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A Second-Site Mutation at Phenylalanine-137 That Increases Catalytic Efficiency in the Mutant Aspartate-27 → Serine *Escherichia coli* Dihydrofolate Reductase†

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ABSTRACT: The adaptability of *Escherichia coli* dihydrofolate reductase (DHFR) is being explored by identifying second-site mutations that can partially suppress the deleterious effect associated with removal of the active-site proton donor aspartic acid-27. The Asp27 → serine mutant DHFR (D27S) was previously characterized and the catalytic activity found to be greatly decreased at pH 7.0 [Howell et al. (1986) *Science 231*, 1123-1128]. Using resistance to trimethoprim (a DHFR inhibitor) in a genetic selection procedure, we have isolated a double-mutant DHFR gene containing Asp27 → Ser and Phe137 → Ser mutations (D27S+F137S). The presence of the F137S mutation increases k_{cat} approximately 3-fold and decreases $K_{m(DHF)}$ approximately 2-fold over D27S DHFR values. The overall effect on $k_{cat}/K_{m(DHF)}$ is a 7-fold increase. The D27S+F137S double-mutant DHFR is still 500-fold less active than wild-type DHFR at pH 7. Surprisingly, Phe137 is approximately 15 Å from residue 27 in the active site and is part of a β-bulge. We propose the F137S mutation likely causes its catalytic effect by slightly altering the conformation of D27S DHFR. This supposition is supported by the observation that the F137S mutation does not have the same kinetic effect when introduced into the wild-type and D27S DHFRs, by the altered distribution of two conformers of free enzyme [see Dunn et al. (1990)] and by a preliminary difference Fourier map comparing the D27S and D27S+F137S DHFR crystal structures.

Dihydrofolate reductase (DHFR;[1] EC 1.5.1.3) catalyzes the NADPH-dependent reduction of dihydrofolate (DHF) to tetrahydrofolate (THF). This activity is necessary in maintaining intracellular pools of THF cofactors that are essential in 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, 1988a,b; Fierke et al., 1987a; Penner & Frieden, 1987). Recently Fierke et al. (1987b) have suggested

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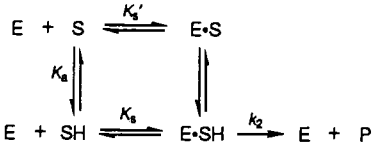
Scheme I



that *Escherichia coli* DHFR is a highly evolved enzyme with a calculated efficiency (E) of 0.15. Enzyme efficiency is defined as $E = v/v^0$, where $1/v$ is the reaction flux (v is the steady-state turnover rate) and $1/v^0$ is the maximum flux (v^0 is $k_{on} \times$ intracellular DHF concentration). By definition, a maximally efficient enzyme has $E = 1$. The conditions for the DHFR analysis approximated the *E. coli* cell, i.e., 1.0 mM NADPH, 1.5 mM $NADP^+$, 0.3 μM DHF, 13 μM THF, and 0.1 M NaCl, pH 7.0, at 25 °C. For comparison, triosephosphate isomerase, a "perfect" enzyme, has a calculated efficiency of 0.6 (Albery & Knowles, 1976).

DHFR has also been studied by NMR spectroscopy (Hammond et al., 1986; Birdsall et al., 1989a,b) and X-ray crystallography techniques (Bolin et al., 1982; Filman et al., 1982; Bystroff et al., 1990). In particular, the X-ray structures have delineated important interactions between ligands in the substrate-binding pocket and the enzyme. These interactions have recently become the focus of site-directed mutagenesis experiments, which have attempted to define the roles of specific amino acids in ligand binding and catalysis (Howell et al., 1986, 1987; Murphy & Benkovic, 1989; Fierke & Benkovic, 1989).

In light of the proposed, high catalytic efficiency of wild-type (WT) *E. coli* DHFR, it is not surprising that all the mutant DHFRs generated by site-directed mutagenesis techniques have greatly decreased enzyme efficiencies (Fierke et al., 1987b). For example, in one mutagenesis experiment the role of Asp27 was investigated by replacing it with asparagine (D27N DHFR; Howell et al., 1986). This mutant DHFR was constructed to investigate the role of a conserved carboxylic acid group in substrate protonation. The resulting D27N 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 an enzyme-mediated protonation step can be bypassed if preprotonated substrate is bound by the enzyme. This bypass occurs more frequently at low pH as the pK_a value of 3.8 for N5 of the pteridine ring of DHF (Poe, 1977) is approached. The D27N mutant enzyme has a calculated efficiency of 0.000056 at pH 7.0 where flux $(1/v) = (K_m + [S])/V_{max}[S]$ (Fierke et al., 1987b).

Even though the proton donor has been removed from the active site of D27N DHFR, the D27N mutant enzyme has proven surprisingly adaptable, catalyzing the reaction at a substantial rate at low pH. We have been intrigued by this adaptability and have embarked on a series of experiments designed to probe the malleability of the enzyme active site. Using a rational design approach based on the crystal structure, we initially moved the position of the proton donor in the active site by constructing the Asp27 → Ser + Thr113 → Glu (D27S+T113E) double-mutant DHFR (Howell et al., 1987). The T113E mutation increases k_{cat} by a factor of 3 at pH 7 (when compared to the "parent" D27S DHFR), and a new pK_a is observed in the pH profile. These results suggest the new glutamic acid at position 113 is contributing to protonation in the mechanism, although with a lower efficiency than WT DHFR.

In an alternate approach aimed toward improving enzyme efficiency, we are using classical genetic techniques to generate and isolate second-site mutations in mutant DHFR genes. Only those revertants expressing an increased DHFR activity are being studied and the double-mutant enzymes are being compared with WT and parent mutant enzymes. Subtle effects involved in the reversion process may be expected to include alterations in the intrinsic binding of substrate and cofactor, the stereochemistry of catalytic events, and/or the mechanism by which catalysis proceeds. This approach is valuable as it focuses our attention on areas of structure that are not normally analyzed. Additionally, this type of experiment may allow us to reevolve, in a stepwise fashion, an alternative active-site configuration capable of substantial activity.

Since the mutant D27N DHFR was well characterized, we started our genetic reversion studies with the corresponding gene. An initial suppressing mutation found was a substitution of serine (AGC codon; D27S mutant DHFR) for asparagine-27 (AAC codon). The effect of the D27S mutation, when compared to the D27N DHFR, is to increase k_{cat} by a factor of 4 (Howell et al., 1986).

In our present study, the mutant D27S DHFR gene was used in an additional genetic selection step and a phenylalanine-137 → serine (F137S) second-site mutation was obtained. The effect of the F137S mutation is to increase k_{cat} 3-fold and decrease $K_{m(DHF)}$ 2–3-fold when compared to the D27S parent mutant enzyme. Therefore, the overall effect on the apparent second-order rate constant (k_{cat}/K_m) is a 7–8-fold increase. Surprisingly, the Phe137 residue is 15 Å from the D27S mutation, and a mutation at this position would not have been predicted to affect DHFR kinetics. It is likely that a conformational change is associated with this mutation which causes a positive effect on catalytic efficiency.

Materials and Methods

A clone of the WT *E. coli* DHFR gene carried in M13mp8 has been previously described (Villafranca et al., 1983). An initial mutation (Asp27 → Asn; D27N) was constructed by site-directed mutagenesis techniques (Villafranca et al., 1983). A mutant serine-27 (D27S) DHFR gene was obtained as a primary-site revertant of the mutant D27N DHFR gene with increased resistance to trimethoprim (TMP) as the genetic selection procedure (Howell et al., 1986).

The F137S single-mutant DHFR gene was constructed from the D27S+F137S double-mutant and WT DHFR genes by recombinant DNA techniques using *Pvu*I. The cutting pattern of this restriction enzyme allows separation of the D27S and F137S mutations. The F137S mutation was verified by dideoxy sequencing of the entire DHFR gene. No additional mutations were found.

To increase protein expression, the DHFR genes were cloned from M13mp8 into pUC8 and transformed into a *fol* strain of *E. coli*. The chromosomal DHFR gene has been deleted in this strain (null allele) and replaced by a kanamycin resistance gene (Howell et al., 1988). This expression system allows purification of mutant DHFRs that are not contami-

[1] Abbreviations: DHFR, dihydrofolate reductase; DHF, dihydrofolate; THF, tetrahydrofolate; TMP, trimethoprim; MTX, methotrexate; D27S DHFR, Asp27 → Ser mutant DHFR; D27N DHFR, Asp27 → Asn mutant DHFR; D27S+F137S DHFR, Asp27 → Ser + Phe137 → Ser double-mutant DHFR; F137S DHFR, Phe137 → Ser mutant DHFR; D27S+T113E DHFR, Asp27 → Ser + Thr113 → Glu double-mutant DHFR; WT DHFR, wild-type DHFR; E165D, H95N, and S96P triosephosphate isomerases, Glu65 → Asp, His95 → Asn, and Ser96 → Pro mutant isomerases; Y175C and G211E tryptophan synthetases, Tyr175 → Cys and Gly211 → Glu mutant tryptophan synthetases; E, enzyme efficiency.

nated by WT DHFR due to expression of the *E. coli* chromosomal gene.

E. coli cells containing the cloned genes were grown at 30 °C in a modified version of TB media (Tartof & Hobbs, 1987) containing 0.017 M KH_2PO_4, 0.07 M K_2HPO_4, 12 g/L Bactotryptone, 24 g/L Bacto yeast extract, 4 mL/L glycerol, and 2 g/L NaCl. Cells were grown to late stationary phase in the presence of 200 μg of ampicillin/mL and 50 μg of kanamycin/mL and lysed by sonication. The soluble lysate was applied to a QAE-ZetaPrep 15 disk (LKB), washed with 50 mM KH_2PO_4 and 1 mM EDTA, pH 8.0, and eluted with the same buffer containing 0.75 M KCl. The D27S and D27S+F137S mutant DHFRs were then purified to homogeneity (as determined by SDS–PAGE) on Sephadex G-75 and DEAE-Fractogel columns as previously described (Villafranca et al., 1983). For the WT and F137S DHFRs, a methotrexate affinity resin was used to purify the proteins to homogeneity (Baccanari et al., 1981). Elution of these DHFRs from the affinity resin required the presence of 2 mM folate, which was subsequently removed by chromatography on a DEAE-Sephacel column.

Steady-state kinetic data were obtained with a Perkin-Elmer λ3A spectrophotometer interfaced with a IBM PS2 (Howell et al., 1986, 1987). The computer program UVSL3 (Softways, Moreno Valley, CA) was used to collect and analyze data. K_m values for DHF were obtained by maintaining NADPH at saturating concentrations and varying DHF at subsaturating concentrations. K_m values for NADPH were obtained by varying both DHF and NADPH at subsaturating concentrations. To eliminate any hysteretic behavior, DHFR was preincubated with either substrate or cofactor prior to initiation of the assay (Penner & Frieden, 1985). Nonlinear regression using ENZFIT (Elsevier Science) was used to determine both k_{cat} and K_m values.

Pre-steady-state kinetic measurements were performed essentially as described by Fierke et al. (1987a). Data were collected at 20 °C with a HI-TECH SF-51 stopped-flow spectrophotometer interfaced with a Datel AT computer. The latter was equipped with a MetraByte Dash-16 A/D converter card operating with a maximum throughput of 50 kHz. The dead time of this system is approximately 2 ms. A solution of enzyme and cofactor was mixed with dihydrofolate to give final concentrations of 15 μM DHFR, 125 μM DHF, and 150 μM NADPH in buffer containing 25 mM Mes, 25 mM acetic acid, 50 mM Tris, and 0.1 M NaCl at pH 5.6 or pH 7.4. Pre-steady-state bursts were followed by a decrease in fluorescence energy transfer to NADPH during enzyme turnover. Fluorescence at 450 nm was detected through an interference filter after excitation at 290 nm with a high-intensity xenon source across a 0.2 × 1.0 cm path. At least 10–20 transmittance traces were averaged and then fit to a single-exponential burst followed by a linear rate by using the program NLIN (Marquardt least-squares algorithm).

NADPD was prepared by reaction of alcohol dehydrogenase from *Leuconostoc mesenteroides* (Boehringer Mannheim) on 1,1-dideuterioethanol (MSD Isotopes) and $NADP^+$ (Sigma), coupled with $NADP^+$ aldehyde dehydrogenase (Sigma) to allow the reaction to approach completion (Stone & Morrison, 1984; Howell et al., 1987). DHF was prepared by the reduction of folic acid by sodium dithionite (Blakley et al., 1960) and stored at −20 °C in 5 mM HCl and 50 mM β-mercaptoethanol.

Concentrations were determined spectrophotometrically by using molar extinction coefficients of 28 000 M^{-1} cm^{-1} at 282 nm for DHF and folic acid (Blakley, 1960; Stone & Morrison, 1986), 6220 M^{-1} cm^{-1} at 340 nm for NADPH (Penner & Frieden, 1985), and 22 100 M^{-1} cm^{-1} at 302 nm, pH 13, or 23 250 M^{-1} cm^{-1} at 258 nm, pH 13, for MTX (Stone & Morrison, 1986). The molar extinction coefficients for dihydrofolate and folate utilization by DHFR were 12 300 and 18 400 M^{-1} cm^{-1} at 340 nm, respectively (Baccanari et al., 1975). Enzyme concentrations were obtained by biuret determination (Gornall et al., 1949) and/or by an enzyme inhibition titration utilizing the active-site inhibitor methotrexate (Williams et al., 1979).

RESULTS

Selection, Screening, and Identification of Revertant Candidates. *E. coli* strain JM109 (*recA1*, *Δlac-pro*, *endA1*, *gyrA96*, *thi-1*, *hsdR17*, *supE44*, *relA1*, *F' traD36*, *proAB*$^+$, *lacIq-ZΔM15*) was infected with M13mp8 carrying the mutant D27S DHFR gene. The infected cells were grown overnight in YT media and 25 μL of the cell suspension was plated onto M9 minimal glucose plates containing 100 μg of trimethoprim/mL (TMP). Trimethoprim is a potent, active-site-directed inhibitor of bacterial DHFR, and its presence in the media allows selection of mutations in DHFR (Burchall et al., 1982; Schimke, 1986; Then & Herman, 1981; Then, 1982). While confluent growth of *E. coli* infected with M13-D27S DHFR (and also with M13-WT DHFR) occurs at ≤50 μg of TMP/mL, when concentrations of TMP greater than 50 μg/mL are used, single colonies are observed after 2–3-days growth at 37 °C. The single colonies contain putative revertants of the D27S DHFR-M13 generated by spontaneous mutational events. Since both WT and D27S DHFR genes confer resistance to similar TMP concentrations, revertants of D27S back to the WT gene sequence are not selected due to their inability to confer a selective advantage.

Prior to screening the DHFR activity associated with these revertant candidates, we separated mutations in the M13-D27S DHFR from mutations associated with the host (e.g., membrane impermeability to TMP, mutations in the chromosomal DHFR gene, *thyA* mutations, etc.; Dewes et al., 1986; Miller, 1972). This step consisted of growing the single colonies overnight in 1 mL of YT media, centrifuging the cells, and retaining the supernatant with infectious virus. A fresh culture of JM109 was then infected with the virus, and the ability of the M13-D27S DHFR phage to confer resistance to an increased level of TMP was reconfirmed.

Next, an overnight culture of JM109 infected with M13-DHFR virus was lysed and the soluble protein screened for DHFR content on a nondenaturing gel using both Coomassie Blue and activity stains (Hiebert et al., 1972). This step facilitates separation of positive mutations on DHFR activity from mutations that weaken DHFR affinity for TMP or overproduce DHFR [e.g., Smith et al. (1982), Smith and Calvo (1982), and Flensburg and Skold (1987)].

By these screening methods, a revertant protein was found that migrated more slowly in native PAGE and was more active than the D27S DHFR control. The virus containing this putative DHFR revertant gene was plaque purified to obtain a single virus and sequenced by dideoxy methods. A new mutation in the D27S DHFR gene sequence was observed; phenylalanine-137 (TTC codon) was replaced by serine (TCC codon). The D27S mutation was still present and no other mutations were found.

We have independently isolated the F137S-suppressing mutation from the D27S DHFR gene two additional times. Independent isolation of the F137S-suppressing mutation three times (out of a total of eight revertant types generated thus far; Howell et al., 1990, and unpublished observations) suggests

the number of suppressing mutations available to the D27S mutant DHFR gene is limited.

Kinetics. The ability of the F137S mutation to affect DHFR catalysis was evaluated. The k_{cat} values at pH 7.0 for WT, D27S, and D27S+F137S DHFRs are 29 ± 1.0, 0.41 ± 0.0082, and 1.3 ± 0.07 s^{-1}; $K_{m(DHF)}$ values are 1.1 ± 0.19, 56 ± 2.4,[2] and 25 ± 2.9 μM; and $K_{m(NADPH)}$ values are 0.94 ± 0.36, 1.3 ± 0.30, and 1.1 ± 0.16 μM, respectively. The presence of the F137S mutation increases k_{cat} of the D27S protein by a factor of approximately 3-fold and concurrently decreases $K_{m(DHF)}$ by a factor of approximately 2-fold. These effects combine to increase the apparent second-order rate constant (k_{cat}/K_m) of the double-mutant DHFR approximately 7-fold (when compared to the D27S enzyme) where the second-order rate constant describes the rate of interaction of dihydrofolate with the binary enzyme–cofactor complex. However, when compared with WT DHFR at pH 7.0, the double-mutant enzyme has a 22-fold lower k_{cat} and a 510-fold lower $k_{cat}/K_{m(DHF)}$.

The pH dependence of k_{cat} and $k_{cat}/K_{m(DHF)}$ was determined for the D27S+F137S mutant enzyme and compared to previously determined values for WT and D27S enzymes (Figure 1; Howell et al., 1986). When compared to the D27S single-mutant enzyme, the D27S+F137S mutant enzyme continues to display a 3-fold increase in k_{cat} throughout the pH range studied and $k_{cat}/K_{m(DHF)}$ of the double mutant remains 7–8-fold higher. When compared to the WT enzyme, the double-mutant DHFR has an equal k_{cat} value at pH 5.0 and the second-order rate constant of the double-mutant enzyme is only decreased 45-fold when compared to the WT DHFR value at pH 5.0. From these values, it can be seen that while the difference between the double-mutant and WT DHFR is substantial at pH 7.0, k_{cat} is converging at low pH.

Trimethoprim Inhibition. As the genetic procedure used to select revertants of D27S DHFR was based on increased resistance to TMP, we briefly assessed TMP binding to the double-mutant DHFR. A kinetic K_i was obtained for the D27S+F137S DHFR by using four different TMP concentrations (1.2, 2.9, 5.8, and 12.8 μM) while [DHF] was varied in the presence of saturating NADPH concentrations. A replot of slope (obtained from a Lineweaver–Burk plot) versus inhibitor concentration gave a K_i of 3.0 μM. For comparison, the TMP K_i's for D27S and WT DHFRs are 2.9 μM and 20 pM, respectively (Howell et al., 1987; Stone & Morrison, 1986). The F137S mutation does not alter binding to TMP to D27S DHFR, suggesting the mutation was genetically selected for increased catalytic efficiency, not for loss in TMP binding affinity.

Isotope Effects. The presence (or absence) of primary isotope effects can be helpful in describing the rate-limiting steps in enzyme mechanisms. Therefore, NADPD effects have been measured for the WT, D27S, and D27S+F137S enzymes. A substantial NADPD isotope effect [$^DV \sim 3$, where $^DV = k_{cat(NADPH)}/k_{cat(NADPD)}$] is observed for WT DHFR from pH 5 to pH 9 with stopped-flow kinetic techniques (Fierke et al., 1987a). However, when steady-state rates are monitored in WT DHFR, a ^DV of ~3 is seen only at pH 9.0 (Chen et al., 1987). As discussed below, these results support a mechanism for WT DHFR where a change in rate-determining step occurs such that hydride transfer is rate determining only at high pH. In contrast, when NADPD isotope effects are measured for the D27S enzyme, a ^DV of approximately 3 is observed from pH 5 to pH 9 (Howell et al., 1987). This result indicates that the hydride-transfer rate has been decreased in the D27S DHFR such that it becomes the rate-determining step. As an increase in K_m for DHF (and presumably for THF) was concurrently observed in the D27S DHFR, this suggests that an increase in the rate of THF release has occurred as well.

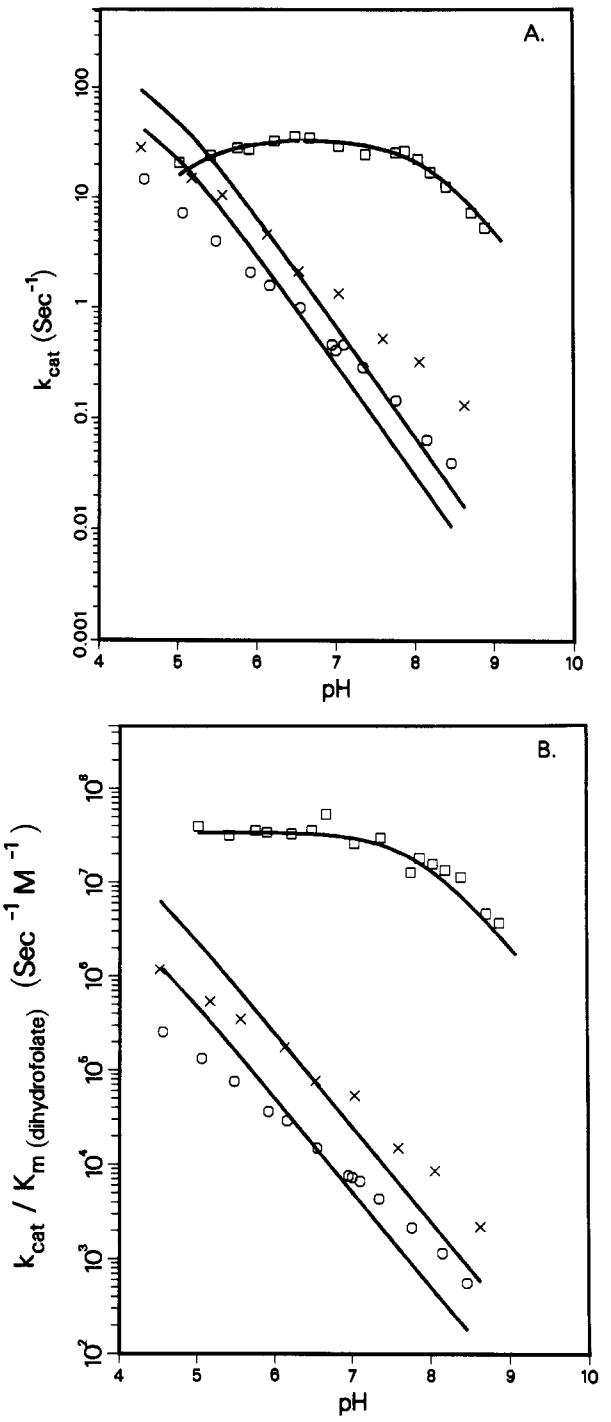


FIGURE 1: pH profiles of (A) k_{cat} and (B) $k_{cat}/K_{m(DHF)}$ for wild-type DHFR (□), D27S mutant DHFR (○), and D27S+F137S mutant DHFR (×). Data were obtained as described under Materials and Methods. Ranges of enzyme concentrations used in the assays were 0.69–6.2 nM for WT, 19–1900 nM for D27S, and 2.1–510 nM for D27S+F137S DHFR. The WT DHFR curve was fit by using eq 5 from Stone and Morrison (1984). D27S and D27S+F137S DHFR curves were fit by using eqs 1 and 2 in Howell et al. (1986). Best fit values for D27S and D27S+F137S DHFRs are given in the text and best fit values for WT DHFR are given in the legend for Figure 2.

[2] The $K_{m(DHF)}$ values for D27S DHFR are lower than those previously reported in Howell et al. (1986).

Since the rate-determining step in the D27S DHFR is hydride transfer, any increase in k_{cat} due to the presence of a suppressing mutation (e.g., F137S) must be due to an increase in the hydride-transfer rate. To determine if hydride transfer is still rate determining in the D27S+F137S DHFR, we measured the effect of NADPD on k_{cat} and $k_{cat}/K_{m(DHF)}$. ^{D}V and $^{D}V/K$ [=($k_{cat}/K_{m(DHF)}$ for NADPH)/($k_{cat}/K_{m(DHF)}$ for NADPD)] values for the double-mutant enzyme are 3.0 and 4.0, respectively, at pH 7.0 and 2.5 and 3.3 at pH 5.0. These results indicate that even though the hydride-transfer rate is increased in this mutant enzyme, it is still the rate-limiting step in catalysis.

Is There an Additive Effect Associated with the F137S Mutation? To assess whether the F137S mutation has any "global" character and can affect the kinetics of the WT DHFR, we constructed the F137S single-mutant DHFR gene using recombinant DNA techniques. From our comparison of D27S and D27S+F137S enzymes, we observe that the presence of the F137S mutation increases the hydride-transfer rate. However, in order to measure the hydride-transfer rate in the F137S single-mutant DHFR, we need to monitor steady-state rates at high pH where hydride transfer is rate determining (not THF release as seen at pH 8 and below; Fierke et al., 1987a) and/or to measure the hydride-transfer rate directly using pre-steady-state techniques.

pH profiles for the F137S single-mutant enzyme are depicted in Figure 2. While there are no observable effects on the steady-state rate at pH 8 and below, above pH 8, a small, but real increase (~1.3–2-fold) in k_{cat} is observed. For example, at pH 9.9, k_{cat} is 1.8 ± 0.083 s^{-1} for WT DHFR and 2.7 ± 0.050 s^{-1} for F137S DHFR. This rate increase has been consistently observed in four different protein preparations. It is also important to note that since determination of protein concentration affects k_{cat} values, we use active-site titrations with methotrexate to quantify the concentration of active enzyme. Finally, a small increase in $K_{m(DHF)}$ is concurrently observed with the small increase in k_{cat} values. Therefore, the F137S DHFR does not have a faster second-order rate constant (k_{cat}/K_m) than WT DHFR.

To corroborate the small increase in k_{cat} associated with F137S DHFR at high pH values, we have monitored pre-steady-state hydride-transfer rates. At pH 7.4, the hydride-transfer rates of WT and F137S DHFRs are 131 ± 6 and 147 ± 6 s^{-1}, whereas at pH 5.6, the rates are 668 ± 30 and 861 ± 54 s^{-1}, respectively. At both pH values, the rates for the F137S DHFR are ~1.2-fold higher than those of WT DHFR, indicating the hydride-transfer rate has increased, albeit slightly.

Finally, we have compared folate utilization rates in WT and F137S DHFRs. Folate is a poor substrate for WT *E. coli* DHFR with a k_{cat} of 0.010 ± 0.00017 s^{-1} and a $K_{m(folate)}$ of 22 ± 1.3 μM at pH 5.0. The F137S mutant DHFR displays a 1.6-fold increase in folate utilization with a k_{cat} of 0.016 ± 0.00028 s^{-1} and a K_m of 28 ± 1.7 μM. To verify that hydride transfer is the rate-determining step during folate reduction in WT DHFR, we have measured the effect of NADPD on reaction rates. The ^{D}V value is 5.2.[3] Therefore, the increased $k_{cat(folate)}$ associated with F137S DHFR is due to an increased hydride-transfer rate.

[3] This ^{D}V value is higher than that observed for dihydrofolate utilization (3.0–3.5) and may reflect a different angle/geometry associated with hydride transfer in the transition state.

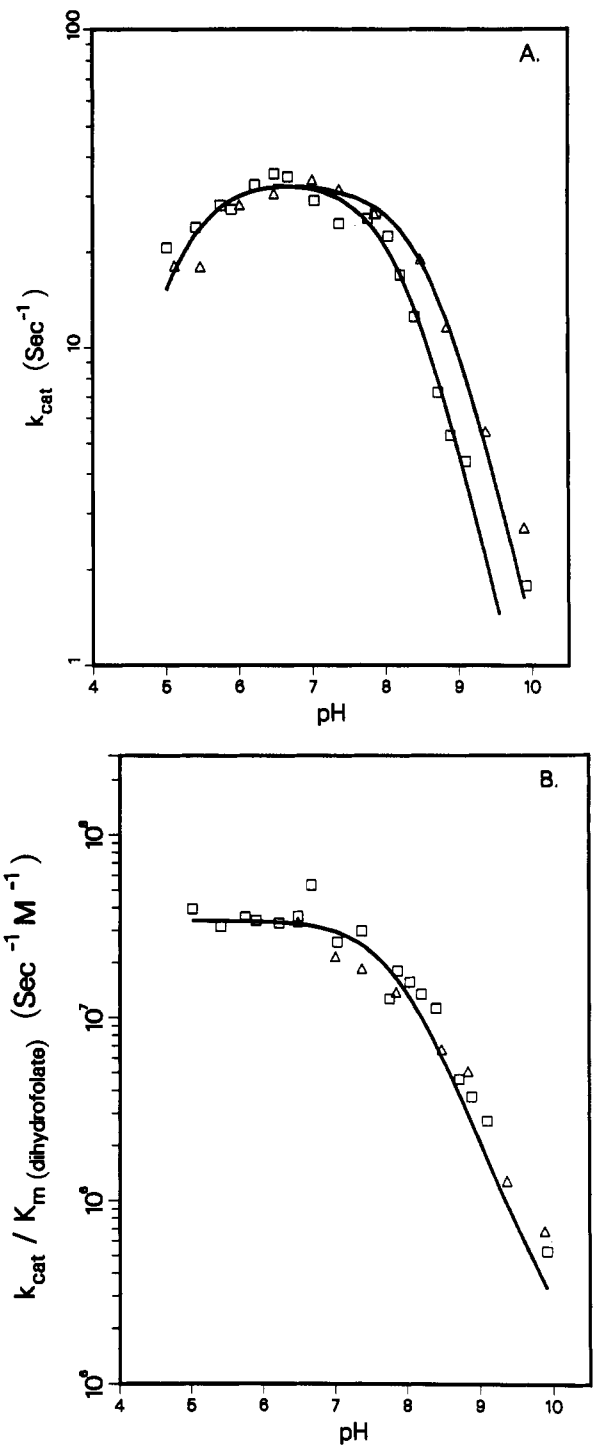


FIGURE 2: pH profiles of (A) k_{cat} and (B) $k_{cat}/K_{m(DHF)}$ for wild-type DHFR (□) and F137S DHFR (△). Enzyme concentrations used for F137S DHFR kinetics were 1.2–11 nM. Both curves were fit by using eq 5 from Stone and Morrison (1984). Best fit values for WT DHFR are k_2 = 34 s^{-1}, K_s = 1 μM, pK_{a1} = 5.1, and pK_{a2} = 8.2. Best fit values for F137S DHFR are k_2 = 34 s^{-1}, K_s = 1.6 μM, pK_{a1} = 5.1, and pK_{a2} = 8.6.

Discussion

Location of the F137S Mutation in the DHFR Structure. The F137 residue in the *E. coli* DHFR structure occurs on the enzyme surface approximately 15 Å from the edge of the DHF binding pocket. F137 is associated with one of four β-bulges present in the *E. coli* reductase structure (Figure 3; Bolin et al., 1982). A β-bulge occurs when an additional amino acid is inserted into a β-strand and disrupts the normal antiparallel hydrogen-bonding pattern (Richardson et al., 1978). The β-bulge at residue 137 is a classic β-bulge involving Val136

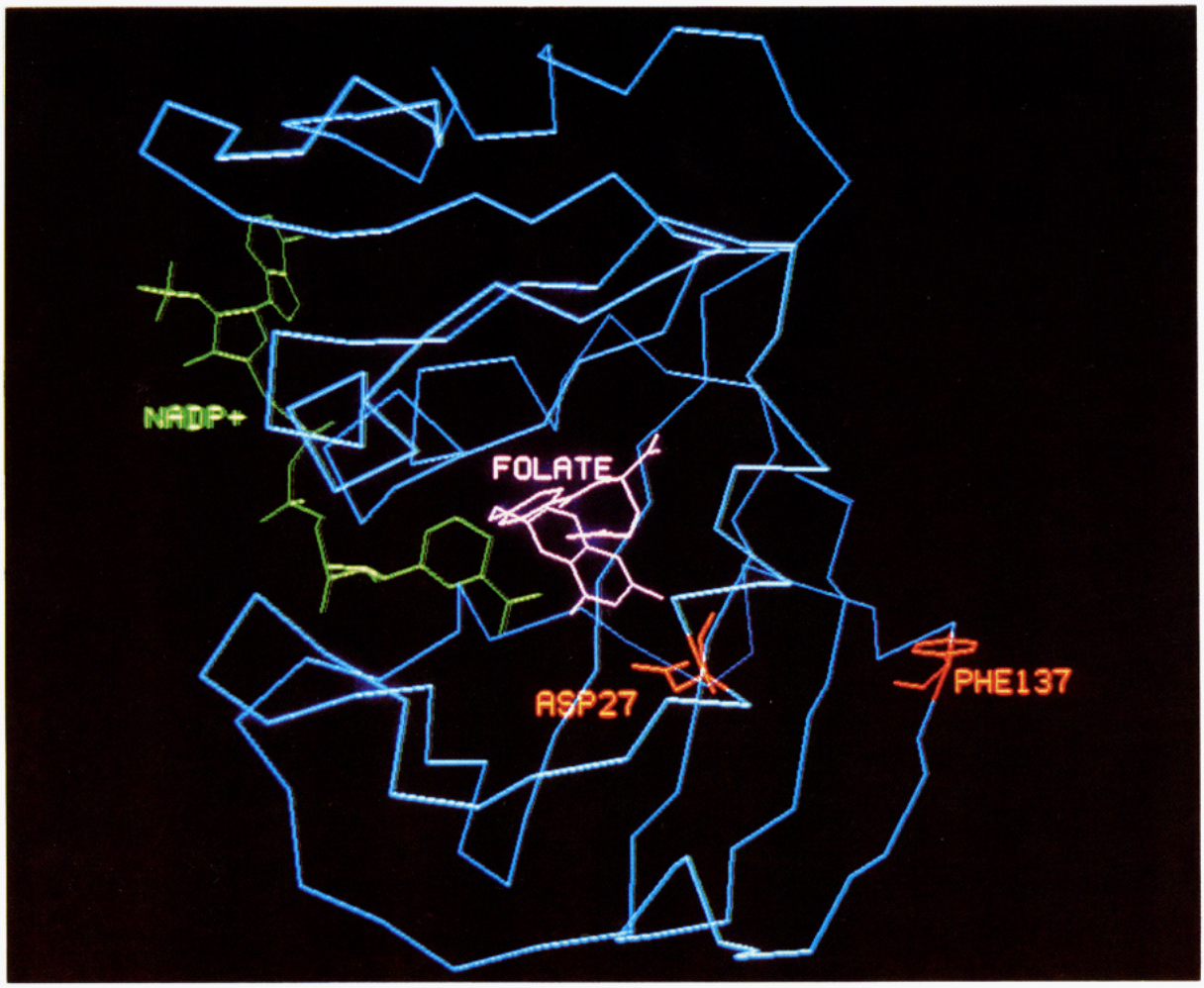


FIGURE 3: Backbone tracing of the crystal structure for WT DHFR from *E. coli* in complex with folate (magenta) and $NADP^+$ (green) (Bystroff et al., 1990). The positions of aspartate-27 and phenylalanine-137 are highlighted in orange. The distance between the α-carbons of Asp27 and Phe137 is 15.2 Å.

and Phe137 at the amino end of the βG strand and Ile155[4] in the antiparallel βH strand. This bulge is conserved in the *Lactobacillus casei* DHFR structure and becomes a "β-blowout" in the chicken liver and human DHFR structures (Bolin et al., 1982; Volz et al., 1982; J. Davies, personal communication). In the β-blowout a six amino acid insertion in the β-strand occurs, also disrupting the hydrogen-bonding pattern. The amino acids involved in these bulge sequences are not conserved.

The function of β-bulges in protein structure is not clear. Richardson et al. (1978) suggest they may be a mechanism for inserting or deleting additional amino acids in protein sequences. Alternatively, β-bulges impart a right-handed twist to the β-strand, which may be important in maintaining specific structures. For example, β-bulges are often found in enzyme active sites due to their ability to specifically orient backbone and side-chain residues. In *E. coli* DHFR, two β-bulges are involved in positioning active-site residues (Bolin et al., 1982). A parallel β-bulge (residues 94–95) followed immediately by a cis peptide bond (residues 95–96) is proposed to position the backbone carbonyl of Ile94 so that it can stabilize a delocalized carbonium ion transition state involving the nicotinamide ring of NADPH. Additionally, a pair of consecutive, classic β-bulges (residues 14, 15 and 122, 123) are proposed to help position the backbone carbonyl of Ile14 so that it can also stabilize the transition state of the nicotinamide ring. The conservation of these β-bulges in the *E. coli*, *L. casei*, chicken, and human DHFR structures may imply functional importance.

The ability of the F137S mutation to affect DHFR activity is unexpected because of its distance from the active site. In order to have a positive effect on enzyme activity, we propose this mutation has likely altered the conformation of the protein. The nonadditive effect of this mutation when introduced into WT and D27S DHFRs, the altered distribution of two DHFR conformers [see Dunn et al. (1990)], and a preliminary difference Fourier map comparing the D27S and D27S+F137S DHFR structures in complex with methotrexate (K. Brown, manuscript in preparation) support this hypothesis. The difference Fourier map suggests that residues 26, 27, 28, 30, and 137 have moved approximately 0.3 Å and also that an active-site water molecule (405) has disappeared. The β-bulge is still present in the D27S+F137S DHFR structure.

D27S+F137S DHFR Kinetics. From Figure 1, we observe that the pH profiles for D27S and D27S+F137S DHFRS are similar and that both mutant enzymes are different when compared to WT DHFR. Simple mechanisms describing both types of profiles have been described as well as a general mechanism which involves all possible combinations of protonated/unprotonated enzyme and substrate species (Howell et al., 1987; Stone & Morrison, 1984). To summarize, a simple mechanism describing WT DHFR kinetics consists of

[4] We have identified another second-site revertant of the D27S gene involving this β-bulge. The mutation is Ile155 → Asn. Kinetic parameters of the D27S+I155N DHFR are currently being determined.

protonated enzyme binding unprotonated substrate, followed by protonation of N5 of the pteridine ring of DHF by the Asp27 residue (eq 1). As N5 is 6.3 Å from Asp27, proton-

$$EH + S \rightleftharpoons EH{\cdot}S \rightarrow E + P \quad (1)$$

ation must be indirect and likely involves an intermediate water molecule (Howell et al., 1987; Morrison & Stone, 1988). Alternatively protonation of O4 on the pteridine ring of DHF via Asp27 may result, by an inductive effect, in depletion of electrons at C6, which in turn facilitates hydride transfer from the nicotinamide ring of NADPH (Gready, 1985). The apparent pK_a observed in the steady-state pH profile for k_{cat} of WT DHFR corresponds to a change in rate-determining step from THF release below pH 8 to hydride transfer above pH 8 (Fierke et al., 1987a). When hydride-transfer rates are measured directly by using rapid kinetic techniques, a pK_a of 6.5 is observed, corresponding to titration of Asp27.

In contrast to WT DHFR, a mechanism describing the kinetic behavior of the D27S-based DHFRs must explain enzyme activity in the absence of the active-site proton donor. Scheme I describes a mechanism where the mutant D27S and D27S+F137S enzymes bind protonated DHF, allowing formation of a productive, protonated transition state. At low pH, protonated substrate is readily available as the pK_a for N5 of the pteridine ring of DHF is ~3.8 (Poe, 1977). This mechanism therefore predicts increased rates at low pH values. Since the presence of the F137S mutation does not alter the shape of the pH profile for the D27S DHFR, it likely does not alter the mechanism.

Kinetic expressions describing these mechanisms have previously been derived (Howell et al., 1986, 1987). Empirically determined best fit values yield $K_s = 10\ \mu M$, $K_s' = 60\ \mu M$, and $k_2 = 80\ s^{-1}$ for D27S DHFR, where K_a is the acid dissociation constant of protonated dihydrofolate (assumed pH 3.8; Poe, 1977), K_s is the dissociation constant for the E·SH Michaelis complex, K_s' is the dissociation constant for the E·S nonproductive complex, and k_2 is the catalytic rate constant. For D27S+F137S DHFR, best fit values are $K_s = 4.5\ \mu M$, $K_s' = 27\ \mu M$, and $k_2 = 177\ s^{-1}$. The difference in k_2 between D27S and D27S+F137S DHFRs suggests a 2-fold difference in the pH-independent k_{cat} value.

An alternate hypothesis that could explain the increase in steady-state rates for D27S+F137S DHFR involves perturbation of the pK_a of bound protonated substrate by the F137S mutation. In this case, the pH-independent hydride-transfer rate would not be altered; instead the concentration of bound protonated substrate would be increased due to an upward shift in the pK_a. As indicated below, we favor the hypothesis that pH-independent hydride-transfer rates are increased.

F137S DHFR Kinetics. Two possibilities emerge to explain the slight increase in steady-state rates in the F137S DHFR when compared to WT DHFR (Figure 2). Either the pH-independent hydride-transfer rate has been increased by a small amount or the pK_a of the Asp27 residue has been shifted upward. For the second case, the increase in hydride-transfer rate would be due to a slight increase in the concentration of protonated Asp27 enzymic species. As we observe the same slight increase in hydride-transfer rates at both pH 5.6 and pH 7.4 (using stopped flow), and since the proposed pK_a for Asp27 is 6.5 (Fierke et al., 1987a), it is likely that the pH-independent hydride-transfer rate has increased slightly.

We additionally favor the latter alternative when we concurrently consider the steady-state rate increase seen in the double-mutant D27S+F137S DHFR (discussed above). That rate increase was potentially explained by either an increase in the pH-independent hydride-transfer rate or a shift in the pK_a of bound protonated substrate. Therefore, one set of hypotheses to explain the rate increases in the F137S and D27S+F137S DHFRS involves pK_a perturbation. However, for the F137S DHFR, the proposed pK_a shift would be due to titration of Asp27, whereas for the D27S+F137S DHFR, the proposed titration would be for N5 in dihydrofolate. As these *differential* pK_a perturbations seem more complicated than necessary, we prefer the simple hypothesis that pH-independent hydride-transfer rates are being affected in both cases.

The above results pose the following question: If the hydride-transfer rate is slightly increased in F137S DHFR, why is it not the "normal WT" DHFR species found in *E. coli*? First, while the k_{cat} value at high pH has increased slightly, $K_{m(DHF)}$ has concurrently increased such that k_{cat}/K_m is equivalent for WT and F137S DHFRs. Additionally, the F137S mutation has destabilized the DHFR molecule as seen in urea gradient gels (not shown) where the midpoint of denaturation for the F137S DHFR has shifted to a lower urea concentration. In a similar vein, the F137S DHFR is not overproduced to the same extent as WT DHFR. A factor of 3–5-fold less production is consistently observed, suggesting the F137S DHFR is more readily proteolyzed. Since enzyme efficiency[5] (which depends on enzyme concentration) is the critical parameter in the cell (Keleti, 1988), any slight increase in protein efficiency or hydride-transfer rate could easily be negated by a larger decrease in the total amount of DHFR produced.

Nonadditive Effect of the F137S Mutation. While the F137S mutation increases hydride-transfer rates approximately 1.3–2-fold when introduced into WT DHFR, it has no effect on $k_{cat}/K_{m(DHF)}$ values. These results suggest that the increase in k_{cat} at high pH for the F137S DHFR is achieved by looser binding of substrate. Transition-state binding is likely not affected as there is no change in k_{cat}/K_m at high pH[6] (Albery & Knowles, 1976; Fersht, 1985). In contrast, a 3-fold increase in k_{cat} and a 7-fold increase in k_{cat}/K_m are observed when F137S is introduced into the D27S "parent" enzyme. These observations suggest the F137S mutation in the D27S DHFR increases binding of the transition state more than it increases ground-state binding (Albery & Knowles, 1976; Fersht, 1985).

The nonadditive character of the F137S mutation strongly suggests that a differential conformational change is associated with the mutant proteins, as previous studies on single- and double-mutant tyrosine-tRNA synthetases have suggested that nonadditive effects occur when protein conformation are altered (Carter et al., 1984). However, another explanation for the nonadditive character of the F137S mutation might be that the mutation could preferentially help the E + SH type of mechanism (Scheme I) over the HE + S mechanism (eq 1). The basis for this explanation lies in the probability that each of these two mechanisms likely has a slightly altered transition state. And any slight differences in the transition state could

[5] To directly compare WT and F137S DHFR efficiencies, we would need to determine k_{on} for DHF addition to F137S DHFR. Since addition of MTX and/or NADPH to D27N, D27S, and F137S DHFRs has changed off rates rather than on rates (Appleman et al., 1988, 1990; Dunn et al., 1990), we have made the assumption that k_{on} for DHF addition to F137S DHFR has not changed from WT values.

[6] Two caveats need to be mentioned in this type of analysis. First, hyride transfer (not product release) should be rate limiting, and second, DHF binding should be to the binary E·NADPH complex. For D27S and D27S+F137S DHFRs, hydride transfer is fully rate determining at all pH values, and for WT and F137S DHFRs hydride transfer is rate determining at high pH. To ensure that DHF addition is to the E·NADPH complex, assays for all four enzymes are typically done at saturating levels of NADPH.

be differentially stabilized by the F137S mutation. We are presently unable to differentiate between these alternatives.

CONCLUSIONS

Second-site mutations are being explored in two other enzyme systems. For example, a second-site revertant of both the E165D and H95N mutant triosephosphate isomerase genes has been genetically selected and identified as S96P (Hermes et al., 1987). The addition of this mutation to the E165D and H95N enzymes increases their efficiencies 24- and 54-fold, respectively. The addition of the S96P mutation to the WT triosephosphate isomerase gene decreases enzyme efficiency 11-fold. As residue 96 occurs in the substrate-binding pocket, replacement of serine by proline likely involves a slight repositioning of the substrate with the catalytic residues. Benner and Ellington (1988) have suggested that since the Glu165 → Asp mutation involves a conservative change, a revertant of this gene is not that surprising. "Had the glutamate been altered to an alanine, one would imagine that a second-site suppressor would have been much more difficult to find".

A similar case has occurred in tryptophan synthetase where a second-site revertant of the Y175C gene has been identified as G211E (Yanofsky & Crawford, 1972). A recent determination of the crystal structure of tryptophan synthetase, followed by site-directed mutagenesis and molecular modeling studies, has suggested the Y175C+G211E double-mutant enzyme possesses partial activity (while neither single mutant does) because a suitable active-site geometry has been restored (Hyde et al., 1988; Nagata et al., 1989).

In contrast to these revertants in triosephosphate isomerase and tryptophan synthetase, the F137S-suppressing mutation in the D27S DHFR does not occur in the binding pocket, but rather on a distant surface of the protein. Therefore, the effect of the F137S mutation on DHFR catalysis must be due to a more global change in protein conformation or dynamics. This result clearly indicates that subtle changes in protein conformation can compensate for mutations in the active site. We further conclude that second-site revertants can be obtained when a conserved and apparently essential catalytic residue has been replaced, although full activity may not necessarily be restored by a single suppressing mutation.

Clearly the advantage of a genetic selection approach to restoration of enzyme activity lies in the ability to obtain mutations that are unexpected. And the unexpected character of the suppressing mutation can then provide new insight into the elements involved in catalysis.

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Dihydrofolate Reductase from *Escherichia coli*: Probing the Role of Aspartate-27 and Phenylalanine-137 in Enzyme Conformation and the Binding of NADPH†

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ABSTRACT: In the absence of ligands, dihydrofolate reductase from *Escherichia coli* exists in at least two interconvertible conformations, only one of which binds NADPH with high affinity. This equilibrium is pH dependent, involving an ionizable group of the enzyme (pK ~ 5.5), and the proportion of the NADPH-binding conformer increases from 42% at pH 5 to 65% at pH 8. The role of specific amino acids in enzyme conformation has been investigated by studying the kinetics of NADPH binding to three dihydrofolate reductase mutants: (i) a mutant in which Asp-27, a residue that is directly involved in the binding of folates and antifolates but not NADPH, has been replaced by a serine, (ii) a mutant in which Phe-137 on the exterior of the molecule and distant from the binding sites has been replaced by a serine, and (iii) a mutant in which both Asp-27 and Phe-137 have been replaced by serines. Mutation of the Asp-27 residue reduces the affinity for NADPH by approximately 7-fold. Kinetic measurements have suggested that this is due mainly to an increase in the rate of dissociation of the initial complex and a slight shift in the enzyme equilibrium to favor the nonbinding conformation. The pH dependence of the conformer equilibrium is also shifted by approximately one pH unit to higher pH (pK ~ 6.5). In addition, the pH profile suggests the involvement of a second ionizable group having a pK of about 8 since, above pH 7, the proportion of the NADPH-binding form decreases. Evidence for the involvement of a second ionizable group with a similar pK has been obtained for the Ser-137 mutant, but otherwise, the kinetics of NADPH binding to this enzyme are not significantly different from those of the wild type. Replacement of both Asp-27 and Phe-137 by serines resulted in an obvious change in the NADPH-binding kinetics. In contrast to the wild type, the proportion of the NADPH-binding form decreased from 54% to 42% between pH 5 and pH 8. This may be explained by changes in the equilibrium constants and pK values of the ionizable group(s) involved in the equilibrium between the two enzyme conformations.

Dihydrofolate reductase (DHFR)[1] is a ubiquitous enzyme that catalyzes the NADPH-dependent reduction of dihydrofolate to tetrahydrofolate. Tetrahydrofolate plays a central metabolic role as a carrier of one-carbon units in the biosynthesis of thymidylate, purines, and some amino acids. The reaction catalyzed by DHFR is therefore ultimately related to DNA synthesis, and the enzyme is a target for both anticancer and antibacterial drugs. Consequently, DHFR has been the subject of intensive structural and kinetic analyses.

Following the cloning and sequencing of the gene for *Escherichia coli* DHFR (Smith & Calvo, 1980), a number of site-directed mutagenesis experiments have been performed

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[1] Abbreviations: DHFR, dihydrofolate reductase; DTT, dithiothreitol; D27N, aspartate-27 → asparagine-27 DHFR mutant; D27S, aspartate-27 → serine-27 DHFR mutant; D27S+F137S, aspartate-27 and phenylalanine-137 → serine-27 and serine-137 DHFR mutant; F137S, phenylalanine-137 → serine-137 DHFR mutant; MES, 2-(*N*-morpholino)-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

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