Effects of Conversion of an Invariant Tryptophan Residue to Phenylalanine on the Function of Human Dihydrofolate Reductase†

Shaoming Huang, Tavner J. Delcamp, Xuehai Tan, Philip L. Smith, Neal J. Prendergast, and James H. Freisheim*
Department of Biochemistry, Medical College of Ohio, Toledo, Ohio 43699
Received July 6, 1988; Revised Manuscript Received August 24, 1988

ABSTRACT: The binding site residue Trp-24 is conserved in all vertebrate and bacterial dihydrofolate reductases of known sequence. To determine its effects on enzyme properties, a Trp-24 to Phe-24 mutant (W-24-F) of human dihydrofolate reductase has been constructed by oligodeoxynucleotide site-directed mutagenesis. The W-24-F mutant enzyme appears to have a more open or flexible conformation as compared to the wild-type human dihydrofolate reductase on the basis of results of a number of studies. These studies include competitive ELISA using peptide-specific antibodies against human dihydrofolate reductase, thermal stability, and protease susceptibility studies of both mutant W-24-F and wild-type enzymes. It is concluded that Trp-24 is important for maintaining the structural integrity of the native enzyme. Changes in relative fluorescence quantum yield indicate that Trp-24 is buried and its fluorescence quenched relative to the other two tryptophan residues in the wild-type human reductase. Kinetic studies indicate that k_{cat} values for W-24-F are increased in the pH range of 4.5–8.5 with a 5-fold increase at pH 7.5 as compared to the wild-type enzyme. However, the catalytic efficiency of W-24-F decreases rapidly as the pH is increased from 7.5 to 9.5. The K_m values for dihydrofolate are also increased for W-24-F in the pH range of 4.5–9.5 with a 30-fold increase at pH 7.5, while the K_m value for NADPH increases only ca. 1.4-fold at pH 7.5 as compared to the wild type. The W-24-F mutant enzyme shows a general reduction in the binding of dihydrofolate, NADPH, folic acid, and trimethoprim as compared to the wild type. Agents at concentrations which show activating effects on the wild-type human enzyme such as organomercurials, KCl, and urea all inactivate W-24-F. The relationship between a conformational change and enzyme activation of DHFR is discussed by comparing the kinetic behavior and responses to KCl and urea of W-24-F to those of organomercurial-activated dihydrofolate reductases.

Dihydrofolate reductase (5,6,7,8-tetrahydrofolate:$NADP^+$ oxidoreductase, EC 1.5.1.3) (DHFR)[1] catalyzes the NADPH-dependent reduction of 7,8-dihydrofolate (FAH_2)[1] to 5,6,7,8-tetrahydrofolate (FAH_4).[1] This enzyme is necessary for maintaining the intracellular pools of FAH_4 and its derivatives, which are essential cofactors in many biosynthetic reactions. DHFR is a target enzyme for a group of anti-folate drugs which are widely used as antitumor and antibacterial agents, such as methotrexate (MTX)[1] and trimethoprim (TMP).[1] Because of its biological and pharmacological importance, DHFR has been the subject of extensive structural and kinetic studies. The development of site-directed mutagenesis techniques has created the opportunity to change any amino acid residue in an enzyme to any other, thereby allowing insights into the functional role of an individual amino acid (Knowles, 1987). In our laboratory, a high-level expression system for human DHFR in *Escherichia coli* has been established (Prendergast et al., 1988) which has allowed the use of site-directed mutagenesis to establish structure–function relationships for the human enzyme.

Features of the structure of human DHFR can be inferred by comparison to the known chicken liver DHFR structure by simply changing amino acid side chains in accord with known differences in the primary sequence (Kraut & Matthews, 1987). Accordingly, the active site of human DHFR is lined with several strictly conserved hydrophobic amino acids. One of these, Trp-24, interacts with the nicotinamide moiety of NADPH through van der Waals contact (Volz et al., 1982) and interacts with Glu-30 and with MTX indirectly via hydrogen bonds through a fixed water molecule (Bolin et al., 1982). Chemical modification by *N*-bromosuccinimide and other reagents has implicated this tryptophan residue in the catalytic activity of DHFRs from several species. In each case, modification of this tryptophan residue leads to the loss of greater than 90% of the enzymic activity (Freisheim & Huennekens, 1969; Warwick et al., 1972; Liu & Dunlap, 1974; Williams, 1975; Freisheim et al., 1977, 1979).

We have constructed a mutant of human DHFR with phenylalanine at position 24 in order to investigate the role of Trp-24 in enzyme function. The mutant W-24-F has been characterized with respect to its protein structure, activation properties, and steady-state kinetics. Our results provide information about the functional role of Trp-24 and the structure–function relationship of human DHFR.

Materials and Methods

Enzymes and Chemicals. Restriction endonucleases, T4 polynucleotide kinase, and T4 DNA ligase were purchased from Bethesda Research Laboratories, P-L Biochemicals, and Boehringer Mannheim, respectively; ddNTPs, dNTPs, and

†This work was supported by a grant to J.H.F. from the National Institutes of Health, National Cancer Institute (CA41461), USPHS, DHHS.
* Correspondence should be addressed to this author.

[1] Abbreviations: MTX, methotrexate (4-amino-10-methyl-4-deoxyfolic acid); DHFR, dihydrofolate reductase; FAH_2, dihydrofolic acid; FAH_4, tetrahydrofolic acid; TMP, trimethoprim; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; $NADP^+$, nicotinamide adenine dinucleotide phosphate (oxidized); NADPH, nicotinamide adenine dinucleotide phosphate (reduced); Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; IPTG, isopropyl β-D-thiogalactopyranoside; pHMB, *p*-(hydroxymercuri)benzoate; MES, 2-(*N*-morpholino)ethanesulfonic acid; MATS, 50 mM Tris, 25 mM acetate, 25 mM MES, and 100 mM NaCl; ELISA, enzyme-linked immunosorbant assay.

0006-2960/89/0428-0471$01.50/0

Klenow large fragment for dideoxy sequencing were purchased from Bethesda Research Laboratories; [α-^{35}S]dATP and [γ-^{32}P]ATP were purchased from DuPont–New England Nuclear. MTX was a generous gift from Dr. John A. R. Mead (Division of Cancer Treatment, NCI). FAH_2 was prepared from commercial folic acid by dithionite reduction (Blakley, 1960).

DNAs and Bacterial Strains. The 18-base oligodeoxynucleotide probe used for mutagenesis, 5′-CCTGCCCTTTCCACCGCT-3′, and the three sequencing primers were synthesized in this laboratory by using an Applied Biosystems Model 380A automated DNA synthesizer, and purified by electrophoresis on a 15% polyacrylamide gel as described by Applied Biosystems (DNA Synthesizer User Bulletin 13, 1984). The plasmid pDFR, an expression vector for the human DHFR cDNA (Prendergast et al., 1988), was isolated by using alkaline lysis or Triton X-100 lysis protocols, followed by cesium chloride–ethidium bromide density gradient centrifugation (Maniatis et al., 1982). Bacterial strains JM101 and JM107 (Yanish-Perron, 1985) were used as hosts in the mutant construction and expression.

Oligodeoxynucleotide-Directed Mutagenesis of Human DHFR cDNA. Conversion of the Trp-24 (TGG) codon to the Phe-24 (TTT) codon was carried out by oligodeoxynucleotide-directed, site-specific mutagenesis (Prendergast et al., 1988). Plasmid pDFR (25 μg) was digested with *Pvu*II, and the digested pDFR (fragment I) then was purified by extraction with phenol/chloroform and ethanol precipitation (Maniatis et al., 1982). The DNA pellet was resuspended in 50 mM Tris-HCl (pH 8.0)/0.1 mM EDTA buffer, dephosphorylated by the addition of calf intestinal phosphatase, and purified by phenol/chloroform extraction and ethanol precipitation. Plasmid pDFR (50 μg) was also digested with *Bam*HI and *Xba*I in a single reaction mixture. The two fragments produced were separated by 0.8% agarose gel electrophoresis. The large fragment (fragment II) was extracted from the gel and purified by electroelution.

Fragment I (0.3 μg) and 0.26 μg of fragment II, 3.75 μL of 5′-phosphorylated primer, and 12 μL of 5× polymerase ligase buffer (500 mM NaCl, 32.5 mM Tris-HCl, pH 7.5, 40 mM $MgCl_2$, and 5 mM 2-mercaptoethanol) were mixed to a final volume of 34.8 μL. The mixture was placed in a boiling water bath for 3 min, then quickly transferred to a 30 °C incubator for 30 min followed by incubation at 4 °C for 30 min, and then kept on ice for 10 min. The mixture (11.6 μL) was then added to 4 μL of the dNTPs (2.5 mM each of dTTP, dCTP, dATP, and dGTP), 2 μL of 10 mM ATP, 0.4 μL of Klenow enzyme (5 units/μL), and 2 μL of T4 DNA ligase (0.5 unit/μL). The mixture was incubated at 12.5 °C overnight. One microliter of the mixture was mixed with 9 μL of H_2O which then was used to transform competent JM101 cells by the method of Maniatis et al. (1982). The cells were then plated onto LB agar plates containing 50 μg/mL ampicillin and allowed to grow at 37 °C overnight.

Colony Screening. Transformants were screened by colony hybridization using 5′-end-labeled probe which was used for the mutagenesis reaction. The nitrocellulose filter replicas were prepared as described by Inouye and Inouye (1987). The hybridization was carried out by the method of Colleaux et al. (1986).

Secondary Transformation. The plasmid of the positive colonies from the first transformation was prepared by using a minipreparative method (Chen & Seeburg, 1985). The plasmid was then used to transform competent JM107 cells. The colonies were screened, and the plasmids of some of the positive clones were prepared as described before. To verify the codon change, the plasmids were digested with *Bal*I, since one of the two sites of this enzyme is missing due to the Trp-24 to Phe-24 codon change. The digests were run on a 1% agarose gel to further verify the mutation.

DNA Sequencing. The plasmid carrying the mutant human DHFR gene was sequenced by the double-stranded dideoxy sequence method (Sanger et al., 1977; Agellon & Chen, 1986).

Expression and Purification of W-24-F DHFR. JM107 cells containing the mutant plasmid were grown in a 14-L culture at 37 °C in the presence of 50 μg of ampicillin/mL to an optical density of 0.75–0.80 (at 600 nm) and induced by the addition of 5 g of isopropyl β-D-thiogalactopyranoside (IPTG).[1] After further growth for 3.5–4 h, the cells were harvested by continuous-flow centrifugation at 15 000 rpm at 4 °C. The cell pellet was stored at −70 °C.

The following steps were carried out at 4 °C. Cells from the 14-L culture were resuspended in 300 mL of buffer A (50 mM potassium phosphate, pH 6.5, 1 mM EDTA, and 10 mM β-mercaptoethanol) with 1 mM phenylmethanesulfonyl fluoride and 2 mg/L leupeptin (lysis buffer). Lysozyme (150 mg/7 mL of lysis buffer) was added to the suspension followed by incubation on ice for 15 min. $MgCl_2$ (1 M, 900 μL) and 24 mg of DNase I were added, and the suspension was placed on ice for another 15 min. The suspension was then centrifuged at 12 000 rpm for 15 min. To the supernatant was slowly added ammonium sulfate to a final concentration of 85% with gentle stirring overnight. Following centrifugation at 18 000 rpm for 30 min, the pellet was resuspended in 150 mL of lysis buffer and loaded onto a 1.5 × 3 cm MTX-(aminohexyl)-Sepharose 4B column equilibrated with buffer A. After the column was extensively washed with buffer B (buffer A + 500 mM KCl) and buffer A, the column was eluted with FAH_2 (2 mg/mL in buffer A, pH 8.0). The fractions containing DHFR activity were pooled and concentrated to 10 mL in an Amicon ultrafiltration apparatus fitted with a YM-10 membrane and subjected to preparative isoelectric focusing (Vesterberg, 1971) on a 110-mL LKB column. After being focused for 84 h, the fractions containing DHFR activity were pooled and passed through a 2 × 86 cm Sephadex G-50 column equilibrated with 0.1 M potassium phosphate buffer, pH 7.5. Enzyme purity and removal of substrate were evaluated by SDS–PAGE and by the UV–vis absorption spectrum, respectively (Delcamp et al., 1983). Purified enzyme was stored at −70 °C.

Amino Acid Analysis and Protein Sequence Determination. Amino acid analysis was performed on a Beckman Model 6300 amino acid analyzer following 24-h hydrolysis in 6 N HCl in vacuo at 110 °C. N-Terminal protein sequence analysis was performed using an Applied Biosystems Model 470A gas-phase protein sequencer.

Protein Determination. Enzyme concentration was determined both by MTX titration (Williams et al., 1979) and by amino acid analysis. MTX concentration was determined spectrophotometrically at 258 nm (ϵ_M = 23 250) and 302 nm (ϵ_M = 22 100) (Seeger et al., 1949).

ELISA. The binding of wild-type and W-24-F enzymes to antibodies against cyanogen bromide peptides 15–52, 53–111, and 140–186 derived from human DHFR was assayed by using the competitive ELISA[1] assay described in Ratnam et al. (1988).

Kinetic Assays. DHFR activity was assayed spectrophotometrically at 340 nm and at 22 °C by using a molar absorbance change at 340 nm of 12 300 cm^{-1} as described in Kempton et al. (1982). Initial rates were derived from the

Table I: Automated Sequence Analysis of W-24-F Mutant DHFR[a]

step	amino acid identified	yield (pmol)	reported cDNA sequence
01	Val	1226	Val
02	Gly	799	Gly
03	Ser	201	Ser
04	Leu	694	Leu
05	Asn	763	Asn
06	ND[b]	ND[b]	Cys
07	Ile	1395	Ile
08	Val	832	Val
09	Ala	497	Ala
10	Val	724	Val
11	Ser	110	Ser
12	Gln	402	Gln
13	Asn	463	Asn
14	Met	406	Met
15	Gly	283	Gly
16	Ile	847	Ile
17	Gly	281	Gly
18	Ile	249	Ile
19	Asn	289	Asn
20	Gly	195	Gly
21	Asp	112	Asp
22	Leu	175	Leu
23	Pro	294	Pro
24	Phe	284	Trp
25	Pro	268	Pro
26	Pro	235	Pro
27	Leu	191	Leu
28	Arg	109	Arg
29	Asn	123	Asn
30	Glu	95	Glu

[a] Automated sequence analysis of ^{14}C-carboxymethylated W-24-F mutant DHFR. Sequence analysis of 1.48 nmol of ^{14}C-carboxymethylated protein. The initial yield was 83%. [b] Not determined.

change in absorbance continuously recorded with a Varian Model 219 spectrophotometer. FAH_2 concentration was determined spectrophotometrically at 280 nm both by using a molar extinction coefficient of 2.8×10^4 M^{-1} cm^{-1} at pH 7.5 (Blakley, 1960) and also by using the enzyme assay described above. NADPH concentration was also determined in both ways by using a molar extinction coefficient of 6.2×10^3 M^{-1} cm^{-1} at 340 nm (Horecker & Kornberg, 1948).

To maintain constant ionic strength over the pH range used in obtaining the pH profiles of k_{cat} and k_{cat}/K_m, a three-component buffer system was used, containing 50 mM Tris, 25 mM acetate, 25 mM MES,[1] and 100 mM NaCl (MATS[1] buffer), with adjustment of pH and ionic strength made by using the computer program described in Williams and Morrison (1981).

Equilibrium Dissociation Constants. Equilibrium dissociation constants (K_d) for both wild-type and W-24-F enzymes with different ligands were determined by fluorescence titration (Blakley et al., 1983) at 24 °C using a Perkin-Elmer Model MPF-66 fluorescence spectrophotometer operated in the integration mode, with a Series 7000 professional computer. The excitation and emission wavelengths were set at 290 and 320 nm, respectively. MATS buffer was used in each determination. To avoid errors caused by thermal denaturation of either protein, fresh enzyme solution was used for each data point, and the volume of added ligand was limited to less than 70 μL (the initial volume was 3.0 mL). Standard tryptophan solutions were used to obtain internal filter factors (Blakley et al., 1983).

Relative Fluorescence Quantum Yield Determination. Excitation and emission monochromators were set at 6.0 nm, and the excitation and emission correction factors were generated by following the manufacturer's instruction using Rhodamine 101 as a quantum counter. The samples were scanned at a rate of 120 nm/min, and the absorbance of the samples was not allowed to exceed a value of 0.03 at the excitation wavelength. Relative fluorescence quantum yield was determined by comparing the fluorescence of the samples with that of a pure tryptophan solution (Teale, 1960).

Data Analysis. Data obtained from K_m determinations by varying FAH_2 or NADPH concentration were fitted to eq 1

$$v = \frac{V_{max}[S]}{K_m + [S]} \quad (1)$$

by a nonlinear regression program to yield values for V_{max} and the Michaelis constant, K_m, as well as V_{max}/K_m.

Data obtained from fluorescence titration were fitted to eq 2 by the FLKIE program which is based on the general data

$$I = I_0 - (I_0 - I_{inf})\frac{X - (X^2 - 4EL)^{1/2}}{2E} \quad (2)$$

fitting program TARSIER (Blakley et al., 1983) where I = fluorescence intensity at ligand concentration L, I_0 = fluorescence intensity in the absence of ligand, I_{inf} = fluorescence intensity at infinite ligand concentration, E = total enzyme concentration, L = total ligand concentration, K_d = dissociation constant, and $X = E + L + K_d$.

Results

Construction and Structural Analysis of the Mutant. The W-24-F mutant plasmid was constructed by using the gapped plasmid protocol (see Materials and Methods). The entire structural gene of the mutant was sequenced and the codon change confirmed. Amino acid sequence data (Table I) demonstrated the complete removal of initiator methionine and, in addition, confirmed the presence of phenylalanine at position 24.

Physicochemical Properties. Table II shows a comparison of physicochemical properties of wild-type and W-24-F reductases. Wild-type and W-24-F had the same isoelectric point (pI = 7.8). W-24-F had a molar extinction coefficient at 280 nm which was 28% less than that of the wild type. This result was expected since the tryptophan content decreased from three residues per molecule in the wild type to two residues per molecule in W-24-F. The fluorescence emission spectra (not shown) of both native and denatured forms of wild type and W-24-F were similar. When excited at 280 nm, the emission spectra of native forms of wild type and W-24-F showed a maximum at 320 nm, while the denatured forms showed a maximum at 350 nm. There was a 1.6-fold difference between the relative fluorescence quantum yield of

Table II: Comparison of Selected Physicochemical Properties of the Wild-Type and W-24-F DHFRs

DHFR	pI	ϵ_M^{280}[a]	fluorescence emission max (nm)		rel fluorescence quantum yield	
			native[b]	denatured[c]	native[b]	denatured[c]
wild-type	7.8	28000	320	350	0.102	0.164
W-24-F	7.8	20200	320	350	0.092	0.111

[a] In 50 mM potassium phosphate, pH 7.5, 25 °C. [b] In 50 mM potassium phosphate, pH 7.5, 24 °C, excitation at 280 nm. [c] In 8 M urea and 50 mM potassium phosphate, pH 7.5, 24 °C, excitation at 280 nm.

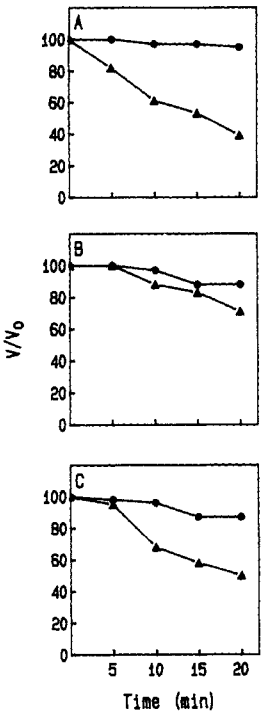

FIGURE 1: Proteolytic susceptibility of wild type (●) and W-24-F (▲) to chymotrypsin (A), *S. aureus* V8 protease (B), and trypsin (C). The enzymes (113 μg/mL) were incubated with 11.3 μg/mL proteases in 0.1 M potassium phosphate in the presence of 90 μM NADPH, pH 7.5, at 30 °C. Aliquots were removed from the incubation mixture at the time intervals indicated and assayed. V_0 = DHFR activity at time 0; V = DHFR activity at the indicated time.

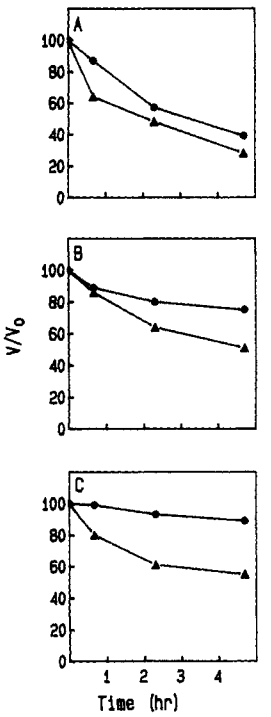

FIGURE 2: Thermal stability of wild type (●) and W-24-F (▲) at 22 °C. The enzymes (0.16 nM) were incubated in 50 mM Tris-HCl, pH 7.5. Aliquots were removed from the incubation mixture at time intervals as indicated and assayed. V_0 = DHFR activity at time 0; V = DHFR activity at the indicated time. (A) Enzymes alone; (B) enzymes + 90 μM NADPH; (C) enzymes + 45 μM FAH_2.

native and denatured forms of wild type with a value of 0.102 for the native form and 0.164 for the denatured form. The differences, however, between native and denatured forms of W-24-F was only 1.2-fold with a value of 0.092 for the native form and 0.111 for the denatured form. The latter value for denatured W-24-F represented a 32% decrease in relative fluorescence quantum yield as compared to the denatured form of the wild type as expected.

Protease Susceptibility and Thermal Stability. The susceptibility of wild type and W-24-F to proteolytic digestion was investigated as an index to qualitatively measure the difference in the overall structure of the two enzyme molecules. The proteases used were *Staphylococcus aureus* V8 protease, bovine trypsin, and chymotrypsin. To ensure that the reductases were not inactivated by thermal denaturation during the course of the protease digestion, 90 μM NADPH was included in the reaction mixture. The control experiment showed that in the absence of protease, both wild type and W-24-F retained 90% activity after 20-min incubation. The results (Figure 1) demonstrated that W-24-F was more susceptible to protease inactivation than wild type. After incubation with trypsin (Figure 1C) and V8 protease (Figure 1B) at 30 °C for 20 min, the wild-type enzyme lost 13% and 12% activity, respectively, whereas the W-24-F mutant lost 50% and 29% activity under the same conditions. In the case of chymotrypsin (Figure 1A), the wild-type enzyme was almost completely resistant to digestion under the experimental conditions used with only 5% inactivation after 20 min. By contrast, W-24-F was inactivated rapidly by this protease with 74% inactivation after 20 min. Analysis of the chymotryptic digests on reversed-phase HPLC revealed no detectable peptides in the case of wild type but a number of peptides in the case of W-24-F (data not shown).

The results of the thermal stability experiment are shown in Figure 2. W-24-F in its apoenzyme form, holoenzyme

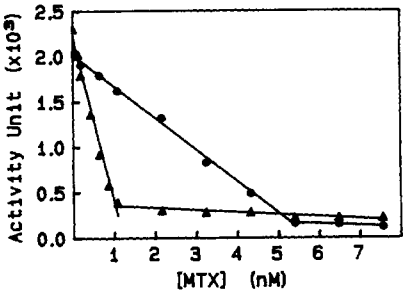

FIGURE 3: MTX titration of wild type (●) and W-24-F (▲). The enzymes (127.4 ng/mL wild type, 24.4 ng/mL W-24-F) were incubated with various concentrations of MTX in 50 mM Tris-HCl and 90 μM NADPH, pH 7.5, for 2 min at 22 °C. The DHFR activities were monitored after addition of 45 μM FAH_2 for at least 1 min.

form, and FAH_2–enzyme complex form were all less stable than the corresponding forms of the wild-type enzyme.

Binding of Peptide-Specific Antibodies to Wild Type and W-24-F. The antibodies used were raised against cyanogen bromide peptides 15–52 (Ab1), 53–111 (Ab2), and 140–186 (Ab4) derived from human DHFR (Ratnam et al., 1988). The binding of antibodies to wild type and W-24-F in solution was monitored by competitive ELISA assays in which denatured human DHFR was immobilized (Ratnam et al., 1988). The binding of antibodies to the immobilized antigen was assayed in the presence of various concentrations of either wild type or W-24-F containing 18 μM NADPH. As indicated in Figure 4, in the case of Ab1, wild type and W-24-F competed for the antibody, inhibiting its binding to the immobilized denatured enzyme to about the same extent. In the cases of Ab2 and Ab4, W-24-F caused 50% and 30% more inhibition, respectively, than did the wild type at the same protein concentration.

Steady-State Kinetics. Specific activities were determined by MTX titration based on the fact that MTX is a stoichiometric, tight binding inhibitor of DHFR (Williams et al.,

Table III: Comparison of Kinetic Properties of the Wild-Type and W-24-F DHFRs

DHFR	k_{cat} (s^{-1})	K_m (μM)		K_d[a] in binary complexes (μM)				
		FAH_2	NADPH	FAH_2	NADPH	FA	TMP	$NADP^+$
wild-type	7.3	0.022	0.26	0.044	0.049	0.111	2.3	1.3
W-24-F	34.5	0.656	0.37	0.196	0.333	0.817	4.1	7.7

[a] The K_d values were determined by monitoring the quenching of protein fluorescence at 320 nm at 24 °C in 50 mM Tris, 25 mM acetate, 25 mM MES, and 100 mM NaCl, pH 7.5, excited at 280 nm. The enzyme concentrations were 0.126 μM wild type and 0.164 μM W-24-F in the FAH_2, NADPH, FA, and $NADP^+$ binding studies and 0.98 μM wild type and 1.13 μM W-24-F in TMP binding studies.

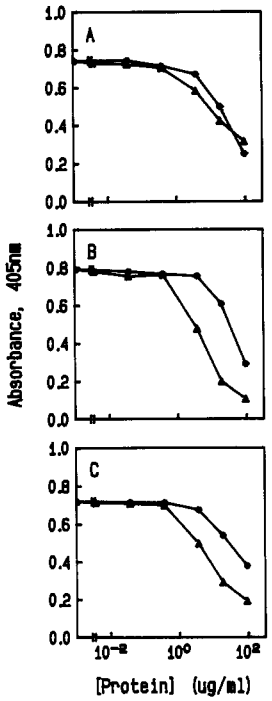

FIGURE 4: Competitive immunoassays using antisera to peptides 15–52 (A), 53–111 (B), and 140–186 (C). ELISA assays were carried out with denatured DHFR as the immobilized antigen. Antisera to cyanogen bromide peptides 15–52, 53–111, and 140–186 were used at 1:100, 1:200, and 1:1000 dilutions, respectively. The antisera (50 μL) were preincubated for 2 h at 22 °C in the absence and in the presence of various concentrations of wild-type (●) and W-24-F (▲) DHFRs with the addition of 18 μM NADPH. The antibody solutions were then applied directly onto the microtiter plate and assayed as described under Materials and Methods.

1979). The titration curves for both wild type and W-24-F are shown in Figure 3. There was a linear relationship between MTX concentration and inhibition of enzyme activity up to an 85% loss of activity for both wild type and W-24-F. The specific activity of W-24-F was 95 units mg^{-1} (corresponding to a k_{cat} value of 34.5 s^{-1}) whereas the specific activity of wild type was 20 units mg^{-1} (corresponding to a k_{cat} value of 7.3 s^{-1}) (Prendergast et al., 1988). The k_{cat} of W-24-F was about 5 times higher than that of wild type.

The K_m values for FAH_2 and NADPH for W-24-F were determined and the results compared to those of wild type as shown in Table III. W-24-F had a K_m value for FAH_2 ca. 30-fold higher and a K_m value for NADPH 1.4-fold higher than those found for wild-type enzyme (Prendergast et al., 1988).

The dissociation constants for different ligands in enzyme–ligand binary complexes were determined by fluorescence titration of the enzyme (Blakley et al., 1983; Dunn et al., 1978). The results are summarized in Table III. W-24-F showed ca. a 4-fold reduction in the binding for FAH_2, ca. a 7-fold reduction in the binding for NADPH, ca. a 7-fold reduction in the binding for folate, and ca. a 2-fold reduction in the binding for trimethoprim as compared to wild type.

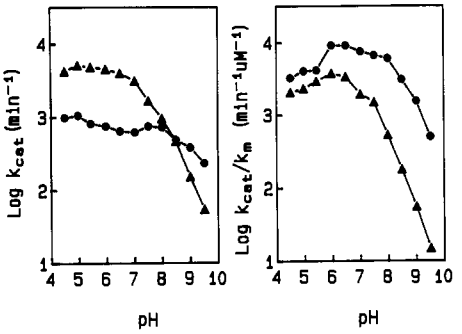

FIGURE 5: pH dependence of k_{cat} and k_{cat}/K_m (FAH_2) for wild type (●) and W-24-F (▲). The reaction conditions were 21 μM NADPH, 0.047–3.8 μM FAH_2, and 0.166–0.179 nM enzyme at 22 °C in MATS buffer.

The catalytic activity of W-24-F using NADH as cofactor was tested in MATS buffer. In the pH range of 5.5–8.5, W-24-F showed no activity when NADH (60 μM) instead of NADPH was included in the assay reaction mixture.

pH Dependence of k_{cat} and k_{cat}/K_m. The pH dependence of k_{cat} and k_{cat}/K_m values for both wild type and W-24-F was determined by varying the FAH_2 concentration at a fixed NADPH concentration. The pH profiles of these values are indicated in Figure 5. The k_{cat} profile of wild type showed two pH optima, one at ca. pH 5 and the other at ca. pH 7.5 (Delcamp et al., 1983). W-24-F showed only one pH optimum at ca. pH 5. In the pH range of 4.5–8.5, W-24-F had higher k_{cat} values than wild type, whereas in the pH 8.5–9.5 range, the k_{cat} values of W-24-F were lower than those of wild type. The k_{cat}/K_m values for W-24-F were lower than those for wild type in the whole pH range tested due to the larger increases in K_m for W-24-F. The k_{cat} values for W-24-F decreased rapidly as the pH increased from 7.0 to 9.5 with a k_{cat} value at pH 9.5 which was only 2% of the k_{cat} value at pH 7.0. Stability tests showed that after incubation for 2 min using the same conditions used for k_{cat} and K_m determinations, W-24-F retained at least 83% activity in the pH range 7.0–9.0 and 70% activity at pH 9.5 (data not shown). Therefore, the instability of the W-24-F enzyme at these pH values was not a major factor for the rapid decrease in k_{cat} with increases in pH.

Effects of DHFR Activating Agents. Human DHFR can be activated to different extents by KCl, urea, and organic mercurial compounds such as *p*-(hydroxymercuri)benzoate (pHMB)[1] (Delcamp et al., 1983). Since W-24-F is already 5-fold more active than wild-type enzyme, the effects of these activating agents were tested on W-24-F. Figure 6 shows the effects of KCl and urea on the enzymic activities of wild type and W-24-F. Wild type was activated to ca. 1.8-fold at 0.6 M KCl, whereas W-24-F was inactivated in the presence of KCl up to 97%. In the case of urea, the wild-type enzyme was activated about 2.8-fold at a 5.4 M concentration whereas W-24-F showed a slight activation at low concentrations with ca. 1.25-fold activation at 1 M urea concentration. Higher than 5 M concentratons of urea inactivated W-24-F almost completely. Figures 7 and 8 show the effects of pHMB on

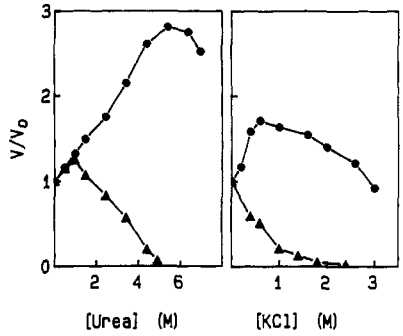

FIGURE 6: Effects of urea and KCl concentration on the enzymic activity of wild-type (●) and W-24-F (▲) DHFRs. The enzymes were assayed in the presence of various concentrations of urea or KCl as indicated in 50 mM Tris-HCl, pH 7.5, at 22 °C. V_0 = DHFR activity in the absence of urea or KCl; V = DHFR activity at the indicated concentration of urea or KCl.

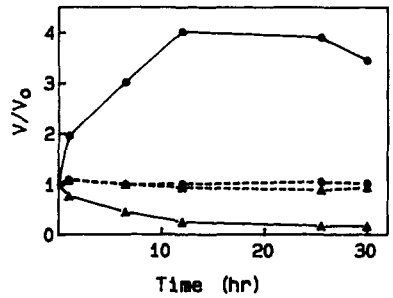

FIGURE 7: Effect of pHMB concentration on wild-type (●) and W-24-F (▲) DHFRs. The enzymes (1.1 μM) were incubated with a 150-fold molar excess of NADPH in 50 mM potassium phosphate, pH 7.5, at 22 °C in the presence of a 10-fold molar excess of pHMB (—) and in the absence of pHMB (---). Aliquots were removed from the incubation mixture and assayed at the times indicated. V_0 = DHFR activity in the absence of pHMB at time 0; V = DHFR activity at the indicated time.

enzyme activity of wild type and W-24-F, and the reversal of such effects by β-mercaptoethanol. As shown in Figure 7, whereas wild type was activated to ca. 4-fold, W-24-F was inhibited to ca. 80% when incubated with a 10-fold molar excess of pHMB in the presence of NADPH. The presence of β-mercaptoethanol prevented such effects; that is, when β-mercaptoethanol was included in the reaction mixtures, neither activation nor inhibition was observed. As indicated in Figure 8, when β-mercaptoethanol was added after the enzymes had been incubated with pHMB for 11 h, the activity of the wild-type enzyme decreased to a level slightly lower than the unmodified enzyme, while the activity of inhibited W-24-F increased slightly from 30% of unmodified activity to 60% of unmodified activity. The lack of complete reversal appears to relate to the instability of the pHMB-modified W-24-F. The controls in these experiments simply indicated that β-mercaptoethanol itself had no effect on either of the enzymic activities and that both wild type and W-24-F were stable during the course of incubation.

Discussion

In studying a mutant protein, one important aspect is the structural change caused by the amino acid substitution. We have employed competitive ELISA using peptide-specific antibodies against human DHFR, thermal stabilities, and protease susceptibilities to address questions of possible structural alterations. The antigenicity of any region of a protein molecule is correlated with the accessibility (Novontny et al., 1986) and conformational flexibility (Westhof et al., 1984) of that region. Since the antibodies used in our study are peptide specific, the stronger binding of W-24-F to anti-

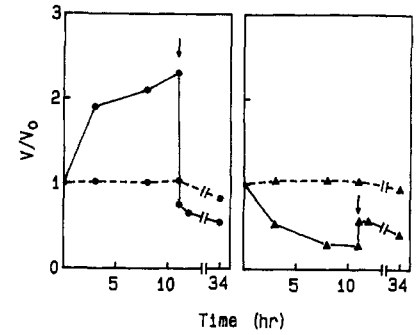

FIGURE 8: Partial reversal of the effects of pHMB on wild-type (●) and W-24-F (▲) DHFRs by β-ME. The enzymes were incubated with pHMB using the same conditions as described in the legend to Figure 7 in the presence of 10 mM β-ME (---) and in the absence of β-ME (—) and following addition of 10 mM β-ME (final concentration) after incubation with pHMB for 11 h as indicated by the arrows. The enzymes were assayed as described in Figure 7.

bodies against peptides 53–111 and 140–186 reflects a higher degree either of accessibility or of conformational flexibility of these two regions in the W-24-F. Thus, W-24-F may be in a more open conformation as compared to the wild-type protein. Protease susceptibility experiments also support this notion. As shown in Figure 1, W-24-F, in general, is more susceptible to proteases than its wild-type counterpart. In the case of chymotrypsin, wild-type reductase is virtually completely resistant to cleavage under the experimental conditions employed, whereas W-24-F is most susceptible to this protease among the three tested. The exposure of more than one chymotryptic site in the W-24-F molecule which is not accessible in the wild type further suggests that W-24-F is in a more open conformation.

A fraction of the population of the wild-type recombinant human DHFR (ca. 40%) contains an initiator Met (Prendergast et al., 1988). Amino acid sequence analysis shows that there is no initiator Met present at the N-terminus of the W-24-F enzyme. This result also suggests that the N-terminal Met in W-24-F is more accessible to aminopeptidase cleavage than in the wild type, perhaps due to the unfolding or loosening of the overall structure. Recent studies have indicated that single amino acid substitutions are, in general, accommodated by very minor, localized readjustment of the tertiary protein structure (Howell et al., 1986; Chen et al., 1985, 1987). The substitution of an indolyl group by a phenyl group is very conservative with respect to size and hydrophobicity. The fact that this conservative change results in significant changes in molecular properties indicates that the tryptophan residue at position 24 is important in maintaining the structural integrity of the enzyme.

With respect to the intrinsic fluorescence properties of W-24-F and wild type, 8 M urea treatment of wild type shifted the emission maximum from 320 to 350 nm and caused an increase in the relative fluorescence quantum yield. This is due to the folding of the peptide backbone of the protein which reduces the fluorescence quantum yield and changes the band position as in the case with other tryptophan-containing proteins (Teale, 1960). W-24-F, on the one hand, has virtually the same fluorescence spectral features as wild type in both native and denatured forms, which reflects the structural similarity of the two molecules. However, in its native form, W-24-F exhibits less of a reduction in relative fluorescence quantum yield compared with its denatured form than does wild type. This suggests that Trp-24 is buried and its fluorescence quenched relative to the other two tryptophans in the wild-type human DHFR.

W-24-F also shows general decreases in the binding affinities for the ligands tested compared to the wild-type enzyme (Table III). It is clear that the structural change in W-24-F has affected the geometry of these overlapping binding sites. The weaker binding of FAH_2 might be due to the loss of the hydrogen bond between N-1 of the indolyl ring and FAH_2 via a fixed water molecule (Bolin et al., 1982). The catalytic efficiency of W-24-F shows a narrower pH dependence than that of wild type. As indicated in Figure 5, both k_{cat} and k_{cat}/K_m of W-24-F decreased more rapidly as the pH was increased than that of wild type, which suggests that the group(s) in W-24-F whose protonation is required for catalysis lose(s) the proton more rapidly compared to wild type. As revealed by study of *E. coli* DHFR, the protonation of the active-site carboxyl group of Asp-27 in the *E. coli* enzyme, which is equivalent to Glu-30 in human DHFR, is required for both catalysis and substrate binding (Stone & Morrison, 1984; Howell et al., 1986). It is possible that loss of the hydrogen bond between N-1 of the indolyl group of the wild type and the carboxyl group of Glu-30 via a fixed water molecule destabilizes the protonated form of Glu-30 in W-24-F.

Human DHFR is one of several DHFRs which can be activated by urea, salts, and organomercurial compounds, such as pHMB (Delcamp et al., 1983). Studies on chicken liver DHFR have suggested that a common feature of enzyme activation is a general loosening of the native structure and that activation of the enzyme is characterized not only by an increase in V_{max} but also by an increase in K_m values for both FAH_2 and NADPH (Barbehenn & Kaufman, 1982; Kaufman, 1968). A similar effect was observed with human WIL2 DHFR (Delcamp et al., 1983). There are similarities between W-24-F and organomercurial-activated DHFRs such as increases in both k_{cat} and K_m for FAH_2 and K_m for NADPH (Table III) and increased sensitivity to thermal denaturation and proteolysis.

The effects of urea and KCl on W-24-F activity also show a similar response to that of organomercurial-activated chicken liver enzyme (Barbehenn & Kaufman, 1982). KCl causes inhibition of enzyme activity, while urea at low concentration stimulates enzyme activity slightly. However, urea at concentrations higher than 2 M inhibits enzyme activity (Figure 6). The effect of pHMB on the activity of W-24-F and its reversal by β-mercaptoethanol is in contrast to that observed for the wild type (see Results and Figures 7 and 8). W-24-F appears to react with pHMB in the same way as wild type, that is, reaction with the sulfhydryl group of Cys-6 (Prendergast et al., 1988). The presence of excess β-mercaptoethanol prevents such a reaction. Rather than stimulating enzyme activity as occurs in the wild type, this reaction results in the inactivation of W-24-F.

Taken together, these experimental results suggest that W-24-F is in a more open conformation as compared to wild type, which facilitates catalysis, and that perturbation of this conformation with salt, urea, or pHMB results in inactivation. The increase in k_{cat} observed by substituting Phe-24 for Trp-24 may be similar in terms of conformational changes to the mechanism of activation by urea, salt, and organomercurials. Therefore, study of the structure and kinetic properties of W-24-F will aid in understanding the mechanism of activation as well as shedding light on individual binding and catalytic steps which occur in the wild-type human DHFR.

Acknowledgments

We thank Kevin Rodeman and William Kohler for excellent technical assistance and Dr. Manohar Ratnam for his helpful suggestions. We also thank Valerie Murphy for typing the manuscript. During the course of this work, use was made of the Harold and Helen McMaster Recombinant DNA Laboratory.

Registry No. Trp, 73-22-3; Phe, 63-91-2; NADPH, 53-57-6; FAH_2, 4033-27-6; FA, 59-30-3; TMP, 738-70-5; NADP, 53-59-8; DHFR, 9002-03-3; pHMB, 1126-48-3; MTX, 59-05-2; KCl, 7447-40-7; urea, 57-13-6.

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Probing the Functional Role of Threonine-113 of *Escherichia coli* Dihydrofolate Reductase for Its Effect on Turnover Efficiency, Catalysis, and Binding†

C. A. Fierke‡ and S. J. Benkovic*,§

Biochemistry Department, Box 3711, Duke University Medical Center, Durham, North Carolina 27710, and Department of Chemistry, 152 Davey Laboratory, The Pennsylvania State University, University Park, Pennsylvania 16802

Received June 21, 1988; Revised Manuscript Received August 30, 1988

ABSTRACT: The role of Thr-113 of *Escherichia coli* dihydrofolate reductase in binding and catalysis was probed by amino acid substitution. Thr-113, a strictly conserved residue that forms a hydrogen bond to the active-site Asp-27 and to the amino group of methotrexate through a fixed water molecule, was replaced by valine. The kinetic scheme is identical in form with the wild-type scheme, although many of the rate constants vary, including a decrease in the association rate constants and an increase in the dissociation rate constants for folate ligands, a decrease in the hydride-transfer rate constant in both directions, and an increase in the intrinsic pK_a of Asp-27. Overall, replacement of Thr-113 by Val decreases the binding of folate substrates by ≈2.3 kcal/mol. These multiple complex changes on various ground and transition states underscore the optimal properties of a strictly conserved residue in the evolution of catalytic function.

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). This enzyme is necessary for maintaining intracellular pools of H_4F and its derivatives, which are essential cofactors in the one-carbon-transfer reactions utilized in the biosynthesis of purines, thymidylate, and several amino acids. In addition 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. Because of its biological and pharmacological importance, dihydrofolate reductase (DHFR) has been the subject of intensive structural and kinetic studies. The structures of the *Escherichia coli*, *Lactobacillus casei*, and chicken liver enzymes have been determined to 1.7-Å resolution for some binary and ternary complexes (Bolin et al., 1982; Filman et al., 1982; Matthews et al., 1985). In addition, a complete kinetic scheme for wild-type DHFR has been derived from pre-steady-state and steady-state kinetics (Fierke et al., 1987a). Despite knowledge of the kinetics and the identities of the amino acids at the active site of DHFR, the function of the amino acids in binding and catalysis is unclear. We have utilized site-directed mutagenesis combined with detailed kinetic analysis to establish structure–function relationships for *E. coli* DHFR.

Site-directed mutagenesis is a powerful tool that has been used to elucidate the functional role of individual amino acids in DHFR (Villafranca et al., 1983, 1987; Chen et al., 1985, 1987; Howell et al., 1985, 1987; Mayer et al., 1986; Taira et al., 1987a; Taira & Benkovic, 1988; Benkovic et al., 1988). The substrate H_2F, binds tightly to dihydrofolate reductase 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 amino acids. One of these, Thr-113, forms one hydrogen bond with Asp-27, the proton-donating residue (Howell et al., 1985), and another with the amino group of methotrexate (and presumably H_2F) indirectly through a bound water molecule. In this work we investigate the importance of these hydrogen

† This work was supported in part by NIH Grant GM24129. C.A.F. is the recipient of a National Institutes of Health postdoctoral fellowship (GM10072).
‡ Duke University Medical Center.
§ The Pennsylvania State University.

[1] Abbreviations: H_2F, 7,8-dihydrofolate; H_4F, 5,6,7,8-tetrahydrofolate; DHFR, dihydrofolate reductase; MTX, methotrexate; TMP, trimethoprim; NADPH, NH, reduced nicotinamide adenine dinucleotide phosphate; NADP, N, nicotinamide adenine dinucleotide phosphate; DAM, 2,4-diamino-6,7-dimethylpteridine; Fol, folate.

0006-2960/89/0428-0478$01.50/0