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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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Received January 17, 1990; Revised Manuscript Received May 22, 1990

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in which Asp-27, a residue that is directly involved in the binding of folates and antifolates but not NADPH, has been replaced by a serine, (ii) a mutant in which Phe-137 on the exterior of the molecule and distant from the binding sites has been replaced by a serine, and (iii) a mutant in which both Asp-27 and Phe-137 have been replaced by serines.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

† This work was supported in part by Grant IN-122 from the American Cancer Society (to S.M.J.D.) and by NIH Grant GM35308 (to E.E.H.).
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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.

0006-2960/90/0429-8569$02.50/0

(Villafranca et al., 1983, 1987; Howell et al., 1986, 1987; Mayer et al., 1986; Chen et al., 1985, 1987; Benkovic et al., 1988; Fierke & Benkovic, 1989; Murphy & Benkovic, 1989; Huang et al., 1989; Andrews et al., 1989). These experiments have been designed to identify specific amino acid residues that are important for ligand binding, enzymic catalysis, and stabilization of protein folding. DHFR is ideally suited for these studies, not only because the kinetic mechanism has been studied in detail (Fierke et al., 1987; Penner & Frieden, 1987) but also because the structures of both the *E. coli* and *Lactobacillus casei* enzymes have been solved to a 1.7-Å resolution for some binary and ternary complexes (Bolin et al., 1982; Filman et al., 1982; Matthews et al., 1985). This greatly facilitates the formulation of precise questions about the DHFR molecule and the design of relevant mutagenesis experiments.

The binding of substrates, coenzymes, and inhibitors to *E. coli* DHFR has been extensively studied in stopped-flow kinetic experiments. In the absence of ligands, the enzyme exists in at least two conformational states (E_1 and E_2) that interconvert only slowly (Cayley et al., 1981; Fierke et al., 1987; Penner & Frieden, 1987). The equilibrium between the enzyme conformations is reflected in hysteretic behavior of the enzyme (Penner & Frieden, 1985) and is likely to be important in enzyme function. A full description of the effects of amino acid mutations on enzyme activity must therefore include a description of possible changes in the equilibrium distribution between the enzymic forms. The conformer equilibrium is pH dependent (Cayley et al., 1981), and NADPH binds preferentially to one of the conformations (E_1). NADPH association kinetics are useful for studying dynamic equilibria between enzyme forms since binary complex formation is accompanied by a biphasic decrease in DHFR fluorescence resulting first from the rapid bimolecular association with E_1, followed by a slower process reflecting the conversion of E_2 to E_1 and further NADPH binding to the newly formed E_1 (Cayley et al., 1981).

The folate binding site of all DHFRs contains an invariant carboxylic acid residue (Asp-27 in *E. coli*), and the role of this residue has been investigated by constructing mutant enzymes in which Asp is replaced by an Asn (D27N; Villafranca et al., 1983) or by a Ser (D27S; Howell et al., 1986). From studies of the crystal structures and catalytic properties of these altered enzymes, it has been concluded that Asp-27 is important for protonation of bound, unprotonated substrate. Although Asp-27 does not interact directly with bound coenzyme, it appears to affect NADPH binding in an indirect manner since Appleman et al. (1990) have studied the kinetics of NADPH binding to D27N DHFR and have shown that the proportion of the NADPH-binding form is decreased in the mutant enzyme. In the present study, we have further examined the role of Asp-27 by investigating the kinetics of NADPH binding to D27S DHFR and show that this mutation alters not only the equilibrium constants but also the pH dependence of the equilibrium.

We also report the kinetics of coenzyme binding to another mutant (F137S) in which Phe-137 has been replaced by a serine. The crystal structure of wild-type DHFR at 1.7 Å shows that DHFR has one antiparallel and seven parallel β-strands (Bolin et al., 1982). These strands form a hydrophobic β-sheet which acts as the structural core of the protein. In *E. coli*, Phe-137 is found in β-sheet G (residues 132–141) and is involved in formation of a β-bulge along with Val-136 and Ile-155, the latter residue being located in antiparallel strand βH (Bolin et al., 1982). This is outside of the active site, and although it would not be expected to be directly involved in the binding of NADPH, it may be important for stabilization of enzyme conformation or the enzyme–ligand complexes. It is curious that, in chicken DHFR, an analogous β-bulge is disrupted by a six amino acid insertion in βG (Volz et al., 1982). In view of the importance of species variation in the inhibitory patterns of antifolate drugs, it is of interest to investigate the role of this part of the molecule in *E. coli* conformation, binding, and catalysis. Mutation of Phe-137 → Ser has little effect on NADPH binding except at high pH (>7). However, if Phe-137 is replaced by a serine in D27S, the pH dependence of the enzyme equilibrium is significantly different from the single D27S mutation, indicating that the equilibrium constants and pK values of the ionizable group(s) involved in the $E_1 \rightleftharpoons E_2$ equilibrium have been further shifted in the double mutant. The finding that the F137S mutation results in altered enzyme conformation is supported by the finding that the double mutant has increased catalytic activity compared to the D27S DHFR "parent" (Howell et al., 1990).

Materials and Methods

Materials. Wild-type (WT), D27S, F137S, and D27S+F137S DHFRs were expressed and purified as described elsewhere [see Howell et al. (1990)]. The concentrations of enzymes were determined by their absorbance at 280 nm ($\epsilon = 3.11 \times 10^4$ M^{-1} cm^{-1}) or by methotrexate titration of enzyme fluorescence. Methotrexate, $NADP^+$, and NADPH were from Sigma Chemical Co. (St. Louis, MO). The concentration of NADPH was determined spectrophotometrically by using an extinction coefficient of 6200 M^{-1} cm^{-1} at 339 nm (P-L Biochemicals, 1961).

Experimental Conditions. Measurements were made in a buffer mixture containing 50 mM Tris, 25 mM MES, 25 mM acetic acid, 100 mM NaCl, 1 mM dithiothreitol (DTT), and 0.02% NaN_3. Over the pH range used (5.0–8.5) the ionic strength of this buffer mixture remains essentially constant (Ellis & Morrison, 1982). Unless otherwise stated the temperature in each experiment was 25 °C.

Equilibrium Dissociation Constants. Dissociation constants for enzyme–NADPH complexes were determined by fluorescence titrations as previously described (Dunn et al., 1978) using a Perkin-Elmer MPF44A fluorometer with excitation and emission wavelengths of 290 and 340 nm, respectively. The enzyme concentration was 0.4 μM and data were analyzed by nonlinear regression techniques using the algorithm of Marquardt (1961) and the equation

$$Fl = 100 - 0.5F_0[E_t + K_d + L_t - [(E_t + K_d + L_t)^2 - 4E_tL_t]^{1/2}]$$

where Fl is the measured fluorescence level expressed as a percentage of the apoenzyme fluorescence, L_t is the known total ligand concentration, and the unknown parameters E_t, K_d, and F_0 were considered as variables and represent the concentration of enzyme binding sites, equilibrium dissociation constant, and fluorescence yield per unit concentration of enzyme, respectively.

Kinetic Measurements. Kinetic data were obtained by using a Durrum D110 stopped-flow fluorometer and data acquisition and analysis instrumentation previously described (Dunn et al., 1980). In most experiments, the excitation wavelength was 290 nm, and the decrease in protein fluorescence that accompanies NADPH binding was monitored by using a Schott Glass Type UG 1 band-pass filter (Melles Griot, Irvine, CA). This filter transmits light of wavelength 300–400 nm and thus eliminates any contribution from NADPH fluorescence (emission maximum ~ 450 nm). The fast and slow

phases in the binding reaction were sufficiently well temporally separated that they could be recorded independently by using either fast (approximately 100 ms) or slow (approximately 100 s) time scales. The fast-phase kinetics, which represent bimolecular association of NADPH and DHFR, were measured under pseudo-first-order conditions, and data were analyzed by a single exponential with a linear rate to correct for the small contribution of the slow phase on these time scales:

$$k_{app} = A_1 \exp(-k_1 t) + k_0 t + A_0$$

where A_1 and k_1 are the amplitude and rate of the fast quench, k_0 is the slope of the base line and A_0 is the fluorescence level at the end of the fast phase. The slow phase in the binding reaction was similarly analyzed by a single-exponential model but without the linear rate. On the slow time scales used, fewer than 10 of a total of 2048 data points collected were attributable to the fast phase and these were excluded from the analysis.

The binding of NADPH to DHFR results not only in a decrease in protein fluorescence at 340 nm but also in an increase in NADPH fluorescence at 450 nm due to energy transfer from the protein (Dunn et al., 1978). In some experiments, this energy-transfer fluorescence was monitored by using an excitation wavelength of 290 nm and recording emission with a GG420 cutoff filter (Melles Griot, Irvine, CA) that excludes light below 380 nm and therefore the protein fluorescence. In the competition experiments to measure NADPH dissociation, 1 μM DHFR was preincubated with 3 μM NADPH for 20 min at room temperature in order to ensure saturation of at least 90% of the binding sites before initiation of dissociation by rapid mixing with 0.5–1.0 mM $NADP^+$. The decrease in energy-transfer fluorescence as NADPH dissociated from the enzyme and was replaced by $NADP^+$ was recorded as described above. The concentration of $NADP^+$ did not significantly affect the measured dissociation rate, and in equilibrium experiments, it has been shown that incubation at room temperature for >20 min does not affect the binding of NADPH to any of the enzymes.

Model Fitting. Equations used for estimation of kinetic and equilibrium constants and pK values are given in the text. All models were examined by nonlinear regression techniques using the algorithm of Marquardt (1961) as previously described (Dunn et al., 1980).

Results and Discussion

Equilibrium Measurements: Binding of NADPH to WT, F137S, D27S, and D27S+F137S DHFRs. The binding of ligands and inhibitors to DHFRs can readily be monitored by following the decrease in protein intrinsic fluorescence that accompanies complex formation. Equilibrium fluorescence titrations have therefore been used to investigate the effects of replacing Asp-27 and/or Phe-137 by serine(s) on the affinity of DHFR for NADPH. At pH 7.5, the binding of NADPH to the wild-type enzyme is characterized by a K_d of 0.05 ± 0.01 μM at 25 °C. Mutation of Phe-137, a residue distant from the binding site, reduces the affinity by approximately 2-fold (K_d = 0.11 ± 0.03 μM). Replacement of Asp-27 by serine led to a greater reduction in affinity, and the measured dissociation constant for the D27S enzyme was 0.57 ± 0.06 μM at pH 7.5. For the double mutant, the measured K_d for NADPH was 0.5 ± 0.1 μM, suggesting that the Phe-137 → Ser mutation, on top of the Asp-27 → Ser mutation, has no further deleterious effect on the equilibrium binding of NADPH. The effect of pH on equilibrium dissociation constants for binding to each enzyme has been investigated over a pH range of 5.0–8.5, and the results of these experiments

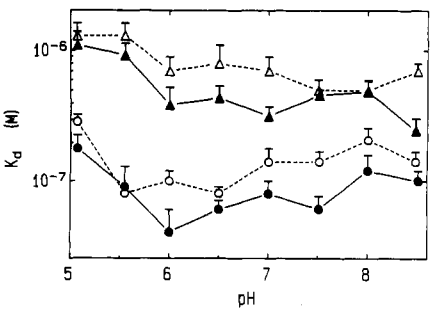


FIGURE 1: Effect of pH on the equilibrium binding of NADPH to WT (●), F137S (○), D27S (▲), and D27S+F137S (△). Equilibrium dissociation constants were estimated in fluorescence titrations using a DHFR concentration of 0.4 μM. The buffer used was 50 mM Tris, 25 mM MES, 25 mM acetic acid, 100 mM NaCl, 1 mM DTT, and 0.02% NaN_3 at 15 °C.

are illustrated in Figure 1. Changes in pH had no significant effect on enzyme affinity between pH 6 and pH 8, and at 15 °C the estimated K_ds for WT, F137S, D27S, and D27S+F137S were 0.06 ± 0.01 μM, 0.11 ± 0.03 μM, 0.5 ± 0.1 μM, and 0.7 ± 0.1 μM, respectively. Thus the increase in K_d as a result of replacing Phe-137, Asp-27, or both by serines was 2-, 7-, and 9-fold, respectively. At lower pH, all enzymes showed a slight decrease in affinity for NADPH, but the paucity of data in this region and the documented instability of reduced pyridine coenzymes at low pH (Kaplan, 1960) preclude quantitative analysis.

The role of Asp-27 in DHFR structure, binding, and catalysis has previously been investigated by mutating this residue to an asparagine (Villafranca et al., 1983) or to a serine (Howell et al., 1986). From changes in the catalytic activity of the mutants, it has been shown that this residue is important as a proton donor for bound, unprotonated substrates. Asp-27 is also important for the binding of methotrexate by forming a pair of hydrogen bonds with N-1 and the 2-amino group of the pteridine ring (Bolin et al., 1982; Filman et al., 1982). Although Asp-27 does not interact directly with bound coenzyme, the present finding that replacement of Asp-27 by a serine residues the affinity for NADPH suggests that this residue must also be indirectly involved in the enzyme–NADPH interaction. A similar finding has recently been reported by Appleman et al. (1990), who showed that replacement of Asp-27 by an asparagine residue causes a reduction in affinity for NADPH by approximately 3-fold.

Kinetics of NADPH Binding to WT, F137S, D27S, and D27S+F137S DHFRs: General Description. The kinetics of ligand and inhibitor binding to DHFRs have previously been studied in detail (Dunn et al., 1978; Dunn & King, 1980; Cayley et al., 1981). For both *L. casei* and *E. coli* DHFRs, binary complex formation with NADPH is characterized by biphasic binding kinetics with a rapid ligand-concentration-dependent phase being followed by a slow ligand-independent process. The kinetics of binding of NADPH to WT DHFR have been monitored by using either the quenching of protein intrinsic fluorescence or the enhancement of NADPH fluorescence resulting from energy transfer upon complex formation. Representative traces obtained for the fast and slow phases are shown in Figure 2. As previously reported for NADPH binding to *L. casei* DHFR (Dunn, 1978), estimates of the rates and relative amplitudes of the two phases were identical for the two monitoring systems.

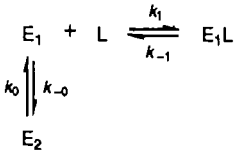
Cayley et al. (1981) have interpreted the biphasic binding kinetics in terms of Scheme I, in which there are two interconvertible conformations of enzyme and NADPH binds to only one of these forms with high affinity.

Table I: Rate and Dissociation Constants for NADPH Binding to WT, F137S, D27S, and D27S+F137S DHFRs according to Scheme I (pH 7.5, 25 °C)

	WT	F137S	D27S	D27S+F137S
k_1 (μM^{-1} s^{-1})	18 ± 1	15 ± 2	16 ± 2	18 ± 1
k_{-1}[a] (s^{-1})	10 ± 4	6 ± 3	38 ± 6	30 ± 5
$K_1 = k_{-1}/k_1$ (μM)	0.6 ± 0.2	0.4 ± 0.2	2.4 ± 0.5	1.7 ± 0.3
k'_{-1}[b]	3.1 ± 0.1	ND[e]	3.2 ± 0.1	4.9 ± 0.2
K_0[c]	0.61 ± 0.02	0.75 ± 0.02	1.4 ± 0.1	1.3 ± 0.1
$K_1(1 + K_0)$ (μM)	0.9 ± 0.3	0.7 ± 0.4	6 ± 1	4 ± 1
K_d[d] (μM)	0.06 ± 0.01	0.14 ± 0.03	0.46 ± 0.05	0.50 ± 0.10
K_z[f]	0.07 ± 0.03	0.25 ± 0.15	0.09 ± 0.02	0.15 ± 0.04

[a] Estimated from dependence of fast-phase rate on NADPH concentration. [b] Estimated from competition experiments. [c] Measured from amplitude data; K_0 = (magnitude of quench occurring in slow phase)/(magnitude of quench occurring in fast phase). [d] Measured in equilibrium fluorescence titrations. [e] ND, not determined. [f] See text.

Scheme I[a]



[a] $K_1 = k_{-1}/k_1$; $K_0 = k_{-0}/k_0 = [E_2]/[E_1]$; $K_{eq} = K_1(1 + K_0)$.

An alternative model that could account for the appearance of the slow phase in the binding kinetics is that it arises from an isomerization of the E_1L binary complex and not the free enzyme. The kinetic data for both the *L. casei* (Dunn et al., 1978) and *E. coli* (Cayley et al., 1981) enzymes have, however, been shown to be inconsistent with this latter model. When the concentration of DHFR was kept constant and the concentration of NADPH was varied, it was found that the amplitude of the fast phase, when expressed as a percentage of the total signal change, was not constant but rather increased toward 100% when less than stoichiometric amounts of NADPH were reacted. Clearly, these results are not consistent with a mechanism where the slow quench represents the isomerization of the binary complex but are consistent with Scheme I.

Provided that the binding of NADPH to E_1 is a simple bimolecular association as depicted in Scheme I, the measured pseudo-first-order rate constant for the fast phase due to NADPH binding to E_1 (k_{app}) is related to rate constants for association (k_1) and dissociation (k_{-1}) by

$$k_{app} = k_1[\text{NADPH}] + k_{-1} \quad (1)$$

Under these conditions, the slope and y-intercept of a plot of k_{app} against [NADPH] yield values for k_1 and k_{-1}. Kinetic simulations (Barshop et al., 1983) have recently been used to show that the pseudo-first-order approximation is valid if the ligand concentration exceeds that of enzyme by ≥8 (Murphy & Benkovic, 1989). Following the rapid association, the slow ligand-concentration-independent process observed in the kinetic traces reflects conversion of E_2 to E_1, followed by further binding of NADPH.

In the present study, qualitatively similar biphasic kinetic behavior has been observed for wild-type and mutant enzymes when binding was monitored by the decrease in protein fluorescence. For each enzyme, the contribution of the fast phase to the total signal change increased when less than stoichiometric amounts of NADPH were reacted as described above for the wild-type enzymes and as predicted by Scheme I. Mutation of Asp-27 and/or Phe-137 does not therefore appear to drastically alter the general mechanism by which NADPH binding may be described, suggesting that no gross change in enzyme structure has occurred as a result of any of the mutations.

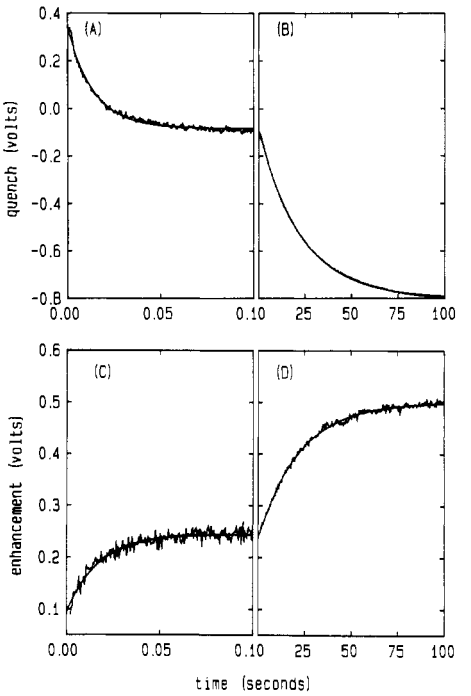


FIGURE 2: Representative kinetic traces for NADPH binding to WT DHFR at pH 5.0 monitored either by quenching of the protein intrinsic fluorescence (A, B) or by NADPH energy-transfer fluorescence (C, D). NADPH (4 μM) was mixed with 0.4 μM WT DHFR at 25 °C and the fast (A, C) and slow phases (B, D) were recorded independently at two different time scales. Solid lines are calculated by using the best fit parameters for a single-exponential model as described under Materials and Methods: (A) Amp = 460 mV; k_{app} = 71 s^{-1}; (B) Amp = 684 mV; k_{app} = 0.044 s^{-1}; (C) Amp = 168 mV; k_{app} = 68 s^{-1}; (D) Amp = 251 mV; k_{app} = 0.045 s^{-1}. Thus the contribution of the fast phase to the total signal change was estimated to be 40.2% from protein quenching and 40.1% from energy transfer (see text).

Fast-Phase Binding Kinetics. The apparent rate constant for the fast phase of NADPH binding to WT and to all three mutant DHFRs increased linearly with NADPH concentration as shown in Figure 3. In all measurements, the concentration of NADPH exceeded that of the enzyme in the E_1 form by at least 10-fold in order to approximate pseudo-first-order conditions. Values of k_1 and k_{-1} obtained from these experiments at pH 7.5 are given in Table I. None of the mutations had a significant effect on the association rate constant (k_1), and the estimated value of 1.8×10^7 M^{-1} s^{-1} for WT DHFR is in good agreement with previous estimates obtained under similar experimental conditions (Fierke et al., 1987; Appleman et al., 1990). Although k_1 was not significantly affected by any of the mutations, k_{-1} was 3–4-fold higher in the case of the D27S and D27S+F137S mutants. Appleman et al. (1990) also reported an apparent increase in k_{-1} by 2.6-fold in the D27N mutant but concluded that this was not statistically significant. We have carried out similar studies of the con-

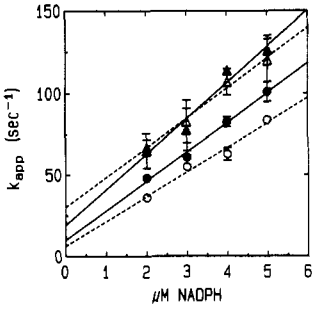


FIGURE 3: Effect of NADPH concentration on the rate of the fast phase for WT (●), F137S (○), D27S (▲), and D27S+F137S (△) DHFRs. Data were obtained in stopped-flow experiments at pH 7.5 and 25 °C. Values obtained for k_1 and k_{-1} (see text) are given in Table I.

Scheme II

$$EL_1 \underset{k_1}{\overset{k_{-1}}{\rightleftharpoons}} E + L_1$$

$$E + L_2 \underset{k_{-c}}{\overset{k_c}{\rightleftharpoons}} EL_2$$

centration dependencies of the fast-phase rates between pH 5 and pH 8.5 at half pH unit intervals. Neither k_1 nor k_{-1} was significantly affected by pH, and average values for k_{-1} were 13 ± 5, 10 ± 5, 49 ± 9, and 38 ± 12 s^{-1} for WT, F137S, D27S, and D27S+F137S, respectively. Estimated dissociation rates for D27S and D27S+F137S were therefore consistently higher than for WT or F137S DHFRs, suggesting that although, as Appleman et al. (1990) have previously noted, these parameters are subject to large experimental error, the increased rate of dissociation for the two mutants does appear to be significant.

Adams et al. (1989) have recently reported that the fast phase of NADPH binding to WT DHFR could be resolved into two kinetically distinguishable components. One component was proposed to represent the bimolecular interaction of NADPH with E_1 (k_{on} = 20 ± 0.2 μM^{-1} s^{-1}; k_{off} = 3 ± 0.3 s^{-1}), similar to the fast-phase kinetics reported here. The second process (k_{on} = 5 ± 0.4 μM^{-1} s^{-1}; k_{off} = 70 ± 3 s^{-1}) was interpreted as arising from the direct binding of NADPH to E_2, to which it bound with approximately 100-fold lower affinity than to E_1. In the present experiments, no better fit of the fast-phase kinetics was obtained by using a two-exponential model. However, Adams et al. (1989) also found that the rapid binding kinetics were monophasic at the concentrations (2–10 μM) used in the present study. Thus the possibility of the direct binding of NADPH to E_2 cannot be excluded and this is discussed further below.

Estimation of Dissociation Rates from Competition Experiments. Dissociation rates can also be measured in competition experiments in which the preformed DHFR–NADPH complex is mixed with a large excess of a second ligand that competes for the NADPH-binding sites (Scheme II). If $k_{-1} \ll k_c[L_2] \gg k_1[L_1]$, the signal due to the disappearance of EL_1 can be interpreted in terms of the rate constant, k_{-1}, for the dissociation of the complex. NADPH dissociation has been measured for the WT, D27S, and D27S+F137S complexes with $NADP^+$ as the competing ligand. Since the binding of NADPH to DHFR is accompanied by an enhancement of NADPH fluorescence due to energy transfer, dissociation can be measured by following the decrease in this fluorescence due to displacement by $NADP^+$ (Dunn et al., 1978). The results of these experiments are shown in Figure 4, and the measured rate constants are given in Table I. It is clear that, particularly in the case of the D27S and D27S+F137S enzymes, there is

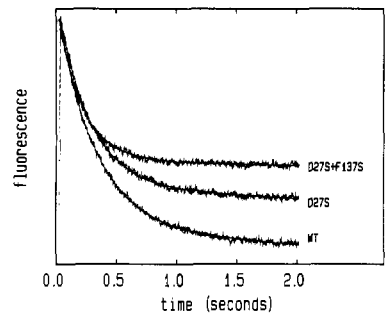


FIGURE 4: Estimation of dissociation constants in competition experiments. DHFR (1 μM) was preincubated with 3 μM NADPH for 20 min at 25 °C, and dissocation was initiated by mixing with an equal volume of $NADP^+$ (final concentration = 1 mM). Experiments were carried out at pH 7.5, and dissociation was measured by monitoring the decline in NADPH energy-transfer fluorescence at >420 nm. Solid lines are calculated from the best fit parameters obtained by nonlinear regression fitting by a single-exponential model. Values for the dissociation rate constants were 3.1 s^{-1} (WT), 3.7 s^{-1} (D27S), and 4.8 s^{-1} (D27S+F137S).

a large discrepancy between the dissociation rates measured in competition experiments and the much higher values estimated from the ligand concentration dependence of the fast-phase association rate.

Slow-Phase Binding Kinetics. The rate of the slow fluorescence quench accompanying NADPH binding to WT and mutant DHFRs has been measured at pH 7.5 with an enzyme concentration of 0.4 μM and variable NADPH concentration between 4 and 10 μM. In this concentration range, there was no measurable dependence of the apparent rate constant on NADPH concentration, and average values were 0.058 ± 0.002, 0.049 ± 0.002, 0.038 ± 0.001, and 0.055 ± 0.005 s^{-1} for WT, F137S, D27S, and D27S+F137S enzymes, respectively. Similar experiments have been carried out between pH 5.0 and 8.5, pH and as was found for the fast-phase kinetics (see above), pH did not have a significant effect on the rate constant of the slow phase for any DHFR mutant. The apparent rate of the slow phase of NADPH binding to WT DHFR is in agreement with that reported by Fierke and Benkovic (1989) but is approximately 6-fold faster than estimated by Appleman et al. (1990). As described above, Adams et al. (1989) have recently resolved a second component in the fast-phase kinetics that was attributed to the direct binding of NADPH to E_2 [see also Murphy and Benkovic (1989)]. Appleman et al. (1990) have also suggested that NADPH binds to E_2 but with much lower affinity (K_d approximately 23 μM) than to E_1. This was suggested from the observation that the apparent rate constant of the slow phase was not independent of NADPH but rather increased hyperbolically with NADPH concentration. We have further investigated this phenomenon in WT, D27S, and D27S+F137S by studying the effect of NADPH concentration between 5 and 100 μM on the slow-phase rate constant. For each enzyme there was some detectable increase in the observed rate constant at NADPH concentrations greater than 20 μM, but this showed no evidence of saturation below 100 μM, and the maximum measured increase (at 100 μM) was less than a factor of 1.4, rendering it too small to be accurately quantitated. At NADPH concentrations greater than 40 μM, there was also a small decrease (by a maximum of 30% at 100 μM) in the amplitude of the fluorescence quench occurring in the slow phase. These results support the interpretation of Appleman et al. (1990) that, at high concentrations, some NADPH is likely to bind to E_2, followed by conversion of $E_2L \rightarrow E_1L$. However, at the concentrations of NADPH used in the experiments reported here (≤10 μM), this pathway is likely

to represent only a minor component of the binding mechanism and it is assumed that any small amount of complex formation as a result of binding of NADPH to E_2 does not significantly influence the interpretation of the data.

Evaluation of the Kinetic Mechanism for WT and Mutant Enzymes. Although Scheme I qualitatively describes the kinetics of NADPH binding to wild-type and mutant enxymes, there are two observations which suggest that the actual kinetic scheme may be more complex. First, as noted above (Table I), there is a clear discrepancy between dissociation rate constants measured in competition experiments and from the association kinetics. Second, the overall equilibrium constant estimated from the kinetic parameters differs substantially from the dissociation constants measured directly in equilibrium fluorescence titration experiments. According to Scheme I, the overall equilibrium constant is given by $K_1(1 + K_0)$, where K_0 is a measure of the conformer equilibrium, $[E_2]/[E_1]$, in the absence of bound ligand. This equilibrium constant can be estimated from the relative magnitudes of fluorescence quench occurring in the fast and slow phases of the reaction traces. Under conditions in which [NADPH] $\gg K_d$ for binding to E_1, the fast phase amplitude relative to the total amplitude of the quench represents the fraction of the total enzyme that is originally present in the E_1 form. An inherent assumption in this analysis is that the fluorescence characteristics of E_1 and E_2 are identical. Some experimental evidence supporting this assumption has been obtained for WT DHFR since the relative amplitudes of the two phases were independent of the monitoring system used, i.e., protein quenching or energy transfer to NADPH (Figure 2). In the energy-transfer experiments, the intrinsic fluorescence of E_1 and E_2 is very small compared to that of the binary complex(es), and the relative amplitude data are likely to give an accurate measure of K_0. The close agreement between these estimates and those obtained by looking at protein quenching appears to validate the assumption that E_1 and E_2 have similar fluorescence properties. In the discussion below, it is assumed that the two conformations of the mutant apoenzymes also have similar fluorescence, but supporting information from energy-transfer experiments is not yet available for these enzymes.

Values of K_0 have been estimated from relative amplitude data obtained by using an enzyme concentration of 0.2–0.4 μM and varying [NADPH] between 4 and 10 μM. Over this range the relative amplitudes were independent of [NADPH] for each enzyme, and the concentration of NADPH exceeded the estimated K_{d1} for binding to E_1 (see below) by at least 20-fold. Results obtained at pH 7.5 are given in Table I. From these values and estimates of K_1 obtained from the fast-phase kinetics, equilibrium constants were calculated from the kinetic data according to Scheme I, and these are included in Table I. For all the enzymes used in the present study, there is clear discrepancy from measured equilibrium constants. A possible explanation for the apparent discrepancy is that in Table I predicted equilibrium constants have been calculated by using values of k_{-1} obtained in the kinetic relaxation experiments. As noted above, these values are subject to large error. If the values of k'_{-1} that were obtained in competition experiments are used instead, the predicted dissociation constants for WT, D27S, and D27S+F137S DHFRs are 0.28 μM, 0.50 μM, and 0.64 μM, respectively. Clearly these are in much better agreement with measured dissociation constants (Table I). Appleman et al. (1990) similarly found good agreement between measured dissociation constants and those predicted from kinetic parameters only if dissociation rates measured in competition experiments were used. Cayley et al. (1981) also reported that rate constants measured in competition experiments were smaller than those determined from concentration-dependence studies and, in order to explain this discrepancy, proposed that Scheme I be extended to include an isomerization of the initial binary complex (Scheme III). For such a mechanism, the dissociation rate constant measured in competition experiments will not equal k_{-1}. An isomerization of E_1L is not directly detected by the fluorescence-quenching technique, although this may be due to all enzyme–ligand complexes having the same fluorescence quantum yield. According to Scheme III, the overall measured dissociation constant, K_d, may be described in terms of the equilibrium constants

Scheme III

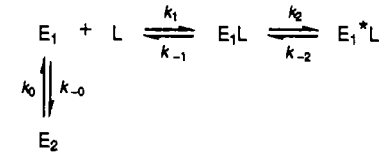


Table II: Effect of pH on the Equilibrium between Enzyme Conformers E_1 and E_2[a]

	WT	F137S	D27S	D27S+F137S
pK_{E1}	5.5 ± 0.1	5.8 ± 0.1	6.3 ± 0.1	6.5 ± 0.2
pK_{E2}	6.0 ± 0.2	6.2 ± 0.2	6.6 ± 0.2	6.1 ± 0.3
K'_0	1.6 ± 0.1	1.6 ± 0.1	2.1 ± 0.2	0.8 ± 0.1
K_0	0.6 ± 0.2	0.7 ± 0.1	1.1 ± 0.1	1.4 ± 0.6
$pK2_{E1}$		8.2 ± 0.2	8.0 ± 0.2	
$pK2_{E2}$		7.8 ± 0.2	7.8 ± 0.1	
$K2_0$		0.3 ± 0.1	0.7 ± 0.2	

[a] Parameters listed were estimated from curve fitting to Scheme IV (WT, D27S+F137S) or V (F137S, D27S) using the amplitude data shown in Figures 4 and 5.

$$K_d = K_1(1 + K_0)/(1 + 1/K_2) \quad (2)$$

where $K_2 = k_{-2}/k_2$ and other constants are as defined above. From the values of K_1, K_0, and K_d given in Table I, estimates of K_2 for WT, F137S, D27S, and D27S+F137S DHFRs were 0.07, 0.25, 0.08, and 0.14, respectively (Table II). It should, however, again be stressed that the occurrence of an isomerization of the initial complex and estimates of kinetic constants for this transition are critically dependent on reliable measurements of k_{-1} and these estimates are subject to large error due to the inherent difficulties associated with their measurement as noted above. With these caveats, the estimated values may be used to calculate apparent K_d values for NADPH binding to E_1 according to the relationship $K_{d1} = K_1/(1 + 1/K_2)$. Estimated values are 0.04 ± 0.02 μM (WT), 0.08 ± 0.06 μM (F137S), 0.18 ± 0.05 μM (D27S), and 0.21 ± 0.07 μM (D27S+F137S). It appears, therefore, that replacement of Phe-137 by a serine reduces the affinity of E_1 for NADPH by about 2-fold but mutation of Asp-27 results in an approximately 5-fold decrease in affinity. This decrease in affinity appears to be due mainly to an increase in the dissociation rate of the initial enzyme–NADPH complex or, in the case of F137S, to a decrease in stability of the E_1*L complex.

Effect of pH on Conformer Equilibrium. It has previously been demonstrated that the equilibrium between the two forms of the wild-type *E. coli* enzyme is pH dependent (Cayley et al., 1981) and that the relative proportion of the NADPH-binding form (E_1) increases with pH. The effect on pH on conformer equilibrium has therefore been investigated for WT and the three mutant DHFRs. In these experiments, the

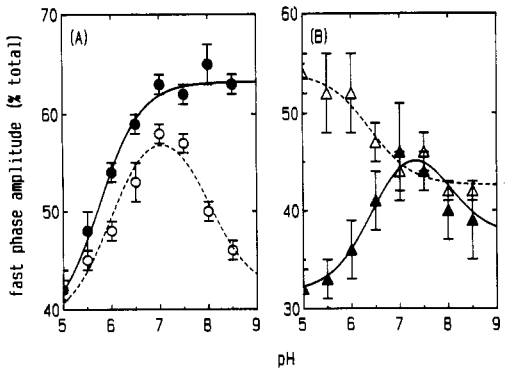
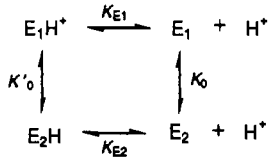


FIGURE 5: Effect of pH on the conformer equilibrium for (A) WT (●) and F137S (○) DHFRs and (B) D27S (▲) and D27S+F137S (△) DHFRs. The percentage of the total fluorescence signal occurring in the fast phase was measured as described in the text, and the lines were calculated by using the best fit parameters, listed in Table II, which were estimated from curve fitting according to Scheme IV (WT, D27S+F137S) or V (F137S, D27S).

Scheme IV



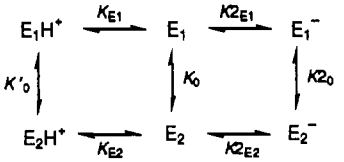
enzyme concentration was constant at 0.4 μM and the NADPH concentration was varied between 4 and 10 μM. The percentage of the enzyme in the E_1 form was estimated from the contribution of the fast-phase quench to the total amplitude change. For each enzyme, this was found to be insensitive to the NADPH concentration over this range. As described above, the assumption in these experiments is that E_1 and E_2 have identical fluorescence properties over the pH range studied and that the relative amplitude data give an accurate measure of K_0. Support for this assumption for WT DHFR has come from energy-transfer experiments at pH 5 (Figure 2), pH 7, and pH 8.5 in which parameter estimates were not significantly different from those obtained in protein-quenching experiments. Similar information is not yet available for the DHFR mutants, and it cannot therefore be excluded that some of the observations reported below arise from a possible pH dependence of the fluorescence properties of E_1 and/or E_2.

Figure 5A illustrates the effect of pH on the relative amplitude of the fast phase (and therefore of E_1) observed when NADPH binds to WT DHFR. These data are similar to those reported by Cayley et al. (1981), and they may be interpreted in terms of a single ionization in the protein which has a different pK in the E_1 form to that in the E_2 form (Scheme IV). Estimated values of the equilibrium constants have been obtained from nonlinear regression curve fitting by using eq 3 and the best fit values are listed in Table II. The conformer

$$\frac{\text{fast amplitude}}{\text{total amplitude}} = \frac{10^{-pH} + K_1}{10^{-pH}(1 + K'_0) + K_1(1 + K_0)} \quad (3)$$

equilibrium, therefore, appears to involve an ionizable group that has a pK of 5.5 ± 0.1 in E_1 and 6.0 ± 0.2 in E_2. A similar pH profile of the conformer equilibrium has been observed from studies of dihydrofolate binding (Cayley et al., 1981), suggesting that an ionizable group of the enzyme, and not of the coenzyme is involved. For the protonated enzyme species, the equilibrium favors the form with little affinity for NADPH, i.e., E_2H^+ (K'_0 = 1.6 ± 0.1), whereas above the pK for the ionization, E_1 is favored (K_0 = 0.6 ± 0.2). Since NADPH binds only to E_1 and E_1H^+ with high affinity, the percentage

Scheme V



of the fast-binding form increases with pH. It should be noted that although the equilibrium is pH dependent, the binding reaction itself is not directly dependent on the degree of ionization of the group conferring this dependency, since the rates of binding were not affected by pH in this range [see above; also see Fierke et al. (1987)].

Replacement of Phe-137 by a serine had little effect on the conformer equilibrium below pH 7 (Figure 5A), but at more alkaline pH the relative amount of fast phase (and therefore of E_1) declined. This suggests that for this mutant a second ionizable group must be involved in the conformer equilibrium and that Scheme IV must be extended as shown in Scheme V. Curve fitting using this model gave the parameters listed in Table II. Equilibrium constants and pK values describing the first ionization were not significantly different from the wild-type enzyme and values for p$K2_{E1}$ and p$K2_{E2}$ were estimated to be 8.2 and 7.8, respectively.

The effects of pH on the equilibrium between the two conformers of D27S were qualitatively very similar to that of F137S, with an obvious quantitative difference in that, at all pH values, the relative abundance of E_1 was decreased (Figure 5B). In a previous study (Appleman et al., 1990) it has been reported that replacement of Asp-27 by an asparagine reduced the relative abundance of E_1 from 63% in WT to 18% in the mutant (pH 7.65). Thus, although the magnitude of the effect appears to be smaller in the D27S DHFR (Table II), these results are consistent in suggesting that the effect of Asp-27 on the conformer equilibrium is to favor the NADPH-binding conformation (E_1). The pK of the first ionizable group was shifted to higher pH, i.e., 6.3 ± 0.1 in E_1 and 6.6 ± 0.2 in E_2. It should be pointed out that the errors in these parameters may be underestimated due both to the complexity of the system under study and to the nature of the experiments, which makes it difficult to obtain large amounts of quantitative data. These results do, however, indicate that Asp-27 is not the residue that confers the pH sensitivity of the conformer equilibrium since an acidic pK is also observed when this is replaced by a serine. At higher pH, there was a decline in the relative amount of E_1 similar to that observed with F137S DHFR, again implicating the involvement of a second ionizable group with a pK of 7.8–8.2 (Figure 5A).

In noticeable contrast to WT, D27S and F137S DHFRs, the relative amount of the double mutant, D27S+F137S DHFR, in the E_1 form appeared to decrease from 54% at pH 5.5 to 42% at pH 8.5 (Figure 5B). These data can be described by a model assuming a single ionization of the protein as shown in Scheme IV. The results of curve fitting are given in Table II. Estimated pK values are similar to the first ionization observed when D27S was used, and it appears that the major contributing factor to the observed difference in conformer equilibrium is a decreased K'_0 (E_2H^+/E_1H^+) and an increased K_0. Thus, unlike the other enzymes, the NADPH-binding form is more favored at lower pH than when the ionizable group involved in the enzymic equilibrium is unprotonated.

The identity of the amino acid residues that confer the pH dependence of the enzymic equilibria has not been established. As discussed above, Asp-27 is unlikely to be involved in conferring the more acidic pK (5.5–6.5) since all mutants dis-

played a similar, albeit shifted, pK in this range. On the basis of the pH dependence, it is tempting to implicate the involvement of a histidine residue [see Cayley et al. (1981)]. However, in the absence of supporting data from additional mutation experiments, this would be only speculative since it has frequently been observed that pK values for titratable side chains in enzymes can be considerably different from their values in isolated amino acids [see Howell et al. (1987)]. Both the D27S and F137S DHFR mutants displayed a more basic pK (approximately 8.0), but as yet the nature of these ionizable groups is unknown.

In earlier studies in which the effects of site-directed mutagenesis on conformer equilibrium has been examined, mutation of several amino acid residues has been shown to alter the equilibrium. As discussed above, replacement of Asp-27 by asparagine markedly decreased the amount of enzyme in the E_1 form at pH 7.65 (Appleman et al., 1990). Thr-113 is also indirectly involved in the conformer equilibrium since replacement by valine increased the amount of E_1 compared to wild-type DHFR at pH 6 (E_1/E_2 = 1.2; Fierke & Benkovic, 1989) and decreased it at pH 7 (E_1/E_2 = 0.4; Chen et al., 1985). On the contrary, replacement of His-45, which interacts directly with NADPH, by glutamine (Chen et al., 1985) or replacement of Leu-54, which interacts with dihydrofolate, by glycine (Meyer et al., 1986) did not apparently change the distribution at pH 7.0. These results must, however, be treated with caution since, as shown above and by Cayley et al. (1981), the distribution of conformers is pH dependent and a result obtained at one pH may not be sufficient to identify a perturbation in the conformer equilibrium.

In the accompanying paper, Howell et al. have studied the catalytic activity of the F137S and D27S+F137S mutant DHFRs. Above pH 8 the F137S enzyme displayed a small (1.3–2-fold) increase in hydride-transfer rate (k_{cat}) compared to that of WT DHFR. However, a concurrent slight loosening in the binding of dihydrofolate (increased K_m) results in no overall change in $k_{cat}/K_{m(DHF)}$. In contrast, a 3-fold increase in k_{cat} and a 7-fold increase in $k_{cat}/K_{m(DHF)}$ were observed when this mutation is added to D27S DHFR. The F137S mutation is therefore nonadditive, suggesting that the effects on catalysis are a result of slight alterations in protein conformation. Furthermore, Howell et al. (1990) have proposed that, since the F137S mutation is distant from the binding sites and occurs on the surface of the protein, the effects of the mutation on catalysis must be due to a more global change in enzyme conformation. This interpretation is supported by the above evidence that the mutations alter the equilibrium distribution of the two conformers of free enzyme.

Acknowledgments

We are especially grateful to Dr. Michael Raftery for lending us his stopped-flow and data acquisition instrumentation for use in these experiments. We thank Deb Remy and Colleen Martin for carrying out some of the equilibrium fluorescence titrations.

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