Probing the Functional Role of Phenylalanine-31 of *Escherichia coli* Dihydrofolate Reductase by Site-Directed Mutagenesis†

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Received October 22, 1986; Revised Manuscript Received February 12, 1987

ABSTRACT: The role of Phe-31 of *Escherichia coli* dihydrofolate reductase in binding and catalysis was probed by amino acid substitution. Phe-31, a strictly conserved residue located in a hydrophobic pocket and interacting with the pteroyl moiety of dihydrofolate (H_2F), was replaced by Tyr and Val. The kinetic behavior of the mutant enzymes in general is similar to that of the wild type. The rate-limiting step for both mutant enzymes is the release of tetrahydrofolate (H_4F) from the E·NADPH·H_4F ternary complex as determined for the wild type. The 2-fold increase in V for the two mutant enzymes arises from faster dissociation of H_4F from the enzyme–product complex. The quantitative effect of these mutations is to decrease the rate of hydride transfer, although not to the extent that this step becomes partially rate limiting, but to accelerate the dissociation rates of tetrahydrofolate from product complexes so that the opposing effects are nearly compensating.

Dihydrofolate reductase (5,6,7,8-tetrahydrofolate:$NADP^+$ oxidoreductase, EC 1.5.1.3) catalyzes the NADPH-dependent reduction of 7,8-dihydrofolate (H_2F)[1] to 5,6,7,8-tetrahydrofolate (H_4F). The enzyme is necessary for maintaining intracellular pools of H_4F and its derivatives, which are essential cofactors in the many important biosynthetic reactions which require the transfer of one-carbon units. It is the target enzyme of a group of antifolate drugs that are widely used as antitumor and antimicrobial agents such as methotrexate (MTX), trimethoprim (TMP), and pyrimethamine (PMA). Because of its biological and pharmacological importance, the enzyme has been the subject of extensive studies, both structural and kinetic, over the past three decades. Although the identities of the amino acids at the active site of dihydrofolate reductase are now known, the function of many of the amino acid residues in binding and catalysis has not been clarified. We have taken a site-directed mutagenesis approach to establish structure–function relationships for *Escherichia coli* DHFR.

Site-directed mutagenesis is a powerful tool to elucidate the functional role of individual amino acids in proteins and to alter enzymic properties such as specificity (Dalbadie-McFarland et al., 1982, 1986; Villafranca et al., 1983; Fersht et al., 1984; Chen et al., 1985; Craik et al., 1985; Estell et al., 1985; Ghosh et al., 1986). *E. coli* dihydrofolate reductase binds tightly to H_2F (Stone & Morrison, 1983) in a conformation with the pteridine ring nearly perpendicular to the *p*-aminobenzoyl group (Bolin et al., 1982; Filman et al., 1982). The binding site is lined with several strictly conserved hydrophobic amino acids. One of these, Phe-31, interacts with the pteroyl moiety through van der Waals contacts with the edge of the phenyl ring toward faces of both the pteridine ring and *p*-aminobenzoyl group. It is of interest to inquire as to the functional role of this strictly conserved amino acid in binding and/or catalysis.

We have investigated both Tyr-31 and Val-31 DHFRs by pre-steady-state and steady-state kinetics and deuterium isotope effect studies. The results provide unique insights into the mechanism of *E. coli* dihydrofolate reductase and the role of Phe-31.

Materials and Methods

DNAs and Transformation. Two *Hae*III fragments (574 and 408 base pairs) containing the trimethoprim-resistant DHFR gene were isolated from pCV29 plasmid (Smith & Calvo, 1980, 1982) and subcloned by Yaegashi and Tu (unpublished results) into the *Bam*HI site of a pBR322 derivative lacking the *Eco*RI site to generate pTY1 (Chen et al., 1985). Plasmid pTY1 was grown in *E. coli* strain HB101 and was purified by the alkaline method (Birnboim & Doly, 1979; Ish-Horowicz & Burke, 1981). Competent cells were prepared, and transformations were done according to the method of Hanahan (1983). Selections were for resistance to ampicillin at 50 μg/mL and/or trimethoprim at 100 μg/mL.

The 21- and 22-base oligonucleotides, 5′-G-A-T-C-T-C-G-C-G-T-G-G-G-T-T-A-A-A-C-G-C-3′ and 5′-G-A-T-C-T-C-G-C-G-T-G-G-T-A-T-A-A-A-C-G-C-A-3′, respectively, were prepared on an Applied Biosystems Model 380A DNA synthesizer and purified by means of high-pressure liquid chromatography on a reverse-phase μBondapak C_{18} column (3.9 mm × 30 cm; Waters Associates, Milford, MA).

Enzymes. E. coli exonuclease III, T4 DNA ligase, *Hae*III, *Bam*HI, *Eco*RI, *Aha*III, *Dde*I, and T4 polynucleotide kinase were obtained from New England Biolabs. *E. coli* DNA polymerase I (Klenow fragment) was purified in our laboratory.

Phosphorylation of Oligonucleotides. Two hundred picomoles of 5′-OH oligonucleotides was treated with 5 units of

† This work was supported in part by NIH Grant GM24129.

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[1] Abbreviations: DHFR, dihydrofolate reductase; H_2F, 7,8-dihydrofolate; H_4F, 5,6,7,8-tetrahydrofolate; NADPH, nicotinamide adenine dinucleotide phosphate, reduced; $NADP^+$, nicotinamide adenine dinucleotide phosphate, oxidized; EDTA, ethylenediaminetetraacetic acid; DTT, *threo*-1,4-dimercapto-2,3-butanediol; DTE, *erythro*-1,4-dimercapto-2,3-butanediol; PMSF, phenylmethanesulfonyl fluoride; Tris, tris(hydroxymethyl)aminomethane; OD, optical density; kb, kilobase(s); TE, 10 mM Tris and 1 mM EDTA, pH 8.0.

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T4 polynucleotide kinase for 50 min at 37 °C in 30 μL of 100 μM ATP/100 mM Tris·HCl, pH 8.0/10 mM $MgCl_2$/5 mM DTT. The enzyme was inactivated by heating to 65 °C for 15 min. The solution was stored at −70 °C.

Preparations of Nicked and Gapped DNA. Fifty micrograms of supercoiled pTY1 DNA and 15 μg of ethidium bromide in 50 μL of 50 mM Tris·HCl, pH 7.5/50 mM NaCl/10 mM $MgCl_2$/1 mM DTT were treated in the dark at room temperature with 40 units of *Eco*RI (10 units/μL). The nicking reaction was stopped by extracting once with an equal volume of phenol, once with $^1/_2$ volume of phenol and $^1/_2$ volume of chloroform, twice with an equal volume of chloroform, and then 4 times with 3–5 volumes of diethyl ether. The DNA was precipitated by adding 0.1 volume of 3 M sodium acetate, pH 7.1, and 2.5 volumes of absolute ethanol and kept at −20 °C for longer than 3 h. After centrifugation at 4 °C for 10 min at 12K rpm, the pellet was washed 3 times with 0.75 mL of cold 70% (v/v) ethanol, dried, and dissolved in 30 μL of TE buffer, pH 7.5. The DNA concentration was estimated to be 1.2 μg/μL, assuming that 1 OD_{260nm} unit = 50 μg of DNA/mL.

About 5 μg (1.4 pmol) of the nicked DNA was treated for 60 min at room temperature with 100 units of *E. coli* exonuclease III in 50 μL of 66 mM Tris·HCl, pH 8.0/90 mM NaCl/5 mM $MgCl_2$/10 mM DTT. Five tubes with the identical reaction condition were combined, and the reaction was stopped by phenol extraction and ethanol precipitation as described in the nicking reaction. This exonuclease III treatment generated about a 700-base gap. The gapped DNA was dissolved in 10 μL of TE buffer, pH 7.5, and stored at −70 °C.

Heteroduplex Formation and Transformation. The gapped DNA (ca. 1.1 pmol) was heated to 65 °C for 7 min in the presence of about 44 pmol of the 5′-phosphorylated synthetic oligonucleotide in 40 μL of 40 mM NaCl/20 mM Tris·HCl, pH 7.5/10 mM $MgCl_2$/1 mM DTT, and then the solution was chilled on ice for 4 h. To the 40 μL of oligonucleotide-annealed DNA solution was added 60 μL of 350 μM ATP/175 μM dATP/175 μM dCTP/175 μM dGTP/175 μM dTTP/40 mM NaCl/20 mM Tris·HCl, pH 7.5/10 mM $MgCl_2$/1 mM DTT containing 7.8 units of *E. coli* DNA polymerase (Klenow fragment) and 2500 units of T4 DNA ligase. The polymerization and ligation reaction was carried out at 10 °C for 10 h. The resulting heteroduplex-containing DNA was isolated by phenol extraction followed by ethanol precipitation.

The concentration of the circular heteroduplex-containing DNA was adjusted to about 0.1 μg DNA per 100 μL of TE buffer, pH 7.5, and 100 μL of this solution was used to transform HB101. The transformed bacterials were then cultured in a 2-L LB medium containing 50 μg/mL ampicillin. Plasmids were amplified and isolated.

Isolation of Mutated DHFR Gene. Two hundred micrograms of the plasmid DNA isolated from the first transformation were digested with both 50 units of *Aha*III and 160 units of *Bam*HI for 16 h at 37 °C in 400 μL of 75 mM NaCl/10 mM Tris·HCl, pH 8.0/10 mM $MgCl_2$/5 mM DTT. (*Bam*HI digestion produces two fragments: a 4.3-kb fragment of a linear pBR322 derivative lacking the *Eco*RI site and a 1-kb fragment containing the DHFR gene. Of this 1-kb fragment only a mutated gene at the Phe-31 site is resistant to the *Aha*III digestion because of the destruction of the *Aha*III recognition sequence.) The digestion mixture was loaded on a 3.5% polyacrylamide gel, and a gel portion containing the 1-kb fragment was cut out from the gel after electrophoresis. (The position of the 1-kb fragment was identified by staining only the control lane containing the 1-kb fragment with ethidium bromide, and the mutant 1-kb fragment was not exposed to the ethidium bromide.) The gel portion containing the mutant 1-kb fragment was placed in a dialysis bag with 0.1 × TBE buffer (5 mM Tris base/6.6 mM boric acid/0.1 mM EDTA), and an electroelution was carried out overnight in the same 0.1 × TBE buffer at 8 mA at 4 °C. The mutant 1-kb fragment was collected on about 0.1 mL of DE52 that had been presoaked in 100 mM NaCl/50 mM Tris·HCl, pH 7.8/1 mM EDTA followed by equilibration with TE buffer and was eventually eluted from the DE52 column by 300 μL of a high-salt solution of 2 M NaCl/50 mM Tris·HCl, pH 7.8/1 mM EDTA. The eluent was dialyzed against 500 mL of 10 mM Tris·HCl, pH 7.5 at 4 °C for 36 h with two changes and concentrated to 250 μL.

Cloning of Mutated DHFR Gene. Dephosphorylation of a pBR322 derivative linearized at the *Bam*HI site was carried out by treating 30 μg of a linear pBR322 derivative (4.3 kb) with 3 units of alkaline phosphatase for 60 min at 37 °C in 150 μL of 50 mM Tris·HCl, pH 8.0/1 mM $MgCl_2$/0.1 mM $ZnCl_2$, in order to avoid self-ligation and/or polymerization of the linear pBR322 derivative upon ligation with the mutant 1-kb fragment. The dephosphorylated pBR322 derivative linearized at the *Bam*HI site was purified by phenol extraction followed by ethanol precipitation. One microgram of the dephosphorylated 4.3-kb fragment and 0.2 μg of the mutant 1-kb fragment were treated with 400 units of T4 DNA ligase at 10 °C for 18 h in 50 μL of 0.5 mM ATP/50 mM Tris·HCl, pH 7.5/10 mM DTT. The enzyme was inactivated by phenol extraction, and the ligated nicked DNA was purified by gel exclusion chromatography on a 1-mL column of Sephadex G25 presoaked in TE buffer, pH 7.5. The total volume of the eluent was about 100 μL, including 50 μL from the TE buffer washing. One-third of the ligated nicked DNA was used to transform *E. coli* HB101.

Colony Screening. Mutant colonies were identified by digestion of the plasmid DNAs, isolated essentially by the method of Birnboim and Doly (1979), with *Bam*HI and *Aha*III. The mutant plasmid showed a unique 1-kb fragment in the gel electrophoresis.

DNA Sequencing. The whole gene of each mutant DHFR was sequenced by the general method of Maxam and Gilbert (1977, 1980) with minor modifications.

Purification of Mutant Enzymes (Val-31 and Tyr-31). Cells for each mutant enzyme were grown in a 2-L culture containing 50 μg of ampicillin/mL to a final optical density of 1.0 at 650 nm and harvested by centrifugation at 6 rpm at 4 °C. The cells were suspended in 20 mL of 40 mM Tris·HCl, pH 8.0/1 mM EDTA and centrifuged at 20K rpm at 4 °C for 30 min to yield about 6 g of precipitate. All steps for the following cell lysis were conducted at 4 °C. The cells (ca. 6 g) were suspended in 7 mL of a lysis buffer (40 mM Tris·HCl, pH 8.0/1 mM EDTA/1 mM DTE), and the following additions were made to the suspension: (1) 0.7 μL of PMSF (18 mg/mL in ethanol), 15 mg of lysozyme in 1 mL of the lysis buffer, and 2.1 mL of EDTA, pH 8.0 (20 mg/mL EDTA in lysis buffer), which was sonicated 3 times for 30 s followed by an 8-min ice cooling; (2) 2.1 mL of Brij 58 (5 g/100 mL of lysis buffer), which was sonicated 3 times for 30 s followed by a 7-min ice cooling; and (3) 330 μL of $MgCl_2$ (5.1 g/25 mL of lysis buffer) and 500 μL of DNase (6.84 mg/3 mL of lysis buffer), which was mixed occasionally for 15 min. The suspension was centrifuged at 20K rpm for 40 min. To the supernatant 1.2 mL of streptomycin sulfate (1 g/10 mL of lysis buffer) was added with gentle stirring at 1-min intervals

(100 μL each time), and the suspension was centrifuged at 20K rpm for 40 min. The supernatant (16.5 mL) was made 40% saturated in ammonium sulfate by gradually adding with gentle stirring 3.85 g of finely powdered $(NH_4)_2SO_4$ over a period of 15 min. The stirring was continued for another 15 min. Unwanted protein was removed by centrifugation at 20K rpm for 1.5 h. The supernatant was then made 90% saturated by gradually adding 5.8 g of $(NH_4)_2SO_4$ over a period of 20 min, and the mixture was gently stirred for an additional 15 min and then kept on ice for 1.5 h. Following the centrifugation at 20K rpm for 30 min, the white pellet was resuspended in 13 mL of 40 mM KH_2PO_4, pH 6.0/0.2 M KCl/1 mM EDTA/1 mM DTE. To this was then added 2 mL of MTX (methotrexate) resin, and the solution was gently agitated for 20 h. The MTX resin was batch washed 4 times with 10 mL of 0.2 M KH_2PO_4, pH 6.0/1 M KCl/1 mM EDTA/1 mM DTE. The MTX resin was transferred to a 10-mL column and washed with the same high-salt buffer (0.2 M KH_2PO_4/1 M KCl/1 mM EDTA/1 mM DTE) until the OD_{280} of the eluent was below 0.1. The mutant DHFR was eluted with 0.2 M K_3BO_3, pH 9.0/1 M KCl/1 mM EDTA/1 mM DTE, dialyzed against 2 L of 10 mM Tris·HCl, pH 7.4/1 mM EDTA/1 mM DTE for 8 h with two changes, and purified on a column (52 × 1.1 cm) of DEAE-Sephacel with a linear gradient of 500 mL each of 10 mM Tris·HCl, pH 7.4/1 mM EDTA/1 mM DTE and 1 M NaCl/1 mM EDTA/1 mM DTE/10 mM Tris·HCl, pH 7.4. About 1 mg of pure DHFR was obtained per 1 g of cells. The pure enzyme solution was made 90% saturated with $(NH_4)_2SO_4$ and stored at −20 °C.

Preparation of A-Side (4R)-4-^2H Reduced Nicotinamide Adenine Dinucleotide Phosphate (NADPD). NADP (ca. 47 mg, 56 μmol) was dissolved in 9.1 mL of 30 mM 3-tris-[[(hydroxymethyl)methyl]amino]propanesulfonic acid (Taps), pH 9.0. After the pH of the solution was adjusted to 9.0 with 1.0 M KOH, [$^2H_6$]ethanol (0.82 mL) and *Leuconostoc mesenteroides* alcohol dehydrogenase (50 units) were added, and the reaction mixture was gently stirred at room temperature for 40 min. Lithium chloride (84 mg) was then added, and the pH of the solution was adjusted to 10 with both 1.0 and 0.1 M KOH. The reaction mixture was stirred at room temperature for another 10 min, then loaded onto a 1.1 × 15.4 cm column of AG MP-1 resin that had been prewashed with 150 mL of 1.0 M LiCl (pH 10.0), and equilibrated with 500 mL of 0.2 M LiCl (pH 10.0) at 4 °C. The chromatography was performed at 4 °C with a step gradient formed from 320 mL of 0.2 M LiCl (pH 10.0) followed by 30 mL of 1.0 M LiCl (pH 10.0) at a flow rate of 0.85 mL/min with 10- and 2-mL fractions collected, respectively. The last four fractions of 0.2 M LiCl (40 mL) and the first nine fractions of the 1.0 M LiCl eluent yielded 38 μmol of NADPD with a 260- to 340-nm ratio of 2.26 ± 0.02. The fraction with the maximum OD at 340 nm was used for kinetic studies of the deuterium isotope effect.

Kinetic Assays. Dihydrofolate was prepared from folic acid by the method of Blakley (1960), and its concentration was estimated spectrophotometrically at 282 nm by using a molar extinction coefficient of 2.8×10^4 M^{-1} cm^{-1} at pH 7.4 (Blakley, 1960). Initial velocities for dihydrofolate reductase reactions were determined at 340 nm and 25 °C with a molar absorbance change of 11.8×10^3 cm^{-1} (Stone & Morrison, 1982) by employing a Cary 219 spectrophotometer. The concentration of NADPH or NADPD ($\epsilon = 6.2 \times 10^3$ M^{-1} cm^{-1} at 340 nm; Horecker & Kornberg, 1948) was maintained constant (60 μM) over the entire pH range tested. The buffer (MTEN) used for the assays contained 50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), 25 mM tris(hydroxymethyl)aminomethane (Tris), 25 mM ethanolamine, and 100 mM NaCl (Stone & Morrison, 1984).

Stopped-Flow Kinetic Measurements. Kinetic data were obtained by using a stopped-flow apparatus, operating in the fluorescence mode, that was built in Dr. K. A. Johnson's laboratory at PSU. Complex formation and ligand trapping studies were followed by excitation at a wavelength of 290 nm and by monitoring the quenching of the protein intrinsic fluorescence by using a 340-nm interference filter. The chemical transformation was monitored by using an excitation wavelength of 290 nm and monitoring the decrease in fluorescence at 450 nm. All experiments were carried out at 25 °C. The buffer system used was MTEN. In most experiments the sum of at least four experimental runs was used for data analysis.

Equilibrium Dissociation Constants. Equilibrium dissociation constants for dihydrofolate reductase–H_2F complexes were determined by fluorescence titration at 25 °C on a SLM 8000 spectrofluorometer with excitation and emission wavelengths of 290 and 340 nm, respectively. The buffer system used was MTEN. Standard tryptophan solutions were used to correct for absorbance by added ligands.

Data Analysis. Data obtained at each pH value by varying the concentration of H_2F were fitted to eq 1 by a nonlinear

$$v = V[H_2F]/(K_{H_2F} + [H_2F]) \quad (1)$$

regression program to yield values for the maximum velocity V and the Michaelis constant K_{H_2F}, as well as $(V/K)_{H_2F}$. The pH–rate profiles were fit to eq 2, where X represents the value

$$X = C/(1 + K_a/[H]) \quad (2)$$

of V/K or V at a particular pH value, C represents the pH-independent value of the parameter, and K_a is the acid dissociation constant. In the case of "hollow" phenomenom, eq 3 was used, where C_1, C_2, and C_3 represent again the pH-in-

$$X = [C_1(1 + C_2K_1/[H])]/[(1 + K_1/[H])(1 + C_3K_1/[H])] \quad (3)$$

dependent values of the parameters and K_1 is the intrinsic acid dissociation constant [see Appendix or Taira et al. (1987)].

The standard errors (σ) for deuterium isotope effects (IE), $^D(V)$ and $^D(V/K)_{H_2F}$, were calculated by eq 4 (Bevington, 1969), where R_H and R_D represent relative standard errors

$$\sigma = IE(R_H^2 + R_D^2)^{1/2} \quad (4)$$

for $^H(V/K)$ or $^H(V)$ and relative standard errors for $^D(V/K)$ or $^D(V)$, respectively.

In the stopped-flow kinetic experiments the data for complex formation and ligand trapping were analyzed according to a single exponential model

$$F(t) = AMP \exp(-k_{obsd}t) + \text{base line} \quad (5)$$

where $F(t)$ is the observed fluorescence signal at time t, AMP is the amplitude of the fluorescence change, and k_{obsd} is the observed rate constant.

RESULTS

Construction of Mutants. Both mutant Tyr-31 and Val-31 plasmids were constructed according to the procedure of Dalbadie-McFarland et al. (1982) with some modifications (see Materials and Methods). The entire structural gene for both mutants has been completely sequenced according to the

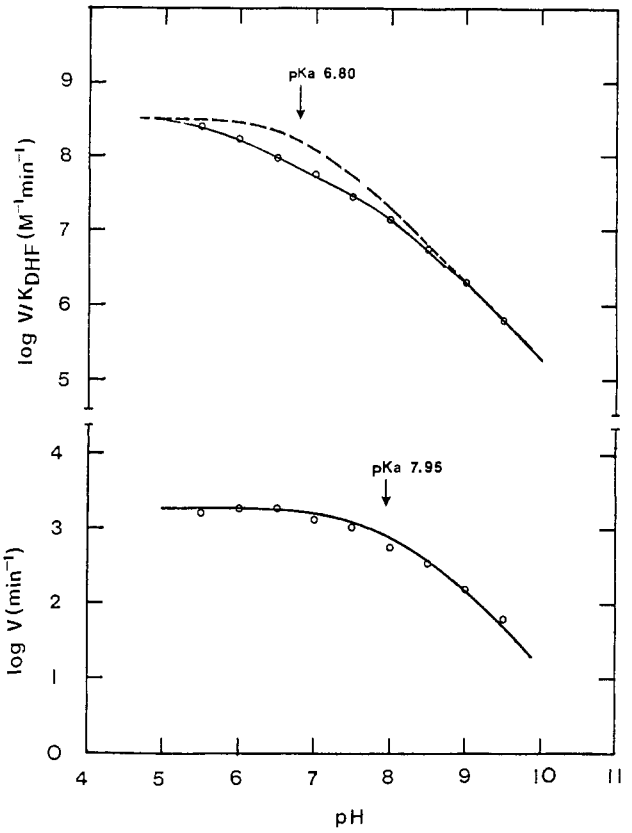


FIGURE 1: pH dependence of V and $(V/K)_{H_2F}$ for Tyr-31 DHFR (O), where the dashed line in the $(V/K)_{H_2F}$ profile and the solid line in the V profile are theoretical curves, drawn on the basis of eq 2, for pK_a values indicated. The solid line in the $(V/K)_{H_2F}$ profile is the theoretical curve, drawn according to eq 3, for the intrinsic pK_a of 6.0 and the condition $k_1 = 10k_7$, $k_6 = 10k_2$, and $k_3 = 10k_2$. Reaction conditions were 1.3–131 nM Tyr-31 DHFR, 2.7–132 μM H_2F, and 60 μM NADPH at 25 °C in MTEN buffer.

Table I: Comparison of NADPH Binding for Native and Mutant DHFRs at pH 6.0[a]

	k_{obsd}[b] (s^{-1})	k_{iso}[c] (s^{-1})	rel[d] amp
Phe-31	200	0.034	0.72
Tyr-31	200	0.032	0.24
Val-31	210	0.036	0.14

[a] The enzymes were excited at 290 nm, and NADPH binding reaction was monitored by fluorescence quenching at 340 nm at 25 °C. The buffer used was MTEN buffer (Stone & Morrison, 1984). [b] The observed first-order rate constant determined when the concentrations of NADPH and the enzymes are 10 and 0.5 μM, respectively. [c] Rate of interconversion of E_2 to E_1 (Caley et al., 1981). [d] Relative amplitudes of the fast phase to the slow phase, i.e., E_1/E_2.

method of Maxam and Gilbert (1977, 1980), and no changes, other than the desired ones, have been detected.

Binding Kinetics of NADPH. Data for the binding of

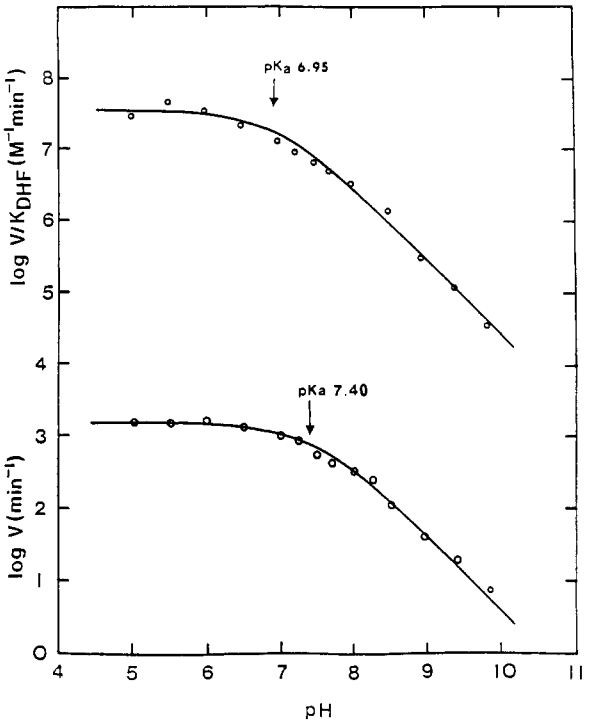
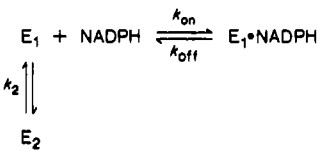


FIGURE 2: pH dependence of V and $(V/K)_{H_2F}$ for Val-31 DHFR (O), where the solid lines are the theoretical curves, drawn on the basis of eq 2, for pK_a values indicated. Reaction conditions were 6.1–730 nM Val-31 DHFR, 3.25–120 μM H_2F, and 60 μM NADPH at 25 °C in MTEN buffer.

NADPH to the wild-type DHFR is biphasic and has been described by (Cayley et al., 1981)



The measured pseudo-first-order rate constant for the fast phase, k_{obsd}, is related to the association and dissociation rate constants k_{on} and k_{off}, respectively, by $k_{obsd} = k_{on}[NADPH] + k_{off}$. The equilibrium concentration of the conformers E_1 and E_2 sets the relative amplitude of the two phases where k_2 is the rate of conversion of $E_2 \rightarrow E_1$ as measured by a decrease in fluorescence at 340 nm of the slow phase. The values for the wild type, Tyr-31 mutant, and Val-31 mutant are listed in Table I. The only departure from parameters characterizing the wild-type enzyme is the 3- and 5-fold decrease in the concentration of E_1 for the Tyr-31 and Val-31 mutants, respectively.

pH Dependence of Isotope Effects. Table II shows the variation with pH of the deuterium isotope effects on V and $(V/K)_{H_2F}$ for the reactions catalyzed by the wild-type, Val-31

Table II: pH Dependence of Deuterium Isotope Effect on V and $(V/K)_{H_2F}$ for *E. coli* Dihydrofolate Reductases at 25 °C[a]

Phe-31 (wild type)						
pH	6.0	8.0	9.0	10.0		
$^D(V)$	1.0	1.8 ± 0.1	2.7 ± 0.3	2.90 ± 0.2		
$^D(V/K)_{H_2F}$		1.9 ± 0.5	3.0 ± 1.4	3.19 ± 0.7		
Val-31 mutant						
pH	5.5	7.0	9.0			
$^D(V)$	1.1 ± 0.1	2.9 ± 0.4	3.4 ± 1.1			
$^D(V/K)_{H_2F}$	2.4 ± 0.9	2.8 ± 0.9	3.2 ± 1.9			
Tyr-31 mutant						
pH	5.5	6.0	7.0	7.5	8.0	9.0
$^D(V)$	0.9 ± 0.1	1.5 ± 0.2	2.1 ± 0.2	2.5 ± 0.5	2.8 ± 0.5	3.0 ± 0.6
$^D(V/K)_{H_2F}$	2.6 ± 1.1	2.5 ± 1.2	3.0 ± 1.0	3.1 ± 1.9	3.4 ± 1.6	3.9 ± 2.2

[a] Conditions: 1–400 nM DHFR, 2.5–125 μM H_2F, 60 μM NADPH, 60 μM NADPD, MTE buffer, 25 °C, and 340 nm.

Table III: Steady-State Kinetic Parameters for Wild Type (Phe-31) and Val-31 and Tyr-31 Mutants under Conditions of Varying $[H_2F]$[a] and Their Equilibrium Dissociation Constants for H_2F

	V (s^{-1})	$(V/K)_{H_2F}$ (M^{-1} s^{-1})	K_{H_2F} (μM)	$K_{D\,H_2F}$[c] (μM)
Phe-31	12	1.2×10^7	1.1[b]	0.2
Tyr-31	30	5.3×10^6	5.7	2.5
Val-31	26	9.7×10^5	27	5.0

	pK_a	
	from V data	from $(V/K)_{H_2F}$ data
Phe-31	8.38 ± 0.02[b]	8.09 ± 0.06[b]
Tyr-31	7.95 ± 0.05	6.80 ± 0.10
Val-31	7.4 ± 0.05	6.95 ± 0.05

[a] Constants (limiting values) are reported for the low pH region of the pH profiles; the concentration of NADPH was maintained at 60 μM. [b] The values were obtained from Stone and Morrison (1984); at pH 6.0, we were unable to measure the K_M value accurately and it was estimated below 0.5 μM. [c] The enzymes were excited at 290 nm, and the fluorescence quenching titrations were performed at 340 nm, 25 °C, and pH 7.0. The buffer used was MTEN buffer (Stone & Morrison, 1984).

mutant, and Tyr-31 mutant dihydrofolate reductases in the presence of saturating NADPH. $^D(V)$ decreases from a value of about 3.0–3.4 at high pH to a value of 1.0 at low pH for all three enzymes. $^D(V/K)_{H_2F}$ also decreases with decreasing pH for all three enzymes: the $^D(V/K)$ value for all three enzymes was about 3.2 at high pH and about 2.5 for both the Val-31 and Tyr-31 mutants at low pH. The value of $^D(V/K)$ for the wild-type enzyme at pH 6.0 was not obtainable with confidence because the K_M for H_2F is below 1 μM.

pH Dependence of V and V/K with $[H_2F]$ Varied. Values for V and $(V/K)_{H_2F}$ for both Val-31 and Tyr-31 mutants were determined by varying the dihydrofolate concentration at a fixed NADPH concentration (60 μM). The pH profiles of these values for these two mutant enzymes are illustrated in Figures 1 and 2. Analysis of the data for the V and $(V/K)_{H_2F}$ profiles for the Val-31 mutant yielded pK_a values of 7.40 ± 0.10 and 6.95 ± 0.10, respectively, and that for the Tyr-31 mutant yielded pK_a values for 7.95 ± 0.05 and 6.80 ± 0.10, respectively. The kinetic constants (V, V/K, K_M, and pK_a) at the pH optimum (the acidic region) for the wild-type enzyme and two mutant enzymes are summarized in Table III. The optimum V values for both the Tyr-31 and Val-31 mutant enzymes are about 2-fold larger than that for the wild type. However, the $(V/K)_{H_2F}$ values for both mutant enzymes are smaller than that for the wild type due to the larger increase in K_M for the mutant enzymes. Above pH 5.5, the $(V/K)_{H_2F}$ for the Tyr-31 mutant enzyme describes a pH dependence that has been termed a "hollow" by Cleland (1977).

Dissociation Rate Constants from Competition Experiments. When dihydrofolate reductase was excited at 290 nm, the fluorescence emission spectrum showed a maximum at 340–350 nm, typical of the fluorescence spectra reported for other proteins (Udenfriend, 1962). Binding of H_2F, NADPH, and their analogues quenched this fluorescence (Dunn et al., 1978; Cayley et al., 1981). The dihydrofolate reductase–ligand complex (EL_1) can be trapped by a second ligand L_2, which competes for the same binding site. If $k_{-1} \ll k_2[L_2] \gg k_1[L_1]$,

$$EL_1 \underset{k_1}{\overset{k_{-1}}{\rightleftharpoons}} E + L_1$$

$$E + L_2 \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EL_2$$

the fluorescence change is attributable to the conversion of EL_1 to EL_2 characterized by a single exponential with a rate constant k_{-1} (Dunn et al., 1978; Cayley et al., 1980). The dissociation rate constants for the DHFR–ligand complexes, binary and ternary, have been measured by this method with MTX and NADPH as the competing ligands since they bind tightly to the enzyme and have high quenching efficiencies at 340 nm. All reaction progress curves fit well to a single exponential, with the results being summarized in Table IV. Enzyme concentration and the concentrations of both bound and competing ligands were chosen so that the observed rates were an accurate reflection of k_{-1}.

Table IV: Rate Constants for Dissociation of $NADP^+$ and H_4F from $E{\cdot}NADP^+{\cdot}H_4F$ and $E{\cdot}NADPH{\cdot}H_4F$ Ternary Complexes[a] and for Hydride Transfer at pH 6.0[b]

	k_9 (s^{-1})[c]	k_{off} (s^{-1})[d]	k_{11} (s^{-1})[e]	k_3[f]
Phe-31	200[g]	2.4[g]	12[g]	690[h]
Tyr-31[a]	280	100	30	400[h]
Val-31		[i]		120[h]

[a] Tyr-31 DHFR (1–3.2 μM) was incubated with H_2F (2.5–10 μM) and $NADP^+$ (40–150 μM) or NADPH (20 μM) and then trapped with MTX (50–150 μM) or NADPH (400 μM) at 25 °C in MTEN buffer; the enzyme was excited at 290 nm, and the velocities of the reactions were monitored at 340 nm. [b] Conditions: DHFRs (4–5 μM) were incubated with 100 μM NADPH or NADPD and then reacted with 100 μM H_2F at 25 °C in MTEN buffer; the enzymes were excited at 290 nm, and the velocities of the reactions were monitored at 450 nm; the range of the time scale chosen for the reactions was 20–50 ms. [c] The rate constant for dissociation of $NADP^+$ from the $E{\cdot}NADP^+{\cdot}H_4F$ ternary complex. [d] The rate constant for dissociation of H_4F from the $E{\cdot}NADP^+{\cdot}H_4F$ ternary complex. [e] The rate constant for dissociation of H_4F from $E{\cdot}NADPH{\cdot}H_4F$ ternary complex. [f] The rate constant for hydride transfer. [g] Taken from Fierke et al. (1987). [h] The deuterium isotope effects (k_3^H/k_3^D) for Phe-31, Tyr-31, and Val-31 are 2.8 ± 0.4, 2.4 ± 0.5, and 2.5 ± 0.5, respectively. [i] Unable to measure, but k_{off} from the binary $E{\cdot}H_4F$ complex is 40 s^{-1} (Mayer, unpublished results).

Table V: Relative Free Energy Changes for Binding of H_2F, Rates for Hydride Transfer, and Dissociation of H_4F from the $E{\cdot}NADPH{\cdot}H_4F$ Ternary Complex for Wild-Type and Mutant Enzymes

	$\Delta\Delta G(K_{D\,H_2F})$	$\Delta\Delta G(k_3)$	$\Delta\Delta G(k_{11})$
Phe-31	0	0	0
Tyr-31	1.5	0.3	−0.6
Val-31	1.9	1.0	−0.5[a]

[a] Estimated from V at low pH.

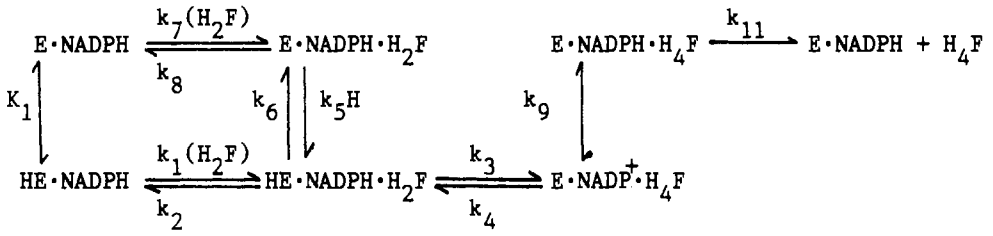
Chemical Transformation and Deuterium Isotope Effects. The rate constant for hydride transfer from NADPH to H_2F can be determined from pre-steady-state experiments. The enzymes (≃4–5 μM) were preincubated with 100 μM NADPH or NADPD and then reacted with 100 μM H_2F. The chemical transformation was followed by using an excitation wavelength of 290 nm and monitoring the decrease in fluorescence at 450 nm (Fierke et al., 1987). The fluorescence at 450 nm arises from the fluorescence energy transfer in $DHFR{\cdot}H_2F{\cdot}NADPH$. No fluorescence energy transfer occurs in $DHFR{\cdot}H_4F{\cdot}NADP^+$. The rate constants for hydride transfer from NADPH to H_2F and associated deuterium isotope effect for the native and mutant enzymes are summarized in Table IV.

Interaction of Dihydrofolate with Dihydrofolate Reductase. The enzymes (1.0 μM) were excited at 290 nm, and the fluorescence quenching (at 340 nm) that accompanied H_2F binding was used to determine the dissociation constant of H_2F. The dissociation constants for the $E{\cdot}H_2F$ binary complexes of the wild-type, Tyr-31 mutant, and Val-31 mutant enzymes at pH 7.0 are listed in Table III, and the values of their relative free energy changes are shown in Table V.

Discussion

In the study of mutant proteins, an important initial question

Scheme I



to address is their structural integrity. Recent crystallographic studies indicate that point mutations are in general accommodated by very minor readjustments of the tertiary protein structure with water molecules occupying the space created by a smaller amino acid α-side chain (Straus et al., 1985; Chothia & Lesk, 1985; Howell et al., 1986). We have employed a kinetic analysis of the conformation states of the DHFR mutants to assess the potential effect of the single amino acid change upon structure. Cayley et al. (1981) had established that the *E. coli* enzyme exists in two interconverting conformational states, designated as the E_1 and E_2 conformers. Their postulate stated that only the E_1 conformer formed a binary complex with NADPH, with the rate for conversion of E_2 to E_1 generally slower than that for ligand binding. The mutation of Phe-31 to Tyr and Val has produced modest changes in the relative E_1 and E_2 distributions (Table I), but the rate constants for the formation of the NADPH binary complex and the conversion of E_2 to E_1 are unaffected. We conclude that the mutant enzymes have the same gross conformation as the wild type and that changes in their binding and catalytic properties can be attributed primarily to the altered amino acid, but we remain aware of potential problems posed by even minor localized conformational changes.

Steady-state kinetic studies leading to V- and V/K-pH profiles for both Tyr-31 and Val-31 DHFRs (Figures 1 and 2, respectively) have revealed that the V/K-pH plot for the Tyr-31 mutant enzyme departed from the dissociation curve anticipated for a single acidic residue and showed a hollow in the vicinity of the apparent pK_a. This phenomenon led us to suggest that the proton on the active-site Asp-27 in the mutant ternary HE·NADPH·H_2F complex exchanges with media water (Taira et al., 1987), in contrast to the conclusion that the active site for the wild-type HE·NADPH·H_2F complex was inaccessible to proton exchange (Stone & Morrison, 1984). Reexamination here of the pH dependence of the deuterium kinetic isotope effects for the wild type, as well as similar measurements on the Tyr-31 and Val-31 mutant enzymes, showed that $^D(V)$ and $^D(V/K)_{H_2F}$ are pH dependent (Table II) but at low pH $^D(V) \rightarrow 1$ for all three enzymes. This constitutes additional evidence that (i) the protonated and free-base ternary complexes involving Asp-27 can equilibrate with media—otherwise $^D(V/K)_{H_2F}$ would be pH independent (Stone & Morrison, 1984)—and (ii) hydride transfer is not rate limiting at low pH but is controlling at high pH. The limiting step at low pH has been subsequently identified by pre-steady-state kinetics as the dissociation of H_4F from the E·NADPH·H_4F complex which has the same value as V, 12 s^{-1} (Fierke et al., 1987). Penner and Frieden (1987) also recently concluded that the rate-limiting step at low pH followed hydride transfer.

The resultant modified kinetic sequence for DHFR for *E. coli* is exhibited in an abbreviated form sufficient for our present analysis in Scheme I (Fierke et al., 1987). Note that k_9 combines the rates of dissociation of $NADP^+$ (200 s^{-1}) and the diffusion-limited association of NADPH. Dissociation of H_4F from E·$NADP^+$·H_4F is slow (2 s^{-1}) and is omitted from this simplified scheme, which pertains to our experimental NADPH levels (60 μM). The steady-state expressions for V/K and V, the relationship between the intrinsic and apparent pK_a's (obtained from the V- and V/K-pH profiles), and the deuterium isotope effects on V and V/K are given in the Appendix.

In order to evaluate the rate coefficients associated with key steps in Scheme I for the mutant enzymes we investigated (i) by steady-state kinetics, the $(V/K)_{H_2F}$ and V parameters as a function of pH and the corresponding deuterium kinetic isotope effects when NADPD replaced NADPH; (ii) by fluorescence quenching, the dissociation of H_2F from E; and (iii) by pre-steady-state methods, the rate coefficients for substrate association (k_1, k_7), for hydride transfer (k_3), and for product release (k_9, k_{11}). The unusual behavior in $(V/K)_{H_2F}$ vs. pH for Tyr-31 DHFR was computer simulated by utilizing eq 3 (equivalent to eq 2 in the Appendix) and the rate coefficients required given in the legend to Figure 1. No unique values could be extracted from the corresponding V-pH profile (eq 2 and the equivalent eq 1 in the Appendix). Stopped-flow studies utilizing fluorescence quenching or energy transfer as a means of monitoring the dissociation step of product and the hydride transfer yielded the rate constants listed in Table IV.

It is apparent that V for Tyr-31 DHFR and probably Val-31 DHFR still is determined by the dissociation of H_4F from the E·NADPH·H_4F complex; i.e., $V \simeq 30\ s^{-1} = k_{11} < k_9$. Although we were unable to measure k_{11} directly for Val-31 DHFR, the rate for the hydride step (k_3) is greater than V; i.e., $k_3 = 120\ s^{-1} > V = 26\ s^{-1}$. At low NADPH levels H_4F will dissociate from the Tyr-31 mutant via the binary E·H_4F complex to a greater extent than Phe-31 DHFR owing to an increased rate of dissociation, 29 vs. 1.4 s^{-1}. The observed hollow in the Tyr-31 V/K-pH profile requires a difference in the association rates (k_1, k_7) to form the ternary complex whereas these rates are pH independent for the Phe-31 enzyme (Fierke et al., 1987). Pre-steady-state single-turnover measurements revealed that this association rate constant for Tyr-31 DHFR has the required pH sensitivity (note that $k_1/k_7 = 10$ in Figure 1) with values of 7.9×10^6 and $4.6 \times 10^6\ M^{-1}\ s^{-1}$ at pH 5.5 and 6.5, respectively. In addition, this is within error of the limiting value of V/K at low pH, so the association of H_2F with E·NADPH constitutes the rate-limiting step for both Phe-31 and Tyr-31 enzymes in the pH region 6.0–8.0.

Although the evaluations of $^D(V/K)$ and $^D(V)$ (Table II) do not furnish unique values for the rate steps in Scheme I, they can serve as a partial check on the consistency of the interpretation of the kinetic data. Complete equations for $^D(V/K)$, $^D(V)$, and various commitment factors derived for Scheme I are given in the Appendix. However, since $C_r \ll 1$ (for Phe-31 DHFR, $C_r \cong 0.6/200 = 0.003$, and the ratio should not change significantly for the mutants), these equations (eq 5 and 6 in the Appendix) simplify to

$$^D(V/K)_{H_2F} = (^Dk_3 + C_f)/(1 + C_f) \qquad (6)$$

and

$$^{D}(V) = (^{D}k_3 + C_{vf})/(1 + C_{vf}) \quad (7)$$

The kinetic isotope effects are more readily evaluated at the low and high pH limits. At high pH, $C_{vf} = C_f \ll 1$ (eq 12 in the Appendix) since k_2:k_6:k_3 is 40:>1000:400 for Tyr-31 DHFR (the value of k_2 is calculated from $k_3/k_2 = 10$, a condition required to describe the data in Figure 1; the k_6 value is a lower limit). Consequently, $^{D}(V/K)$ and $^{D}(V)$ should converge at the high pH limit at values for the intrinsic isotope effect $^{D}k_3$. The data in Table II suggest that $^{D}k_3$ for this process ranges from 3 to 4, for all three enzymes. It is likely that the evaluation of $^{D}k_3$ by steady-state kinetics yields an artificially high value owing to experimental limitations imposed by the low K_{H_2F}. On the other hand, the measured $^{H}k_3/^{D}k_3$ observed in the pre-steady-state should be a direct measure of $^{D}k_3$ (Table IV). At low pH, $C_{vf} \neq C_f$ but can be evaluated from eq 10 and 11 (Appendix) and the data in Table IV. For Tyr-31 DHFR setting $^{D}k_3 \simeq 3$, the predicted values of $^{D}(V/K)$ and $^{D}(V)$ are 1.2 and 1.1 vs. observed values of 2.5 and 0.9, respectively, with, as expected, the agreement in $^{D}(V)$ being more satisfactory.

The effect of mutations at Phe-31 should be assessed in terms of this residue's locus at the enzyme active site. Phe-31 is located in a hydrophobic pocket and interacts with the pteroyl moiety of H_2F through van der Waals contacts in such a way that the edge of the phenyl ring is oriented toward faces of both the pteridine ring and *p*-aminobenzoyl group (Bolin et al., 1982). The edge-to-face aromatic–aromatic interaction is very common in proteins and plays a role in tertiary structure stabilization (Burley & Petsko, 1985). We will consider in turn the quantitative effect of the mutation on substrate binding and catalysis. Values for the corresponding calculated free energy changes are given in Table V.

A change in binding energy of 1.90 kcal/mol for H_2F was observed upon mutation of Phe-31 to Val. The decrease in binding may be attributed to the loss of an aromatic–aromatic interaction between the side chain of Val and the pteroyl moiety of H_2F reflected to some extent in the reduced hydrophobicity. According to Nozaki and Tanford (1971), replacement of Phe with Val decreases hydrophobicity by about 1.0 kcal/mol (measured by partitioning). It may be estimated from our observed binding energy change that ca. 0.9 kcal/mol is attributed to a single aromatic pair interaction, a value that agrees with the calculated values for biphenyl compounds (Burley & Petsko, 1985). The unfavorable free energy change for binding of H_2F to Tyr-31 DHFR is 1.5 kcal/mol (Table V). The decrease in binding may result from a steric effect caused by the *p*-hydroxyl group since the aromatic interaction should have been retained.

The effect of the Phe-31 to Tyr mutation and Val mutation is to make chemical catalysis, specifically the k_3 step, less efficient at pH 6.0.[2] The difference in the rate of hydride transfer is about 1.7- and 5.8-fold (0.3 and 1.0 kcal/mol, respectively) less favorable with respect to the wild type for Tyr-31 and Val-31 mutant enzymes, respectively. In striking contrast is the ca. 2.5-fold increase in V for the Tyr-31 and Val-31 mutant enzymes (−0.5 kcal/mol) owing to the faster dissociation of H_4F from either the E·NADPH·H_4F or E·H_4F complexes, clearly illustrating the balance struck between the need for catalytic efficiency, k_3, and competitive product dissociation rates (k_{11}) (Jencks, 1969). Although in principle the mutant enzymes have superior V values, the smaller $(V/K)_{H_2F}$ coefficients lower their catalytic efficiency particularly in a cellular milieu where the concentration of H_2F is estimated as between 0.02 and 0.06 μM (Jackson & Harrap, 1973).

[2] The hydride-transfer rate is pH dependent, and the rate-constant ratio of the mutants relative to the wild-type enzyme for this step may decrease as the pH is lowered owing to a lower intrinsic pK_a for the mutants.

Conclusions

We have used pre-steady-state and steady-state methods to study the Tyr-31 and Val-31 mutants created by the technique of site-directed mutagenesis. The kinetic behavior of the mutant enzymes is similar to that of the wild type. The rate-limiting step for the mutant enzymes at physiological pH is the release of H_4F from the enzyme–NADPH–H_4F ternary complex as determined for the wild type. The 2-fold increase in V for the two mutant enzymes arises from faster dissociation of H_4F from the enzyme–product complex. The quantitative effect of these mutations is to decrease the rate of hydride transfer, although not to the extent that this step becomes partially rate limiting, but to accelerate the dissociation rates of H_4F from product complexes so that the opposing effects are nearly compensating.

Acknowledgments

We thank Drs. Kenneth A. Johnson and Carol A. Fierke for many helpful suggestions and Dr. Lawrence F. Courtney for the preparation of tetrahydrofolate. We also thank Kaye Yarnell for typing the manuscript.

Appendix

The steady-state expressions for V and V/K for the reaction given by Scheme I are described by eq 1 and 2. The pro-

$$V = [C_1'(1 + C_2'K_2/[H])]/\{[1 + C_3'K_2/[H] + C_4'(K_2/[H])^2]\} \quad (1)$$

$$V/K = [C_1 (1 + C_2K_1/[H])]/[(1 + K_1/[H])(1 + C_3K_1/[H])] \quad (2)$$

ton-transfer step between E·NADPH and HE·NADPH was treated as an equilibrium step in the derivation, where $K_2 = k_6/k_5$, $[H] = [H^+]$

$$C_1' = k_3k_9k_{11}/[k_{11}(k_4 + k_9) + k_3(k_9 + k_{11})]$$

$$C_2' = k_8(k_2 + k_6)/(k_2k_6)$$

$$C_3' = [k_{11}(k_6 + k_8)(k_4 + k_9) + k_3k_8(k_9 + k_{11})]/[k_6k_{11}(k_4 + k_9) + k_3k_6(k_9 + k_{11})] + k_8/k_2$$

$$C_4' = \{k_8k_{11}[(k_2 + k_6) \times (k_4 + k_9) + k_3k_9]\}/\{k_2k_6[k_{11}(k_4 + k_9) + k_3(k_9 + k_{11})]\}$$

$$C_1 = k_1k_3k_9/(k_2k_4 + k_2k_9 + k_3k_9)$$

$$C_2 = (k_7/k_1)(1 + k_2/k_6)$$

$$C_3 = (k_2k_7/k_6k_1)\{1 + k_6/[k_2 + k_3k_9/(k_4 + k_9)]\}$$

The relationship between the apparent pK_a obtained from the V/K profile and the intrinsic pK_a (pK_1) is

$$pK_{a\,app} = pK_1 + \log (C_2/C_3) = pK_1 + \log \frac{(1 + k_2/k_6)}{(k_2/k_6)\{1 + k_6/[k_2 + k_3k_9/(k_4 + k_9)]\}} \quad (3)$$

The relationship between the apparent pK_a obtained from the V profile and the intrinsic pK_a of the ternary complex (pK_2, where $K_2 = k_6/k_5$) is

$$pK_{a\,app} = pK_2 + \log (C_2'/C_4) = pK_2 + \log \frac{(k_2 + k_6)[k_{11}(k_4 + k_9) + k_3(k_9 + k_{11})]}{k_{11}[(k_2 + k_6)(k_4 + k_9) + k_3k_9]} \quad (4)$$

or, since $K_2 = K_1\ (k_2k_7/k_1k_8)$ in Scheme I

$$pK_{a\,app} = pK_1 + \log \frac{k_1k_8(k_2 + k_6)[k_{11}(k_4 + k_9) + k_3(k_9 + k_{11})]}{k_2k_7k_{11}[(k_2 + k_6)(k_4 + k_9) + k_3k_9]}$$

The deuterium isotope effects, ${}^D(V/K)_{H_2F}$ and ${}^D(V)$ in Scheme I, have the general forms of

$${}^D(V/K) = ({}^Dk_3 + C_f + C_r{}^DK_{eq})/(1 + C_f + C_r) \quad (5)$$

$${}^D(V) = ({}^Dk_3 + C_{vf} + C_r{}^DK_{eq})/(1 + C_{vf} + C_r) \quad (6)$$

where ${}^Dk_3$ represents the intrinsic deuterium isotope effect $(k_3{}^H/k_3{}^D)$ and ${}^DK_{eq} = k_3{}^Hk_4{}^D/(k_4{}^Hk_3{}^D)$. C_f, C_r, and C_{vf} are defined by the relationships given in eq 7, 8, and 9, respectively.

$$C_f = \{(k_3/k_2)[1 + (k_1k_6/k_7k_2)([H]/K_1)]\}/\{1 + (k_6/k_2)[1 + (k_1/k_7)([H]/K_1)]\} \quad (7)$$

$$C_r = k_4/k_9 \quad (8)$$

$$C_{vf} = (k_3/k_9)[k_7k_9 + (1 + k_9/k_{11})(k_5k_7K_1 + k_1k_8)([H]/K_1) + k_1k_5K_1(1 + k_9/k_{11})([H]/K_1)^2]/\{k_7(k_2 + k_6) + [k_5k_7K_1 + k_1(k_6 + k_8)]([H]/K_1) + k_1k_5K_1([H]/K_1)^2\} \quad (9)$$

At low pH, C_f and C_{vf} reduce to eq 10 and 11, respectively.

$$C_f = k_3/k_2 \quad (10)$$

$$C_{vf} = (k_3/k_9)(1 + k_9/k_{11}) \quad (11)$$

At high pH, both C_f and C_{vf} have the same expression as given by

$$C_f = C_{vf} = (k_3/k_2)/(1 + k_6/k_2) \quad (12)$$

Registry No. DHFR, 9002-03-3; $NADP^+$, 53-59-8; NADPH, 53-57-6; H_2F, 4033-27-6; H_4F, 135-16-0; L-Phe, 63-91-2; L-Tyr, 60-18-4; L-Val, 72-18-4; D_2, 7782-39-0.

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