Critical Role of Phenylalanine 34 of Human Dihydrofolate Reductase in Substrate and Inhibitor Binding and in Catalysis†

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Received March 28, 1994; Revised Manuscript Received May 25, 1994®

ABSTRACT: Directed mutagenesis has been used to construct five variants of human dihydrofolate reductase in which smaller residues are substituted for phenylalanine 34, a residue participating in the binding of substrate and methotrexate by interaction with their pteridine rings. The variant enzymes are stable and have decreased affinities for methotrexate (by factors of 2700–60000 at pH 7.65) due to a decreased rate of methotrexate association and a much larger increase in the rate constant for dissociation. However, the catalytic efficiencies of the variants are also lowered by factors of 160–5000, so that it is doubtful whether these enzymes are capable of conferring methotrexate resistance on the cells harboring them. High concentrations of dihydrofolate cause marked inhibition of all the variants, which complicates the determination of kinetic parameters. By the use of stopped-flow spectrophotometry and fluorimetry and other methods, it has been shown that, like the wild-type enzyme, the variants have a branched reaction pathway, but in contrast to the wild-type enzyme, the distribution of flux between alternate pathways is dependent on the concentration of dihydrofolate. This different branch point is a consequence of the very rapid dissociation of tetrahydrofolate from the ternary product complexes of the variant enzymes. Inhibition by dihydrofolate is due to its combination with the enzyme–NADP complex and the slow dissociation of NADP from the resulting abortive complex. When steady state kinetics for this model are simulated using the experimentally determined rate and dissociation constants for the alanine 34 variant, most steady state experimental results are closely approximated.

Phenylalanine 34 is one of the hydrophobic residues lining the active site of human dihydrofolate reductase (hDHFR).[1] In the crystal structure of the folate complex of hDHFR, the plane of the phenyl ring of Phe^{34} is inclined at about 45° to the plane of the pteridine ring of bound folate, and several atoms of the two rings are in van der Waals contact (Oefner et al., 1988; Davies et al., 1990). In particular, atoms of the phenyl ring make close contacts with N8, N1, C8a, and C4a of the pteridine ring. There is a similar spatial relation between the side chain of Phe^{34} of chicken DHFR and the pteridine ring of bound biopterin in the crystal structure of the ternary complex of chicken DHFR with biopterin and NADP (McTigue et al., 1992). Atoms of the phenyl ring, especially CD1, again make close contacts with N1, N8, and C8a of biopterin. Interaction between the phenyl ring of Phe^{34} and the pteridine ring of a bound MTX derivative is also similar, with close contacts between atoms of the phenyl ring and C2, N3, C4, and N4 of the pteridine ring (Chunduru et al., 1994). From this spatial relationship of Phe^{34} to bound folate, biopterin, and inhibitor, it is to be expected that mutations resulting in the substitution of other amino acids for Phe^{34} would greatly affect the binding of substrates and of inhibitors like MTX that can occupy the substrate binding site.

We have previously investigated the role of Phe^{31}, which is another hydrophobic residue in the active site and is also located close to the pteridine ring of bound folate (Davies et al., 1990) and bound MTX (Chunduru et al., 1994), but with a spatial relation to the pteridine ring different from that of Phe^{34}. Replacement of Phe^{31} by residues with smaller side chains results in decreases in the affinity of MTX by factors of up to 100. On the other hand, the affinity of H_2folate for the unligated enzyme is *increased* up to 67-fold. K_m for dihydrofolate for these variants is increased by a small factor (3.5–7.2), partly as a consequence of the greatly decreased rate of the chemical transformation. The net result is that several of these Phe^{31} variants seem likely to have potential for conferring resistance to MTX on cells that contain even a single copy of the gene or of the cDNA stably incorporated into the genome.

We report here the kinetic behavior of variants of hDHFR in which Phe^{34} is replaced by smaller residues. The affinity for MTX is decreased by much larger factors than for the corresponding Phe^{31} variants, but at the cost of larger decreases in catalytic efficiency.

† This research was supported in part by U.S. Public Health Service Grant R01 CA 31922 (R.L.B.), Cancer Core Grant P30 CA 21765 (R.L.B.), and National Research Service Award T32 CA09346 (H.T.S.) from the National Cancer Institute, National Institutes of Health, by a Postdoctoral Research Fellowship from St. Jude Children's Research Hospital (T.N.), and by the American Lebanese Syrian Associated Charities.

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® Abstract published in *Advance ACS Abstracts,* July 15, 1994.

[1] Abbreviations: DHFR, dihydrofolate reductase (EC 1.5.1.3); hDHFR, recombinant human DHFR; WT, wild-type hDHFR, that is, with Phe at position 34; F34X hDHFR, variants of hDHFR with another amino acid residue, X, substituted for phenylalanine 34; H_2folate, 7,8-dihydrofolate; H_4folate, 5,6,7,8-tetrahydrofolate; MATS, 25 mM 2-morpholinoethanesulfonic acid, 25 mM acetic acid, 50 mM Tris, 100 mM NaCl, and 0.02% sodium azide; MTX, methotrexate; NADPD, (4*R*)-[4-^{2}H]NADPH; $k_{cat} = V_{max}/[E]$, where V_{max} is the steady state maximum velocity for the reaction pathway predominating at low substrate concentration and [E] is the enzyme concentration; k_{cat}', a similar constant relevant to the reaction pathway predominating at high substrate concentration; k_{on}, association rate constant; k_{off}, dissociation rate constant; k_{chem}, rate constant for the chemical transformation step; ^Dv, primary deuterium isotope effect on reaction velocity due to substitution of NADPD for NADPH.

0006-2960/94/0433-9945$04.50/0

MATERIALS AND METHODS

Materials. Unless otherwise indicated, the materials used were as previously described (Chunduru et al., 1994). Q-Sepharose was obtained from Sigma, and hydroxylapatite was from Calbiochem. Solutions of crystalline dihydrofolate (Blakley, 1960) were prepared daily, and the purity was checked by the characteristics of the ultrviolet spectrum.

Construction and Expression of Mutants of cDNA for hDHFR. This was carried out as described previously (Chunduru et al., 1994).

Purification of Variant hDHFRs. Crude extracts were prepared from M15 (pREP4) (pDS5/hDHFR) *E. coli* as previously described (Chunduru et al., 1994). These variant enzymes have very low affinities for MTX, so that affinity chromatography on MTX–Sepharose was modified from the procedure used for wild-type hDHFR (Prendergast et al., 1988). In most cases, the enzyme bound weakly to the affinity column, so that it could be eluted simply by a change in buffer without the use of a displacing ligand such as folate or H_2-folate. After precipitation with ammonium sulfate (40–85% saturation), the pellet was dissolved in 50 mM potassium chloride/50 mM potassium phosphate buffer, pH 6.4, and the solution was applied to the MTX–Sepharose affinity column. Washing was performed with a KCl buffer solution of the same composition until the enzyme started to appear in the eluate, at which point washing with the eluting buffer (50 mM KCl/50 mM potassium phosphate, pH 8.65) was commenced. Fractions containing the enzyme were pooled and passed through a Q-Sepharose column (1.5 × 55 cm) equilibrated with 50 mM potassium phosphate buffer, pH 7.5. The column was washed with the same buffer until the enzyme was all off, and the enzyme-containing solution was then concentrated by ultrafiltration. Since folate was not used for elution, contamination by the products of folate decomposition, which are difficult to remove from WT hDHFR, was avoided. The concentrated solution (about 10 mL) was applied to a Sephadex G-75 column (2.5 × 100 cm) equilibrated with 50 mM potassium phosphate buffer, pH 7.5, and elution was performed with the same buffer. In some cases, a small amount of residual protein impurities was removed by chromatography on a hydroxylapatite column (1.5 × 30 cm) equilibrated with 25 mM potassium phosphate buffer, pH 7.5. After application of the sample, the column was washed with 200 mL of the same buffer. A linear phosphate concentration gradient that was commenced at this point was produced from 400 mL of 25 mM potassium phosphate, pH 7.5, in the mixer and 400 mL of 200 mM potassium phosphate, pH 7.5, in the reservoir. Fractions containing enzyme were pooled and concentrated by ultrafiltration. The F34S variant did not bind to the MTX–Sepharose column and was purified only by the other procedures described. The purity of the final preparation of this variant was about 64%. The other variants were obtained in homogeneous form as indicated by gel electrophoresis and by comparison of the concentration of active sites (determined by titration of the protein fluorescence with MTX) with protein concentration, as determined from absorbance at 280 nm (Margosiak et al., 1993).

The mutant cDNAs were well expressed, except in the case of the F34S cDNA, so that variant hDHFRs constituted 2–5% of the soluble protein in the bacterial cell extract. The yield of purified enzyme was satisfactory, and 8–43 mg of purified enzyme was obtained from a 12-L bacterial culture, depending on the particular variant.

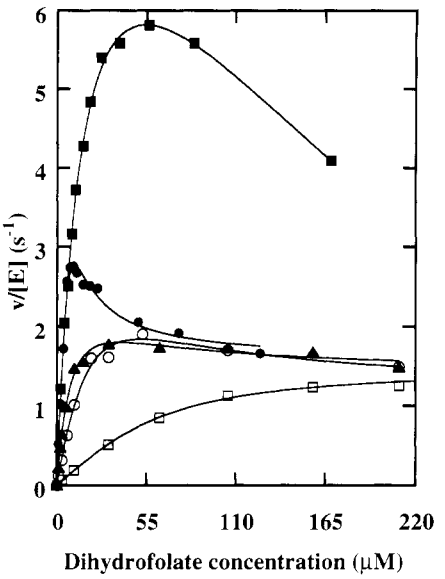


FIGURE 1: Effect of the concentration of H_2folate on the rate of its reduction by F34 variants of hDHFR. Determinations were carried under standard conditions with 100 μM NADPH: ■, F34I; ●, F34V; ○, F34T; □, F34S; ▲, F34A. The F34S curve is fit to rates at 630 and 1260 μM H_2folate, as well as to those shown.

Determination of Rate Constants, Dissociation Constants, and Inhibition Constants. The methods were, in general, the same as those we have used previously (Chunduru et al., 1994; Margosiak et al., 1993).

Experimental Conditions and Reporting of Results. Unless otherwise stated, experiments were conducted at 20 °C in MATS buffer, pH 7.65. Results reported are the means of duplicate experiments followed by the differences between the means and individual values.

Data Fitting. Curves shown in the figures are least-squares fits of the data to the appropriate equation. They were obtained by use of the Kaleidagraph program on a MacIntosh computer.

RESULTS

Steady State Kinetic Constants. When steady state reaction velocity was determined as a function of H_2folate concentration (Figure 1), it was apparent that all F34 variants showed substrate inhibition and that in many cases it was very marked. Even in the case of the Ala^{34}, Ser^{34}, and Thr^{34} variants, for which the substrate inhibition is less obvious, the data fit poorly to the simple Michaelis–Menten equation but fit well to eq 1 for substrate inhibition. This precludes the determination of K_m for H_2folate or k_{cat} in the usual way and in fact means that there are two values for each of these constants: K_m and k_{cat} corresponding to the reaction pathway predominating at low H_2folate concentration, and K_m' and k_{cat}' corresponding to the pathway predominating at high substrate concentration. The data in Figure 1 for each variant were fit to the following equation for substrate inhibition:

$$v/[E] = \frac{c_1S + c_2S^2}{1 + c_3S + c_4S^2} \quad (1)$$

where S is the substrate concentration, and c_1, c_2, c_3, and c_4 are constants that are functions of various rate constants in the reaction pathway, as described in the Appendix. The curves in Figure 1 are the least-squares fits of the data to this equation. As described in the Appendix, $k_{cat}/K_m = c_1$, and $k_{cat}' = c_2/c_4$. This permitted calculation of the values for these terms, shown in Table 1.

Table 1: Steady State Kinetic Constants

enzyme	k_{cat}/K_m[a] (μM^{-1} s^{-1})	relative[c]	k_{cat}' [a,b] (s^{-1})	K_m(NADPH) (μM)
WT	91.7[d]	1		0.16, 4.2[d]
F34I	0.57 ± 0.06[e]	0.0062	2.65 ± 0.05	ND
F34V	0.49 ± 0.09	0.0053	1.53 ± 0.03	0.96 ± 0.09
F34T	0.11 ± 0.01	0.0012	1.16 ± 0.02	0.40 ± 0.06
F34S	0.017 ± 0.001	0.0002	1.4 ± 1.5[f]	0.94 ± 0.11
F34A	0.24 ± 0.04	0.0026	1.34 ± 0.06	0.74 ± 0.14

[a] Derived from plots of velocity versus dihydrofolate concentration by least-squares fit to the equation for substrate inhibition (eq 1). K_m is for H_2folate. [b] $k_{cat}' = v/[E]$ at saturating H_2folate. [c] Relative to WT = 1. [d] From Appleman et al. (1990). [e] Results in this and other tables are the means from duplicate experiments ± difference of the mean from individual values. [f] Standard deviation from the fit of the data to the equation in one of two duplicate experiments. The other experiment did not give a value of k_{cat}'.

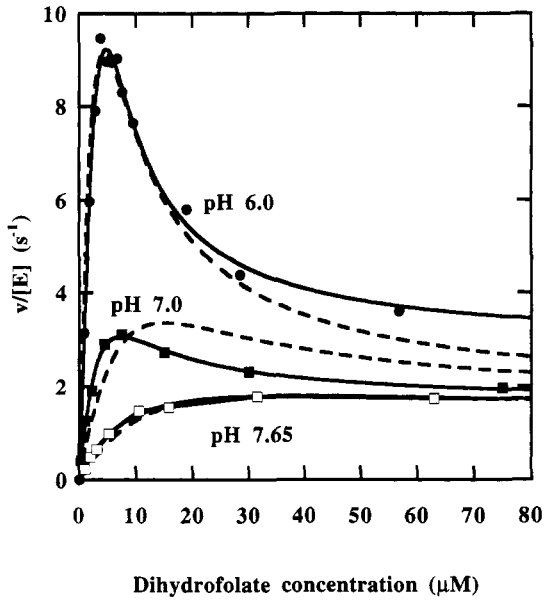


FIGURE 2: Effect of pH on the dependence of the activity of F34A hDHFR on substrate concentration: ●, pH 6.0; ■, pH 7.0; □, pH 7.65. Rates were determined under standard conditions, apart from pH and substrate concentration, and in the presence of 100 μM NADPH. Continuous lines show the least-squares fit of the data to eq 1. The curve for pH 7.65 was fit to rates at 105, 157, and 210 μM H_2folate, as well as to those shown, and the pH 6.0 curve was fit to the rates at 94.7 and 189 μM H_2folate, as well as to those shown. Broken lines show the velocities obtained by simulation as described in the Discussion.

The calculated values of k_{cat}/K_m give a measure of the catalytic efficiency of the enzymes at low concentrations of H_2folate, such as are present in mammalian cells. It may be seen from Table 1 that all of the variants have very low efficiencies compared with WT hDHFR, so that they may be unable to provide cell needs for reduced folates unless they are greatly overproduced. It is also noteworthy that k_{cat}' is considerably lower than the rate at the highest concentrations of H_2folate used, an observation indicating that at these concentrations there was still a considerable contribution to the rate by the faster pathway that is predominant at lower substrate concentrations.

The results illustrated in Figure 2 show that, in the case of the F34A variant, substrate inhibition becomes much more marked as the pH is decreased from 7.65 to 6. At pH 7.65, 7.0, and 6.0, the values of k_{cat}/K_m are 0.24, 1.2, and 3.0 μM^{-1} s^{-1}, respectively. Values of k_{cat}' are 1.3, 1.6, and 2.8 s^{-1}, respectively. The marked change in the shape of the curves

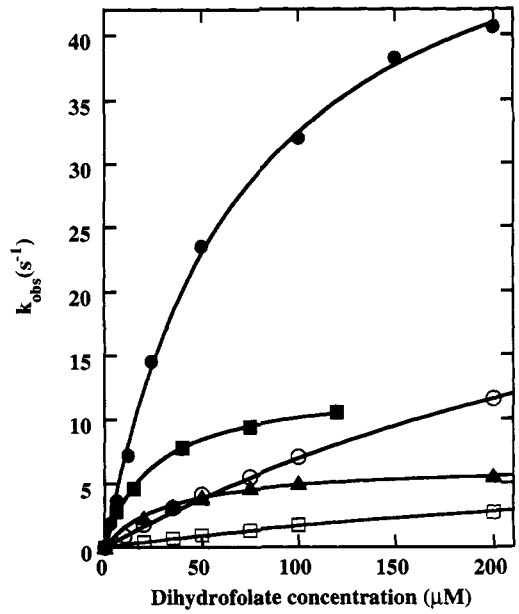


FIGURE 3: Effect of H_2folate concentration on the observed rate constant for the conversion of enzyme-bound substrates to enzyme-bound products by F34 variants of hDHFR. The determinations were carried out with a stopped-flow spectrophotometer as described in Materials and Methods. After mixing, the concentration of NADPH was 2.25 μM and that of enzyme was 1.75 μM: ■, F34I; ●, F34V; ○, F34T; □, F34S; ▲, F34A. The curves for F34T, F34S, and F34A are fit to rates at 350 and 500 μM H_2folate, as well as to those shown.

in Figure 2 as the pH is lowered is therefore due to the increase in k_{cat}/K_m (probably primarily due to an increase in k_{cat}) relative to k_{cat}'.

In contrast to the results with varied dihydrofolate concentrations, rates at varied NADPH concentrations fit well to the Michaelis–Menten equation (results not shown) to give a single K_m for NADPH (Table 1), which differed little from one variant to another. This is consistent with the two different NADPH complexes formed in the two pathways having dissociation constants that do not greatly differ, which proved to be the case. For all the variants, K_m for NADPH was several times higher than the lower of the two values for WT hDHFR.

Single-Turnover Experiments. These were able to give estimates not only for k_{chem}, the rate constant for the chemical transformation step, but also for the dissociation constant for H_2folate dissociation from the enzyme–NADPH–H_2folate ternary complex. The data for these experiments (Figure 3) were fit to the following equation:

$$k_{obs} = \frac{k_{chem}[H_2folate]}{K_d + [H_2folate]} \quad (2)$$

where K_d is the ternary dissociation constant. In these stopped-flow experiments, one syringe contained enzyme and a substoichiometric concentration of NADPH, and the other syringe contained H_2folate at one of a series of concentrations higher than that of the enzyme. Equation 2 is valid only if k_{on} and k_{off} for H_2folate are fast compared with k_{chem}, and if the dissociation of NADPH is negligible during the observation period. However, this is only approximately true. Consequently, estimates for k_{chem} are somewhat lower than the true values, and the K_d estimates are probably subject to some error also. The values calculated are given in Table 2, from which it may be seen that all of the amino acid substitutions for Phe[34] greatly reduce the rate of the chemical transforma-

Table 2: Determination of Kinetic Constants from the Rate of a Single Turnover of Enzyme-Bound Substrates to Enzyme-Bound Products

	k_{chem}			K_d[a]		k_{cat}[b]	
enzyme	(s^{-1})	relative	$^Dk_{chem}$	(μM)	relative	(s^{-1})	relative
WT	1360[c]	1		0.96[c]	1	11.0[c]	1
F34I	12.5 ± 0.3	0.0092		24 ± 2	25	13.7	1.25
F34V	54.6 ± 2.2	0.04		63 ± 6	66	30.7	2.79
F34T	31 ± 5	0.023	3.5	300 ± 120	310	33 ± 3	3.0
F34S	6.7 ± 1.7	0.0049	3.5	350 ± 60	360	6.0 ± 0.2	0.55
F34A	6.8 ± 0.4	0.005	3.3	36 ± 5	38	8.4 ± 1.3	0.78

[a] Thermodynamic dissociation constant for the dissociation of H_2folate from the enzyme–NADPH–H_2folate complex. [b] From $K_d k_{cat}/K_m$. Values of k_{cat}/K_m are from Table 1. [c] From Appleman et al. (1990).

tion. In the instances where it was measured, the rate of the chemical transformation shows a high isotope effect ($^Dk_{chem}$) when NADPD is substituted for NADPH. This indicates that the rate of the chemical transformation was completely limited by the rate of hydride transfer, in contrast to the case of WT enzyme (Beard et al., 1989). Furthermore, in all cases the binding of H_2folate in the productive substrate complex was considerably weakened compared with binding by WT, as shown by dissociation constants higher than those for WT by a factor of 25–360.

Since, as we shall discuss later, the rate of the chemical reaction is much slower than that of the subsequent dissociation of H_4folate, K_d may be used as an approximation for K_m, the Michaelis constant for H_2folate for the pathway involving the enzyme–NADPH–H_2folate complex. Thus, this Michaelis constant for H_2folate is much higher than that for WT hDHFR (360-fold higher in the case of the F34S variant).

By using these approximations to K_m and the values of k_{cat}/K_m from Table 1, it is possible to calculate approximate values for k_{cat}, which are also shown in Table 2. These indicate that the pathway through enzyme–NADP, unligated enzyme, and enzyme–NADPH is almost as fast as for the WT enzyme, and in some cases (F34V and F34T) it is much faster. This is explained by the fact that H_4folate release is the rate-limiting step for the WT enzyme and is much slower for WT than for the variant enzymes. However, it should be noted that these values of k_{cat} are never approached by $v/[E]$ at any substrate concentration (Figure 1), because of the existence of a slower alternate pathway.

Effect of pH on the Activity of Variant Enzymes. Whereas pH has little effect over the range pH 6–9 on k_{cat} for WT enzyme (Beard et al., 1989) and only begins to decrease k_{cat} for the F31G variant as the pH is raised above 7 (Chunduru et al., 1994), the activity of the F34A variant decreases as the pH is raised above 5.5 (Figure 4A) with an apparent pK_a of 6.36 ± 0.07. This pK_a corresponds to the pH at which the rate of hydride transfer (which declines as the pH is raised) is equal to the rates of other processes that limit the rate of the overall reaction pathway. To confirm that the rate of hydride transfer decreases with increasing pH, the pH dependence of ^Dv was determined (Figure 4B). ^Dv is the primary isotope effect on the steady state rate due to the substitution of NADPD for NADPH. Analysis of the data gave an apparent pK value of 5.8 ± 0.3 for the effect of pH on the rate of hydride transfer, but this is accurate only if the rate of the isotope-independent rate-limiting steps (product release) is pH independent. The fit gave an isotope effect of 3.6 ± 1 for hydride transfer (limiting value at high pH).

Constants for the Binding of Substrates and Release of Products. Since the determination of steady state kinetic parameters gives a very incomplete understanding of an enzyme reaction pathway, it is desirable to obtain transient state kinetic parameters also, particularly the rate constants

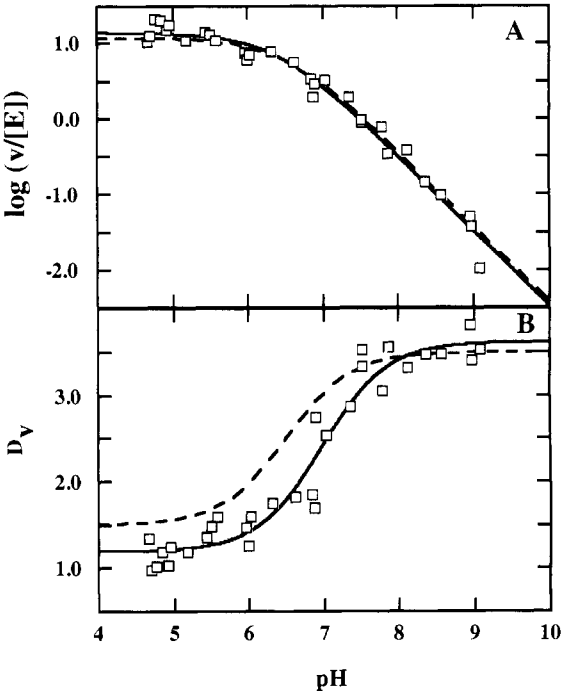


FIGURE 4: pH dependence of the steady state catalytic rate for F34A hDHFR and its isotope effect, ^Dv, resulting from the use of NADPD instead of NADPH. The concentrations of reactants were as follows: NADPH or NADPD, 50 μM; H_2folate, 5 μM; enzyme, 1–830 nM, depending on the pH. pH values were determined for each reaction mixture after rate determination. In A, the continuous line shows the least-squares fit of the data to $k = k'/[1 + 10^{(pH - pKa)}]$, where k is $v/[E]$ and k' is the limiting value of k at low pH. In B, the continuous line shows the least-squares fit of the data to $^Dv = [r + {}^Dv'(1 + 10^{(pH - pK)})]/[r + 1 + 10^{(pH - pK)}]$, where r is the ratio of the rate constant at low pH for the isotope-sensitive step (hydride transfer) to the rate constant for the isotope-insensitive step (NADP release, assumed pH independent), $^Dv'$ is the isotope effect for the isotope-sensitive reaction, and pK describes the pH dependence of the isotope-sensitive rate constant. The broken lines show the results of simulation as described in the Discussion.

Table 3: Rate Constants for the Formation and Dissociation of Dinucleotide Binary Complexes with hDHFR and Its Variants[a]

	NADPH	NADP		
enzyme	k_{on} (μM^{-1} s^{-1})	k_{on} (μM^{-1} s^{-1})	k_{off} (s^{-1})	k_{off}/k_{on} (μM)
WT	38[b]	17[b]	35.6[b]	2.1[b]
F34T	31 ± 1	15.6 ± 1	87 ± 5	5.6
F34S	33 ± 3	17.3 ± 1.7	85 ± 11	4.9
F34A	34 ± 1	15.0 ± 0.9	92 ± 8	6.1

[a] From binding kinetics determined by stopped-flow fluorimetry. [b] From Appleman et al. (1990).

for ligand binding and release. We were able to obtain k_{on} and k_{off} for NADP and k_{on} for NADPH for binding to several of the unliganded variant enzymes (Table 3). The binding of either dinucleotide is little affected by the amino acid

Table 4: Thermodynamic Dissociation Constants, K_d, for Binary Complexes of hDHFR Variants[a]

enzyme	NADPH (nM)	NADP (μM)	H_2folate (μM)	H_4folate (μM)
Wt	50[b]	2.3[b]	0.12[b]	0.05[b]
F34T	12 ± 3		45 ± 15	
F34S	17 ± 6		140 ± 10	
F34A	16 ± 2	6.4	15 ± 4	56 ± 2

[a] From the effect of ligand binding on fluorescence. [b] From Appleman et al. (1990).

substitutions, and the release of NADP is increased only modestly. In measurements of k_{on} for NADP, an initial fast phase accounted for 70% of the total amplitude change and therefore corresponded to binding of NADP to the tightly binding conformer. The fast phase was followed by two slower phases that were not characterized further, but one of them probably corresponds to conversion of the weak binder to the strong binder, as in the case of the WT enzyme (Appleman et al., 1990). These slower phases were not observed in measurements of k_{on} for NADPH, but this may have been due to the fact that observations were made over 0.4 s, and during this period contributions by the slower phase(s) would be small. If the occurrence of these slower phases in the binding process is ignored, $k_{off}/k_{on} \approx K_d$. That this is a reasonable approximation is indicated by the good agreement between k_{off}/k_{on} for F34A binding NADP (5.1 μM) and the value of K_d in Table 4 (6.4 μM). From the values of K_d for NADPH in Table 4 and those for k_{on} in Table 3, the values of k_{off} for NADPH are 0.37, 0.56, and 0.54 s^{-1} for F34T, F34S, and F34A, respectively. These were too low to be measured accurately from the binding kinetics and are 3-5 times lower than for WT enzyme. We were also able to obtain k_{on} for NADPH binding to the H_2folate complex of F34A hDHFR. Final concentrations of H_2folate and enzyme were 70 and 2 μM, respectively, which results in 85% of the enzyme being complexed with H_2folate. Final concentrations of NADPH were 2.5–20 μM. A value of 44 ± 5 μM^{-1} s^{-1} was obtained for k_{on}.

Attempts were made to obtain rate constants for the association of H_2folate with F34A hDHFR or with its NADP complex, but the transient was too fast to be measured by the stopped-flow fluorimeter. Since the latter has a dead time of less than 2 ms, the effective rate constant for the process must be very fast. Since k_{on} is unlikely to be altered by as much as k_{off} in the variants [see, for example, Chunduru et al. (1994)], it is likely that k_{off} is greatly increased. This increases the value of k_{obs}, which is related to k_{off} under pseudo-first-order conditions as follows: $k_{obs} = k_{off} + k_{on}[L]$. In confirmation of this interpretation, the thermodynamic dissociation constant for the binary complex of F34A hDHFR is 130 times higher than that for WT enzyme (Table 4), and K_d is even higher for the F34S and F34T variants. If it is assumed, as is likely, that the increase in K_d primarily is due to an increase in k_{off}, then it can be calculated that the half-time for the reaction is about 0.3 ms for the F34A variant and even shorter for the others, so that the measurement of binding kinetics for H_2folate by stopped flow clearly is impossible.

In the case of F34A hDHFR, the value of K_d for the binary complex with H_4folate is 1100 times higher than that for WT (Table 4). It is therefore probable that k_{off} for H_4folate is much higher for F34A than for WT, so that determination of the rate constants for H_4folate binding by stopped flow is probably impossible, and this was not attempted.

Binding of MTX to F34 Variants. Since all of the variants show marked substrate inhibition by H_2folate, it is not possible to obtain an accurate value of K_i for MTX from measurements of its inhibition of catalytic activity under any reaction conditions. We therefore determined K_d for MTX dissociation from the enzyme–MTX–NADPH complex by measuring the quenching of the fluorescence of the enzyme–NADPH complex by MTX. The results (Table 5) indicate a very greatly decreased affinity of MTX compared with WT, the greatest decrease being in the case of the F34S variant (by a factor of 60 000). The latter result is in reasonable agreement with the value previously reported for F34S hDHFR at pH 7.0 (Schweitzer et al., 1989). K_d for the binary complex of MTX with F34A hDHFR was also determined and found to be 6.4 ± 0.6 μM, a value 5300 times higher than that reported for WT (Chunduru et al., 1994).

Table 5: Binding of MTX to Variants of hDHFR in the Presence of NADPH

enzyme	ternary K_d[a] (nM)	relative	$K_d k_{cat}/K_m$ (s^{-1})	relative
WT	0.0035[b]	1	0.000321	1
F34I	13 ± 2	3700	0.0074	23
F34V	10 ± 1	2800	0.0049	15
F34T	9.6 ± 1.3	2700	0.0011	3.4
F34S	210 ± 20	60000	0.0036	11
F34A	34 ± 10	9700	0.0081	25

[a] Dissociation constant for MTX dissociation from the enzyme–NADPH–MTX complex. [b] $k_{off,app}/k_{on}$ from Chunduru et al. (1994).

In order to determine whether the decreased binding of MTX was due to slower association of MTX or faster dissociation, we determined the binding kinetics of MTX to unliganded F34A hDHFR and to its complex with NADPH. The results (Table 6) indicate that the weak affinity is due to both a decrease in k_{on} (by factors of 30 and 40) and a much larger increase in k_{off} (253- and 460-fold).

Stability of Variants of hDHFR. The replacement of Phe^{34} by much smaller side chains, or by less hydrophobic side chains, has little effect on the stability of the enzyme at 37 °C in the presence of 1 mM NADPH and 100 mM potassium phosphate buffer at pH 7.4, with none of the half-lives falling below that for WT by more than 35%.

DISCUSSION

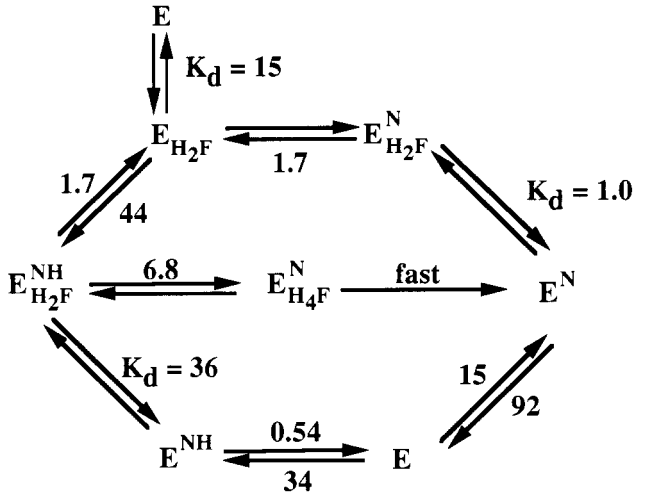
Reaction Pathway for F34A hDHFR. Since H_4folate dissociation is extremely rapid, all of the ternary product complex is rapidly converted to enzyme–NADP (Scheme 1). Two pathways are then possible. One involves NADP dissociation followed by NADPH binding and then H_2folate binding (the lower pathway in Scheme 1). The reverse order of binding of the substrates to unliganded enzyme occurs to a much smaller extent because of the higher ratio of concentration to K_d for NADPH than for H_2folate under experimental or physiological conditions. The rate constants shown for this pathway in Scheme 1 and the K_d shown for H_2folate are taken from the tables. It may be noted that at high substrate concentrations at pH 7.65, all steps in this lower pathway in Scheme 1 will be rapid compared with the chemical transformation, so that for this pathway $k_{cat} = k_{chem}$, as was found to be the case for the overall reaction (Table 2). Furthermore at pH 7.65, the isotope effect due to the use of NADPD should be a maximum for the lower pathway. This was approximately the case for ^Dv for the overall reaction in the presence of 5 μM H_2folate (Figure 4B), the lower than maximum value being due to some contribution to the total flux by the alternate pathway under these conditions. In the alternate pathway (the upper loop in Scheme 1), H_2folate

Table 6: Association and Dissociation Constants for Complexes of MTX with WT and F34A hDHFR

enzyme	species	k_{on} (μM^{-1} s^{-1})	k_{on} relative[a]	k_{off} (s^{-1})	k_{off} relative[a]	k_{off}/k_{on} (nM)	k_{off}/k_{on} relative[a]
WT[b]	E	155	1	0.186	1	1.2	1
	E·NADPH	139	1	0.00035	1	0.00025	1
F34A	E	4.9 ± 0.9[c]	0.032	47.1 ± 5.3[c,d]	253	9600	8000
	E·NADPH	3.5 ± 1.1	0.025	0.161 ± 0.007[e]	460	46	18000

[a] Relative to the corresponding WT value. [b] Values for WT from Chunduru et al. (1994). [c] Mean and standard deviation from three determinations. [d] From binding kinetics. [e] $k_{off,app}$ was determined by competition with edatrexate carried out as previously described (Chunduru et al., 1994).

Scheme 1: Kinetic Scheme for F34A hDHFR at pH 7.65 and 20 °C in MATS Buffer[a]



[a] E, F34A hDHFR; NH, NADPH; H_2F, H_2folate; N, NADP; H_4F, H_4folate. Units are as follows: k_{on}, μM^{-1} s^{-1}; k_{off}, s^{-1}; K_d, μM.

Table 7: Kinetic Parameters for the Slow Alternate Reaction Pathway

parameter	F34T	F34A
k_{off} for NADP from E–NADP–H_2folate (s^{-1})	1.21 ± 0.01	1.67 ± 0.09
K_d for H_2folate from E–NADPH–H_2folate (μM)	1.82 ± 0.35	1.03 ± 0.35
K_m' for H_2folate (μM)	1.75 ± 0.34	0.82 ± 0.27

binds to enzyme–NADP prior to NADP dissociation, and finally NADPH binds rapidly. This pathway becomes significant when the concentration of H_2folate becomes high enough that its rate of binding to enzyme–NADP becomes comparable with the rate of NADP dissociation from the enzyme–NADP complex.

Only two of the rate constants for steps that occur only in this pathway were determined directly, but in addition, certain kinetic parameters could be calculated as follows. As indicated in the Appendix, k_{off} for NADP dissociation from the enzyme–NADP–H_2folate complex is given by $c_2k_{chem}/(c_4k_{chem} - c_2)$, where c_2 and c_4 are constants obtained from fitting the data in Figure 1 to eq 1. The value of K_d for H_2folate dissociation from the enzyme–NADP–H_2folate complex is given by c_1k_{off}/c_2k_{off}', where k_{off} and k_{off}' are the rate constants for NADP dissociation from its complex with enzyme–H_2folate and from its complex with unliganded enzyme, respectively. K_m' for H_2folate is obtained from the relationship $K_m' = c_1/c_4k_{off}'$. The resulting parameter values are shown in Table 7. k_{off} for NADPH dissociation from the ternary substrate complex was determined indirectly. K_d for NADPH dissociation from this complex can be calculated from the K_d values for the binary complexes of H_2folate and of NADPH and from the K_d for H_2folate dissociation from the ternary substrate complex. Since k_{on} for NADPH formation of the ternary substrate complex was determined, k_{off} could be calculated from this and the K_d for NADPH dissociation.

The very low k_{off} for dissociation of NADP from the abortive complex accounts for substrate inhibition. The overall reaction rate decreases as the concentration of H_2folate is increased because more of the reaction flux is forced into the slower upper loop in Scheme 1. The absence of substrate inhibition for WT hDHFR is due to the fact that, for WT, the branch point for the alternate pathways occurs not at the enzyme–NADP complex but at the enzyme–NADP–H_4folate complex (Appleman et al., 1990). The different branch point for the F34 variants is in turn due to a much higher k_{off} for H_4folate dissociation from the ternary product complex of the variant enzymes, as indicated by the high K_d (Table 4). In the case of variants of hDHFR with Phe^{31} replaced by smaller residues, the preferred pathway is neither of those shown in Scheme 1, but rather the pathway from enzyme–NADP–H_4folate to enzyme–H_4folate, enzyme–H_4folate–NADPH, and enzyme–NADPH. This a consequence of a *slower* dissociation of H_4folate from the ternary product complex than in the case of WT hDHFR (Chunduru et al., 1994). Because of this pathway preference, variants with residues other than Phe^{31} do not show inhibition at high concentrations of H_2folate.

Simulation of Catalytic Behavior from Rate Constants. Although it was not possible to estimate all of the relevant rate constants for the kinetic scheme for F34A hDHFR, and several estimates were not obtained by direct measurements, it was still possible to use those that were available, together with the equilibrium dissociation constants (K_d), to simulate steady state kinetic behavior of this variant by the method previously used for WT enzyme (Appleman et al., 1990). In Figure 2, the broken lines represent the simulated dependence of steady state velocity on H_2folate concentration at the three pH values, with the assumption of a pK_a of 5.8 for the pH dependence of the rate of the chemical transformation. It may be seen that in general the dependence is reasonably well predicted. The deviation of the curve for the simulated rates from the experimental data at pH 6 and 7, at high concentrations of H_2folate, is very likely due to the underestimation of k_{chem} mentioned in the Results section, with possible additional contributions from the overestimation of k_{off} for NADP from the enzyme–NADP–H_2folate complex and the assumption that this rate constant is pH independent. In Figure 4A, the agreement between simulation and observation is very close, and the deviation in Figure 4B of the dependence of ^Dv on pH with the assumption of a pK_a of 5.8 for hydride transfer is probably due to the same causes as in the case of Figure 2. The dependence of steady state velocity on NADPH concentration was also simulated and gave a normal hyperbolic plot (not shown), but the corresponding K_m was 0.063 μM, which is much lower than the value of 0.74 μM obtained experimentally. This discrepancy probably arises from the fact that k_{off} for NADPH dissociation from the ternary substrate complex was obtained indirectly, as previously

discussed, and is therefore subject to the cumulative effect of errors in the constants from which it was calculated, especially errors in K_d for H_2folate dissociation from the ternary substrate complex. Despite this discrepancy, the simulation results in general support the model shown in Scheme 1 and indicate that the constants shown are probably close to the true values.

Molecular Interpretation of the Effects of the Mutations. Since the replacement of Phe[34] by residues with smaller side chains causes very large changes in k_{chem} and K_d for MTX, the relation of these property changes to the change in the volume of the side chain of residue 31 is of some interest. When they are buried in proteins, the average volumes (Å^3) for the residues at position 34 of WT and variants of hDHFR W are as follows: Phe, 203.4; Ile, 168.8; Val, 141.7; Thr, 122.1; Ser, 99.1; Ala, 91.5 (Chothia, 1975). Since the average volume for a buried Gly is 66.4 Å^3, the volume of the backbone atoms is approximately 61 Å^3. By correcting the total residue volumes for the volume of the backbone atoms, the side chain volumes are as follows (Å^3): Phe, 142; Ile, 108; Val, 81; Thr, 61; Ser, 38; Ala, 31. When the changes in the values of k_{chem} (Table 2) or K_d for MTX binding (Table 5) with residue are examined, however, it may be seen that the major change is from Phe[34] to Ile[34], with relatively minor changes from Ile[34] to Ala[34], despite the fact that most of the side chain volume decrease occurs in the latter series. In WT hDHFR, the αC of Phe[34] is 3.26 and 4.51 Å, respectively, from ring atoms CD1 and CE1 of this residue, the atoms in closest contact with the pteridine ring of bound folate or bound inhibitor. Since CD1 of Ile can extend 3.97 Å from its αC, the Ile[34] side chain could also make contact with atoms of the pteridine rings of these ligands. Perhaps the greater flexibility of the Ile side chain enables it to adopt a conformation in which contact with the pteridine ring is avoided, thus causing the large change in behavior. It is noteworthy that, in the series of Phe[31] variants, there is no decrease in MTX binding with substitution by Leu, Val, or Thr, and it is only with Ser, Ala, or Gly that a major decrease occurs (Chunduru et al., 1994). Once again, the decrease in affinity does not parallel the decrease in side chain bulk, but the major decrease occurs at a point in this series quite different from the decrease point in the Phe[34] series.

The three substitutions investigated in detail (Table 4) cause large *increases* (125–1200-fold) in K_d for the binary complexes of H_2folate, and for the Ala[34] variant, K_d for the binary complex of H_4folate is 1100 times higher than that for WT. By contrast, substitution of Ala for Phe[31], a residue also at the active site in close proximity to the pteridine ring of bound substrate, causes K_d for H_2folate to *decrease* 33-fold (Chunduru et al., 1994). These clear and major differences in the effects on reduced folate binding of replacing the two phenylalanine rings by small side chains must reflect a major difference in either (a) structural changes induced by the substitutions or (b) the role played by the two Phe residues in the binding of reduced folates. Presently, it is not possible to compare the respective structural changes at the binding site induced by substitutions of the two Phe residues. While it has been possible to obtain crystallographic evidence that substitution of Phe[31] causes little perturbation of other residues at the active site or bound ligands (Chunduru et al., 1994), no information is yet available regarding the structural effects of substitutions for Phe[34].

In regard to the second possibility, crystallographic structures of complexes of WT hDHFR with folate (Oefner et al., 1988; Davies et al., 1990) show that the interactions of the two Phe side chains with bound folate are significantly different. The Phe[34] phenyl ring lies "above" the pteridine ring of bound folate (that is, on the opposite side from the NADPH binding site), and the plane of the phenyl ring is at an angle to that on the pteridine ring. The carbon atoms of one side of the phenyl ring make close contacts (down to 3.18 Å) with N1, C8a, and N8 of the pteridine ring. This residue therefore serves to maintain the pteridine ring in close proximity to the nicotinamide ring of NADPH, and it is not surprising that its replacement by residues with smaller side chains weakens the binding of pteridine substrate, product, and inhibitor and greatly decreases the efficiency of hydride transfer. The phenyl ring of Phe[34] also interacts with the benzene ring of folate, the closest approach of their atoms (CE2 of Phe[34] and C12 of folate) being 3.8 Å, and loss or weakening of these interactions in the variants must also weaken the binding of pteridine ligands. On the other hand, the interaction of the phenyl ring of Phe[31] with the pteridine ring of bound folate is not a face to face interaction, since the Phe[31] phenyl is located at the edge of the pteridine ring and makes its closest contact with O4 (3.4 Å). Like the phenyl of Phe[34], it makes strong interactions with a number of atoms in the benzoyl glutamate moiety of folate. The significant differences in the interactions of the two phenyl rings with the pteridine ring of bound ligands therefore may well account for at least some of the differences in the effects of the respective substitutions.

As in the case of substitutions of Phe[31], substitutions of Phe[34] weaken the binding of MTX (Table 5) much more than the binding of H_2folate (Table 2). Although the crystallographic structures for the F31G, F31A, and F31S variants are available (Chunduru et al., 1994), the molecular basis for this differential effect for these variants is still unclear. Whether structural information about the Phe[34] variants will explain the much greater decreases in MTX binding observed for them remains to be seen.

Overall Capacity of Variants To Confer MTX Resistance. The capacity of a variant hDHFR to confer MTX resistance on a cell in which it is produced depends on the stability of the enzyme in the cell, the extent of the decrease in its binding of MTX, and its catalytic efficiency. If their stability is good, as seems to be the case for these variants, a guide to their resistance-conferring capacity is provided by K_dk_{cat}/K_m, where K_d is for MTX dissociation from its ternary complex. Values for this quantity for the Phe[34] variants are moderately increased (Table 5) because the very large increases in K_d more than offset the large decreases in catalytic efficiency. The original estimates of k_{cat} and K_m for the F34S variant (Schweitzer et al., 1989) indicated that their ratio was decreased by a factor of 69 compared with WT. However, our analysis indicates that k_{cat}/K_m for the F34S variant is actually 5000 times less than that for WT. The discrepancy between our findings and those of Schweitzer et al. (1989) may have been due in part to the fact that substrate inhibition and the complications it introduces into kinetic analysis were apparently overlooked by these authors. We have, in fact, observed very large decreases in k_{cat}/K_m for all of the substitutions at Phe[34] that we studied (Table 1).

When values of K_dk_{cat}/K_m for the Phe[34] variants are compared with the corresponding K_ik_{cat}/K_m values for Phe[31] variants (Chunduru et al., 1994), there is considerable similarity in the numbers. However, an important difference is that for the Phe[31] variants a modest increase in $K_i \leq 100$-fold is combined with quite small decreases in k_{cat}/K_m.

Our results for F34I and F34V hDHFR are in contrast to those reported for the F34L variant of mouse hDHFR by

Thillet et al. (1988). This variant was reported to have a K_i for MTX that was 200 times that of wild-type mouse DHFR, an unchanged k_{cat}, and a K_m for dihydrofolate about 100 times that of the wild-type mouse enzyme. Although we did not examine the F34L variant of hDHFR, it seems unlikely that its properties would be much different from those of the F34I and F34V variants. This raises the intriguing possibility that there are significant differences in the effects of substitutions of active site residues in the mouse and human DHFRs, despite their general similarity in primary, secondary, and tertiary structure.

Values of k_{cat}/K_m for the Phe[34] variants of hDHFR are 160–5000 times lower than those for WT (Table 1). It therefore may be fairly questioned whether the expression of these variants by a single copy of the gene or the cDNA would provide the cell with adequate levels of reduced folates. A definitive answer to this question must await the result of appropriate analyses presently ongoing in our laboratory. In the meantime, since the decreased catalytic efficiency is mostly due to an increase in K_m, it seems likely that adequate rates of reduction of H_2folate or its polyglutamates would occur only if the intracellular concentrations of these substrates approached their K_m's for the variant. As explained earlier in this paper, K_d for H_2folate dissociation from its complex with enzyme–NADPH approximates the K_m operational at likely intracellular concentrations of H_2folate, and values of K_d are in the range 24–350 μM (Table 2). It seems likely, therefore, that levels of H_2folate approaching this range would need to accumulate in the cell before rapid reduction would occur. However, H_2folate, and especially its poyglutamates, are powerful inhibitors of several folate enzymes, such as methylenetetrahydrofolate reductase (Matthews & Baugh, 1980), thymidylate synthase (Dolnick & Cheng, 1978; Barum et al., 1988), and amido phosphoribosyltransferase (Sant et al., 1992). Although accurate calculations are not possible from the available data, the concentrations of H_2folate necessary for its reduction by Phe[34] variants of hDHFR may well be highly toxic to the cell.

APPENDIX

The kinetic scheme for substrate inhibition for which the equation was derived is shown in Scheme 2. Then by the method of Indge and Childs (1976), it can be shown that

$$c_1 = k_1k_3/(k_2 + k_3) = k_{cat}/K_m$$

$$c_2 = k_1k_3k_5k_7/(k_2 + k_3)k_4(k_6 + k_7) = k_{cat}k_{cat}'/K_mK_m'k_4$$

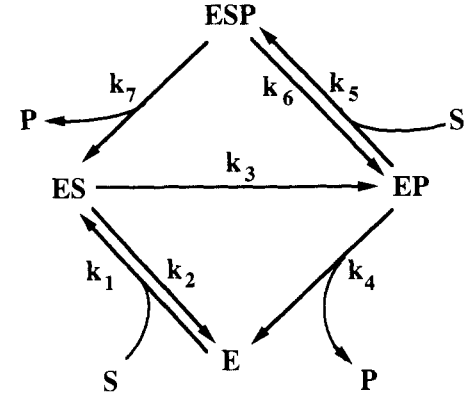
$$c = k_1(k_3 + k_4)/k_4(k_2 + k_3) + k_2k_5k_7/k_4(k_2 + k_3)(k_6 + k7)$$

$$= 1/K_m + (k_{cat}k_{cat}'/K_mK_m')(k_2/k_1k_3k_4)$$

$$c_4 = k_1k_5(k_3 + k_7)/k_4(k_2 + k_3)(k_6 + k_7) = k_{cat}/K_mK_m'k_4$$

As applied to the variant DHFRs, S represents H_2folate and P represents NADP in Scheme 2. Scheme 1 collapses to Scheme 2 under the following conditions: (1) NADPH is saturating, so that its binding is always fast and essentially irreversible. The conversions of enzyme to enzyme–NADPH and enzyme–H_2folate to enzyme–H_2folate–NADPH are therefore omitted from Scheme 2. (2) The concentration of free NADP is essentially zero, so that there is no rebinding, and rebinding reactions are therefore omitted from Scheme 2. (3) k_{off} for NADP from enzyme–H_4folate–NADP $\ll k_{off}$ for H_4folate from this complex. The step for the dissociation of H_4folate from the ternary complex is therefore combined with the chemical transformation step in Scheme 2. (4) This combined chemical transformation and dissociation of H_4folate is essentially irreversible, and the reverse reaction is therefore omitted from Scheme 2.

Scheme 2: Kinetic Scheme for Which the Substrate Inhibition Equation Was Derived[a]



[a] E, enzyme; S, substrate; P, product.

The first two of these conditions apply in determinations of initial steady state rates with 100 μM NADPH and no NADP. The data reported in this paper indicate that, with zero H_4folate concentration as in the initial steady state measurements, the last two conditions also apply.

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