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INVESTIGATION OF MUTANTS IN THE ACTIVE SITE AND THE β -BULGE
REGION OF DIHYDROFOLATE REDUCTASE

A Thesis
Presented for the
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Degree
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Guisheng Zhang
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DEDICATION

To my father Zheng Zhang and my mother Rui-lin Ren. To my sister and my brother.

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ABSTRACT

Aspartic acid 27 is conserved in all bacterial dihydrofolate reductases (DHFR) and it serves as a proton donor in the catalytic mechanism (Howell et al., Sci 23:1123). Not surprisingly, the D27S mutant protein has a greatly decreased k_{cat} at pH 7.0. Second site suppressor mutations have been generated in the D27S DHFR gene by genetic selections and site-directed mutagenesis techniques. These mutations partially suppress the effect caused by removal of the proton donor.

To investigate whether these individual suppressing mutations have additive effects, a D27S+E17G+D127G+F137S quadruple mutant gene was constructed. The k_{cat} and K_m (DHF) of the quadruple mutant DHFR are similar to those of the D27S+F137S double mutant, thus an additive effect is not found in the quadruple mutant DHFR.

Additionally, the D27S+T113E+F153S and D27S+T113E+F137S and D27S+T113E+I155N triple mutant DHFR genes were generated to test the relationship between the active site and a β -bulge region. The T113E mutation puts a potential proton donating group in the active site, although at a different position. The triple mutants have different mobilities on a nondenaturing polyacrylamide gel when compared to wild-type and D27S DHFRs, indicating altered conformations. The k_{cat} and K_m values of the D27S+T113E+F153S triple mutant DHFR are

slightly better than those of the D27S+T113E double mutant but they are worse than those of the D27S+F153S double mutant DHFR. The triple mutant DHFR does not show additive effects.

TABLE OF CONTENTS

CHAPTER	PAGE
I. Introduction	1
II. Purification and Kinetic Analysis of a Quadruple Mutant DHFR	9
III. Construction and Overproduction of a Triple Mutant DHFR	16
IV. Purification and Kinetic Analysis of D27S+T113E+F153S DHFR	25
List of References	39
Appendix	44
Vita	68

LIST OF TABLES

TABLES	PAGE
I. A comparison of kinetic parameters for D27S+E17G, D27S+D127G, D27S+F137S and D27S+E17G+D127G+F137S mutant DHFRs at different pH values	65
II. A comparison of kinetic parameters for D27S, D27S+T113E, D27S+F153S and D27S+T113E+F153S mutant DHFRs at different pH values	66
III. Coupling energies of D27S+T113E+F153S triple mutant DHFR at several pH values	67

LIST OF FIGURES

FIGURE	PAGE
1. The carbon backbone structure of <u>E. coli</u> DHFR-methotrexate binary complex	45
2. The crystal structure of the active site and β -bulge region of DHFR	46
3. SDS & native PAGE of purified quadruple mutant DHFR	47
4. pH profiles for the quadruple mutant DHFR	50
5. Oligonucleotide sequences	51
6. D27S+T113E+F137S triple mutant DHFR DNA sequencing gel	52
7. D27S+T113E+F153S triple mutant DHFR DNA sequencing gel	53
8. Western blot of inclusion bodies	54
9. Construction of PT7-5"-WT and PT7-5" mutant DHFR plasmids	55
10. Analysis of the growth curve for D27S+T113E+F153S triple mutant DHFR	57
11. SDS & native PAGE of purified D27S+T113E+F153S triple mutant DHFR	59
12. pH profiles of $\log k_{cat}/K_m$ for D27S, D27S+T113E, D27S+F153S and D27S+T113E+F153S mutant DHFRs	61
13. pH profile of K_m for different mutant DHFRs	62
14. Backbone tracing of the crystal structure for WT DHFR and D27S+F137S double mutant DHFR from <u>E. coli</u>	63

LIST OF ABBREVIATIONS

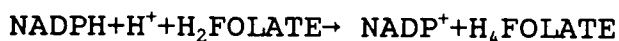
Abs ₂₈₀	absorbance at 280 nm
AMP	ampicillin
D27S	Asp27→ser single mutation
DHF	dihydrofolate
DHFR	dihydrofolate reductase
DNase	deoxyribonuclease
DS	double stranded DNA
<u>E. coli</u>	Escherichia coli
EDTA	Ethylenediaminetetraacetic acid
T113E	Thr113→glu single mutation
F137S	Phe137→ser single mutation
F153S	Phe153→ser single mutation
I155N	Ile155→asn single mutation
IPTG	Isopropyl-1-chloro-3-indolyl-β-D-galactoside
MES	2-[N-Morpholino]ethanesulfonic acid
MET	methionine
MTT	3-[4,5-Dimethylthiazol-2-yl] 2,5-diphenyltetrazolium bromide
MTX	methotrexate
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
RNase	ribonuclease
PAGE	polyacrylamide gel electrophoresis
PSMF	phenylmethanesulfonyl fluoride
PE	50 mM potassium phosphate and 1mM Ethylenediaminetetraacetic acid

P	50 mM potassium phosphate
SS	single stranded DNA
THF	tetrahydrofolate
Trp	tryptophan
TMP	trimethoprim
X-gal	5 Bromo-4-chloro-3-indolyl-B-D-galactoside
dTMP	deoxythymidine5'-monophosphate

CHAPTER I

INTRODUCTION

Dihydrofolate reductase (DHFR, EC 1.5.1.3.) catalyzes the reduction of 7,8-dihydrofolate (DHF) to 5,6,7,8-tetrahydrofolate (THF) using the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor.



DHFR is found in almost all organisms as it plays a vital role in the metabolism of proliferating cells (Blakley, 1984). It is not only required for the maintenance of adequate levels of fully reduced folate but also when coupled with thymidylate synthetase, produces dTMP, a precursor in DNA synthesis. The link between its biological role and clinical usefulness of its inhibitors in treatment of a variety of diseases such as certain cancers and bacterial infections (Roth & Cheng, 1982; Jolviet et al., 1983; Hitchings & Baccanari, 1984) has made DHFR the most thoroughly studied enzyme in folate metabolism. In addition, DHFR has served as a model for investigating broad questions concerning the relationship between molecular structure and functional properties in enzymes (Kraut & Matthews, 1987). DHFR is a monomer with molecular weight range 18,000-22,000 daltons. The Michaelis constant for DHF binding to DHFR ranges from 0.2

to 24 μ M depending on the tissues or organisms (Blakley, 1984). The specific activity of the enzyme reported also varies from less than 1 μ mole to more than 200 μ mole of DHF reduced per minute per mg of protein. It has been reported that DHFR from many sources increases its activity in the presence of certain agents including salts, urea and guanidine that are known to unfold or disrupt hydrophobic, hydrogen and ionic bonds, and also reagents that react with thiol groups such as mercurial, 5,5,-dithiobis-(2-nitrobenzoic acid) and iodine. Both types of reagents are believed to produce activating effects by causing changes in the conformation of the enzyme (Blakley, 1984). The current understanding of DHFR comes from studies of DHFR from perspectives of pharmacology and medicinal chemistry. DHFR is the target of a few commonly used, very potent anticancer and antibacterial reagents such as methotrexate (MTX), trimethoprim (TMP) and pyrimethamine (Hitchings & Baccanari, 1984). Some inhibitors of DHFR demonstrate marked species selectivity, i.e., they tend to inhibit DHFRs from some species more effectively than others. The most notable example is trimethoprim (TMP). Unlike the folate analog methotrexate (MTX) (a universal inhibitor of chromosomal DHFRs), TMP selectively inhibits bacterial DHFRs (Kraut & Matthews, 1987). In basic research, MTX is commonly used in the process of DHFR purification either as an enzyme stabilizer or the specific ligand for DHFR in affinity-based purification.

The complete sequences of DHFR from three bacterial, one avian and three mammalian sources were compared and much greater homology was observed between DHFRs from vertebrate sources than between bacterial DHFR. In the seven sequences compared, there are 18 invariant residues and in addition many changes are highly conservative (e.g. Ser→Thr, Val→Ile, Asp→Glu, Ser→Gly). Greater homology is observed in the amino terminal region than in the C-terminal (Blakley, 1984).

X-ray structural studies of DHFR were first carried out on crystals of the E. coli enzyme containing bound MTX (Matthews et al., 1977). Shortly thereafter crystallographic results were reported for a L. casei DHFR ternary complex with MTX and NADPH (Matthews et al., 1978, 1979) and later the structure of the ternary complex of chicken liver DHFR with NADPH and a methoxyphenyltriazine inhibitor was solved (Volz et al., 1982). Recently Bystroff and Kraut (1990, 1991) have determined the crystal structure of the E. coli apo enzyme, a binary complex with NADP⁺ and a Folate·NADP⁺ ternary complex. Overall backbone folding is very similar between bacterial and vertebrate DHFRs, even though the sequence identity is only about 25%. The overall structure consists of an eight-stranded β -sheet, four α -helices and loops that join the elements of secondary structure (Fig 1) (all figures appear in the Appendix). The active site is a pronounced cavity some 15 Å deep cutting across one face of the enzyme and is lined by hydrophobic side chains, a clear indication that much of the

binding of substrates and inhibitors must be due to hydrophobic and van der Waals interactions. The only polar side chain in this cavity is Asp27 in the case of bacterial enzymes, or Glu 30 in the case of vertebrate DHFR (Volz et al., 1982).

NADPH binds to DHFR in an extended conformation and occupies a shallow groove that stretches across the terminal ends of five strands of β -sheet. Adenine binds in a hydrophobic pocket in van der Waals contact with five residues. The nicotinamide ring is inserted through the entrance of the major hydrophobic cavity where it makes hydrophobic and hydrogen-bonding interactions with residues on the bottom and sides of the cavity. The pyrophosphate bridge of NADPH leads around and over the edge of the sheets, there are numerous hydrogen bonds between pyrophosphate oxygen atoms and backbone amido groups and side chains of residues at the amino terminal ends of two α -helices (α C and α F) (Kraut & Matthews, 1987).

MTX binds to the enzyme in a open conformation with the pteridine ring nearly perpendicular to the benzoyl ring. The pyrimidine edge of the pteridine ring is deeply buried in the active site cavity, but the β -aminobenzoylglutamate side chain extends out of the cavity to the surface, and the glutamate fragment is draped across α B. The α -carboxylate is positioned to interact with Arg-57 (Blakley, 1984).

Based on X-ray structure, NMR analysis (Blakley et al., 1978) and kinetic measurements (Stone & Morrison, 1984 a,b; Fierke et al., 1987; Penner & Frieden, 1987), a reaction mechanism for DHFR has been proposed. DHF binding in the active site differs from MTX, such that the pteridine ring is rotated 180°. The pteridine ring of bound DHF is thought to be activated at C6 for hydride transfer by protonation at N5. The proton donor, Asp27, uses an intermediate water molecule, #403, to deliver the proton (Howell et al., 1986). Hydride transfer then occurs from C4 of the A face (re) of NADPH to C6 of the (si) face of the protonated pteridine ring of dihydrofolate.

To test the essential function of conserved amino acids of DHFR to further our understanding of DHFR, a series of oligonucleotide directed mutagenesis experiments have been conducted (Villafranca et al., 1983; Howell et al., 1986; Howell, personal communication). The mutants obtained include Trp22→His and Phe, Asp27→Asn and Ser, Thr46→Val, Ser63→Glu and Gly95→Ala and Val, Phe137→Ser, Phe153→Ser, Ile155→Asn. The mutants were all inactive or partially active, suggesting important functional or structural roles for these amino acids.

The Asp27 mutants (D27N, D27S) have drawn much more attention than others. The Asp27 residue is conserved in all bacteria DHFR and the carboxylic acid of Asp27 is proposed to serve as the proton donor in the catalytic mechanism by

activating the pteridine ring of dihydrofolate for hydride transfer. So it is not surprising that the mutant D27S and D27N proteins have greatly decreased activity at pH 7.0 (Howell et al., 1986). Surprisingly the efficiency of these two mutant proteins increases rapidly with decreasing pH. At pH 5.0, the k_{cat}/K_m of the D27S mutant protein is 18 fold higher than at pH 7.0. Therefore it has been proposed that productive binding of protonated DHF (pK_a N5 DHF = 3.84, Poe 1977) allows catalysis in the mutant enzymes. This data suggests that protonated DHF can act as a substrate in the mutant enzyme because it has already been protonated at N5 in solution. Accordingly, the proton donor in WT enzyme (Asp27) can be replaced by protonated DHF in the D27N, D27S mutant DHFRs.

Based on the information derived from the characterization of D27N and D27S mutant DHFRs, second site revertants of DHFR which could increase the catalytic efficiency were genetically selected and also rationally designed. A second site revertant obtained from the Ser27 (D27S) mutant DHFR gene using trimethoprim selection was a mutation at Phe 137 to Ser (D27S+F137S) (Howell et al., 1990). This mutation increases k_{cat} (pH 7.0) by a factor of 3 and decreases K_m by a factor of 5 compared to the parent D27S single mutant. Therefore the overall effect is to increase the efficiency (k_{cat}/K_m DHF) of the D27S DHFR by a factor of 15. The mutation does not affect the shape of the pH profile.

At pH 4.5, the efficiency of the double mutant enzyme is decreased only 30 fold when compared to the wild type DHFR and increased 78 fold when compared to D27N. Therefore after only two genetic selection steps from the D27N mutant gene, an enzyme has been evolved (D27S+F137S) capable of fairly efficient catalysis at low pH. The effect of the F137S mutation is surprising because it is approximately 15 Å away from the D27S mutation and therefore would not have been predicted to affect the kinetics, Phe 137 is also associated with a β -bulge structure (Howell et al., 1980). The function of the β -bulge is not clear. This structure disrupts the normal antiparallel hydrogen bonding pattern in β -sheets. It also imparts a right-handed twist to the strand. β -bulges are often found in enzyme active sites due to their ability to specifically orient backbone and side chain residues. The β -bulge involving the D27S+F137S mutant DHFR is a classic β -bulge involving Val136, Phe137 and Ile155 residues (Volz et al., 1982).

Another double mutant DHFR produced was D27S+T113E. Glu was introduced to replace Thr 113 in order to evaluate whether the stereochemistry of proton donation in the DHFR active site could be altered. It was found in this double mutant that the enzyme has three-fold greater k_{cat} and two fold higher K_m (DHF) at pH 7.0 as compared to the single mutant D27S enzyme. Of special interest, the D27S+T113E double mutant enzyme appears to donate a proton to bound DHF as a new pKa appears in the pH

profile (Howell et al., 1987). The results imply there are at least two different proton donation routes for activation of the pteridine ring of DHF for hydride transfer and that the two routes have different efficiencies.

The significance of these results is that they have contributed new knowledge concerning the mechanism of action of DHFR which could not have been predicted based on knowledge of active site residues identified by the X-ray structure. Also these results represent a "second generation" research effort aimed at restoration of activity to an inactive/impaired enzyme. Therefore the purpose of this thesis project was to test the effect of adding these mutations in the active-site and β -bulges region (Fig 2) on catalysis. We hoped to further restore the activity towards WT DHFR levels.

CHAPTER II

PURIFICATION AND KINETIC ANALYSIS OF A QUADRUPLE MUTANT DHFR

A. Introduction

When using resistance to TMP as a genetic selection, three mutants of the D27S DHFR gene were found, i.e. D27S+E17G, D27S+F137S and D27S+D127G. Glutamic acid 17 is on the edge of the active site. Aspartic acid 127 is distant from the active site, and Phe 137 is a member of the β -bulge mentioned previously. The D27S+E17G double mutant DHFR decreases K_m (DHF) by a factor of 2 when compared to the parent D27S DHFR mutant. The D27S+D127G mutant DHFR increases k_{cat} by a factor of 2 when compared to D27S DHFR. And the D27S+F137S mutant DHFR increases k_{cat} by a factor of 2-3 and decreases K_m by a factor of 2-3. Mutations are potentially additive if they do not change the conformation of a protein or if the mutations increase activity by different mechanisms. Because the F137S, E17G and D127G suppressing mutations may work by different mechanisms and are spread across the DHFR structure and these individual mutations give positive results on the mutant enzyme activity, they might give additive effects if they are put together. To test this hypothesis, the D27S+E17G+D127G+F137S quadruple mutant DHFR gene was

previously constructed. In this thesis a kinetic analysis of this mutant DHFR is reported.

B. Materials and Methods

1. Purification of D27S+F17G+D127G+F137S DHFR

(a) Media, clones, columns and gels

TB media, in which bacteria were grown, contains 12g/L Bacto-tryptone, 24 g/L Bacto yeast extract, 0.4% v/v glycerol, 2.3 g/L potassium phosphate monobasic and 12.5 g/L potassium phosphate dibasic.

A gene encoding D27S+E17G+D127G+F137S mutant DHFR was made available in our lab as a 1100 base pair BamHI fragment cloned into pUC8 and expressed by a mutant (up) promoter for DHFR (constitutive).

A QAE-Zeta Prep 15 disk (LKB), DEAE-Fractogel column and Sephadex G-75 (Villafranca et al., 1983) column were used according to directions supplied by the manufacturer (Pharmacia Fine Chemicals). FPLC using a Mono Q column was performed according to the directions of the manufacturer (Pharmacia Fine Chemicals).

Non-denaturing and denaturing polyacrylamide gels were carried out using standard protocols (Smith, 1989). Proteins were stained with Coomassie brilliant blue (Sasse, 1989), and an activity stain using MTT (3-(4,5-Dimethylthiazol-2-yl) 2,5 diphenylterazolium bromide) dye (Hiebert, et al., 1972).

(b) Quadruple mutant DHFR purification

A fol minus strain of E. coli (Δ fol::kan) expressing the pUC8 plasmid carrying the quadruple mutant DHFR gene was cultured overnight at 30 °C in 20 ml of TB media containing 20 ug/ml trimethoprim (TMP), 200 ug/ml ampicillin (AMP) and 200 ug/ml kanamycin. 12 liters of TB media containing 20ug/ml TMP, 200 ug/ml AMP and 200 ug/ml kanamycin were innoculated with 12 ml of the overnight culture. Liter cultures were incubated at 30 °C with shaking until a ten-fold dilution attained an absorbance at 660 nm (Abs660) of approximately 0.36. This typically took 3 days. Cells were lysed by sonication, the soluble lysate was applied to a QAE-Zeta Prep 15 disk, washed with PE buffer (50mM potassium phosphate, 1mM EDTA pH 8.0) and eluted with the same buffer containing 0.75 M KCl. The partially purified lysate was dialyzed overnight against PE buffer. The mutant DHFR was applied to an DEAE column (30cm x 3.5cm). Proteins were eluted using a 0-0.8 M KCl gradient. Fractions were analyzed using activity and Coomassie blue stains of nondenaturing polyacrylamide gels, selected fractions were pooled, concentrated and applied to a Sephadex G-75 column (88cm x 3.5cm) that had previously been equilibrated with PE buffer. Fractions were eluted using PE buffer and analyzed using denaturing polyacrylamide gels. Selected fractions were pooled and concentrated, and applied to an FPLC Mono Q column that had previously been equilibrated with P (50 mM potassium phosphate pH 8.0) buffer.

Fractions were eluted using 0-1 M KCl salt gradient and again analyzed using activity and Coomassie blue stains of nondenaturing polyacrylamide gels. Selected fractions were pooled and concentrated.

The concentration of the mutant DHFR was determined by MTX titration. Quenching of the DHFR fluorescence was performed as described by Cayley et al., (1981). Fluorescence (excitation 290 nm, emission 340 nm) was monitored in a Perkin-Elmer LS-5B fluorometer. Quenching of the fluorescence of 1 μ M DHFR in a 2-ml solution (in 50 μ M acetic acid + 50 μ M MES +100 μ M Tris +10 mM β -mercaptoethanol polybuffer, pH 7.0 Ellis & Morrison, 1982) was observed by the addition of small (2.5 μ l) volumes of a concentrated ligand solution. Effects due to absorption of light by the ligand were assessed by parallel titration of tryptophan control solutions. The data were analyzed by using a nonlinear least-squares fitting program (Dunn et al., 1978). Purified quadruple mutant DHFR was extensively dialyzed against deionized water and lyophilized for storage at 4°C.

2. Kinetic analysis of the quadruple mutant DHFR

(a) Ligands

7,8- Dihydrofolate (DHF) was synthesized from folic acid using the method of Blakley, (1961) and stored at -70°C in 5 mM HCl and 50 mM β -mercaptoethanol. The reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) was

purchased from Sigma Chemical Company. The concentrations of ligands were determined spectrophotometrically using the following molar extinction coefficients: DHF, $28,000 \text{ M}^{-1} \text{ cm}^{-1}$ at 282 nm (Blakley, 1961) and NADPH, $6220 \text{ M}^{-1} \text{ cm}^{-1}$ (Penner M. et al., 1987) MTX, $22,100 \text{ M}^{-1} \text{ cm}^{-1}$ at 302 nm pH 13 and $23,250 \text{ M}^{-1} \text{ cm}^{-1}$ at 258 nm pH 13 (Stone & Morrison 1986). The molar extinction coefficient for dihydrofolate utilization by DHFR was $12300 \text{ M}^{-1} \text{ cm}^{-1}$ at 340nm. (Baccanari et al., 1975).

(b) Kinetics

Steady-state kinetic data were obtained on a Perkin-Elmer lambda 3a spectrophotometer linked to a IBM PC (Howell et al., 1986; Hood & Robert 1987; Stone & Morrison, 1983). Rates were measured by a change in absorbance at 340 nm. Initial rates were calculated using the Softways' UVS program. All assays were performed at 30°C in a polybuffer containing 44 μM imidazole, 33 μM succinic acid, 44 μM diethanolamine and 10 mM β -mercaptoethanol. This buffering system maintains a constant ionic strength between pH 4.5 and 9.5 (Ellis & Morrison, 1982).

The ligand ranges were 20 μM to 120 μM for DHF while NADPH concentration was maintained at a saturating concentration (30-50 μM). Values for k_{cat} and K_m (DHF) were calculated using a nonlinear regression analysis of the Michaelis-Menten equation using Elsevier's Enzfitter program. For the calculation of K_m (DHF) and k_{cat} , the enzyme was first

preincubated with a saturating concentration of NADPH. Assays were initiated by adding 10 ul of enzyme-NADPH solution to a cuvette containing 610 ul buffer plus DHF. The reference cuvette contained the substrate and cofactor and served to subtract any background rate that might be present due to decomposition of the cofactor, which is a source of error at lower pH values (Hillcoat et al., 1967).

C. Results and Discussion

Fig. 3 A shows a denaturing polyacrylamide gel of purified quadruple mutant DHFR. The Abs 280/260 ratio was >1.5. The yields of the quadruple mutant protein were 16-21 mg of protein /liter of E. coli culture. There was a small contaminating band in the preparation. Fig. 3 B and C are nondenaturing polyacrylamide gels with Coomassie blue and activity stains. They indicated the contaminating band is not E. coli chromosomal DHFR.

Kinetic analysis of the quadruple mutant DHFR has been performed. The goal of this study was to see if the mutations were additive. Initial work therefore focused upon determining the k_{cat} and K_m for DHF at pH 7.0 by first saturating the enzyme with NADPH and then measuring the rate of the reaction at varying concentrations of DHF. Values determined for k_{cat} , K_m (DHF) using this method at different pH values, are shown in Table I (all tables appear in the

Appendix). pH profiles for the D27S+E17G, D27S+D127G, D27S+F137S and quadruple mutant DHFRs are shown in Fig. 4 for comparison. The K_d (MTX) of quadruple mutant DHFR is $0.16 \mu\text{M}$ and that of D27S single mutant is $0.2 \mu\text{M}$.

Based on these results, the quadruple mutant DHFR does not show an additive effect. The k_{cat}/K_m of the quadruple mutant DHFR is better than the D27S+E17G, D27S+D127G double mutants, but it is the same as the D27S+F137S double mutant DHFR. The hypothesis of the construction of the quadruple mutant DHFR is based on the supposition that the conformation of the mutant DHFRs is not changed by these individual mutations. Fig. 3 B and C show clearly that the quadruple mutant enzyme migrates differently from WT and D27S single mutant DHFR. The different migration on the native gel compared to the wild type is due to the charge difference as well as to potential conformational changes of the enzyme introduced by the quadruple mutation. On the other hand, the nonadditivity associated with the quadruple mutant strongly suggests that differential conformational changes are associated with the mutant proteins. We note previous studies on single-and double-mutant tyrosine-tRNA synthetase have suggested that nonadditive effects occur when protein conformations are altered (Carter et al., 1984).

CHAPTER III

CONSTRUCTION AND OVERPRODUCTION OF A TRIPLE MUTANT DHFR

A. Introduction

The T113E mutation in the D27S+T113E DHFR is proposed to restore a proton donor to the DHFR active site. A β -bulge region at residues 136, 137 and 155 is far from the active site area but several mutations (F137S, F153S, I155N) have been found to increase the activity of the D27S mutant enzyme. The F137S mutation is proposed to work by a conformational change. A crystal structure of the D27S+F137S DHFR binary complex with MTX shows Trp 30 flips into the active site and water #403 is lost. Since F153S and I155N also flank W30 they might work in a similar fashion to increase the activity of D27S DHFR. Because individual double mutants increase the catalytic activity of D27S DHFR and they may work by different mechanisms, we are interested in whether any combination of these mutations will have an additive effect on enzyme activity. Since the expression level of a triple mutant DHFR (D27S+T113E+F153S) was low, a T7 polymerase promoter system was used as a means to increase protein expression.

B. Material and Methods

1. Construction of D27S+T113E+F137S, D27S+T113E+F153S and D27S+T113E+I155N triple mutant DHFR genes

(a) Bacteria, viruses, plasmids and media

Plasmid 101-4 WT DHFR-pUC8 (in E. coli strain SK383) and E. coli strains JM 107, E. coli 7-27(Δ fol::kan) and RZ 1032 ung dut strains were already available in our lab. The single-stranded form of the mutant D27S+T113E DHFR gene inserted in M13mp8 was available in our lab. YT media consists of 8 g/L Bacto-tryptone, 5 g/L Bactoyeast extract, and 5 g/L NaCl. YT agar plates with antibiotics were prepared as described by Lech and Brent (1989). A few drops of concentrated acetic acid were added to solutions containing trimethoprim (TMP) in order to get the antibiotic to dissolve. Agar plates containing X-gal (5 Bromo-4-chloro-3-indolyl-B-D-galac-toside) (80 ug/ml) and IPTG(Isopropyl-1-Thio-B-D-galactoside) were prepared (Lech & Brent., 1989).

(b) Enzymes and oligonucleotides

Restriction enzymes, calf-intestinal phosphatase, T4 polynucleotide kinase and T4 DNA ligase were purchased from Pharmacia or Promega and used as directed by the supplier. Oligonucleotides (Fig. 5) were synthesized and purified by the molecular biology resource facility at the University of Tennessee.

(c) Mutagenesis

RZ1032 ung dut cells were infected by an M13mp8 virus containing the mutant D27S+T113E DHFR gene. The single stranded DNA was purified (Treco., 1989). Three different oligonucleotides designed to change Phe 137 (TTC codon) to Ser (TCC), Phe 153 (TTT codon) to Ser (TCT) and Ile 155 (ATT codon) to Asn (AAT) were annealed separately. Mutagenesis was performed as per standard protocols (Smith, 1982; Zoller & Smith, 1982). The synthesized double stranded DNA was transfected into E. coli JM107 cells. Individual plaques were picked and grown. The three triple mutants were identified by dot blot hybridization techniques, using ³²P labeled oligos. The entire DHFR gene were sequenced by the Sanger dideoxy method (Sanger et al., 1977, 1980) to ascertain that no additional mutations had occurred.

(d) Cloning of these triple mutant DHFR genes into pUC8

To increase protein expression, the mutant DHFR genes were cloned from M13mp8 into pUC8. These triple mutant DHFR genes in M13mp8 and pUC8 were digested with SalI restriction endonuclease. Individual fragments were obtained via fragment elution following 6% polyacrylamide gel electrophoresis. The excised SalI fragment of pUC8 plasmid was dephosphorylated using calf intestinal phosphatase. The fragments were ligated and transformed into E. coli JM107. Colonies were screened for their ability to grow on YT agar plates containing

antibiotics (200 ug/ml ampicillin and 20 ug/ml trimethoprim). Colonies containing the different triple mutant DHFR genes were identified respectively. Then the DHFR genes were transformed into a fol minus strain of E. coli Δ fol::kan (7-27). The chromosomal DHFR gene has been deleted in this strain and replaced by a kanamycin resistance gene (Howell et al., 1988). This expression system allows purification of mutant DHFRs that are not contaminated by WT DHFR due to expression of the E. coli chromosomal gene.

(e) Purification of D27S+T113E+F153S triple mutant DHFR

Materials and method are same as that of quadruple mutant DHFR (see chapter II, section b, subsection b).

2. Cloning of the D27S+T113E+F153S mutant DHFR gene into a T7RNA polymerase promoter system

(a) Bacteria, Plasmids, and Media

Plasmid pGP1-2 and PT7-5 were from Stan Tabor (Harvard Medical School). E. coli strain K38 was from Stan Tabor.

(b) Enzymes

SmaI, HindIII, SnaBI restriction enzymes and T4 DNA ligase were purchased from Pharmacia or Promega and used as directed by the supplier.

(c) Construction of PT7-5"-WT and PT-7-5"-MUTANT DHFR Plasmid

Vector PT7-5 was digested by SmaI and HindIII. Plasmid pUC8 which contain WT DHFR gene and the D27S+T113E+F153S triple mutant DHFR gene were digested by SnaBI and HindIII respectively. Polyacrylamide gel electrophoresis of DNA, (Chory, 1989) fragment elution (Ibid. Section 2.7) transformations (Seidman, 1989) were all achieved using previously described protocols. The newly constructed PT7-5" plasmid was transformed into E. coli strain K38 containing the pGP1-2 plasmid. Colonies were screened for their ability to grow on YT agar plates containing antibiotics (50ug/ml ampicillin, 20ug/ml trimethoprim and 50ug/ml kanamycin). The insertion size of PT7-5"-mutant DHFR plasmid was checked on an 1% agrose gel.

3. Expression of the D27S+T113E+F153S triple mutant DHFR gene

(a) Bacteria, Media

Plasmid PT7-5" was constructed as described above. Plasmid pGP1-2 and E. coli strain K38 were obtained from Stan Tabor. Lysozyme and deoxyribonuclease (DNase) were purchased from Sigma Chemical Company.

(b) Induction of the D27S+T113E+F153S triple mutant DHFR gene and WT DHFR gene

E. coli strain K38 carrying pGP1-2 and PT7-5"-MUTANT DHFR and PT7-5"-WT DHFR were cultured overnight at 30°C in 10 ml YT respectively. The culture was diluted 1:40 into fresh

YT medium plus 50ug/ml ampicillin and 50 ug/ml kanamycin and grown several hours at 30°C to an O.D.₅₉₀=0.4. The culture was transferred into a 42°C water bath for 30 mins. The gene for T7 RNA polymerase was induced by raising the temperature to 42°C. Then the cells were grown at 37°C for 30 mins with shaking (Tabor, 1990), and the DHFR inhibitor, TMP, was added at concentrations of 20ug/ml to stabilize the mutant DHFR protein.

(c) Cell lysis

Cells were harvested 90 minutes after induction. Cell pellets were resuspended in 75 ul of lysis buffer (3.3 mg/ml lysozyme, 24 mM Tris-HCl pH 8.0, 0.8 mg/ml EDTA) and allowed to sit at 4°C for twenty minutes. 15 ul of 5% Brij 58 was added. The resulting solution was vortexed, and allowed to sit at 4°C for twenty minutes. 10 ul of DNase solution (0.67 mM MgCl₂, 5 mg/ml DNase) was then added. The solution was vortexed, and allowed to sit at 4°C for twenty minutes. Extracts were separated from cell debris by spinning (13,000 rpm) in an epifuge for 15 minutes at 4°C.

(d) Polyacrylamide gel electrophoretic analysis of protein samples

Nondenaturing gel electrophoresis was performed according to previously described protocols (Smith, 1989). Proteins separated by gel electrophoresis were examined using an MTT

activity stain (Hiebert et al., 1972), or Coomassie brilliant blue protein stain (Sasse, 1989).

C. Results

Identification of triple mutant DHFR genes. The D27S+T113E+F137S and D27S+T113E+F153S triple mutant DHFR genes were identified by dot blot hybridization techniques and the entire DHFR gene were sequenced by the Sanger dideoxy method (Fig. 6 and Fig. 7) to ascertain that no additional mutations had occurred. The mutagenesis yields were about 2%, in contrast to normal yields of about 50% using ung dut E. coli protocols (supplied by Bio Bad).

Physical characterization of these triple mutant proteins. The amount of D27S+T113E+F137S and D27S+T113E+F153S mutant protein produced from E. coli containing these genes cloned in pUC8 was found to be very greatly reduced when compared to WT DHFR protein production under similar conditions. This behavior is also seen in the parent D27S+T113E double mutant DHFR. While typical yields of WT and D27S DHFR are 8-25 mg of protein /L of E. coli culture, the yield of D27S+T113E is 0.4-2.5 mg/L (Howell et al., 1987). The amount of the triple mutant enzyme obtained was small even though purified by the same protocol and enzyme activity could be monitored during the purification process. One of the possible mechanisms for reduction of mutant enzyme production

is that the enzyme was quickly digested by proteases due to a decreased stability. To test this hypothesis, we have included TMP (5uM) in culture medium and all buffers used in purification process, in addition to the protease inhibitor (phenylmethylsulfonyl fluoride). Unfortunately, still not enough mutant enzyme for kinetic measurement was obtained from 12 liters E. coli culture. Furthermore, the purity of the enzyme was not satisfactory (data not shown).

Another possibility associated with low enzyme production might have been due to the accumulation of protein in inclusion bodies. Inclusion bodies are commonly found in E. coli cells when recombinant proteins are expressed. In order to check for formation of inclusion bodies in our system, cells were observed under a phase contrast microscope (800 x). Approximately 30% of the cells were found to bear inclusion bodies at log phase. The inclusion bodies were then purified (Glover, 1987) and protein was checked by Western blot analysis (Fig. 8) (Haelow & Lane, 1988). The gel shows that DHFR was not found in the inclusion bodies. The antibody against WT DHFR was generated by a standard immunization protocol using rabbits (Harlow & David Lane 1988). A third possibility for the low production of triple mutant DHFR may be because the mutant gene needs a strong promoter. The T7 RNA polymerase promoter system, which can transcribe mRNA several times without stopping, was therefore used.

Vector construction. The vectors PT7-5"- WT (WT DHFR gene was put in this system as a positive control) and PT7-5"- MUTANT (Fig. 9) were generated by either SnaBI-HindIII or SmaII- HindIII restriction cuts. The size of PT7-5"-MUTANT vector was checked on an 1% agarose gel (data not shown). The DNA possessing the correct size insertion were used for protein expression.

Protein expression curves. The WT and D27S+T113E+F153S protein growth curve were performed in 1 Liter YT medium. After induction, the samples were collected at different times. Fig. 10 is a polyacrylamide gel with both activity and Coomassie blue stain. It indicates WT DHFR was produced very well and the triple mutant was expressed reasonably well. Further a good harvesting time would be less than 8 hours after induction.

CHAPTER IV

PURIFICATION AND KINETIC ANALYSIS OF D27S+T113E+F153S DHFR

In the previous chapter, the construction and overproduction of the D27S+T113E+F153S triple mutant DHFR were described. In this chapter, purification and kinetic analysis of the triple mutant enzyme will be described.

A. Materials and Methods

(a) Columns, enzymes, gels, ligands

Chromatography on DEAE and Sephadex G-75 columns were carried out according to the directions of the manufacturer (Pharmacia Fine Chemicals). A MTX affinity column was used to purify the mutant protein (Baccanari et al., 1981). Deoxyribonuclease (DNase), ribonuclease (RNase) and trimethoprim (TMP) were purchased from Sigma Chemical Company. Phenylmethylsulfonyl fluoride (PMSF) was purchased from Boehringer Mannheim Biochemicals. Non-denaturing polyacrylamide gels with activity and Coomassie Blue stains were performed as previously described.

7,8-Dihydrofolate (DHF) was synthesized from folic acid using the method of Blakley (1961) and stored at -70°C in 5 mM HCl and 50 mM β -mercaptoethanol. The reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) was

purchased from Sigma Chemical Company. The concentrations of ligands were determined spectrophotometrically using the following molar extinction coefficients: DHF, $28,000 \text{ M}^{-1} \text{ cm}^{-1}$ at 282 nm (Blakely 1961) and NADPH, $6220 \text{ M}^{-1} \text{ cm}^{-1}$ at 340 nm (Penner et al., 1987), MTX, $22,100 \text{ M}^{-1} \text{ cm}^{-1}$ at 302nm pH 13 and $23,250 \text{ M}^{-1} \text{ cm}^{-1}$ at 258 nm pH 13 (Stone & Morrison, 1986) respectively.

(b) Purification of D27S+T113E+F153S mutant DHFR protein

24 liters K38/pGP1-2/ PT7-5"-MUTANT DHFR culture were grown overnight at 30°C. The induction of the triple mutant DHFR was the same as previously described (see Chapter III, section B, subsection c) except the cells were harvested at 7 hours after their induction. The triple mutant enzyme was first subjected to ammonium sulfate precipitation. The partially purified lysate was then applied to a Sephadex G-75 column (88cm x 3.5cm) that had previously been equilibrated with PE (50 mM potassium phosphate and 1 mM EDTA pH 8.0) buffer. Fractions were eluted using PE buffer and analyzed using polyacrymide gels with activity and Coomassie blue stains. Selected fractions were pooled and applied to a DEAE column (30 cm x 3.5cm) that had been rinsed with PE buffer.

Proteins were eluted using a 0-0.8 M KCl gradient. Fractions were again analyzed using polyacrylamide gels with activity and Coomassie blue stains. Selected fractions were pooled, concentrated and dialyzed against PE buffer. The

sample was next applied to a MTX affinity column (2cm x 0.6cm). Fractions were eluted with a 0-0.5 M KCl gradient and analyzed on polyacrylamide gels. The purified triple mutant protein was stored as an ammonium sulfate precipitate.

The concentration of mutant DHFR was determined by MTX titration. Quenching of the DHFR fluorescence was performed as described by Cayley et al., (1981). Tryptophan fluorescence (excitation 290 nm, emission 340 nm) was monitored in a Perkin-Elmer LS-5B fluorometer. Quenching of the fluorescence of a 2-ml solution (1 μ M DHFR in 50 μ M acetic acid + 50 μ M MES + 100 μ M Tris + 10 mM β -mercaptoethanol polybuffer, pH 7.0) (Ellis & Morrison, 1982) was observed by the addition of small (2.5 μ l) volumes of a concentrated ligand solution. Effects due to absorption of light by the ligand were assessed by parallel titration of tryptophan control solutions. The data was analyzed by a nonlinear fit to the following equation (Dunn, et al., 1978).

$$F = F(L)L_T + F(E)E_T + (F(C) - F(L) - F(E))[C]$$

$$\text{given that } [C] = B - \text{SQRT}(B^2 - E_T L_T) \quad \text{and}$$

$$B = (L_T + E_T + K_D) / 2$$

where F is the observed fluorescence, F(L), F(E), and F(C), are the fluorescence yields per unit concentration of ligand, enzyme and complex, respectively, L_T is the total concentration of ligand, E_T is the total concentration of enzyme, [C] the concentration of enzyme-ligand complex, and K_D the ligand dissociation constant.

B. Kinetic Analysis of the Triple Mutant DHFR

Steady-state kinetic data were obtained on a Perkin-Elmer lambda 3a spectrophotometer linked to a IBM PC computer. (Howell et al., 1986; Hood & Robert 1978; Stone & Morrison 1983). Rates were measured as the change in Abs 340. Initial rates were calculated using the UVS softways program. All assays were performed at 30°C in a polybuffer containing 44 μ M imidazole, 33 μ M succinic acid, 44 μ M diethanolamine and 10 mM β -mercaptoethanol. This buffering system maintains a constant ionic strength between pH 4.5 and 9.5 (Ellis & Morrison, 1982).

The ligand ranges utilized were 20-120 μ M for DHF and 30-80 μ M for NADPH. Values for k_{act} and K_m were calculated by nonlinear regression of the Michaelis Menten equation (Enzfitter program). For the calculation of K_m (DHF) and k_{cat} (DHF), the enzyme was first preincubated with a saturating concentration of NADPH. Assays were initiated by adding 10 μ l of enzyme-NADPH solution to a cuvette containing 610 μ l buffer and DHF. The reference cuvette contained the substrate and cofactor and served to subtract any background rate that might be present due to decomposition of the cofactor, which is a source of error at lower pH values (Hillcoat et al., 1967).

C. Results and Discussion

Protein purification. During the protein purification process, DNase (15 ug/ml) and RNase (20 ug/ml) were added to remove unusually large DNA and RNA contamination. Protease inhibitor, PMSF, (0.05-1 μ M) was added to protect mutant protein from protease digestion. The DHFR inhibitor trimethoprim (TMP 5 μ M) was added to stabilize the triple mutant protein. Bound TMP was subsequently removed by passing the triple mutant protein over a methotrexate affinity column. This affinity chromatography step also removes any contaminating wild type DHFR which may be present due to expression of the E. coli chromosomal DHFR gene in strain K38. While the mutant DHFR is eluted by a 0.5 M KCl wash, elution of WT DHFR requires 1 M KCl and 2 μ M folate at pH 9.0.

The possibility of WT DHFR contamination is low. Some separation of WT DHFR and triple mutant DHFR can be done on a DEAE column due to charge difference between WT and triple mutant enzymes. These enzymes can be separated by repetitive loading and elution on an FPLC Mono Q column (data not shown). This separation, however, is not optimized with respect to time, so a MTX affinity column was used to remove any remaining WT DHFR and any bound TMP. At this point the triple mutant enzyme is free of WT DHFR contamination. The enzyme purity was checked by electrophoresis (Fig. 11). The major band on the gel corresponded to the mutant enzyme. There are

other bands on the protein gel, but the relative quantities are small. The yield of the triple mutant protein is about 0.2-0.5mg/Liter.

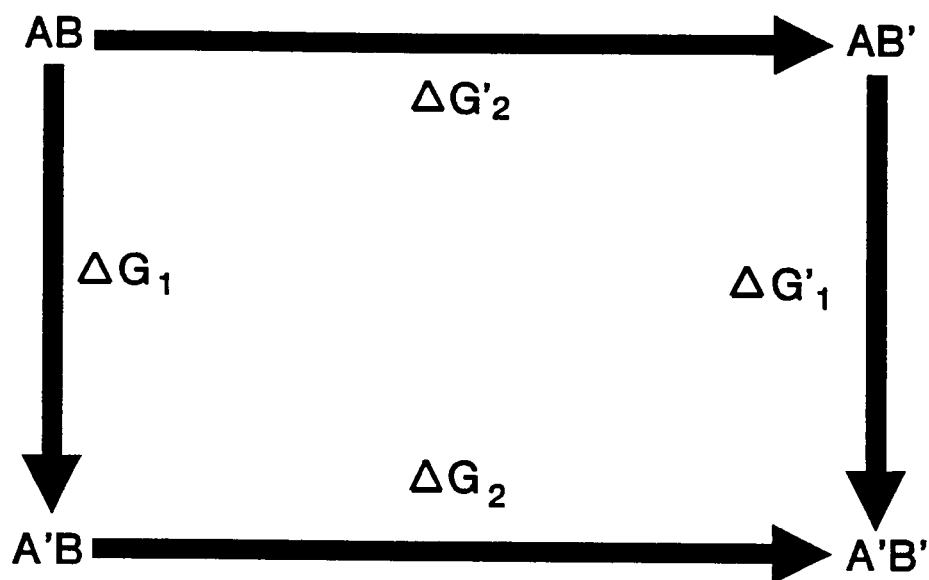
Kinetic analysis. The concentration of enzyme was determined by fluorescence titration using MTX. This is an active site titration procedure which allows us to calculate the concentration of active DHFR. The K_d of MTX was also determined by this method. The K_d of this triple mutant enzyme is 0.4-0.56 μM . It is higher than the K_d associated with other mutant enzymes (the K_d for D27S is 0.12-0.2 μM , the K_d for the D27S+F153S is 0.1-0.3 μM).

Steady-state kinetic parameters obtained for the D27S+T113E+F153S DHFR at pH 7.0, 30° C, were $k_{\text{cat}} = 1.48 \text{ s}^{-1}$ K_m (DHF)=77.18 μM . These values may be compared with kinetic parameters previously obtained for D27S, D27S+T113E and D27S+F153S DHFRs, listed in Table II. A 3-fold increase in both k_{cat} and k_{cat}/K_m which is the second-order rate constant describing the rate of interaction of dihydrofolate with the binary enzyme-cofactor complex is seen for the triple mutant enzyme as compared with the D27S mutant enzyme at this pH. There are slight decreases in K_m seen for the triple mutant enzyme as compared with the D27S+T113E double mutant enzyme at pH 7.0, while k_{cat} is unaffected. However, when compared with the D27S+F153S double mutant DHFR at pH 7.0, the triple mutant enzyme has 1.8 fold lower k_{cat} and 2 fold higher K_m (DHF).

The mechanism for WT DHFR catalysis consists of protonated enzyme binding unprotonated substrate, followed by protonation of N5 of the pteridine of DHF by Asp27. This is described by the equation $\text{EH} + \text{S} \rightleftharpoons \text{EH}'\text{S} \rightarrow \text{E} + \text{P}$. At low pH, the substrate can be protonated at N5 in solution ($\text{pK}_a = 3.8$, Poe, 1977) allowing catalysis in the D27S mutant enzymes. An enzyme-mediated protonation step can be bypassed if preprotonated substrate is bound by the enzyme. This bypass occurs more frequently at low pH. This is described by the equation $\text{E} + \text{SH} \rightleftharpoons \text{E}'\text{SH} \rightarrow \text{E} + \text{P}$ (Howell et al., 1990). Thus the D27S based mutant DHFRs depend upon protonated substrate to carry out their reaction. Lowering the pH increases the concentration of protonated substrate, thus enhancing catalysis. So the enzyme activity is pH dependent. The pH dependence of k_{cat} and k_{cat}/K_m (DHF) are illustrated in Fig. 12 for the D27S, D27S+T113E, D27S+F153S and D27S+T113E+F153S mutant DHFRs. The k_{cat}/K_m of different mutant enzymes varies with pH. At pH 5.0 and pH 7.0 the K_m of the triple mutant is less than the D27S+T113E double mutant but it is higher than that of the D27S and D27S+F153S mutant DHFRs. At pH 8.0 the K_m of the triple mutant DHFR is highest. At pH 5.0 k_{cat} of the triple mutant DHFR is the same as that of D27S+T113E and is 2 fold, and 10 fold lower than those of the D27S and D27S+F153S mutant DHFRs. At pH 8.0, k_{cat} of the triple mutant DHFR is the same as that of D27S+F153S and is 7 fold higher than that of D27S, and 1.4 fold lower than that of the D27S+T113E mutant DHFRs.

These values suggested the triple mutation can either affect substrate binding and/or catalytic efficiency. It can be seen that the efficiency of the D27S+T113E+F153S triple mutant enzyme is better than D27S and D27S+T113E mutant enzyme at certain pH values, but it is worse than the D27S+F153S double mutant enzyme throughout the pH range studied.

It is clear that the D27S+T113E+F153S triple mutant DHFR does not show an additive effect due to the T113E and F153S mutations. Any additive effects should correlate with an increase in the k_{cat} or the k_{cat}/K_m value for the protein. The quantitative effects of mutations on kinetic and thermodynamic parameters of enzymes are most conveniently expressed as changes in free energy of activation and binding compared to those of the parent enzyme. Generally, individual mutations in a protein can act either independently or interactively in a double mutant. If replacement of different side chains do not induce structural changes in the enzyme or enzyme-substrate complex, their effects will be independent and the overall change in interaction energy of the enzyme-substrate transition state in double mutants will be simply additive. For example, consider two mutations, A and B, where amino acid side chain A is mutated to A' and B to B':



$$\Delta G = -RT \ln \frac{k_{cat}/K_m(\text{mutant})}{k_{cat}/K_m(\text{wt})}$$

In thermodynamic terms:

$$\Delta G_1 = \Delta G'_1 \quad \text{and} \quad \Delta G_2 = \Delta G'_2$$

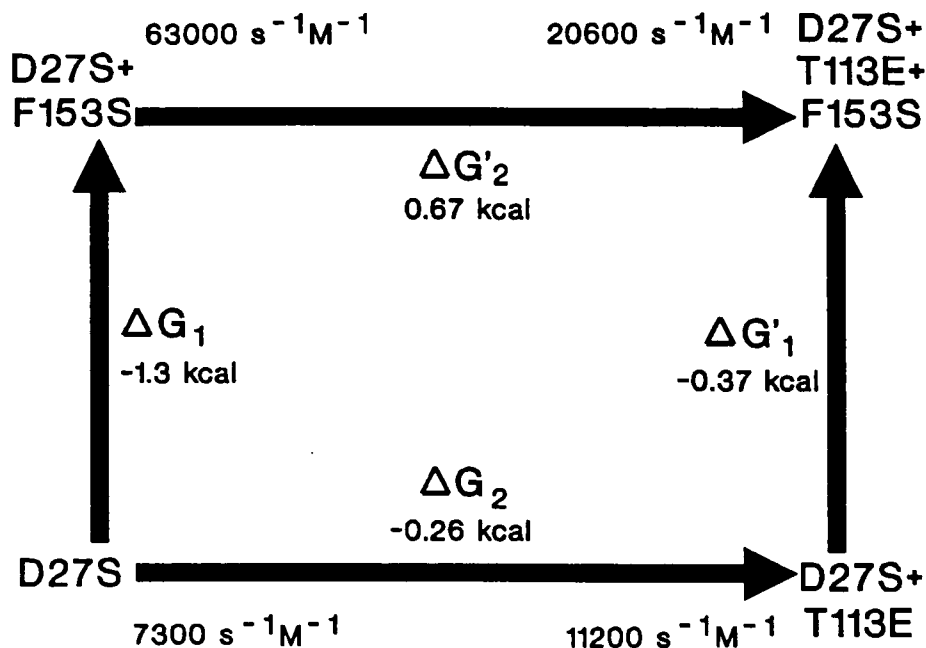
If replacements of amino acid side chains were accompanied by extensive structural changes in the enzyme or the enzyme

substrate transition state, calculations for one mutation could vary depending on the presence of the other mutations. In this situation:

$$\Delta G_1 \neq \Delta G_1' \text{ and } \Delta G_2 \neq \Delta G_2'$$

$$\text{So } \Delta G_1 + \Delta G_2' = \Delta G_1' + \Delta G_2$$

The $|\Delta G_1 - \Delta G_1'|$ term, which is defined as the coupling energy, represents an interaction energy between sites (Carter 1984). When the coupling energy is near or equal to zero, the sites behave independently and the double mutant enzyme displays simple additivity. When the coupling energy differs significantly from zero, the mutations are interactive in the double mutant. If the functional property is enhanced in the double mutant relative to that predicted from simple additivity, then the mutations act synergistically. When the k_{cat}/K_m is diminished relative to the predicted value, then the mutations behave antagonistically (Blacklow et al., 1991). The interaction of each side chain with the transition state (k_{cat}/K_m) in the D27S+T113E+F153S mutant DHFR is as follows:



The coupling energies of the triple mutant are 0.39, 0.93 and 1.96 Kcal/mol at pH 5.0, 7.0 and 8.0 respectively (Table III). It is reasonable for us to think that site interdependence exist. The structure of the mutant enzyme might be changed when these three mutations are put together. These changes prohibit the sites from functioning independently.

Specific interactions are expected when residues are close to one another. The distance between the α -C of the Thr113 and the CE1 of Phe153 is 5.19 Å and the α -C of the

Thr113 is 5.09 Å away from OG of D27S. The CE1 of Phe153 is 4.17 Å away from α -C of the D27S. It is reasonable to suppose that these side chains interact with each other in the triple mutant.

As these results suggest the secondary structure of the triple mutant DHFR might have changed, we note in Fig. 11 A that the triple mutant enzyme shows an altered electrophoretic mobility as compared to WT DHFR under nondenaturing conditions. The triple mutant migrates more slowly than both WT DHFR and D27S DHFR. This mobility difference suggests an alteration in charge and/or conformation associated with the triple mutant DHFR.

It is clear from Table II that the triple mutant DHFR can affect substrate binding and catalysis. As pH increases, the efficiency of the triple mutant DHFR is decreased. Because at low pH, DHF becomes protonated at N5 of the pteridine ring, protonated DHF should act as a productive substrate with the mutant enzyme. In contrast, at higher pH, DHF is predominately unprotonated and Glu113 can now serve as a proton donor. However its efficiency is low when compared to WT DHFR.

As mentioned earlier, the binding of substrate to enzyme is predominately due to hydrophobic and van der Waals interaction (Blakley, 1984). Since our data in Table II shows that the K_m of the triple mutant enzyme increases with pH, it is likely that deprotonation of Glu 113 at high pH causes the

anionic form of Glu 113 to disrupt binding between substrate and mutant enzyme. Another possibility is the normal DHF binding site is disturbed if Trp30 flips into the active site and occupies substrate binding space (see below). These situations could decrease the binding affinity.

Fig. 13 is a comparison of K_m 's for different mutations at several pH values. The K_m 's for D27S, D27D+T113E, and D27S+F153S mutant DHFRs do not show a substantial change as pH increases but the K_m of the D27S+T113E+F153S mutant DHFR increases dramatically. At pH 8.0, the K_m of the triple mutant DHFR is 4-fold higher than that of D27S DHFR, 2-fold higher than that of D27S+T113E DHFR and 5-fold higher than that of D27S+F153S mutant DHFR. This might indicate that any conformational change induced by these mutants can interfere with substrate binding. We note the coupling energy increases as pH increases (Table III), suggesting that the interactions of these side chains are dependent one another.

The ratio of A_{280} over A_{260} for most of our purified DHFRs is about 1.5-1.8, but the ratio for the triple mutant enzyme is about 0.8-1.0. A UV absorbance scan of the mutant protein at this range indicates a shift in the absorbance peak from what is normally observed (data not shown). We believe this is not due to contamination by DNA or RNA and is likely due to a conformational change possibly involving Trp30. The Trp30 position might be altered and flipped 180° into the active

site as seen in the D27S+F137S double mutant DHFR-MTX structure (Fig. 14).

These results show that large deviations from simple additivity can occur when mutation sites strongly interact with one another. This factor must be considered when designing an enzyme with new or improved catalytic activities. To determine the new conformation of this mutant DHFR, we would need to use X-ray diffraction techniques.

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APPENDIX

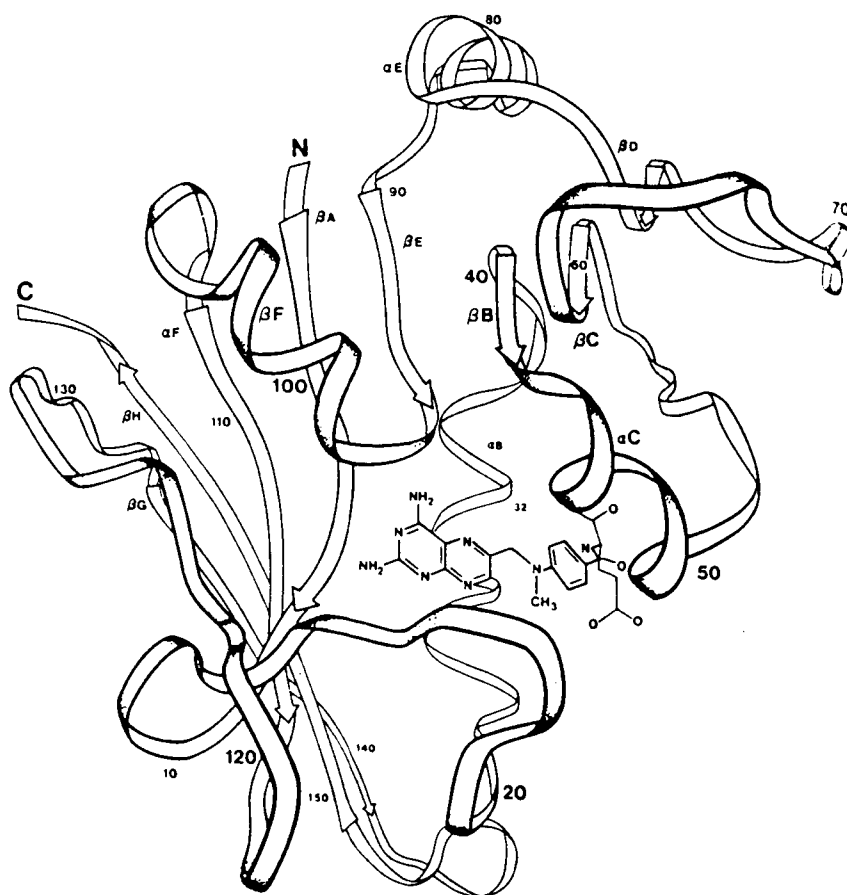


Fig 1. The carbon backbone structure of E. coli DHFR-methotrexate binary complex. β -Strands (represented by arrow) and α -helices are labeled. The approximate position of every tenth residue is indicated (Blakley 1984).

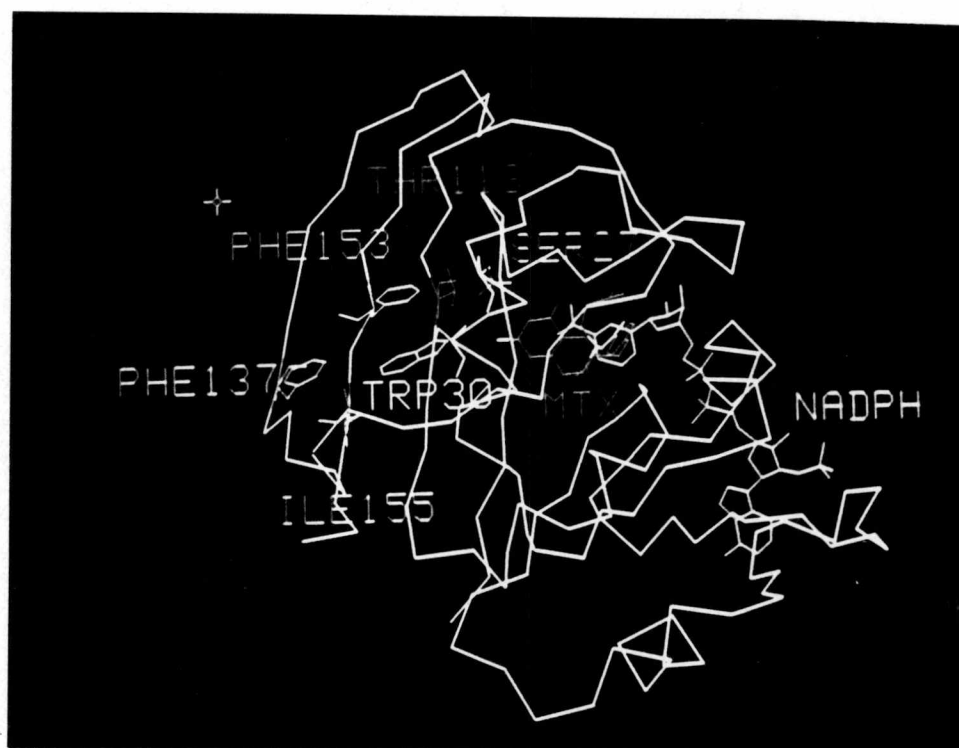


Fig 2. The crystal structure of the active site and β -bulge region of DHFR.

MTX is shown in red and NADP⁺ shown in yellow. The D27S mutation is located on the α -helix in the foreground of the photo. The distance from the β -bulge at position 136, 137, 155 to the active site is 15-20 Å. Mutations in the bulge can affect the active site from a distance. The β -bulge is proposed to play a role in stabilizing specific conformations.

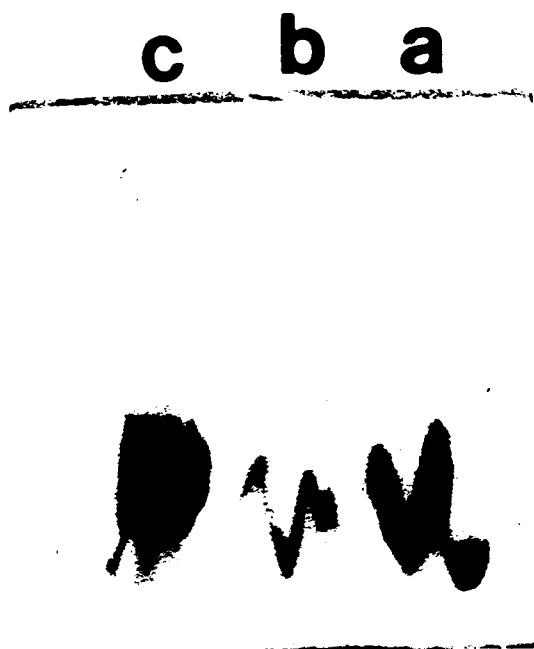


Fig 3. SDS & native PAGE of purified quadruple mutant DHFR. A. A 13.8% denaturing (SDS) polyacrylamide gel of purified D27S+E17G+D127G+F137S quadruple mutant DHFR (a-WT standard, b-D27S single mutant DHFR, c-quadruple mutant DHFR). The $Abs_{280/260}$ was > 1.5 . There were small contaminating bands in the preparation. The contaminating bands were not E. coli chromosomal DHFR because the molecular weight is not same as that of chromosomal E. coli DHFR.

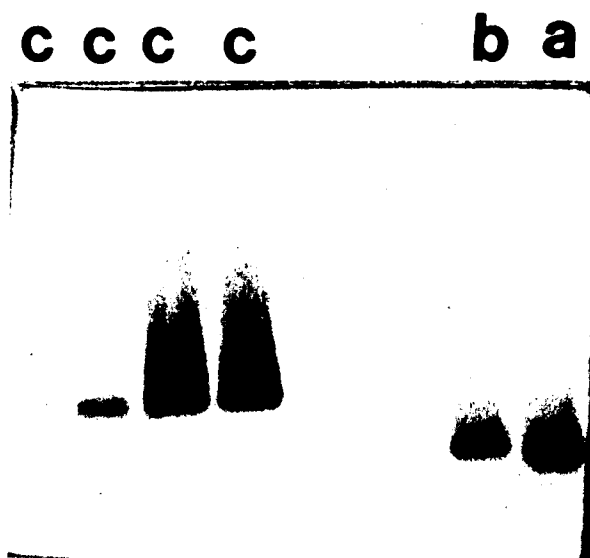


Fig 3 B. A nondenaturing 13.8% polyacrylamide gel of purified D27S+E17G+D127G+F153S quadruple mutant DHFR with Commassie blue stain (a-WT standard, b-D27S single mutant DHFR, c-quadruple mutant DHFR).

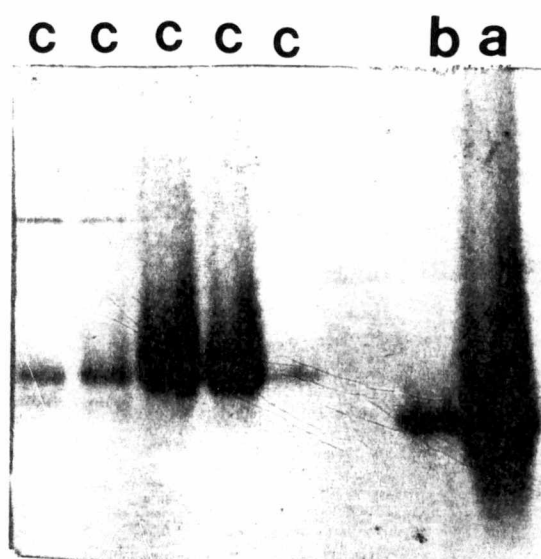


Fig 3 C. A nondenaturing 13.8% polyacrylamide gel of purified D27S+E17G+D127G+F153S quadruple mutant DHFR with activity stain (a-WT standard, b-D27S single mutant DHFR, c-quadruple mutant DHFR).

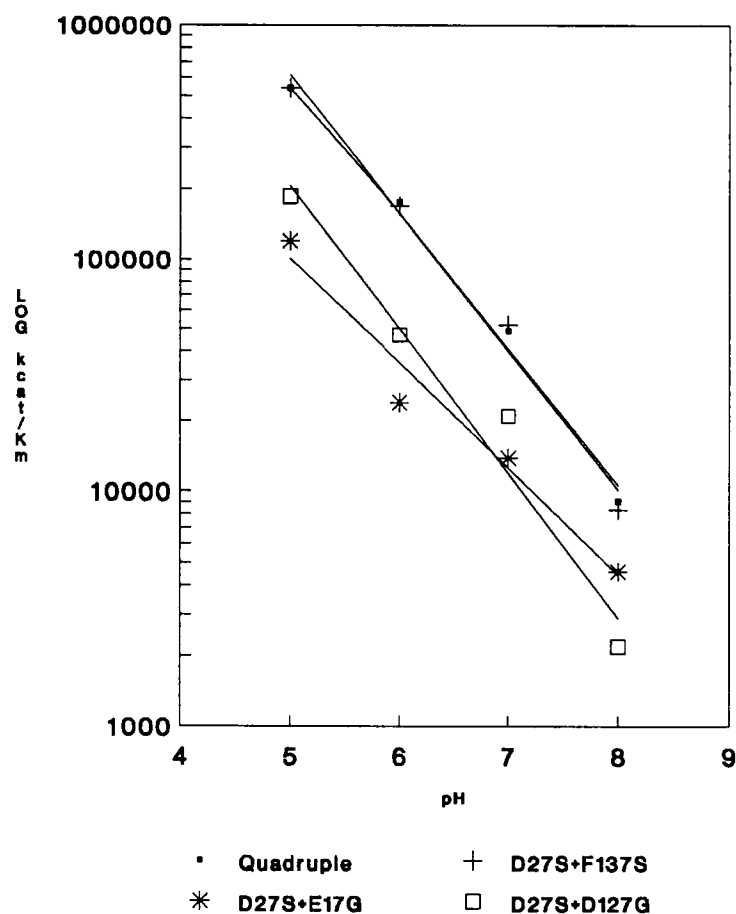


Fig 4. pH profiles for the quadruple mutant DHFR. pH profiles of $\log k_{cat}/K_m$ (DHF) were obtained using a saturating concentration of NADPH and varying the dihydrofolate concentration. The efficiency of the quadruple mutant DHFR is higher than either D27S+E17G or D27S+D127G DHFRs, but it is similar to that of the D27S+F137S double mutant DHFR.

oligonucleotide to produce the F153S mutation

5' AAT CTC AGA GCA ATA 3'

oligonucleotide to produce the I155N mutation

5' CCG CTC CAG ATT CTC AAA GCA3'

oligonucleotide to produce the F137S mutation¹

5'TTC GCT GGA TAC CCG ATT CCC A 3'

Fig 5. Oligonucleotide sequences.

These oligonucleotides were synthesized and purified by the molecular biology resource facility at The University of Tennessee. They were used to generate triple mutant DHFR by site-directed mutagenesis techniques.

¹. This oligonucleotide was made available in our lab.

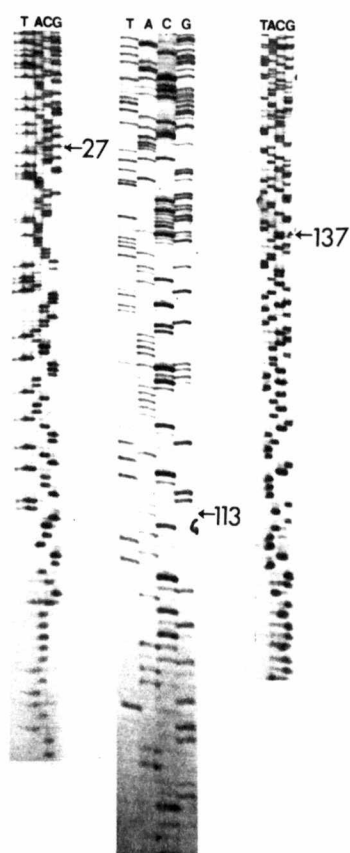


Fig 6. D27S+T113E+F137S triple mutant DHFR DNA sequencing gel.

The codon of Aspartic acid 27 in WT DHFR is GAT. The codon of D27S is AGC. The codon of Threonine 113 in WT DHFR is ACG. The codon of T113E is GAA. The codon of Phenylalanine 137 in WT DHFR is TTC. The codon of F137S is TCC.

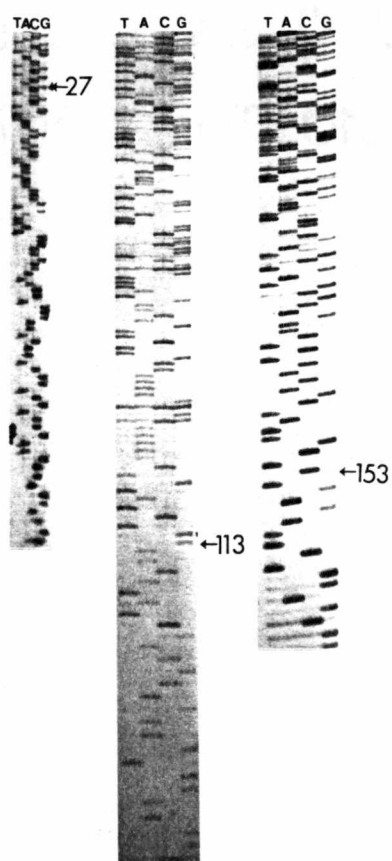


Fig 7. D27S+T113E+F153S triple mutant DHFR DNA sequencing gel.

The codon of Aspartic acid 27 in WT DHFR is GAT. The codon of D27S is AGC. The codon of Threonine 113 in WT DHFR is ACG. The codon of T113E is GAA. The codon of Phenylalanine 153 in WT DHFR is TTT. The codon of F153S is TCT.

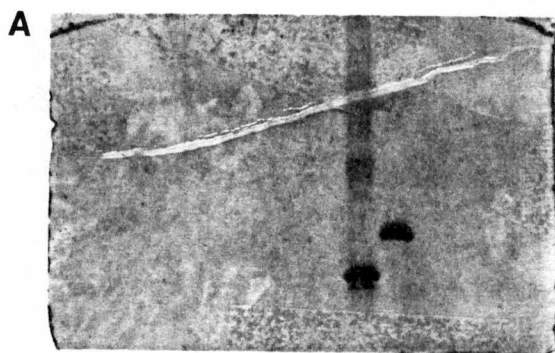


Fig 8. Western blot of inclusion bodies.
 A. Denaturing protein gel with Coomassie Blue stain.
 Left lane- protein from inclusion bodies.
 Right lane- WT DHFR protein.
 B. Western blot using antibody against WT DHFR.
 Left lane- protein from inclusion bodies (Antibody did not bind any of the protein from the inclusion bodies).
 Middle lane- WT DHFR protein.
 Right lane- molecular weight standards.

A

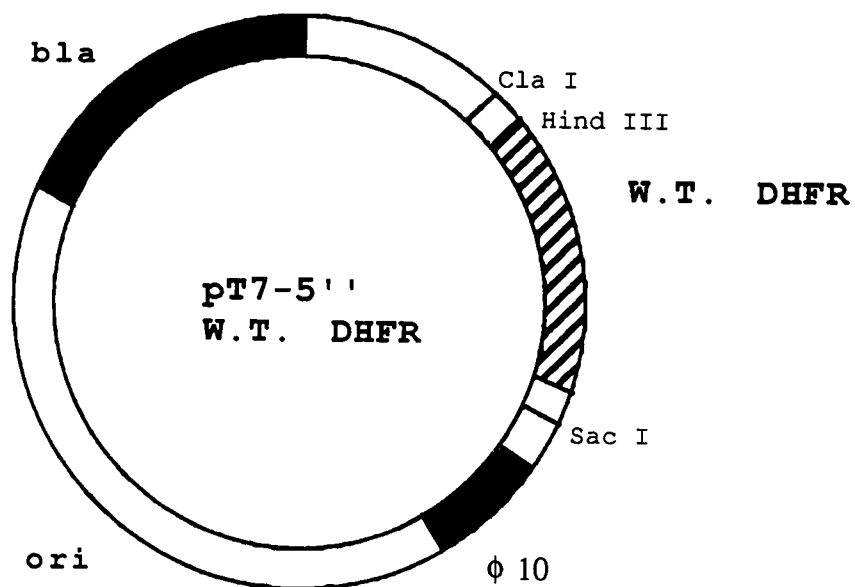


Fig 9. Construction of PT7-5"-WT and PT7-5" mutant DHFR plasmids.

A. vector PT7-5"-WT DHFR. Vector PT7-5 was cut by **SmaI** and **HindIII**. WT DHFR (in Puc8) was cut by **SnaBI** and **HindIII**. Polyacrylamide gel electrophoresis of DNA and fragment elution and ligation (**SmaI/SnaBI** joint neither site retained upon ligation) were performed as described under materials and methods.

B

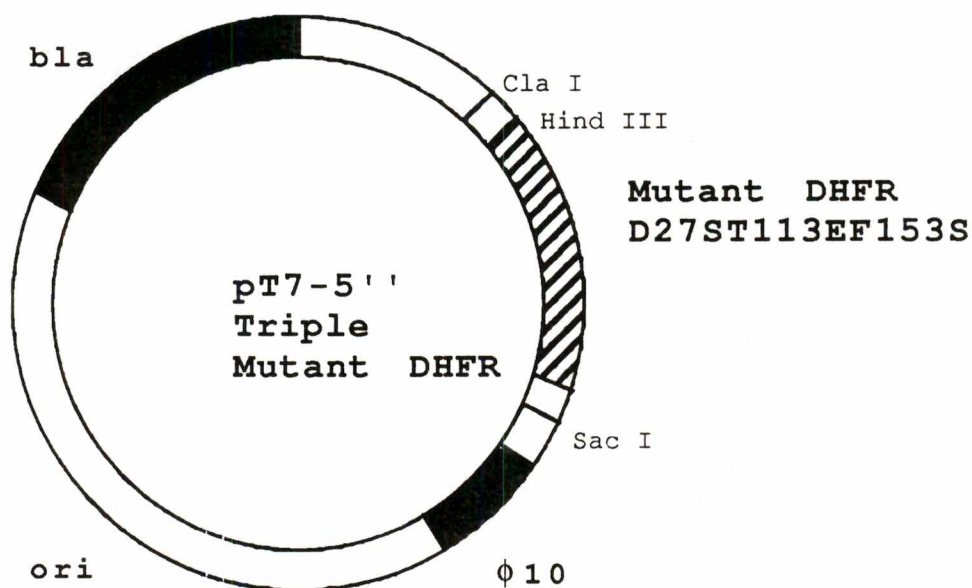


Fig 9 B. vector PT7-5''-Triple mutant DHFR. Vector PT7-5 was cut by *Sma*I and *Hind*III. D27S+T113E+F153S triple mutant DHFR (in pUC8) were cut by *Sna*BI and *Hind*III. Polyacrylamide gel electrophoresis of DNA and fragment elution and ligation (*Sma*I/*Sna*BI joint neither site retained upon ligation) were performed as described under materials and methods. The size of PT7-5''-Triple mutant DHFR was checked on a 1% agarose gel (data not shown).

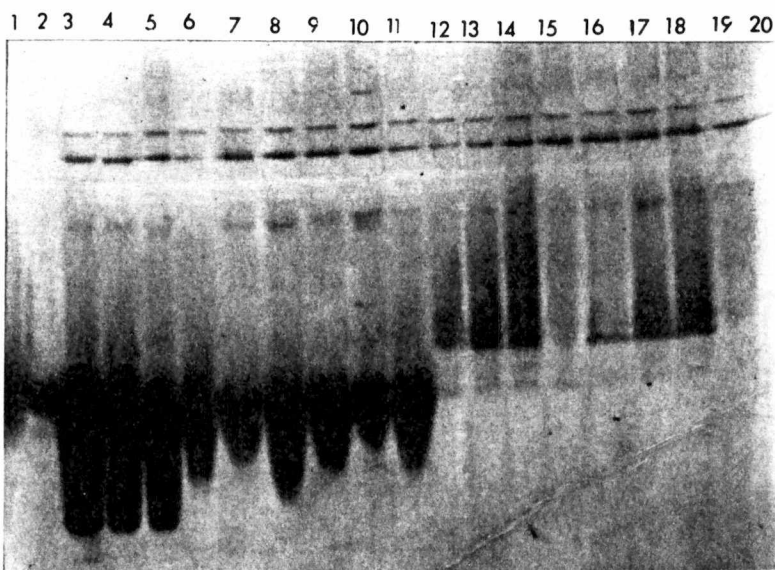


Fig 10. Analysis of the growth curve for D27S+T113E+F153S triple mutant DHFR.

A. 13.8% Polyacrylamide gel with activity stain. The culture conditions were described under materials and methods. Lane 1: WT DHFR. Lane 2: D27S single mutant DHFR. Lane 3-6: Wild type DHFR in PT7-5" in K38 cell line was grown in YT and samples were collected at 2,4, 8, 16 hours. Lane 7-10: Wild type DHFR in PT7-5" in K38 cell line was grown in TB and samples were collected at 2,4, 8, 16 hours. Lane 11: Wild type DHFR in PT7-5" in K38 cell line was grown in TB 16 hours. It was not induced by heating. Lane 12-15: D27S+T113E+F153S mutant DHFR in PT 7-5" in K38 cell line was grown in YT and samples were collected at 2,4, 8, 16 hours. Lane 16-19: D27S+T113E+F153S mutant DHFR in PT7-5" in K38 cell line was grown in TB and samples were collected at 2,4, 8, 16 hours. Lane 20: D27S+T113E+F153S mutant DHFR in PT7-5" in K38 cell line was grown in TB 16 hours. It was not induced by heating.

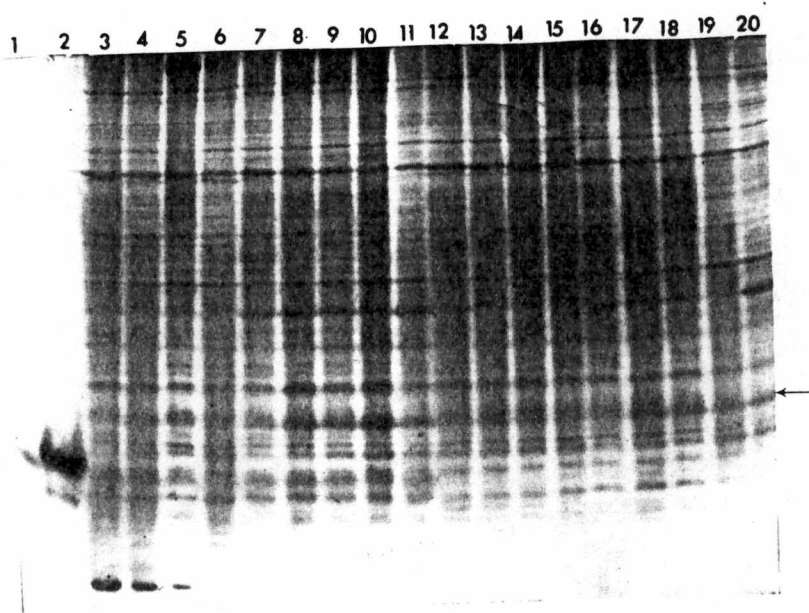


Fig 10 B. 13.8% Polyacrylamide gel with Coomassie blue stain. The culture conditions were described under materials and methods.

Lane 1: WT DHFR. Lane 2: D27S single mutant DHFR. Lane 3-6: Wild type DHFR in PT7-5" in K38 cell line was grown in YT and samples were collected at 2, 4, 8, 16 hours. Lane 7-10: Wild type DHFR in PT7-5" in K38 cell line was grown in TB and samples were collected at 2, 4, 8, 16 hours. Lane 11: Wild type DHFR in PT7-5" in K38 cell line was grown in TB 16 hours. It was not induced by heating. Lane 12-15: D27S+T113E+F153S mutant DHFR in PT7-5" in K38 cell line was grown in YT and sample were collected at 2, 4, 8, 16 hours. Lane 16-19: D27S+T113E+F153S mutant DHFR in PT7-5" in K38 cell line was grown in TB and samples were collected at 2, 4, 8, 16 hours. Lane 20: D27S+T113E+F153S mutant DHFR in PT7-5" in K38 cell line was grown in TB 16 hours. It was not induced by heating. → is the position of the triple mutant DHFR.

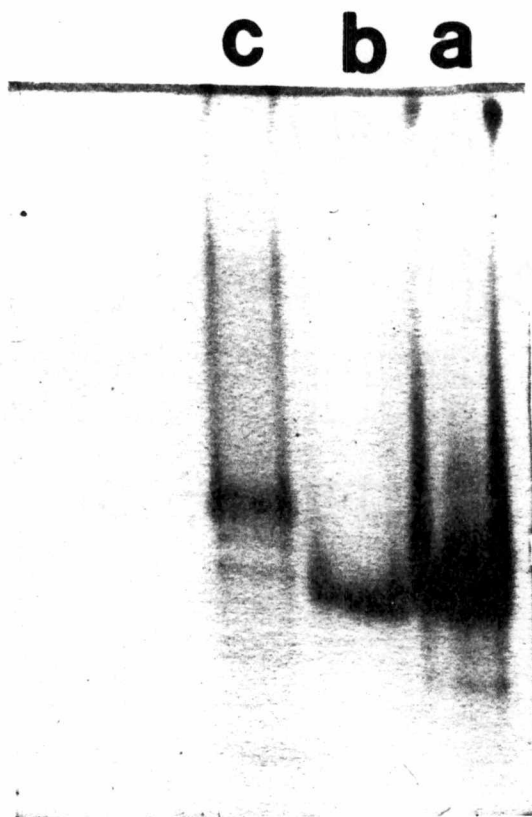


Fig 11. SDS & native PAGE of purified D27S+T113E+F153S triple mutant DHFR.

A. Coomassie blue stained 13.8 % nondenaturing polyacrylamide gel of purified D27S+T113E+F153S mutant DHFR. (a-WT DHFR, b-D27S single mutant DHFR, c-D27S+T113E+F153S triple mutant DHFR). The $Abs_{280/260}$ was 0.8-1.0. Contamination bands appear on the gel. The contamination band was not the WT DHFR (data not shown).

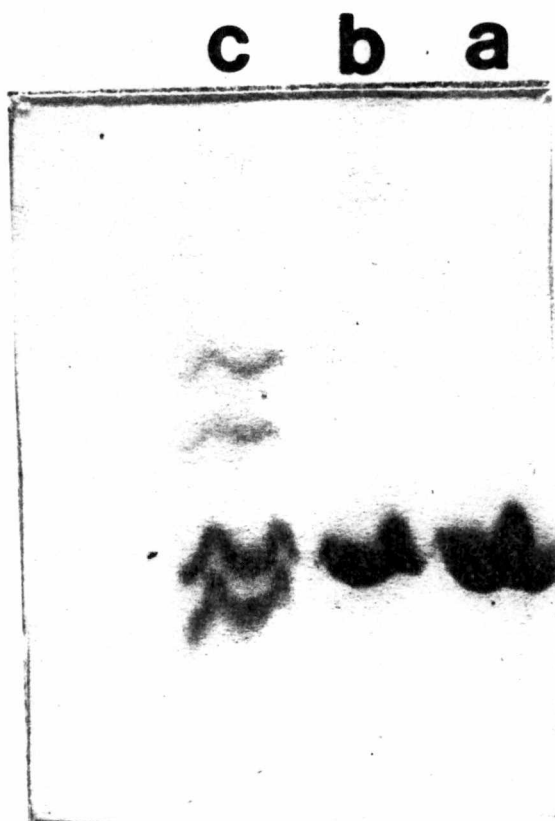


Fig 11 B. Coomassie blue stained 13.8 % denaturing polyacrylamide gel of purified D27S+T113E+F153S mutant DHFR. (a-WT DHFR, b- D27S single mutant DHFR, c-D27S+T113E+F153S triple mutant DHFR).

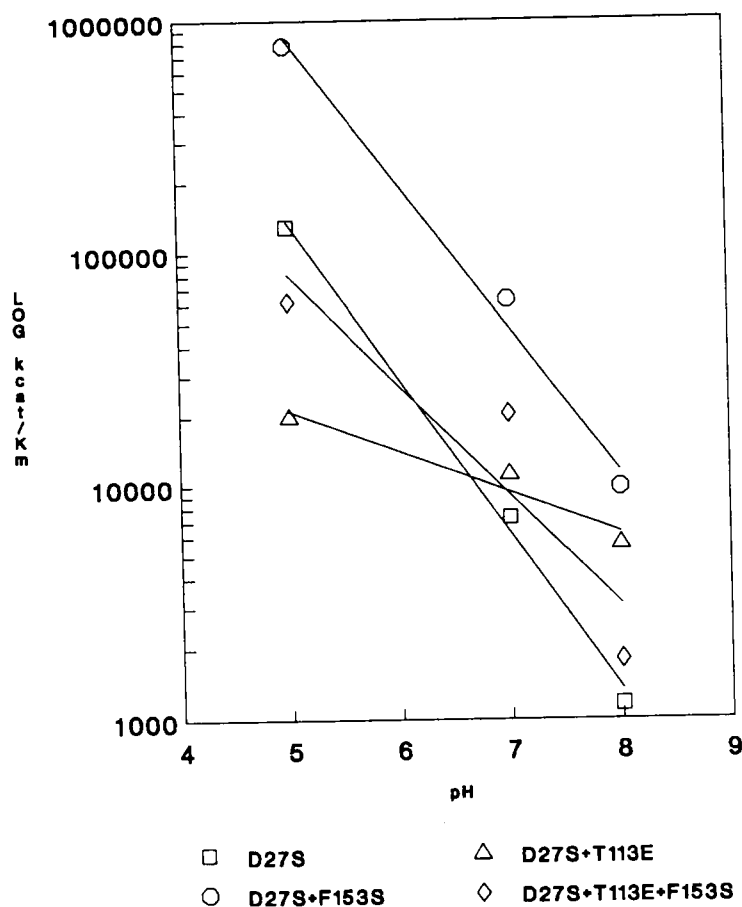


Fig 12. pH profiles of $\log k_{cat}/K_m$ for D27S, D27S+T113E, D27S+F153S and D27S+T113E+F153S mutant DHFRs. Data were obtained as described under materials and methods.

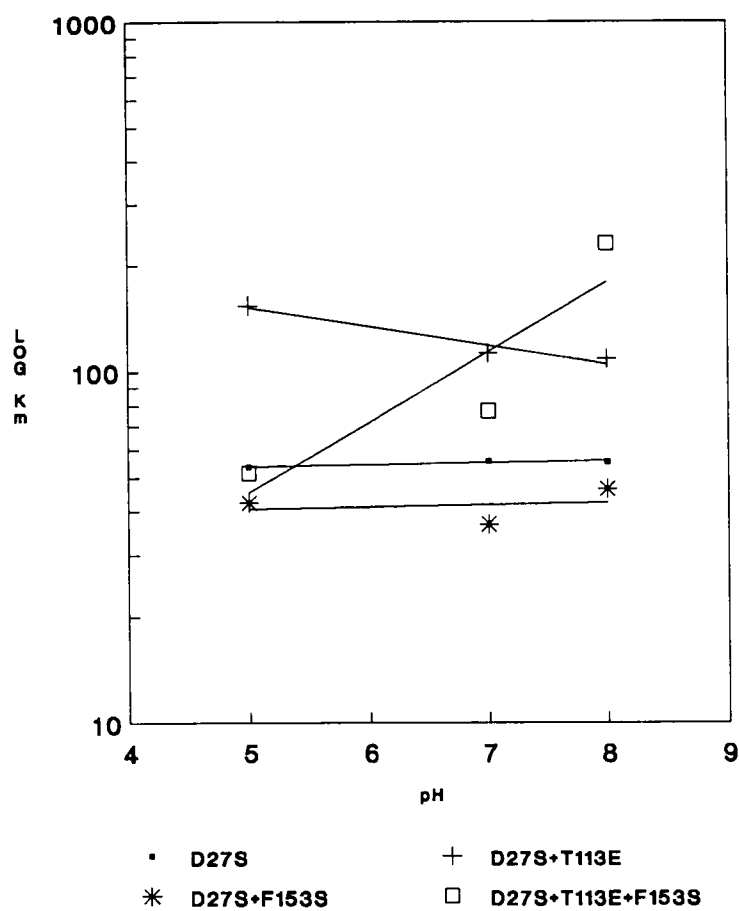


Fig 13. pH profile of K_m for different mutant DHFRs. Data were obtained as described under material and methods.

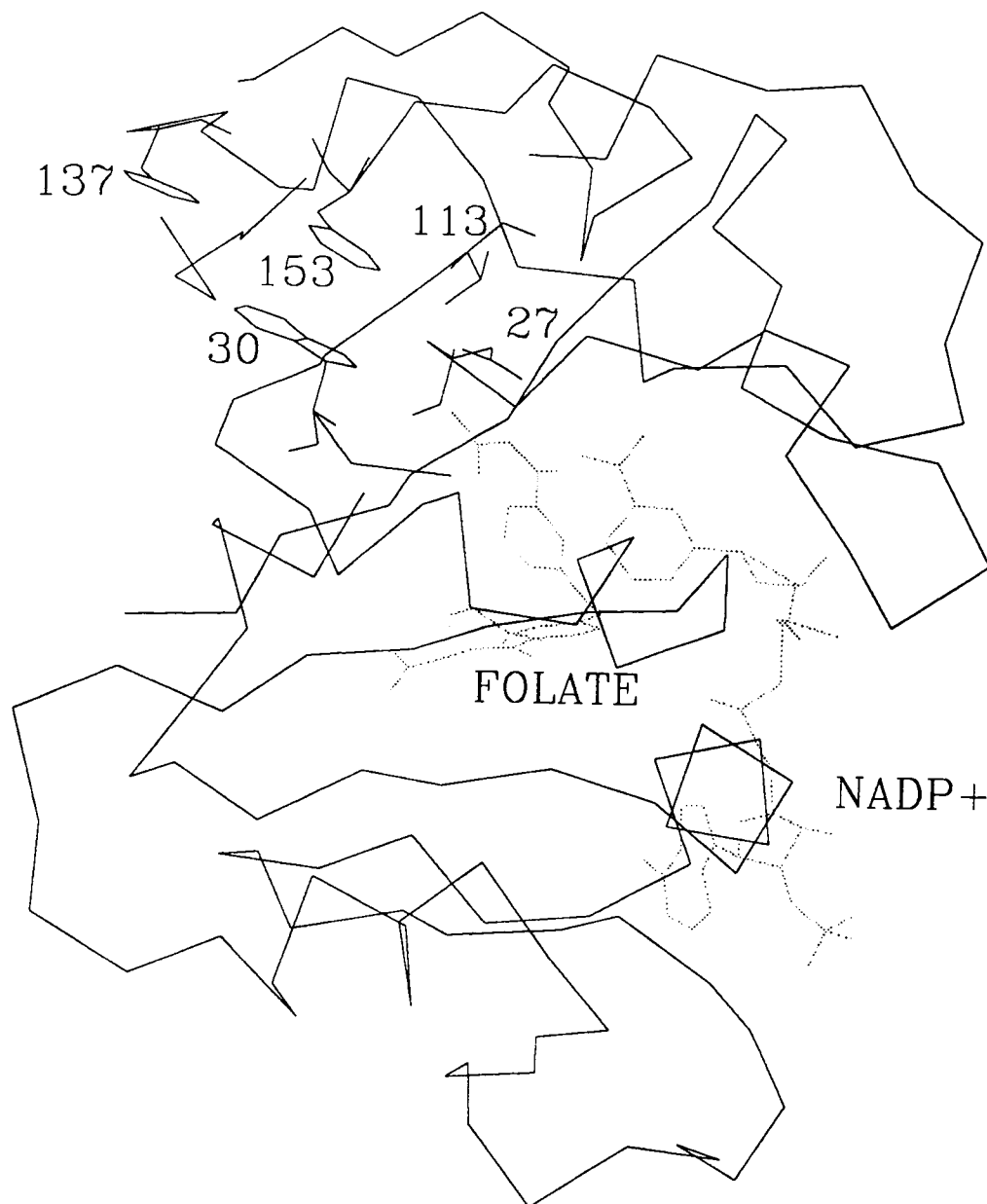


Fig 14. Backbone tracing of the crystal structure for WT DHFR and D27S+F137S double mutant DHFR from E. coli.
A. Backbone tracing of the crystal structure for WT DHFR from E. coli. 27 represents Asp 27 residue. 30 represents Trp 30 residue. 113 represents Thr 113 residue. 137 represents Phe 137 residue. 153 represents Phe 153 residue.

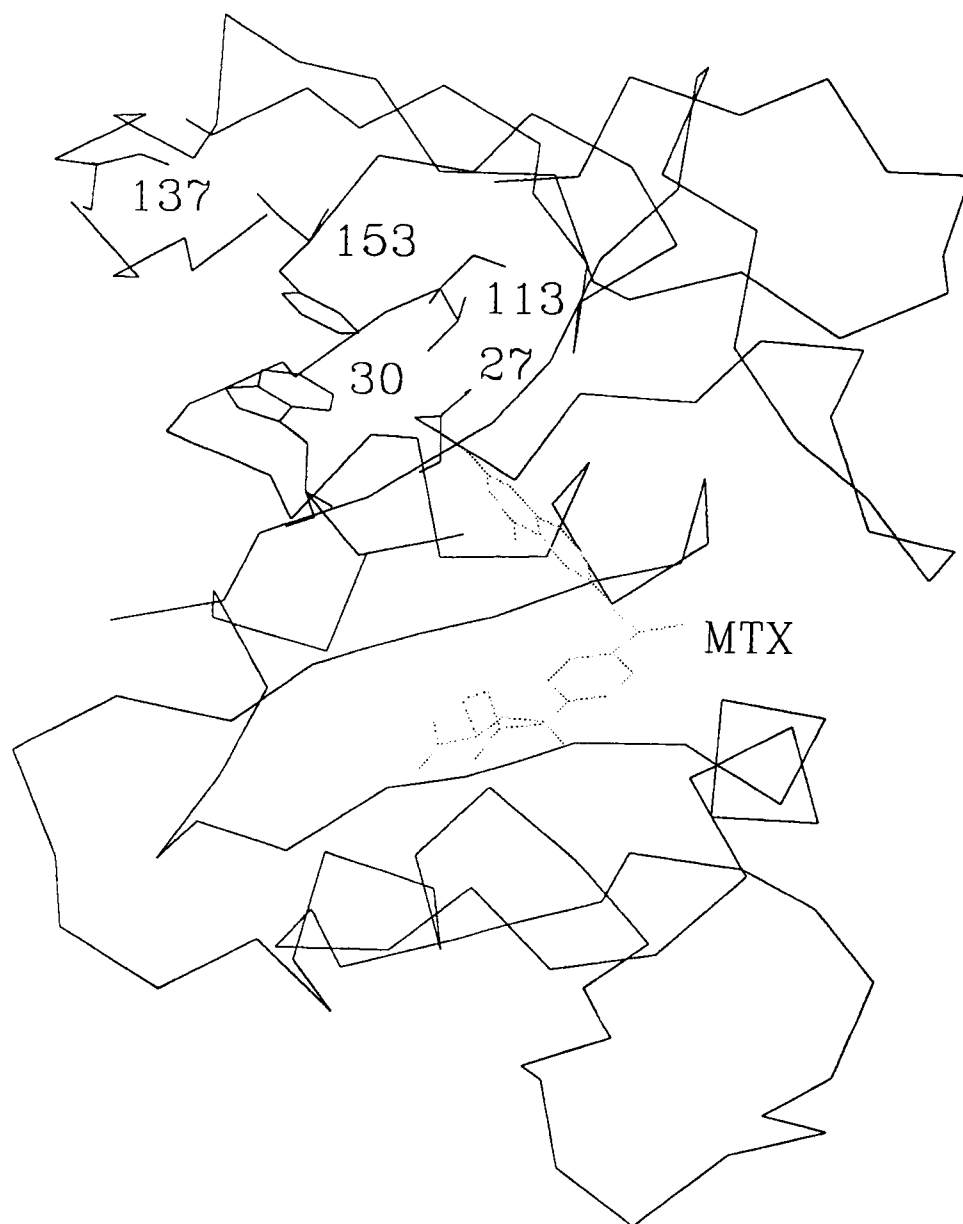


Fig 14 B. Backbone tracing of the crystal structure for the D27S+F137S double mutant DHFR from *E. coli* in complex with MTX. 27 represents D27S. 30 represents Trp 30 residue. 113 represents Thr 113 residue. 137 represents F137S. 153 represents Phe 153 residue.

Table I. A Comparison of Kinetic Parameters for D27S+E17G, D27S+D127G, D27S+F137S and D27S+E17G+D127G+F137S Mutant DHFRs at Different pH Values.

pH	k_{cat} (sec^{-1})	K_m (μM)	k_{cat}/K_m ($\text{s}^{-1}\text{M}^{-1}$)
(5.0)			
D27S+E17G	7.60	60.0	1.2×10^5
D27S+D127G	12.20	65.0	1.9×10^5
D27S+F137S	14.90	27.0	5.5×10^5
D27S+E17G+D127G+F137S	19.00	35.0	5.4×10^5
(6.0)			
D27S+E17G	1.50	61.0	2.4×10^4
D27S+D127G	2.80	58.7	4.7×10^4
D27S+F137S	4.60	26.4	1.7×10^4
D27S+E17G+D127G+F137S	4.53	26.3	1.7×10^4
(7.0)			
D27S+E17G	0.60	43.0	1.4×10^4
D27S+D127G	1.00	42.7	2.1×10^4
D27S+F137S	1.32	24.7	5.2×10^4
D27S+E17G+D127G+F137S	1.10	22.4	4.9×10^4
(8.0)			
D27S+E17G	0.15	32.0	4.6×10^3
D27S+D127G	0.14	62.3	2.2×10^3
D27S+F137S	0.32	37.9	8.4×10^3
D27S+E17G+D127G+F137S	0.27	29.6	9.1×10^3

Table II. A Comparison of Kinetic Parameters for D27S, D27S+T113E, D27S+F153S and D27S+T113E+F153S Mutant DHFRs at Different pH Values.

pH	k_{cat} (sec^{-1})	K_m (μM)	k_{cat}/K_m ($\text{s}^{-1}\text{M}^{-1}$)
(5.0)			
D27S	7.20	54.0	1.3×10^5
D27S+T113E	3.16	154.	2.0×10^4
D27S+F153S	33.40	42.4	7.8×10^5
D27S+T113E+F153S	3.20	51.5	6.2×10^5
(7.0)			
D27S	0.40	55.7	7.3×10^3
D27S+T113E	1.20	112.	1.1×10^4
D27S+F153S	2.34	36.7	6.3×10^4
D27S+T113E+F153S	1.48	77.2	2.1×10^4
(8.0)			
D27S	0.06	55.4	1.2×10^3
D27S+T113E	0.61	108	9.8×10^3
D27S+F153S	0.45	46.3	9.8×10^3
D27S+T113E+F153S	0.48	231	1.8×10^3

TABLE III. Coupling Energies of D27S+T113E+F153S Triple Mutant DHFR at Several pH Values.

pH	Coupling Energy
5.0	0.39 Kcal/mol
7.0	0.93 Kcal/mol
8.0	1.96 Kcal/mol

VITA

Guisheng Zhang was born in Beijing, China on October 14, 1955. She attended Jincuang 11th Elementary School, Jincuang 9th Junior High School, and graduated from Jincuang 1st High School in February, 1973. The following 4 years she worked in both the countryside and industry. She entered Lanzhou University (Gansu, China) in February, 1978 and received a Bachelor of Arts degree in Biochemistry & Microbiology in January, 1982. After graduation, she worked in the Commercial College Lanzhou. She came to the United States in March, 1986. She was admitted by The University of Tennessee, Knoxville in August, 1989. She was awarded a Master of Science degree in Biochemistry in December, 1991.