Hydrophobic Interactions via Mutants of *Escherichia coli* Dihydrofolate Reductase: Separation of Binding and Catalysis

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ABSTRACT: The strictly conserved residue leucine-54 of *Escherichia coli* dihydrofolate reductase forms part of the hydrophobic wall which binds the *p*-aminobenzoyl side chain of dihydrofolate. In addition to the previously reported glycine-54 mutant, isoleucine-54 and asparagine-54 substitutions have been constructed and characterized with regard to their effects on binding and catalysis. $NADP^+$ and NADPH binding is virtually unaffected with the exception of a 15-fold decrease in NADPH dissociation from the Gly-54 mutant. The synergistic effect of NADPH on tetrahydrofolate dissociation seen in the wild-type enzyme is lost in the isoleucine-54 mutant: little acceleration is seen in tetrahydrofolate dissociation when cofactor is bound, and there is no discrimination between reduced and oxidized cofactor. The dissociation constants for dihydrofolate and methotrexate increase in the order Leu < Ile < Asn < Gly, varying by a maximum factor of 1700 for dihydrofolate and 6300 for methotrexate. Despite these large changes in binding affinity, the hydride transfer rate of 950 s^{-1} in the wild-type enzyme is decreased by a constant factor of ca. 30 (2 kcal/mol) *regardless* of the mutant. Thus, the contributions of residue 54 to binding and catalysis appear to have been separated.

Dihydrofolate reductase (5,6,7,8-tetrahydrofolate:$NADP^+$ oxidoreductase, EC 1.5.1.3; DHFR)[1] catalyzes the NADPH-dependent reduction of 7,8-dihydrofolate (H_2F) to 5,6,7,8-tetrahydrofolate (H_4F). The enzyme is necessary for maintaining intracellular pools of H_4F and its derivatives which are essential cofactors in several important biosynthetic reactions which require the transfer of one-carbon units. As a result, inhibition of the enzyme by compounds such as methotrexate (MTX) and trimethoprim (TMP) in vivo has proven to be clinically efficacious. Studies of mutants of the *Escherichia coli* DHFR (Appleman et al., 1988; Howell et al., 1986, 1987; Taira & Benkovic, 1988; Chen et al., 1985, 1987; Taira et al., 1987a–c; Mayer et al., 1986; Villafranca, 1983) have been facilitated by high-resolution crystal structures (Bolin et al., 1982; Filman et al., 1982; Matthews et al., 1985) and detailed analysis of the kinetic mechanism (Fierke et al., 1987; Morrison, 1988).

The strictly conserved residue leucine-54 forms part of the hydrophobic wall which binds the *p*-aminobenzoyl side chain of dihydrofolate (Figure 1). Despite being >10 Å from the site of hydride ion transfer, removal of the isobutyl side chain by mutagenesis to glycine (L54G) has a dramatic effect on the catalytic reaction (Mayer et al., 1986). In the wild-type enzyme, hydride transfer is rapid, and product release is rate determining (at physiological pH); in L54G, the reverse situation holds: hydride transfer is rate determining, and product release is rapid.

Two more mutants at this position have been constructed which involve more subtle changes in side chain structure to further elucidate the nature of this hydrophobic interaction: the isomeric and isosteric isoleucine-54 (L54I) and the sterically smaller asparagine-54 (L54N). The net effect of the *sec*-butyl side chain is to remove one methyl group from interaction with the substrate (or inhibitor); the carboxamide introduces a polar function into a formerly apolar–apolar pairing. The glycine substitution most likely involves a combination of water filling the cavity and the protein collapsing into it.

Materials and Methods

Substrates. Dihydrofolate (H_2F) was prepared by the reduction of folate by the method of Blakely (1960), and (6*S*)-tetrahydrofolate (H_4F) was prepared by using wild-type dihydrofolate reductase to reduce H_2F (Mathews & Huennekens, 1963) and purified on DE-52 resin eluting with a triethylammonium bicarbonate linear gradient (Curthoys et al., 1972). [4′(*R*)-^{2}H]NADPH was prepared by using *Leuconostoc mesenteroides* alcohol dehydrogenase (Stone & Morrison, 1982) obtained from Research Plus Inc., and purified by the method of Viola et al. (1979). 6-(Ethoxymethyl)tetrahydropterin was the generous gift of Dr. G. K. Smith of the Wellcome Research Laboratories and oxidized to the dihydro form by the method of Smith et al. (1987).

Enzymes. T4 DNA ligase, *Bam*HI, *Eco*RI, *Apa*I, and T4 polynucleotide kinase were purchased from New England Biolabs. T4 gene 32 protein and AMV reverse transcriptase were purchased from Boehringer Mannheim. T4 DNA polymerase was purified in our laboratory by T. Capson and Klenow polymerase by R. D. Kuchta.

DNA. The 1-kilobase *fol* fragment containing the L54G mutation was obtained by *Bam*HI digestion of the pBR322-derived plasmid pTY1 (Chen et al., 1987, 1985) and isolation from low-melting type VII agarose (Bethesda Research Laboratories).

Plasmid pTz19R including M13K07 helper phage was obtained from Pharmacia Inc. The 20-base oligonucleotides 5′-GGT-CGT-CCG-ATC-CCA-GGA-CG and 5′-GGT-

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[1] Abbreviations: DHFR, dihydrofolate reductase; H_2F, 7,8-dihydrofolate; H_4F, 5,6,7,8-tetrahydrofolate; NADPH, nicotinamide adenine dinucleotide phosphate, reduced; $NADP^+$, nicotinamide adenine dinucleotide phosphate; MTX, methotrexate; TMP, trimethoprim; L54I, leucine-54 → isoleucine-54 mutant of DHFR; L54N, leucine-54 → asparagine-54 mutant of DHFR; L54G, leucine-54 → glycine-54 mutant of DHFR.

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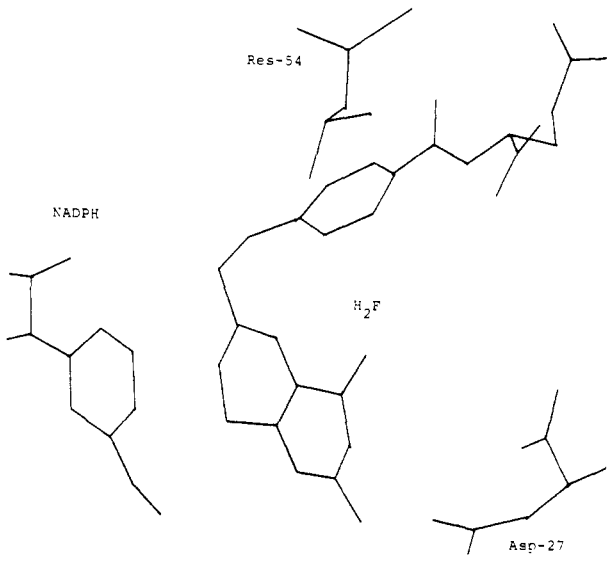


FIGURE 1: Representation of the active site of DHFR highlighting Leu-54 and Asp-27 residues with NADPH and H_2F.

CGT-CCG-AAC-CCA-GGA-CG were synthesized on an Applied Biosystems Model 380A DNA synthesizer and purified by means of high-performance liquid chromatography on a reverse-phase μ-Bondapak C_{18} column (3.9 mm × 30 cm; Waters Associates).

Phosphorylation of Oligonucleotides. 5′-OH oligonucleotide (280 pmol) was treated with 1 unit of T4 polynucleotide kinase for 30 min at 37 °C in 20 μL of 1.5 mM ATP, 70 mM Tris-HCl, pH 9.0, 10 mM $MgCl_2$, and 20 mM DTT. The enzyme was heat-inactivated at 65 °C for 10 min and the solution stored at −20 °C.

Preparation of pTZ/Gly-54. Isolated *fol* fragment (0.08 pmol) was mixed with 0.02 pmol of *Bam*HI-linearized pTz19R and ligated in 20 μL of solution containing 50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 20 mM DTT, 1 mM ATP, and 10 units of T4 DNA ligase for 18 h at 16 °C.

The ligation mixture was transformed into JM109 utilizing the New England Biolabs "M13 Sequencing" protocol onto x-gal/IPTG agarose plates. All cultures containing Amp plasmids were grown on plates containing 50 μg/mL ampicillin and in solution with 200 μg/mL ampicillin.

Preparation of Single-Stranded Template. A 1:100 dilution of a stationary-phase culture of JM109 containing pTz/Gly-54 was superinfected with an equal volume of 10^{12} pfu/mL M13K07 phage and grown for 8 h with good aeration. The cells were centrifuged at 3900*g* for 15 min (Sorvall SS34 rotor, 18 000 rpm). Twenty volume percent of 2.5 M NaCl/20% poly(ethylene glycol) was added to the supernatant and the solution allowed to stand for 20 min at 4 °C. The phage were pelleted at 3900*g* for 30 min, redissolved in 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA, and exhaustively extracted with buffer-saturated 1:1 phenol/chloroform followed by ether extraction and ethanol precipitation.

Annealing and Polymerization. To 0.5 μg (0.5 pmol) of single-stranded pTz/Gly-54 template was added 14 pmol of 5′-phosphorylated oligonucleotide in 10 μL of water. The solution was heated to 85 °C for 2 min, allowed to cool to room temperature, and then put on ice. To this solution was added 10 μL of a solution 2.5 mM in dATP, dCTP, dGTP, and TTP, 5 μL of 1 mM ATP, 1 μL of 7.5 mg/mL T4 gene 32 protein, 1 μL of 0.1 mg/mL T4 polymerase (ca. 2 units), 1 μL of 2 units/μL T4 DNA ligase, 5 μL of 10× buffer [10× = 200 mM Hepes, pH 7.8, 20 mM DTT, and 100 mM $MgCl_2$ (Kunkel, 1986)], and 17 μL of water for a total volume of 50 μL. The reaction was kept at 0 °C for an additional 5 min, warmed to room temperature for 5 min, and then heated at 37 °C for 30 min.

Transformation and Screening. The polymerization reaction mixture was transformed into HB2154 cells (Carter et al., 1985) (5 μL of reaction mixture per 200 μL of cells) and grown on agarose plates. The cells were scraped from the plates; the plasmid DNA was isolated by miniprep (Birnboim & Doly, 1979) and retransformed into JM109 cells. Twelve single colonies were picked from the "asparagine" and "isoleucine" plates. The plasmid DNA was isolated and screened for the absence of the *Apa*I site.

Sequencing. Single-stranded DNA was isolated for candidates of the Asn and Ile mutants as described above and used as the template for dideoxy sequencing (Sanger, 1981) utilizing three primers to cover the structural gene. AMV reverse transcriptase and Klenow polymerase were used for polymerization.

Reactant Concentrations. The concentration of tetrahydrofolate was determined spectrophotometrically by using a molar extinction coefficient of 28 000 M^{-1} cm^{-1} at 297 nm (Kallen & Jencks, 1966) or enzymatically by using a molar absorptivity change for the 10-formylsynthetase reaction of 12 000 M^{-1} cm^{-1} at 312 nm (Smith et al., 1981). The concentration of dihydrofolate was determined enzymatically by using a molar absorbancy change for the dihydrofolate reductase reaction of 11 800 M^{-1} cm^{-1} at 340 nm (Stone & Morrison, 1982). The concentration of purified L54I DHFR (see below) was determined by methotrexate titration (Williams et al., 1979). The concentrations of L54G and L54N mutants were determined by the molar extinction coefficient of 31 100 M^{-1} cm^{-1} at 280 nm.

Enzyme Purification. The L54I mutant was grown in *E. coli* strain SF32 (Singer et al., 1985) which is deficient in the chromosomal wild-type dihydrofolate reductase, and containing the plasmid pTz/Ile-54. L broth medium was used and supplemented with 0.2% glucose, 50 μg/mL thymidine, and 15 μg/mL tetracycline. The L54G and L54N mutant enzymes were grown in *E. coli* strain JM109 containing the plasmids pTz/Gly-54 and pTz/Asn-54, respectively. All three mutants were purified by using a methotrexate affinity resin (Pierce Biochemicals) (Baccanari et al., 1977) with the following modifications. The L54I and L54N mutants were eluted from the methotrexate resin with pH 9 borate buffer only. The L54G mutant was eluted with 1 M KCl buffer, pH 6, only and then, following the DEAE-Sephacel column, eluted through a G-75 Sephadex column.

Buffers. All kinetic and equilibrium measurements were performed at 25 °C in buffer containing 50 mM 2-morpholinoethanesulfonic acid (Mes), 25 mM tris(hydroxymethyl)aminomethane (Tris), 25 mM ethanolamine, and 100 mM sodium chloride (MTEN buffer, pH 5–10) or 25 mM Mes, 50 mM Tris, 25 mM sodium acetate, and 100 mM sodium chloride (MTAN buffer, pH 4–5) (Ellis & Morrison, 1982).

Stopped-Flow Measurements. Kinetic data were obtained by using a stopped-flow instrument built in the laboratory of K. A. Johnson (Johnson, 1986) as described in Fierke et al. (1987). For reactions utilizing tetrahydrofolate, the solutions were vacuum-evacuated at room temperature, followed by argon addition. This cycle was repeated at least 5 times.

Steady-State Measurements. Initial velocities (v) for the dihydrofolate reductase reactions were determined by measuring the rate of enzyme-dependent decrease at 340 nm with a molar absorbancy change of 11 800 M^{-1} cm^{-1} employing a

Cary 219 spectrophotometer. The concentration of NADPH or NADPD was initially maintained at a saturating level of 100 μM. The enzyme was preincubated in the presence of 100 μM NADPH or NADPD at pH 8.0 for 15 min to remove the hysteretic behavior (Penner & Frieden, 1985) and used over the course of the kinetic runs (1–2 h). The reaction was initiated by adding 5–10 μL of the enzyme–cofactor solution to a 0.99-mL solution of 100 μM cofactor and varying concentrations of H_2F in MTEN or MTAN buffer at the appropriate pH. The maximum velocity and Michaelis constants were determined by utilizing a nonlinear least-squares fitting program, NLIN (Marquardt method) [SAS Users Guide, 1985; see also Taira et al. (1987c)]. The pH–rate profiles were fitted to the functions $V = \log V_{max} - \log (1 + \chi^{pH-pK_a})$, where $V = k_{cat}$ or k_{cat}/K_M and $\chi = 10$, 6, or 5 for slopes of 1.0, 0.6, and 0.5, respectively. The reverse reaction was carried out as described in Fierke et al. (1987) under the conditions of 600 μM $NADP^+$, 225–450 μM H_4F, pH 10.0, MTEN buffer. The lack of change in observed rate with the concentration of H_4F showed these conditions to be saturating.

Fluorescence Titration. Thermodynamic dissociation constants were determined by fluorescence titration on an SLM 8000 spectrofluorometer as described in Taira and Benkovic (1988).

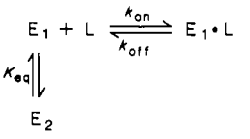
Results

Subcloning and Construction of Mutant Enzymes. The pTz/Gly-54 plasmid was constructed by inserting the L54G mutant *fol* fragment (Mayer et al., 1986) into the *Bam*HI site of the polylinker of the vector pTz19R. Insertion into the polylinker of the *lac Z'* gene should interrupt production of the α-protein of β-galactosidase and thus yield colonies which are "white" when grown on agar plates containing x-gal and IPTG. That the colonies containing the *fol* insert were in fact blue is believed due to an in-frame start codon following the *fol* gene. The orientation of the *fol* gene was determined from the 141- and 3724-base fragments obtained after *Eco*RI digestion. The opposite orientation would have yielded 883 and 3970 bp fragments. The observed orientation results in expression of the anticoding strand of the *fol* gene when packaged as a single strand using M13K07 helper phage. The pTz/Gly-54 construct allows (i) manipulation of the vector as a double-stranded plasmid, (ii) facile mutagenesis reactions and sequencing on the single-stranded template, and (iii) direct expression of subsequent mutant protein via the native *fol* promoter.

Construction of Mutant Enzymes. Both the L54I (isoleucine-54) and L54N (asparagine-54) mutant proteins were constructed by annealing the mutagenic oligonucleotide to the single-stranded template and polymerizing "all-the-way-around" with T4 polymerase and T4 gene 32 single-stranded binding protein. The addition of gene 32 protein proved to be crucial for complete polymerization. Agarose gel electrophoresis showed only nicked or covalently closed relaxed double-stranded plasmids by comparison with authentic markers. No single-stranded template was detectable. (We have found that reducing the amount of ethidium bromide in the gel to 0.1 μg/mL dramatically increased the sensitivity of detection.)

Transformation into the repair-deficient cell line HB2154 (Carter, 1985), isolation of the plasmid DNA, and retransformation into JM109 yielded single colonies which were genotypically homogeneous. Plasmid DNA was isolated from 12 single-colony picks for each potential mutant and screened by the absence of the *Apa*I site. One positive in 12 was found for each the L54I and L54N mutants. Although the mutagenesis did not utilize any selection against wild type (in this case "wild type" equals L54G), two factors are believed important for its success: (i) the polymerase conditions were optimized, and (ii) the repair-deficient cell line removes the selection against mismatches near GATC sites (of which there are five in the *fol* gene).

Scheme I[a]



[a] $K_{eq} = [E_2]/[E_1]$.

Dideoxy sequencing was performed on the single-stranded template (Sanger, 1981). In addition to the desired changes at the 54-position, both the L54I and L54N mutants showed a degenerate C to T change in the third position of the codon for arginine-33.

Protein Purification. The L54I mutant was grown in the *E. coli* cell line SF32 to avoid the possibility of contamination by wild-type DHFR in such a structurally similar mutant; the L54N mutant was grown in JM109 cells. Both these mutants required only 0.2 M borate (pH 9.0)/1 M KCl buffer for elution from the methotrexate affinity resin, whereas the wild-type enzyme requires the above buffer plus folate for elution. The L54G mutant was purified as previously reported (Mayer et al., 1986) and further purified by elution down a G-75 Sephadex sizing column. All three proteins were homogeneous by polyacrylamide gel electrophoreses.

Binding Kinetics Relaxation and Competition Methods. The data for binding and dissociation of ligands and inhibitors to DHFRs have been described in detail previously (Dunn et al., 1978; Dunn & King, 1980; Cayley et al., 1981; Blakley & Cocco, 1985a,b; Fierke et al., 1987). Briefly, the binding of NADPH to the wild-type *E. coli* enzyme is biphasic and has been described by Cayley et al. (1981) (Scheme I). The measured pseudo-first-order rate constant for the fast, ligand-dependent phase (k_{obsd}) is related to the association (k_{on}) and dissociation (k_{off}) rate constants by eq 1. The slope and

$$k_{obsd} = k_{on}[\text{NADPH}] + k_{off} \quad (1)$$

y intercept of the plot of k_{obsd} versus [NADPH] yield the values for k_{on} and k_{off} if a simple association, dissociation mechanism is operative. The slow, ligand-independent phase represents the conversion of E_2 to E_1. Kinetic simulations (Barshop et al., 1983) have shown the pseudo-first-order approximation to be valid when $[S] \geq 8[E]$.

An independent measure of the dissociation rate constant was obtained by competition experiments (Birdsall, 1980). The dihydrofolate reductase–ligand complex (EL_1) was trapped with a second ligand (L_2) which competes for the same binding site (Scheme II). If $k_{rev} \ll k_{trap}[L_2] \gg k_{on}[L_1]$, then the fluorescence change is due to the conversion of EL_1 to EL_2 characterized by a single exponential with a rate constant k_{rev} (Dunn & King, 1980; Cayley et al., 1981). If there are unimolecular isomerization steps preceding the dissociation step, k_{rev} will not necessarily equal k_{off}.

Scheme II

$$E{\cdot}L_1 \underset{k_{on}}{\overset{k_{rev}}{\rightleftharpoons}} E + L_1 + L_2 \underset{k_{diss}}{\overset{k_{trap}}{\rightleftharpoons}} E{\cdot}L_2$$

Thermodynamic Dissociation Constants. The binding of methotrexate, dihydrofolate, and NADPH to free DHFR was determined by following the decrease in enzyme fluorescence that occurs upon formation of DHFR–ligand complexes. The

Table I: Ligand Binding

	K_D[a]		
	[NADPH] (μM)	[H_2F] (μM)	[MTX] (nm)
Leu-54	0.33 ± 0.06[c]	0.21 ± 0.03[c]	0.02 ± 0.007[b]
Ile-54	0.30 ± 0.08	1.9 ± 0.3	1.8 ± 0.3
Gly-54	0.02 ± 0.01	350 ± 50[d]	125 ± 20[e]
Asn-54	0.59 ± 0.02	75 ± 15[d]	60 ± 10[e]

[a] Values recorded at pH 6.00, 25 °C, MTEN buffer unless otherwise noted. [b] Taira et al. (1986). [c] Fierke et al. (1987). [d] The K_D is assumed to be equal to the K_m for the L54G and L54N mutants. The steady-state isotope effects are consistent with this assumption: $^D(V/K)$ = 3.35 ± 0.5 for L54G and 5 ± 1 for L54N, pH 5.00, 25 °C, MTAN buffer. [e] Values recorded at pH 5.00, 25 °C, MTAN buffer.

data were fit according to Taira and Benkovic (1988). The equilibrium dissociation constants for various ligands to mutant and wild-type enzymes are given in Table I. The binding of NADPH to mutant relative to wild-type enzyme is generally unchanged except for the 17-fold tighter binding to L54G. As expected, the changes at leucine-54 are reflected primarily in changes in affinity at the folate site as evidenced by the approximately 70-fold and 3000-fold increase in the dissociation of H_2F and methotrexate, e.g., the L54N mutant.

Ligand Dissociation Rates. Dissociation rates of various ligands from mutant and wild-type enzymes are summarized in Table II. Since the association rate (40 μM^{-1} s^{-1}) for H_2F binding to the wild-type enzyme is at or near diffusion control, the 10-fold increase in K_D for the L54I mutant is reflected in the dissociation rate. The association rate for L54I and both association and dissociation rates for L54N and L54G exceed those that can be measured by stopped-flow fluorometry. The association rate (25 μM^{-1} s^{-1}) for H_4F to L54I is the same as with wild-type enzyme.

For H_4F, there is a 40-fold increase in the rate of dissociation from the two possible product complexes, E·H_4F and E·$NADP^+$·H_4F, for the L54I enzyme. A smaller increase (7×) is seen in the mixed ternary complex, $E^{(NH)}_{H_4F}$. The effect of this mutation is to also attenuate the effect of H_4F on NADPH dissociation [compare $E^{NH}_{(H_4F)}$ and E^{NH}]. On the other hand, the $NADP^+$ dissociation from the L54I product ternary complex E·$NADP^+$·H_4F or from the binary E·$NADP^+$ complex is unchanged from the respective wild-type values of 200 and 300 s^{-1}.

Steady-State Kinetics. The steady-state kinetics are diagnostic of the changes in rate-limiting steps between wild-type and mutant enzymes. The pK_a of 6.52 ± 0.08 in V and 6.49 ± 0.08 in V/K for L54I (Figure 2) matches the pK_a governing hydride transfer in the wild-type enzyme (Fierke et al., 1987), suggesting that hydride transfer is rate limiting in the steady state for L54I. This is confirmed by the deuterium isotope effects. While product release masks the isotope effect in the wild-type enzyme at low pH (^DV = 1.0 ± 0.1, pH 6.0), a large effect is seen for L54I, L54G, and L54N where ^DV = 2.26 ± 0.16, pH 6.0; 3.35 ± 0.3, pH 5.0; and 4.4 ± 0.4, pH 5.0, respectively, consistent with rate-limiting hydride transfer for all the mutants (Table III). The V for L54N and L54G is

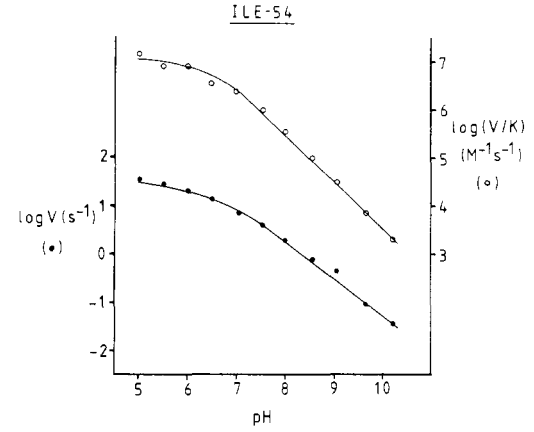


FIGURE 2: pH–rate profiles for V/K and V as a function of pH for the Ile-54 mutant.

associated with a pK_a of 5.60 ± 0.16 and 5.72 ± 0.10, respectively (data not shown). In all three mutants, the limiting slope in log plots of V vs pH are 0.5–0.65, similar to the results obtained in the Asp-27 → Ser-27 mutant (Howell et al., 1986, 1987).

The reaction of the truncated substrate 6-(ethoxymethyl)-dihydropterin, which is reduced to its tetrahydro form at pH 6.0 (Smith et al., 1987), gave values of k_{cat} = 1.1 ± 0.1 s^{-1} and K_M = 300 ± 25 μM for the wild-type enzyme and k_{cat} = 1.0 ± 0.1 s^{-1} and K_M = 300 ± 70 μM for the L54I enzyme.

Discussion

Binding Kinetics and Thermodynamics. The K_D values for ligand binding to wild-type enzyme generally are predicted by eq 2, in accord with a binding/dissociation step that is

$$K_D = (k_{off}/k_{on})(1 + 1/K_{eq}) \quad (2)$$

modified by the presence of a nonbinding enzyme form E_2 (Scheme I) (Cayley et al., 1981). However, recent evidence indicates that in fact NADPH binding is to both enzyme forms (Adams and Benkovic, unpublished results) and resembles that postulated for methotrexate (Taira et al., 1988). Consequently, the rate constants obtained from relaxation experiments may not correspond to single steps. This subject will be treated in a future publication; for the present cases with the exception of L54G, the effect of the mutation is not reflected in NADPH or $NADP^+$ binding.

For H_2F, the thermodynamic dissociation constant increases in the order Leu < Ile < Asn < Gly. For the leucine to isoleucine mutation, the differences in polar and steric effects are minimal (Table III) yet there is a 10-fold change in K_D for L54I. It is conceivable that the shifting of a single methyl group away from the phenyl ring of the substrate removes a potentially stabilizing hydrophobic interaction. Alternatively, the β-branching of the isoleucine side chain could cause a subtle reorganization of protein structure whose cumulative binding surface is less favorable for binding. That the effect of the leucine to isoleucine mutation is primarily due to changes

Table II: Ligand Dissociation Rates

	k_{off} (s^{-1})[a]					
	E_{H_2F}	E^{NH}[b]	$E^{NH}_{(H_4F)}$	E_{H_4F}	$E^{(N)}_{H_4F}$[b]	$E^{(NH)}_{H_4F}$
Leu-54[c]	22 ± 5	3.6 ± 0.5	85 ± 10	1.4 ± 0.2	2.5 ± 0.02	12.3 ± 2
Ile-54	300 ± 10	15 ± 2	21 ± 1	60 ± 3	100 ± 2	90 ± 10
Gly-54	≫300			>300		
Asn-54	≫300			>300		

[a] Dissociating ligand not in parentheses. All values determined by the competition experiment at pH 6.0, 25 °C, MTEN buffer. [b] NH = NADPH; N = $NADP^+$. [c] Fierke et al. (1987).

Table III: Hydride Transfer Rates

	forward k_{hyd} (s^{-1})[a]	reverse k_{hyd} (s^{-1})	volume[c] (Å^3)
Leu-54[b]	950 ± 50	0.6 ± 0.1	166.7
Ile-54	31 ± 4	0.1 ± 0.02	166.7
Gly-54	29 ± 4		60.1
Asn-54	42 ± 8		137.7

[a] pH-independent values. [b] Fierke et al. (1987). [c] Zamyatinin (1972).

in side chain binding is demonstrated by the results with 6-(ethoxymethyl)dihydropterin which bears an ethoxy group in place of the *p*-aminobenzoylglutamate of H_2F. Both the wild type and L54I mutant process this substrate with the same k_{cat} value of 1 s^{-1}.

The L54G mutant features a significantly smaller side chain volume, creating a space that may be occupied by water. Similarly, water may be hydrogen bonded to the side chain carboxamide in the L54N mutant. A significant factor in the weaker binding of H_2F to the L54G and L54N proteins therefore may be due to a requirement for increased desolvation upon forming the binary complex (Singh & Benkovic, 1988; Bash et al., 1987). Alternatively, the NH of the asparagine carboxamide could hydrogen bond to the carbonyl of dihydrofolate, forcing the substrate into an unfavorable conformation. The L54N mutant is a particularly telling example of the importance of hydrophobic interactions in ligand binding since in this case the H_2F binding is decreased by 350-fold (3.5 kcal/mol).

The rate constant for dissociation of H_2F from the enzyme directly reflects alterations in the structure of residue 54 [presuming the association rate remains close to diffusion limited (k_{on} = 40 μM^{-1} s^{-1})]. Thus, k_{off} would be increased by 14-, 360-, and 1700-fold for L54I, L54N, and L54G, respectively. The dissociation of H_4F (Table II) from the binary complex exhibits a similar sensitivity. As noted previously (Fierke et al., 1987), the increase in the dissociation rate of tetrahydrofolate caused by the binding of NADPH influences the course of the wild-type kinetic sequence. At saturating NADPH, a portion of the favored kinetic pathway is that shown in eq 3 (Fierke et al., 1987) rather than a path where

$$E^{N}_{H_4F} \rightarrow E_{H_4F} \rightarrow E^{NH}_{H_4F} \rightarrow E^{NH} \quad (3)$$

both products dissociate to form free E. The synergistic effects of NADPH and tetrahydrofolate are complementary: the presence of NADPH (but not $NADP^+$) increases k_{off} for H_4F by 9-fold from free enzyme, and the presence of H_4F increases k_{off} for NADPH by 24-fold (Table II). The L54I mutant behaves much differently: binding of NADPH assists H_4F dissociation by only a factor of 1.5, in marked contrast to the wild-type enzyme. The differential effect of reduced versus oxidized cofactor on H_4F dissociation is also lost in L54I. Moreover, tetrahydrofolate has little effect on the rate constant for NADPH dissociation: k_{off} = 15–20 s^{-1} from either E·NADPH or E·NADPH·H_4F. Because of the faster release of H_4F from the product ternary complex (E·$NADP^+$·H_4F), the net result is that 33% of the L54I enzyme partitions to the binary E·$NADP^+$ complex and 67% forms the other binary E·H_4F complex during steady-state turnover; less than 0.7% of the enzyme forms the E·$NADP^+$ complex in the wild type.

The conclusion to be reached from these data is that the substrate synergism has been structurally *disconnected* by this mutation. Examination of X-ray crystal structures, particularly those of the *E. coli* MTX and *Lactobacillus casei* MTX·$NADP^+$ enzyme complexes, reveals that leucine-54 is at the C-terminal end of the C α-helix whose N-terminal end has residues that interact with the adenine moiety of the NADPH. Although movement of this unit upon NADPH binding has been suggested from NMR studies on the *L. casei* enzyme, studies of other mutants of the N-terminus of the α-helix do not lead to uncoupling (Adams and Benkovic, unpublished results). It is possible that the NADPH effect is a result of direct repulsive interactions between the pterin and nicotinamide rings of the two substrate ligands. Loss of the leucine-54 may alleviate such behavior in the ternary complex.

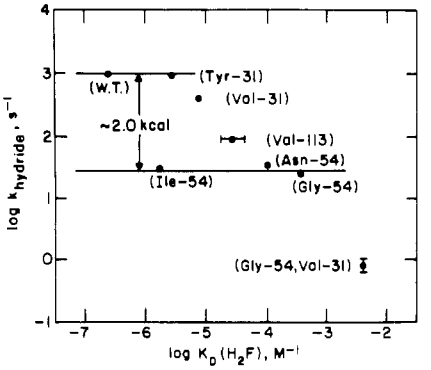


FIGURE 3: Plot of log $k_{hydride}$ versus log K_D (the dissociation of dihydrofolate) for a series of mutants at the folate site of DHFR.

Catalysis. The pH-independent maximum velocities of the residue 54 mutant reductases are all faster than the wild-type enzyme due to an increase in the rate of product loss without a corresponding totally compensating reduction in the rate of the chemical step; nevertheless, the mutants are inferior catalysts when H_2F is subsaturating. The parameter which is functionally important to cell viability is V/K_{H_2F}, which for wild type is 18 μM^{-1} s^{-1} (Stone & Morrison, 1984), but for L54I, the value is 1.2 μM^{-1} s^{-1} under similar conditions. Thus, the catalytic efficiency of the L54I mutant is an order of magnitude less than wild type. This suggests (i) that the efficiency of the enzyme is under the control of natural selection and (ii) that a decrease of a single power of 10 in V/K_{H_2F} is selectively disadvantageous.

The pK_a of 8.4 in V for the wild-type enzyme (Stone & Morrison, 1984) does not reflect the actual pK_a of the ionizing aspartate-27 but is instead the point at which the pH-dependent hydride transfer rate and pH-independent product release rate intersect (Fierke et al., 1987). The steady-state pK_a (6.5) of the L54I mutant is identical with the wild-type value for pre-steady-state hydride transfer (Fierke et al., 1987). This reflects rate-limiting hydride transfer in the L54I mutant under steady-state conditions, which is confirmed by the steady-state deuterium kinetic isotope effects. The steady-state isotope effects for the L54G and L54N mutants likewise infer rate-limiting hydride transfer followed by rapid product release. However, in these two cases, the controlling pK_a's are 5.60 and 5.74 for L54N and L54G, respectively, implicating the transmission of structural change to alter the environment of aspartate-27. This change in active-site structure may be a subtle modification of the microenvironment surrounding this group; it, however, is not responsible for the shallower than expected slope (0.5–0.6 vs 1.0) in the V vs pH profiles, a phenomena also manifest in the Ser-27 mutant (Howell et al., 1986, 1987) whose X-ray crystal structure virtually overlays that of wild type.

The most striking aspect of the mutant enzyme's hydride transfer rates is their similarity (Table III). While the binding constants for H_2F and MTX vary by factors $\leq 10^3$, k_{hyd} (the rate of hydride transfer) does not. Thus, there is no correlation between the substrate binding affinity and the rate of hydride transfer (Figure 3). Removal of the leucine-54 residue results

in a constant loss of 2.0 kcal/mol, reducing k_{hyd} by a factor of ca. 30-fold regardless of the mutant. In particular, the inability to substitute leucine with isoleucine (and to a lesser extent asparagine and glycine) implies that this region of the active site is responsible for a unique interaction with the folate in order to gain selective transition-state stabilization of ca. 2.0 kcal/mol. This mandate may not be met by side chains of similar volume, with or without differing polarity, as far as transition-state stabilization is concerned, but is relaxed in ground-state binding of ligand as manifested in the changes in K_D for H_2F and MTX. The primary role of this residue in transition-state stabilization is also seen in the reverse direction: $k_{hyd-rev}$ is decreased by a factor of 6 (1.0 kcal/mol) in the L54I mutant. If these data are added to our earlier linear free energy relationship relating k_{hyd} to K_{D,H_2F} (Benkovic et al., 1988), it is apparent that there is no global relationship that rationalizes the behavior of all the mutants (Figure 3). Although it may be argued on the basis of the observed pK_a changes in Asp-27 that these amino acid substitutions are disruptive (Fersht, 1987; Lowe, 1987), nevertheless their behavior underscores the complexity of mutant behavior and the absence, as expected, of simple relationships between binding of ligand and enzyme turnover.

Leucine-54 may achieve its unique role by limiting unproductive conformations of dihydrofolate or by precisely positioning dihydrofolate for reaction. The identical kinetic behavior of the wild-type and L54I enzymes toward the abbreviated pterin substrate that lacks the *p*-aminobenzoylglutamate moiety further localizes this effect to a specific interaction in the side chain region. The fact that leucine-54 is totally conserved throughout all species sequenced suggests that it cannot be readily replaced by compensating residues. It will be of considerable interest to determine whether other strictly conserved residues likewise contribute a fixed free energy increment to the overall free energy change for the reactive step and whether such contributions will be additive. There are examples of both effects in other mutants: in the case of subtilisin, mutations within the catalytic triad of histidine, aspartate, and serine are synergistic (Carter & Wells, 1988); in the case of tRNA tyrosyl synthetase, a number of double mutants within the ATP binding site are additive in their binding and catalytic properties (Ho & Fersht, 1986). It seems likely that the critical determinant will be whether the multiple substitutions are at amino acids sufficiently remote that the structural alteration is not propagated or at amino acids that are not part of a network.

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Registry No. DHFR, 9002-03-3; H_2F, 4033-27-6; H_4F, 135-16-0; NADPH, 53-57-6; $NADP^+$, 53-59-8; MTX, 59-05-2; Leu-54, 61-90-5; Ile-54, 73-32-5; Gly-54, 56-40-6; Asn-54, 70-47-3.

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Myeloperoxidase-Mediated Inhibition of Microbial Respiration: Damage to *Escherichia coli* Ubiquinol Oxidase†

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ABSTRACT: A microbicidal system, mediated by neutrophil myeloperoxidase, inhibits succinate-dependent respiration in *Escherichia coli* at rates that correlate with loss of microbial viability. Succinate dehydrogenase, the initial enzyme of the succinate oxidase respiratory pathway, catalyzes the reduction of ubiquinone to ubiquinol, which is reoxidized by terminal oxidase complexes. The steady-state ratio of ubiquinol to total quinone (ubiquinol + ubiquinone) reflects the balance between dehydrogenase-dependent ubiquinone reduction and terminal oxidase-dependent ubiquinol oxidation. Myeloperoxidase had no effect on total quinone content of *E. coli* but altered the steady-state ratio of ubiquinol to total quinone. The ratio doubled for organisms incubated with the myeloperoxidase system for 10 min, suggesting decreased ubiquinol oxidase activity, which was confirmed by observation of a 50% decrease in oxidation of the ubiquinol analogue 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinol. Despite inhibition of ubiquinol oxidase, overall succinate oxidase activity remained unchanged, suggesting that succinate dehydrogenase activity was preserved and that the dehydrogenase was rate limiting. Microbial viability was unaffected by early changes in ubiquinol oxidase activity. Longer (60 min) exposure of *E. coli* to the myeloperoxidase system resulted in only modest further inhibition of the ubiquinol oxidase, but the ubiquinol to total quinone ratio fell to 0%, reflecting complete loss of succinate dehydrogenase activity. Succinate oxidase activity was abolished, and there was extensive loss of microbial viability. Early myeloperoxidase-mediated injury to ubiquinol oxidase appeared to be compensated for by higher steady-state levels of ubiquinol which sustained electron turnover by mass effect. Later myeloperoxidase-mediated injuries eliminated succinate-dependent ubiquinone reduction, through inhibition of succinate dehydrogenase, with loss of succinate oxidase activity, effects which were associated with, although not clearly causal for, microbicidal activity.

The microbicidal effects of neutrophils and monocytes are mediated, in part, by myeloperoxidase, a granule-bound enzyme that is released into the phagosome and extracellular space during phagocytosis (Klebanoff & Clark, 1978). Myeloperoxidase, together with phagocyte-derived hydrogen peroxide and chloride, generates hypochlorous acid (Harrison & Schultz, 1976) and derivative oxidants (Thomas, 1979) that mediate a potent microbicidal effect. The microbicidal mechanisms of the oxidants are incompletely understood. Multiple sites of microbial injury have been described, most of them located in the microbial cytoplasmic membrane [for review see Hurst and Barrette (1989)]. Among these is the loss of succinate-dependent respiration mediated by the succinate oxidase system (Rosen et al., 1987) which is comprised of several components that vary depending on growth conditions. Under appropriate conditions, the electron-transfer pathway can be schematized as (Lorence et al., 1987; Ingledew & Poole, 1984; Poole & Ingledew, 1987; Pudek & Bragg, 1974)

$$\text{succinate} \xrightarrow{\text{succinate dehydrogenase}} Q_8 \xrightarrow{\text{cytochrome } d \text{ complex}} O_2$$

Myeloperoxidase rapidly inactivates the succinate dehydrogenase component (Rosen et al., 1987). Because of this inactivation, succinate cannot be employed as a probe of the

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