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Site-Specific Mutagenesis of Dihydrofolate Reductase From *Escherichia coli*

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Two site-specific mutations of dihydrofolate reductase from *Escherichia coli* based on the x-ray crystallographic structure were constructed. The first mutation (His-45 → Gln) is aimed at assessing the interaction between the imidazole moiety and the pyrophosphate backbone of NADPH. The second (Thr-113 → Val) is part of a hydrogen bonding network that contacts the dihydrofolate substrate and may be involved in proton delivery to the N5-C6 imine undergoing reduction. The first mutation was shown to alter both the association and dissociation rate constants for the cofactor so that the dissociation constant was increased 6–40-fold. A corresponding but smaller (fourfold) effect was noted in V/K but not in V compared to the wild-type enzyme. The second was demonstrated to increase the dissociation rate constant for methotrexate 20–30-fold, and presumably dihydrofolate also, with a corresponding 20–30-fold increase in the dissociation constant. In this case an identical effect was noted on V/K but not in V relative to the native enzyme. Thus, in both mutant enzymes the decrease in binding has not been translated into a loss of catalytic efficiency.

Key words: steady state, site-specific mutagenesis, kinetics, dihydrofolate reductase, binding

Dihydrofolate reductase (DHFR), which catalyzes the reduction of dihydrofolate (H_2-folate) to tetrahydrofolate (H_4-folate) with nicotinamide adenine dinucleotide phosphate (NADPH) as cofactor, is one of a few proteins whose x-ray crystal structure has been solved to a resolution better than 2 Å [1,2]. Thus, the relationship between enzyme structure and function can be studied in detail. The structure of the methotrexate-NADPH-ternary complex of *Lactobacillus casei* DHFR is shown schematically in Figure 1, with two sites of particular interest, Arg-44 and Thr-116, indicated. We report here the effect of specific mutations at the two equivalent sites in the *Escherichia coli* DHFR, His-45 and Thr-113.

From the proximity of His-45 to the pyrophosphate bridge of NADPH, this residue was anticipated to interact with the nicotinamide 5′-phosphate, perhaps via a salt bridge as well as by a hydrogen bond. By replacing His-45 with Gln, the possibility for an ionic interaction would be eliminated and the role of His-45 in

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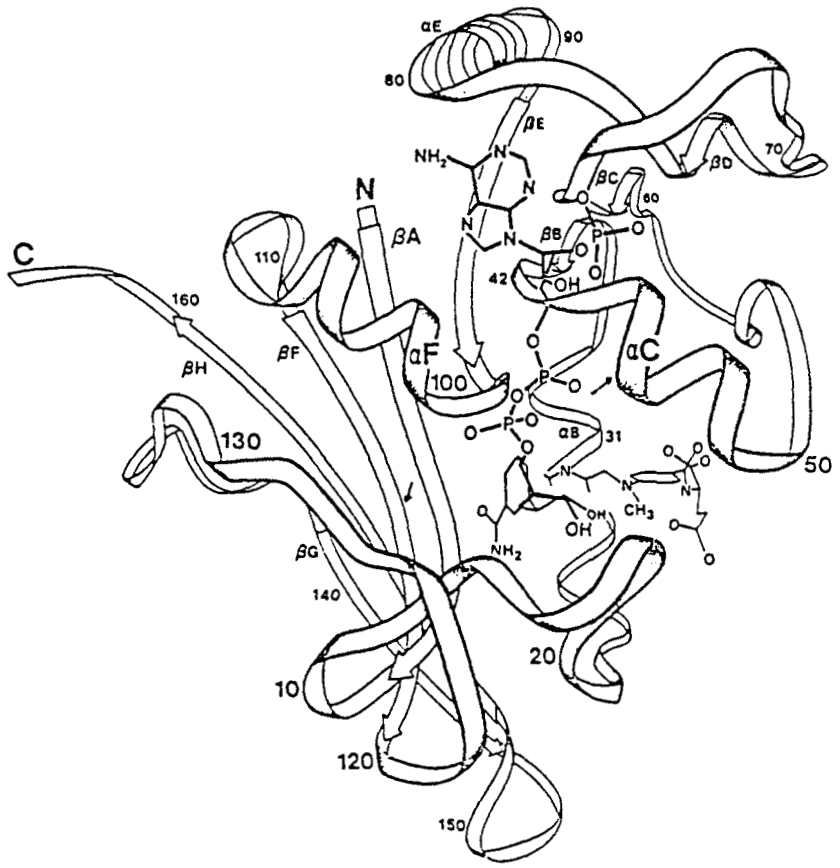

Fig. 1. The general features of the *Lactobacillus casei*-NADPH-methotrexate ternary complex structure [2] with the mutation sites indicated by the arrows.

binding and/or catalysis elucidated. Thr-113 is a strictly conserved residue at the H_2-folate binding site, interacting with Asp-27 via one hydrogen bond and with methotrexate (and by analogy, H_2-folate) indirectly through water 405. Asp-27 is also conserved and involved in catalysis [3], suggesting that Thr-113 could be required for proton transfer to the N5-C6 imine undergoing reduction; alternatively, it might only be required for substrate binding. Again, removing the hydrogen bond by replacement with Val allows these hypotheses to be tested.

These particular mutations also address the more general question of the relationship between binding energy and catalysis. It has been proposed that intrinsic binding energy from interactions between an enzyme and substrate are expressed maximally in the transition state to provide catalysis [4]. It is possible to test this hypothesis using the mutant enzymes by measuring the effect of single-site amino acid replacements on catalysis and binding.

MATERIALS AND METHODS

Plasmid pTY1 (a construction of a derivative of pBR322 with the fol gene cloned into the BamHI site [5,6]) was grown in *Escherichia coli* strain HB101 and

was purified by standard procedures. Mismatches were created essentially by the method of Dalbadie-McFarland et al [7] using the unique EcoRI site in the fol gene to generate nicked plasmid DNA. Oligonucleotides were synthesized on an Applied Biosystems synthesizer and were generously provided by Wojciech J. Stec (Polish Academy of Science, Boczna 5, Poland). Mutants were identified after transformation either by colony hybridization [8] or by direct observation of a new restriction fragment. The mutation was confirmed by sequencing the fol gene.

Mutant dihydrofolate reductases were purified by methotrexate affinity chromatography [9]. The Val-113 mutant was observed to bind slightly less tightly to the resin than the wild-type enzyme (that is, it could be eluted at a rate about five times faster than wild-type without 1 mM folate in the elution buffer), but this difference in binding was not sufficient to be useful in purification.

The enzymes were characterized by stopped-flow fluorescence quenching at 340 nm using the method of Cayley et al [10] with equipment constructed in the laboratory of K.A. Johnson [11]. Nuclear magnetic resonance (NMR) spectroscopy experiments were based on the technique of Poe et al [12]. Kinetic parameters as a function of pH and binding constants for methotrexate were determined under conditions similar to those used by Stone and Morrison [13] using the same buffer system (25 mM Tris, 50 mM Mes, 25 mM ethanolamine, 0.1 M sodium chloride—TME buffer). Acetylpyridine adenine dinucleotide phosphate (APADPH) was synthesized as previously described [13].

RESULTS AND DISCUSSION

Several criteria were used to establish that no gross changes in conformation had occurred as a result of the amino acid replacement. These included (1) the equilibrium distribution between two principal conformational states of the enzyme as established by Cayley et al [10], (2) the chemical shifts of the five histidine C-2 protons as reflective of their environment [12], and (3) the effect of mutagenesis on binding of the substrate that is remote from the amino acid change.

Data for the binding of NADPH to the wild-type DHFR is biphasic and has been described by the following Scheme:

$$E_1 + \text{NADPH} \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} E_1{\cdot}\text{NADPH}$$

$$\downarrow\uparrow k_2$$

$$E_2$$

The equilibrium concentration of the conformers E_1 and E_2 sets the relative amplitude of the two phases where k_2 is the rate of conversion of $E_2 \rightarrow E_1$ as measured by the decrease in fluorescence at 340 nm of the slow phase. The values for the wild-type, Gln-45, and Val-113 mutants are listed in Table I. The only departure for the parameters associated with the wild-type enzyme is the twofold increase in the concentration of E_2 for the Val-113 mutant.

TABLE I. Effect of Mutations on DHFR Conformation*

	Rel Amp	k_2 (s^{-1})
His-45, Thr-113	1.2 : 1	0.030
Gln-45	1.1 : 1	0.027
Val-113	1 : 2.5	0.035

*The relative amplitude and k_2 values were determined according to Scheme 1. Conditions were: 0.46–2 μM DHFR, 10–100 μM NADPH, TME buffer (pH 7.0), 25°C, 340 nm.

TABLE II. Effect of the Mutation on Binding of the Substrate Remote From the Amino Acid Change*

	K_M (DHF) (μM)	k_{on} (NADPH) (M^{-1} s^{-1})
His-45, Thr-113	1	1.6×10^7
Gln-45	1	—
Val-113	—	2×10^7

*The value of k_{on} for NADPH was determined from the NADPH dependence for the rate of the fast phase under conditions identical to those in Table I. The K_M parameter for H_2-folate was determined in TME buffer, pH 7.0, 25°C, at saturating 100 μM NADPH and variable 4–30 nM DHFR and 0.8–12 μM H_2-folate levels.

Further evidence that the amino acid replacement has a restricted, local effect on conformation is given in Table II. Column 1 lists the Michaelis constant for H_2-folate at saturating NADPH. Column 2 shows the rate constant for NADPH binding (k_{on}) measured from the NADPH dependence of the rate of the fluorescence change for the initial phase (Scheme I). No significant difference was found for the three proteins.

Finally, the [^{1}H]NMR spectra of the wild-type and Gln-45 mutant were obtained in the region of the histidine resonances (Fig. 2) . All five histidines in the wild-type enzyme can be identified; a single resonance at 7.79 is absent in the Gln-45 mutant. The close correspondence in the chemical shifts of the C-2 proton of these histidines is consistent with no major change in their environments. Collectively, the data support our conclusion that the major structural elements of the wild-type and mutant enzymes are the same.

Gln-45 Mutant

Figure 3 shows the pH profile of k_{on} and k_{off} for NADPH binding to the wild-type and Gln-45 enzymes. The decrease in k_{on} below pH 7.0 for both enzymes may be partially explained by the protonation of the 2′-phosphate group in the bulk solution, removing some electrostatic attraction between the ligand and the binding site. The pK_a value for the 2′-phosphate group of the coenzyme in free solution is 6.1, and the charge state of the 2′-phosphate group is important in the equilibrium binding of NADPH and NADP [14].

Since estimates of the pK_a for His-45 within the binary complex range from pH 5.3 to pH 6.4 [12], the state of protonation of the imidazole moiety does not appear

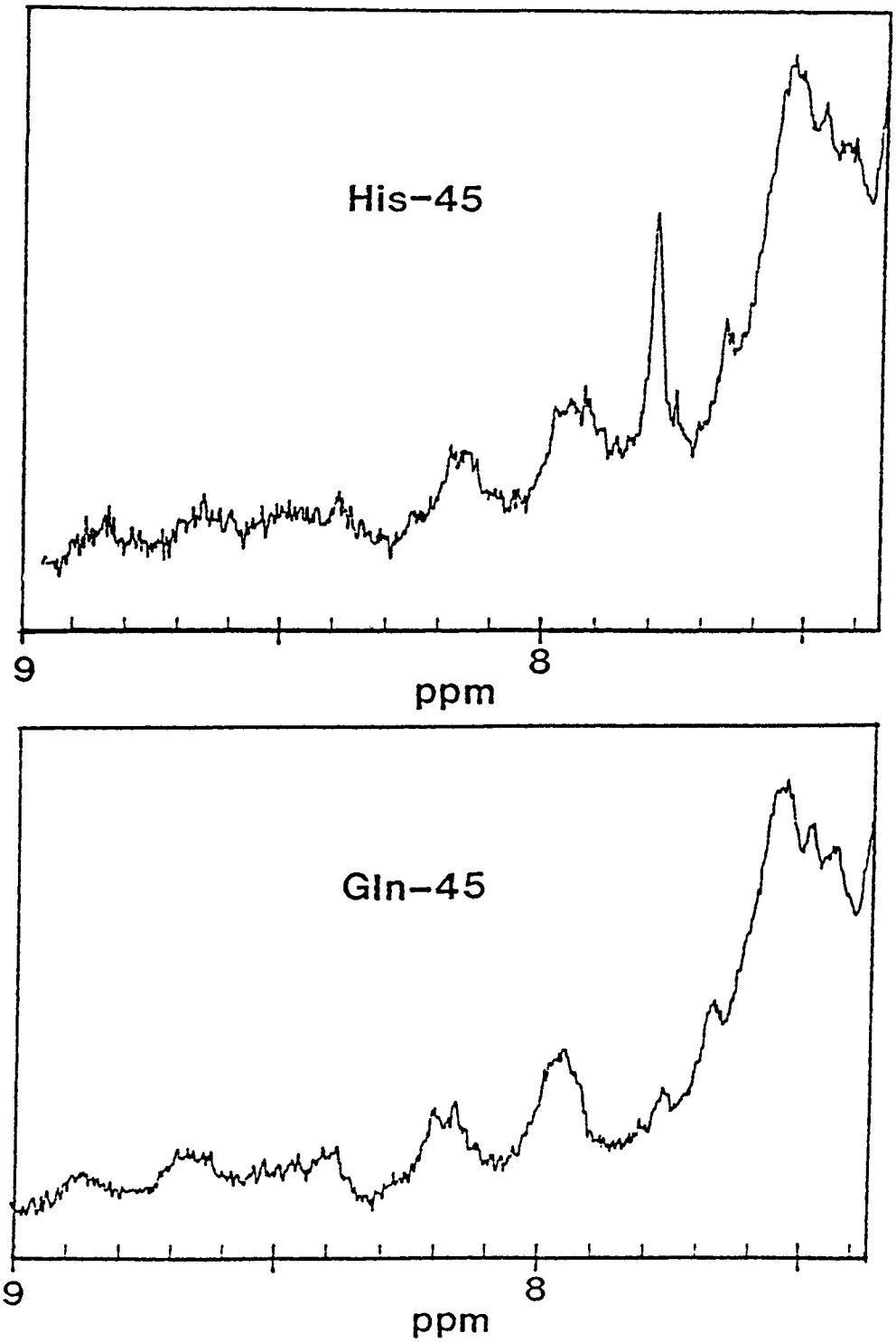

Fig. 2. The lowest field portions of 360 MHz [^{1}H]NMR spectra of *Escherichia coli* DHFR are depicted for His-45 (wild-type enzyme) and Gln-45 (mutant enzyme). The conditions used were as follows: 0.4–0.5 mM DHFR in 0.05 M sodium chloride, 1 mM dithiothreitol, and 0.05 M Tris, pH 8.0, 12°C. The ^{1}H chemical shifts are expressed relative to 4,4-dimethyl-4-silapentane sulfonate.

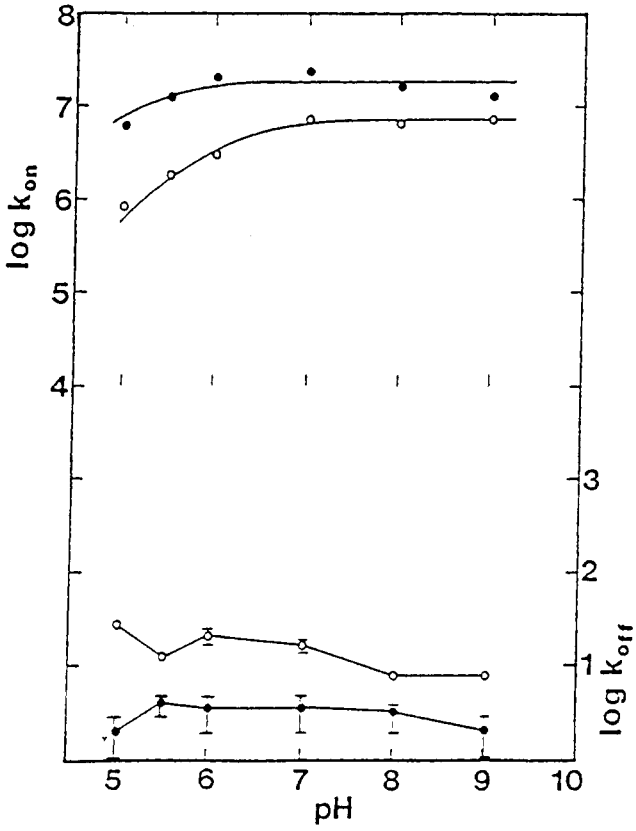

Fig. 3. The dependence of k_{on} ($M^{-1}S^{-1}$) and k_{off} (S^{-1}) on pH for NADPH binding to His-45 DHFR (●) and Gln-45 DHFR (○) measured under the conditions of Table I.

to alter k_{on} markedly. Likewise, there is no apparent pH dependence on k_{off} for the wild-type enzyme, suggesting that the equilibria for binding NADPH is insensitive to the protonation state of His-45.

The ionic contribution of His-45 to binding within the binary complex might be manifest in the His-45 to Gln mutation. However, values of either k_{on} or k_{off} do not reflect any unexpected pH dependence, particularly between pH 5 and pH 6, but are generally threefold and three- to tenfold different than wild-type for k_{on} and k_{off}, respectively. Since there are subtle changes in both rate constants, the value of K_D for NADPH dissociation from Gln-45 is sixfold (pH > 7) and 40-fold (pH < 7) greater than for wild-type enzyme. Thus, a salt bridge derived from the His-45 within the binary complex does not possess binding properties superior to that presumed for a hydrogen bond; but overall, either interaction is stronger than that with Gln-45. The ineffectiveness of this salt bridge in binding is consistent with the His-45 being exposed to water as suggested by the crystal structure [1,2].

It was of considerable interest to determine whether the weaker binding of the nicotinamide cofactor was reflected in the steady state kinetic parameters for the mutant enzyme. Values for these terms at the pH extremes are shown in Table III for saturating H_2-folate and varying the coenzyme analog, APADPH. This cofactor was

selected on the basis that it purportedly gave rise to a rapid equilibrium random sequence [13,15]. From Table III, there is, at most, a twofold change in V, but a fourfold decrease in V/K for the mutant enzyme. This decrease can be ascribed mainly to an increase in K_{APADPH}, paralleling the increase in K_D for NADPH. It is possible that the smaller effect of this mutation on K_M compared to K_D may reflect a difference in the binding of the cofactor to free enzyme and the enzyme-H_2-folate binary complex.

Val-113

The Val-113 mutant DHFR was characterized primarily by its kinetic parameters. A typical set of data (pH 7.0) and the method of graphical analysis for obtaining V and K_{DHF} are shown in Figure 4. The nonzero intercept in the Michaelis-Menten plot and the distinct break in the reciprocal plot are a result of contaminating wild-type enzyme, presumably from the *Escherichia coli* chromosomal gene. The concentration of this enzyme is 2% of the total, calculated from the reciprocal plot by the method of Spears et al [16], which is consistent with this enzyme being a product of the chromosomal gene. The values for V and K_M for the Val-113 mutant are summarized in Table IV. For these relative velocities and K_M's, the graphical analysis provides results accurate to 5% for the high K_M enzyme [16].

The V for the reaction is comparable to that of wild-type enzyme, at pH 7.0. The K_M for H_2-folate, on the other hand, is 25-fold larger than that for the Val-113 mutant. This increase in K_M is most likely an effect on substrate binding in analogy with the effect of this mutation on the methotrexate binding as discussed below. Cayley et al [10] report a value for the K_D for methotrexate of $< 10^{-8}$ M, which is consistent with fluorescence titration data that yield a maximum value for K_D of 10^{-8} M (data not shown). Fluorescence titration of the Val-113 mutant yields a K_D value of 0.3 ± 0.1 μM, a 20–30-fold increase in the equilibrium constant. The value of k_{on} for the Val-113 mutant, determined by stopped-flow fluorescence quenching, was found to be $3 \pm 1 \times 10^6$ M^{-1} s^{-1}, in very good agreement with the rate constant

TABLE III. Kinetic Constants for the Gln-45 Mutant Under Conditions of Varying Acetylpyridine Adenine Dinucleotide Phosphate (APADPH)*

	V (s^{-1})	V/K_{APADPH} (M^{-1} s^{-1})	K_{APADPH} (μM)
pH 5.0			
His-45	3.5	27.2×10^5	1.3
Gln-45	2.1	5.9×10^5	3.5
pH 9.0			
His-45	1.2	1.2×10^5	10
Gln-45	1.2	0.3×10^5	40
pK_a			
His-45	8.8	7.6	
Gln-45	9.0	7.5	

*Constants are reported for the pH extremes measured as well as the pK_a's describing the pH dependence of the V and V/K values. The conditions were: TME buffer, 25°C, 3–17 nM DHFR, 0.8–60 μM APADPH, and 25 μM H_2-folate. The pK_a values were determined as in Stone and Morrison [13].

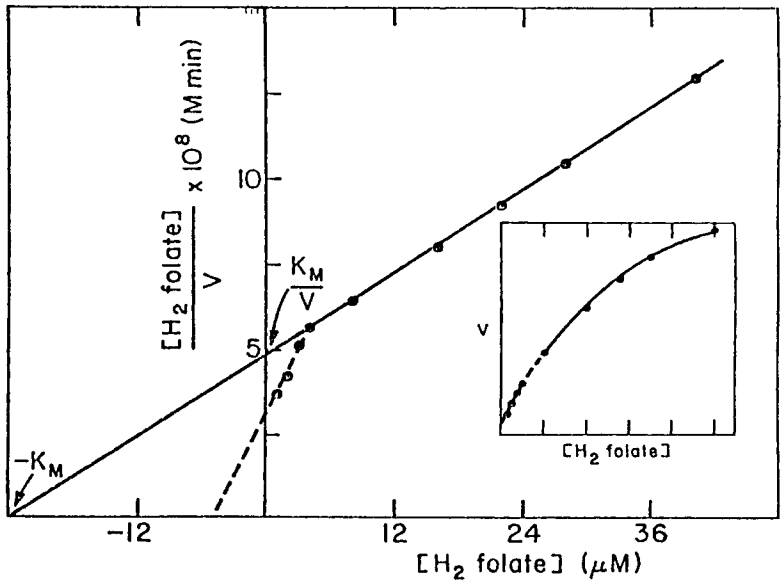

Fig. 4. An example of the reciprocal plot used to obtain the Michaelis-Menten parameters for the Val-113 mutant (pH 7.0) with values for the kinetic constants indicated. The inset shows the Michaelis-Menten plot as described in the text. The conditions are as indicated in Table IV.

TABLE IV. Kinetic Constants for the Val-113 Mutant Under Conditions of Varying H_2-Folate*

	V (s^{-1})	V/K_{DHF} ($M^{-1}\,s^{-1}$)	K_{DHF} (μM)
Thr-113	17.0	2.0×10^7	1.0
Val-113	14.0	5.6×10^5	25

*These kinetic parameters were determined using TME buffer, 25°C, 9–30 nM DHFR, 100 μM NADPH, and 1–50 μM H_2-folate.

found for the wild-type [10]. Thus, the entire increase in K_D must be a result of a faster off rate.

The pH dependence for both V and V/K from pH 5.0 to pH 9.5 reveals pK_a's 0.4 pH units lower than the native enzyme [13] (Fig. 5). One simple interpretation is that the hydrogen bonding interaction between Thr-113 and Asp-27 has been removed, thereby destabilizing the acid form of Asp-27. Alternatively, the water molecule originally hydrogen bonded to Thr-113 may in its absence directly solvate Asp-27, provided the water is still there. The loss of a single hydrogen bond (2–3 kcal/mol) also can account for the effect on K_M for H_2-folate.

The analysis of the kinetic data for the two mutants also impacts on the kinetic sequence ascribed to DHFR. The fact that the pK_a observed (Table III) in the V-pH profile when H_2-folate is saturating and APADPH is varied differs (8.8 vs 7.9 [13]) from that observed when APADPH is saturating and is not consistent with a random, equilibrium model at all pH values, even for this cofactor. Secondly, the finding that V is certainly not increased by amino acid changes, which increase the dissociation

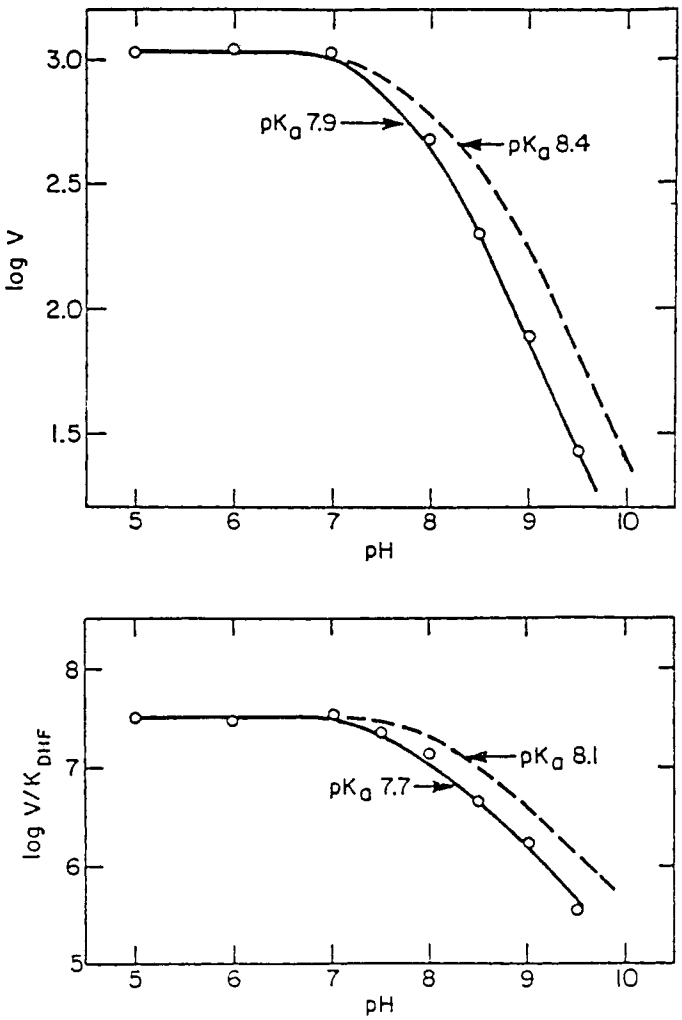

Fig. 5. The pH dependence of V (min^{-1}) and V/K ($M^{-1}S^{-1}$) for the Val-113 (○), where the solid lines are the theoretical curve for the pK_a value indicated as determined in ref [13]. The dashed lines are the theoretical curves expected if the pK_a were the same as that of the wild-type enzyme [13] and were calculated using the pH-independent values (V, V/K) obtained for the Val-113 mutant. The conditions are as indicated in Table IV.

rate for substrate or cofactor, suggests that the loss of the product forms of these reactants does not limit the rate of turnover. This deduction presumes that the behavior of the product species is similar.

Finally, we note that these two mutations have affected the binding properties of the enzyme with respect to both cofactor and substrate. The decrease in binding, however, has not been translated into a loss of catalytic efficiency—V is relatively unchanged. Thr-113, which is totally conserved in all species, may be required for maintaining a low K_M value. In short, these are active site residues whose interaction

with substrate species is limited simply to binding but is not involved in their processing.

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