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Nonadditivity of Mutational Effects at the Folate Binding Site of *Escherichia coli* Dihydrofolate Reductase†

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ABSTRACT: The function of the hydrophobic residues Leu28, Phe31, Ile50, and Leu54 at the folate binding site in *Escherichia coli* dihydrofolate reductase (5,6,7,8-tetrahydrofolate:NADP$^+$ oxidoreductase, EC 1.5.1.3) has been studied by a combination of site-specific mutagenesis and reaction kinetics. Studies suggest that the overall protein structure and kinetic sequence for the reaction did not change for the mutant proteins compared to the wild-type enzyme. Two sets of mutated reductases have been constructed. The first set, in which the side chains of the targeted amino acids are spatially well separated (~8 Å), includes two single mutants (L28Y and L54F) and a double mutant (L28Y-L54F). This set features residues that increased the side chain surface area and the potential for substrate interactions. Unexpectedly, nonadditivity in the free energy changes for the thermodynamics of ligand binding and in the rates of hydride transfer and product release is observed. The progressive increase in dihydrofolate binding is reversed for the sterically more crowded double mutant, with $\Delta\Delta G$ ca. 3 kcal mol^{-1} less favorable than anticipated. On the other hand, the decrease in the rate constant for hydride transfer noted with the single mutants relative to the wild-type enzyme is reversed for the double mutant, so that $\Delta\Delta G^{\ddagger}$ is ca. 2 kcal mol^{-1} more favorable. The second set of mutant proteins includes two double mutants (L28A-F31A and I50A-L54G) in which the selected amino acids are separated by three to four intervening amino acids and a quadruple mutant (L28A-F31A-I50A-L54G) in which the two sets L28A-F31A and I50A-L54G are spatially distinct. This set deleted the side chain surface area to lower the opportunity for substrate interactions. Nonadditivity in the free energy changes associated with key kinetic and thermodynamic parameters is again observed. The decrease in dihydrofolate binding found with the two double mutants is not observed with the quadruple mutant, which binds the substrate with $\Delta\Delta G$ ca. 6.5 kcal mol^{-1} more favorable than expected. Similarly, the quadruple mutant has a larger rate constant for hydride transfer ($-\Delta\Delta G^{\ddagger} \simeq 1.7$ kcal mol^{-1}) than predicted. One interpretation for the nonadditivity is that these residues interact through binding of the folate substrate, which serves to link molecularly remote side chain moieties within the active site. The resiliency of the active site to tolerate such damage suggests that there must be sufficient flexibility and redundancy built into the site and its framework to retain a high population of active enzyme–substrate complexes.

Dihydrofolate reductase (5,6,7,8-tetrahydrofolate:NADP$^+$ oxidoreductase, EC 1.5.1.3, DHFR) catalyzes the reduction of 7,8-dihydrofolate (H_2F) to 5,6,7,8-tetrahydrofolate (H_4F) using NADPH as a cofactor. DHFR is necessary for maintaining intracellular pools of H_4F and its derivatives, which are essential cofactors in many important biosynthetic reactions that require the transfer of one-carbon units. It is, therefore, the target enzyme of a group of antifolate drugs widely used as antitumor and antimicrobial agents, such as methotrexate (MTX) and trimethoprim (TMP). Because of its biological and pharmacological importance, DHFR has been the subject of intensive structural and kinetic studies.

High-resolution X-ray structures of the *Escherichia coli*, *Lactobacillus casei*, chicken liver, and human enzymes have been determined for several binary and ternary complexes, as well as for the apoenzyme (Matthews et al., 1978; Bolin et al., 1982; Filman et al., 1982; Bystroff et al., 1990; Bystroff & Kraut, 1991), and complete kinetic schemes for wild-type *E. coli*, *L. casei*, and murine DHFR have been derived from steady-state and pre-steady-state kinetic studies (Penner & Frieden, 1987; Fierke et al., 1987; Andrews et al., 1989; Thillet et al., 1990). The folate binding site for the *E. coli* enzyme is a cavity 15 Å deep, lined with the hydrophobic side chains Leu28, Phe31, Ile50, and Leu54, which principally interact with the *p*-aminobenzoyl-L-glutamate (PABG) moiety of folate, as shown in Figure 1. Mutagenesis coupled with kinetic analysis implicated Asp27 as the essential proton-donating residue during the chemical step (Howell et al., 1986).

In order to understand more about the relationship between active site structure and catalytic function, several *E. coli* DHFR mutants have been constructed by site-directed mutagenesis and examined kinetically. Studies of single mutants L28F, F31Y, F31V, L54I, L54N, and L54G and the double mutant F31V-L54G, which involve the amino acids constituting the PABG binding site, revealed the influence of these residues on the binding of H_2F and on modulating the rate of the chemical step of hydride transfer (Mayer et al., 1986; Chen et al., 1987; Taira et al., 1987a,b; Benkovic et al., 1988; Murphy & Benkovic, 1989; Wagner et al., 1992). The K_D (μM) values for the dissociation of H_2F in the series wild type:F31V:L54G:F31V-L54G are 0.22:5.0:350:2000, revealing the quantitative importance of these residues in substrate binding. Similarly, the rate constants for hydride transfer (s^{-1}) decrease across the series in the same order: 950:400:29:0.9.

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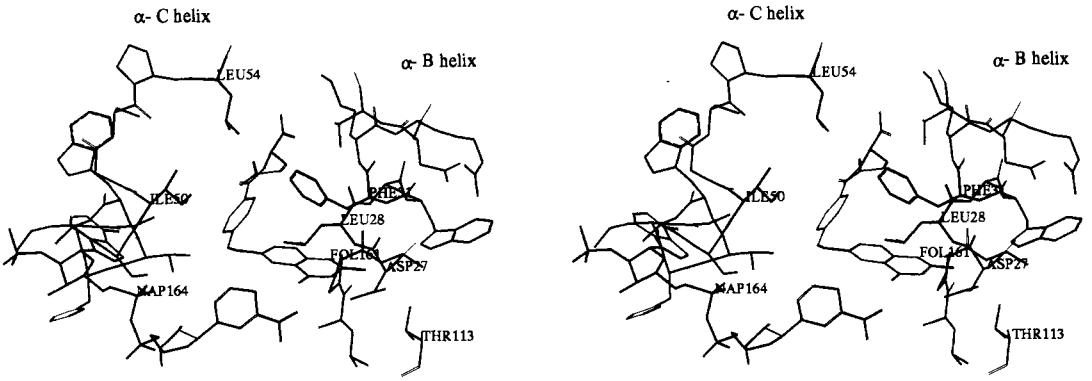
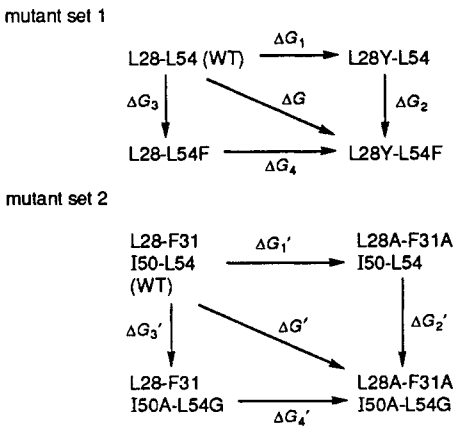


FIGURE 1: Active site of the ternary complex of *E. coli* dihydrofolate reductase with folate and $NADP^+$.

To determine the collective interactions between these side chains, we have generated single, double, and quadruple mutants that are interrelated through two sets of mutagenic cycles:



The choice of the amino acid substitutions reflects our interest in the impact on binding and catalysis of increasing (leucine to phenylalanine or tyrosine) or decreasing (leucine, isoleucine, and phenylalanine to alanine or glycine) side chain surface area.

For amino acids acting independently (in the absence of major alterations in protein structure and reaction mechanism), the free energy change for a particular kinetic or thermodynamic parameter observed for a double mutant should be simply the sum of that observed in the two single mutants (Horovitz & Fersht, 1990; Wells, 1990; Mildvan et al., 1992). Therefore, if the two residues Leu28 and Leu54 are not coupled electronically or sterically, the free energy change observed should be additive: $\Delta G_1 + \Delta G_2 = \Delta G_3 + \Delta G_4 = \Delta G$. If they do interact, the free energy change observed should be nonadditive: $\Delta G_1 + \Delta G_2 \neq \Delta G_3 + \Delta G_4$. Similar reasoning should apply to the two double mutants (L28A-F31A and I50A-L54G) and the related quadruple mutant (L28A-F31A-I50A-L54G). We found that no cycle is in fact additive either in terms of the free energy changes observed for the thermodynamics of ligand binding (NADPH and H_2F) or for the kinetics of the chemical step (hydride transfer) and the product release step (H_4F off-rate). In both cases, the mutated residues are not close enough spatially to interact directly through van der Waals contacts or indirectly via bound water clusters. Moreover, our analysis indicates that neither the overall protein structure nor the key steps within the reaction mechanism have changed. Our observation of nonadditivity is consistent with cooperative interactions between these distant residues that may come into play only when substrates either are bound or are being processed.

MATERIALS AND METHODS

Substrates and Buffers

7,8-Dihydrofolate (H_2F) was prepared by the dithionite reduction of folic acid (Blakley, 1960). (6*S*)-5,6,7,8-Tetrahydrofolate (H_4F) was prepared in the presence of β-mercaptoethanol from H_2F by enzymatic conversion with the wild-type DHFR, using glucose 6-phosphate, $NADP^+$, and glucose-6-phosphate dehydrogenase to regenerate NADPH (Mathews & Huennekens, 1960). Purification of H_4F was achieved from a DE-52 resin eluted with a linear triethylammonium bicarbonate gradient (Curthoys et al., 1972). NADPH, $NADP^+$, MTX, TMP, and folic acid were purchased from Sigma. [4′(*R*)-^{2}H]NADPH (NADPD) was prepared by using *Leuconostic mesenteroides* alcohol dehydrogenase purchased from Research Plus, Inc. (Stone & Morrison, 1982) and purified on a Pharmacia FPLC Mono Q column that was eluted with a triethanolammonium bicarbonate gradient (Viola et al., 1979). TE buffer contained 10 mM Tris·HCl (pH 8.0) and 1 mM EDTA. Ligand concentrations were determined spectrophotometrically using the following extinction coefficients: H_4F, 28 000 M^{-1} at 297 nm, pH 7.5 (Kallen & Jencks, 1968); H_2F, 28 000 M^{-1} at 282 nm, pH 7.4 (Dawson et al., 1969); MTX, 22 100 M^{-1} at 302 nm in 0.1 KOH (Seeger et al., 1949); NADPH, 6200 M^{-1} at 339 nm, pH 7.0 (P-L Biochemicals, 1961); $NADP^+$, 18 000 M^{-1} at 259 nm, pH 7.0 (P-L Biochemicals, 1961).

All kinetics and equilibrium measurements were conducted at 25 °C in a buffer that contained 50 mM 2-morpholinoethanesulfonic acid, 25 mM Tris, 25 mM ethanolamine, and 100 mM NaCl (MTEN buffer, pH 5–10). Over the pH range used in these experiments, the ionic strength of this buffer remained constant (Ellis & Morrison, 1982).

E. coli Strains and Biochemicals

The following strains were obtained as gifts from Dr. P. Stanssens: BMH-71 (*muts215:Tn10*), W71-18 (*su⁻:hpsl*), and MK30-3 (*recA⁻*, *su⁻*). Strains DH5α and DH5αF′IQ were purchased from BRL/Gibco Inc. Strain JM109 was obtained from New England Biolabs, Inc. Helper phage M13KO7, Ultra-pure, SeaKem, and NuSieve agarose were purchased from Pharmacia Inc. and FMC Inc. Plasmid pTZwt1-3 was obtained as a gift from Dr. C. R. Matthews, and plasmid pMC-5 was obtained as a gift from Dr. P. Stanssens. Plasmid pRWA-1 was constructed by Dr. C. R. Wagner (Wagner et al., 1992). T4 DNA ligase and T4 polynucleotide kinase were purchased from Boehringer

Mannheim and HK phosphatase was purchased from Epicentre Technologies Inc. Calf intestinal phosphatase was purchased from Sigma. *E. coli* DNA polymerase pol I (Klenow fragment) was purified in this laboratory by Dr. B. Eger. All restriction endonucleases (*Nco*I, *Eag*I, *Sma*I, *Bam*HI, and *Cla*I) were obtained from New England Biolabs, Inc. *Taq* polymerase was purchased from Perkin-Elmer Cetus Inc. Antibiotics (ampicillin and chloramphenicol) were purchased from Sigma. Oligonucleotide purification cartridges (reverse phase cartridge) were purchased from Applied Biosystems. Ultrafree-MC membrane filters were obtained from Millipore Inc. All transformations were carried out using the Hanahan method (Hanahan, 1983).

Oligonucleotide-Directed Mutagenesis

Construction of E. coli DHFR Mutants L28Y and L54F. Mutagenesis was carried out as previously described by Wagner et al. (1992). For the L28Y mutant, the mutagenic oligonucleotide GGAACCTGCCGGCCGATTACGCCTGG (L28Y) was obtained from and HPLC purified by the Penn State Biotechnology Oligonucleotide Synthesis Facility. The primer was designed to replace the codon for leucine (CTC) with that for tyrosine (TAC) and incorporate a unique *Eag*I restriction site. The efficiency of mutagenesis was found to be 58%. Construction of the mutant L54F was carried out similarly with the oligonucleotide CGGTCGTCCGTTCCCGGGACGC (L54F) designed to replace the codon for leucine (TTG) with that for phenylalanine (TTC) and to incorporate a unique *Sma*I restriction site. The efficiency of mutagenesis was 95%.

Construction of E. coli DHFR Mutants L28Y-L54F, I50A-L54G, and L28A-F31A-I50A-L54G. The double mutant L28Y-L54F was constructed using the PCR (polymerase chain reaction) method developed by Scharf et al. (1986). The oligonucleotide PCR primers were obtained from American Synthesis Inc. (ASI) and purified by a reverse phase cartridge. For the mutant L28Y-L54F, we used a 51-mer (forward primer) in which a codon for leucine (CTC) was replaced by a codon for tyrosine (TAC), GCCATGCCATGGAACCTGCCTGCCGATTACGCCTGGTTTAAACGCAACACC, and a 39-mer (reverse primer) in which a codon for leucine (TTG) was replaced by a codon for phenylalanine (TTT), ATTTTTGCTGCCGGGAAAAGGCCTACCGATTGATTCCCA (complementary strand).

PCR reaction mixtures contained 1 μM selected forward and reverse primers, 1 μg of pTZwt, 250 μM each dATP, dTTP, dGTP, and dCTP, 50 mM KCl, 25 mM $MgCl_2$, 100 mM Tris·HCl (pH 8.3), and 5 units of AmpliTaq DNA polymerase. The reaction mixture was overlaid with mineral oil and subjected to 40 cycles of amplification using a Perkin-Elmer thermal cycler. Each PCR cycle consisted of 94 °C for 1 min (denaturation), 52 °C for 2 min (annealing), and 72 °C for 3 min (extension). The products were purified on a 1% agarose (SeaKem) gel, isolated by electroelution, and resuspended in TE.

Within the plasmid pTZwt, the DNA sequence encoding residues 23–57 is flanked by the unique restriction sites *Nco*I and *Eag*I. Linearized pTZwt was prepared by double digesting with *Nco*I and *Eag*I followed by treatment with HK phosphatase. The larger DNA fragment was purified on a 1% agarose (NuSieve) gel, isolated by membrane filtration, and resuspended in TE.

*Nco*I and *Eag*I digestion of the PCR products produced a DNA insert fragment encoding the L28Y-L54F mutation. The linearized pTZwt and mutant insert fragment were ligated using T4 ligase. The ligation mixture was then used to transform *E. coli* strain DH5α. Transformants were plated onto LB/agar plates containing 100 μg/mL ampicilin. Randomly selected clones were sequenced by the method of Sanger et al. (1977).

The double mutant I50A-L54G and the quadruple mutant L28A-F31A-I50A-L54G were constructed similarly. The primers used for the PCR reactions were a 24-mer (wt), which is the same as the wild-type DHFR gene GCCATGCCATGGAACCTGCCTGCC, a 51-mer (L28A-F31A), in which codons for leucine (CTC) and phenylalanine (TTT) were replaced by those for alanine (GCC and GCT), GCCATGCCATGGAACCTGCCTGCCGATGCCGCCTGGGCTAAACGCAACACC, and a reverse 39-mer (I50A-L54G) in which codons for isoleucine (ATC) and leucine (TTG) were replaced by those for alanine (GCT) and glycine (GGA), ATTTTTGCGGCCGGGTCCAGGCCTACCAGCTGATTCCCA. The wt and the reverse I50A-L54G primers were used for construction of the double mutant I50A-L54G. Primer L28A-F31A and the reverse I50A-L54G primer were used for the L28A-F31A-I50A-L54G quadruple mutant. All of the PCR reaction conditions were identical to those used in the construction of L28Y-L54F, except that 30 cycles were used instead of 40 cycles. Ligation, transformation, and sequencing were also performed as described for preparation of the L28Y-L54F mutant.

Construction of E. coli DHFR Mutant L28A-F31A. Mutagenesis was carried out by the PCR method (Sarkar et al., 1990). The following oligonucleotide PCR primers were obtained from American Synthesis Inc. (ASI) and purified through a reverse phase cartridge: a 27-mer (PCR-2), CACCGAAACGCGCGAGGCAGAGCTTGG; a 25-mer (PCR-1), GGAACCTCTTACGTGCCGATCAACG; and a 34-mer (L28A-F31A) in which codons for leucine (CTC) and phenylalanine (TTT) were replaced by those for alanine (GCC and GCT), GCCTGCCGATGCCGCCTGGGCTAAACGCAACACC.

PCR reaction mixtures contained 1 μM primers PCR-1 and L28A-F31A and 1 μg of pF28 [a plasmid encoding the *E. coli* DHFR L28F mutation (Wagner et al., 1992)], under conditions identical to those above. To produce the full length mutant DHFR DNA, a reaction mixture was prepared that contained 200 ng of the L28A-F31A megaprimer, which was the product of the first PCR reaction and subjected to the same reaction conditions. After isolation of the products on a 2% agarose gel and purification by electroelution, the mutant DNA was digested with the restriction endonuclease *Bam*HI to obtain the mutated insert fragment.

The plasmid pRWA-1 (Wagner et al., 1992), which carries the *E. coli* wild-type gene flanked by two *Bam*H1 restriction sites, was digested with *Bam*HI, dephosphorylated with HK phosphatase, purified on a 0.8% agarose gel, isolated by electroelution, and resuspended in TE. The *Bam*HI-digested DNA insert, carrying the L28A-F31A mutations, and pRWA-1 were subsequently ligated with T4 ligase. *E. coli* strain DH5α was then transformed with the ligation reaction mixture and plated onto LB/agar plates containing 25 μg/mL chloramphenicol. Randomly selected clones for the L28A-F31A mutant were screened for the presence of the inserted mutant DNA by restriction digestion with *Bam*HI. Potential mutant DNA was then fully sequenced by the method of Sanger (Sanger et al., 1977), thus ensuring that no additional mutations had been incorporated.

Table 1: Steady-State Parameters in MTEN Buffer at 25 °C

			mutant set 1			mutant set 2		
		WT[a]	L28Y	L54F	L28Y L54Y	L28A F31A	I50A L54G	L28A L31A I50A L54G
pH 6.0	k_{cat} (s^{-1})	12.3 ± 0.7	15 ± 3	6.3 ± 0.1	13.2 ± 3.0	7.5 ± 0.7	0.64 ± 0.09	0.12 ± 0.02
	K_M (μM)	0.7 ± 0.2	1.0 ± 0.2	0.7 ± 0.1	61 ± 17	18 ± 3	350 ± 80	23 ± 8
	^DV	1.0 ± 0.1	1.6 ± 0.3	1.2 ± 0.2	1.4 ± 0.2	3.0 ± 0.3	3.2 ± 0.2	1.7 ± 0.3
pH 9.0	k_{cat} (s^{-1})	2.3 ± 0.2	1.4 ± 0.3	1.4 ± 0.2	1.2 ± 0.2	0.44 ± 0.19		0.029 ± 0.005
	K_M (μM)	1.8 ± 0.2	10 ± 3	75 ± 13	186 ± 27	23 ± 6		10 ± 5
	^DV	2.9 ± 0.08	3.0 ± 0.2	2.7 ± 0.5	3.0 ± 0.2			2.2 ± 0.5

[a] Data taken from Fierke et al. (1987).

Protein Purification

Strain MK30-3 or W71-18 was transformed with the plasmid for the *E. coli* DHFR mutants. The 10 mL overnight LB culture supplemented with antibiotic (ampicillin or chloramphenicol) and trimethoprim was diluted to 1 L of LB supplemented with the same antibiotic, and the cells were harvested by centrifugation. All protein purifications were done by a standard procedure for DHFR as previously described using an MTX affinity column (Baccanari et al., 1977), with the exception of the quadruple mutant. Since the quadruple mutant does not bind to an MTX column, this affinity column was used to remove the wild-type DHFR contamination produced by the *E. coli* chromosomal *folA* gene. The quadruple mutant protein was then purified by two passes through a Pharmacia FPLC DEAE-Sephacel column. SDS–PAGE showed a single band, indicating that the protein was pure after the Pharmacia FPLC column procedure. Concentrations of the purified proteins were determined by MTX titration on an SLM 8000 spectrofluorimeter for mutants L28Y, L54F, and L28Y-L54F and by the absorbance at 280 nm or the Bradford method (Bradford, 1976) for mutants L28A-F31A and I50A-L54G and the quadruple mutant.

Steady-State Kinetics. Initial velocities for the enzyme reactions were measured by monitoring the rate of enzyme-dependent absorbance decrease at 340 nm (ϵ = 11 800 M^{-1}; Stone & Morrison, 1982) on a Cary 219 UV–vis spectrophotometer. Proteins showing a hysteretic effect (Penner & Frieden, 1985) were preincubated with either substrate or cofactor. Data were fit to the Michaelis–Menten equation to obtain k_{cat} and K_M values using a nonlinear least-squares fitting program (RS1) run on a VAX microcomputer.

Equilibrium Dissociation Constants. The thermodynamic dissociation constants (K_D) for ligands from DHFR were measured by fluorescence titration. In this experiment, the quenching of the intrinsic enzyme fluorescence at 340 nm upon excitation at 290 nm was monitored as a function of ligand concentration. In a separate control experiment, a known quantity of tryptophan was titrated to correct for the absorbance caused by added ligands (Taira & Benkovic, 1988). Typically, the enzyme concentration used was either at or slightly less than the K_D value of the ligand. The data were fit using the RS1 program run on a VAX microcomputer (Taira & Benkovic, 1988).

Transient Binding and Pre-Steady-State Kinetics. Transient binding and pre-steady-state kinetic experiments were performed on a stopped-flow spectrophotometer purchased from Applied Photophysics Limited. Ligand binding and trapping experiments were monitored by excitation of the intrinsic fluorescence of DHFR at 290 nm and then observation of the degree of emission quenching at 340 nm through an output filter with a range of 250–400 nm. Hydride transfer rates were measured by monitoring coenzyme fluorescence at 450 nm through a 400 nm output filter (Velick, 1958; Dunn & King, 1980; Fierke et al., 1987). The basis for this technique is the excitation by energy transfer of the nicotinamide portion of NADPH at 340 nm, which in turn emits at 450 nm. Since the oxidized nicotinamide of $NADP^+$ displays no emission in this spectral region, the consumption of NADPH is monitored directly. Absorbance measurements were conducted at 340 nm using the 250–400 nm output filter. Typically, 5–8 traces were recorded and averaged for data analysis.

Data were collected by an Archimedes computer over a preselected time range from 5 ms to 15 min. Kinetic data were fit using a nonlinear least-squares computer program provided by Applied Photophysics Inc. that analyzes the transients as either single or double exponentials or a single exponential followed by a linear rate. For rapid binding experiments, data were then transferred to a VAX microcomputer to resolve associating and dissociating rates for the cofactor using the computer program RS1.

RESULTS

Steady-State Kinetics

The Michaelis–Menten parameters, k_{cat} and K_M, were determined for the mutants L28Y, L54F, L28Y-L54F, and L28A-F31A by varying [H_2F] at saturating [NADPH] at several different pH values. For the double mutant I50A-L54G and the quadruple mutant L28A-F31A-I50A-L54G, k_{cat} and K_M were measured only at the limiting pH values of pH 6.0 and 9.0 due to the small amount of protein obtained. Preincubation of the enzyme with NADPH eliminated the hysteretic effect (Fierke et al., 1987; Penner & Frieden, 1987) for all of the mutants except the quadruple mutant, for which a lag phase was still observed upon adding H_2F to initiate the reaction. The deuterium isotope effect, ^DV, of all mutants was determined at pH 6.0 and 9.0. All of the data were computer fitted to the Michaelis–Menten equation. The steady-state kinetic parameters for the mutant proteins at pH 6.0 and 9.0 are shown in Table 1.

It is convenient to group the mutants into two sets: set 1 is L28Y, L54F, and L28Y-L54F, and set 2 is L28A-F31A, I50A-L54G, and L28A-F31A-I50A-L54G. For each set, the ultimate mutant protein is a combination of the first two. Within set 1, there are negligible changes in the k_{cat} values and an approximately 60-fold change in the K_M value (pH 6.0) for H_2F across the single and double mutants. A partial deuterium isotope effect (^DV) of 1.2–1.6 at pH 6.0 and a maximal value for ^DV of 3.0 at pH 9.0 were observed. These data are consistent with the rate of the chemical step being comparable to the rate of H_4F release at low pH values. Within set 2, the k_{cat} values (pH 6.0) decreased from 1.6- to 100-fold for the mutant enzymes relative to the wild-type enzyme, while the K_M values (pH 6.0) for H_2F increased by 26- to 350-fold. The appearance of a maximal kinetic isotope effect for the

Table 2: Thermodynamic Dissociation Constants[a]

		mutant set 1			mutant set 2		
	WT[b]	L28Y	L54F	L28Y L54F	L28A F31A	I50A L54G	L28A L31A I50A L54G
K_D (μM) (NADPH)	0.33 ± 0.06	0.15 ± 0.01	0.07 ● 0.02	0.60 ± 0.15	<1.1	0.70 ± 0.09	0.87 ± 0.02
K_D (μM) (H_2F)	0.22 ± 0.06	0.11 ± 0.02	0.10 ± 0.02	11 ± 3	35 ± 5	350 ± 80[c]	0.97 ± 0.04

[a] The K_D's for NADPH were measured at pH 7.0, and the K_D's for H_2F were measured at pH 6.0. All experiments were carried out at 25 °C. [b] Data taken from Fierke et al. (1987). [c] The K_M value is used, since the K_D was not obtainable by titration.

Table 3: Kinetic Binding Constants for NADPH: Relaxation Method (pH 6.0, 25 °C)

		mutant set 1			mutant set 2		
	WT	L28Y	L54F	L28Y L54F	L28A F31A	I50A L54G	L28A L31A I50A L54G
k_{on} (μM^{-1} s^{-1})	20 ± 1[a]	7.7 ± 0.5	13.2 ± 0.1	9.0 ± 0.2	20.8 ± 0.9	8.1 ± 0.8	8.8 ± 1.5
k_{off} (s^{-1})	3.5 ± 1.5[a]	5.4 ± 0.3[c]	3.3 ± 0.1	6.4 ± 4.1	10.7 ± 3.5	65 ± 10	43 ± 21
E_1/E_2	1.0[a]	1.8 ± 0.3	4.4 ± 0.3	2.1 ± 0.2	0.8 ± 0.1	0.7 ± 0.1	0.6 ± 0.2
k_1 (s^{-1})	0.032[b]	0.058 ± 0.002	0.039 ± 0.002	0.062 ± 0.003	0.054 ± 0.001	0.028 ± 0.001	0.032 ● 0.003

[a] Data taken from Fierke et al. (1987). [b] Data taken from Adams et al. (1989). [c] Determined by competition experiment.

two double mutants at pH 6.0 implies that the chemical step is rate limiting throughout our pH range; the less than maximal value of ^DV for the quadruple mutant at pH 6.0 and 9.0 suggests a change in the chemical nature of the hydride transfer step.

Thermodynamic Dissociation Constants

The binding of ligands to DHFR was measured by following the quenching of the intrinsic enzyme fluorescence upon ligand titration. The data were computer fit to obtain the overall thermodynamic dissociation constant (K_D) for the desired ligand (Taira & Benkovic, 1988). K_D values for H_2F (pH 6.0) and NADPH (pH 7.0) are listed in Table 2. Titrations performed at pH 9.0 showed that the K_D values were independent of pH (data not shown). The K_D value for H_2F binding to the I50A-L54G double mutant was too large to be measured accurately by fluorescence titration and therefore was estimated from the K_M value. This estimation is reasonable because the binding of H_2F to the E·NH complex could be treated as a rapid equilibrium step, since both the hydride transfer rate (see Table 5 and pre-steady-state kinetics) and the affinity of H_2F for E·NH are low. Therefore, K_D (H_2F to E·NH) $\cong K_M$ is expected. Furthermore, there is no significant cooperativity noted in the binding of H_2F to free E or the E·NH complex (Fierke et al., 1987), so that $K_M \cong K_D$ (H_2F to E). For the mutant L28A-F31A, the fluorescence quenching by added NADPH was too small to obtain an accurate K_D value. The upper limit for the value of K_D (NADPH) for this double mutant was estimated from the rate constants for assocation and dissociation of the cofactor to the protein (Adams et al., 1989). Overall, the binding of NADPH was nearly invariant, whereas the binding of H_2F was destabilized from 160-fold (L28A-F31A) to 1600-fold (I50A-L54G) relative to the wild-type enzyme.

Pre-Steady-State Kinetics

Binding Kinetics: Relaxation Method. The rate constants for association and dissociation of NADPH to the mutant proteins were also measured by following the quenching of the intrinsic enzyme fluorescence. Generally, the enzyme exists as an equilibrium mixture of two slowly interconverting forms, E_1 and E_2, of which only one form, E_1, binds NADPH (Cayley et al., 1981; Fierke et al., 1987). The mechanism, which is illustrated in Scheme 1, gives rise to a rapid ligand-dependent phase and a slower ligand independent phase in the ligand binding curve.

Scheme 1

$$\begin{array}{ccc} E_1 + L & \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} & E_1{\cdot}L \\ k_1 \big\uparrow \big\downarrow k_{-1} & & \\ E_2 & & \end{array}$$

Scheme 2

$$E{\cdot}L_1 \underset{k_1}{\overset{k_{-1}}{\rightleftharpoons}} E + L_1 \qquad k_2[L_2] >> k_1[L_1]$$

$$E + L_2 \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E{\cdot}L_2 \qquad k_2[L_2] >> k_{-1}$$

Under pseudo-first-order conditions ([L] ≫ [E]), the observed rate constant (k_{obs}) for the binding of L to E_1 is given by $k_{obs} = k_{on}[L] + k_{off}$, where k_{on} and k_{off} are the association and dissociation rate constants obtained from the plot of k_{obs} as a function of [L] (Fierke et al., 1987). The rate constant for the enzyme interconversion (k_1) was determined from the slow phase, while the equilibrium constant ($K_{eq} = k_1/k_{-1}$) was determined from the ratio of the relative amplitudes of the fast and slow phases. Values of the association and dissociation rate constants for NADPH binding and the equilibrium constants for E_1/E_2 are listed in Table 3. For set 1 mutants, the k_{on} values were generally slightly lower than that observed for the wild-type enzyme by 1.5–2.6-fold, while values for k_{off} were comparable to that for the wild-type enzyme. For set 2 mutants, the k_{on} values did not change dramatically, while the k_{off} values for I50A-L54G and the quadruple mutants increased by 20- and 12-fold, respectively. Except for the L54F mutant, the ratio of E_1 to E_2 for all of the mutant proteins remained close to unity. Over the range of NADPH concentrations (2.5–30 μM), the ratios of the relative amplitudes of the fast and the slow phases were invariant, implying that conformer E_2 did not appreciably bind to NADPH at NADPH levels less than 30 μM.

Ligand Dissociation Constants: Competition Method. The dissociation rates of enzyme-bound ligand were measured by competition between L_1 and L_2 (Birdsall et al., 1980) as described in Scheme 2.

The rate constants for H_4F dissociating from the binary E·H_4F and ternary E·H_4F·NADPH complexes and for $NADP^+$ dissociating from the E·H_4F·$NADP^+$ species are listed in Table 4. For all mutant proteins, the off-rates of H_4F from both the binary and ternary complexes increased. Further-

Table 4: Dissociation Rate Constants (k_{off} (s^{-1}) at pH 6.0, 25 °C): Competition Method

				mutant set 1			mutant set 2		
ligand	enzyme species	trapping ligand	WT[a]	L28Y	L54F	L28Y L54F	L28A F31A	I50A L54G	L28A L31A I50A L54G
H_4F	E·H_4F	MTX	1.4 ± 0.2	37 ± 4	2.1 ± 0.8	21 ± 5	41 ± 8	366 ± 60	
H_4F	E·H_4F·NADPH	MTX	12 ± 2	21 ± 4	13 ± 3	21 ± 3	85 ± 5	479 ± 30	535 ± 100
$NADP^+$	E·H_4F·$NADP^+$	NADPH	200 ± 20	103 ± 3	232 ± 59	40 ± 3	200 ± 20		

[a] Data taken from Fierke et al. (1987).

Table 5: Hydride Transfer Rates (pH 6.0, 25 °C)

		mutant set 1			mutant set 2		
	WT[a]	L28Y	L54F	L28Y L54F	L28A F31A	I50A L54G	L28A L31A I50A L54G
k_{hyd} (s^{-1})	950 ± 50	109 ± 5	20.0 ± 0.2	77 ± 15	10 ± 2	0.64 ± 0.09[b]	0.12 ± 0.02[b]

[a] Data taken from Fierke et al. (1987). [b] Steady-state k_{cat} at pH 6.0.

more, except for L54F, the H_4F off-rates were not accelerated significantly by NADPH binding, a key feature in the kinetic scheme of the wild-type enzyme. The set 1 mutants appeared to have H_4F off-rates from the ternary ligand complex within 2-fold of that observed for the wild type, whereas set 2 mutants exhibited dissociation rate constants that were 7–44-fold faster than the value of wild-type DHFR. The dissociation rate constants for $NADP^+$ from the initial product complex were approximately equal to the value for the wild-type enzyme, except for the L28Y-L54F double mutant in which a 5-fold decrease was observed.

Hydride Transfer Rates. The hydride transfer rates for all mutant proteins except the double mutant I50A-L54G and the quadruple mutant in set 2 were measured directly using pre-steady-state fluorescence energy transfer experiments.

Two kinds of experiments were done in order to measure the hydride transfer rates. In the first, the enzyme was preequilibrated with excess NADPH before the addition of H_2F to remove the hysteresis associated with ligand binding. A single-exponential burst followed by a linear steady-state turnover was generally observed, correlating with the conventional steady-state rate measurements (Fierke et al., 1987). In the second type of experiment, excess enzyme was preequilibrated with NADPH before the addition of H_2F. A single-exponential burst was generally observed corresponding to the hydride transfer rate (Wagner et al., 1992). In each experiment, NADPD was substituted for NADPH to verify whether the hydride transfer step was actually being observed, as signaled by the observation of a maximal kinetic isotope effect. The hydride transfer rates, k_{hyd}, are listed in Table 5. The chemistry step was depressed for both mutant sets.

In two cases, the hydride transfer rate was estimated from steady-state kinetic data. For the I50A-L54G mutant in set 2, a steady-state ^DV value of 3.0 was observed at pH 6.0, which is consistent with k_{cat} being equal to the hydride transfer rate (k_{hyd} = 0.64 ± 0.09 s^{-1}). For the quadruple mutant in set 2, a ^DV value of 2.0 at both pH 6.0 and 9.0 under steady-state conditions indicated that k_{cat} is probably a measure of the hydride transfer step for the quadruple mutant (see Discussion).

DISCUSSION

The creation and testing of a series of proteins interrelated through multiple mutations provides a means for probing the importance of molecular interactions between specific amino acids within a protein framework (Carter et al., 1984; Ackers & Smith, 1985; Horovitz & Fersht, 1990). For enzymes, the changes may be limited to active site residues from which one can assess the effects of the substitution on the kinetic and thermodynamic parameters associated with catalysis. In general, the effect of a mutation on a functional property of the protein can be expressed in terms of a free energy relationship:

$$\Delta\Delta G_{(x,y)} = \Delta\Delta G_{(x)} + \Delta\Delta G_{(y)} + \Delta G_I$$

where $\Delta\Delta G_{(x)}$ is the change in free energy of a functional property caused by a mutation at site x compared to the wild-type protein, and $\Delta\Delta G_{(y)}$ and $\Delta\Delta G_{(x,y)}$ represent a similar change indexed to the wild-type protein for the y and x,y mutants (Wells, 1990). The ΔG_I term is the coupling energy: if sites x and y function independently, $\Delta G_I \approx 0$, and if they interact, $\Delta G_I \not\approx 0$. The relationship may be easily expanded to encompass higher order mutations. If the effect of mutation x alone is greater than that of mutation y alone, $[\Delta\Delta G_{(x)} > \Delta\Delta G_{(y)}]$, then the second mutation, y, may be either antagonistic $[\Delta\Delta G_{(x,y)} < \Delta\Delta G_{(x)}]$, absent $[\Delta\Delta G_{(x,y)} = \Delta\Delta G_{(x)}]$, partially additive $[\Delta\Delta G_{(x)} < \Delta\Delta G_{(x,y)} < \Delta\Delta G_{(x)} + \Delta\Delta G_{(y)}]$, additive $[\Delta\Delta G_{(x,y)} = \Delta\Delta G_{(x)} + \Delta\Delta G_{(y)}]$, or synergistic $[\Delta\Delta G_{(x,y)} > \Delta\Delta G_{(x)} + \Delta\Delta G_{(y)}]$ with respect to the first mutation, x (Mildvan et al., 1992). Its classification may, however, depend on the functional property being measured. Previous studies on different systems showed that additivity is the main trend, if the residues modified are sufficiently far from each other (not within van der Waals contact range) (Wells, 1990).

There are a wide variety of hydrophilic (i.e., Arg44) and hydrophobic (i.e., Leu54) side chains that constitute the ensemble of active site side chains in DHFR. Inspection of the X-ray crystal structure data for the apo-, binary, and ternary enzyme complexes coupled with mechanistic studies by mutagenic and kinetic techniques have revealed that, due to the myriad of hydrogen bonds available in the active site, the substitution of polar residues can lead to complex and unpredictable mechanistic ramifications (Adams et al., 1991; Wagner & Benkovic, 1992). Point site mutations, however, of the hydrophobic side chains Leu28, Phe31, Ile50, and Leu54 have generally resulted in localized perturbations of binding and catalysis (Chen et al., 1987; Li et al., 1992; Murphy & Benkovic, 1989; Wagner et al., 1992). Consequently, we have chosen to vary the hydrophobicity and surface area of the residues at positions 28, 31, 50, and 54 in order to define the nature of the interactions that exist between them during substrate binding and processing.

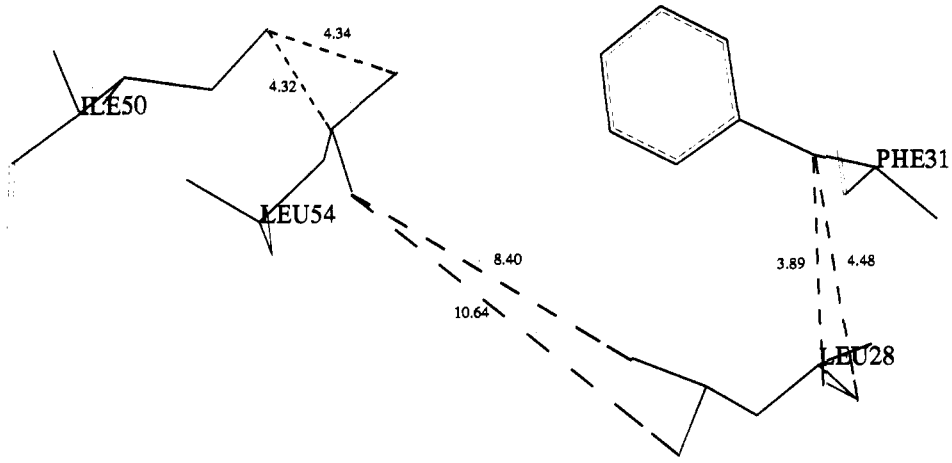


FIGURE 2: Distances (Å) between the side chains of Leu28, Phe31, Ile50, and Leu54 in apo *E. coli* dihydrofolate reductase.

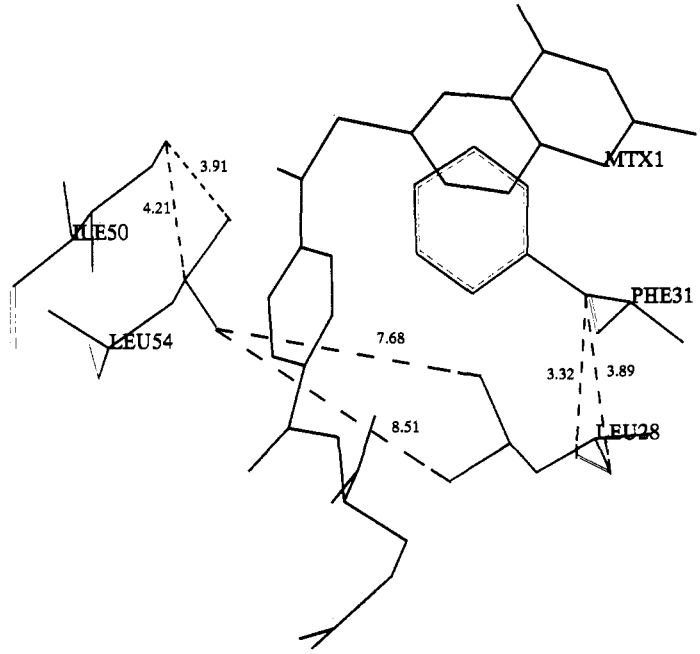


FIGURE 3: Distances (Å) between the side chains of Leu28, Phe31, Ile50, and Leu54 in the binary complex of *E. coli* dihydrofolate reductase with MTX.

Two sets of mutant proteins have been constructed and their kinetic parameters determined. Set 1 contains the mutant proteins designated L28Y, L54F, and L28Y-L54F. Position 28 exhibits species variations and is occupied by either leucine, tyrosine, or phenylalanine. The leucine at position 54, however, is invariant across species. Previous studies on the influence of position 54 through site-directed mutagenesis (Mayer et al., 1986; Murphy & Benkovic, 1989) found that the free energy barrier for hydride transfer was increased by approximately 2 kcal mol^{-1}, regardless of whether the substitution was a hydrophobic or hydrophilic residue (L54I, L54F, L54N) or a side chain deletion (L54G). The side chains at positions 28 and 54 are approximately 8–9 Å apart in the apoenzyme (Figure 2) and separated by at least 7–8 Å in the binary MTX·enzyme complex (Figure 3) (Bolin et al., 1982; Bystroff & Kraut, 1991). Consequently, they should not interact through van der Waals contacts, and one might expect $\Delta\Delta G_I$-(Y,F) $\cong$ 0 for the double mutant protein (L28Y-L54F). Set 1 consequently retains the opportunity for side chain/substrate interactions.

In the second set of mutant proteins, we deleted specific side chains by introducing alanine or glycine, thereby reducing the potential for molecular interactions. The double mutant proteins L28A-F31A and I50A-L54G were constructed. Position 31 is a conserved phenylalanine across species; position 50 is an isoleucine in all eukaryotic species but a phenylalanine in *L. casei*. In the MTX·enzyme binary complex, the side chains in positions 28 and 31 and in positions 50 and 54 are approximately 3–4 Å apart and consequently are within van der Waals contact range (Figure 3). Nevertheless, these two pairs should not directly interact with one another in the quadruple mutant protein (L28A-F31A-I50A-L54G).

Four critical reaction parameters were assessed in establishing criteria for the free energy relationship: the two thermodynamic dissociation constants for H_2F and NADPH from their binary complexes, E·ligand; the rate constants for release of H_4F from the E·NADPH·H_4F ternary complexes (the rate-limiting step in steady-state turnover for the wild-type enzyme; Fierke et al., 1987); and the hydride transfer rate constants, the chemical step for the enzymic reaction.

For set 1, the differences in $\Delta\Delta G$ for the mutant proteins compared to wild-type DHFR for these four reaction parameters are shown in Figure 4. Since $\Delta\Delta G$ for L28Y-L54F is not the sum of the $\Delta\Delta G$ values for L28Y and L54F, $\Delta G_I \neq 0$ for either the binding of NADPH and H_2F, the rate constant for hydride transfer, or the rate constant for the

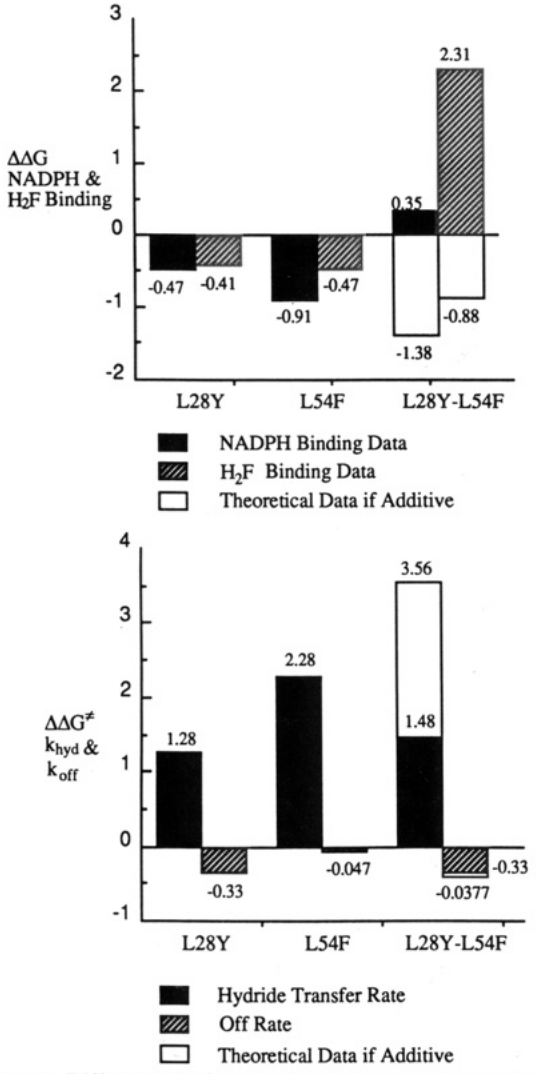


FIGURE 4: Differences in free energy changes ($\Delta\Delta G$ or $\Delta\Delta G^{\ddagger}$) for the binding of NADPH or H_2F or for the rate constants for hydride transfer and tetrahydrofolate dissociation relative to the wild-type enzyme for the L28Y, L54F, and L28Y-L54F mutants. Calculated from the following relationships: $\Delta\Delta G = -RT \ln K_D^{wt}/K_D^{M}$ and $\Delta\Delta G^{\ddagger} = -RT \ln k^{wt}/k^{M}$, where wt and M are wild-type and mutant proteins, respectively.

release of H_4F. For L28Y-L54F, the binding of NADPH follows a 1:1 stoichiometry (data not shown), and the relative magnitudes and ordering of the kinetic steps probed with deuterium kinetic isotope effects are collectively identical to those of the wild type. Therefore, there is no change in the overall kinetic and chemical mechanism of action.

The introduction of aromatic residues for aliphatic side chains strengthens the enzyme's affinity for both H_2F and NADPH (Table 2). The simultaneous replacement of both leucines in the L28Y-L54F mutant protein disfavors substrate and cofactor binding, so that the effect is antagonistic (Table 2). The interaction energy is approximately 3 kcal mol^{-1} for the binding of H_2F (Figure 4). Both single mutant proteins show 1–2 kcal mol^{-1} increases in the free energy barrier for hydride transfer. Surprisingly, the L28Y-L54F mutant exhibits a more favorable free energy than predicted (approximately 2.0 kcal mol^{-1}) for the same kinetic step (partial additivity). The effect of the mutations on the off-rate constants for H_4F from the E·NADPH·H_4F complex is negligible.

The crystal structure of the L54F·MTX binary complex (Brown and Kraut, personal communication) revealed that the active site locally accommodates the substitution of the larger phenylalanine for the leucine without any significant effect on the position of ligand binding. The two binding complexes (mutant and wild type) are virtually superimposable. The substrate, which interacts with Leu28 and Leu54 on opposite faces of the benzyl ring, must provide the linkage between these two residues since they normally do not interact through space. Likewise, no changes in the positioning of side chains within the NADPH binding site were observed. Despite the lack of insights furnished by the crystallographic structure, we can only presume that the conversion from antagonism to partial additivity observed in this series for the terms $\Delta\Delta G$ and $\Delta\Delta G^{\ddagger}$ associated with ligand binding and the rate of hydride transfer, respectively, must arise because of improved transition vs ground-state interactions within the double mutant protein, abetted by the aromatic side chains in the substituted amino acids.

Data on the second set of mutant proteins likewise demonstrated that the binding of NADPH was well behaved (Table 2). The dissociation rate constants from the binary E·NADPH complex increase by 3–20-fold in this series (Table 3). In contrast to these values, the dissociation of H_2F from E·H_2F is markedly sensitive to side chain deletions: for L28A-F31A, the K_D increased by a factor of 160 relative to the wild type, while for I50A-L54G, the K_D increased by 1600-fold (Table 2). Surprisingly, for the quadruple mutant protein, L28A-F31A-I50A-L54G, the magnitude of the K_D value is similar to that of the wild type, which corresponds to an antagonistic effect of over 6 kcal mol^{-1} (Figure 5). Apparently the binding site has sufficient conformational flexibility to restore most of its binding capacity for folate, despite the fact that the mutated side chains have a smaller volume. Any such conformational changes are probably localized to the H_2F binding site. In summary, however, all of the set 2 mutants, owing to the reduced side chain–substrate interactions, have weakened the affinity of the enzyme for its substrates.

Measurement of the rate constants for hydride transfer at low and high pH values revealed that the steady-state turnover was limited by this chemical step ($^{D}V \cong 3.0$, pH 6.0 and 9.0) for the set 2 mutants. For the quadruple mutant protein, the maximal value for the deuterium isotope effect is 2.0 rather than 3.0 noted for the wild-type enzyme. The free energy change for this step is only partially additive relative to the double mutant proteins (Figure 5). Similarly, the free energy change associated with off-rates for H_4F release is partially additive, with the value for the L28A-F31A-I50A-L54G mutant protein being ca. 1 kcal mol^{-1} less than expected (Figure 5). It appears, therefore, that despite the change in the magnitude of the deuterium isotope effect, which may indicate a shift in the transition state for the hydride transfer relative to the reaction coordinate (Page, 1984), the nonadditivity of the free energy changes is a characteristic of this set of mutants (Figure 5). Again one may argue for both ground- and transition-state effects that are mediated through the binding of substrate. There is no indication that the overall drop in the rate constant for hydride transfer is due to nonproductive substrate binding, since the k_{cat}/K_M values (Table 1) for the set 2 mutants differ significantly from that for the wild-type enzyme.

CONCLUSION

This work has focused on two sets of mutants at the folate binding site of *E. coli* DHFR. One of them (L28Y, L54F, and L28Y-L54F) has retained hydrophobic surfaces similar to those found in other species, while the second set (L28A-

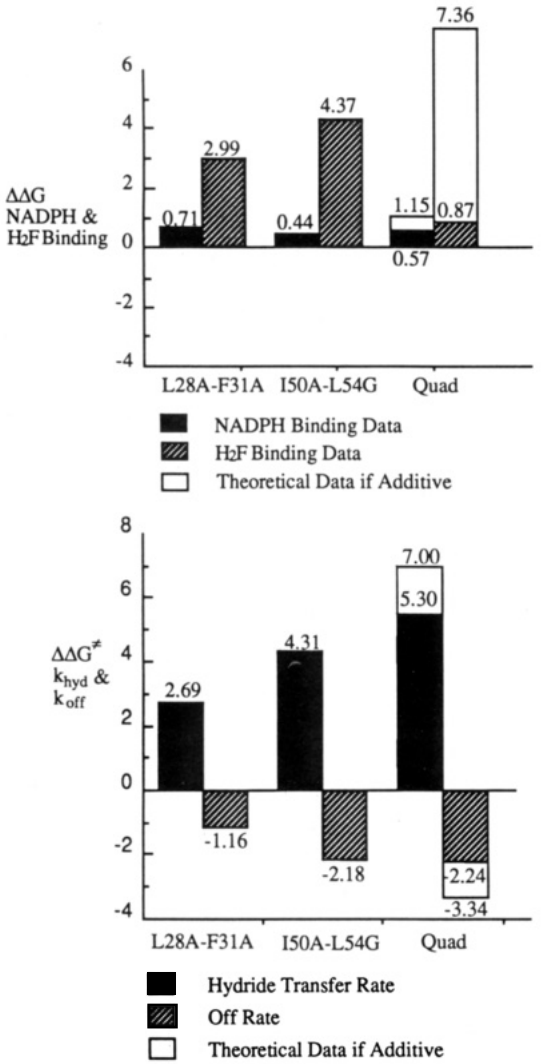


FIGURE 5: Differences in free energy changes ($\Delta\Delta G$ or $\Delta\Delta G^{\neq}$) for the binding of NADPH or H_2F or for the rate constants for hydride transfer or tetrahydrofolate dissociation relative to the wild-type enzyme for the L28A-F31A, I50A-L54G, and L28A-F31A-I50A-L54G mutants. Calculated as shown in the legend to Figure 4.

F31A, I50A-L54G, and L28A-F31A-I50A-L54G) has the side chains deleted by replacing amino acids using alanine and glycine. Generally, nonadditivity in the free energy changes associated with key steps of the enzyme's turnover is observed for both sets of mutants.

The net effect is to keep the evaluated thermodynamic and kinetic parameters closer to the original wild-type values than anticipated. Only in the case of H_2F and NADPH binding to the double mutant L28Y-L54F does the protein active site not compensate for the mutations. What is remarkable is how well the active site tolerates such mutations and retains significant catalytic activity. Clearly, if we accept the premise that subangstrom tolerances are required for optimal catalytic activity by DHFR (Benkovic et al., 1988), there must be sufficient redundancy and flexibility built into the active site and the surrounding framework to retain a sufficient population of active enzyme complexes, despite perturbations at key substrate–side chain contacts. Although the residue pairs Leu28 and Phe31 are 8-9 Å from the pairs Ile50 and Leu54, these residues are sensitive to changes in one anothers' side chain volume in the presence of a substrate (H_2F) that bridges the distance between the residue pairs.

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