Importance of a hydrophobic residue in binding and catalysis by dihydrofolate reductase

(site-directed mutagenesis)

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ABSTRACT A conserved residue at the dihydrofolate binding site of dihydrofolate reductase (EC 1.5.1.3), leucine-54, was replaced with glycine to ascertain the role of this hydrophobic amino acid. The effect of the mutation is both to increase the dissociation rate of dihydrofolate and decrease the rate of hydride transfer thus changing the rate-limiting step in catalysis from product loss (leucine-54) to hydride transfer (glycine-54). The total stabilization by leucine-54 of the transition state for hydride transfer is *ca.* 10^4 -fold ($\Delta\Delta G \approx 5.4$ kcal/mol) at subsaturating dihydrofolate levels relative to free enzyme despite its location some 10 Å from the site of chemical reaction.

Classical mechanistic studies on enzyme catalysis have focused on active site residues involved as acid-base catalysts or in the formation of covalent intermediates. The importance of hydrophobic amino acids remote from or hydrophobic residues directly at the active site could not be readily ascertained. Site-directed mutagenesis provides a means to define the role of hydrophobic amino acids in enzymic catalysis. To date, the focus of most studies using site-directed mutagenesis remains on active site residues, deleting them or replacing amino acids with potentially more efficient catalysts (1-3). One striking mutagenesis study on t-RNA^{Tyr} ligase (formerly t-RNA^{Tyr} synthetase) (4) suggests the critical importance of remote amino acids in catalysis. Replacing two hydrogen-bonding residues in the binding site for the γ -phosphate of ATP (nucleophilic attack occurs at the α -phosphate) decreased the rate of catalysis by a factor of 10^5 , while only weakening ATP binding by a factor of 5. We report here the effect on catalysis and binding of a mutation in dihydrofolate reductase (DHFR; EC 1.5.1.3) at the substrate binding site, but remote from the site of chemical transformation.

DHFR catalyzes the reduction of dihydrofolate (H₂F) to tetrahydrofolate (H₄F) by direct hydride transfer from NADPH. Close inspection of the x-ray crystal structure of the ternary complex with the *Lactobacillus casei* enzyme (5, 6) does not suggest a direct mediation of the hydride transfer by, for instance, hydrogen bonding or by charge stabilization of the NADP⁺ product.

DHFR from *Escherichia coli* binds H₂F tightly (7) (K_d , 1 μ M) in a conformation with the *p*-aminobenzoyl side chain almost at right angles to the pterin ring (5, 6). The binding site is lined with several hydrophobic, highly conserved amino acids that could contribute to product stabilization thereby affecting catalysis. One of these, Leu-54, interacts with the benzoyl glutamate portion of H₂F, and is more than 10 Å distant from the site of hydride transfer. We have monitored the effect of the mutation Leu-54 $\rightarrow$ Gly by pre- and steady-state kinetics as well as equilibrium binding studies

and have interpreted our results in light of the kinetic mechanism of the wild-type enzyme. We also have compared selected properties of two other mutants, Phe-31 $\rightarrow$ Val and (Leu-54 $\rightarrow$ Gly, Phe-31 $\rightarrow$ Val) to clarify the properties of the Gly-54 enzyme ([Gly⁵⁴]DHFR).

MATERIALS AND METHODS

The Gly-54 and Val-31 mutants were constructed by primer extension of oligonucleotides using as the template partially single-stranded pTY1 (8, 9), a derivative of pBR322 containing a 1-kilobase insert encoding for *E. coli* DHFR. The double mutant was constructed in the same manner, but using the Val-31 encoding plasmid as the template. The oligonucleotide sequence introduced a new restriction site (Fig. 1), so that mutants could be selected by restriction enzyme analysis, followed by isolation of the fragment by gel electrophoresis and recloning of this DNA fragment. The entire structural gene was then sequenced to verify that no other mutations had occurred (10). The mutant proteins were expressed in *E. coli* strain HB101, and wild-type enzyme was removed during purification of [Gly⁵⁴]- and [Val³¹]DHFR by virtue of the higher affinity of the wild-type enzyme for the methotrexate (MTX) affinity resin used in the purification (11). The double mutant was purified by ion-exchange (DEAE-Sephacel) and gel-filtration (Sephadex G-100) chromatography rather than affinity chromatography, but the extract was first mixed batchwise with the MTX resin to remove any wild-type DHFR. Steady-state kinetic parameters were obtained under the conditions of Stone and Morrison (12). Stopped-flow fluorescence quenching was carried out using the method of Cayley *et al.* (13) using equipment constructed in the laboratory of K. A. Johnson (14).

RESULTS AND DISCUSSION

The steady-state parameters for the wild-type and [Gly⁵⁴]-DHFR, shown in the first two columns of Table 1, demonstrate that while the Gly-54 mutation has a large effect on K_m (increasing it more than 200 times) the steady-state turnover rate, *V*, is not affected. Nevertheless, a more detailed investigation of the kinetics of this mutant enzyme demonstrates that the catalytic step involving hydride transfer is impaired. Specifically, we have shown that *V* does not represent the same rate-limiting step in the wild-type and [Gly⁵⁴]DHFR.

The rate-limiting step at neutral pH in the wild-type enzyme, under conditions where NADPH is saturating, is the dissociation of H₄F from the enzyme-NADPH-H₄F ternary complex (unpublished results). The measured NADPH-assisted H₄F dissociation rate is more rapid than the dissociation of H₄F from either enzyme or enzyme-NADP⁺. Their analysis is further supported by the fact that at pH values

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Abbreviations: DHFR, dihydrofolate reductase; H₂F, dihydrofolate; H₄F, tetrahydrofolate; MTX, methotrexate.

<u>Leu-54 --> Gly</u>	
Wild-type	TCGGTCGTCGGTTGCCAGGA
Mutant	TCGGTCGTC <u>CCCGGG</u> CCAGGA
Apa I	
<u>Phe-31 --> Val</u>	
Wild-type	GATCTCGCCTGGTTTAAACGC
Mutant	GATCTCGCCTGGGTTAAACGC
Dra I removed	

FIG. 1. Oligonucleotides and restriction enzyme sites used for mutagenesis.

below the apparent pK_a in the pH rate profiles ($pH \approx 8.0$), no deuterium isotope effect is observed using $NADP^2H$ (ref. 12; unpublished results). However, this kinetic scheme does not apply to $[Gly^{54}]DHFR$. The rate of dissociation of H_4F from free enzyme, as measured by stopped-flow fluorescence quenching at 340 nm using MTX as a competitive trap (16), is greater than $300 s^{-1}$, in contrast to the $1.4 s^{-1}$ observed for wild type, and in parallel with the weak binding of H_2F (Table 1 and see Table 3). Secondly, the deuterium isotope effect in V at pH values below the pK_a for $[Gly^{54}]DHFR$ is 3.0, in contrast to a deuterium isotope effect in V of 1.1 for wild type. Together, these two observations indicate that the observed V of $14 s^{-1}$ is not the rate of product dissociation, as in wild type, but rather is largely hydride transfer. Thus, the similar turnover rates observed for the two enzymes in the steady-state measurements are purely coincidental (Table 1).

We must also address the question of protein integrity: Are the properties of $[Gly^{54}]DHFR$ due solely to the loss of the Leu-54 or did the mutation seriously affect the enzyme conformation so that function was impaired? It has been found in various studies that point mutations are in general accommodated by very minor readjustments of the protein structure and that "space" created by introducing smaller amino acids can be occupied by water molecules (17–19). Experimental evidence for $[Gly^{54}]DHFR$ supports this generalization. The binding and dissociation rates for NADPH binding were determined from the pseudo-first order rate constant measured by stopped-flow fluorescence quenching. The binding of NADPH results in a biphasic curve, with the fast phase yielding the pseudo-first order rate constant for NADPH binding and the slow phase yielding a rate for the interconversion of two conformers of the enzyme. As shown in Table 2, the binding of NADPH, the rates of conformer interconversion, and their relative concentrations are all in good agreement with wild-type values, consistent with localized structural changes caused by the mutation.

Table 1. Comparison of kinetic parameters for wild type and $[Gly^{54}]DHFR$

Enzyme	K_m , [*] μM	V , [*] s^{-1}	$k_{off}(H_4F)$, [†] s^{-1}	$(V)^{2H\ddagger}$
Wild type DHFR	0.5	12	1.4	1.1
$[Gly^{54}]DHFR$	140	14	>300	3.0

*Reaction conditions were as follows: 50 mM Mes/25 mM Tris/25 mM ethanolamine/100 mM NaCl, pH 5.5 (MTEN buffer), 25°C, 100 μM NADPH/enzyme/ H_2F according to K_m and V values.

†Reaction conditions were as follows: MTEN buffer, pH 7.0, 25°C.

‡Kinetic deuterium isotope effect with $NADP^2H$, under same conditions as *. $NADP^2H$ prepared according to ref. 15 and unpublished results.

Table 2. Comparison of NADPH binding for native and $[Gly^{54}]DHFR$

Enzyme	k_{on} , [*] $M^{-1} s^{-1}$	k_{off} , [*] s^{-1}	k_c , [†] s^{-1}	Rel. amp. [‡]
Wild type DHFR	2.0×10^7	4	0.030	1.2
$[Gly^{54}]DHFR$	1.9×10^7	10	0.029	1.2

*Determined from dependence on NADPH concentration of k_{obs} , fluorescence quenching at 340 nm, pH 7.0.

†Rate of interconversion of $E_2 \rightarrow E_1$ (16), k_{obs} for slow phase; conditions as in *.

‡Relative amplitudes of two phases, conditions as in *.

The properties of a double mutant, with the second mutation in the same spatial area of the protein as the first, provide another indication of the structural localization of the single mutants. If the effects were not local, the structural effects of two proximal mutations would not be simply additive. Phe-31, another conserved amino acid at the H_2F binding site, is spatially on the opposite face of the p -aminobenzoyl moiety from the Leu-54. The closest carbon atoms of these amino acids are only 4.4 Å apart in the *E. coli*-MTX binary complex (5). If a mutation at Phe-31 and a mutation at Leu-54 have effects on binding energy that are strictly additive in a double mutant, it is reasonable to conclude that the effects of each single mutant on structure are local. $[Val^{31}]DHFR$ (unpublished results) was used in conjunction with $[Gly^{54}]DHFR$ and the double mutant $[Val^{31}, Gly^{54}]DHFR$ in a comparison of binding energies for MTX and H_2F . As listed in Table 3, the decreased binding energy due to each single mutation is indeed additive in the double mutant. Despite the poor affinity for H_2F , the double mutant is still active (V is $0.7 s^{-1}$ at pH 6.0).

The absolute binding energy change for $[Val^{31}]$ - and $[Gly^{54}]DHFR$ is also of interest, as relatively few mutations at hydrophobic residues have been reported. Since the Val-31 is a more conservative change than the Gly-54 (the volume decrease from the original to the substituted amino acid is smaller) the smaller effect of the Val-31 mutation might have been anticipated. The change in binding energy may be attributed directly to lost hydrophobic interaction between enzyme and substrate by poorer desolvation of substrate upon binding. There may be other contributions as well, such as entropy differences because a different number of water molecules are displaced upon binding. The transfer free energy, or hydrophobicity, of amino acids varies from 1.5 kcal/mol for valine to 3.4 kcal/mol for tryptophan (20), so the observed binding energy differences for mutations at hydrophobic amino acids from 1.6 to 2.9 kcal/mol seem quite reasonable. The difference in energy due to a hydrophobic mutation for an enzyme substrate complex is probably neither predictable nor general, but with a number of mutations a pattern may emerge.

Table 3. Comparison of binding energy changes for H_2F and MTX for $[Val^{31}]$ -, $[Gly^{54}]$ -, $[Val^{31}, Gly^{54}]DHFR$

Enzyme	$K_d(H_2F)$, μM	Δ kcal/ mol	$K_d(MTX)$, μM	Δ kcal/ mol
Wild type DHFR	1.0*	—	0.004 [§]	—
$[Val^{31}]DHFR$	15 [†]	1.6	0.3 [†]	2.2
$[Gly^{54}]DHFR$	140 [‡]	2.9	1.2 [†]	2.9
$[Val^{31}, Gly^{54}]DHFR$	2000 [‡]	4.5	9 [†]	4.5

*Calculated from k_{off} and k_{on} determined by stopped-flow fluorescence quenching, pH 6.0.

†Determined by fluorescence titration using the analysis as in (7) MTEN buffer, pH 7.0.

‡Determined as K_S under steady-state conditions.

§Calculated as *, k_{off} determined using trimethoprim as a competitive trap.

The effect of the Leu-54 → Gly mutation then is both to decrease the binding interaction and to make chemical catalysis less efficient. The primary effect of removing the isobutyl side chain is a 140 times, or 2.9 kcal/mol, decrease in the free energy change obtained upon binding substrate. However, the interaction between Leu-54 and substrate in the transition state for the chemical step must also be important despite the remoteness of the residue from the reaction center. We estimate the rate of the hydride transfer step for wild type at 1000 s^{-1} so that the decrease would be at least 70 times to 14 s^{-1} (unpublished results). The transition state may be destabilized either by H₂F misalignment relative to the NADPH, or by loss of a hydrophobic interaction that preferentially stabilizes the developing H₄F product. In terms of observed free energy changes, at saturating H₂F levels, the Leu-54 residue stabilizes the transition state for hydride transfer *ca.* 70-fold ($\Delta\Delta G \approx 2.5\text{ kcal/mol}$) relative to the ternary complexes. At subsaturating H₂F levels, one would observe both the 2.9 kcal/mol of binding energy and the 2.5 kcal/mol of transition state stabilization. Thus the total stabilization by Leu-54 of the transition state for hydride transfer is *ca.* 10^4 -fold ($\Delta\Delta G \approx 5.4\text{ kcal/mol}$) relative to free enzyme, assuming both mutant and wild type have identical free energies.

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