Functional Role of Aspartic Acid–27 in Dihydrofolate Reductase Revealed by Mutagenesis

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The crystal structures and enzymic properties of two mutant dihydrofolate reductases (*Escherichia coli*) were studied in order to clarify the functional role of an invariant carboxylic acid (aspartic acid at position 27) at the substrate binding site. One mutation, constructed by oligonucleotide-directed mutagenesis, replaces Asp^{27} with asparagine; the other is a primary-site revertant to Ser^{27}. The only structural perturbations involve two internally bound water molecules. Both mutants have low but readily measurable activity, which increases rapidly with decreasing *p*H. The mutant enzymes were also characterized with respect to relative folate: dihydrofolate activities and kinetic deuterium isotope effects. It is concluded that Asp^{27} participates in protonation of the substrate but not in electrostatic stabilization of a positively charged, protonated transition state.

DIRECTED MUTAGENESIS IS AN INCREASINGLY POPULAR technique for examining the relation between molecular structure and functional properties in enzymes (*1–5*). We have described (*2*) the construction of three mutations derived from the cloned wild-type *Escherichia coli* dihydrofolate reductase gene (*fol*) and presented the results of our initial solution studies on the mutant enzymes. One of the mutants, $Asp^{27} \rightarrow$ Asn, had about 1/300th the activity of wild-type dihydrofolate reductase (DHFR) at neutral *p*H, demonstrating that Asp^{27} must serve an important catalytic function. What that function may be, however, remains to be determined.

We now present the results of detailed solution and crystallographic studies of the $Asp^{27} \rightarrow$ Asn mutant, and of a new mutant, $Asp^{27} \rightarrow$ Ser. The most important conclusion concerning the mechanism of the enzyme's activity is that the Asp^{27} side chain participates in proton transfer between solution and substrate, but does not contribute to catalysis by electrostatic stabilization. If we assume that DHFR is representative, a further finding is that enzyme molecules appear to accept localized alterations in their active sites without propagating distortions to other parts of the structure. This is true even when a hole is created by substituting a smaller side chain for a larger one as in the $Asp^{27} \rightarrow$ Ser mutation, where the hole is simply occupied by a water molecule. Our present results support the view that directed mutagenesis can be a powerful technique for understanding enzymes, but they underscore the importance of following through with intensive studies on the newly created enzyme species if the full potential of the method is to be realized.

Production of mutant DHFR's. The Asn^{27} mutant DHFR gene was generated by oligonucleotide-directed mutagenesis of the cloned wild-type *E. coli* gene. The Ser^{27} mutant gene was obtained as a primary-site revertant of the Asn^{27} gene in which the spontaneous transition AAC (Asn) to AGC (Ser) had occurred. The Ser^{27} mutant gene was genetically selected for its ability to confer increased resistance to the DHFR inhibitor trimethoprim (TMP) on an *E. coli* host when compared to the "parent" Asn^{27} DHFR gene. The mutant enzymes were expressed and purified as described (*2*).

X-ray structures. Our principal objective in applying mutagenesis techniques to enzymology is to examine the effect of exactly defined structural changes on molecular properties. Obviously, however, merely specifying the replacement of one amino acid residue by another does not adequately define the geometry of the new molecule. Although previous structural determinations of hemoglobin and T4 lysozyme variants suggest that extensive structural perturbations do not result from single-residue substitutions (*6–8*), it is not yet known how tolerant the typical enzyme molecular structure may be to this kind of tampering when the mutation involves a catalytic residue. In fact, the available examples of naturally occurring protein variants probably give a somewhat biased view because they are all mutants that have survived some sort of selection for their ability to fold and function more or less normally.

We have therefore crystallized and determined the x-ray structures of both the Asn^{27} and the Ser^{27} mutant DHFR's as the binary complexes with the inhibitor methotrexate (MTX). The crystal structures of the mutant enzymes are isomorphous with each other and with that of the wild type; all three have been refined at 1.9 Å resolution for detailed comparison. These structures provide a firm basis for interpreting the properties of the new DHFR molecules, as described below.

The electron-density difference map of Asn^{27} DHFR-MTX *minus* wild-type DHFR-MTX was nearly featureless except in the vicinity of two fixed water molecules close to the MTX binding site. The pteridine ring of MTX and some surrounding enzyme groups and fixed water molecules in the refined wild-type DHFR structure are shown in Fig. 1 (solid lines). Refinement of the Asn^{27} structure showed that the largest changes from the wild type were a shift in position of fixed water molecule Wat^{403} by 0.9 Å and of Wat^{567} by

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1.4 Å toward the surface of the molecule (toward the observer in Fig. 1). In the wild-type enzyme Wat^{403} is hydrogen bonded to OD2 of Asp^{27}, which, on the basis of hydrogen bonding considerations (see below), must be replaced by the side chain amido group of Asn^{27} in the mutant. The resulting alteration in the hydrogen bonding network involving Wat^{403} could well account for its 0.9 Å movement. In its new position, Wat^{403} is also firmly hydrogen bonded (2.8 Å) to a second fixed water molecule (not shown in Fig. 1) to which it had been more weakly bonded in the wild-type enzyme (3.2 Å). The second water molecule, however, is unperturbed by the mutation, remaining hydrogen bonded to an adjacent segment of backbone chain. Wat^{567}, in turn, probably moves in order to maintain its interaction with Wat^{403}. Only minor structural perturbations, corresponding to shifts in position of 0.2 Å or less, were observed elsewhere in the molecule. Thus neither the MTX binding geometry nor the detailed three-dimensional topography of the DHFR molecule were altered by the mutation.

The difference map for Ser^{27} DHFR-MTX *minus* wild-type DHFR-MTX was somewhat more complicated. In this case the distribution of negative and positive difference density indicated that the gamma oxygen (OG) of Ser^{27} now occupies a position just below where the delta oxygen (OD1) of Asp^{27} had been in the wild-type DHFR, and that a new water molecule, Wat^{885}, takes the place of OD2. In other words, Asp^{27} has actually been replaced by a serine hydrate. Again, the geometry of the bound MTX molecule is unchanged. Comparison of refined coordinates for wild-type DHFR and the Ser^{27} mutant reveals no other coordinate changes greater than 0.2 Å in MTX or in any backbone or side chain atoms.

Figure 1 shows the exact positioning of the Ser^{27} side chain and Wat^{885} in the mutant structure (broken lines) superimposed on the wild-type structure (full lines). Both the 2.7 Å distance between OG of Ser^{27} and Wat^{885} and the local geometry suggest that they are hydrogen-bonded to one another. Wat^{885} is 2 Å from the site occupied by Wat^{403} in the wild-type enzyme; however, in the Ser^{27} mutant the Wat^{403} site is vacant, and instead the electron density for Wat^{885} is elongated in the direction of the Wat^{403} site. Another peculiar feature of the mutant structure is that Ser^{27} OG is 4.0 Å from the 2-amino group (NA2) of MTX, much too far for normal hydrogen bonding. In fact, a van der Waals surface display shows an empty gap of about 1 Å between MTX and the surrounding enzyme at this location.

Binding of methotrexate. Methotrexate is an extremely strong inhibitor of almost all chromosomally encoded DHFR's. It is a close structural analog of folate, differing only in that a 4-amino group in MTX replaces the 4-oxo group of folate, and in that the nitrogen at position 10 (N-10) of MTX carries a methyl substituent. From the binding properties of a large number of DHFR inhibitors, it is evident that a 4-amino substituted pteridine or other heterocyclic ring is important for strong binding, but that the methyl substitution at N-10 is not (*9*). However, despite this seemingly slight difference in molecular structure between MTX and folate or dihydrofolate, MTX binds several orders of magnitude more strongly to all species of DHFR: the K_D (dissociation constant) for MTX is typically in the range 10^{-9} to $10^{-12}M$ as compared with a K_D of about 10^{-5} to $10^{-7}M$ for folate or 10^{-6} to $10^{-7}M$ for dihydrofolate (see table 5.2 in *10*) (*10, 11*). For this among other reasons, the nature of the interaction between DHFR and MTX has attracted considerable interest.

A great deal is already known about this interaction (*12*). The crystal structures of two bacterial DHFR-MTX complexes reveal that the side chain of a buried aspartic acid residue (Asp^{27} in the *E. coli* enzyme) closely approaches the pteridine ring of bound MTX, forming a pair of hydrogen bonds with N-1 and the 2-amino group (*13–16*). An analogous interaction is seen in the crystal structure of chicken DHFR where the carboxylate side chain of Glu^{30} hydrogen bonds to the substituted heterocycles of a variety of bound inhibitors (*17, 18*). It is also known that the pteridine ring of MTX is flipped over in the substrate binding site, as compared with the orientation of the pteridine ring of the substrate itself (*19–21*), and that DHFR-bound MTX is protonated at N-1 whereas bound substrate is not protonated (*22–26*). Moreover, the proton affinity of N-1 is greatly enhanced by interaction with the enzyme, as evidenced by the observation that enzyme-bound MTX exhibits a pK_a of >10, to be compared with a pK_a of 5.73 in solution (*22, 23*). It has thus been inferred that the enhanced binding of MTX and other 2,4-diamino heterocyclic inhibitors is due in large part to a favorable ionic interaction between the carboxylate side chain of Asp^{27} and a protonated, positively charged N-1, and this view has been supported by theoretical considerations (*27*).

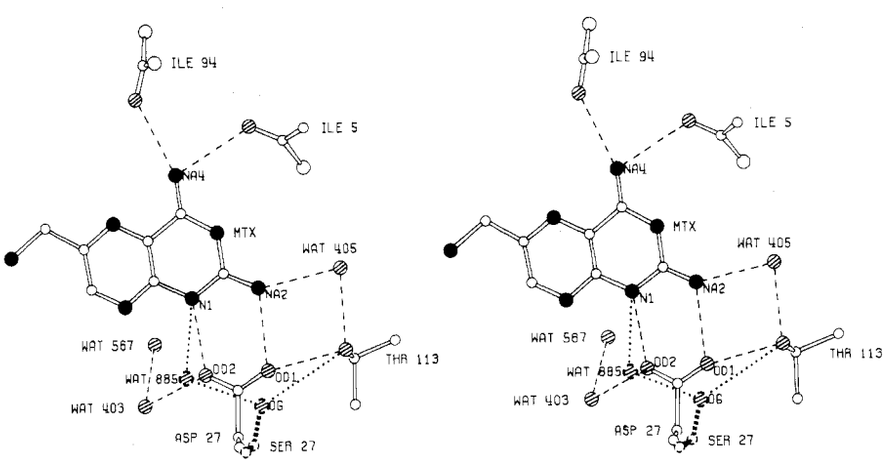

Fig. 1. Stereopair comparing neighborhood of Asp^{27} and MTX in wild-type DHFR (solid lines) and in the Ser^{27} mutant DHFR (broken lines). Dashed and dotted single lines represent hydrogen bonds. Filled spheres are nitrogen atoms, striped spheres are oxygen atoms, and open spheres are carbon atoms. Wild-type and both mutant DHFR's in the binary complex with MTX were crystallized by the same procedure as was used for *E. coli* MB1428 DHFR and are isomorphous to within 0.5 percent with crystals of the latter (*13–15*). The *E. coli* MB1428 DHFR differs from the wild type by having a lysine at position 154 instead of glutamic acid. Diffraction data to 1.9 Å resolution were collected (Xuong-Hamlin multiwire-area-detector diffractometer) (*43*). Final R_{sym} values were less than 0.06. A model of the wild-type enzyme was obtained by substitution of a lysine residue for Glu^{154} in the MB1428 structure; 11 cycles of restrained-parameter least-squares refinement (*44*) were performed to give a model with an R value of 0.163 for the 30295 reflections to 1.9 Å. The root-mean-square (rms) deviation of bond lengths in the final model from their dictionary values is 0.013 Å. Difference electron density maps for the Asn^{27} and Ser^{27} mutants (mut) were computed with coefficients $(F_{mut} - F_{wt})e^{i\alpha}$ where α is the phase computed for the refined wild-type (wt) structure. The maps were displayed and interpreted on an Evans and Sutherland Picture System II and a PS300 graphics system. Models of the mutant DHFR's were each subjected to ten cycles of restrained-parameter least-squares refinement, resulting in improved coordinates which gave R values of 0.167 and 0.166 for the Asn^{27} and Ser^{27} mutants, respectively. The rms deviations in bond lengths were 0.012 Å in both cases.

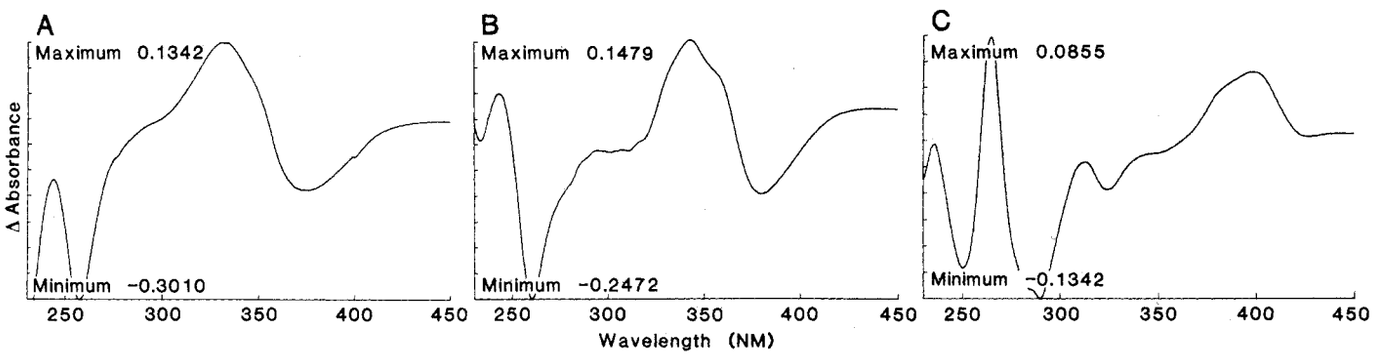

Fig. 2. Methotrexate difference spectra. (A) Free MTX in solution, *p*H 1.8 versus *p*H 7.0; (B) MTX bound to wild-type DHFR versus free enzyme plus free MTX in solution, *p*H 7.0; (C) MTX bound to Asn^{27} DHFR versus free enzyme plus free MTX in solution, *p*H 7.0. Difference spectra were obtained at 30°C in a buffer consisting of 50 m*M* KH_2PO_4 plus 1 m*M* EDTA, *p*H 7.0. Enzyme concentrations were 4 to 8 μ*M*, MTX concentration was 24 μ*M*. Difference spectra were generated by subtracting spectra of unmixed solutions from spectra of mixed solutions.

We chose initially to characterize the MTX-mutant enzyme interactions by monitoring the protonation state of bound MTX by difference spectroscopy (*24, 25*). The protonation difference spectrum of free MTX and the difference spectrum observed upon binding of MTX to wild-type DHFR are shown in Fig. 2, A and B. The two are obviously similar, which has previously been interpreted as indicating that MTX is protonated when bound to the wild-type enzyme (*24, 25*). In contrast, the difference spectrum obtained when MTX binds to the Asn^{27} mutant enzyme (Fig. 2C) bears no resemblance to the MTX protonation difference spectrum, indicating that MTX is not protonated in this environment (*28*). A similar result was also obtained for the difference spectrum of Ser^{27} mutant plus MTX. Thus, MTX is unprotonated when bound to either the Asn^{27} or Ser^{27} mutant even though the conformation of MTX is the same as when bound to wild-type DHFR.

How is the binding affinity of DHFR for MTX affected by these two mutations? A K_D value for the binding of MTX to the Ser^{27} mutant DHFR was obtained by fluorescence quenching techniques (*29, 30*); however, it was necessary to use equilibrium dialysis with ^3H-labeled MTX (*31, 32*) in the cases of both the wild-type enzyme and the Asn^{27} mutant because the inhibitor is much more tightly bound to the wild type and the Asn^{27} mutant. The K_D values at *p*H 7.0 and 4°C were 0.07 n*M* for the wild type; 1.9 n*M* for the Asn^{27} mutant; and 210 n*M* for the Ser^{27} mutant. The results to note are that, in comparison with the wild-type enzyme, the MTX dissociation constant of the Asn^{27} mutant has increased by only a factor of 27 (corresponding to a decrease in binding energy of 1.8 kcal mol^{-1}) whereas that of the Ser^{27} mutant has increased by a factor of 3000 (a binding energy decrease of 4.4 kcal mol^{-1}).

Returning to our x-ray structural results, and taking into account that MTX is protonated when bound to the wild-type enzyme but unprotonated when bound to either mutant, we can now draw some plausible inferences about the hydrogen-bonded interactions involved and attempt to explain the relative binding energies of our three DHFR variants. These inferences are based on the assumption that all interactions between protein and ligand are unchanged except at the site of the amino acid substitution. This assumption is supported by the x-ray structures. The most likely hydrogen bondings are shown in Fig. 3, A to C. The point to be emphasized is how little difference there is between the wild-type enzyme and the Asn^{27} mutant (Fig. 3, A and B); an ionic interaction has been abolished and an NH–O hydrogen bond has been replaced by an NH–N hydrogen bond. If we can assume, from the ab initio calculations by Dill *et al.* (*33*), that the energy difference between these two kinds of electrically neutral hydrogen bonds is slight, an immediate but surprising conclusion is that elimination of the ionic interaction between protonated MTX and Asp^{27} sacrifices only about 1.8 kcal per mol of binding energy.

An indirect result obtained by Stone and Morrison (*34*) supports this conclusion. On the basis of their overall profiles of K_D plotted as a function of *p*H for MTX bound to wild-type *E. coli* DHFR, they calculate K_D = 0.011 n*M* for the interaction between protonated MTX and ionized (presumably Asp^{27}) DHFR, and K_D = 0.12 n*M* for the interaction between unprotonated MTX and the nonionized

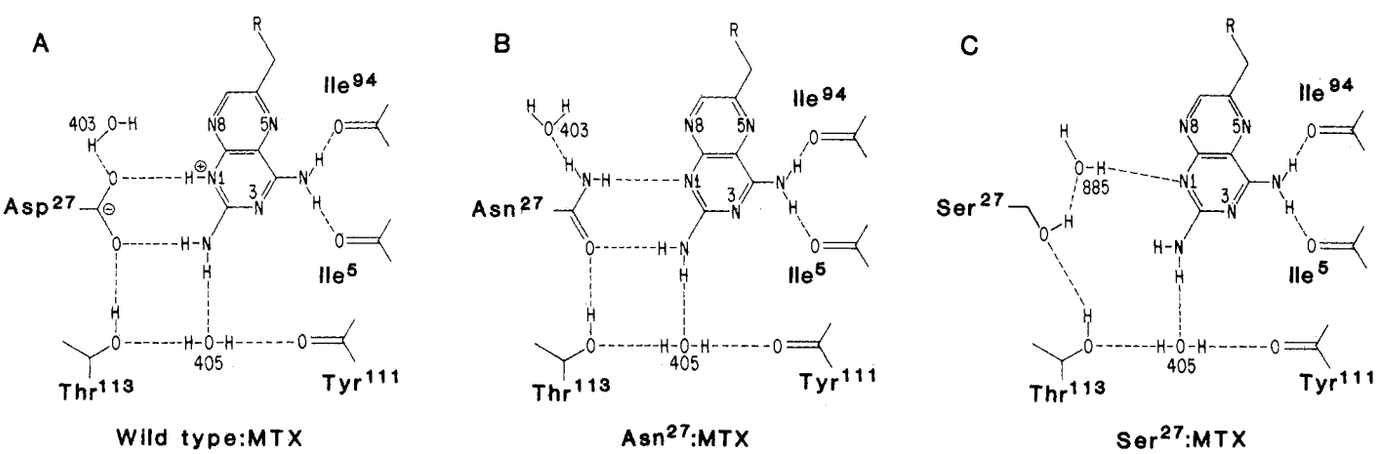

Fig. 3. Probable hydrogen bondings between MTX and (A) wild-type DHFR; (B) Asn^{27} mutant; (C) Ser^{27} mutant.

enzyme. Thus the results of Stone and Morrison indicate a factor of 11 due to the ionic interaction, roughly in agreement with our directly measured factor of 27.

The much greater decrease in binding energy for the Ser^{27} mutant is also understandable in structural terms, although the structural perturbations caused by the mutation are more complicated. The probable arrangement of hydrogen bonds is shown in Fig. 3C. As mentioned earlier, we note three important differences between the wild-type and Ser^{27} enzymes with respect to MTX binding. One is the loss of an ionic interaction between N-1 and the side chain of Asp^{27}. A second is the loss of a hydrogen bond with the 2-amino group. And the third (not obvious in Fig. 3C) is the van der Waals gap that is left between the 2-amino group and the surrounding enzyme. Taken together, it is reasonable to suppose that these differences would account for a 4.4 kcal mol^{-1} decrease in binding energy, as observed. If we allow 1.8 kcal mol^{-1} for the ionic interaction, as deduced from the Asn^{27} mutant, an additional 2.6 kcal mol^{-1} may be explained by the lost hydrogen bond and the van der Waals gap.

If the ionic interaction between Asp^{27} and protonated MTX is only worth 1.8 kcal mol^{-1} or a factor of about 27 in the K_D, how then can we account for the fact that K_D for MTX bound to the wild-type enzyme is less than $10^{-11}M$ while K_D for folate or dihydrofolate is around $10^{-6}M$? The answer may lie in formation of an extra pair of hydrogen bonds between the 4-amino group of MTX and the buried backbone carbonyls of Ile^{94} and Ile^{5}. These two carbonyls are not otherwise hydrogen-bonded to any part of the enzyme structure. Certainly they were not designed through evolution to bind MTX; however, they could play some as yet undefined role in the catalytic mechanism.

Possible functions of Asp^{27}. DHFR catalyzes the reduction of dihydrofolate at the N-5–C-6 imine bond in a net reaction that amounts to transfer of a hydride ion from the dihydronicotinamide ring of NADPH plus a proton from solution. Since C-6 is the hydride acceptor, and since protonation at N-5 would enhance carbonium ion character at C-6, the generally accepted and most plausible view is that protonation at N-5 precedes hydride transfer (*35*). Two relevant fundamental questions are: (i) what is the path of the proton from solution onto N-5?; and (ii) how does the enzyme stabilize the presumed positively charged, protonated transition state?

The obvious candidate for involvement in both of these functions, given its evolutionarily invariant presence at the substrate binding site, is the side chain carboxyl of Asp^{27} (*13, 36*). One would thus expect to find this group interacting with N-5 or at least close to it. However, in our current model for productive binding of substrate to DHFR (*14*) the Asp^{27} side chain is hydrogen-bonded to the 2-amino group and to N-3 of the substrate but is more than 5 Å from N-5. Nevertheless, it is reasonable to suppose that (i) the Asp^{27} side chain is implicated in transfer of a proton from solution onto some group of the substrate's dihydropteridine ring, perhaps via a structural water molecule (Wat^{403}); and (ii) the Asp^{27} side chain, in the ionized state, contributes to transition state stabilization by ionic interaction with the protonated, positively charged dihydropteridine ring. In fact, as related below, the results of our kinetic studies on the Asn^{27} and Ser^{27} mutants suggest that supposition (i) is correct but supposition (ii) is not.

An analysis (*11*) of the kinetic behavior of DHFR reveals that the reaction approximately conforms to a random, rapid equilibrium mechanism. A priori, either hydride transfer or proton transfer or product release could in principle be rate limiting. But, since no kinetic isotope effect is observed with deuterated cofactor (*11, 37*), hydride transfer can be ruled out as the slow step for the wild-type enzyme. It has been suggested (*37*) that the slow step is a conformational isomerization associated with protonation of N-5. That product release is at least partially rate limiting appears unlikely from the study of two mutant DHFR's ($Thr^{113} \rightarrow Val$ and $His^{45} \rightarrow Gln$) by Chen *et al.* (*38*). These mutations decrease binding of dihydrofolate by 25-fold and that of NADPH (nicotinamide adenine dinucleotide phosphate, reduced form) by 3-fold respectively, but do not affect k_{cat} (observed first-order rate constant for conversion of Michaelis complex to products). On the assumption that binding of product is similarly decreased, Chen *et al.* (*38*) conclude that product release is unlikely to be the rate-determining step in the wild-type mechanism.

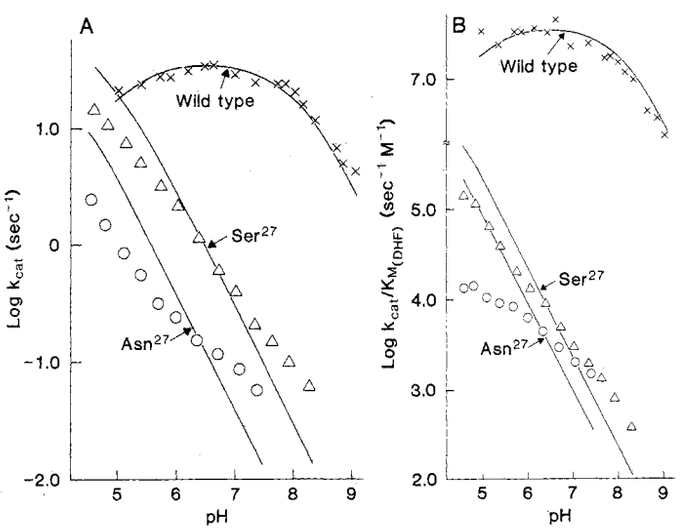

Fig. 4. *p*H profiles of (A) log k_{cat} and (B) log k_{cat}/K_m (dihydrofolate) for wild-type DHFR (x), Asn^{27} mutant DHFR (○), and Ser^{27} mutant DHFR (△). Assays were performed at 30°C in 0.033*M* succinic acid + 0.044*M* imidazole + 0.044*M* diethanolamine + 10 m*M* β-mercaptoethanol buffer (*45*). Ranges of enzyme concentrations were 0.69 to 6.2 n*M* for wild type, 80 to 600 n*M* for Asn^{27}, and 19 to 1900 n*M* for Ser^{27} DHFR. The dihydrofolate concentration was varied and the NADPH concentration was saturating at all *p*H values. Assays were started by the addition of enzyme. Each point was done in triplicate and the data analyzed by plots of the ratio of substrate to velocity as a function of substrate. Curves giving best fit to the data were generated with the use of equation 5 in (*37*) for the wild type or by Eqs. 1 and 2 in our article for the Asn^{27} and Ser^{27} mutants. Best-fit parameters are given in the text.

Kinetics of the Asn^{27} and Ser^{27} mutants. Because we are expressing low-activity mutant DHFRs in an *E. coli* host system, the possibility of contamination by wild-type DHFR is an important concern in kinetic studies. Moreover, it is necessary to guard against the possibility of deamidation of Asn^{27} in that mutant. Thus a number of precautions were observed. Care was taken to remove any contaminating wild-type DHFR by isoelectric focusing procedures (Asn^{27} mutant) (*2*) or by MTX affinity chromatography (Ser^{27} mutant) (*31*). We also tested for the slow appearance of any deamidation product in a solution of the Asn^{27} mutant enzyme stored at *p*H 7, 4°C, for more than a month. No measurable deamidation was observed. As a final precaution, all kinetic data were analyzed by the method of Spears *et al.* (*39*), which has the advantage of showing biphasic behavior in plots of S/V as a function of S if contaminating wild-type enzyme is present (S, substrate concentration; V, velocity). No such behavior could be detected with the purified mutant enzymes.

At *p*H 7.0, observed values of k_{cat} for the wild type, Asn^{27}, and Ser^{27} enzymes are 30, 0.10, and 0.44 sec^{-1}, respectively, and values for the Michaelis constant K_m (dihydrofolate) are 1.2, 44, and 140 μ*M*. The evident conclusion is that while Asp^{27} must perform an important function with respect to both catalysis and binding of

substrate in the wild-type enzyme, this function can to some degree be circumvented.

A clue as to what the function of Asp^{27} might be is given by the *p*H dependence of the kinetic parameters, which were determined for the wild type and both mutant enzymes over the full *p*H range for which reliable measurements could be made. Experimentally determined values for k_{cat} and k_{cat}/K_m (dihydrofolate) are plotted logarithmically as a function of *p*H (Fig. 4, A and B) and compared with predicted curves derived for a kinetic mechanism discussed below.

Our results for the wild-type enzyme agree well with those published by Stone and Morrison (*11, 37*). Activity apparently depends on some group with a pK_{a1} of about 8 that must be protonated and on another group (or groups) with an acidic pK_a that must be unprotonated. Experimental points for the wild-type enzyme are well fitted by the solid curves representing equation 5 from Stone and Morrison (*37*) with $pK_{a1} = 8.0$, and a single acidic $pK_{a2} = 5.0$, $K_s = 1.0\ \mu M$, and $k_2 = 37.0\ sec^{-1}$. The identity of the groups involved in the kinetic pK_a's evident in the plots is not known, but Stone and Morrison (*37*) have suggested that Asp^{27} is responsible for the pK_a of 8.0.

The *p*H profiles of the kinetic parameters for the two mutant enzymes are quite different from those of the wild type. In both cases k_{cat} increases rapidly with decreasing *p*H, while K_m (dihydrofolate) is more or less constant (above *p*H 5, at any rate). These observations can be interpreted, as a first approximation, in terms of the simple kinetic mechanism shown in scheme 1 (Fig. 5), with K_s of the same order of magnitude as K_s'. According to this scheme only protonated dihydrofolate is a substrate for the mutant enzymes, although both protonated and unprotonated dihydrofolate bind with roughly equal affinity. The relation between the experimentally determined k_{cat} and K_m (dihydrofolate) at any *p*H and the molecular parameters according to scheme 1 would be

$$k_{cat} = \frac{k_2 K_s'[H^+]}{K_s K_a + K_s'[H^+]} \quad (1)$$

$$K_m = \frac{K_s K_s'(K_a + [H^+])}{K_s K_a + K_s'[H^+]} \quad (2)$$

For present purposes, an adequate fit to the Asn^{27} data is obtained with $pK_a = 3.8$ (the pK_a of dihydrofolate in solution) (*40*); $K_s = K_s' = 40\ \mu M$; and $k_2 = 60\ sec^{-1}$. Similarly, for the Ser^{27} mutant we find $pK_a = 3.8$; $K_s = 17\ \mu M$; $K_s' = 135\ \mu M$; and $k_2 = 60\ sec^{-1}$.

Several observations about scheme 1 are in order. First, we have ignored whatever complication has caused slopes of −0.6 to −0.7 in the *p*H profiles, compared with predicted slopes of −1. It may be the same phenomenon that causes the gradual decrease in wild-type activity observed at low *p*H, perhaps a *p*H-dependent isomerization. The *p*H dependence of a kinetic hysteresis seen by Penner and Frieden (*41*) may also be related. They observed a rate increase when DHFR was incubated first with substrate or NADPH. This result is consistent with a model (*29, 30*) describing a *p*H-dependent equilibrium between two conformers of the enzyme, one of which is incapable of binding substrate or NADPH. Second, the increase in K_s values for the mutants as compared with the wild type is generally in accord with the increase in K_D for the inhibitor MTX, though it is not as great for the Ser^{27} enzyme as might have been expected. We conclude that substrate binding to the wild-type enzyme does not depend on ionization of Asp^{27}. And third, the values of k_2 for the two mutants are about the same and 1.6 times larger than for the wild-type enzyme. This last observation is particularly significant for assigning a functional role to Asp^{27} in wild-type DHFR. It implies that if we could present the mutant

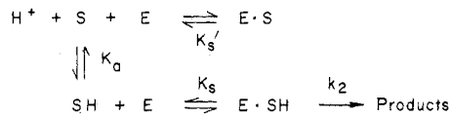

Fig. 5. Scheme 1. Kinetic mechanism proposed to explain *p*H profiles of kinetic parameters for mutant DHFR's. E is the holoenzyme (NADPH bound); S and SH are, respectively, unprotonated dihydrofolate and dihydrofolate protonated at N-5; K_a is the acid dissociation constant of protonated dihydrofolate; K_s is the dissociation constant for the E·SH Michaelis complex; and K_s' is the dissociation constant for the E·S nonproductive complex.

enzymes with totally protonated dihydrofolate, their activity would actually somewhat exceed that of the wild-type enzyme toward unprotonated dihydrofolate. Incorporating this finding into scheme 1, we can conclude that Asp^{27} is required for protonation of bound substrate, but is not essential for hydride transfer or the other events included under the rate constant k_2.

Mutant DHFR's are inactive toward folate. Corroboration of scheme 1 is provided by the inability of either mutant enzyme to catalyze the reduction of folate (in contrast to dihydrofolate) at low *p*H. The key fact here is that wild-type *E. coli* DHFR catalyzes the reduction of folate at a readily measurable rate, about one-thousandth that of dihydrofolate reduction (*42*). However, since N-5 of folate has a pK_a of less than −1.5 as compared with $pK_a = 3.8$ for dihydrofolate (*40*), essentially no protonated folate will be present in solution at *p*H 5. Thus scheme 1 predicts that both mutant enzymes will be completely inactive toward folate, even at *p*H 5 where the activity of the Ser^{27} mutant toward dihydrofolate is almost as high as the wild type. This expectation is borne out by experiments showing that at *p*H 5 neither the Ser^{27} nor the Asn^{27} mutant enzymes have any detectable activity with respect to the reduction of folate.

Deuterium isotope effects. Further confirmation of these interpretations is afforded by our observation of deuterium isotope effects on the kinetic parameters of the mutant enzymes. If we have in fact blocked the protonation step and then circumvented that block by presenting the mutant enzymes with protonated dihydrofolate as substrate, it may be that either hydride transfer or release of product has now become rate limiting. Accordingly, we examined the kinetic effects of utilizing deuterated cofactor, NADPD. At *p*H 5.1, the ratio of k_{cat} for undeuterated compared to deuterated cofactor (^DV) was 2.2 and 3.5 for the Asn^{27} and Ser^{27} mutants, respectively. For k_{cat}/K_m (cofactor) the corresponding ratios ($^DV/K$) were 3.4 and 1.8. These values are significantly greater than 1, and show that hydride transfer is at least partially rate determining in the mechanism of the mutant enzymes. For the wild-type enzyme at *p*H 5.1, on the other hand, we find minimal deuterium isotope effects, namely $^DV = 1.2$ and $^DV/K = 1.1$, again in agreement with the small effects found by Stone and Morrison (*37*) at higher *p*H's.

The simplest interpretation of these results is that, for the wild-type enzyme, the individual rates increase in the order proton transfer < hydride transfer < product release. However, we cannot yet rule out the possibility that the order is different (three other permutations remain possible) and that the mutations have simultaneously accelerated product release while blocking proton transfer. This complication is unlikely to apply if the product in question is $NADP^+$, since we find K_m for the cofactor to be nearly the same for the wild type and both mutants. At *p*H 7, the observed K_m's for cofactor are 0.9 μM for wild type, 1.5 μM for Asn^{27}, and 1.7 μM for Ser^{27}. A clear implication is that the mutations have not much affected cofactor binding.

Conclusions. Enzyme molecules are surprisingly accommodating in the substitutions they can tolerate without structural distortion.

Although disabling the catalytic-site proton relay in DHFR by the mutations $Asp^{27} \rightarrow Asn$ or Ser does substantially diminish the enzyme's activity at *p*H 7, the mutant enzymes can nevertheless function at full catalytic efficiency by utilizing preprotonated substrate from solution at sufficiently low *p*H.

In addition, the x-ray structures of the mutant enzymes in complex with MTX show only minimal changes in geometrical detail. These changes involved neither the backbone chain, the unmutated side chains, nor the conformation of the bound ligand. Rather, certain water molecules bound at the active site have moved slightly to accommodate the substitutions. It is perhaps not surprising that such bound waters are the most readily adjustable structural features of the enzyme molecule. An incidental implication of this observation is that the conformational equilibriums detected in the function of DHFR (and other enzymes) may simply reflect movement of bound waters at the active site.

Finally, our experiments establish that the joint application of directed mutagenesis, x-ray crystallography, and classical binding and kinetic techniques provides a powerful approach to elucidating structure-function relationships in enzyme molecules.

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