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Cis/Trans Isomerization at Proline: Desolvation and Its Consequences for Protein Folding

Peptide bonds to the nitrogen atom of proline undergo *cis/trans* isomerization slowly, in a process that limits the rates of refolding of many denatured proteins *in vitro*. At 333 K, acetylproline *N*-methylamide undergoes *cis*-to-*trans* isomerization 46-fold more rapidly in toluene than in water, consistent with the idea that the transition state for isomerization is less polar than that for either the *cis* or the *trans* isomer. The catalytic action of peptidyl–prolyl isomerases, whose active sites appear to be relatively nonpolar, may thus be explained at least in part by desolvation. These results imply that protein folding is most likely to be impeded by isomerization at exposed proline residues that remain exposed to solvent in the transition state for refolding, whereas peptidyl–prolyl linkages in a protein's interior, or at a nonpolar protein–protein interface, probably undergo rapid isomerization without the assistance of an external catalyst.

In a slow process that often governs the rate of protein folding (*1*), peptide bonds to the nitrogen atom of proline undergo *cis/trans* isomerization with typical half-times of 0.5–1.0 min in water. Enzymes with peptidyl–prolyl isomerase activity (*2*), which may serve as catalysts for the folding of denatured or newly synthesized polypeptides *in vivo*, include proteins that bind the immunosuppressive drugs cyclosporin A, rapamycin, and FK506 (*3*). In considering how these enzymes might act, it was originally suggested that the reaction might be initiated by addition of a nucleophile at the carbonyl group of the substrate, to generate a tetrahedral adduct in which bond rotation might occur more easily than in the ground state (*2*). Later evidence from kinetic isotope effects (*4*), the insensitivity of the reaction rate to changing pH (*4*), the inhibitory activity of a compound that is bound in a form resembling a twisted substrate (*5*), and the negligible effect of replacing amino acids that might serve as nucleophiles (*6, 7*) appears to render addition–elimination mechanisms unlikely for this enzyme.

If enzyme-catalyzed *cis/trans* isomerization does not involve addition or elimination, then some other mechanism must facilitate rotation of the peptide bond out of its original plane. Peptide bonds are among the most hydrophilic uncharged groups that occur in biological molecules (*8*), and the free energies of solvation of their *cis* and *trans* isomers are similar in magnitude (*9*). Furthermore, peptides to the nitrogen atom of proline are more strongly solvated than noncyclic peptides of similar size (*10*). When either the *cis* or the *trans* isomer is distorted from planarity, the hybridization of its nitrogen atom changes from sp^2 to sp^3, the C–N bond becomes elongated, and the dipole moment is reduced significantly (*11, 12*). Isomerization might therefore be promoted by transfer from water to less polar surroundings as shown in Scheme 1, furnishing one possible mechanism for catalysis by isomerases (*13*). Earlier work had shown that *cis/trans* isomerization of *N,N*-dimethylacetamide occurs more rapidly in cyclohexane than in water (*14*).

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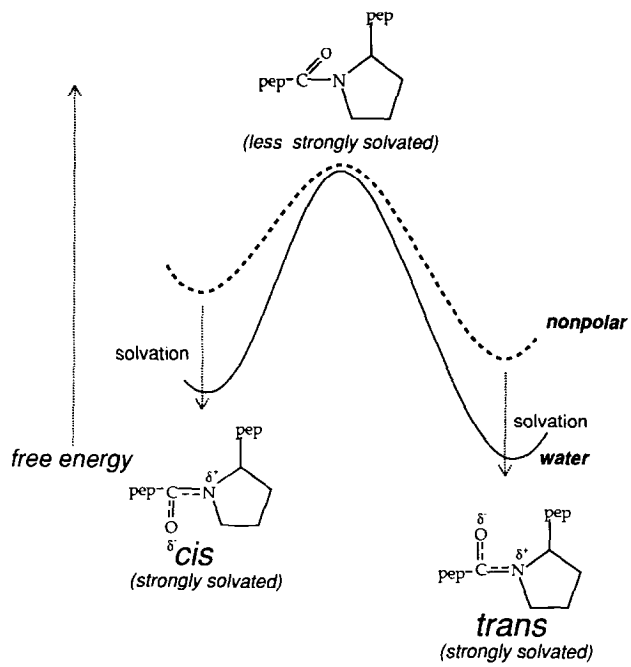

SCHEME 1. Affinity for nonpolar surroundings changes during peptide rotation. By interfering with resonance, bond rotation is expected to reduce the polarity and strength of solvation of a peptide bond by water. Accordingly, the barrier to rotation is expected to be lower in a nonpolar environment (broken line) than in water (solid line).

Thus, it seemed desirable to examine the isomerization properties of proline peptides directly.

To determine whether isomerization of peptide bonds to proline nitrogen is susceptible to catalysis by desolvation, as shown in Scheme 1, we used ^{1}H NMR inversion–transfer experiments to examine equilibria and rates of interconversion between isomers of acetylproline *N*-methylamide in water and in toluene. At 333 K, the temperature at which these experiments were conducted, equilibrium *cis* : *trans* ratios for this compound were 1 : 3 in water and 1 : 15 in toluene (*15*). To observe the rate of interconversion of isomers, one of the NMR signals arising from the *cis* or *trans* isomer was inverted, and the amplitudes of signals from both isomers were then monitored as a function of the time that had elapsed since inversion (*16*). In water, signals from the *N*-methyl protons were suitable for this purpose, whereas in toluene, signals from the α-proton proved to be more useful. Rate constants were then obtained by numerical integration of differential equations describing the time dependence of signal amplitudes for a two-site chemical exchange process, using the program NONLIN84 (*17*).

Overall rate constants observed for isomerization at 333 K were 0.59 ± 0.04 s^{-1} in D_2O and 21.3 ± 13 s^{-1} in D_8-toluene. In toluene, the rapid interconversion of isomers, together with the low abundance of the *cis* isomer (*18*), resulted in a

TABLE 1

Rate Constants for *Cis/Trans* Isomerization of Acetylproline *N*-methylamide, 333 K

Solvent	$k_{cis \rightarrow trans}$	$k_{trans \rightarrow cis}$	$k_{overall}$	Equilibrium constant
Water[a]	0.44 s^{-1}	0.15 s^{-1}	0.59 ± 0.04 s^{-1}	(*Trans*)/(*cis*) = 3
Toluene[b]	20 s^{-1}	1.33 s^{-1}	21 ± 13 s^{-1}	(*Trans*)/(*cis*) = 15
Toluene rate enhancement	46-fold	9-fold		

[a] In D_2O, the *N*-methyl proton was monitored at 2.81 ppm for the *cis* isomer and 2.76 ppm for the *trans* isomer, downfield from a TSP reference.

[b] In toluene-D_8, the proton on the α-carbon of proline was monitored at 3.88 ppm for the *cis* isomer and 4.82 ppm for the *trans* isomer, using the residual methyl protons of toluene at 2.09 ppm as a reference.

substantial experimental error. These rate ratios should therefore be regarded as semiquantitative, although this does not affect the general conclusions drawn below. In order to estimate the individual rate constants for *cis*-to-*trans* and *trans*-to-*cis* conversion, overall rate constants in water and in toluene were combined with the equilibrium constants observed in these solvents, using the following conventional equations for a first order process:

$$k_{overall} = k_{cis \rightarrow trans} + k_{trans \rightarrow cis}$$

$$K_{eq}(cis \rightarrow trans) = (k_{cis \rightarrow trans})/(k_{trans \rightarrow cis})$$

Table 1 shows that when acetylproline *N*-methylamide was transferred from aqueous solution to toluene, its rate of *cis*-to-*trans* isomerization was enhanced by a factor of approximately 46, whereas the rate of the reverse process was enhanced by a factor of 9. As noted above, this difference in rate enhancements is associated with a difference in the abundance of the *trans* isomer at equilibrium.

The active sites of peptidyl–proline isomerases have been found to be lined with nonpolar aliphatic and aromatic residues (*19*), for which solvent toluene may furnish a reasonable model (*20*). The present findings support the possibility that isomerase catalysis can be explained, at least in part, by stabilization of the distorted substrate relative to the more polar ground states, at a nonpolar active site. The half-time for refolding of denatured proteins, limited by isomerization of a single peptide bond to proline, appears typically to be in the neighborhood of 50 s (*1*). A nonpolar environment, if it produced a rate enhancement of ca. 50-fold (similar to that observed in the present experiments), would reduce the half-time for isomerization to roughly 1 s, eliminating any serious impediment to *cis/trans* isomerization at proline nitrogen (*21*). Such a nonpolar environment could be furnished by a region of contact with a peptidyl–prolyl isomerase, another member of a multiprotein complex, or a chaperonin.

The relative rate constants in Table 1 lead to more specific inferences about the environment in which rate-limiting proline residues are likely to be found. Since most proline residues are expected to be in the *trans* configuration in the unfolded state, those that remain solvated in the transition state for isomerization, but

appear in the *cis* configuration in the native protein, seem especially likely to retard folding (*22*). The reverse process, involving conversion of exposed *cis* to *trans* isomers, is inherently more rapid but is also likely to limit the rate of folding of some proteins: *trans* residues are especially abundant in folded proteins, and some of these are expected to have been in the *cis* configuration in the unfolded structure (*23*). Both of the above processes involve residues that were initially exposed to solvent, and presumably accessible to the approach of peptidyl–prolyl isomerases.

Finally, it should be noted that a nonpolar environment, conducive to isomerization, could also be furnished by the interior of the very protein molecule in which isomerization occurs. Thus, acylproline linkages that are inaccessible to isomerases because they are buried in the relatively nonpolar environment of a protein's interior, or at a nonpolar protein–protein interface, seem likely to be capable of isomerizing relatively easily. For peptide bonds of this kind, assistance from an external catalyst is probably unnecessary.

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and 1 : 18 in cyclohexane. In nonpolar solvents, the carbonyl oxygen of the *trans* form of peptidyl-prolyl amide is probably H-bonded intramolecularly to the next peptidic hydrogen in a γ-turn, accounting for its greater thermodynamic and kinetic stability [HUNSTON, R. N., GEROTHANASSIS, I. P., AND LAUTERWEIN, J. (1985) *J. Am. Chem. Soc.* **107,** 2654–2661]. Esters, unlike peptides, are devoid of peptidic hydrogen and cannot form a γ-turn type H-bond and their *cis/trans* ratios appear to be relatively insensitive to solvent polarity [EBERHARDT E. S., LOH S. N., HINCK A. P., AND RAINES R. T. (1992) *J. Am. Chem. Soc.* **114,** 5437–5439].

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21. In a preliminary communication, that appeared while this paper was being written, a similar but somewhat smaller rate enhancement was reported for *cis/trans* isomerization of Ac–Gly–Pro methyl ester (EBERHARDT E. S., LOH S. N., HINCK A. P., AND RAINES R. T. (1992) *J. Am. Chem. Soc.* **114,** 5437–5439).
22. For example, mutation experiments indicate that conversion of exposed *cis* Pro-76 to Ala eliminates a slow phase in the refolding of thioredoxin [KELLEY, R. F., AND RICHARDS, F. M. (1987) *Biochemistry* **26,** 6765–6774]. Likewise, conversion of exposed *cis* Pro-55 to Asp reduces the rate of refolding of RNase T1 by a factor of 10 [KIEFHABER, T., AND SCHMID, F. X. (1992) *J. Mol. Biol.* **224,** 231–240].
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ANNA RADZICKA*,[1]
SCOTT A. ACHESON†
RICHARD WOLFENDEN*

* *Department of Biochemistry and Biophysics, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599-7260; and* † *Pacific Hemostasis, 11515 Vanstory Drive, Huntersville, North Carolina 28078*

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[1] To whom correspondence should be addressed.