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Protein Engineering of Dihydrofolate Reductase. Improved Catalytic Step of Mutant-Enzymes

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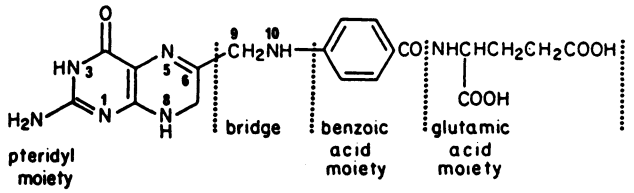
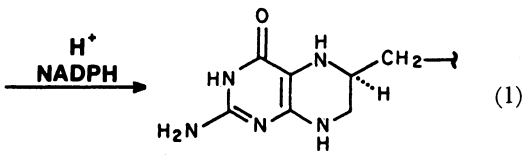
Two site-specific mutations on dihydrofolate reductase from *Escherichia coli* have been carried out at the Phe-31 site. From the crystallographic structure the Phe-31 is located at the dihydrofolate binding site and interacts with both the pteridine ring and the *p*-aminobenzoyl moiety of the substrate. Two mutant enzymes (Phe-31→Tyr and Val) have been purified to homogeneity and characterized by steady-state kinetics. The two mutations are aimed at assessing the hydrophobic interaction between the phenyl ring and the aromatic ring moiety of the substrate. Despite the fact that the first mutation has introduced a polar Tyr-group into a hydrophobic binding site, the Michaelis constant K_{DHF} (K_M with saturating NADPH and varying dihydrofolate) has increased only five-fold. The second mutation (Phe-31→Val-31) results in a 25-fold increase in K_{DHF}. More importantly, the maximum velocity of both mutant enzymes has increased more than 100%, indicating that these are better enzymes under the condition of [substrate]$>K_{DHF}$. Thus, in both mutant enzymes the decrease in binding has not been translated into a loss of catalytic efficiency.

Site-directed mutagenesis is becoming an increasingly powerful technique for elucidation of the role of specific amino acids in proteins.[1-6] Dihydrofolate reductase (DHFR) catalyzes the reduction of dihydrofolate (H_2-folate) to tetrahydrofolate (H_4-folate) with NADPH as the cofactor. The metabolic importance of



DHF (H_2-folate)



(1)

THF (H_4-folate)

DHFR, which stems from the fact that derivatives of its product, H_4-folate, have coenzyme functions in one-carbon transfer reactions, has inspired extensive research on inhibitors of the enzyme, several of which are widely used as antibacterial or antitumor agents.[7] This enzyme is one of a few proteins whose X-ray crystal structure has been solved to a resolution better than 2 Å.[8,9] It is a single polypeptide chain of 159 amino acids (17800 daltons) and possesses an extensive secondary structure consisting of eight β strands, seven of which are parallel, and four α helices.[8,9] Thus, this richness of structural information enables us to study the relationship between enzyme structure and function in detail by means of site-directed mutagenesis on this enzyme.

The structure of the *E. coli* DHFR-methotrexate binary complex is shown schematically in Fig. 1, with

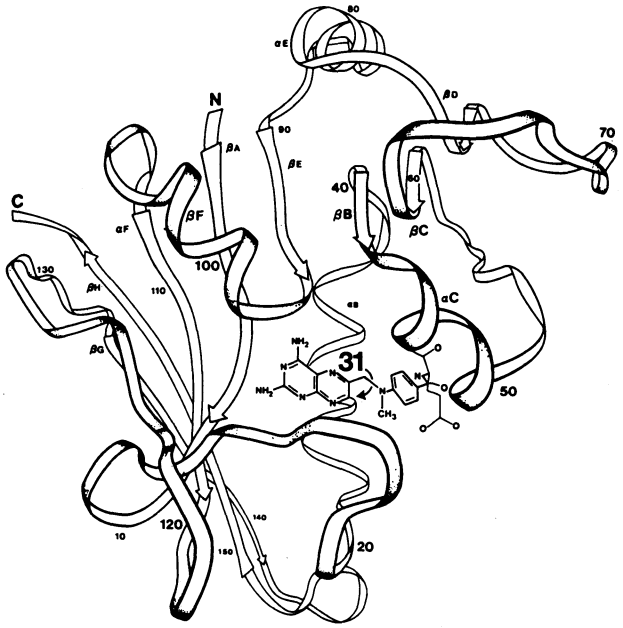
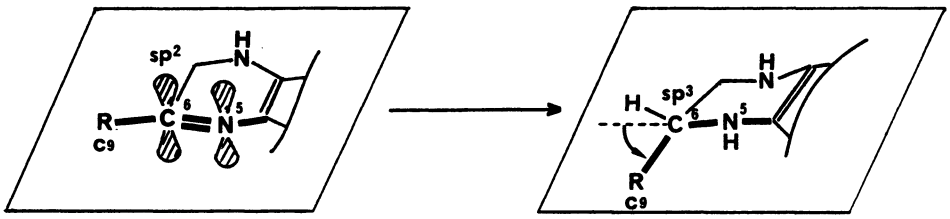


Fig. 1. Backbone ribbon drawing of the *E. coli* DHFR-methotrexate binary complex[8] with the mutation site, Phe-31, indicated by the arrow.

the site of particular interest, Phe-31, indicated by an arrow. This phenylalanine, strictly conserved in all known sequences of DHFR, interacts with both the pteridine ring and the *p*-aminobenzoyl moiety. The *p*-aminobenzoyl moiety is almost perpendicular to the pteridine ring with the Phe-31 being at the corner of the bend providing van der Waals contacts with both aromatic rings. Considering the fact that the hybridization of C6 changes from sp^2 to sp^3 so that C9 swings away from the plane of the pteridine ring upon reduction of the C6=N5 imino bond, it is of interest to inquire whether the interactions with Phe-31 change during product formation.

One might also imagine that the imino double bond



(2)

in H_2-folate may be activated towards reduction through a degree of strain on the imino bond, possibly caused by the bend. By replacing the Phe-31 by a smaller amino acid such as Val, one can assess to what degree reduced substrate-enzyme interactions alter catalysis. In addition we have chosen Tyr for the replacement of Phe-31, which still possesses aromatic character but may be able to facilitate the protonation of N5 prior to reduction since no amino acid exists at the active site that is capable of direct proton transfer.[8]

This approach is made possible through the technique of site-directed mutagenesis. We have successfully created two new proteins (Tyr-31 and Val-31) via site-directed mutagenesis which have been purified to homogeneity and offer significant mechanistic insights which could not be gained merely by studying the wild-type enzyme.

Methods

The method of Dalbadie-McFarland et al. was employed to generate specific mutations using a chemically synthesized oligodeoxynucleotide that directs the mutationl.[1] Figure 2(a) shows the nucleotide sequence of the synthetic 22-mer oligodeoxynucleotide, with asterisks indicating mismatches, together with the corresponding base sequence of the wild-type *E. coli* DHFR. Figure 2(b) illustrates a restriction map of the plasmid DNA, pTY1,[10] in which a 1 Kb fragment containing the DHFR gene (*fol*)[11] is inserted into the *Bam*H I site of a 4.4 Kb pBR322 derivative DNA lacking the *Eco*R I site, as well as the restriction fragments formed upon treatment with *Aha* III and *Bam*H I. The recognition sequence of the *Aha* III is TTTAAA which is found in the wild-type DHFR gene corresponding to Phe-31 and Lys-32 (see Fig. 2(a)). Upon mutation of the Phe-31 the TTT codon is replaced by a corresponding codon for each mutant enzyme, in case of the Tyr-31 mutant, the corresponding codon is TAT. Thus, any successful mutation destroys the *Aha* III recognition site in *fol*, resulting in the resistance of a 1 Kb fragment toward *Aha* III digestion. The isolation of the *Aha* III resistant 1 Kb fragment followed by religation with the 4.4 Kb pBR322

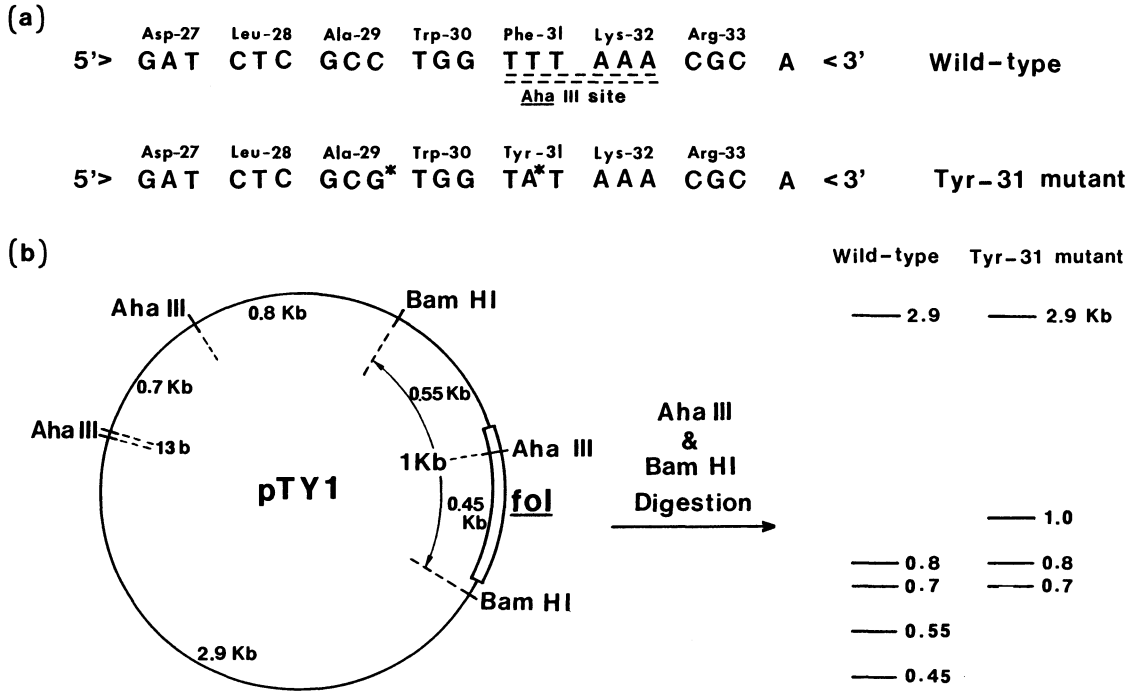


Fig. 2. (a) Nucleotide sequence of the syuthetic oligodeoxynucleotide for Tyr-31 mutant and the corresponding sequence for the wild-type *E. coli* DHFR. The base pair mismatches are indicated by asterisks and the amino acids encoded by both mutant and wild-type sequences are shown. (b) Restriction map of the plasmid DNA, pTY1, showing the location of the DHFR gene (*fol*) by the open box. Gel electrophoresis results of both wild-type and mutant DNAs upon *Aha* III/*Bam*H I digestion are schematically shown.

derivative completes the preparation of mutant plasmid DNA. The exact experimental conditions employed[10-15] are described in the following section.

Experimental

Oligodeoxyribonucleotides. The syntheses of two oligodeoxyribonucleotides were performed on an Applied Biosystems Model 380A DNA synthesizer. Base sequences of the two oligodeoxyribonucleotides were as follows, with asterisks indicating mismatchs.

Tyr (22-mer):
5'>GAT CTC GCG* TGG TA*T AAA CGC A<3'
Val (21-mer):
5'>GAT CTC GCG* TGG G*TT AAA CGC<3'

The oligodeoxyribonucleotides were purified by means of HPLC on a reverse phase μBONDAPAK C18 column employing a 10—30% gradient of CH_3CN in 0.1 M§ triethylammonium acetate (TEAA) pH 7. After detritylation with 3% CH_3COOH at room temperature for 3 min the deblocked oligonucleotides were subject to a second HPLC purification under the above conditions.

Purity of the primers was confirmed by repeating the HPLC procedure as well as running electrophoresis on ^{32}P-end-labelled primers prepared as follows.[12] To an Eppendorf tube were added 4 μl H_2O, 2.5 μl 5X Kinase buffer (0.5 M Tris·HCl, pH 8.0, 0.05 M $MgCl_2$, 0.025 M DTT), 1 μl deoxyribonucleotide (4.0 OD_{260}/ml, ≈12 pmol), 3 μl [γ-^{32}P]-ATP (2500 Ci/mmol, New England Nuclear), and 2 μl T4 Kinase (2 u/μl, New England Biolabs). This solution was incubated at 37 °C for 1 1/4 h, mixed with 10 μl 0.5 M EDTA to stop the reaction, and the solution spin desalted through a G25 fine Sephadex column. The column was washed with 50 μl TE (10 mM Tris·HCl, pH 7.5, 1 mM EDTA). The recovered ^{32}P-end-labelled primer (20×10^3 CPM in 1 μl) was mixed with 9 μl of a sequencing dye and ca. 1 μl of this sample per lane was loaded onto a denaturing 20% polyacrylamide gel calibrated with appropriate standards. This electrophoresis analysis confirmed the purity as well as the length of each primer.

Plasmid pTYl. Two *Hae* III fragments (574 base pairs and 408 base pairs long) containing the DHFR gene were isolated from pCV29 DNA[11] and subcloned by Yaegashi et. al.[10] into the *Bam*H I site of a pBR322 derivative lacking the *Eco*R I site to generate pTYl.

Nicking. Fifty micrograms of supercoiled pTYl DNA and 15 μg of ethidium bromide in 50 μl of 50 mM Tris·HCl, pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$, and 1 mM dithiothreitol (DTT) were treated in the dark at room temperature with 4.0 μl of *Eco*R I (10 units/μl) for 4.5 h.[1] (The reaction was assayed, after stopping the reaction by an addition of 1 μl of 0.5 M EDTA, by electrophoresis on 0.8% agarose gel in 40 mM Tris acetate, pH 8.0, 1 mM EDTA; conditions that separate nicked, linear, and supercoiled DNA molecules.) The solution was extracted once with an equal volume of phenol, which had been equilibrated with TE buffer, once with 1/2 volume of phenol and 1/2 volume of chloroform, twice with equal volumes of chloroform, and then four times with 3—5 times volumes of diethyl ether. The DNA was precipitated by adding 1/10 volume of 3 M sodium acetate, pH 7.1, and 2.5 volumes of absolute ethanol and the mixture maintained at −20 °C for longer than 3 h. After centrifugation, the pellet was washed three times with 70% (vol/vol) ethanol, dried and dissolved in 30 μl of TE, pH 7.5. The concentration of the DNA was calculated to be 1.24 μg/μl, assuming that one OD_{260} equaled 50 μg DNA/ml.

Gapping. Prior to each exonuclease treatment the activity of the enzyme was checked by digesting a linear double-stranded pTYl plasmid and following the formation of two single-stranded fragments by electrophoresis on 0.7% agarose containing 1 μg ethidium bromide per ml. About 5 μg (1.4 pmol) of the nicked DNA was treated for 60 min at room temperature with 100 units of exonuclease III in 50 μl of 66 mM Tris·HCl, pH 8.0, 90 mM NaCl, 5 mM $MgCl_2$, and 10 mM DTT. The reaction was stopped by carrying out phenol extraction followed by ethanol precipitation as described above. This exonuclease III treatment created an approximately 600 bp gap. The gapped DNA was dissolved in 2 μl of TE and stored at −70 °C.

Phosphorylation of Oligonucleotides. Two hundreds pmoles of 5'-OH oligodeoxyribonucleotide (6.7 μM) were treated with 5 units of polynucleotide kinase for 50 min at 37 °C in 30 μl of 100 μM ATP, 100 mM Tris·HCl, pH 8.0, 10 mM $MgCl_2$, and 5 mM DTT.[12] The enzyme was inactivated by heating to 65 °C for 15 min. The final solution was stored at −70 °C.

Annealing of Oligonucleotide, Polymerization, and Ligation. The nicked and exonuclease-treated DNA (1.1 pmol of gapped circular pTY1) was heated to 65 °C for 7 min in the presence of about 44 pmol of the 5'-phosphorylated synthetic oligonucleotide in 40 μl of 40 mM NaCl, 20 mM Tris·HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, and then the solution was chilled on ice for 4 h.

This oligonucleotide annealed DNA was treated with 7.8 units of Klenow fragment and 2500 units of T4 DNA ligase at 10 °C overnight in 60 μl of 350 μM ATP, 175 μM dATP, 175 μM dCTP, 175 μM dGTP, 175 μM dTTP, 40 mM NaCl, 20 mM Tris·HCl, pH 7.5, 10 mM $MgCl_2$, and 1 mM DTT.[1-5] The resulting heteroduplex containing DNA was isolated as before by phenol extraction followed by ethanol precipitation.

Competent HB101 Cells. Five hundred mls of LB medium (5.0 g of Bacto-trypton, 2.5 g of Bacto-yeast extract, and 5.0 g of NaCl in 500 ml of H_2O) was sterilized and 5.0 ml of this LB medium was transferred to a sterile 16×100 mm Kimble culture tube, inoculated with a single colony of *E. coli* strain HB101, and shaken at 37 °C overnight. Two 250 ml flasks containing 100 ml of LB media with 0.2% glucose were autoclaved, inoculated with 1.0 ml of the overnight culture of HB101, shaken at 250 rpm at 30 °C for 8 h until the OD_{600} had reached 0.65, and then left on ice for 2 h. The intact flask of HB101 (the one not used for the OD measurements) was opened and the cell suspension was centrifuged at 6000 rpm for 5—8 min at 4 °C. After decantation of the supernatant fluid the cells were resuspended in ca. 50 ml of a cold sterile solution of 100 mM $CaCl_2$, 70 mM $MnCl_2$, and 40 mM sodium acetate, pH 5.5, left on ice for 30 min, and centrifuged again at 6000 rpm at 4 °C. The pellet was resuspended in 10 ml of cold 100 mM $CaCl_2$, 70 mM $MnCl_2$, and 40 mM sodium acetate, pH 5.5, and then 1.4 ml of sterile 80% glycerol was added. The competent cells were dispensed as 0.3 ml aliquots into about 34 culture tubes (Kimble, 16×100 mm), and stored at −70 °C.[10,12] These competent cells could

§1 M=1 mol dm^{-3}.

be kept more than 6 months without losing much of the transformation frequency (≈10^5).

Transformation, Growth of Bacteria, and Amplification of Plasmid. The concentration of the heteroduplex containing circular DNA was adjusted to ca. 0.1 μg DNA per 100 μl TE (pH 7.5). One hundred μl of this solution were incubated with 0.3 ml of competent HB101 cells, kept on ice for 60 min and then heat shocked at 42 °C for 2 min. To this was then added 2.6 ml of LB medium and the solution agitated at 37 °C for 60 min. In order to confirm the transformation, 100 μl of this solution was plated on two agar plates containing 50 μg of ampicillin per ml of medium and the remaining 2.8 ml used to inoculate a one liter LB medium containing the same concentration of ampicillin. The latter was incubated at 37 °C with agitation at 250 rpm until the culture had reached an OD_{650} of 0.5. At this point 4 ml of a solution of chloramphenicol (40 mg/ml in ethanol) was added for amplification of plasmids and the incubation was continued with agitation at 250 rpm at 37 °C for 15 h.[12]

Harvesting, Lysis, and Isolation of Plasmid DNA. Bacterial cells in the one liter culture were harvested, lysed, and the plasmid DNA isolated as in Ref. 12.

***Aha* III and *Bam*H I Digestion of Plasmid DNA and Isolation of Mutant 1 Kb Fragment.** Two hundreds micrograms of the plasmid DNA were digested with both 50 units of *Aha* III and 160 units of *Bam*H I for 16 h at 37 °C in 400 μl of 75 mM NaCl, 10 mM Tris·HCl, pH 8.0, 10 mM $MgCl_2$, and 5 mM DTT. (*Bam*H I digestion produces two fragments: 4.4 Kb fragment of a linear pBR322 derivative lacking the *Eco*R I site and 1 Kb fragment containing the DHFR gene. Of this 1 Kb fragment only a mutated gene at the Phe-31 site is resistant to the *Aha* III digestion because of the destruction of the *Aha* III recognition sequence (see Fig. 2).) The digestion mixture was loaded on a 3.5% polyacrylamide gel and a gel portion containing the 1 Kb fragment was cut out from the gel (the position of the 1 Kb fragment was identified by staining only the control lane containing the 1 Kb fragment with ethidium bromide and the mutant 1 Kb fragment was not exposed to the ethidium bromide). The gel portion containing the mutant 1 Kb fragment was placed in a dialysis bag with 0.1X TBE buffer (5 mM Tris base, 6.6 mM boric acid, 0.1 mM EDTA) and an electroelution[12] was carried out overnight in the same 0.1X TBE buffer at 8 mA at 4 °C. The mutant 1 Kb fragment was absorbed onto 0.1 ml of DE52 which had been prequilibrated in 100 mM NaCl, 50 mM Tris·HCl, pH 7.8, and 1 mM EDTA followed by equilibration with TE, and was eluted from the DE52 by running 300 μl of a high salt solution of 2 M NaCl, 50 mM Tris·HCl, pH 7.8, and 1 mM EDTA. The eluent was dialyzed against 500 ml of 10 mM Tris·HCl, pH 7.5 at 4 °C for 36 h with two changes and concentrated to 250 μl.

Ligation of Mutant 1 Kb Fragment with pBR322 Derivative at *Bam*H I Site. Dephosphorylation of a pBR322 derivative linearized at the *Bam*H I site was carried out, by treating 10 μg of a linear pBR322 derivative with 1.0 unit of alkaline phosphatase for 60 min at 37 °C in 50 μl of 50 mM Tris·HCl, pH 8.0, 1 mM $MgCl_2$, and 0.1 mM $ZnCl_2$, in order to avoid self-ligation and/or polymerization of the linear pBR322 derivative upon ligation with the mutant 1 Kb fragment. The dephosphorylated pBR322 derivative linearized at the *Bam*H I site was purified by phenol extraction followed by ethanol precipitation. One μg of the dephosphorylated 4.4 Kb fragment and 0.2 μg of the mutant 1 Kb fragment were treated with 400 units of T4 DNA ligase at 10 °C for 18 h in 50 μl of 0.5 mM ATP, 50 mM Tris·HCl, pH 7.5, 10 mM $MgCl_2$, and 10 mM DTT. The enzyme was inactivated by phenol extraction and the ligated nicked DNA was purified on a Sephadex G25 fine column. The eluent was used for the second transformation.

Second Transformation and Colony Screening. The second transformation was carried out using the ligated nicked DNA and the same procedure described above. Well-separated single colonies were numbered and seven colonies were arbitrarily chosen. For a rapid isolation of plasmid DNA the transformed HB101 cells were grown on a much smaller scale (15 ml LB medium) without amplification and the isolated DNA was partially purified by a treatment with DNase-free RNase in place of the cesium chloride-ethidium bromide gradients purification. These plasmid DNAs were digested with *Aha* III and *Bam*H I and those 1 Kb DHFR gene fragments which were resistant to the digestion were identified as mutant colonies.

Isolation of Mutant Plasmid DNA and Purification of *Bam*H I 1 Kb Fragment. Five ml of LB medium containing 50 μg of ampicillin per one ml of medium was inoculated with a mutant single bacterial colony. The bacteria were eventually grown in 2 l medium and each mutant plasmid DNA was isolated and purified by the cesium chloride-ethidium bromide gradients as described earlier. The purified mutant plasmid DNA was digested with *Bam*H I and the 1 Kb fragment was isolated as described earlier.

***Dde* I Digestion of 1 Kb Fragment, End-Labelling, and Sequencing.** About 1 μg of the *Bam*H I 1 Kb fragment was treated with 4 units of *Dde* I at 37 °C for 4 h in 20 μl of 100 mM Tris·HCl, pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, and 6 mM 2-mercaptoethanol. To this were then added 14 μl of H_2O, 4 μl of [α-^{32}P] dTTP (3200 Ci/mmol), and 0.7 μl of reverse transcriptase (16 units/μl), and the solution was kept at room temperature for 6.5 h. The reaction mixture was mixed with a loading dye and two fragments were isolated from a 3.5% polyacrylamide gel (the position of each fragment could be located by an autoradiogram). After both fragments were cut from the gel, they were eluted out (10 mA, 4 °C, overnight), collected on a DE52 column (≈0.05 ml resin). The column was eluted with 400 μl of 2 M NaCl, 50 mM Tris·HCl, PH 7.8, and 1 mM EDTA, the eluent then mixed with 1 μl of *t*-RNA (30 μg/μl), and the DNA fragment precipitated at −20 °C (6 h ethanol precipitation). The pellet was washed with 0.5 ml of cold 70% ethanol three times, dried in a speed vac for 10 min, and dissolved in 50 μl of TE and 5.5 μl of 3 M NaAc, pH 7.1. After an addition of 140 μl of ethanol, the mixture was kept at −20 °C for 1 h and centrifuged. The pellet was washed with 70% ethanol three times, dried in a speed vac, and redissolved in 50 μl of H_2O. The radioactivity of each sample varied from 700 to 3000 cps/5 μl. The DNA sequence of each fragment was determined by the general method[12,13] of Maxam and Gilbert with minor modifications. The DNA sequences were confirmed by autoradiogram.

Isolation of Mutant Enzymes. Five ml of LB medium containing 50 μg of ampicillin per one ml medium were inoculated with a single mutant colony. Cells for each mutant enzyme were grown in 2 l culture containing 50 μg of ampicillin/ml to a final optical density of 1.0 at 650 nm and harvested by centrifugation at 6000 rpm at 4 °C. The cells were suspended in 20 ml of 40 mM Tris·HCl, pH 8.0, 1

mM EDTA and centrifuged at 20000 rpm at 4 °C for 30 min to yield about 6 g of precipitate. All steps for the following cell lysis were conducted at 4 °C. The cells (ca. 6 g) were suspended in 7 ml of buffer (40 mM Tris·HCl, pH 8.0, 1 mM EDTA, 1 mM DTE (dithioerythritol)) with 0.7 μl of PMSF (phenylmethanesulfonyl fluoride) (17.4 mg/ml) and the following additions were made to the suspension: 1) 15 mg of lysozyme in 1 ml of the above buffer and 2.1 ml of EDTA (20 mg/ml in the above buffer) followed by sonication of the mixture for 30 sec then 8 min on ice (this sonication-cooling process was repeated two more times.); 2) 2.1 ml of Brij 58 (5 g/100 ml above buffer) followed by sonication for 30 s then 7 min on ice (this process was repeated two more times.); and 3) 330 μl of $MgCl_2$ (5.1 g/25 ml above buffer) and 500. μl of DNase (6.84 mg/3 ml above buffer) with occasional mixing for 15 min. The suspension was centrifuged at 20000 rpm for 40 min. To the supernatant 1.2 ml of streptomycin sulfate (1 g/10 ml above buffer) was added at 1 min intervals (100 μl/each time) with gentle stirring and the suspension then was centrifuged at 20000 rpm for 40 min. The supernatant (16.5 ml) was made 40% saturated in ammonium sulfate by gradually adding 3.85 g of finely powdered $(NH_4)_2SO_4$ over a period of 15 min with stirring. The stirring was continued for another 15 min. Unwanted protein was removed by centrifugation at 20000 rpm for 1.5 h. The supernatant was then made 90% saturated by gradually adding 5.8 g of $(NH_4)_2SO_4$ over a period of 20 min, and the mixture was gently stirred for additional 15 min and then kept on ice for 1.5 h. Following the centrifugation at 20000 for 30 min, the white pellet was resuspended in 13 ml of 40 mM KH_2PO_4, pH 6.0, 0.2 M KCl, 1 mM EDTA, and 1 mM DTE. To this was then added 2 ml of MTX (methotrexate) resin[14] and the solution gently shook for 20 h. The MTX resin was batch washed each time with 10 ml of 0.2 M KH_2PO_4, pH 6.0, 1 M KCl, 1 mM EDTA, and 1 mM DTE total four times. The MTX resin was transferred to a 10 ml column and washed with the same high salt buffer (0.2 M KH_2PO_4, 1 M KCl, 1 mM EDTA, 1 mM DTE) until the OD_{280} of the eluent was below 0.1. The mutant DHFR was eluted with 0.2 M K_3BO_3, pH 9.0, 1 M KCl, dialyzed against 2 l of 10 mM Tris·HCl, pH 7.4, 1 mM EDTA, and 1 mM DTE for 8 h with two changes, and purified on a column (52×1.1 cm) of DEAE-Sephacel with a linear gradient of 500 ml each of a solution containing 10 mM Tris·HCl, pH 7.4, 1 mM EDTA, 1 mM DTE, and a solution containing 1 M NaCl, 1 mM EDTA, 1 mM DTE. About 1 mg pure mutant DHFR was obtained per 1 g cells. The pure enzyme was made 90% saturated with $(NH_4)_2SO_4$ and stored at −20 °C. Although this DHFR is a Form I (Leu-28) isozyme, it is not necessary to make the eluting buffer 1 mM in folic acid since the mutant enzymes (Tyr-31 and Val-31) bind to the MTX resin less tightly than the wild-type DHFR. This affinity difference for the MTX resin enabled us to separate the mutant DHFR from possible contamination by the wild-type DHFR. The purity of the mutant DHFR was confirmed by the observation of a single band on a 12% polyacrylamide protein gel. There was no contamination by nucleic acids since the OD_{280}/OD_{260} ratio was 1.9 for both mutant enzymes.

Kinetics and Data Analysis. Initial velocities for dihydrofolate reductase were determined at 25 °C following the disappearance of NADPH and DHF at 340 nm (ε=11800 M^{-1} cm^{-1}). The buffer used for the assays contained 50 mM 2-morpholinoethanesulfonic acid (MES), 25 mM tris(hydroxymethyl)aminomethane (Tris), 25 mM 2-aminoethanol, and 100 mM NaCl (MTEN buffer).[15] The concentration of NADPH was maintained constant at 60 μM. The concentrations of DHF and the enzymes are listed in Table 1.

Data obtained at each pH value by varying the concentration of DHF were fitted to Eq. 3 by a nonlinear computer fitting program to yield values for the maximum velocity (V) and the Michaelis constant (K_a) as well as for V/K_a.

$$v = \frac{V[\mathrm{DHF}]}{K_a + [\mathrm{DHF}]} \tag{3}$$

Results

Two mutant plasmid DNAs (Tyr-31 and Val-31) have been constructed by the procedure illustrated in

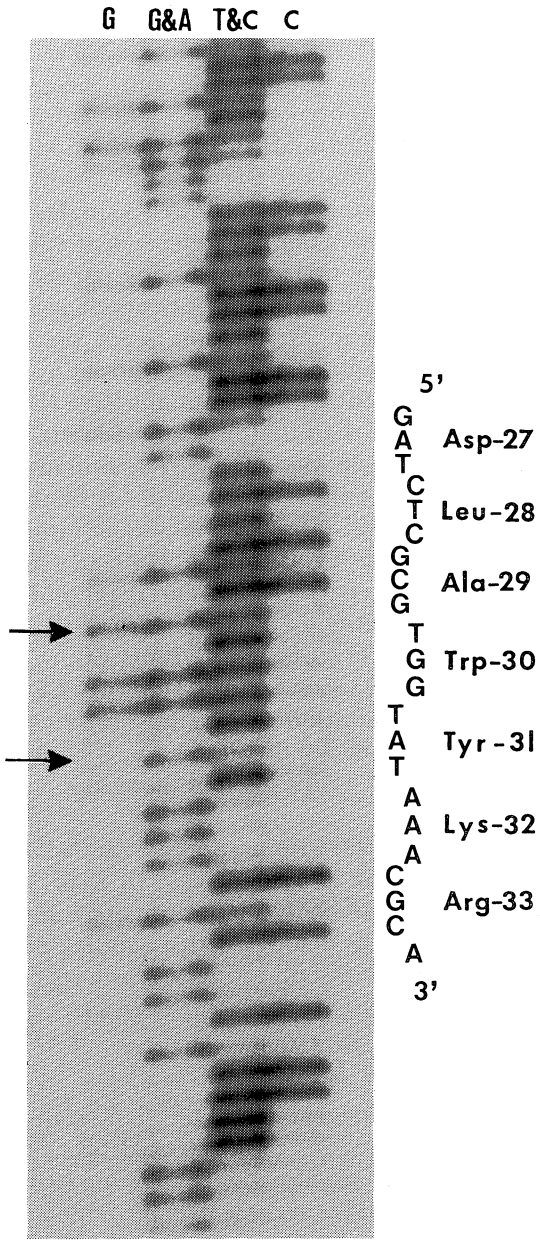


Fig. 3. Maxam-Gilbert sequencing ladder of Tyr-31 plasmid around the mutated *Aha* III site. Two replacements (TA→A and C→G, see Fig. 2(a)) are indicated by arrows.

Table 1. Summary of pH-Independent Values of V, V/K_{DHF}, and K_{DHF}, for Tyr-31 and Val-31[a]

	V/s^{-1}	V/K / $10^{-6}\,M^{-1}\,s^{-1}$	$K_{DHF}/\mu M$
Wild-type	12	11	1.1[b]
Tyr-31	30	5.3	5.7
Val-31	26	0.97	27

a) Conditions: 60 μM NADPH, 3.25—120 μM H_2-folate, 1.1—500 nM (Tyr-31)-DHFR, 6.1—730 nM (Val-31)-DHFR, MTEN buffer, 25 °C, 340 nm. b) Taken from Ref. 15.

Fig. 2. The DHFR coding sequences in these plasmids have been determined by the chemical method of Maxam and Gilbert[12,13] and the sequencing ladder of Tyr-31, as an example, around the mutated *Aha* III site is shown in Fig. 3 with the replacements marked by arrows. The whole structural genes have been completely sequenced and no mutations, other than designed ones, have been detected. The two mutant enzymes were isolated and purified to homogeneity. The purity of the mutant DHFR was confirmed by SDS gel electrophoresis which showed a single band.

Table 1 summarizes the pH independent values of V, V/K, and K_M (see the accompanying paper) determined for the purified mutant enzymes, Tyr-31 and Val-31. The kinetic experiments were performed under conditions similar to those employed by Stone and Morrison.[15] Both of these mutants have V values significantly larger than that of wild-type (wt), while the V/K values are smaller. The Tyr-31 mutant has a 2.5 fold higher V value than that of wt in the pH independent region, indicating that, under saturating DHF conditions, the Tyr-31 mutant is a better catalyst than the wild-type enzyme. The Tyr-31 mutant has a two-fold smaller value for V/K_{DHF} owing to a 5-fold increase in K_{DHF} at low pH. At high pH values, however, the K_{DHF} value increases to 300 μM (pH 9.5), possibly due to the ionization of Tyr, drastically decreasing the hydrophobicity of the side chain. The detailed pH-rate profile will be discussed in the accompanying paper.

The effect of the Val-31 mutation is again a 2.2-fold increase in V and a 25-fold increase in K_{DHF}. This increase in K_{DHF} is probably a reflection of a higher K_D value for H_2-folate, by analogy with a parallel increase in the dissociation constant estimated from its behavior during purification from the methotrexate resin.

Discussion

Site-Directed Mutagenesis. The first controlled modifications of proteins were reported two decades ago using chemical reactions to change the hydroxyl group of a serine residue at the active site of the proteolytic enzyme subtilisin into a mercapto group.[16,17] The problem with chemical modification is that it is harsh and nonspecific. Unless great care is taken, an attempt for example to exchange a serine for a cysteine at the active site may result in exchanging all serines on the surface of the enzyme for cysteines with unpredictable results. Furthermore, many desired changes, as in our Phe-31 → Tyr and Val mutations, cannot be achieved with chemical techniques, beside the fact that many of these residues are in the interior of the enzyme and inaccessible to a reagent. Advances in genetic engineering technology in the last few years, however, have made amino acid substitutions "almost routine."[1-6]

The procedure described in Fig. 2 takes advantage of destroying a restriction site upon successful incorporation of a synthetic oligodeoxynucleotide primer and isolating a new restriction fragment. Even if such a convenient restriction site is not available, the colony hybridization technique,[1,18] with the same oligodeoxynucleotide labeled with ^{32}P as a hybridization probe, allows identification of the desired mutant colony regardless of its phenotype. After the second transformation, the plasmid DNA of each mutant was characterized not only in the region of the mutation but also the entire structural gene and shown to have the desired sequence (see Fig. 3).

DHF Binding Site. Nonpolar interactions between the pteridine ring and the enzyme originate with the side chains of residues Ile-5, Ala-7, Phe-31, and Ile-94 providing most of the van der Waals contacts.[8] Among these residues only Ala-7 and Phe-31 are strictly conserved, although all of them are hydrophobic in all the known sequences. The *p*-aminobenzoyl moiety occupies a hydrophobic pocket formed by the helix αB on one side and by the loop (Gly-51 to Gly-56) connecting αC to βC on the other. In particular, the side chains of Leu-28 and Phe-31 extending from αB interact with the inner face of the *p*-aminobenzoyl group. This Phe-31, but not the Leu-28, is strictly conserved in all known sequences.[8]

A closer examination of the DHF (dihydrofolate) binding site reveals that the *p*-aminobenzoyl moiety is almost perpendicular to the pteridine ring with the Phe-31 at the corner of the bend. We felt that this bend could be important for the catalysis because of the following reasons. The enzyme catalyzes its reaction by having an active site that is complementary in structure to the structure of the transition state of the substrate rather than the substrate itself.[19] During the reduction of H_2-folate to H_4-folate (Eq. 1), the hybridization of C-6 carbon changes from sp^2 to sp^3 accompanying the movement of the C-9 carbon of the side chain from its original in-plane position at sp^2 to the out of plane from the pteridine ring at the sp^3 state (see Eq. 2). If the DHF binding site is designed such that it stabilizes the transition state or product more favorably than the substrate, H_2-folate, some of the intrinsic binding energy is utilized for catalysis.[20] The Phe-31, which is located at the corner of the bend interacting with both the pteridine ring and the *p*-aminobenzoyl moiety, could be a part of an interacting network.[21-25]

Replacement of Phe-31 by a smaller group as in the site-directed mutagenesis experiment of Phe-31 → Val-31, should decrease the affinity of the mutant enzyme for the substrate to some extent depending on the intrinsic binding energy of the mutant enzyme, but the affinity for the transition state would be decreased to a greater extent. This means that the value of K_{DHF} may be increased but the value of k_{cat} will fall. The steady-state kinetic parameters for the Tyr-31 and Val-31 mutants listed in Table 1 are, at first glance, not supportive of the transition state stabilization hypothesis. That the increase in V for the Tyr-31 mutant may result from the facilitated protonation of N5 necessary for reduction is unlikely since a similar increase in V is observed for the Val-31 mutant. However, additional experiments have revealed that the steady-state parameters mask any changes in the hydride transfer step. Pre-steady state stopped-flow measurements convincingly implicate the rate of dissociation of H_4-folate as mainly rate-limiting at this pH.[29] Consequently the increase in V noted in the two mutations arises simply from an increased rate of product dissociation that is reflected in the higher K_D of H_2-folate. Because the hydride transfer step is still not rate-limiting, one cannot readily assess the effect of the mutations in terms of the transition state stabilization hypothesis. The interpretation of the effects of mutations based on steady state parameters is always subject to ambiguities unless one known step in the kinetic sequence is dominant.

Although the mutants we have generated are not necessarily the evolutionary precursors of the present native enzyme, they are possible forms of the enzyme that have been rejected during evolution, since the rate of spontaneous mutation is sufficiently high for them to have arisen and hence been tested. For the sake of the argument, however, let us assume that each of the mutant enzymes, Tyr-31 and Val-31, is an evolutionary precursor of the native Phe-31 enzyme. Various proposals have been made as to how enzymic rate constants should respond to selective pressure in evolution to maximize the overall rate of reaction[30,31] by stabilizing both transition state and substrate-enzyme complexes. This kind of evolutional replacement leads to an increase in rate as long as [S] remains below K_M; other wise ([S]>K_M), stabilization of (ES) and (ES)$^{\neq}$ equally has a neutral effect (no change in rate).[31] Since the concentration of dihydrofolate in vivo is estimated to be below 1 μM, the Tyr-31 and Val-31 mutant enzymes, despite their higher turnover numbers, would be less effective in maintaining H_4-folate levels owing to their greater K_{DHF} values.

In conclusion, the present study has demonstrated that a protein engineering at the level of DNA is a powerful technique for elucidation of the structure-reactivity relationship in an enzyme. Specifically, in both Tyr-31 and Val-31 mutant enzymes the decrease in binding has not been translated into a loss of catalytic efficiency and both the mutant enzymes have generated an improved k_{cat}, most probably by facilitating the product-release step without significantly altering the hydride transfer step.

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