for wild-type rhDHFR are calculated from the appropriate rate constants from Appleman et al. (1990a). [d] Weighted average and standard deviation of weighted average as defined by Beers (1957). [e] Determined as described under Materials and Methods.

Scheme III: Kinetic Scheme for W24F rhDHFR at 20 °C and pH 7.5[a]



[a] First- and second-order rate constants have the units s^{-1} and μM^{-1} s^{-1}, respectively. Values in parentheses are for wt enzyme (Appleman et al., 1990a), and those without parentheses are for W24F rhDHFR. Rate constants with an asterisk were calculated to balance binding equilibria or otherwise as described under Materials and Methods. The major steps in the catalytic cycle at steady state in the presence of saturating substrate concentrations are depicted by solid arrows and are like that determined for wild-type rhDHFR (Appleman et al., 1990a). E = rhDHFR, NH = NADPH, N = NADP, H_2F = H_2-folate, and H_4F = H_4folate.

increased significantly for mutant enzyme: wt, k_1 = 0.11 min^{-1}, $k_2 = 6 \times 10^{-4}$ min^{-1}; W24F, k_1 = 0.26 min^{-1}, $k_2 = 1.6 \times 10^{-3}$ min^{-1}. The stability of both enzymes was unchanged in the presence of 50 mM mercaptoethanol in addition to NADPH.

Discussion

Trp^{24} is conserved in all bacterial and vertebrate DHFRs that have been sequenced. It is part of a loop that connects elements of secondary structure (strand A of the β sheet and α-helix B). In all vertebrate DHFRs there is a conserved

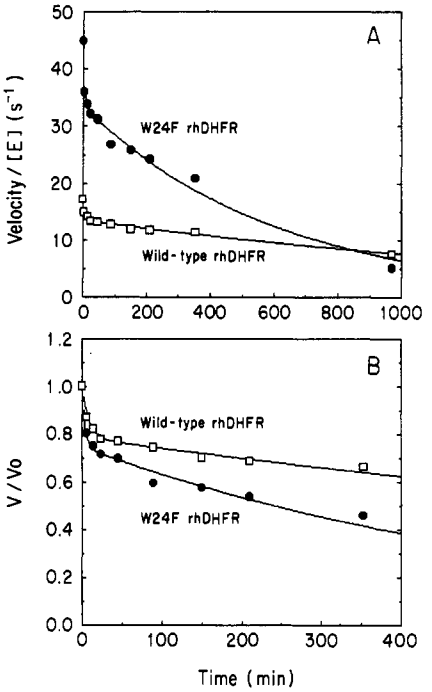


FIGURE 4: Stability of wt and W24F rhDHFR at 37 °C. Enzymes (250 nM) were incubated in 100 mM potassium phosphate with 2 mM NADPH, pH 7.4. Aliquots were removed from the incubation mixture at time intervals and assayed for activity at 37 °C in the presence of 50 μM substrates. Panel A illustrates the loss of activity (velocity/[E]) with time, and panel B is a replot of velocity (V) during the initial 400 min normalized to velocity at zero time (V_0). The solid lines represent the best fit of the data to a two-exponential decay.

segment of this loop, Pro^{23}-Trp^{24}-Pro^{25}-Pro^{26}, and in chicken DHFR this segment forms an almost ideal polyproline 3-fold helix (Volz et al., 1982). The effect of this structure on the mobility of TRP^{24} is unclear. The loop of which this segment is a part forms one wall of the active site cavity, and the Trp^{24} side chain constitutes part of this wall, which is composed of hydrophobic residues with the exception of Glu^{30}. The indole nitrogen of Trp^{24} is hydrogen bonded to a fixed water molecule which in turn is hydrogen bonded to the C^4 oxygen of bound folate and to the carboxyl of Glu^{30} (Oefner et al., 1988). In the crystal structures of the MTX·NADPH ternary complexes with lcDHFR (Filman et al., 1982) and with chicken DHFR (Volz et al., 1982) C^2 of Trp^{24} makes van der Waals and hydrophobic interactions with the carboxamide nitrogen of bound NADPH. Although the crystal structure of the corresponding complex of rhDHFR has not been determined, it seems reasonable to assue a similar interaction between Trp^{24} of rhDHFR and the nicotinamide ring of bound NADPH. Such an interaction appears likely from the hypothetical NADPH·MTX·rhDHFR ternary structure constructed by Oefner et al. (1988). This model was obtained by a least-squares fit of selected C_α coordinates of the human enzyme

structure (mainly located in elements of secondary structure) with those in the ternary complex of lcDHFR with NADPH and MTX.

Effect of the Trp → Phe Substitution on Ligand Binding. A substituted amino acid may alter protein behavior either directly or through secondary changes in protein conformation. In our study, as for most other mutants of DHFR that have been reported, it is unknown whether conformational changes occur that have significant effects on enzyme properties. In the case of the only mutant DHFRs that have been examined by X-ray crystallography negligible changes in atomic positions were found in any part of the backbone or in side chains other than that of the substitute amino acid (Howell, 1986). Accordingly, as a first approach we assume in what follows that the most significant effects are due to the substituted amino acid side chain itself.

It is to be anticipated that because of the probable interactions of Trp^{24} with bound pteridine substrates via the fixed water molecule and with bound NADPH, replacement of Trp^{24} by Phe would result in decreased affinity for these ligands. Although none of the association rate constants changed by a factor greater than 2.7 (Table III), almost all the dissociation rate constants are increased, and some by substantial factors (7- to 10-fold). The result is that the energy of binding to the tight-binding conformer, as measured by k_{off}/k_{on}, is decreased by 1–2 kcal/mol in these cases. The slightly greater decreases in binding energy for NADP compared with the decreases for NADPH in corresponding complexes suggest that the interaction of Trp^{24} with the nicotinamide ring of NADPH contributes less to binding of the latter than in the case of NADP. This is in contrast to the report of Andrews et al. (1989) that the Trp^{24} → Leu mutation in lcDHFR causes a decrease in NADPH binding of 3.44 kcal/mol (at 25 °C) whereas binding of NADP is slightly increased. The contrast in the effect of the two mutations on NADPH binding may be due in part to the smaller volume of the Leu side chain (168 Å^3) compared to that of Phe (203 Å^3), but this alone does not account for the divergent observations. The different effects on NADP binding may be related to the report that in at least some complexes with lcDHFR NADP binds in two conformational states, in one of which the nicotinamide ring is probably not bound in the active site cavity but extends into the solution (Grogenborn et al., 1981; Birdsall et al., 1989). There is no evidence for such behavior by NADP in complexes with vertebrate DHFR.

Although the dissociation rate constants for binding of ligands in a number of binary complexes (Table VI) and ternary complexes (Table VII) are significantly increased, the increases are not enough to suggest that weakened ligand binding is a prime reason for conservation of Trp^{24}.

Effect of the Mutation on the Rate of Hydride Transfer. The Trp → Phe substitution decreases the effective rate constant for hydride transfer at 20 °C and pH 7.5 by a factor of 38 (Table II). This is presumably due to unfavorable geometry for hydride transfer from C^4 of the nicotinamide ring to C^6 of the pteridine ring. A decreased rate of proton transfer to N^5 of H_2folate in the formation of the transition-state complex cannot be responsible because of the high value of $^Dk_{burst}$. The lower rate of hydride transfer may be due to a decrease in the pK_a for the pH dependence of the rate of hydride transfer, or in the pH-independent rate of hydride transfer. The decrease in hydride-transfer rate at any pH for the Trp^{24} → Leu mutant of *L. casei* DHFR is primarily attributable to the latter, with the pH-independent rate decreasing from 430 to 4.3 s^{-1} whereas the pK_a is relatively unperturbed (6.0 for wt and 5.85 for the mutant) (Andrews et al., 1989). The rate of hydride transfer to H_2folate is too rapid at pH <6 with W24F rhDHFR for measurement by stopped-flow techniques. The pK_a describing the pH dependence of k_{cat} (Figure 1) cannot be used, since it is an apparent value indicating change in rate-determining step. A pK_a value of 6.4 is observed in the profile of k_{cat}/K_m for the substrate H_2biopterin (data not shown). That hydride transfer is rate-limiting throughout the entire pH range of this profile is indicated by a large isotope effect when NADPH is substituted for NADPD (3.1 at pH 5.4 and 3.2 at 8.0). A similar pK_a, 6.2, is observed in the ratio of k_{hyd}/K_d for H_2biopterin with wt rHDHFR (Appleman and Blakley, unpublished results). These pK_as are not necessarily that for hybride transfer but are instead for a group on the W24F rhDHFR·NADPH complex [see Cleland (1982) for discussion]. Presumably this group is Glu^{30}, the active site carboxylic acid.

However, the pH dependence of the rate of hydride transfer over the accessible pH range (>6) with H_2folate as substrate and W24F as catalyst indicates that the pK_a for this process must be well below 6.1. This low pK_a of undetermined value is for protonation of the W24F hDHFR·NADPH·H_2folate complex, rather than that of the W24F rhDHFR·NADPH complex determined from k_{cat}/K_m for H_2biopterin. It may be that the pK_as of the binary and ternary complexes are indeed different or represent different protonations (i.e., Glu^{30} in the binary complex, N^5 of the pteridine ring in the ternary). It would be of interest to determine the pK_a of the W24F rhDHFR·NADPH·H_2bioptern complex by analysis of the pH dependence of k_{cat}. However, because of the low affinity of this substrate for W24F·NADPH, it was not possible to measure k_{cat} for H_2biopterin (only k_{cat}/K_m), and the pK_a of hydride transfer within the W24F·NADPH·H_2biopterin complex also remains unknown. Thus, although the amino acid substitution does not grossly perturb the pK_a of Glu^{30} in the rhDHFR·NADPH complex, the possibility that the pK_a governing hydride transfer is altered sufficiently to account for the altered rate of hydride transfer in the W24F mutant compared to wt cannot be excluded.

However this may be, it is clear that the decrease in the rate of hydride transfer is sufficiently great to suggest that it has some relation to the conservation of Trp^{24}, even though k_{cat} under assay conditions is increased compared with wt enzyme.

It has been proposed that the function of the active site side-chain carboxyl (Glu^{30} in rhDHFR: Asp in ecDHFR) is to assist proton donation to N^5 of H_2folate (Howell et al., 1986), and a detailed mechanism of how this might occur has been proposed (Taira et al., 1987; Morrison & Stone, 1988; Uchimaru et al., 1989). The mechanism operates via a network of hydrogen bonds between atoms of the ligand, the protein, and bound water molecules. One of the bound water molecules is hydrogen bonded to the indole nitrogen of Trp^{24}, so that it seemed possible that absence of the indole ring might result in some distortion of this hydrogen bond network. However, our data provide no evidence that this is the case, and if the mechanism is correct, the bound water molecules must participate with unimpaired efficiency.

Effect of the Mutation on Conformational Equilibria. As noted above, the Trp → Phe substitution does not appear to decrease the rate of the obligatory isomerization accompanying hydride transfer. However, it does displace the equilibrium between apoenzyme conformers (Scheme II) toward the nonbinding form, so that for the mutant enzyme 24% is in the ligand-binding form as compared with 80% of wt rhDHFR (Appleman et al., 1990a). Values of $t_{1/2}$ for conversion of the

nonbinding to the binding form are ≈5 and 50 s for wt and W24F rhDHFR, respectively. A similar conformer equilibrium in *E. coli* is perturbed in many mutant enzymes [see Appleman et al. (1990b) for discussion].

Effect of the Mutation on Stability. It seems unlikely that the displacement of the equilibrium between apoenzyme conformers by the the $Trp^{24} \rightarrow$ Phe influences the rate of catalysis within cells, since the high intracellular NADPH concentration (≈2 mM) would maintain enzyme in the binding conformation. A more significant result of the amino acid substitution appears to be a decrease in stability even in the presence of a saturating concentration of NADPH, such as would be present in the cell. The loss of activity occurs in two phases, both of which occur at about twice the rate in the mutant enzyme. The two-phase loss of activity may be related to the two-phase urea-induced unfolding of ecDHFR (Touchette et al., 1986). The destabilizing effect of Phe^{24} is in contrast to that of Thillet et al. (1988), who found that the $Trp^{24} \rightarrow$ Arg mutation in mouse DHFR actually increased stability. This mutation is obviously much less conservative than our $Trp^{24} \rightarrow$ Phe and, as might be expected, caused major changes in kinetic parameters including a large decrease in k_{cat} and a very large increase in $K_{m(H_2folate)}$. The mechanism for the stabilizing effect of Arg^{24} in mouse DHFR is unclear, but the destabilizing effect of Phe^{24} rhDHFR seems probably due to decreased hydrophobic interaction with other nonpolar side chains in the region. The half-life for the slower phase of activity loss is decreased from 19.2 h in wt to 7.2 h in W24F rhDHFR, which may be sufficient to constitute a selective disadvantage.

Mechanistic Scheme for Mutant Enzyme. Some of the rate constants (indicated by asterisks) for the complete mechanism (Scheme III) could not be determined directly because of experimental difficulties, and estimates were made as described under Materials and Methods. A check on the general correctness of the rate constants in Scheme III can be made by the prediction of steady-state kinetic parameters from the appropriate differentiated equations as previously described for wt rhDHFR (Appleman et al., 1990a). Predicted values, obtained by simulation as described under Materials and Methods, which agree well with the experimental values in Table I were as follows: $K_{m(H_2folate)}$, 1.6 μM; $K_{m(NADPH)}$, 2.0 μM; k_{cat}, 40 s^{-1}; $K_{i(NADP)}$, 3.8 μM; $K_{i,app(H_4folate)}$, 60 μm. In addition, there was good agreement between measured and simulated full time courses of the reaction over 5 s with 1 μM W24F rhDHFR and 50–100 μM substrates (data not shown).

The mechanistic scheme for W24F rhDHFR resembles that for wt enzyme, but with some quantitative differences corresponding to changes in rate constants. The rate constants for dissociation from the ternary product complex are the same for NADP and for H_4folate, so that from the onset of the reaction, distribution between the two major loops is more nearly equal than for wt (Figure 5). For wt, flux through the upper loop by way of E·NADP·H_2folate is limited by the slow dissociation of NADP from this complex, with resulting accumulation of increasing amounts of enzyme in the form of this complex and increasing flux through the upper loop (Appleman et al., 1990a). This switch from a high flux (95%) through the lower loop to 65% at steady state is partly responsible for the marked hysteresis in transient-state kinetics for wt enzyme. In the case of W24F rhDHFR dissociation of NADP from E·NADP·H_2folate is over 10 times faster, there is much less accumulation of this complex, and excess flux through the lower loop is smaller and more transient (Figure 5), so that hysteresis is much less marked (Figure 2). At

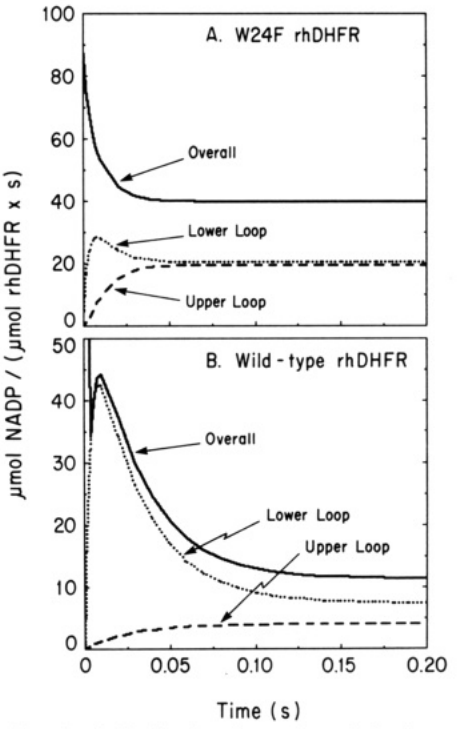


FIGURE 5: Simulated distribution of reaction velocity between upper and lower pathways illustrated in Scheme III. In the simulations, the concentrations of rhDHFR and substrates were 0.001 and 1000 μM, respectively, and at $t = 0$ all enzyme is complexed with NADPH. (A) W24F rhDHFR; (B) wild type rhDHFR.

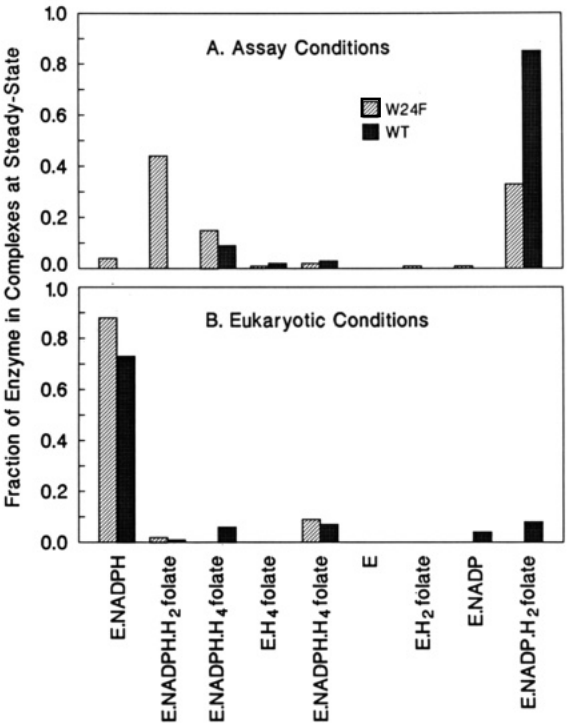


FIGURE 6: Distribution of W24F and wt rhDHFR among complexes at steady-state computed from rate constants in Scheme III. Concentrations were as follows: (A) 100 μM NADPH and 100 μM H_2folate at pH 7.5; (B) 2000 μM NADPH, 0.1 μM H_2folate , 20 μM NADP, and 1 μM H_4folate at pH 7.4. Concentrations were chosen to be representative of eukaryotic cells in B (Appleman et al., 1990a).

steady state under assay conditions flux through the two major loops is almost equal (Figure 5), and the major enzyme complexes present are E·NADPH·H_2folate, E·NADP·H_2folate, and E·NADP·H_4folate (Figure 6A). Accumulation of the first

of these is a consequence of the much slower rate of hybride transfer.

The higher values of K_m result from weaker binding of both substrates in all their complexes, but especially in the ternary complexes. The higher k_{cat} (Table I) is due to increases in the rate constants for all four product dissociation steps that are rate-limiting in the wt mechanism.

The above considerations apply to assay conditions where no substrate addition steps are rate-limiting. Under intracellular conditions, however, the comparison with wt enzyme is quite different. Although the distribution of the mutant enzyme among its complexes at steady state is not greatly changed (Figure 6B), the steady-state velocity under these conditions is 2.0 μmol of product/(μmol of enzyme·s), compared with 6.9 for wt enzyme. The efficiency of the enzyme decreases correspondingly to 21% (wt 69%), where efficiency is defined as the velocity expressed as a percent of that determined by the diffusion-limited rate of substrate binding to enzyme (Albery & Knowles, 1976). This decrease is caused by the high rate at which H_2folate dissociates from W24F rhDHFR·NADPH·H_2folate compared with the rate of formation of products within this complex (350 s^{-1} vs 112 s^{-1} at pH 7.4). Conversely for wt, H_2folate dissociation is much slower than is product formation (94 s^{-1} vs 1360 s^{-1} at pH 7.65).

Conclusion. The very conservative amino acid substitution $Trp^{24} \rightarrow$ Phe changes the binding of substrates and products to rhDHFR, especially in ternary complexes. Although the increases in several dissociation rates have only modest effects on the mechanistic behavior of the mutant enzyme either before or after steady-state conditions have been achieved under assay conditions (i.e., near saturating substrate concentrations), they produce a marked decrease in catalytic efficiency under intracellular conditions. This, coupled with a significant decrease in enzyme stability, appears to account for why even this highly conservative change has not been tolerated during the evolution of higher organisms.

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