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Intramolecular Catalysis of a Proline Isomerization Reaction in the Folding of Dihydrofolate Reductase†

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ABSTRACT: The cis/trans isomerization of the peptide bond preceding proline residues in proteins can limit the rate at which a protein folds to its native conformation. Mutagenic analyses of dihydrofolate reductase (DHFR) from *Escherichia coli* show that this isomerization reaction can be intramolecularly catalyzed by a side chain from an amino acid which is distant in sequence but adjacent in the native conformation. The guanidinium NH_2 nitrogen of Arg 44 forms one hydrogen bond to the imide nitrogen and a second to the carbonyl oxygen of Pro 66 in wild-type DHFR. Replacement of Arg 44 with Leu results in a change of the nature of the two slow steps in refolding from being limited by the acquisition of secondary and/or tertiary structure to being limited by isomerization. The simultaneous replacement of Pro 66 with Ala (i.e., the Leu 44/Ala 66 double mutant) eliminates this isomerization reaction and once again makes protein folding the limiting process. Apparently, one or both of the hydrogen bonds between Arg 44 and Pro 66 accelerate the isomerization of the Gln 65–Pro 66 peptide bond. The replacement of Arg 44 with Leu affects the kinetics of the slow folding reactions in a fashion which indicates that the crucial hydrogen bonds form in the transition states for the rate-limiting steps in folding.

There is a growing body of evidence that the cis/trans isomerization of the peptide bond preceding proline residues can limit the rate at which a protein can fold to its native conformation. As originally formulated, the proline isomerization hypothesis (Brandts et al., 1975) held that only the fraction of the unfolded protein with the same set of isomers found in the native conformation can fold directly to native. The remaining populations must first isomerize all of the Xaa–Pro peptide bonds to the native isomeric forms before folding can begin. Mutagenic analyses of bovine pancreatic trypsin inhibitor (BPTI;[1] Hurle et al., 1991), a yeast cytochrome *c* (Wood et al., 1988), ribonuclease T1 (Kiefhaber et al., 1990), and thioredoxin (Kelley & Richards, 1987) all clearly show that specific proline residues are critical to the existence of kinetically distinct folding species. Their removal by site-directed mutagenesis eliminates slow folding phases detected in the wild-type proteins.

However, it has also become clear that not all prolines are essential in folding. The removal of Pro 71, a conserved proline in the cytochrome *c* from yeast, has no significant effect on the slow folding phases (Ramdas & Nall, 1986; White et al., 1987). Ribonuclease A can refold to an enzymatically active, nativelike conformation with one of the proline peptide bonds in the nonnative isomeric form (Schmid & Baldwin, 1978; Schmid, 1981). Levitt (1981) examined this possibility in BPTI and predicted that while some sites in the native conformation would be tolerant of the incorrect isomer, others would not. The difference depended upon the steric strain required to introduce the nonnative isomer into the native conformation. Recent experimental results on the folding of proline mutants in BPTI (Hurle et al., 1991) are consistent with his predictions.

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[1] Abbreviations: BPTI, bovine pancreatic trypsin inhibitor; DHFR, dihydrofolate reductase; K_2EDTA, ethylenediaminetetraacetic acid dipotassium salt; SDS, sodium dodecyl sulfate; R44L, Arg 44 → Leu; P66A, Pro 66 → Ala.

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An interesting aspect of the ribonuclease nativelike intermediate is that the proline isomerization reaction which converts this species to the native species is 40-fold faster than the same reaction in the unfolded protein at 0 °C (Cook et al., 1979). Creighton (1978) considered possible ways in which these isomerization reactions could be accelerated and suggested that intramolecular catalysis, perhaps by adjacent amino acids in the sequence, could be involved. Until now, there has been no specific evidence to support this hypothesis. In this paper, we present mutagenic evidence that a proline isomerization reaction in dihydrofolate reductase (DHFR) is intramolecularly catalyzed by an arginine side chain which is distant in sequence but adjacent in the three-dimensional structure.

Materials and Methods

Protein Source. Construction and isolation of the R44L mutant have been described previously (Perry et al., 1987). The P66A mutant was constructed from plasmid pBF2 (Kuwajima et al., 1991) by oligonucleotide-directed mutagenesis according to the protocol included in the Muta-Gene Phagemid Kit (Bio-Rad). The oligonucleotide 5′-CAGTCAAGCGGGTACCGACGATC-3′, containing a C to G substitution in the first base of the codon for amino acid 66, produces the Pro → Ala mutation. Substitution of C for G in the third base of the codon for amino acid 68 introduces a *Kpn*I restriction site which provides a convenient screen for selection of the mutant. The R44L/P66A double mutant was constructed from plasmid pBF2.P66A using the same oligonucleotide as for the R44L mutant (Perry et al., 1987). Substitution of T for G in the middle base of the codon for amino acid 44 resulted in removal of an *Hae*III restriction site, facilitating selection of the double mutant. For both mutants, the entire DHFR gene was sequenced to confirm the mutations.

Protein Purification. All proteins were purified by the procedure of Baccanari et al. (1975, 1977). Purity of protein samples was confirmed by the observation of single bands on Coomassie-blue-stained SDS and native polyacrylamide gels. Protein concentration was measured by UV absorption spectroscopy at 280 nm using a molar extinction coefficient of 3.11 × 10^4 M^{-1} cm^{-1}. Enzymatic activity was determined as described previously (Hillcoat et al., 1967). Specific activities measured for wild-type and mutant DHFR proteins were wild-type, 70 units mg^{-1}; R44L, 32 units mg^{-1}; P66A, 113 units mg^{-1}; and R44L/P66A, 75 units mg^{-1}.

Spectroscopic Methods. Equilibrium unfolding data from difference UV absorbance spectroscopy were obtained and analyzed according to a two-state transition model as described previously (Garvey & Matthews, 1989). Kinetic unfolding and refolding jumps, initiated by manual mixing techniques, were monitored by UV absorbance spectroscopy and were fit to a sum of exponentials as described previously (Garvey & Matthews, 1989). All experiments were carried out in buffered solutions of 10 mM potassium phosphate, 0.2 mM K_2EDTA, and 1 mM 2-mercaptoethanol, pH 7.8, at a temperature of 15 °C.

Reagents. Oligonucleotides were obtained from the Penn State Biotechnology Institute DNA/Protein Facility. The Sequenase DNA sequencing kit was obtained from U.S. Biochemicals. Ultrapure urea was obtained from Schwarz/Mann and was used without further purification. All other chemicals were of reagent grade.

Results

DHFR from *Escherichia coli* (159 residues; MW = 17 680) is a single subunit protein which has been proposed to fold via four parallel channels to a pair of native conformers or a pair of nativelike intermediates (Figure 1; Touchette et al., 1986). In this model, the two slowest folding reactions correspond to the formation of two native conformers which are highly populated in the absence of denaturant. The slower of these two reactions was designated the τ_1 reaction, and the faster was designated the τ_2 reaction.

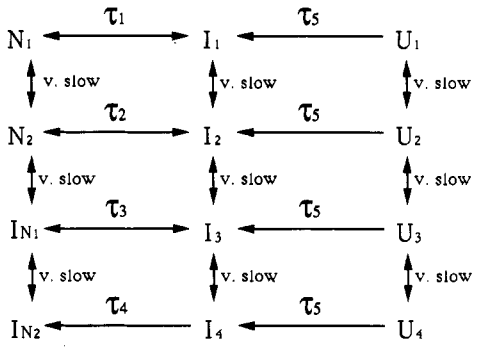

FIGURE 1: Proposed kinetic folding model for DHFR (Touchette et al., 1986).

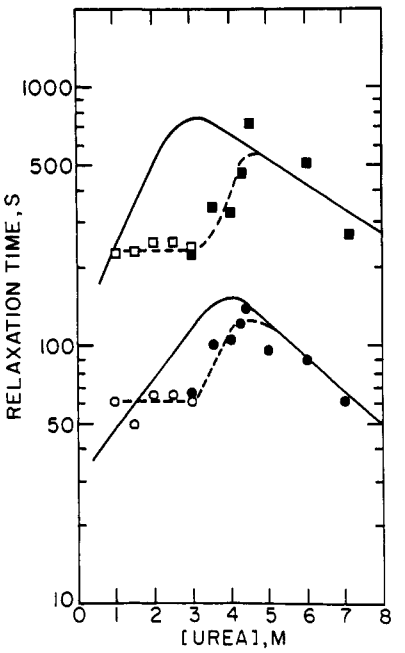

FIGURE 2: A semilog plot of the urea dependence of the relaxation times for the τ_1 (□, ■) and τ_2 (○, ●) folding reactions in R44L DHFR from *E. coli*. Closed symbols show the results of unfolding reactions beginning at 0 M urea and ending at the indicated final urea concentration, while open symbols show refolding relaxation times obtained following unfolding at 5.4 M urea. The solid lines indicate the behavior of wild-type DHFR under the same conditions. Reprinted with permission from Perry et al. (1987). Copyright 1987 American Chemical Society.

An important property of both of these slow phases in the wild-type protein is that the relaxation times in both unfolding and refolding depend upon the denaturant concentration in a fashion expected for reactions limited by protein folding (Matthews, 1987). The relaxation times for unfolding decrease exponentially with *increasing* denaturant concentration while those for refolding decrease exponentially with *decreasing* denaturant concentration. In the unfolding transition zone these relaxation times pass through a maximum and are equivalent, indicating that the process is kinetically reversible (Figure 2).

Previously, a mutation was constructed in DHFR in which Arg 44 was replaced with Leu (R44L; Perry et al., 1987). Although the unfolding relaxation times are not significantly

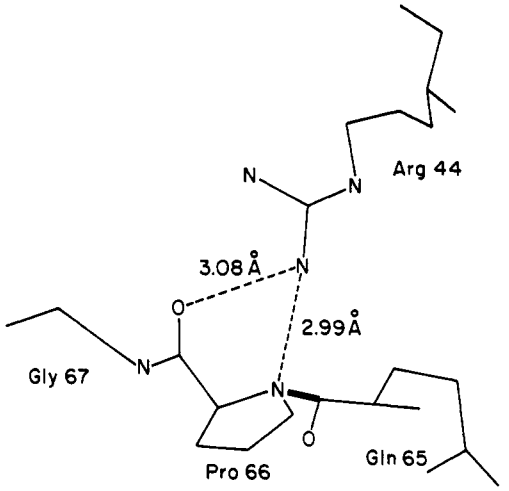

FIGURE 3: A portion of the X-ray crystal structure of ligand-free DHFR (Bystroff & Kraut, 1991) showing the relative positions of residues Arg 44, Gln 65, Pro 66, and Gly 67. Distances from the NH_2 nitrogen of Arg 44 to the imide nitrogen and carbonyl oxygen of Pro 66 are indicated. The bold line represents the Gln 65–Pro 66 peptide bond which is of interest in the present study.

Table I: Stabilities and Transition Midpoints for the Urea-Induced Unfolding of Wild-Type DHFR and Three Mutant Proteins

protein	$\Delta G^{H_2O}{}_{app}{}^a$ (kcal mol^{-1})	$C_m{}^b$ (M, urea)	$-A^c$ [kcal mol^{-1} (M urea)$^{-1}$]
wild type	5.9 ● 0.3	3.1 ● 0.1	1.9 ± 0.1
R44L	7.3 ± 0.4	3.2 ± 0.1	2.3 ± 0.1
P66A	7.2 ● 0.5	3.0 ± 0.1	2.4 ± 0.2
R44L/P66A	6.8 ± 0.6	3.0 ± 0.1	2.3 ± 0.2

[a] Apparent free energy of folding in the absence of urea at pH 7.8 and 15 °C. Errors are 95% confidence intervals from the nonlinear least-squares fits. [b] Midpoint of the urea-induced unfolding transition. The value of C_m is calculated from $C_m = -\Delta G^{H_2O}{}_{app}/A$. The errors were calculated by propagation of errors analysis. [c] The parameter A describes the cooperativity of the unfolding transition. Errors are 95% confidence intervals from the nonlinear least-squares fits.

altered, this mutation has a unique effect on the slow refolding kinetics. Between 4.5 and 3 M urea, both the τ_1 and the τ_2 relaxation times decrease with decreasing urea concentration (Figure 2). Below 3 M urea, however, the relaxation times for both of these reactions become independent of the denaturant concentration. Apparently, the rate-limiting step changes below 3 M urea to a process which, unlike folding, does not depend on the denaturant concentration. Although proline isomerization is a possible candidate, Arg 44 is five residues removed from the closest proline residue, Pro 39. Proline isomerization rates are sensitive to the structure of the preceding side chain but are less so to more distant amino acids (Grathwahl & Wuthrich, 1981).

Examination of the X-ray crystal structure of ligand-free DHFR (Bystroff & Kraut, 1991) showed that the NH_2 nitrogen in the guanidinium moiety of Arg 44 forms one hydrogen bond with the backbone imide nitrogen of Pro 66 and a second with the carbonyl oxygen of this same residue (Figure 3). Note that the first of these hydrogen-bond acceptors is a part of the Gln 65–Pro 66 peptide bond and the second a part of the Pro 66–Gly 67 peptide bond. Reasoning that one or both of these interactions could be catalyzing the isomerization of the Gln 65–Pro 66 peptide bond in the wild-type protein, we constructed the double mutant R44L/P66A. If Pro 66 is indeed responsible for the urea-independent slow folding reactions in R44L, where the guanidinium moiety is absent at position 44, its replacement with an alanine residue in the double mutant should lead to a recovery of denaturant-dependent slow folding reactions.

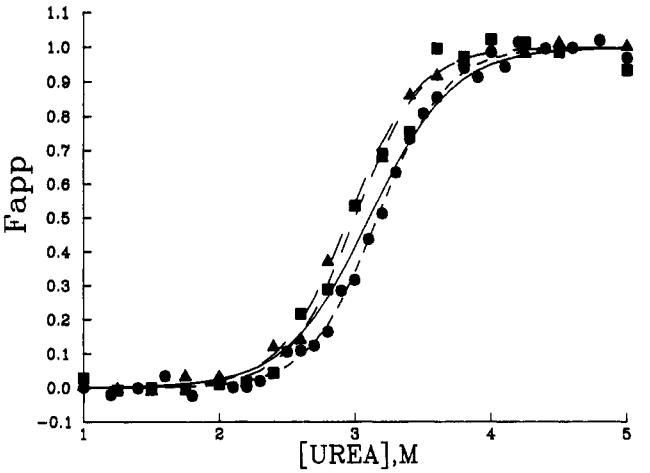

FIGURE 4: Dependence of the apparent fraction of unfolded protein, F_{app}, on the urea concentration for DHFR mutants R44L (●), P66A (■), and R44L/P66A (▲). The solid line represents the urea-dependent unfolding transition for wild-type DHFR.

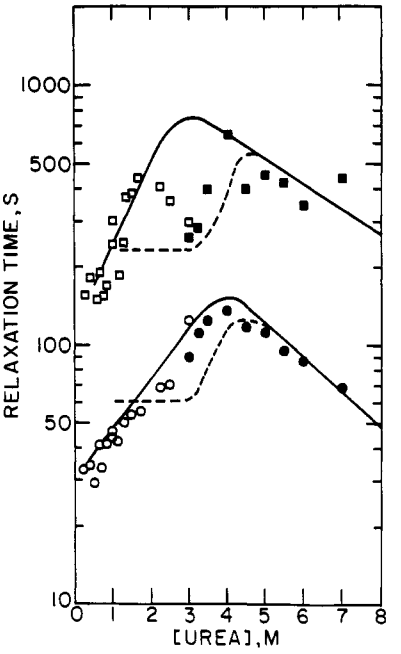

FIGURE 5: A semilog plot of the urea dependence of the relaxation times of the τ_1 (□, ■) and τ_2 (○, ●) folding reactions in R44L/P66A DHFR. Closed symbols show the results of unfolding reactions beginning at 0 M urea and ending at the indicated final urea concentration, while open symbols show refolding relaxation times obtained following unfolding at 5.4 M urea. The solid lines indicate the behavior of wild-type DHFR and the dashed lines the behavior of R44L DHFR depicted in Figure 2.

The equilibrium unfolding transitions for the wild type and R44L, P66A, and R44L/P66A mutants are shown in Figure 4. When these data were fit to a two-state unfolding model (Tanford, 1968), it was found that the effects of the single replacements are to increase the stability by 1.3 (P66A) and 1.5 kcal/mol (R44L; Table I). The double mutation, R44L/P66A, only increases the stability by 0.9 kcal/mol. The nonadditivity of the individual effects on stability is prima facie evidence for interaction between the side chains at positions 44 and 66 (Hurle et al., 1986). It is interesting to note that the increases in the values of the free energy difference in the absence of denaturant for all three mutant proteins are almost solely due to increases in the magnitudes of the cooperativity parameter, A; the midpoints of the transitions are very similar (Table I).

As can be seen in Figure 5, the τ_2 refolding relaxation time in R44L/P66A DHFR depends upon the denaturant concentration in the same fashion as that of the wild-type protein. The τ_1 reaction is more complex. Under strongly unfolding and refolding conditions, i.e., those above 5 M and below 2 M urea, this relaxation time behaves the same as that for the wild-type protein. In the transition zone, the deviation from the inverted "V" behavior is similar to that obtained previously for a slow folding reaction in the α subunit of tryptophan synthase at low temperature (Tweedy et al., 1990). Because those results were successfully modeled by including faster, preceding folding reactions, it is possible that the same phenomenon is occurring in the slowest folding channel of DHFR as well.

Relevant to the issue of catalysis of the isomerization reaction, the relaxation times for both refolding phases in R44L/P66A are 2-fold faster than the value observed in the R44L mutant protein under strongly refolding conditions (0.54 M urea). These results are most easily explained by proposing that the isomerization of the Gln 65–Pro 66 peptide bond can limit the folding of DHFR *only if* the arginine side chain is not present at position 44. If Pro 66 is replaced or if the residue at position 44 is arginine, urea-dependent protein folding is rate limiting. The behavior of the refolding phases in the P66A mutant is quantitatively similar to that of the wild-type DHFR (data not shown).

When does the arginine 44 side chain form the hydrogen bonds with the Pro 66 carbonyl oxygen and imide nitrogen in the wild-type protein? Inspection of the effects of the R44L mutation on the refolding relaxation times between 3 and 4.5 M urea (Figure 2), where both unfolding and refolding rate constants contribute to the relaxation time (Matthews, 1987), shows that these values are considerably shorter than those for the wild-type protein. In contrast, the unfolding relaxation times, i.e., those above 5 M urea, for both phases are identical to those of the wild-type protein within experimental error. Taken together, these observations imply that the refolding reaction in the R44L mutant is selectively accelerated relative to that of the wild-type protein. The conclusion to be drawn from these results is that the transition state for the rate-limiting step in folding in both of these channels resembles the native conformation in the vicinity of the side chain at position 44 (Matthews, 1987). Therefore, the hydrogen bonds which occur in the native conformation of the wild-type protein between Arg 44 and Pro 66 must also be present in the transition state.

Discussion

There are two aspects of the replacement of Arg 44 with Leu on the slow folding kinetics of DHFR which require further consideration.

(1) If the replacement of Arg 44 results in the slow cis/trans isomerization of the Gln 65–Pro 66 peptide bond, what happens to the folding of that fraction of the protein in which this peptide bond was trans in the unfolded form? Studies on the distribution of isomers in proline-containing peptides have shown that both forms are likely to be significantly populated in the unfolded protein, roughly 80% trans and 20% cis (Grathwohl & Wuthrich, 1981; Brandts et al., 1975). Because only the trans form appears at this position in the native conformation of DHFR (Bystroff & Kraut, 1991), the majority of the protein should be limited in folding only by the formation of secondary and tertiary structure, not by isomerization. The answer may lie in the observation that the τ_1 and τ_2 phases only account for 6% and 13%, respectively, of the binding of methotrexate, a tight binding inhibitor of DHFR (Touchette et al., 1986). Taken together, these two phases may represent the folding of that fraction of the unfolded protein which has the cis isomer at Pro 66. Another possibility is that the significant acceleration of the refolding reaction seen for the R44L mutant in Figure 2 means that the population containing the trans isomer folds within the dead time of the manual mixing experiments at low denaturant concentration. Only the cis population is detected in the slow time range.

(2) How can two slow folding steps with different urea-independent relaxation times, i.e., 230 s for τ_1 and 60 s for τ_2 at 0.54 M urea, be limited by the same proline isomerization reaction in R44L DHFR? The factor of 4 difference in these refolding relaxation times may reflect the influence of different local folding in the two channels (Nall et al., 1978). This proposal is supported by measurements of the activation energies of these two urea-independent reactions under refolding conditions. At 2 M urea, the activation energies were found to be 9 and 13 kcal/mol for the τ_1 and τ_2 reactions, respectively (data not shown). These values are less than expected from measurements of proline isomerization reactions in model compounds, 16–20 kcal/mol (Brandts et al., 1975; Grathwohl & Wuthrich, 1981). Coupling between folding and isomerization could decrease the apparent activation energy.

The proposal that the isomerization of the Gln 65–Pro 66 peptide bond can be a limiting reaction in the folding of DHFR can, in principle, be tested by a double-jump experiment (Brandts et al., 1975). In this experiment, the protein is rapidly unfolded, allowed to incubate for various times, and then refolded to the native conformation. With increasing delay times, the fraction of nonnative proline isomer should increase and result in a larger amplitude for the slow refolding phase. Unfortunately, this experiment was not feasible for DHFR because it was not possible to find conditions under which the protein could be unfolded rapidly compared to proline isomerization. The activation energies for unfolding via the τ_1 and τ_2 channels (Touchette et al., 1986) are comparable to that of proline isomerization, precluding the search for an alternative temperature which would discriminate folding and isomerization rates. Solubility problems at acidic pH, chemical damage at alkaline pH, and complexities with chloride ion binding in guanidine hydrochloride induced unfolding eliminate alternative destabilizing conditions for selectively accelerating the unfolding reactions.

Earlier studies on the protonation of amides by strong acids (Berger et al., 1959; Perrin, 1989) provide a possible rationale for the mechanism of catalysis of the peptide bond isomerization by the arginine side chain. Although protonation occurs primarily on the carbonyl oxygen in *N*-methylacetamide, the minor species in which the nitrogen is protonated is responsible for catalyzing the rotation about the C–N bond. The catalysis of proline-containing peptide bonds in proteins by strong acids is well documented (Steinberg et al., 1960; Schmid & Baldwin, 1978). In an analogous manner, the hydrogen bond between the NH_2 nitrogen in the guanidine moiety of Arg 44 and the imide nitrogen in the peptide bond between Gln 65 and Pro 66 in DHFR could serve to accelerate the isomerization reaction by withdrawing electron density from the critical C–N bond (Figure 3).

It is also possible that the hydrogen bond to the Pro 66 carbonyl oxygen, involved in the Pro 66–Gly 67 peptide bond, could assist in this process by stabilizing the trans isomeric form of Gln 65–Pro 66 in the transition state. This putative indirect effect would be propagated through the backbone atoms of the proline residue. Proline is an especially favorable

candidate for such a long-range effect because rotation can only occur around the C–C bond; the pyrrolidine ring prevents rotation about the C–N bond. Grathwohl and Wuthrich (1981) suggested a role for steric strain to account for their observation of a 6-fold acceleration of the rate of proline isomerization in a cyclic pentapeptide compared to the rate in the corresponding linear pentapeptide.

Is the intramolecular catalysis of the proline isomerization reaction by the hydrogen bonds to the imide nitrogen and/or the carbonyl oxygen of the proline likely to be unique or could it have some generality? Examination of the hydrogen-bonding patterns in 42 proteins with high-resolution X-ray structures shows that there are eight examples of side-chain nitrogens in strongly basic residues (Lys, Arg, His) as hydrogen bond donors within 4 Å of the proline nitrogen. There are 23 examples of hydrogen bonds between the carbonyl oxygen of a proline residue and donors in strongly basic side chains (D. S. Stickle and G. D. Rose, personal communication). If the argument is extended to neutral donors, then 12 additional hydrogen bonds to the nitrogen and 22 to the carbonyl oxygen of proline can be found in this same set of proteins. Thus, there are a significant number of candidates for this type of acceleration of the proline isomerization reaction which can be tested by mutagenesis.

The proposal that an arginine side chain can catalyze the cis/trans proline isomerization reaction during protein folding can be compared to an alternative mechanism recently suggested for cyclophilin. This enzyme is the target of the immunosuppressive drug cyclosporin (Handschumacher et al., 1984) and catalyzes the proline isomerization reaction in peptide and protein substrates (Takahashi et al., 1989; Fischer et al., 1989). The X-ray and NMR structures of cyclophilin show that the binding site contains both a histidine and an arginine residue (Kallen et al., 1991). The guanidinium group in Arg 55 forms a hydrogen bond with the carbonyl oxygen of the Ala–Pro peptide bond in a model tetrapeptide. The direct activation of the amide carbonyl by a cationic hydrogen bond to the oxygen (presumably accompanied by the attack of a nucleophile on the carbonyl carbon) appears to be an important aspect of the enzymatic mechanism. The results of the present paper suggest that the acceleration of the proline isomerization reaction can also be achieved by the formation of intramolecular hydrogen bonds with the imide nitrogen and/or carbonyl oxygen in the proline residue. Further studies are required to determine the relative contribution of each to the process.

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Registry No. DHFR, 9002-03-3; Arg, 74-79-3; Pro, 147-85-3.

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