Expression and Site-Directed Mutagenesis of Human Dihydrofolate Reductase†

Neal J. Prendergast, Tavner J. Delcamp, Philip L. Smith, and James H. Freisheim*
Department of Biochemistry, Medical College of Ohio, Toledo, Ohio 43699
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ABSTRACT: A procaryotic high-level expression vector for human dihydrofolate reductase has been constructed and the protein characterized as a first step toward structure–function studies of this enzyme. A vector bearing the *tac* promoter, four synthetic oligodeoxynucleotides, and a restriction fragment from the dihydrofolate reductase cDNA were ligated in a manner which optimized the transcriptional and translational frequency of the enzyme mRNA. The reductase, comprising ca. 17% of the total soluble protein in the host bacteria, was purified to apparent homogeneity as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and characterized by amino acid composition, partial amino acid sequence, and steady-state kinetic analysis. This expression vector has been used as a template for double-stranded plasmid DNA site-specific mutagenesis. Functional studies on a Cys-6 → Ser-6 mutant enzyme support the contention that Cys-6 is obligatory for organomercurial activation of human dihydrofolate reductase. The Ser-6 mutant enzyme was not activated to any extent following a 24-h incubation with *p*-(hydroxymercuri)benzoate and nicotinamide adenine dinucleotide phosphate (reduced) (NADPH), whereas the k_{cat} for Cys-6 reductase increased 2-fold under identical conditions. The specific activities of the Cys-6 and Ser-6 enzymes were virtually identical as determined by methotrexate titration as were the K_m values for both dihydrofolate and NADPH. The Ser-6 mutant showed a decreased temperature stability and was more sensitive to inactivation by α-chymotrypsin when compared to the wild-type enzyme. These results suggest that the Ser-6 mutant reductase is conformationally altered relative to the Cys-6 native enzyme.

The nicotinamide adenine dinucleotide phosphate (reduced) (NADPH)[1] -dependent reduction of dihydrofolate, catalyzed by dihydrofolate reductase (DHFR) (5,6,7,8-tetrahydrofolate:$NADP^+$ oxidoreductase, EC 1.5.1.3), is a key reaction in folate metabolism. Consequently, DHFR has been an important target for anti-folate drugs aimed at inhibiting nucleic acid biosynthesis in some pathogenic bacteria and certain cancer cells.

The major emphasis of detailed structural studies including X-ray crystallography of DHFR and its ligand complexes has been on the enzymes from *Lactobacillus casei* (Matthews et al., 1977, 1978, 1979, 1985), from *Escherichia coli* (Bolin et al., 1982; Filman et al., 1982), and from chicken liver (Volz et al., 1982; Matthews et al., 1985). Alignment of DHFR primary sequences (Freisheim & Matthews, 1984) shows greater homology among vertebrate enzymes (73–93%) than among bacterial DHFR's (20–30%) and little homology between vertebrate and bacterial enzymes (<30%). Despite limited sequence homology between *E. coli*, *L. casei*, and chicken liver DHFR's, all display highly conserved α-carbon backbone geometries, i.e., four major α-helices and seven to eight β-sheets in corresponding positions of an 18K–22K monomer. Furthermore, bacterial and vertebrate reductases possess an active site constructed of predominantly identical or analogous amino acid residues.

Investigations designed to determine the functional roles of individual amino acid residues using site-directed mutagenesis have recently been reported for the *E. coli* DHFR (Villafranca et al., 1983; Howell et al., 1986; Chen et al., 1987). Similar amino acid replacement studies have not been done on a vertebrate enzyme. Described herein are the first reports of a human DHFR studied by this approach. The amino acid sequence of the human enzyme has been reported on the basis of its cDNA sequence (Masters & Attardi, 1983), but detailed studies of this enzyme have been hindered due to its low abundance both in normal human tissue and in methotrexate-resistant, DHFR-overproducing human cell lines (Delcamp et al., 1983). The results reported in the present study describe the development of a recombinant system for generating human DHFR in *E. coli*, purification and characterization of the expressed enzyme from the bacterial host, and production of a site-specific mutation in the DHFR cDNA. The properties of the mutant enzyme, Ser-6, designed to address the role of a unique cysteine residue located at position 6 in the wild-type DHFR are presented.

Experimental Procedures

Oligodeoxynucleotides. Synthetic oligodeoxynucleotides were prepared by using an Applied Biosystems Model 380A automated DNA synthesizer. Oligodeoxynucleotides used in the construction of the DHFR expression vector, pDFR, and those used for site-directed mutagenesis experiments were purified by preparative polyacrylamide gel electrophoresis. Phosphorylation of oligodeoxynucleotides was performed in a manner similar to that described by Chen et al. (1987).

Plasmids, E. coli Strains, and Construction of pDFR. Plasmids pKK-223-3 (P-L Biochemicals) and pDFR, constructed as described below, were grown in *E. coli* strain JM107. The plasmid pHD84 carrying the full-length human DHFR cDNA was kindly provided by Dr. Giuseppe Attardi (California Institute of Technology) and was maintained in

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* 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; 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; CIP, calf intestinal alkaline phosphatase; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; bp, base pair(s).

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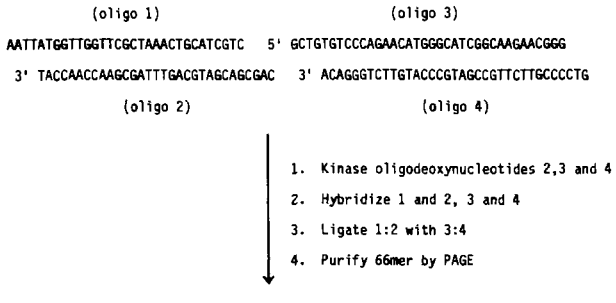

FIGURE 1: Construction of a 66-mer synthetic duplex prepared with four oligodeoxynucleotides. Oligodeoxynucleotides 2, 3, and 4 were phosphorylated at 37 °C for 45 min in a reaction containing 500 pmol of each oligodeoxynucleotide, 50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 15 mM DTT, and 1 mM ATP. Oligo 2 was heated to 90 °C for 5 min after which time 500 pmol of oligo 1 was added. This mixture was heated an additional 2 min at 90 °C and slowly cooled to 4 °C. Oligonucleotides 3 and 4 were added together after the phosphorylation reaction, heated to 90 °C for 5 min, and slowly cooled to 4 °C. The duplex molecules 1·2 and 3·4 were mixed together with 1 μL of 80 mM ATP and 2 μL of T4 DNA ligase to a final volume of 60 μL. This mixture was incubated at 12 °C for 16 h and the sample loaded onto an 8% nondenaturing polyacrylamide gel. The 66-mer synthetic product was identified following staining of the gel by ethidium bromide and comparing the ligation reaction products to molecular weight standards (data not shown).

E. coli strain HB101. The plasmid pDFR was constructed from a restriction fragment of the DHFR cDNA, a 66-mer synthetic duplex, and the expression vector pKK-223-3, which carries the hybrid *tac* promoter. Four oligodeoxynucleotides were used to construct the 66-mer as described in the legend for Figure 1. The 66-mer contained the first 63 nucleotides of the DHFR cDNA coding region and was synthesized to replace the coding region of the cDNA lost following its treatment with *Ava*II (see below). A restriction fragment containing the remaining coding sequence, stop codon, and 3′-noncoding region of the DHFR cDNA was prepared as shown in Figure 2. The plasmid pHD84 was digested with *Pst*I and the cDNA isolated. The cDNA was then digested with *Ava*II to remove the 5′-noncoding region, and, in addition, the first 63 nucleotides of the coding sequence are lost during this cleavage step. The large fragment from this cleavage was then isolated and treated with calf intestinal alkaline phosphatase (CIP). Alkaline phosphatase treatment limited the formation of undesired ligation products in the pDFR ligation reaction mixture (see below). pKK-223-3 was treated with both *Eco*RI and *Pst*I and the large fragment isolated. A ligation mixture containing 1.7 pmol of each component (Figure 2) was incubated overnight at 12 °C, and then various dilutions of this mixture were used to transform competent *E. coli* strain JM107. In all experiments, competent cells were prepared using $CaCl_2$ as described by Maniatis et al. (1982). Bacteria were plated onto LB agar plates containing ampicillin (50 μg/mL) and trimethoprim (250 μg/mL). Bacteria not expressing human DHFR activity are sensitive to trimethoprim and will grow poorly if at all under these conditions.

Oligodeoxynucleotide-Directed, Site-Specific Mutagenesis. Double-stranded plasmid DNA was used in preparing the Ser-6 mutant using a modification of the protocol described

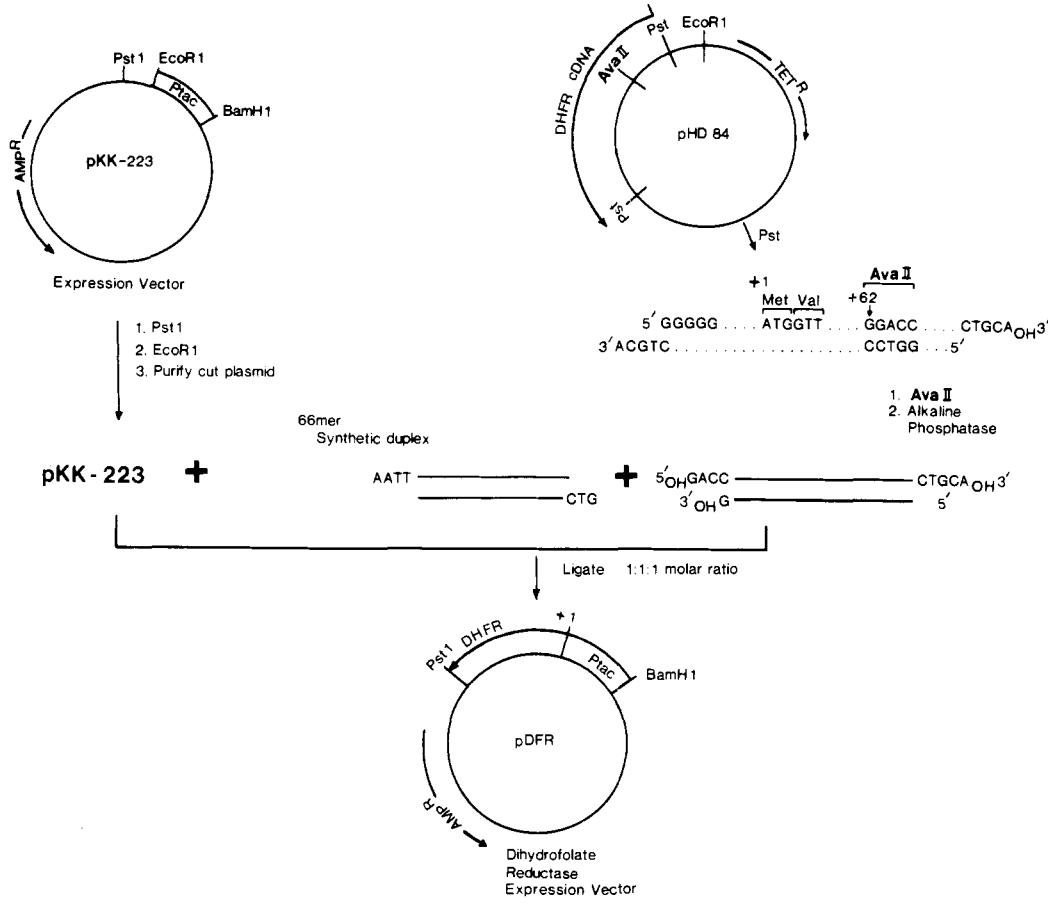

FIGURE 2: Schematic depiction of the steps involved in the construction of pDFR (see text for details).

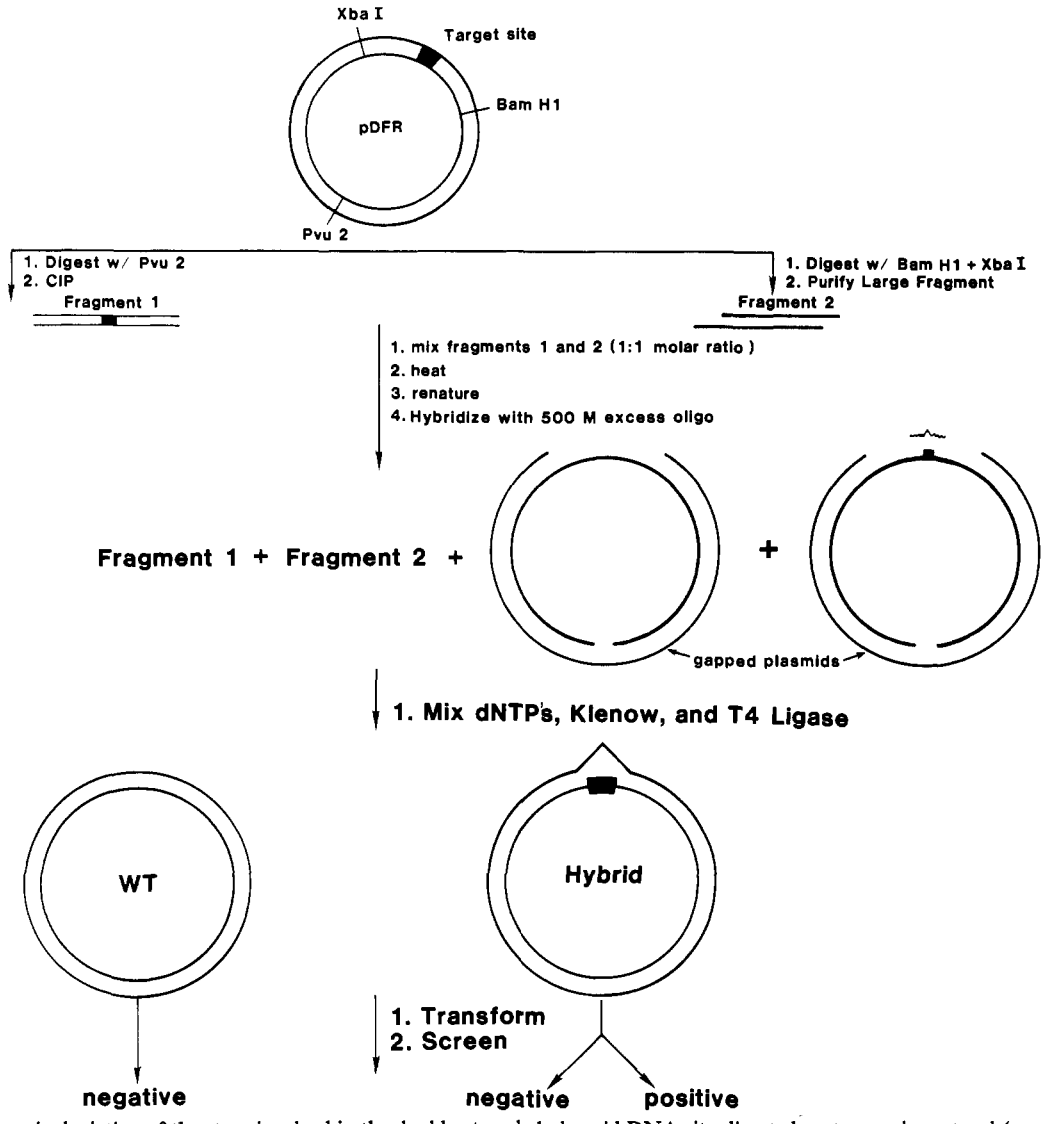

FIGURE 3: Schematic depiction of the steps involved in the double-stranded plasmid DNA site-directed mutagenesis protocol (see text for details).

by Inouye and Inouye (1987). A schematic depiction of the steps involved in this procedure is shown in Figure 3. Fragments 1 and 2, prepared as shown in Figure 3, were mixed in equimolar amounts, and 1 volume of 80% formamide, 10 mM Tris-HCl, and 20 mM EDTA (pH 8.0) was added. The sample was boiled for 3 min and the solution immediately placed at 37 °C for 15-30 min. An aliquot of the gapped plasmid mixture (0.1 pmol) was mixed with 20 pmol of phosphorylated oligodeoxynucleotide together with T4 DNA ligase buffer (2.6 μL) and H_2O to yield a final volume of 26 μL. The oligodeoxynucleotide in this experiment was 19 nucleotides in length and contained a single mismatch designed to convert the Cys codon (TGC) to a Ser codon (TCC). The gapped plasmid oligodeoxynucleotide mixture was incubated 30–60 min at 30 °C and chilled on ice. One microliter of 10 mM ATP, 1 μL of 10 mM in all four dNTP's, 0.2 μL of DNA polymerase large fragment, and 1 μL of T4 DNA ligase were added, and the mixture was incubated 3–6 h at 12 °C. Various dilutions of this reaction mixture were used to transform *E. coli* strain JM101. Bacteria were plated onto LB agar plates containing ampicillin (50 μg/mL). Transformants carrying the mutant DHFR plasmid were distinguished from those carrying the wild-type plasmid by colony hybridization analysis using the ^{32}P-labeled oligodeoxynucleotide (the same oligodeoxynucleotide used in the mutagenesis reaction mixture) as a probe. Prehybridization and hybridization conditions used in these experiments were determined essentially as described by Inouye and Inouye (1987).

DNA Sequence Analysis. DNA sequence analysis of the promoter region and partial coding region of pDFR was originally determined by the base-specific chemical cleavage method of Maxam and Gilbert (1977). Subsequently, dideoxy sequence analysis (Sanger et al., 1977) of double-stranded DNA was used to obtain the entire coding region of both Cys-6 and Ser-6 genes. In these studies, plasmid DNA purified by ethidium bromide–cesium chloride density gradient centrifugation was subjected to alkaline denaturation as described by Chen and Seeburg (1985). The DNA pellet from this step (2 μg) was resuspended in 1.0 μL of polymerase reaction buffer [BRL (Bethesda Research Laboratory) Cloning and Sequencing Manual], 10 μL of H_2O, and 1.4 μL of primer solution (6 ng of primer in H_2O). This mixture was placed at 42 °C for 15 min and slowly chilled to 4 °C. The subsequent steps in this protocol were identical with those described in BRL's sequencing manual for sequencing with [α-^{35}S]dATP with two exceptions. The incubation temperature for the sequencing reaction was 42 °C. The concentrations of the ddNTP solutions, prepared before addition to the sequencing reaction mixture, were 0.2 mM ddATP, 0.35 mM ddCTP, 0.7 mM ddGTP, and 1.4 mM ddTTP.

Expression and Purification of Wild-Type and Ser-6 DHFR's. Quantitation of DHFR expression from pDFR is

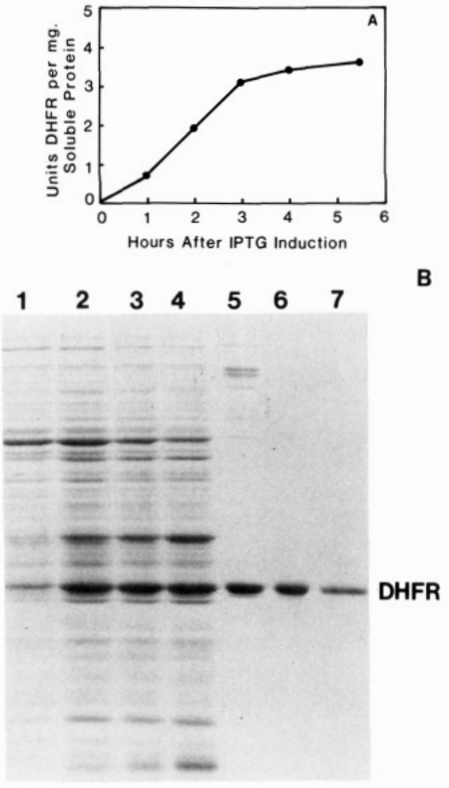

FIGURE 4: (A) Measurement of DHFR levels in JM107 carrying the plasmid construct pDFR following induction with IPTG. *E. coli* carrying pDFR were grown to an OD_{600nm} = 0.6, and IPTG was added to a final concentration of 5 mM. Cells were harvested at various times after induction (time 0 = immediately after IPTG addition). Bacterial extracts were prepared by freeze–thawing cells in 50 mM Tris-HCl (pH 7.5) 3 times. The soluble component was separated from cellular debris by centrifugation. DHFR levels were measured enzymatically, and total protein levels were measured by the Bio-Rad method. (B) Expression and purification of pDFR: Samples after 1, 2, 3, and 4 h of induction were subjected to 12.5% SDS–PAGE (lanes 1–4, respectively). Purification of pDFR DHFR: lane 5, MTX affinity chromatography; lane 6, Sephadex G-75 gel filtration; lane 7, human DHFR purified from a WIL2/M4 cell line (Delcamp et al., 1983).

described in Figure 4. The purification procedure for both wild-type and Ser-6 enzymes was identical. JM107 carrying the expression plasmids were grown overnight in 100 mL of M9 minimal media supplemented with 0.2 mg/L thiamin and 50 μg/mL ampicillin. This culture was used to inoculate 14 L of LB medium containing 50 μg/mL ampicillin. *E. coli* cells were grown to an OD_{600} = 0.6, and isopropyl β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM. After 4 h, cells were harvested by centrifugation at 8000*g* for 10 min at 4 °C. Cell pellets were resuspended in 250 mL of ice-cold 5 mM EDTA, 1 mM phenylmethanesulfonyl fluoride, 2 mg/L leupeptin, 10 mM β-mercaptoethanol, and 50 mM imidazole, pH 6.5. Lysozyme was added (0.5 mg/mL) and the solution kept on ice for 20 min. DNase and $MgCl_2$ were added to final concentrations of 80 μg/mL and 3 mM, respectively, and kept on ice an additional 20 min. The mixture was frozen in a dry ice–acetone bath and thawed to optimize cell lysis. Once thawed, the mixture was centrifuged at 27000*g* for 20 min at 4 °C. The supernatant was collected and assayed for DHFR activity as described below. The pellet from a 0–85% ammonium sulfate precipitation was resuspended in 100 mL of ice-cold 1 mM EDTA, 10 mM β-mercaptoethanol, 10% glycerol, and 50 mM imidazole, pH 6.5 (buffer A), containing 100 mM KCl and applied to a 2.5 × 5 cm methotrexate (MTX)–Sepharose-4B column (Kaufman, 1974) equilibrated with the same buffer. The column was washed with 200 mL of buffer A + 100 mM KCl, 100 mL of buffer A + 500 mM KCl, and 500 mL of buffer A + 100 mM KCl or until the OD_{280} <0.10. The column then was reequilibrated with 100 mL of buffer A + 100 mM KCl adjusted to pH 8.0. Dihydrofolate (FAH_2) (60 mg) was dissolved in 20 mL of this buffer; the solution was adjusted to pH 8.0 and passed over the MTX column at 0.5 mL/min. Five-milliliter fractions were collected and assayed for DHFR activity, and those fractions containing enzyme activity were concentrated in an Amicon ultrafiltration unit using a YM-10 membrane to a final volume of 5 mL. This material was then applied to a 2.5 × 85 cm Sephadex G-75 (fine) column equilibrated with buffer A + 200 mM KCl adjusted to pH 7.5. Fractions containing DHFR activity were pooled, concentrated as described above, and subjected to preparative isoelectric focusing to remove bound FAH_2 and to obtain a p*I* value for the recombinant enzyme (Delcamp et al., 1983).

Enzyme Assay. DHFR activity was measured spectrophotometrically at 22 °C unless otherwise indicated. The assay monitors a decrease in absorbance at 340 nm which occurs when NADPH and FAH_2 are converted to $NADP^+$ and tetrahydrofolate (FAH_4). Standard assay conditions for enzyme activity measurements were 50 mM Tris-HCl (pH 7.5), 60 μM NADPH, and 50 μM FAH_2. The molar extinction coefficient for the reaction at 340 nm is 12 300 (Hillcoat et al., 1967). One unit of enzyme activity is defined as the amount of enzyme required to convert 1.0 μmol of FAH_2 to FAH_4 in 1 min at 22 °C. All assays were initiated with FAH_2 unless otherwise indicated. Steady-state kinetic studies for the determination of K_m values for NADPH and FAH_2 were performed as described by Delcamp et al. (1983). Analysis of initial velocity data was performed by a computer program which uses nonlinear least-squares analysis (Cleland, 1967) to generate K_m values together with standard errors of these kinetic constants.

Protein Sequence and Amino Acid Analysis Determinations. N-Terminal protein sequence analysis was performed by using an Applied Biosystems Model 470A gas-phase protein sequencer. Reductive carboxymethylation for the determination of cysteine was performed according to Kaufman et al. (1980). Amino acid analysis was performed on a Beckman Model 6300 amino acid analyzer following a 24-h hydrolysis in 6 N HCl in vacuo at 110 °C. Cysteine content of wild-type and Ser-6 DHFR's was determined by performic acid oxidation and amino acid analysis (Moore, 1963).

Results and Discussion

Expression Vector Construction. The human DHFR expression vector was constructed from the DHFR cDNA, four oligodeoxynucleotides, and a vector carrying the *tac* promoter. The scheme outlined in Figure 2 and described under Experimental Procedures depicts how removal of the *Ava*II fragment and replacement with the 66-mer positions the start codon of the cDNA 9 bp downstream from the Shine–Dalgarno sequence. It is well established that high-level expression of recombinant gene products in *E. coli* requires appropriate spacing between Shine–Dalgarno and start codon sequences. A procaryotic system was chosen to express this enzyme since human DHFR is a single polypeptide containing no disulfide linkages or posttranslational modifications. In addition, *E. coli* which express human DHFR can be identified phenotypically by their ability to survive trimethoprim selection. The presumed arrangement of the promoter and coding sequence of pDFR is shown at the bottom of Figure 2. Restriction endonuclease mapping and dideoxy sequence analysis confirmed this arrangement. In subsequent experiments, the entire

Table I: Amino Acid Composition of Cys-6 and Ser-6 DHFR's[a]

amino acid	Cys-6 (residues)	Ser-6 (residues)	reported cDNA sequence (residues)
Asx	19.0	19.2	19
Thr	5.8	5.9	7
Ser	8.3	8.5	12
Glx	22.1	22.1	23
Pro	11.1	11.7	12
Gly	14.0	12.7	13
Ala	5.0	5.0	5
Cys[b]	0.7	0.0	1
Val	12.7	13.3	14
Met[b]	5.1	6.1	6
Ile	7.6	7.9	9
Leu	17.0	17.5	19
Tyr	ND[c]	ND[c]	6
Phe	7.8	8.1	9
His	2.4	2.7	3
Lys	14.9	15.4	17
Arg	6.9	7.0	8
Trp[c]	ND[c]	ND[c]	3

[a] Amino acid composition of Cys-6 and Ser-6 enzymes following 24-h hydrolysis in 6 N HCl at 110 °C assuming five alanines per mole of protein. The values shown are the average of two analyses. [b] Determined as cysteic acid and methionine sulfone, respectively, according to Moore (1963). [c] Not determined.

coding region of the DHFR cDNA in pDFR was sequenced by double-stranded dideoxy sequence analysis and found to be identical with that reported earlier for the human DHFR cDNA (Masters & Attardi, 1983).

Expression and Characterization of Human DHFR. The recipient vector for the reconstructed DHFR cDNA, pKK-223-3, has been shown to function effectively in generating high levels of foreign gene products in *E. coli* (Amann et al., 1983). This plasmid contains the hybrid trp-lac (*tac*) promoter (Amann et al., 1983) which can be regulated when grown in *E. coli* strains overexpressing the *lac* repressor. DHFR synthesis from pDFR grown in lac I^Q strains of *E. coli* (e.g., JM107) was repressed and could be induced by the addition of IPTG (Figure 4A). Following 4 h of induction, DHFR levels had reached 17% of the total soluble protein. Bacterial DHFR activity was found to be insignificant relative to the total DHFR activity in JM107 carrying pDFR (data not shown). Human DHFR expressed from pDFR was purified to homogeneity as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (Figure 4B). The electrophoretic mobility of the purified recombinant enzyme determined from SDS–PAGE was identical with that of human DHFR purified from the WIL2/M4 lymphoblastoid cell line (Delcamp et al., 1983) (Figure 4B). Amino acid analysis (Table I) and N-terminal protein sequence analysis (Table II) were performed on pDFR DHFR. The results from these experiments were in agreement with those predicted from the nucleotide sequence of the DHFR cDNA. N-Terminal protein sequence analysis of the recombinant DHFR revealed that ca. 40% of the total enzyme contained an initiator methionine. The pDFR enzyme was further characterized to determine the isoelectric point, K_m values for substrate and cofactor, and specific activity of the recombinant enzyme as compared with the human enzyme isolated from the WIL2/M4 lymphoblastoid cell line. Table III summarizes the results obtained from these studies and those conducted on the WIL2/M4 DHFR (Delcamp et al., 1983). The properties examined for pDFR DHFR were in close agreement with those obtained for the WIL2/M4 enzyme.

Mutant Construction. A modification of the method described by Inouye and Inouye (1987) has been used to generate

Table II: Automated Sequence Analysis of Human Recombinant DHFR[a]

step	amino acid identified	yield (nmol)	amino acid identified	yield (nmol)
01	Met	6.4	Val	8.8
02	Val	7.4	Gly	5.4
03	Gly	3.6	Ser[b]	*c*
04	Ser[b]	*c*	Leu	8.5
05	Leu	7.6	Asn	3.7
06	Asn	2.8	Cys	*d*
07	Cys	*d*	Ile	4.9
08	Ile	4.8	Val	5.5
09	Val	4.7	Ala	4.7
10	Ala	4.6	Val	5.5
11	Val	4.9	Ser[b]	*c*
12	Ser[b]	*c*	Gln	3.0
13	Gln	2.8	Asn	1.7
14	Asn	2.2	Met	2.6
15	Met	2.3	Gly	2.3
16	Gly	2.4	Ile	2.9
17	Ile	2.9	Gly	2.6
18	Gly	2.5	Lys	1.6
19	Lys	1.5	Asn	1.7
20	Asn	1.6	Gly	2.6
21	Gly	2.3	Asp	1.2
22	Asp	1.2	Leu	1.9
23	Leu	0.9	Pro	0.5
24	Pro	0.4	Trp	0.4
25	Trp	0.4	Pro	0.6

[a] Automated sequence analysis of ^{14}C-carboxylated human DHFR. Sequence analysis of 27 nmol of ^{14}C-carboxymethylated protein. The initial yield was 56.4%. The repetitive yield was 91.9%. [b] Serine was identified as a DTT adduct of PTH-dehydroalanine. [c] δ-Ser. [d] ^{14}C-Carboxymethylated Cys.

Table III: Properties of Human Dihydrofolate Reductases[a]

source	Michaelis constants (μM) NADPH	Michaelis constants (μM) FAH_2	pI[b]	sp act. (units/mg)
WIL2/M4[c]	0.25	0.036	7.7	16.1
Cys-6 DHFR	0.26	0.022	7.9	20.0
Ser-6 DHFR	0.28	0.014	ND[d]	20.0

[a] Properties of native and recombinant human DHFR's. For each enzyme, Michaelis constants and specific activity measurements were determined spectrophotometrically at 22 °C in 50 mM Tris-HCl, pH 7.5. [b] Isoelectric point. [c] Reference (Delcamp et al., 1983). [d] Not determined.

site-specific mutations in the human DHFR cDNA. As described under Experimental Procedures, JM101 was used in place of JM107 as the host for transformation with the mutagenesis reaction mixture. It has been determined in this laboratory that the Klenow enzyme greatly reduces the transformation efficiency of both JM101 and JM107 (data not shown). However, JM101 is affected to a lesser degree than JM107 and as such was selected as the host of choice for these experiments. The entire coding region of the Ser-6 mutant enzyme was determined by double-stranded dideoxy sequence analysis. This analysis demonstrated that the expected substitution had been introduced and that no other alteration in the coding region of the DHFR cDNA had occurred.

Expression and Structural Analysis of the Ser-6 Mutant DHFR. The functional role of the single cysteine residue in human DHFR, Cys-6, was evaluated by replacing this residue with serine and measuring the effects on the kinetic, activation, and structural properties of the enzyme. The expression and purification of the Ser-6 reductase were monitored by SDS–PAGE. Ser-6 DHFR was efficiently expressed from the plasmid and could be purified in a manner identical with that described for the wild-type enzyme (data not shown). Amino

Table IV: Automated Sequence Analysis of Ser-6 DHFR[a]

step	amino acid identified	yield (nmol)	amino acid identified	yield (nmol)
01	Val	0.50	Met	0.20
02	Gly	0.30	Val	0.20
03	Ser[b]	c	Gly	0.12
04	Leu	0.41	Ser[b]	c
05	Asn	0.15	Leu	0.10
06	Ser[b]	c	Asn	0.11
07	Ile	0.61	Ser[b]	c
	Val	0.13	Ile	0.59

[a] Automated sequence analysis of 1.7 nmol of the Ser-6 enzyme. The initial yield was 40.9%. The repetitive yield was 82.9%. [b] Ser was identified as a DTT adduct of PTH-dehydroalanine. [c] δ-Ser.

acid analysis was performed on the Ser-6 protein following performic acid oxidation (Table I). No cysteic acid was detected in the Ser-6 mutant whereas the wild-type enzyme contained approximately 1 mol of cysteic acid per mole of protein. N-Terminal protein sequence analysis of the Ser-6 mutant (Table IV) identified serine at position 6 and, in addition, identified a fraction of the enzyme population containing an initiator methionine. The percentage of the Ser-6 enzyme molecules still containing initiatior methionine (30% of the total) was similar to that observed for the wild-type enzyme.

Kinetic Studies. The protein sequence information available for animal and bacterial DHFR's (Blakley, 1984) shows that in all of the animal DHFR's a cysteine residue is located at position 6 or 11. Bacterial DHFR's, by contrast, do not contain a cysteine residue within the first 50 amino acids of the molecule and in some cases contain no cysteine. From these comparisons, it has been tentatively concluded that cysteine residues are not required for direct substrate binding or involved in the catalytic mechanism of the DHFR reaction (Kaufman et al., 1980). The results presented in Table III summarizing the steady-state kinetic properties of the Cys-6 and Ser-6 enzymes support this notion. The Cys-6 and Ser-6 reductases displayed similar K_m values for both substrate and cofactor and have identical catalytic coefficients. Thus, DHFR catalysis, measured under the stated conditions, proceeds unaffected by the absence of the sulfhydryl group. However, it cannot be stated unequivocally that in the native DHFR cysteine has no influence on the binding or catalytic properties of the enzyme, since serine could replace that function.

Activation Studies. One of the well-documented and unusual properties of vertebrate DHFR's is their response to organic mercurials. These compounds react specifically with available thiol groups of proteins (Means & Feeney, 1971) and in the case of vertebrate DHFR's react with the single cysteine at either position 6 or position 11 on the DHFR molecule. Several lines of evidence suggest that activation of vertebrate DHFR's by mercurial agents is a consequence of a conformational change induced by covalent attachment of the mercurial to the sulfhydryl moiety on Cys-6 or -11 (Freisheim & Matthews, 1984). Mercurial activation is reversible upon addition of 2-mercaptoethanol to the activated enzyme. In addition, the activated DHFR, unlike the native form, exhibits an increased sensitivity to heat, to proteolysis, and to its ionic environment (Barbehenn & Kaufman, 1982). Duffy et al. (1987) have recently reported the rate of chemical modification of the murine L1210 DHFR with the mercurial *p*-(hydroxymercuri)benzoate (pHMB). These authors report that the rate of Cys-6 modification by pHMB exceeded the rate of activation and proposed that mercurial activation occurs in a stepwise manner in which pHMB first attaches to the sulfhydryl group and subsequently induces a conformational

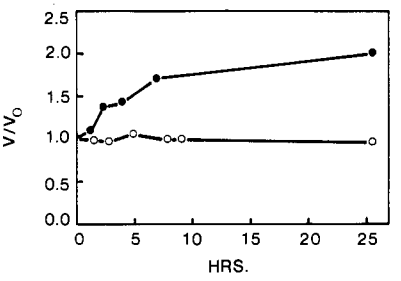

FIGURE 5: Effect of pHMB concentration on the mutant Ser-6 enzyme (○) and the native Cys-6 enzyme activity (●). Both enzymes were incubated with a 50-fold molar excess of NADPH and a 10-fold molar excess of pHMB in 50 mM Tris-HCl (pH 7.5) at 22 °C. Aliquots from the reaction mixture were removed at various times and assayed under the standard enzyme assay conditions. Substrate and cofactor were dissolved in 50 mM Tris-HCl (pH 7.5) without 2-mercaptoethanol. V_0 = DHFR activity at time 0. V = DHFR activity at the indicated time.

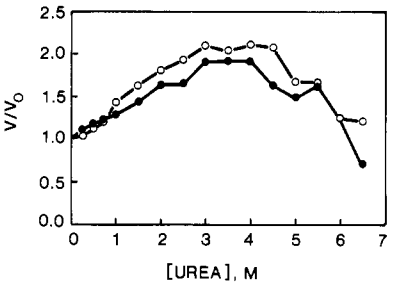

FIGURE 6: Effect of urea concentration on the Ser-6 mutant enzyme (○) and on the native Cys-6 enzyme (●) activity. The assay buffer contained the indicated amount of urea in 50 mM Tris-HCl, pH 7.5. NADPH concentration (initial), 7.6×10^{-5} M. FAH_2 concentration (initial), 5.4×10^{-5} M. Ser-6 enzyme concentration, 3.8×10^{-8} M. Cys-6 enzyme concentration, 5.6×10^{-8} M. V_0 = DHFR activity in the absence of urea. V = DHFR activity at the indicated urea concentration.

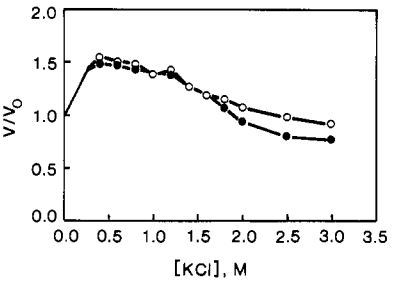

FIGURE 7: Effect of KCl concentration on the mutant Ser-6 enzyme (○) and the native Cys-6 enzyme (●) activity. The assay buffer contained the indicated amount of KCl in 50 mM Tris-HCl, pH 7.5. NADPH concentration (initial), 8.6×10^{-5} M. FAH_2 concentration (initial), 6.7×10^{-5} M. Ser-6 enzyme concentration, 3.7×10^{-8} M. Cys-6 enzyme concentration, 3.4×10^{-8} M. V_0 = DHFR activity in the absence of KCl. V = DHFR activity at the indicated KCl concentration.

change in the enzyme, which in turn produces the activated state. The data indicated in Figure 5 support this contention. Failure of the Ser-6 mutant enzyme to respond to the mercurial under conditions where the Cys-6 enzyme is activated ca. 2-fold demonstrates that Cys-6 is the site of attachment for pHMB. Furthermore, these results agree with the activation pathway proposed by Duffy et al. (1987) regarding the mechanism of mercurial activation.

In this study, experiments were also conducted to determine if the presence of Cys-6 is essential for the activation of human DHFR by chaotropes such as KCl and urea. In contrast to pHMB, urea and KCl both activate the Ser-6 mutant enzyme in a manner similar to that observed for the Cys-6 reductase (Figures 6 and 7, respectively). Thus, substitution at this

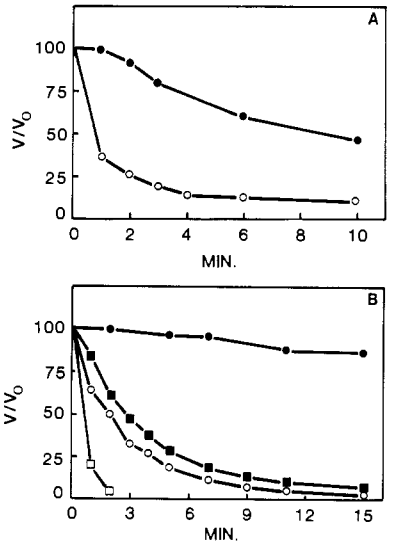

FIGURE 8: (A) Temperature stability of apoenzyme forms of Ser-6 (○) and Cys-6 (●) at 30 °C. Ser-6 enzyme (2.1×10^{-7} M) and Cys-6 enzyme (2.2×10^{-7} M) were incubated in 50 mM Tris-HCl, pH 7.5, at 30 °C. Samples were removed at various times and placed on ice. After 30 min, these samples were assayed under standard conditions. (B) Thermal stability of the Cys-6 and Ser-6 holoenzyme complexes at 50 °C. NADPH–DHFR thermal stability profiles for Cys-6 (●) and Ser-6 (■). Incubation conditions: Cys-6 enzyme (2.1×10^{-6} M) + NADPH (9.4×10^{-4} M) in 50 mM Tris-HCl, pH 7.5; Ser-6 enzyme (2.0×10^{-6} M) + NADPH (9.4×10^{-4} M) in 50 mM Tris-HCl, pH 7.5. Enzyme–NADPH complexes were incubated at 50 °C for various times and immediately placed on ice. After 30 min, samples were assayed under the standard conditions. FAH_2–DHFR thermal stability profiles for the Cys-6 native enzyme (○) and the Ser-6 mutant enzyme (□). Incubation conditions: Cys-6 enzyme (2.6×10^{-6} M) + FAH_2 (3.9×10^{-4} M) in 50 mM Tris-HCl, pH 7.5; Ser-6 enzyme (3.0×10^{-6} M) + FAH_2 (3.9×10^{-4} M) in 50 mM Tris-HCl, pH 7.5. Enzyme–FAH_2 complexes were incubated at 50 °C for various times and immediately placed on ice. After 15 min, samples were assayed under the standard conditions. V_0 = DHFR activity at time 0. V = DHFR activity at the indicated times.

position does not alter the enzyme's ability to assume a more active conformation in the presence of general chaotropic agents. These results suggest that the presence or absence of a cysteine residue does not influence the conformation assumed by the enzyme in reaching the activated state. An alternative explanation for these findings could be that serine is capable of replacing the function which cysteine may have in these processes. Additional mutagenesis experiments at this position, less conservative in nature, might distinguish between these two possibilities.

Thermal and Protease Susceptibility Studies. A series of experiments were conducted on the Ser-6 mutant enzyme to determine if this substitution had altered its general conformation. These experiments were undertaken following the observation that Ser-6 lost activity much more rapidly when stored at −20 °C than Cys-6 stored under similar conditions. Two criteria, temperature stability and protease sensitivity, were used to compare the Ser-6 and Cys-6 reductases. The most dramatic change in the Ser-6 enzyme was in its temperature stability properties. Both apoenzyme and holoenzyme forms show a marked increase in sensitivity to temperature (panels A and B, respectively, of Figure 8). It is interesting to note that DHFR's modified at cysteine show similar temperature-sensitive properties. Such results suggest that at least part of the temperature-sensitive properties displayed by mercurial-modified enzymes are the result of the breaking of a critical noncovalent bond between cysteine and another portion of the molecule. The nature of this bond is uncertain, but a possible candidate is a hydrogen bond. The temperature-sensitive behavior of the Ser-6 mutant enzyme is somewhat puzzling since serine should be capable of forming the same hydrogen bond as cysteine, assuming that this substitution has not produced a conformational change in the enzyme. That the Ser-6 enzyme is preferentially inactivated by α-chymotrypsin in contrast to Cys-6 (Figure 9) suggests that a conformational change has in fact resulted as a consequence of this substitution to expose one or more α-chymotrypsin-sensitive cleavage sites. It is interesting to note that Ser-6 did not show increased sensitivity to trypsin. In both cases, the experimental data were reproducible. The nature of the differences in tertiary structure between the Ser-6 and Cys-6 enzymes remains to be determined. It is possible that replacement of cysteine with serine alters the three-dimensional structure of human DHFR such that serine can no longer replace the chemical bonding properties of cysteine. This would occur if substitution of cysteine with serine produced an enzyme that no longer followed the same folding pathway as that taken by Cys-6 during and following synthesis of the polypeptide. This would follow if the final conformation assumed by the polypeptide positions the hydroxyl group on serine in the Ser-6 mutant reductase differently from that of the sulfhydryl group in the Cys-6 native enzyme. A second possibility is that the difference in the van der Waals radius of oxygen on serine as compared to sulfur on cysteine is large enough to prevent hydrogen-bond formation from occurring in the Ser-6 mutant. In this case, it is the absence of a hydrogen bond which defines the conformational differences qualitatively measured in these studies. A third possibility involves the ionization of the sulfhydryl moiety of cysteine. The pK_a value for the sulfhydryl group in proteins has been reported to range between 8.5 and 8.8 (Cantor & Schimmel, 1980). It is possible that the sulfhydryl group in the Cys-6 enzyme may ionize and form a salt bridge with another residue and in so doing stabilize and protect the enzyme against temperature and protease denaturation. In contrast, the hydroxyl group of serine in the Ser-6 enzyme would not be expected to ionize and thus could explain the altered properties of this mutant.

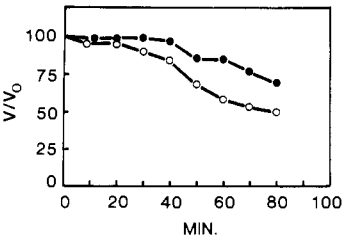

FIGURE 9: Proteolytic susceptibility of the Cys-6 native enzyme (●) and the Ser-6 mutant enzyme (○) to α-chymotrypsin. Cys-6 (5.8×10^{-7} M) and Ser-6 (6.9×10^{-7} M) enzymes were incubated at 22 °C with NADPH (6.6×10^{-5} M), 129 μg/mL α-chymotrypsin, 100 mM Tris-HCl, and 1 mM $CaCl_2$, pH 8.0. Aliquots were removed from the incubation mixture at various times and assayed under standard conditions. V_0 = DHFR activity at time 0. V = DHFR activity at the indicated times.

In summary, recombinant DNA techniques have been used to produce milligram quantities of human DHFR and to examine the effects of the replacement of Cys-6 with Ser-6 generated by site-specific mutagenesis. The production of sufficient quantities of both native and mutant proteins will facilitate future structure–function studies of this enzyme.

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Registry No. DHFR, 9002-03-3; Cys, 52-90-4.

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