Effects of Multiple Replacements at a Single Position on the Folding and Stability of Dihydrofolate Reductase from *Escherichia coli*†

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ABSTRACT: We have made multiple replacements (alanine, arginine, cysteine, histidine, isoleucine, serine, tyrosine) of valine-75 in dihydrofolate reductase from *Escherichia coli* to examine the relative importance to protein folding of the *position* that is substituted and the *specific character* of the amino acid replacement. Valine-75 is part of the eight-stranded β sheet that forms the structural core of the protein. The isopropyl side chain participates in van der Waals interactions with a number of nonpolar residues, helping to establish a large hydrophobic cluster. Equilibrium studies showed that arginine, histidine, isoleucine, serine, and tyrosine destabilize the protein by 1.9–2.8 kcal mol^{-1}. Alanine and cysteine substitutions have little or no effect. Contrary to other recent studies of the effect of multiple replacements at a hydrophobic site, there is no observed correlation between the changes of the free energy of folding and the changes of the free energy of transfer for the individual amino acids from water to an organic solvent when they are inserted into this site. The effects observed in kinetic studies are both consistent with and extend the equilibrium results; these data indicate that *position* 75 participates in a rate-limiting step of folding. Some of the equilibrium and kinetic properties of the tyrosine-75 mutant deviated significantly from those of wild-type protein and the other mutants at position 75. (1) The tyrosine variant displayed a complex banding pattern when analyzed by native gel electrophoresis; the wild-type protein and all other mutants at position 75 migrated as single, discrete bands. (2) Comparison of the difference ultraviolet and circular dichroism transition curves showed that a third species is populated at equilibrium; the wild-type protein and all other mutants at position 75 follow a two-state model involving only native and unfolded forms. (3) A third kinetic phase appeared in the unfolding reaction; the wild-type protein and all other mutants at position 75 only showed two kinetic phases in unfolding. Properties 1 and 3 suggest that the tyrosine mutation significantly alters the distribution of native conformers in the protein. These effects on the equilibrium and kinetic data readily display an overriding pattern: residues that would require hydrogen bonding or lead to an expansion of the tightly packed hydrophobic environment in which valine-75 resides destabilize the protein and alter relaxation times of kinetic phases in a consistent manner. Thus, for the most part, these results show that it is the role of *position* 75 in the folding of dihydrofolate reductase that has been examined by mutagenesis. However, the tyrosine substitution produced striking effects in both equilibrium and kinetic studies that were not detected in the other mutant proteins. Therefore, a *specific* amino acid replacement can result in unique effects when compared with other replacements at the same site.

Our laboratory has studied single amino acid replacements to identify residues that participate in rate-limiting steps of protein folding in order to understand this complex reaction on a structural level. These studies have allowed us to extend the molecular description of the rate-limiting step in the folding of the α-subunit of tryptophan synthase (Beasty et al., 1986; Hurle et al., 1986) and to propose a structural model for the slow steps of folding of dihydrofolate reductase (Perry et al., 1987). To date, most of the information that we have obtained from mutant proteins has come from studying a single substitution at a given site. In such studies where only a single replacement has been examined, one must be concerned with whether the observed effects reflect the position under examination (in terms of its location in secondary or tertiary structure) or the specific replacement for a given residue.

One can address this concern by examining the effects of multiple replacements at a single position on the equilibrium and kinetic properties of folding and noting whether an overriding pattern emerges from the data. If a trend does appear that can be understood in terms of a common disruption of one or a few noncovalent interactions, then one can argue mutagenesis has probed the role of the *position* in the folding process. However, if various substitutions result in effects that are distinctly different from one another, then the effects would reflect the *specific characters* of the amino acids that have been introduced into the site.

Multiple replacements have recently been examined in several systems to address issues concerning protein stability (Yutani et al., 1987; Alber et al., 1987; Matsumura et al., 1988a,b). For the most part, these studies reveal clear patterns in the data and thus indicate that the position plays the dominant role. B. W. Matthews and colleagues examined 13 substitutions of Thr-157 in T4 lysozyme and found that the replacements which best mimicked the hydrogen-bonding network in the wild-type protein least effected the protein in terms of loss of stability (Albers et al., 1987). Yutani and colleagues (Yutani et al., 1987) made all 19 replacements of Glu-49 in the α-subunit of tryptophan synthase; they observed a correlation between the change in the free energy of folding (i.e., the stability) and the free energy of transfer of most of the substitutions from water to an organic solvent. However, replacements that were either charged or aromatic did not show this correlation, demonstrating that specific mutations can elicit alternative effects. To date, no study has been

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reported on the effects of multiple replacements on the kinetics of protein folding.

We have chosen position 75 in dihydrofolate reductase (DHFR,[1] EC 1.5.1.3) from *Escherichia coli* to study the effects of multiple replacements on the equilibria and kinetics of folding. This position was chosen for three reasons. (1) Residue 75 in DHFR is positioned in a β strand, and we have focused our attention on mutations within recognized elements of secondary structure. The rationale for this decision is based upon the *framework model* (Kim & Baldwin, 1982) which hypothesizes that elements of secondary structure form early in the folding reaction; rate-limiting steps in folding might involve rearrangements of these intermediate structures. (2) The crystal structure of DHFR is known to 1.7 Å resolution (Bolin et al., 1982), and the immediate locale of this buried valine is well-defined by other hydrophobic side chains. Thus, mutations might be expected to alter the local interactions and perturb the equilibrium and kinetic properties of folding. This site also provides another opportunity to determine, when substitutions are made at a hydrophobic position, if changes in the free energy of folding correlate with the free energy of transfer of the given amino acid replacements from water to an organic solvent. (3) The particular DNA sequence near the codon for residue 75 greatly simplified the task of making multiple replacements. We report here the effects on folding of seven mutant DHFRs, each substitution at position 75.

Materials and Methods

Oligonucleotide-Directed Mutagenesis. Construction of the expression and mutagenesis vector pTY1 containing DHFR from *E. coli* was described previously (Perry et al., 1987). Multiple replacements at position 75 were made by a two-step process. First, the sequence was altered so that the codon for the wild-type valine (GTG) was changed to that for alanine (GCC); the same alteration created a unique *Bst*XI restriction site. Second, mutagenesis was performed using this plasmid pTY1.V75A and an oligonucleotide mix that was degenerate in the first and second positions of the codon for position 75; the new sequences resulted in nine possible amino acid codons and the removal of the *Bst*XI site.

Plasmid mutagenesis of wild-type pTY1 was performed as described by Dalbadie-McFarland et al. (1982). The initial oligonucleotide 5′-GTGGG*CC*AAGTCGGTGGA-3′ was synthesized on an Applied Biosystems DNA Synthesizer, Model 380A (the positions of the mutagenic mismatches are italicized). The wild-type plasmid was nicked at a unique *Eco*RI site, and single-strand DNA was exposed by digestion with exonuclease III. After the oligonucleotide was annealed and the double-stranded plasmid was resynthesized using the Klenow fragment of DNA polymerase, HB2154 *E. coli* were transformed with the heteroduplex plasmid. The mutant pTY1.V75A was identified by screening plasmid DNA from isolated colonies for the novel *Bst*XI site. The efficiency of mutagenesis was about 5%. The entire gene for DHFR was sequenced to confirm that the desired mutations were the only changes in nucleotide sequence.

The oligonucleotide 5′-GTAACGTGG*12*CAAGTCGGT-3′ was synthesized by injecting an equal molar mixture of T, C, and A at position 1 and an equal molar mixture of T, G, and A at position 2 so that the resulting solution was a mixture of nine different oligonucleotides. The sequence of each 18-mer would effectively remove the *Bst*XI site and also introduce codons for one of the following amino acids: Arg, Asn, Cys, His, Ile, Leu, Phe, Ser, Tyr. Mutagenesis of pTY1.V75A was then performed in a similar manner as described above except for the following modification. After transformation of HB2154 cells with the mutagenized pTY1.V75A, the heterogeneous population of plasmid was isolated, digested with *Bst*XI, and then used to transform MC294 *E. coli*. Approximately 50% of the plasmid DNA isolated from colonies at this point of the procedure was not linearized by digestion with *Bst*XI. These DNAs were screened for the desired mutations by sequencing the area of interest; out of 20 DNAs that were not linearized by digestion with *Bst*XI, 7 different amino acid codons were obtained. The seven additional amino acids were Arg, Cys, His, Ile, Phe, Ser, and Tyr. The remainder of each gene was also sequenced to ensure that no other changes had occurred.

Protein Purification and Characterization. All mutant DHFRs were purified by the procedure of Baccanari et al. (1975, 1977). Purity was ascertained by $NaDodSO_4$ (Laemmli, 1970) and native (Davis, 1964) polyacrylamide gel electrophoresis. The native gel and the native electrophoresis running buffer were buffered by 25 mM Tris/190 mM glycine, pH 8.4. Native gel electrophoresis was performed at 4 °C for 6 h at 20 mA. Native gels were also performed in the presence of methotrexate; 20 μM MTX was included in both the sample buffer (10 min prior to loading) and the electrophoretic buffer. The lagging edge of the diffused band of V75Y DHFR which comigrated with the wild-type protein was eluted from native polyacrylamide gels that had been run in the presence of MTX by the following technique. Approximately 20 μg of mutant protein was loaded in 10 consecutive lanes. After electrophoresis was performed, the inner four lanes were cut and stored at 4 °C, while the remaining six lanes were rapidly stained with Coomassie blue and destained (30 min). The gel was realigned, and the stained gel was used to mark where V75Y migrated on the unstained gel; care was taken to excise the protein above the faster migrating, leading edge of this diffuse band. The gel containing the major band was minced, and the protein was allowed to diffuse out of the gel into 10 mM potassium phosphate, pH 7.8, 1 mM EDTA, and 1 mM 2-mercaptoethanol, in a 1.5-mL microcentrifuge tube, overnight at 4 °C. Protein was then concentrated in a Centricon-10 (Amicon) prior to reelectrophoresis under the same conditions as described above.

Protein concentration was determined by the absorbance at 280 nm ($\epsilon_{280} = 3.11 \times 10^4$ M^{-1} cm^{-1}; D. Baccanari, personal communication). No detectable change in extinction coefficient was observed for V75Y DHFR when ϵ_{280} was redetermined by assaying the protein concentration by the Bradford method (Bradford, 1976); wild-type DHFR contains five Phe, four Tyr, and five Trp residues. Enzymatic activity was monitored as previously described (Hillcoat et al., 1967) in 0.1 M imidazole chloride (pH 7.0) at 30 °C.

Folding Studies. The equilibrium unfolding reaction was monitored by difference ultraviolet spectroscopy (Beasty et al., 1986) at 293 nm on a Cary 118CX spectrophotometer, modified by AVIV Associates. Equilibrium studies were also performed by monitoring the change in ellipticity at 220 nm on a Jasco J-20 spectropolarimeter. Difference UV monitors the tertiary structure when tyrosine and tryptophan residues are exposed to solvent by addition of denaturant, while circular dichroism (CD) spectroscopy at 220 nm monitors the presence of secondary structure in proteins. All samples were fully equilibrated prior to collection of spectra. The unfolding

[1] Abbreviations: DHFR, dihydrofolate reductase; CD, circular dichroism; K_2EDTA, ethylenediaminetetraacetic acid potassium salt; MTX, methotrexate; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

transitions for each of the mutants were judged to be reversible by the recovery of both the native absorption spectrum and at least 90% of the enzymatic activity after 6 M urea was removed by dialysis.

Kinetic experiments were done by manual mixing methods and monitored by difference ultraviolet spectroscopy for unfolding and refolding reactions with relaxation times longer than 15 s. For faster reactions, data were collected on a Durrum 110 stopped-flow spectrophotometer in the fluorescence mode. Fluorescence was measured by exciting at 290 nm with a slit width of 5 mm. Emission intensity was monitored at wavelengths greater than 340 nm with a Corning C. S. O-52 ground-glass filter. It has been previously demonstrated that difference ultraviolet and fluorescence spectroscopies monitor the same folding reactions in DHFR (Touchette et al., 1986).

The buffer used for all folding experiments was 10 mM potassium phosphate, pH 7.8, 0.2 mM K_2EDTA, and 1 mM 2-mercaptoethanol. The temperature was maintained at 15 °C. Final protein concentrations ranged from 0.15 to 0.70 mg/mL, depending on the availability of the particular protein.

Computer Fitting of Equilibrium and Kinetic Data. Equilibrium data were converted to plots of the apparent fraction of unfolded protein, F_{app}, by $F_{app} = (\epsilon_{obsd} - \epsilon_N)/(\epsilon_U - \epsilon_N)$ where ϵ_{obsd} is the observed extinction coefficient and ϵ_N and ϵ_U are the extinction coefficients of the native and unfolded forms, respectively. Values for ϵ_N and ϵ_U in the transition region were obtained by linear extrapolation from the base-line regions. For most unfolding transitions, a two-state model of N ↔ U (where N and U are native and unfolded forms of protein) adequately fit the data. The data for the dependence of F_{app} on the concentration of urea were fit to the equation (Tanford, 1968):

$$F_{app} = \exp(-\Delta G_{app}/RT)/[1 + \exp(-\Delta G_{app}/RT)] \quad (1)$$

where ΔG_{app} is the apparent free energy difference between the native and unfolded forms, R is the gas constant, and T is the absolute temperature. ΔG_{app} was assumed to depend linearly on the urea concentration (Schellman, 1978):

$$\Delta G_{app} = \Delta G_{app}^{H_2O} + A[\text{urea}] \quad (2)$$

where ΔG_{app} is the apparent free energy difference at a given concentration of urea, $\Delta G_{app}^{H_2O}$ is the free energy difference in the absence of urea, and A is a parameter that describes the cooperativity of the unfolding transition. When a three-state model for unfolding, N ↔ I ↔ U (where I is a stable intermediate), was required, the data were fit to the equation (Beasty et al., 1986):

$$F_{app} = [\exp(-\Delta G_{NI}/RT)][Z + \exp(-\Delta G_{IU}/RT)]/[1 + \exp(-\Delta G_{NI}/RT) + \exp(-\Delta G_{IU}/RT)]$$

where ΔG_{NI} and ΔG_{IU} are the apparent free energy differences between native and intermediate protein species and between intermediate and unfolded species, respectively, and $Z = (\epsilon_I - \epsilon_N)/(\epsilon_U - \epsilon_N)$. The fits were done by using a nonlinear least-squares fitting program, NLIN (SAS Institute Inc., Cary, NC).

The kinetic data were fit to a sum of exponentials:

$$A(t) = \sum A_i \exp(-t/\tau_i) + A_\infty \quad (3)$$

where $A(t)$ is the total amplitude at time t, A_∞ is the amplitude at infinite time, A_i is the amplitude corresponding to the individual phase, i, at zero time, and τ_i is the associated relaxation time. Again, data were fitted by using NLIN.

Reagents. Ultrapure urea was purchased from Schwarz/Mann and used without further purification; fresh solutions were prepared on the day of experimentation. The methotrexate–agarose affinity resin used in the protein purification was obtained from Pierce. All other chemicals were reagent grade.

Results

Protein Production and Purification. When cultures of MC294 *E. coli* containing the variant pTY1 plasmids were assayed, a wide variation of DHFR activities was detected. The Ala, Cys, His, and Tyr mutant DHFRs were each present at levels comparable to wild-type protein. However, the levels of activity in crude extracts containing the Arg, Ile, Phe, and Ser variants were each at least an order of magnitude lower. Corresponding differences in the level of mutant DHFRs were also observed when crude extracts were electrophoresed on either native or $NaDodSO_4$–polyacrylamide gels (data not shown). Thus, the low levels of activity appeared to reflect low levels of protein and not specific effects of a given mutation upon the activity of DHFR.

Because of this variation in yields, not all mutant DHFRs were obtained in enough quantity to be completely characterized. V75A, V75C, V75H, and V75Y DHFRs were all purified in amounts sufficient to allow complete equilibrium and kinetic studies. V75I DHFR was studied by equilibrium methods and partly by kinetic techniques. V75R and V75S DHFRs were characterized only by equilibrium studies. V75F was not obtained in purified form.

Most of the purification of DHFR is achieved by affinity chromatography on methotrexate (MTX)–agarose resin. MTX is an analogue of the substrate dihydrofolate and results in a tight binary complex with DHFR ($K_d \sim 10$ nM; Cayley et al., 1981). Wild-type DHFR is eluted from the MTX resin through a combination of high salt (1 M KCl), high pH (pH 9), and presence of a substrate (folic acid) (Baccanari et al., 1975, 1977). It has been shown that a single amino acid replacement within the active site can sufficiently perturb the binding of DHFR to MTX so that DHFR will elute from MTX resin simply in the presence of high salt and high pH (Baccanari et al., 1981). All variant DHFRs in the present study also eluted from MTX–agarose in the absence of folic acid, demonstrating that these mutations caused changes in the binding pocket even though position 75 is ~14 Å removed from the nearest contact with MTX (Bolin et al., 1982). It should be added that although V75F DHFR was not obtained in purified form, V75F DHFR activity also eluted from the resin in the absence of substrate.

It is noted that the elution of these mutant DHFRs in the absence of folic acid affords a clean separation from the small amount of wild-type protein that is produced by the chromosomal DNA of the host *E. coli* cells.

The structural changes that caused the alteration of MTX binding in the mutant DHFRs did not result in any dramatic changes in the specific activity of the purified proteins. Wild-type DHFR has reported specific activity of 85 units mg^{-1} (Baccanari et al., 1977); the value in our laboratory has been observed to range from 70 to 95 units mg^{-1}. The specific activities of V75A, V75C, V75H, V75I, V75S, V75R, and V75Y DHFRs were 70, 73, 86, 82, 75, 95, and 62 units mg^{-1}, respectively.

Whereas each purified mutant DHFR migrated as a single discrete band on $NaDodSO_4$–polyacrylamide gels (Figure 1A), V75Y DHFR displayed more than one band upon electrophoresis on native gels, and, in addition, the banding was quite diffuse compared with wild type and the other mutant DHFRs (Figure 1B). The single band on $NaDodSO_4$ gels and the absence of contamination from the preparations of all other mutant DHFRs (which all originated from the same bacterial

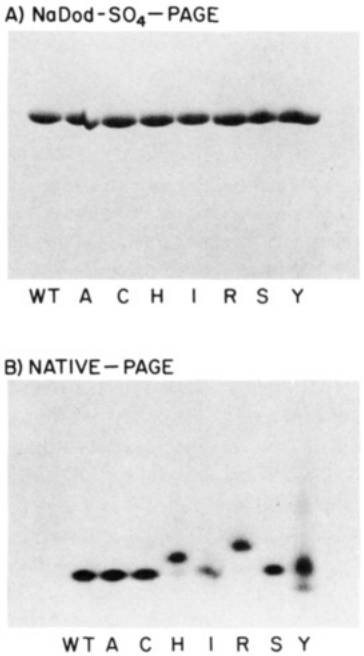

FIGURE 1: Gel electrophoresis of wild-type and mutant DHFRs: (A) $NaDodSO_4$ and (B) native polyacrylamide gel electrophoresis. Approximately 10 μg of protein was loaded per lane. Electrophoresis procedures are described under Materials and Methods. Letters are the one-letter code for amino acids and represent the mutation at position 75 in DHFR.

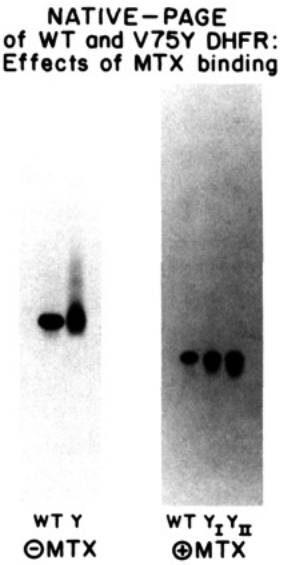

FIGURE 2: Native gel electrophoresis of wild-type and V75Y DHFRs in the absence and presence of methotrexate. Approximately 10 μg of protein was loaded per lane. Electrophoresis was performed as described under Materials and Methods. In the gel that was run in the presence of MTX, Y_I refers to the initial electrophoresis of the tyrosine mutant, and Y_{II} refers to the reelectrophoresis of the tyrosine mutant that had been isolated from a native gel and allowed to come to equilibrium in solution. The technique for removing the V75Y DHFR from a native gel is described under Materials and Methods.

strain) suggested that this electrophoretic behavior did not result from contamination. Rather, the diffuse banding could represent a slow equilibrium between two or more native conformers of V75Y DHFR.

To test this hypothesis, we conducted the following experiments. First, native polyacrylamide electrophoresis was performed in the presence of MTX (Figure 2). When wild-type and V75Y DHFRs were preincubated with MTX and electrophoresis was performed with MTX in the running buffer, V75Y DHFR migrated as a more compact but still diffuse band near that for the wild-type protein; the two slower bands were not present. To test if this diffuse band was due to a nearly comigrating protein contaminant or to equilibration between native conformers, the lagging edge of the tyrosine variant which comigrated with the wild-type protein was eluted from the gel under native conditions, as described under Materials and Methods. When this isolated protein sample was again electrophoresed on native gels in the presence of MTX, it displayed the same diffused banding pattern as the original sample of V75Y DHFR run in the presence of MTX (Figure 2). Thus, the minor bands and the diffused banding pattern of all V75Y species can be attributed to multiple species of V75Y DHFR that slowly equilibrate with each other.

V75R and V75H DHFRs moved as single bands with slower mobilities than wild-type DHFR upon native gel electrophoresis (Figure 1B). The relatively slow mobility of V75R is consistent with the addition of a formal positive charge. The intermediate mobility of V75H may reflect either a partial positive charge at pH 8.5 or a significant change in the native conformation of the protein.

Equilibrium Studies. Our laboratory has previously reported that the urea-induced unfolding transition of wild-type DHFR is best described by a two-state model (Touchette et al., 1986). At pH 7.8, 15 °C, the midpoint of the transition occurs at 3.1 M urea, and the free energy of unfolding in the absence of denaturant is 5.9 kcal mol^{-1}. We wished to know if multiple replacements at a single position would alter this folding model or result in systematic effects on the stability. Therefore, the unfolding transition of the seven mutant DHFRs at position 75 was monitored by difference UV spectroscopy. Residue 75 in *E. coli* DHFR is a valine and is positioned in a short strand at the edge of the β-sheet [βD in the nomenclature of Bolin et al. (1982)] (Figure 3). The valine side chain points into the interior of the protein and intimately participates in a large hydrophobic cluster (Figure 4).

The unfolding transition curves for the Cys and Ile mutant DHFRs are shown in Figure 5 and are representative of the data collected from the equilibrium studies. As was found with wild-type (Touchette et al., 1986) and the previously described mutant DHFRs (Perry et al., 1987), the data are best described by a two-state model of unfolding for all but the Tyr mutant (see below). These two data sets depict the two general effects observed from mutations at position 75: either silent or destabilizing. Both V75C and V75A replacements have little or no effect upon the stability of the protein, and their curves are virtually superimposable upon that of the wild-type protein. The transition curves for the other substituted DHFRs all show large shifts to lower concentrations of urea, similar to the shift observed for V75I DHFR (Figure 5). The midpoints for V75I, V75H, V75R, and V75Y range from 2.0 to 2.2 M urea. The midpoint for V75S occurs at 2.4 M urea and may represent a somewhat intermediate effect upon stability.

The changes in stability caused by the replacements at position 75 are presented in Table I. In each study, the data were fitted to a two-state model, i.e., eq 1. The free energy of folding in the absence of denaturant, $\Delta G_{app}^{H_2O}$, was determined by a linear extrapolation of the transition to 0 M urea (Schellman, 1978). Because the errors incurred in extrapolation to 0 M urea are similar to the alterations in the free energy of folding observed in many mutant proteins, Cupo and Pace (1983) have suggested that the use of the midpoint of the wild-type protein as a reference allows for a more accurate evaluation of the change in stability. Therefore, we prefer to use the concentration of urea at the midpoint of the transition

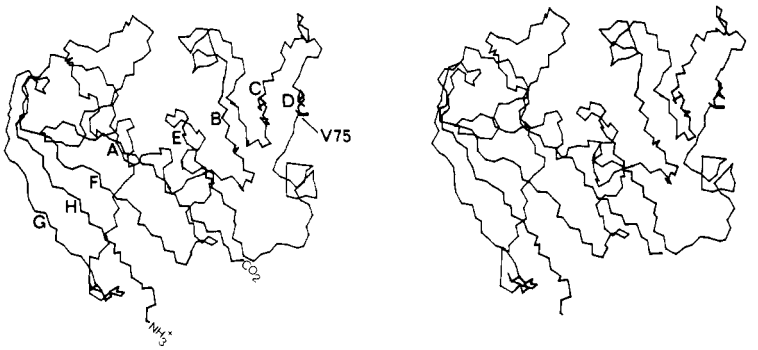

FIGURE 3: Position of residue 75 in wild-type DHFR. The eight strands that comprise the β-sheet in DHFR are labeled in this α-carbon tracing. Val-75 is marked by an arrow. The X-ray crystal structure was determined by Bolin et al. (1982).

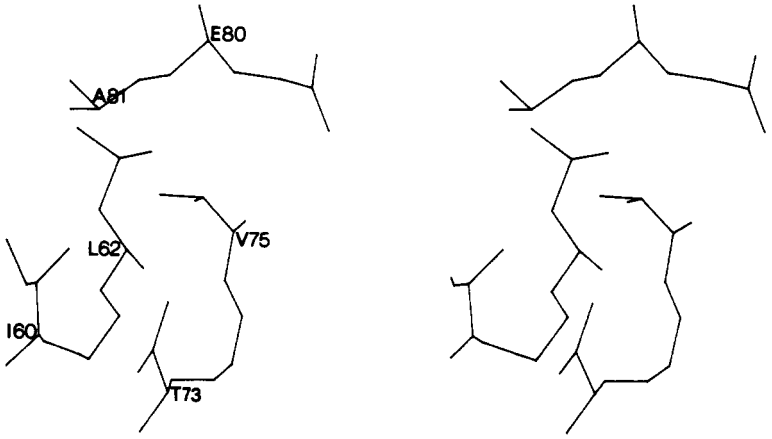

FIGURE 4: Direct van der Waals contacts with Val-75. Residues which have at least one distance that is 4 Å or less between one of its carbons and a carbon of Val-75 are presented. The X-ray crystal structure was determined by Bolin et al. (1982).

Table I: Effects of the Urea-Induced Unfolding of DHFR Due to Substitutions at Position 75

protein	volume[a] of residue (Å^3)	$\Delta G_{app}^{H_2O}$[b] (kcal mol^{-1})	C_m[c] (M, urea)	A[d] [kcal mol^{-1} (M, urea)$^{-1}$]	$\Delta\Delta G$[e] (kcal mol^{-1})
Val (wild type)	140	5.9 ± 0.3	3.1 ± 0.1	−1.9 ± 0.1	
Ala	88.6	6.0 ± 0.5	3.0 ± 0.1	−2.0 ± 0.2	−0.2 ± 0.1
Cys	108	6.0 ± 0.8	3.1 ± 0.1	−2.0 ± 0.3	−0.2 ± 0.2
Ser	89.0	6.2 ± 0.6	2.4 ± 0.1	−2.6 ± 0.2	−1.9 ± 0.1
His, pH 7.8	153	3.9 ± 0.3	2.2 ± 0.1	−1.8 ± 0.1	−1.7 ± 0.1
His, pH 6.5		4.6 ± 0.6	2.2 ± 0.1	−2.1 ± 0.3	−1.9 ± 0.1
Ile	167	4.6 ± 0.5	2.2 ± 0.1	−2.1 ± 0.2	−2.0 ± 0.1
Arg	173	4.7 ± 0.5	2.0 ± 0.1	−2.4 ± 0.3	−2.8 ± 0.1
Tyr UV	194	3.5 ± 0.3	2.1 ± 0.1	−1.6 ± 0.2	−1.5 ± 0.1
Tyr CD		4.6 ± 0.7	2.1 ± 0.1	−2.2 ± 0.3	−2.3 ± 0.2

[a] Zamyatnin (1972). [b] The apparent free energy of unfolding in the absence of urea at pH 7.8 (except where noted), 15 °C. Errors are 95% confidence intervals from nonlinear least-squares fits. [c] Midpoint of the urea-induced unfolding transition. The value of C_m is calculated from $C_m = -\Delta G_{app}/A$. The errors were calculated by propagation of errors analysis. [d] The parameter A is defined in eq 2. Errors are 95% confidence intervals from nonlinear least-squares fits. [e] $\Delta\Delta G$ is calculated from $\Delta\Delta G = \Delta G$(mutant) − ΔG(wt), where $\Delta G = \Delta G_{app}^{H_2O} + A$[urea] and [urea] is the midpoint of the unfolding transition for wild-type DHFR, 3.1 M. Errors were calculated by propagation of errors analysis.

for wild-type DHFR (3.1 M urea), instead of 0 M urea, as the reference state.

The Ala and Cys substitutions both resulted in little or no changes in stability (−0.2 kcal mol^{-1}). However, each of the other replacements greatly destabilized DHFR (−1.6 to −2.8 kcal mol^{-1}). A second observation is that the slopes (parameter A in Table I) of all unfolding transitions except those for V75Y DHFR are, within experimental error, equal to or greater than that of wild-type DHFR.

Comparison of the observed data and fitted transition curve for the V75Y mutant (Figure 6A) suggested that the more shallow slope observed for the Tyr variant resulted from the inappropriate use of the two-state unfolding model. The data are better fit by a three-state unfolding model (Figure 6A), where the third species of protein (I) is a stable unfolding intermediate: N ↔ I ↔ U.

As another test of whether the tyrosine mutation had indeed caused a change in the equilibrium folding model, we examined the unfolding transition by CD spectroscopy. Coincidence of the folding transitions monitored by two different spectroscopic techniques implies that a two-state model is appropriate; noncoincidence implicates other stable species. Figure 6B shows the data collected by CD spectroscopy and the two-state fit to that data; the data from UV difference spectroscopy and the three-state fit are shown as a reference. The data from the two techniques and the respective fits are coincident for approximately the first two-thirds of the curve and noticeably noncoincident for the remainder of the transition. Therefore,

Table II: Fits of Equilibrium Data for V75Y DHFR to a Three-State Unfolding Model[a]

protein	$\Delta G^{H_2O}_{appNI}$ (kcal mol^{-1})	C_{mNI} (M, urea)	A_{NI} [kcal mol^{-1} (M, urea)$^{-1}$]	$\Delta\Delta G^b$ (kcal mol^{-1})	$\Delta G^{H_2O}_{appIU}$ (kcal mol^{-1})	C_{mIU} (M, urea)	A_{IU} [kcal mol^{-1} (M, urea)$^{-1}$]
Tyr	4.3 ± 0.3	2.0 ± 0.1	−2.2 ± 0.2	−2.6 ± 0.1	9.3 ± 5.0	3.2 ± 0.8	−2.8 ± 1.5

[a] ΔG_{app}, C_m, and A values when data are fit to the three-state model of N ↔ I ↔ U, where N, I, and U are the native, intermediate, and unfolded forms of the protein. Parameters and determination of errors are defined in Table II. [b] $\Delta\Delta G$ is calculated from $\Delta\Delta G = \Delta G_{NI}$(mutant) − ΔG(wt), where $\Delta G = \Delta G^{H_2O}_{app} + A$[urea] and [urea] is the midpoint of the unfolding transition for wild-type DHFR, 3.1 M.

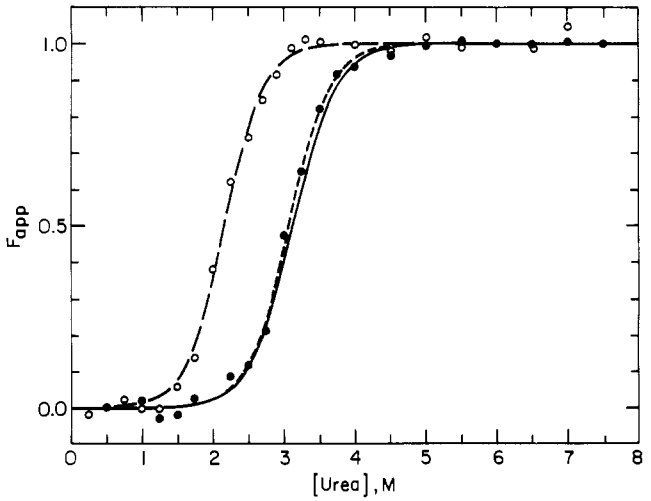

FIGURE 5: Dependence of the apparent fraction of unfolded protein, F_{app}, on the urea concentration for V75C (●---●) and V75I (○—○) DHFRs. Data were collected by difference UV spectroscopy at 293 nm, and the buffer was 10 mM potassium phosphate, pH 7.8, 0.2 mM K_2EDTA, and 1 mM 2-mercaptoethanol; the temperature was 15 °C. For reference, the unfolding transition curve for the wild-type protein under the same conditions is shown as a solid line (Touchette et al., 1986). The dashed lines through the data for the two mutants and the solid line for wild-type DHFR are all fits to a two-state unfolding model.

it appears that the replacement of Val at position 75 by Tyr results in the population of another stable conformation.

The parameters that result from fitting the Tyr data to a three-state model are presented in Table II. Not only does a three-state model fit the data better (Figure 6A), but also the slope for the N ↔ I transition corresponds well with those of the mutant DHFRs which were fit to two-state models. In addition, for the tyrosine mutation, $\Delta\Delta G_{NI}$ observed by UV spectroscopy agrees well with $\Delta\Delta G_{NU}$ obtained from CD spectroscopy. This contrasts with the differences observed when the UV data are fit to a two-state model (Table I).

Finally, the unfolding transition for V75H DHFR was examined at a lower pH in an attempt to differentiate between size and charge effects. Unfortunately, V75H DHFR precipitated at a pH equal to or lower than 6.0; 6.5 was the lowest pH at which the equilibrium transition was studied. There were no significant differences observed in the unfolding transition at this pH when compared with data collected at pH 7.8 (Table I). Thus, *if* this histidine is appreciably protonated at pH 6.5 and unprotonated at pH 7.8, the addition of a positive charge does not appear to contribute to the destabilizing effect.

Kinetic Studies. The kinetic folding model proposed for wild-type DHFR is shown in Figure 7. Briefly, two phases, designated τ_1 and τ_2, are detected by difference UV spectroscopy when unfolding or refolding is initiated by a manual mixing technique. These phases are proposed to represent the unfolding and refolding of the two native forms of DHFR, N_1 and N_2 (Touchette et al., 1986). For unfolding reactions, the amplitudes of the τ_1 and τ_2 phases are 15% and 85% of the

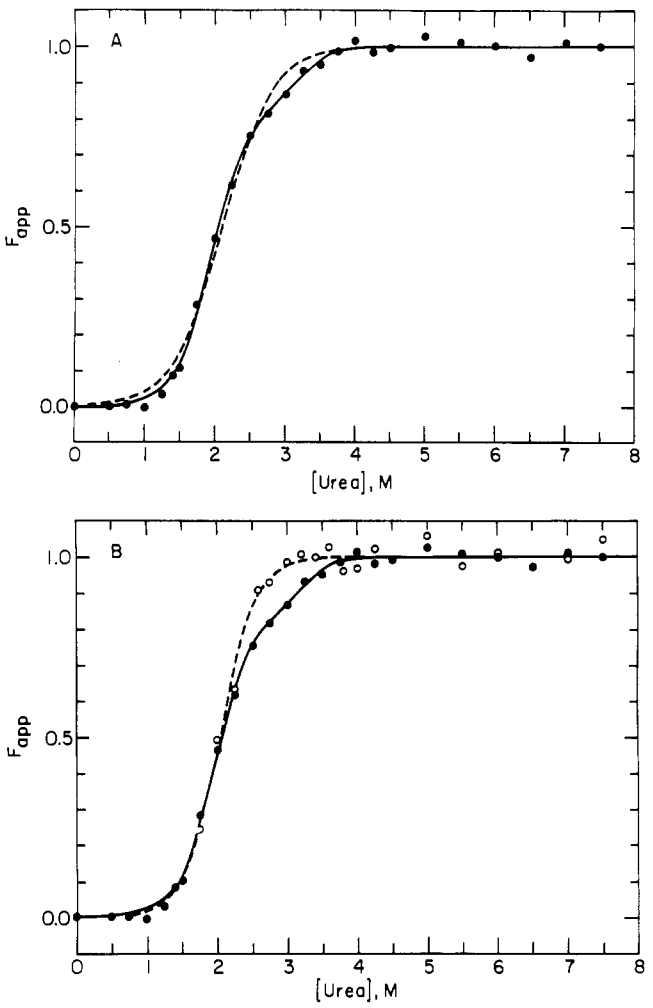

FIGURE 6: Equilibrium unfolding intermediate in V75Y DHFR. (A) Dependence of the apparent fraction of unfolded protein, F_{app}, on the urea concentration for V75Y DHFR and fits to a two-state (---) and to a three-state (—) unfolding model. Data collected by difference UV spectroscopy. (B) Dependence of the apparent fraction of unfolded protein, F_{app}, on the urea concentration for V75Y DHFR monitored by difference UV spectroscopy at 293 nm (●—●) and by CD spectroscopy at 220 nm (○---○). The fitted line through UV data results from a three-state model; that through the CD data results from a two-state model. The buffer in both experiments was 10 mM potassium phosphate, pH 7.8, 0.2 mM K_2EDTA, and 1 mM 2-mercaptoethanol; temperature was 15 °C.

expected absorbance change and no faster phases are observed, even with stopped-flow instrumentation. However, three faster phases *are* observed when refolding is performed by stopped-flow techniques and monitored by fluorescence spectroscopy. The fastest phase, τ_5, is noteworthy because the fluorescence intensity *increases* with time. The other four phases show decreases in intensity and are consistent with the change in fluorescence intensity expected for the refolding of DHFR (Touchette et al., 1986). The τ_5 phase was hypothesized to represent the collapse of unfolded DHFR to an intermediate species that is highly hydrophobic in structure (Touchette et al., 1986).

Table III: Amplitudes of Various Kinetic Phases for the Urea-Induced Unfolding and Refolding of Wild-Type and Substituted DHFRs

	refolding[a]						unfolding[b]		
protein	A_1[c] (%)	A_2[c] (%)	A_2[d]	A_3[d]	A_4[d]	A_5[d]	A_1[c] (%)	A_2[c] (%)	A_3[c] (%)
WT	9	11	−0.53	−0.14	−0.89	1.13	14	78	n.o.[e]
V75A	5	14	−0.79	nd[f]	−1.03	0.88	13	67	n.o.
V75C	3	9	−0.63	nd[f]	−0.92	1.14	11	63	n.o.
V75H	11	14	−0.40	−0.26	n.o.	0.14	13	80	n.o.
V75I	3	7	np[g]	np	np	np	18	64	n.o.
V75Y	10	25	−0.35	−0.12	n.o.	0.07	8	52	22

[a] The amplitudes of various kinetic phases when refolding from 5.4 to 1.0 M urea. [b] The amplitudes of various phases when unfolding from 0 to 4.0 M urea. [c] Percentage of expected amplitude for a given phase relative to ΔA_{293} observed in equilibrium experiments; data collected by manual mixing and monitored by difference UV spectroscopy. [d] Total voltage change observed for a given phase at 11 μM protein; data collected by stopped-flow and monitored by fluorescence spectrscopy. [e] Kinetic phase not observed. [f] Kinetic phase detected but amplitude too low to generate reasonable fit. [g] Stopped-flow study not performed on V751 DHFR.

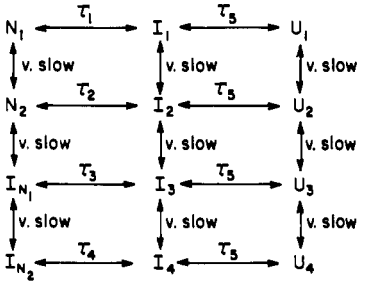

FIGURE 7: Proposed kinetic folding model for DHFR (Touchette et al., 1986).

The effects of three mutations, Cys, His, and Tyr, on the kinetics of folding are shown in Figures 8–10. The effects of the Ala replacement were similar to that of Cys and the effects of the Ile replacement to that of His; these data are not shown. The unfolding and refolding reactions were monitored both by difference UV and by fluorescence spectroscopies and were initiated both by manual mixing and by stopped-flow techniques.

The kinetic data collected for V75C DHFR are presented in Figure 8. The relaxation times for all kinetic phases for V75C DHFR are within experimental error of those for wild-type DHFR. Whereas slight perturbations were seen in the relaxation times for unfolding of τ_2 and refolding of τ_1 for V75A DHFR, the differences range between 20% and 40% of the values for the wild-type protein and are not viewed as significant (data not shown). All other relaxation times for V75A DHFR were the same as those for the parent protein. Likewise, the relative amplitudes of all kinetic phases observed for both mutant proteins were similar to the corresponding amplitudes for the wild-type DHFR (Table III). It is noted that the amplitudes of kinetic phases are exceedingly difficult to measure accurately for low-amplitude phases. Therefore, the amplitudes reported for the two slow phases in refolding (columns 2 and 3 in Table III) are deemed to be equal, within experimental error. The relaxation times corresponding to the τ_3 refolding phase were not included due to poor fits. The amplitude of this phase is extremely low even in wild-type DHFR. Although the plots of the residuals indicated that the τ_3 phase was present in the refolding of these mutants, attempts to obtain consistent parameters for this phase were unsuccessful.

The urea concentration dependence of the relaxation times for V75H DHFR is shown in Figure 9. The alterations in the relaxation times for several of the kinetic phases contrast with the lack of alterations in the Ala and Cys variants. The largest alterations detected were in the values of τ_2. The relaxation times for unfolding were decreased by factors of 2–3, and the relaxation times for refolding increased by factors of 2–4. In contrast, the relaxation times for τ_1 were very

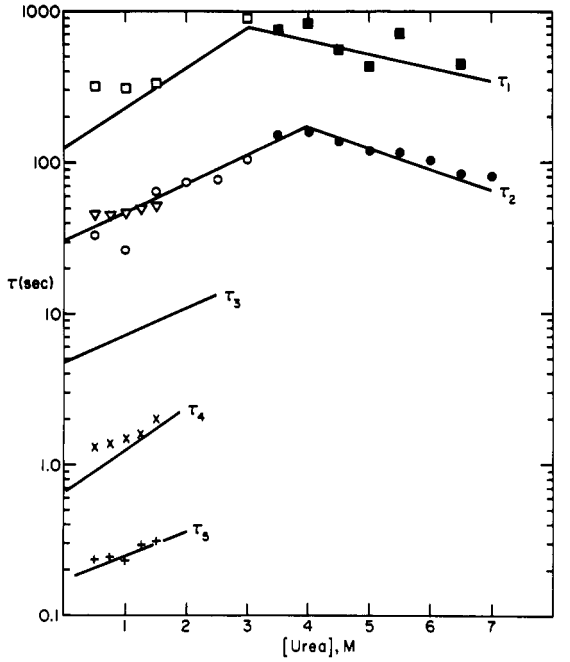

FIGURE 8: Dependence of relaxation times on the final urea concentration for V75C DHFR. The τ_1 (□, ■) and τ_2 (○, ●) phases were monitored by difference UV spectroscopy after manual mixing. Closed symbols represent unfolding jumps from 0 M urea to the indicated final concentrations of urea. Open symbols represent refolding jumps; protein was incubated in 5.4 M urea for 3 h at 15 °C prior to initiation of the reaction by dilution to indicated concentrations of urea. The τ_2 (▽), τ_4 (×), and τ_5 (+) refolding phases were monitored by fluorescence spectroscopy coupled with stopped-flow instrumentation; again, the jumps were from 5.4 M urea to the indicated concentrations of urea. It is noted that the τ_1 phase is too slow to be monitored by stopped-flow techniques. The buffer was 10 mM potassium phosphate, pH 7.8, 0.2 mM K_2EDTA, and 1 mM 2-mercaptoethanol; the temperature was 15 °C. The solid lines are nonfitted lines through the kinetic data for wild-type DHFR, collected under the same conditions (Touchette et al., 1986).

similar to those for wild-type DHFR in refolding. Minor changes in unfolding for the τ_1 phase appear to represent a change in slope of the dependence of the relaxation times on urea concentration. There were no significant changes in the relative amplitudes of the two phases detected by difference UV when compared with data from wild-type DHFR (Table III).

Additional alterations were observed when refolding of V75H DHFR was initiated by a stopped-flow technique and monitored by fluorescence spectroscopy (Figure 9). The relaxation times for either for τ_3 or the τ_4 phase were significantly increased. For simplification, we have labeled this phase τ_3 in the refolding kinetics of V75H because its amplitude corresponds more closely with the amplitude of τ_3 in wild-type DHFR (Table III). The second alteration observed by stop-

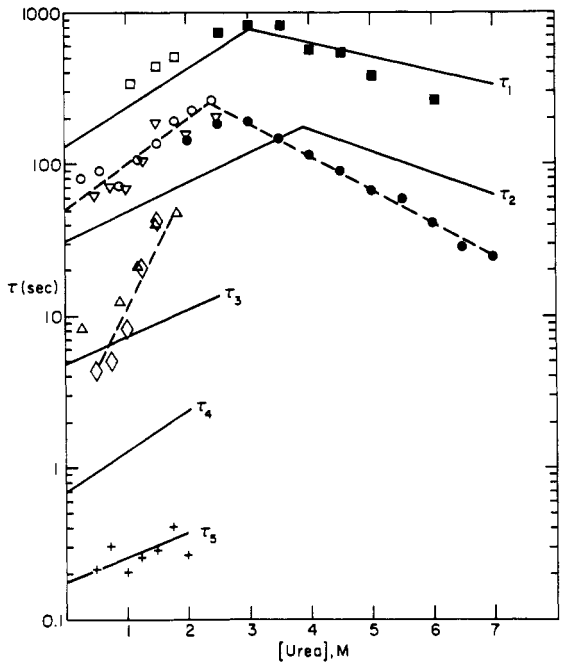

FIGURE 9: Dependence of relaxation times on the final urea concentration for V75H DHFR. Definition of symbols and description of conditions are the same as in the legend for Figure 8, except for the following: the τ_3 phase in refolding was detected both by difference UV (△) and by fluorescence (◇) spectroscopies, and the dashed lines are nonfitted lines through the data that deviate from that of wild-type DHFR.

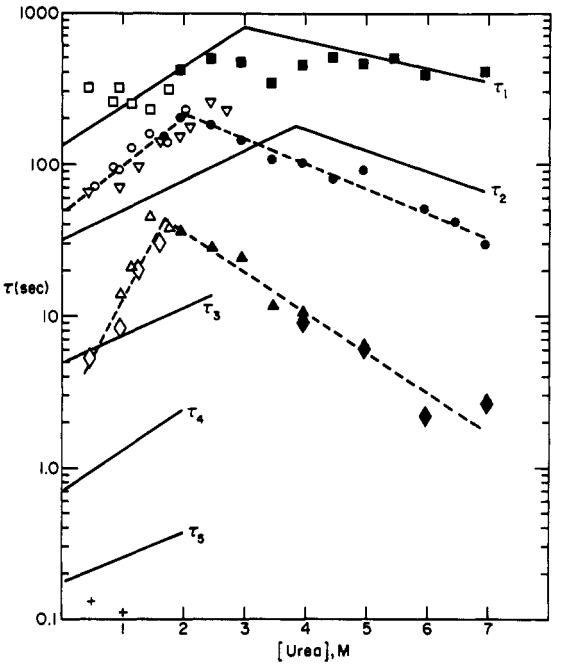

FIGURE 10: Dependence of the relaxation times on the final urea concentration for V75Y DHFR. Definition of symbols and description of conditions are the same as in the legend for Figure 8, except for the following: the τ_3 phase in both refolding *and* unfolding was detected both by difference UV (△) and by fluorescence (◇) spectroscopies, and the dashed lines are nonfitted lines through the data that deviate from that of wild-type DHFR.

ped-flow experiments was a large change in the relative amplitude of τ_5 (Table III). Whereas the ratio for the amplitudes of τ_5 to τ_3 to τ_2 is approximately 8:1:4 for the wild-type protein, the ratio for the V75H protein is approximately 1.5:2:4. There is roughly a 5-fold decrease in the relative amplitude of τ_5 in the V75H protein.

UV kinetic studies on V75H DHFR at pH 6.5 demonstrated no significant differences either in the relaxation times or in the corresponding amplitudes, compared to data collected at pH 7.8 (data not shown).

The kinetics of folding for V75I DHFR were studied only by difference UV spectroscopy and manual mixing techniques. The relaxation times for τ_1 and τ_2 for both unfolding and refolding were within error of those of V75H DHFR (data not shown). Likewise, the Ile mutation had no significant effect on the amplitudes of these two phases detected by difference UV (Table III).

The relaxation times for V75Y DHFR are presented in Figure 10. The relaxation times for τ_2 are virtually identical with those for both V75H and V75I; thus, the Tyr, His, and Ile mutations appear to have affected the major native species of DHFR in a similar fashion. This mutation may have also altered the urea concentration dependence of the τ_1 phase, but the relative amplitude of this slow phase has decreased to such a level (8%) that it is difficult to draw any firm conclusions on this point. V75Y DHFR showed the largest changes in the relative amplitudes for unfolding (Table III); the amplitude of τ_2 decreased by 26%. In fact, a third phase in unfolding was detected both by difference UV and by fluorescence spectroscopies. Again, for simplification, we have labeled this unfolding phase τ_3 because it appears to connect smoothly with the relaxation times of the refolding phase designated τ_3. The amplitude of this τ_3 phase in unfolding accounts for the loss in amplitude of the τ_2 phase.

As observed in the stopped-flow study of V75H DHFR, the tyrosine mutation has also noticeably affected the relaxation times of refolding for the τ_3 phase (Figure 10); the relaxation times are identical with those produced by the histidine mutation. In addition, the effect of the tyrosine substitution on the amplitude of τ_5 is even greater than that observed for the histidine mutation (Table III). There is an approximately 10-fold decrease in the relative amplitude of τ_5 compared with the 5-fold decrease observed in V75H DHFR. In fact, the mutation also appears to have altered the relaxation times for τ_5 (Figure 10). However, the amplitude of this phase in V75Y DHFR was so low that the resulting error in these values does not allow us to state if the differences are significant.

Discussion

Description of the Environment Surrounding Position 75. DHFR contains an eight-stranded β-sheet that can be divided into two halves by the lengths of the strands: strands G, H, F, and A have lengths of 8–10 amino acids, and strands E, B, C, and D have lengths of 3–6 (viewing from left to right in Figure 3). Valine-75 in wild-type DHFR is the last of three amino acids which comprise the short edge βD strand (Thr-Trp-Val-) (Bolin et al., 1982). The isopropyl side chain of valine points into the interior of the protein and intimately participates in the formation of a large hydrophobic core of DHFR. This cluster of amino acids is approximately 13 × 16 × 14 Å and has no buried waters or unfilled cavities. Approximately half of this cluster is formed by Val-75 and the residues that make direct van der Waals contact with its side chain. The contacts with Val-75 are the γ-methyl groups of Ile-60, Leu-62, and Thr-73, the β-methylene group of Glu-80, and the α-methenyl group of Ala-81 (Figure 4).

Patterns in the Data from Equilibrium Studies. Each of the four substitutions at position 75 that have a larger side chain than valine in wild-type DHFR, Arg, His, Ile, and Tyr (153–194 vs 140 Å^3; Table I), destabilizes the protein by a rather similar amount, −2.0 to −2.8 kcal mol^{-1}. Two of the three replacements, Ala and Cys, that are smaller in volume

than valine (89 and 108 vs 140 Å^3) have little or no effect upon the free energy of folding. The lone exception to this apparent correlation with the volume of the side chain is the V75S mutant, where the serine side chain, although considerably smaller (89 Å^3) than valine, results in a significantly destabilized protein (−1.9 kcal/mol). However, burying the β-hydroxyl of serine without an obligatory hydrogen-bonding acceptor would be energetically expensive (Baker & Hubbard, 1984); the fraction of serines found in the interior of proteins is relatively low (Chothia, 1976). Because of this exception and because the four larger residues, Arg, His, Ile, and Tyr, display a variety of hydrophobicity, polarity, and charge, the pattern in the equilibrium data might be better defined as *any* substitution that disrupts this tightly packed hydrophobic environment or requires hydrogen bonding would significantly destabilize the free energy of folding.

The absence of effects of the smaller replacements and the seemingly all or none effect of the larger substitutions at this hydrophobic site demonstrate that there is no simple correlation between the changes in stability and the theoretical free energy of transfer of the various residues from water to ethanol. These results are similar to those observed in a series of replacements at position 146 in T4 lysozyme. Replacements of Gly, Val, Thr, Ile, and Phe for the wild-type Ala have shown that the stability of lysozyme did not correlate with the volume of the substitution at this mostly buried site (D. E. Tronrud and B. W. Matthews, personal communication). In contrast, multiple replacements of Glu-49 in the α-subunit of tryptophan synthase (Yutani et al., 1987), of Asp-80 in kanamycin nucleotidyltransferase (Matsumura et al., 1988a), and of Ile-3 in T4 lysozyme (Matsumura et al., 1988b) have shown a correlation between the stability of the protein and the free energy of transfer of the side chains from water to ethanol. Although the environment of Asp-80 in the transferase is not known, the other two positions have been described. The wild-type Glu at position 49 in the α-subunit appears to be a partly buried residue in the active site based on the high pK_a of the wild-type Glu (7.5) (Yutani et al., 1984) and the preliminary results of the crystal structure (Hyde et al., 1988). The side chain of Ile-3 in T4 lysozyme is approximately 80% buried and makes direct contact with three other nonpolar side chains and also part of the main chain (Remington et al., 1978).

These results suggest that hydrophobic sites might be divided into two general classes: (1) sites in which the effect of the replacement on the stability of the protein is correlated with the free energy of transfer of the side chain from water to an organic solvent and (2) sites in which there is no such correlation. Crystallographic studies of mutants from these two different types of sites will be needed for a definitive structural understanding. However, it is tempting to hypothesize that the first class of hydrophobic sites actually mimics the environment of a hydrocarbon liquid in the sense of being sufficiently flexible to accommodate the introduction of nonpolar side chains, regardless of size. This malleable class of hydrophobic sites contrasts with the second class of sites which might be viewed as being relatively rigid. In a strict definition, such a site is not tolerant to changes of size, hydrophobicity, or polarity; hypothetically, these sites are much more constrained within the local environment of the native protein.

These descriptions of sites focus upon the structure of the folded protein; it is possible that the data in the above studies reflect alterations in both the folded and unfolded protein. Presumably, the effect of the substitution on the free energy of the unfolded form for both classes of sites is similar. Although this assumption may be true to a first approximation, the possibility of residual structure in the unfolded form is a complicating issue. Whether these two classes exist and, if they do, what particular structural differences separate them await further examinations of hydrophobic positions.

The results from these equilibrium studies underline two general phenomena that have been observed in many studies of the effect of single amino acid replacements on protein stability [for reviews, see Dill (1987), Matthews (1987), and Goldenberg (1988)]: (1) protein stability is a delicate balance of many interactions, and (2) protein structure is highly adaptable. This apparent dichotomy of protein structure as both fragile and flexible is demonstrated by the isoleucine and arginine replacements at position 75. In the V75I protein, the introduction of a single additional methyl group at this site produces a significant decrease in protein stability (−2.0 kcal/mol). Thus, the stability of DHFR is disturbed by a relatively small alteration of 1 of its 159 amino acids. On the other hand, replacing a buried valine with the fully charged δ-guanido group of arginine only decreases the stability by −2.8 kcal mol^{-1}. Because Arg is almost never found in the interior or proteins (Chothia, 1976), one might have expected that this mutant protein would not fold to a stable conformation. However, position 75 is in an edge strand in the sheet. The long and flexible aliphatic chain of arginine may allow both a partial filling of the hydrophobic cavity and the exposure of the δ-guanido group to solvent. Consistent with this hypothesis is the slower migration of V75R DHFR compared with wild-type protein on native polyacrylamide gels, suggesting that the mutant is more positively charged than wild-type DHFR. Although an X-ray structure is required to validate this hypothesis, the V75R mutant is an impressive example of the adaptability that proteins can display.

It is extremely interesting that these substitutions at position 75 resulted in changes in methotrexate binding, as viewed by their altered elution from MTX–agarose (compared to wild-type protein), even though the shortest distance between the valyl side chain and MTX is 14 Å. This qualitative data indicate that amino acid replacements can have long-range effects through the protein matrix. Recent NMR studies of mutations in *Staphylococcus* nuclease have demonstrated that substitutions can disturb the chemical shifts of residues up to 30 Å from the mutated residue (Fox et al., 1986; Hibler et al., 1987; Wilde et al., 1988). Thus, one must be concerned with the possibility of long-range effects which can be induced by single amino acid replacements.

Variations in the Patterns in Equilibrium Data: V75Y DHFR. Whereas the multiple replacements at position 75 resulted in a general pattern in terms of their effects upon stability, the substitution with tyrosine also produced unique effects. First, the unfolding transition for V75Y DHFR monitored by difference UV is best described by a three-state model. The inflection in the transition curve could represent either a stable intermediate along the unfolding pathway or a native conformer that unfolds at a slightly higher concentration of urea. Regardless of the identity of the third species, it is interesting that this species was detected by difference UV and not by CD. Previous examples of noncoincidence in these transition curves show the tertiary structure unfolding prior to the secondary structure [e.g., see Holladay et al. (1974) and Robson and Pain (1976)]. This is primary evidence for the framework model of protein folding (Kim & Baldwin, 1982); units of secondary structure form first and are inherently more stable. One possible explanation of this oddity is that the tyrosine at position 75 interacts with the tyrptophan at position 74 such that exposure to solvent of Trp-74 is delayed.

The second significant difference is that the tyrosine variant had a significantly different electrophoretic mobility than either wild-type or other mutant DHFRs on native polyacrylamide gels. The disappearance of the slow-migrating diffuse bands in the presence of methotrexate suggests a slow equilibrium between more than one native conformer; presumably, the equilibria between these native conformers are shifted to the form that binds this potent inhibitor most tightly. The less diffuse band that persists in the presence of methotrexate was also shown to result from an equilibrium between two or more native conformers. It seems likely that these multiple native conformers which remain in the presence of MTX all bind MTX to the same relative degree and remain in slow equilibrium with each other.

Patterns in the Data from Kinetic Studies. The effects of multiple replacements at position 75 on the kinetics of folding generated patterns that are consistent with patterns in the equilibrium data. The decreases in stability observed for the His, Ile, and Tyr mutants were paralleled by decreases in the relaxation times for unfolding and increases in the relaxation times for refolding for the phase that describes the folding of the predominant native conformer, N_2. The Ala and Cys replacements did not alter the stability or the relaxation times for unfolding and refolding. As was observed in the equilibrium results, unique effects were observed in the kinetics of folding of the tyrosine mutant.

The alterations in the kinetics of folding can be characterized by a formulism previously described (Beasty et al., 1986) and used to characterize the effects of mutations at six other positions in DHFR (Perry et al., 1987). Because this formalism requires that the variant be compared with the wild-type protein for *both* unfolding and refolding kinetic phases, this discussion will focus on the effects on the τ_1 and τ_2 phases of folding. These two phases describe the unfolding/refolding channels of the two native conformers of DHFR, N_1 and N_2 (Figure 7).

As noted above, the His, Ile, and Tyr substitutions decreased the relaxation times of τ_2 for unfolding and increased the relaxation times of τ_2 for refolding. Such effects have been classified as mixed equilibrium–kinetic effects (Beasty et al., 1986) and implicate the given position in participating in a rate-limiting step of protein folding. In contrast to these effects on the τ_2 phase, there are no or only slight changes in the relaxation times of τ_1 for both unfolding and refolding. Two conclusions can be drawn from these results. First, multiple replacements indicate that, when the specific interactions of residue 75 are disrupted, *position* 75 is seen to play a key role in the folding of DHFR. In addition, the differences in the effects on τ_1 and τ_2 imply that the structural differences between N_1 and N_2 may incorporate position 75.

On the basis of the initial examination of mutations in DHFR (Perry et al., 1987), only mutations in the β-sheet appeared to generate kinetic effects. Therefore, it was hypothesized that a slow step in folding of DHFR was limited by the maturation of the β-sheet. Because position 75 is in the edge-strand βD and the two previously studied kinetic mutations are in the other edge strand βG and its neighboring strand βF (Figure 3), this maturation process might be a sheet-wide phenomenon. However, in addition to the study reported here, our laboratory has recently made and studied a mutation near the end of the αF helix, F103I, that produced mixed equilibrium–kinetic effects (B. Finn and C. R. Matthews, unpublished data). Therefore, although the formation/rearrangement of the β-sheet could be a central component, the structural view of the rate-limiting step in the folding of DHFR must include at least part of the αF-helix. We are actively examining the positions that have yielded kinetic variants and the residues near these positions in attempts to better understand the molecular explanation for kinetic effects.

The formulism used to categorize these effects is based upon the transition-state theory and rationalizes the effects on rate constants ($k = \tau^{-1}$) in terms of effects on the free energies of native, intermediate, or transition-state species. Although we cannot determine the absolute free energies of any given species of protein, we can readily identify changes in free energy of a stable species (native or intermediate) relative to the transition-state species. Such changes in the energy of *intermediate* relative to transition state (i.e., changes in rates of *refolding*) are important because these alterations infer that the given position plays a role in the structure of the intermediate. The previous information concerning the structure of the kinetic intermediate in DHFR includes (1) the kinetic phase in fluorescence studies of refolding which describes its formation is opposite in amplitude from the remaining DHFR refolding reactions, and (2) the intermediate does not bind the potent inhibitor methotrexate (Touchette et al., 1986). It was hypothesized that the structure of the intermediate is similar to a hydrophobic aggregate and that the active site is not fully formed. The data presented in this report are consistent with this hypothesis. Mutations that disrupt a tightly packed hydrophobic core in the *native* protein have also significantly altered the free energy of the intermediate (which is thought to have its hydrophobic structure at least partly established) relative to the transition state. Thus, the hydrophobic cluster to which the polypeptide initially collapses during refolding may be a nativelike, well-packed structure close to that which Val-75 helps form in the native protein. Supporting this hypothesis is the lack of effect on τ_1 and τ_2 phases in refolding by the Ala and Cys mutations; i.e., the intermediate structure appears to have the same response to amino acid replacements as does the native structure.

Finally, both of the variants which destabilize the protein and which were studied by stopped-flow techniques, V75H and V75Y, displayed large decreases in the amplitude of the burst phase in refolding. As stated above, this phase has been described as the initial collapse of unfolded DHFR to a hydrophobic aggregate. The unexpected direction in the change of fluorescence intensity was attributed to the change in the environment of the Trp residues from solvent to the interior of the protein during refolding; i.e., the fluorescence of transiently buried indole rings is not quenched by solvent. It was assumed that most, if not all, of the five Trp residues contributed to this signal. However, because of the drastic reduction in the amplitude of τ_5 and because residue 74 is a tryptophan, these results suggest that Trp-74 may be the major, if not sole, contributor to this fluorescence change of τ_5. We are currently constructing the mutant DHFR Trp74 → Phe to investigate this possibility.

Variation in the Patterns in Kinetic Data: V75Y DHFR. As in the equilibrium experiments, the kinetic studies uncovered a difference in the effects of the tyrosine mutation when compared with the effects of the other substitutions. V75Y DHFR was the only variant that showed a significant alteration in the amplitude of τ_2 in unfolding; this alteration was complemented by the appearance of a third phase in unfolding. These data imply that three native conformers exist in V75Y DHFR. Because the log τ of this unfolding phase appears to smoothly connect with that of the refolding phase which is tentatively identified as τ_3, we label this third native conformer N_3. This implies that a nativelike intermediate

previously proposed for wild-type DHFR (possibly I_{N1}, Figure 7) is, in fact, populated in this mutant. Why the tyrosine mutation at position 75 has uniquely altered the equilibrium constant or rate constants for interconversion is not known; however, X-ray data may provide some insight.

Conclusions. We undertook this project with the intention of clarifying the procedure for identifying positions that participate in key steps of protein folding. Multiple replacements at position 75 in DHFR result in an easily understood pattern; this pattern was observed in the effects on both equilibria and kinetics of folding. Replacements of the buried valine with side chains that would either expand a tightly packed hydrophobic core or require hydrogen bonding both destabilize the protein and also affect the relaxation times of folding in a consistent manner. These data implicate position 75 in playing a key role in a rate-limiting step of folding.

Although general trends were observed in the data for the mutants at position 75 in DHFR, the Tyr mutation clearly produced additional alterations that were not present in the data from the other mutants. If the only replacement that was studied had been the tyrosine mutation, one might suggest that the appearance of the third unfolding phase was part of a general pattern. Therefore, we must be cautious in concluding that all effects resulting from a single substitution can be attributed to the *position*; clearly, some effects must be attributed to the *specific* residue that has been inserted. These results emphasize the need to make multiple replacements in probing the role of a given position in the folding mechanism.

Registry No. DHFR, 9002-03-3; Val, 72-18-4; Tyr, 60-18-4; Ala, 56-41-7; Cys, 52-90-4; Ser, 56-45-1; His, 71-00-1; Ile, 73-32-5; Arg, 74-79-3.

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