Construction of an Altered Proton Donation Mechanism in *Escherichia coli* Dihydrofolate Reductase†

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ABSTRACT: We have explored the substrate protonation mechanism of *Escherichia coli* dihydrofolate reductase by changing the location of the proton donor. A double mutant was constructed in which the proton donor of the wild-type enzyme, aspartic acid-27, has been changed to serine and simultaneously an alternative proton donor, glutamic acid, has replaced threonine at position 113. The active site of the resulting variant enzyme molecule should therefore somewhat resemble that proposed for the R67 plasmid-encoded dihydrofolate reductase [Matthews, D. A., Smith, S. L., Baccanari, D. P., Burchall, J. J., Oatley, S. J., & Kraut, J. (1986) *Biochemistry 25*, 4194]. At pH 7, the double-mutant enzyme has a 3-fold greater k_{cat} and an unchanged $K_{m(dihydrofolate)}$ as compared with the single-mutant Asp-27 → Ser enzyme described previously [Howell, E. E., Villafranca, J. E., Warren, M. S., Oatley, S. J., & Kraut, J. (1986) *Science (Washington, D.C.) 231*, 1123]. Additionally, its activity vs pH profiles, together with observed deuterium isotope effects, suggest that catalysis depends on an acidic group with a pK_a of 8. It is concluded that the dihydropteridine ring of a bound substrate molecule can indeed be protonated by a glutamic acid side chain at position 113 (instead of an aspartic acid side chain at position 27), but with greatly decreased efficiency: at pH 7, the double mutant still has a 25-fold lower k_{cat} (1.2 s^{-1}) and a 2900-fold lower $k_{cat}/K_{m(dihydrofolate)}$ (8.6×10^3 s^{-1} M^{-1}) than the wild-type enzyme.

Dihydrofolate reductase (DHFR)[1] (EC 1.5.1.3) catalyzes the following reaction: dihydrofolate (DHF) + NADPH + H^+ → tetrahydrofolate (THF) + $NADP^+$. This activity is necessary to maintain intracellular pools of THF cofactors essential for biosynthetic reactions involving the transfer of one-carbon units. Inhibitors of DHFR are clinically important and include the antitumor drug methotrexate (MTX), the antibacterial trimethoprim (TMP), and the antimalarial pyrimethamine.

DHFR has been extensively studied by kinetic methods (Stone & Morrison, 1982, 1984a,b; Fierke et al., 1987), NMR spectroscopy (Hammond et al., 1986; Searle et al., 1986; London et al., 1986), and X-ray crystallography (Matthews et al., 1977, 1985; Bolin et al., 1982; Filman et al., 1982). In particular, the X-ray structures have delineated important interactions between ligands in the substrate binding pocket and the enzyme molecule. These interactions have recently become the focus of site-directed mutagenesis experiments which attempt to define the precise role of specific amino acids in ligand binding and catalysis (Villafranca et al., 1983; Chen et al., 1985; Howell et al., 1986; Mayer et al., 1986).

It is generally believed that the dihydropterin ring of substrate must be protonated at N5 to facilitate hydride transfer from NADPH to C6. A conserved carboxylic acid side chain, Asp-27, in the *Escherichia coli* enzyme seems to be a logical candidate for the enzymic group involved in substrate protonation as no other proton-donating group in the enzyme is near N5. However, the carboxylic acid side chain of Asp-27 is 6.3 Å away from the N5–C6 double bond and forms hydrogen bonds not to N5 but to N3 and the 2-amino group of the pteridine ring (Bolin et al., 1982; Chris Bystroff, personal communication). In one mutagenesis experiment, the role of Asp-27 was investigated by replacing it with asparagine (Villafranca et al., 1983; Howell et al., 1986). The resulting Asp-27 → Asn DHFR suffers a 300-fold decrease in k_{cat} at pH 7.0 but has a drastically altered pH profile such that catalysis increases rapidly as pH decreases. These results argue that Asp-27 is indeed important for protonation of bound, unprotonated substrate but that an enzyme-mediated protonation step can be bypassed if preprotonated substrate is bound. This bypass occurs more frequently at low pH as the pK_a value of 3.8 for N5 of the pteridine ring of DHF is approached.

While the identity of the enzymic proton donor was confirmed by the Asp-27 → Asn mutagenesis experiment, the route of protonation in the wild-type (wt) enzyme was not further clarified. Because of their relative positions, a mechanism involving direct protonation of N5 by Asp-27 does not seem possible. However, the proton might be relayed from Asp-27 to N5 via a fixed, invariant water molecule, water-403. Further, an unresolved question is whether the Asp-27 side chain becomes anionic at any stage, providing Coulombic stabilization for a positively charged, protonated transition state of the substrate.[2] In fact, it is possible to imagine a pro-

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[1] Abbreviations: DHFR, dihydrofolate reductase; DHF, dihydrofolate; THF, tetrahydrofolate; wt, wild type; TMP, trimethoprim; MTX, methotrexate; D27S DHFR, Asp-27 → Ser mutant DHFR; D27S + T113E DHFR, Asp-27 → Ser + Thr-113 → Glu double-mutant DHFR; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)-aminomethane; MES, 2-(*N*-morpholino)ethanesulfonic acid.

[2] Our earlier conclusion (Howell et al., 1986) that an ionized Asp-27 does not contribute to stabilization of the transition state for hydride transfer must be withdrawn in light of the finding by Fierke et al. (1987) that the rate-limiting step in DHFR at pH 7.0 is THF release. A more probable alternative is that either mutation (Asp-27 → Asn or Asp-27 → Ser) simultaneously causes an increase in the rate of THF release and a decrease in the rate of hydride transfer. The latter rate now becomes limiting, but the net rate for turnover of preprotonated substrate in the mutants is increased by a factor of 1.6.

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tonation mechanism involving keto–enol tautomerization at the pteridine ring whereby protons are shifted in an eight-membered ring that includes water-403, O4, N3, and Asp-27, and Asp-27 is left in the protonated state. It is not clear, however, how the proton might then get from O4 to N5. A recently suggested alternative is that N5 need not be protonated in the first place; protonation of O4 may be sufficient to achieve the transition state for hydride transfer. Ab initio quantum mechanical calculations on a model compound, dihydropterin, indicate that protonation at O4 results in significant π electron depletion at C6, which could facilitate hydride transfer from NADPH in the enzymic transition state (Gready, 1985).

With the above as background, we can now ask the following: Can the proton donor be moved to a different position in the active site and still facilitate enzyme-mediated protonation of the substrate? In this report, we describe our efforts to answer this question. We also report preliminary studies on the effects of such alterations on ligand binding and molecular stability.

We have constructed a double-mutant gene which changes the location of the proton donor in the pteridine binding pocket of the enzyme molecule. Although a location near N5 would be one obvious candidate for this experiment, an already existing alternative model can be found in the recently determined X-ray structure for the R67 plasmid DHFR (Matthews et al., 1986). This unusual, nonchromosomally encoded DHFR is reported to be a tetramer of four identical subunits (Smith et al., 1979). The 78-residue monomer has no sequence homology with any known chromosomal DHFRs. The structure of a dimeric form of R67 DHFR without bound ligands has been determined (Matthews et al., 1986) and compared with the structure of the chromosomally encoded DHFR from *E. coli*. While *E. coli* DHFR is a 159-residue monomer composed of an eight-stranded β sheet with four α helices connecting successive β strands, the crystallized R67 DHFR dimer is composed of two β barrels which form a third β barrel at the subunit interface. Comparison of the pteridine binding pocket of *E. coli* DHFR with a *proposed* pteridine binding pocket in R67 DHFR has shown no structural similarities except for two amino acids which may interact with N3 and the 2-amino group of the pteridine ring. These amino acids are Asp-27 and Thr-113 in the *E. coli* DHFR structure, and their positions coincide geometrically with Thr-48 (subunit I) and Glu-59 (subunit II) in the R67 DHFR structure. This comparison suggests that the positions of the acidic group and the threonine side chain relative to the pteridine ring of bound substrate may be reversed in the two different enzyme molecules. Matthews et al. (1986) have therefore suggested that "while a proton-donating group in the neighborhood of bound substrate is essential for ring protonation preceding hydride transfer, perhaps the exact location of this side chain with respect to the dihydrofolate molecule may be less crucial".

In order to ascertain if the carboxylic acid and hydroxyl side chain functionalities can be geometrically reversed in the *E. coli* DHFR active site and still result in an enzyme molecule capable of protonating substrate, a double-mutant DHFR gene was constructed which contains the following two mutations: Asp-27 → Ser and Thr-113 → Glu (D27S + T113E). The catalytic properties of the double-mutant enzyme were compared with those of the wt and also with those of the single-mutant Asp-27 → Ser (D27S) DHFR which was previously characterized (Howell et al., 1986). An initial comparison was also made with the single-mutant Thr-113 → Glu (T113E) DHFR and with the R67 DHFR.

Materials and Methods

Mutant and wt DHFRs were expressed and purified as previously described (Villafranca et al., 1983; Howell et al., 1986). Prior to all binding or kinetic studies on mutant enzymes, any contaminating wt DHFR due to expression of the *E. coli* chromosomal gene was removed by differential elution from a methotrexate affinity column (Baccanari et al., 1981).

Ultraviolet difference spectroscopy was performed using split cell cuvettes in a λ3a Perkin-Elmer spectrophotometer linked to a 3600 data station (Hood & Roberts, 1978; Stone & Morrison, 1983; Howell et al., 1986). Enzyme and ligand were placed in separate compartments of the cuvette, and a base-line spectrum was recorded. Subsequently, the contents of one cuvette were mixed, and the mixed spectrum was recorded. The base-line spectrum was subtracted from the mixed spectrum to give the difference spectrum associated with ligand binding to the enzyme. Experimental conditions for the wt DHFR + methotrexate spectrum were given previously (Howell et al., 1986). The enzyme concentration used for the D27S + T113E spectrum was 10 μM, and the methotrexate concentration was 45 μM.

Equilibrium dialysis experiments to determine K_d values for MTX were performed as described by Pattishall et al. (1976) utilizing modifications suggested by Baccanari et al. (1981). In this technique, the enzyme and various concentrations of [^{3}H]MTX (New England Nuclear) were mixed in 0.1 M Na_2HPO_4, pH 7.0, buffer containing 2 mM dithiothreitol, 0.1 mM EDTA, and sufficient bovine serum albumin to give an 8:1 molar ratio of albumin to the enzyme. One-milliliter aliquots of enzyme + MTX were loaded into one side of the dialysis chamber, and 1 mL of buffer was loaded into the second chamber. The apparatus was mixed on a platform shaker (120 rpm at 4 °C). Equilibration of the label typically occurred in 24 h. The radioactivity distribution in both chambers was monitored in triplicate in Aquasol 2. The K_d and stoichiometry of binding were calculated by using the equation:

$$[L]/[EL] = K_d/n[E] + [L]/n[E] \quad (1)$$

where [L], [E], and [EL] are the concentrations of the free ligand, total enzyme, and enzyme–ligand complex, respectively, K_d is the dissociation constant for the EL complex, and n is the stoichiometry of binding (Pattishall et al., 1976). Linear regression of a [L]/[EL] versus [L] plot yielded a slope of $1/n[E]$ and an x intercept of $-K_d$.

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 MPF44A fluorometer. Quenching of the fluorescence of a 2-mL solution [1 μM DHFR in 50 μM acetic acid + 50 μM MES + 100 μM Tris + 10 mM β-mercaptoethanol polybuffer, pH 7.0 (Ellis & Morrison, 1982)] was observed by the addition of small (0.005-mL) 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 the nonlinear least-squares program MINPACK (Argonne National Laboratory).

Steady-state kinetic data were obtained on a Perkin-Elmer λ3a spectrophotometer linked to a 3600 data station. The Perkin-Elmer IFL3 programs were utilized to collect the data and calculate initial rates. 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 substrate ranges utilized were 3–250 μM for DHF and 1–100

μM for NADPH. Assays were initiated by the addition of 0.01 mL of E·NADPH solution to 0.41 mL of buffer + dihydrofolate. 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 becomes problematic at low pH values (Hillcoat et al., 1967). Initial rates were obtained in triplicate and analyzed by substrate/velocity versus substrate plots.

NADPD was formed by the reaction of alcohol dehydrogenase from *Leuconostoc mesenteroides* (Boehringer Mannheim) on 1,1-dideuterioethanol (MSD Isotopes) and $NADP^+$ (Stone & Morrison, 1984a). The alcohol dehydrogenase reaction was coupled with $NADP^+$ aldehyde dehydrogenase (Sigma) to allow the reaction to approach completion (Viola et al., 1979; Hermes et al., 1984). The dehydrogenases were removed from the NADPD by ultrafiltration through a YM-10 membrane. Any unreacted $NADP^+$ was removed by chromatography on a DEAE Fractogel column (1 × 14 cm) using a 0–0.4 M KCl gradient (Vanoni & Matthews, 1984). The NADPD was subsequently desalted by chromatography on a Bio-Gel P-2 column (0.8 × 57 cm) (Viola et al., 1984) and used immediately and/or lyophilized. The A_{260}/A_{340} ratio of any pooled fractions was <2.2. A portion of the NADPD was analyzed by proton magnetic resonance spectroscopy in a 360-MHz hand-fabricated instrument (Wright et al., 1980) in order to evaluate the ^1H content at the 4-position of NADPD. The NADPD was found to contain >95% ^2H (Oppenheimer et al., 1978).

Dihydrofolate was prepared by the reduction of folic acid with sodium dithionite (Blakley, 1961) and stored at −20 °C in 5 mM HCl and 50 mM β-mercaptoethanol. The concentrations of dihydrofolate, NADPH, TMP, and MTX were determined spectrophotometrically using molar extinction coefficients of 28 000 M^{-1} cm^{-1} at 282 nm (Blakley, 1961), 6220 M^{-1} cm^{-1} at 340 nm (Penner & Frieden, 1985), 7250 M^{-1} cm^{-1} at 287 nm pH 13 (Stone & Morrison, 1986), and 22 100 M^{-1} cm^{-1} at 302 nm (pH 13) (or 23 250 M^{-1} cm^{-1} at 258 nm, pH 13) (Stone & Morrison, 1986), respectively.

Results and Discussion

Mutagenesis. The single-stranded form of the mutant Ser-27 DHFR gene inserted in M13mp8 (Howell et al., 1986) was purified, and a 25-base-long oligonucleotide designed to change Thr-113 (ACG codon) to Glu (GAA) was annealed. Mutagenesis proceeded as per standard protocol (Smith, 1982; Zoller & Smith, 1982). The double mutant was identified by dot blot hybridization techniques, and the entire DHFR gene was sequenced by the Sanger dideoxy method (Sanger et al., 1977, 1980) to ascertain that no additional mutations had occurred. The presence of the Glu-113 mutation could also be monitored by the loss of Hga 1 (GACGC) restriction site. The double-mutant DHFR gene was then cloned into pUC8 for protein expression.

Physical Characterization of the Double-Mutant Ser-27 + Glu-113 Protein. The amount of D27S + T113E protein produced from *E. coli* containing the gene cloned in pUC8 was found to be greatly reduced when compared to wt DHFR protein production under similar conditions. While typical yields of wt and D27S DHFR are 8–25 mg of protein/L of *E. coli* culture, the yield of D27S + T113E DHFR is only 0.4–2.5 mg/L.

Addition of the DHFR inhibitor trimethoprim (TMP, 5 μM) was found to stabilize the double-mutant protein throughout both the cell growth cycle and the purification protocol. Bound TMP was subsequently removed by passing the double-mutant protein over a methotrexate affinity column.

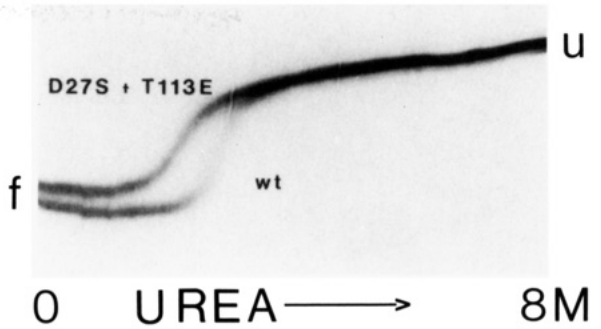


FIGURE 1: Urea gradient polyacrylamide gel showing the denaturation profiles of a 1:1 mixture of wt and D27S + T113E DHFRs. The letters f and u indicate positions of folded and unfolded forms, respectively. Electrophoresis was performed as described by Villafranca et al. (1987). The bottom of the gel is the anode end.

This affinity chromatography step also removes any contaminating wt DHFR which may be present due to expression of the *E. coli* chromosomal DHFR gene. While the double-mutant DHFR (and also the D27S) is eluted with a 0.5 M KCl wash, the wt enzyme requires substrate and high-pH/high-salt conditions in order for substantial elution to occur (Baccanari et al., 1981).

The stability of the double-mutant DHFR was investigated by urea gradient gel electrophoresis. A 1:1 mixture of wt DHFR and D27S + T113E DHFR was applied to a 0–8 M urea gradient gel. Figure 1 shows a substantial decrease in stability associated with the double-mutant enzyme. The D27S enzyme (not shown) has the same stability as wt DHFR in this gel system even though it has been found, by differential scanning calorimetry (J. Sturtevant, personal communication), that the D27S enzyme is slightly more stable than the wt with respect to thermal denaturation.

Also apparent from Figure 1 is the altered electrophoretic mobility of the double-mutant enzyme as compared to wt DHFR under nondenaturing conditions. The double mutant migrates more slowly than wt DHFR, and D27S DHFR migrates at an intermediate position between these two (not shown). This mobility difference implies an alteration in charge and/or conformation associated with the double mutation.

In order to evaluate a possible charge change, the isoelectric points (p*I*'s) of the various DHFRs were monitored by marker analysis in nondenaturing isoelectric focusing slab gels. At 10 °C, the wt DHFR p*I* value was 4.72, and p*I* values for D27S and D27S + T113E enzymes were both approximately 4.88. A mixture of D27S and D27S + T113E does resolve into two bands, indicating that the p*I*'s are not equivalent. However, the difference between them is slight.

The observation that D27S has a higher p*I* than wt DHFR is expected as an acidic residue, Asp-27, has been replaced by a neutral residue. However, restoring an acidic group (Glu-113), albeit at a different position, does not result in a decrease in p*I* for D27S + T113E to 4.72, the wt value. This result suggests that the active-site environment in DHFR stabilizes a higher pK_a for the Glu-113 residue in the double mutant as compared to the pK_a of Asp-27 in the wt enzyme.

Inhibitor Binding. The capacity of D27S + T113E DHFR to protonate N1 of MTX was investigated by difference spectroscopy. MTX bound to wt DHFR (Figure 2A) has a spectrum similar to protonated MTX, indicating N1 protonation of bound MTX at pH 7.0 (Poe et al., 1974; Hood & Roberts, 1978; Howell et al., 1986). The interaction between Asp-27 and MTX in the wt enzyme is strong, resulting in elevation of the N1 pK_a of bound MTX by at least 4 pH units,

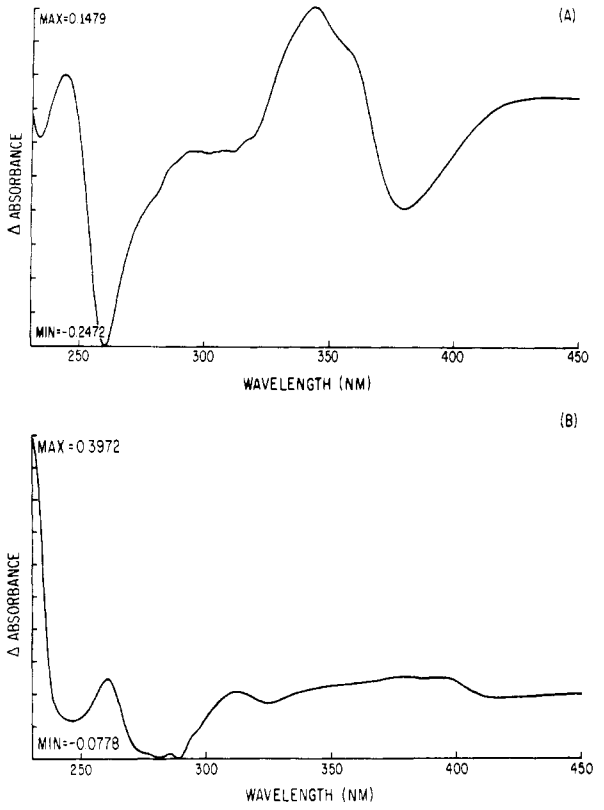


FIGURE 2: Methotrexate (MTX) difference spectra. (A) MTX bound to wt DHFR versus free enzyme plus free MTX in solution. (B) MTX bound to D27S + T113E DHFR versus free enzyme plus MTX in solution. Difference spectra were obtained as described by Howell et al. (1986).

from 5.8 (free) to >10 (bound) (Cocco et al., 1981; Stone & Morrison, 1983). As expected, the D27S mutant enzyme has previously been shown *not* to protonate bound MTX (Howell et al., 1986; London et al., 1986). The D27S + T113E double-mutant enzyme also does not protonate bound MTX, as shown by the dissimilarity between its difference spectrum (Figure 2B) and the protonated difference spectrum seen in Figure 2A. Although this double-mutant enzyme has an acidic group, Glu-113, in its active site, this particular configuration does not result in a stable ionic interaction between protonated N1 of MTX and anionic Glu-113.

The dissociation constant describing binding of [^{3}H]MTX to D27S + T113E DHFR was obtained by equilibrium dialysis methods at 4 °C. This value is 60 nM at pH 7.0 in phosphate buffer. Comparison with the previously obtained wt DHFR and D27S DHFR K_d values of 0.068 and 55 nM, respectively (Howell et al., 1986), shows an 880-fold increase from wt DHFR and virtually no change from D27S. From these results, it can be seen that the Thr-113 → Glu mutation, on top of the Asp-27 → Ser mutation, has no further deleterious effects on MTX binding at pH 7.0. The loss of affinity of D27S + T113E DHFR for MTX is due to the presence of the Ser-27 substitution.

Binding of TMP to the D27S + T113E DHFR was evaluated by obtaining kinetic K_i values which describe binding of TMP to DHFR·NADPH to form a ternary complex. TMP inhibition of the D27S + T113E mutant enzyme was monitored at pH 7.0, and Figure 3 illustrates the competitive nature of the inhibition. A K_i value of 17 μM was obtained. This compares with 20 pM and 1.2 μM K_i values previously obtained for the wt (Stone & Morrison, 1986) and D27S (London et al., 1986) enzymes, respectively. Again, the major loss in binding affinity for TMP is due to the Asp-27 → Ser

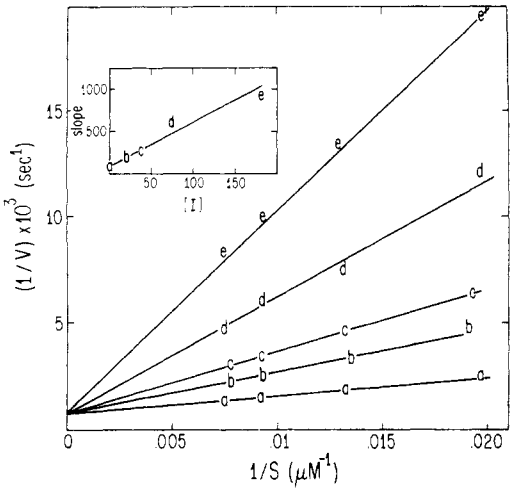


FIGURE 3: Inhibition of D27S + T113E DHFR by trimethoprim (TMP). Lineweaver–Burk plots (reciprocal velocity versus reciprocal substrate concentration) showing inhibition of the double-mutant enzyme at various concentrations of TMP. The (a) points indicate no TMP; (b) 18.9 μM TMP; (c) 37.8 μM TMP; (d) 76.4 μM TMP; and (e) 185 μM TMP. The inset plots inhibitor concentration versus slope to obtain an x intercept of $-K_i$.

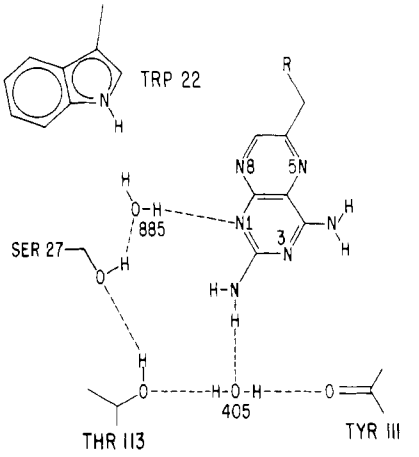


FIGURE 4: Schematic representation of hydrogen bonding between MTX and active-site residues in Asp-27 → Ser DHFR; based on 1.9-Å crystal structure (Howell et al., 1986).

substitution. The 14-fold difference between D27S and D27S + T113E DHFR is slight in comparison with the (6 × 10^4)-fold difference seen between D27S and wt DHFR.

Molecular Modeling. Because MTX binding at pH 7.0 is unaffected by the presence of a Glu-113 mutation, the D27S DHFR·MTX crystal structure (depicted in Figure 4) was used as the initial structure for modeling the active site of the D27S + T113E double mutant (Howell et al., 1986). Using the INSIGHT (Biosym) program on an Evans and Sutherland PS300 picture system, we replaced Thr-113 in the D27S DHFR·MTX structure with glutamic acid. Two plausible positions were found for the Glu-113 side chain. First, it can displace a conserved water molecule, 405. This water molecule normally forms hydrogen bonds with the C2 amine of MTX, with OG of Thr-113, and with the backbone carbonyl of Tyr-111. In this model, OE2 of the Glu-113 substitution can potentially form a new hydrogen bond to the C2 amine group of MTX. This model would therefore predict no net loss of hydrogen bonds to MTX and could be taken to imply there should be no change in the tightness of MTX binding to D27S + T113E DHFR as compared with binding to D27S DHFR. This is in accord with the results cited above.

In the second plausible model for the active-site geometry of the double-mutant enzyme, the Glu-113 residue can par-

tially occupy the van der Waals gap observed in the D27S DHFR·MTX crystal structure. This positioning does, however, require that both the Ser-27 OG and water-885 move toward Trp-22 by approximately 1.2 Å to minimize any overlaps between their van der Waals surfaces. In this position, the OE2 of Glu-113 can form an additional hydrogen bond with NA2 of MTX, which could be taken to predict tighter binding of MTX to the double-mutant enzyme. This is not in agreement with the observations described above.

Kinetic Analysis. Since both the Ser-27 and Glu-113 mutations occur in the DHF binding site, a preliminary evaluation of the effect of these mutations on the overall conformation of the DHFR molecule is provided by monitoring the binding of NADPH. The K_m values obtained for NADPH binding to the wt, D27S, and D27S + T113E DHFRs at pH 7.0 are 0.9, 1.7, and 2.6 μM, respectively. These values were obtained from steady-state kinetic data (both NADPH and DHF concentrations varied) and describe binding of NADPH to the enzyme–DHF complex to form a ternary complex. Thus, the D27S + T113E enzyme shows a 3-fold increase in K_m for NADPH when compared to wt, indicating a minor effect on ternary complex formation. Additionally, K_d values for NADPH binding to wt, D27S, and D27S + T113E DHFRs at pH 7.0 are 0.043, 0.069, and 2.2 μM, respectively. These values were obtained by fluorescence quenching and describe binary complex formation between DHFR and NADPH. Comparison indicates that the D27S + T113E mutations have a 50-fold weakening effect on NADPH binding to the wt apoenzyme. This 50-fold effect on K_d implies that there may be slight conformational changes in the double-mutant enzyme.

From the above, it can be seen that both the wt and D27S DHFRs have K_d's for NADPH binding which are less than K_m's. This binding difference in the formation of binary versus ternary complexes has been seen previously for the wt enzyme (Baccanari et al., 1977, 1981; Cayley et al., 1981; Stone & Morrison, 1982, 1984b; Penner & Frieden, 1987) and indicates that NADPH is a sticky substrate (Cleland, 1982). Since the D27S + T113E enzyme has a K_d equal to its K_m, NADPH is not a sticky substrate for this double-mutant enzyme. Such behavior ($K_{d,NADPH} = K_{m,NADPH}$) has also been observed for the wt enzyme at higher ionic strength conditions (0.1 M NaCl; Fierke et al., 1987), indicating that the behavior of the D27S + T113E enzyme still lies within a wide "normal" range.

Steady-state kinetic parameters obtained for D27S + T113E DHFR at pH 7.0, 30 °C, were $k_{cat} = 1.2\ s^{-1}$, $K_{m(dihydrofolate)} = 140\ \mu M$, and $k_{cat}/K_{m(dihydrofolate)} = 8.6 \times 10^3\ s^{-1}\ M^{-1}$. They may be compared with kinetic parameters previously obtained for wt and D27S DHFRs and also for the R67 DHFR, which are listed in Table I. A 3-fold *increase* in both k_{cat} and k_{cat}/K_m is seen for the D27S + T113E mutant enzyme as compared with the D27S mutant enzyme at this pH. (At pH 8, a 7.5-fold *increase* in k_{cat} is noted when the D27S + T113E enzyme's activity is compared to the activity of D27S DHFR.) No effect on $K_{m(dihydrofolate)}$ is observed. Obviously, the double-mutant enzyme is still much slower (25-fold lower k_{cat}) and less efficient (2900-fold lower k_{cat}/K_m) than the wt enzyme. The k_{cat} value for the R67 DHFR is 2-fold lower than for D27S + T113E, but the k_{cat}/K_m value is 17-fold higher owing to tighter binding of dihydrofolate (Pattishall et al., 1977).

The pH dependence of k_{cat} and $k_{cat}/K_{m(dihydrofolate)}$ is illustrated in Figure 5 for the wt, D27S, and D27S + T113E enzymes. Both the wt and D27S + T113E exhibit kinetic pK_a values of around pH 8, signifying titration of some group affecting binding or catalytic activity. Since this pK_a is not apparent in the D27S profile, yet is present in the D27S + T113E profile, it is likely that the observed pK_a in the latter is due to Glu-113 of the double mutant. As this titration appears in both the k_{cat} and k_{cat}/K_m profiles, it corresponds to a group that titrates both in the enzyme–substrate–cofactor complex and in the enzyme–cofactor complex (Fersht, 1985). Note that the activity of D27S + T113E DHFR is *higher* between pH 6.5 and 9 than the D27S enzyme. We conclude that Glu-113 is indeed able to contribute to protonation of the substrate.

Table I: Kinetic Parameters at pH 7.0

DHFR	k_{cat} (s^{-1})	$K_{m(dihydrofolate)}$ (μM)	$k_{cat}/K_{m(dihydrofolate)}$ ($s^{-1}\ M^{-1}$)
wild type	30	1.2	2.5×10^7
D27S	0.43	140	3.1×10^3
T113E	0.11	1100	1.0×10^2
D27S + T113E	1.2	140	8.6×10^3
R67	0.6[a]	4.1[b]	1.5×10^5

[a] Smith et al. (1979). [b] Pattishall et al. (1977).

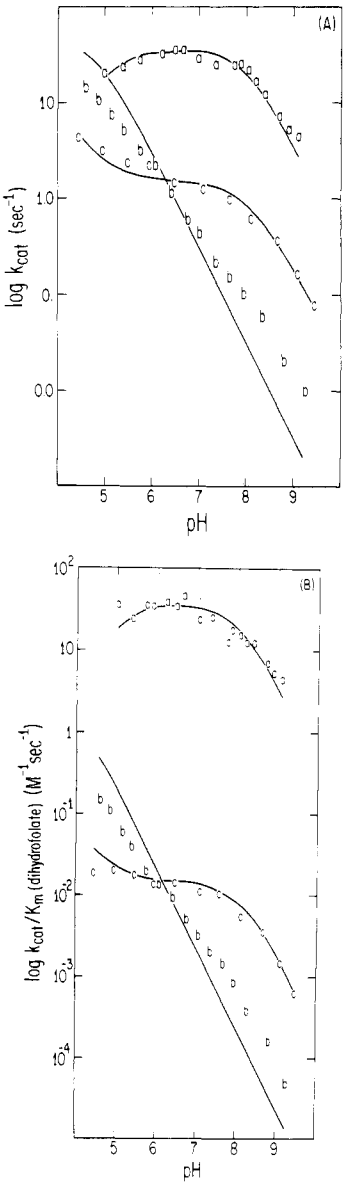
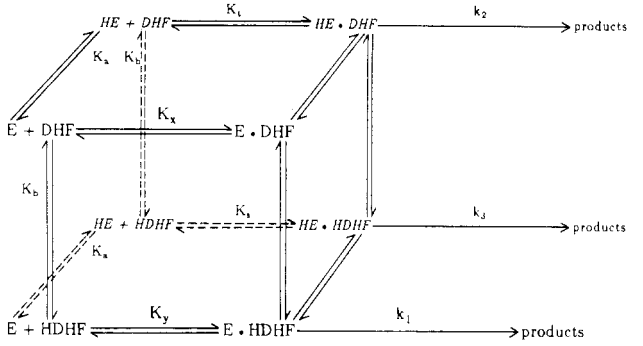


FIGURE 5: pH profiles of (A) log k_{cat} and (B) log $(k_{cat}/K_{m(dihydrofolate)})$ for wt DHFR (a), D27S DHFR (b), and D27S + T113E DHFR (c). The data were obtained as described under Materials and Methods, and the D27S + T113E data were fitted according to eq 2 and 3 in the text. The wt and D27S DHFR data were fit according to Howell et al. (1986).

Scheme I



While the pK_a for the wt enzyme is an apparent value arising from a change in the rate-determining step from THF release to hydride transfer (Fierke et al., 1987), the pK_a for the D27S + T113E enzyme probably represents a real titration of the Glu-113 residue. This conclusion is primarily supported by observation of a full NADPD isotope effect from pH 5 to 9 (see below), indicating hydride transfer is the rate-determining step in the double-mutant enzyme throughout the pH range studied.

We have previously hypothesized that D27S DHFR requires preprotonated substrate (pK_a of N5 = 3.8) as it does not have a proton donor in its catalytic site (Howell et al., 1986). Such a mechanism is consistent with an increasing rate with decreasing pH as the pK_a for N5 is approached and the concentration of available, protonated substrate increases. Both the wt and the double-mutant D27S + T113E enzymes can additionally use nonprotonated DHF as substrate and catalyze the reaction by supplying a proton via an active-site carboxylic acid group. In the wt, the latter is Asp-27, while in the D27S + T113E, it is the Glu-113 residue. The various pH-dependent reactions available to the wt, D27S, and D27S + T113E enzymes are depicted in Scheme I, which is an expanded version of the scheme we used previously[3] to analyze the pH dependence of steady-state kinetic values for the D27S mutant enzyme (Howell et al., 1986). The present scheme additionally includes protonated enzyme.

The kinetic equations describing the pH dependence of k_{cat} and K_m according to Scheme I are

$$k_{cat} = \frac{k_1}{1 + K_yK_b/K_tK_a + K_yK_b/K_x[H^+] + [H^+]K_y/K_sK_a} + \frac{k_2}{1 + K_t[H^+]/K_sK_b + K_aK_t/K_x[H^+] + K_aK_t/K_yK_b} + \frac{k_3}{1 + K_sK_b/K_t[H^+] + K_aK_s/K_y[H^+] + K_aK_sK_b/K_x[H^+][H^+]} \quad (2)$$

$$K_m = \frac{(K_yK_a + [H^+]K_y)(1 + K_b/[H^+])}{K_a(1 + K_yK_b/K_x[H^+] + K_yK_b/K_tK_a + [H^+]K_y/K_sK_a)} \quad (3)$$

where k_1, k_2, and k_3 are catalytic rate constants, K_a and K_b are acid dissociation constants, and K_s, K_t, K_x, and K_y are dissociation constants for loss of substrate from the enzyme–substrate–cofactor complexes. The empirically determined best-fit values for the D27S + T113E enzyme are $k_1 = 70\ s^{-1}$, $k_2 = 1.5\ s^{-1}$, $k_3 = 70\ s^{-1}$, pK_a = 8.1, pK_b = 3.8, $K_s = 500\ \mu M$, and $K_t = K_x = K_y = 100\ \mu M$. The higher values for k_1 and k_3 as compared with k_2 suggest that attainment of the transition state occurs more readily with bound, preprotonated DHF than by protonation via the active-site carboxylate group of bound, unprotonated DHF.

These best-fit values for k_1, k_2, k_3, K_s, K_t, K_x, and K_y explain why the D27S + T113E enzyme is less active than the D27S DHFR between pH 4.5 and 6.5. For example, at pH 6.0, the D27S + T113E enzyme will preferentially bind the major solution species (unprotonated DHF) and catalyze its reduction at a low rate. Only when a substantial proportion of the substrate is present as a protonated species (pK_a = 3.8) will the overall rate increase substantially to reflect contributions from k_1 and k_3.

A subset of the above scheme pertains to D27S DHFR for which only unprotonated enzyme exists. Therefore, catalysis can proceed only via k_1. Fitting of the pH profile data for D27S DHFR and for the wt enzyme was done previously (Howell et al., 1986; Stone & Morrison, 1984a). A comparison of the rate constants for the various DHFRs shows $k_2 = 1.5\ s^{-1}$ for the D27S + T113E enzyme and $k_2 = 37\ s^{-1}$ for the wt DHFR (Howell et al., 1986). This 25-fold difference in rate constants clearly indicates a lower capacity of the double-mutant enzyme to protonate dihydrofolate when compared to the wt DHFR. In the case of k_1 and k_3, the D27S enzyme gave $k_1 = 60\ s^{-1}$ (Howell et al., 1986), and the D27S + T113E enzyme gave $k_1 = k_3 = 70\ s^{-1}$. These rates are comparable and indicate similar capacities to utilize preprotonated substrate at low pH.

In addition, the capacity of D27S + T113E DHFR to utilize folate as a substrate was monitored. Even at high concentrations of the double-mutant enzyme, no reduction of folate was observed at either pH 5 or pH 7. This observation suggests that activation of the pteridine ring by the Glu-113 residue is much more difficult in the case of folate than in the case of dihydrofolate. Similarly, k_{cat} for folate utilization by wt DHFR is 1000-fold lower than for dihydrofolate (Baccanari et al., 1975). The concentration of protonated folate available in solution will be extremely low even at pH 5 since the pK_a for N5 is <−1.5 (Poe, 1977) and the pK_a for N8 is estimated to be 1.7 (Rokos & Pfleiderer, 1971; Gready, 1985).

Isotope Effects. Primary deuterium isotope effects on steady-state kinetic parameters can reveal whether hydride transfer is at least partially rate limiting. Moreover, the pH dependence of such isotope effects can show whether the steady-state pK_a is a true pK_a of the group being titrated or is an apparent pK_a arising from a change in the rate-limiting step. Fierke et al. (1987) have measured the NADPD isotope effect on k_{cat} (often designated $^DV = k_{cat(NADPH)}/k_{cat(NADPD)}$) for wt DHFR at several pH values. They find $^DV = 1.2$ at pH 7.0 and below, indicating that hydride transfer is not significantly rate limiting at neutral and low pH. At pH 9.0, however, ^DV increases to 3.0. Therefore, the apparent pK_a of about 8 in the steady-state pH profile for the wt enzyme is due to a change in the rate-limiting step. By stopped-flow kinetics, Fierke et al. (1987) have found that tetrahydrofolate (THF) release is the rate-limiting step at pH 7 and below. Thus, at pH 9, the rate-limiting step must have changed from THF release to hydride transfer, which occurs less readily at high pH because Asp-27 is unprotonated and cannot facilitate protonation of the substrate. Fierke et al. (1987) also reported a primary isotope effect of 3 for the directly observed hydride transfer step. This value may in fact characterize the intrinsic isotope effect on hydride transfer.

In order to ascertain if the rate-limiting step for D27S or D27S + T113E DHFR is hydride transfer, primary NADPD

[3] Note that the symbols used here differ from those of Howell et al. (1986).

Table II: Kinetic Isotope Effects

DHFR	pH	^{D}V	$^{D}(V/K_{m(dihydrofolate)})$
wild type	5.1	1.2	1.1
	6.0[a]	1.2	
	9.0[a]	2.7	3.0
D27S	5.1	3.2	3.6
	7.0	3.3	3.1
	9.0	3.2	3.3
D27S + T113E	5.1	2.1	3.8
	7.0	3.1	3.5
	9.4	3.0	3.8

[a] Fierke et al. (1987).

isotope effects were measured at several pH values for both mutant enzymes (see Table II). Both the D27S and D27S + T113E DHFRs exhibit substantial isotope effects from pH 6 to 9.5, indicating that hydride transfer is indeed rate limiting *throughout* this range of pH, in contrast to what is observed for wt DHFR. Of particular relevance, the essentially pH-independent isotope effects observed for the D27S + T113E enzyme suggest that the pK_a of 8.0 seen in the kinetic pH profile directly reflects ionization of the Glu-113 residue, since no change in the rate-limiting step occurs as the pH is increased. While this is an abnormally high pK_a for a carboxylic acid, our isoelectric focusing experiments indicate that Glu-113 in the double-mutant enzyme must have a higher pK_a than Asp-27 in wt DHFR. Previous determinations of the pK_a for Asp-27 in the wt enzyme yielded a value of 6.5, based on titration of MTX binding (Stone & Morrison, 1983b) and direct observation of the hydride transfer step over a range of pH values (Fierke et al., 1987). A pK_a perturbation of this magnitude has been seen previously for the Glu-35 residue in lysozyme where a pK_a of 6.0 was observed in the free enzyme and a pK_a of 8.0–8.5 was observed in the glycol chitin–lysozyme complex (Parsons & Raftery, 1972a,b).

The D27S and D27S + T113E enzymes, which bind DHF more weakly than wt (see Table I), will presumably also bind THF more weakly. However, as the rate-determining step in wt catalysis is THF release, weakening of THF binding in the double mutant would result in an increase in the catalytic rate at pH 7.0 if no other changes in enzymic properties had occurred. Obviously, however, the hydride transfer rate has been concurrently reduced in the double-mutant enzyme because the hydride transfer rate is 950 s^{-1} in the wt enzyme (at pH 5; Fierke et al., 1987), whereas in the D27S + T113E mutant, hydride transfer must be about 3.2 s^{-1} at this pH (deduced from steady-state rates and a presumably full isotope effect of $^{D}V = 3$). Therefore, the Asp-27 → Ser + Thr-113 → Glu mutations likely affect *both* hydride transfer and THF release rates.

Glu-113 Single-Mutant DHFR Kinetics. To complete this study, the other single-mutant DHFR gene, Thr-113 → Glu, was obtained by cloning a *Pvu*I fragment from the D27S + T113E DHFR gene into the *Pvu*I site of the wt DHFR gene in M13mp8. The T113E single mutation was verified by sequencing the entire gene.

This single mutant protein was characterized to a very limited extent. The T113E DHFR must be expressed in a *lon* (protease-deficient) strain of *E. coli* in order to obtain even minimal quantities of protein. The existence of at least two bands as well as a decreased stability is observed in 0–8 M urea gradient gels (Figure 6). Possibly the juxtaposition of two acidic groups destabilizes the molecular structure. Obviously, any interconversion between the species represented by these bands must be slow.

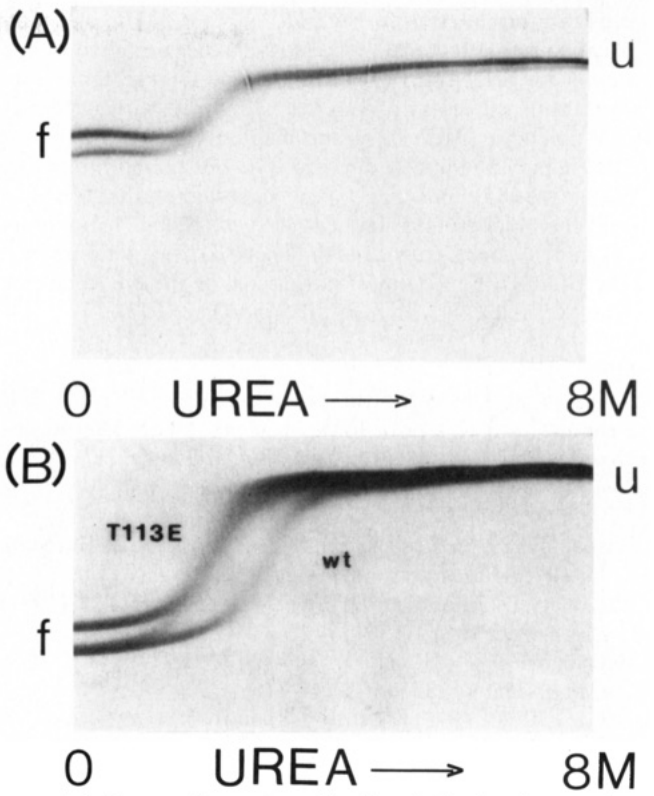


FIGURE 6: Urea gradient polyacrylamide gels showing denaturation profiles of (A) the T113E DHFR alone and (B) a 1:1 mixture of wt and T113E DHFRs. The letters f and u indicate the positions of the folded and unfolded forms, respectively. The gels were run according to Villafranca et al. (1987). The bottom of the gel is the anode end.

Initial kinetic data obtained at pH 7.0 for the T113E mutant DHFR give a k_{cat} value of 0.11 s^{-1} and a $K_{m(dihydrofolate)}$ of approximately 1100 μM (see Table I). This 1000-fold increase in $K_{m(dihydrofolate)}$ suggests that the active site is severely crowded in the T113E single-mutant enzyme. Since the glutamic acid substitution at position 113 involves insertion of a bulkier side chain, such a result is not surprising. The Glu-113 substitution probably does not affect the K_m of the D27S + T113E enzyme as drastically because of the additional space available in that active site.

Other amino acids, including hydrophobic residues, have replaced Thr-113 without seriously affecting the enzyme's function. The insertion of a valine residue in place of Thr-113 in *E. coli* DHFR has previously been evaluated (Chen et al., 1985). No effect on k_{cat} and a 25-fold increase in $K_{m(dihydrofolate)}$ were observed.

The kinetic parameters of the T113E mutant also exhibit an altered pH dependence. The observed k_{cat} at pH 6.0 for the mutant enzyme is increased over the rate observed at pH 7.0. These two data points give a slope of about 0.7 for the pH profile, indicating that only one group is likely to be involved in proton donation even though two acidic groups are available, namely, Asp-27 and Glu-113.

Conclusions

These results indicate that the protonation mechanism at the active site of DHFR can be modified but at the cost of greatly decreased enzyme efficiency. Not only can the enzyme still function with the proton donor completely eliminated but it can also protonate dihydrofolate when a proton donor is inserted in the active site in an alternative position. In other words, a proton can come from Asp-27 (in wt), from Glu-113 (in D27S + T113E), or from solution (in D27S). What do these observations suggest with respect to the importance of

substrate protonation for hydride transfer? The clear implication is that protonation is essential to attain the transition state for hydride transfer but that the proton can come from a variety of sources, by various routes, and with varying degrees of efficiency. While a proton derived from Asp-27 in wt DHFR may be initially donated to O4 of the pteridine ring (Gready, 1985), modeling experiments suggest that this is an unlikely initial protonation site in the D27S + T113E mutant enzyme. A more likely candidate is N1 of the pteridine ring. This atom is the next most basic atom of the pteridine ring after N5 and O4, with a pK_a of 1.38 (Poe, 1977).

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