Intramolecular Catalysis of the Cis−Trans Isomerization of Proline Peptide Bonds in Cyclic Disulfide-Containing Peptides

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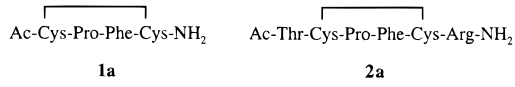
The peptide bond normally exists exclusively in the trans-conformation, the most common exception being the Xaa-prolyl peptide bond. Because of the pyrollidine ring, the energy difference between the cis and trans isomers is smaller for the Xaa-prolyl peptide bond, and both are normally populated in small peptides and denatured proteins.[1] Cis−trans isomerization is a slow process due to the partial double bond character of the Xaa-Pro peptide bond,[2,3] one consequence of this being that cis−trans isomerization of Xaa-prolyl peptide bonds can be the slow step in the folding of proline-containing proteins.[4−6]

Peptidyl prolyl isomerases (PPIases), including the FK506 binding proteins (FKBPs) and cyclophilin, catalyze *cis−trans* isomerization of the Xaa-prolyl peptide bond in proteins.[7−9] It has been proposed on the basis of theoretical studies that catalysis by the FKBPs results, in part at least, from the binding of substrate protein in the active site of FKBP in a type VIa proline turn which brings the prolyl imide nitrogen into close proximity with the N−H proton of the next peptide bond.[10] As the Xaa-Pro peptide bond is twisted and the imide nitrogen lone pair shifts from a p_z orbital to an sp^3 orbital, the pyramidalized imide nitrogen forms a hydrogen bond with the NH proton.[3] The result is a lowering of the relative energy of the transition state and an increase in the rates of both cis-to-trans and trans-to-cis isomerization.

We report here that the rates of cis-to-trans and trans-to-cis isomerization of the Cys-Pro peptide bonds of peptides **1a** and **2a** are significantly accelerated, and we propose that the rate enhancement provides experimental evidence for the intramolecular hydrogen bonding mechanism proposed for FKBP-catalyzed cis−trans isomerization of the Xaa-Pro peptide bond in proteins. Between 5 and 12% of peptides **1a** and **2a**, and their



1a **2a**

acyclic dithiol forms (**1b** and **2b**, respectively), exist as the cis conformation across the Cys-Pro peptide bond, as determined from the relative intensities of resonances for the cis and trans isomers in their ^{1}H NMR spectra.[11] Isomerization equilibrium constants (K = [trans]/[cis]) are reported in Table 1.

Rate constants for cis-to-trans (k_{ct}) and trans-to-cis (k_{tc}) isomerization were determined by the inversion-magnetization transfer method for peptides **1a**, **1b**, **2a**, and **2b**.[12,13] Inversion-magnetization transfer data sets are presented in Figure 1 for peptides **2a** and **2b**. The inversion-transfer data for **2b** are normal in that an elevated temperature is necessary to observe magnetization transfer.[14] However, the rates of cis−trans isomerization are considerably faster for the cyclic peptide, i.e., incorporation of the proline into the cyclic tetrapeptide sequence has the unexpected effect of causing the rate of cis−trans isomerization to increase. Rate constants were derived from the dependence of the intensity of the cis resonance on mxing time,[13] for data measured over a range of temperatures. Activation enthalpies and entropies were calculated using the Eyring equation. The results are summarized in Table 1.

The results indicate that, at 25 °C, k_{ct} is 10 times larger for peptide **1a** than for **1b**, k_{tc} is 7 times larger, and $\Delta H^{\ddagger}_{ct}$ is less by 2.63 kcal/mol. Likewise, k_{ct} is 8 times larger for peptide **2a** than for **2b**, k_{tc} is 4.2 times larger, and $\Delta H^{\ddagger}_{ct}$ is less by 2.87 kcal/mol.[15] For comparison, k_{ct} is 0.042 and 0.067 s^{-1} for rotation around the Cys^6-Pro peptide bonds of oxytocin and vasopressin, respectively, while k_{tc} is 0.0035 and 0.0046 s^{-1}.[14] Both oxytocin and vasopressin are cyclic disulfide-containing peptides; however, the Cys^6-Pro peptide bond is located in the acyclic part of each peptide. The cis−trans isomerization rate constants for the acyclic peptides **1b** and **2b** are very similar to those for oxytocin and vasopressin, which indicates that their rates are normal for Cys-Pro peptide bonds, while those for **1a** and **2a** are significantly accelerated. The activation entropies for all four peptides are close to zero, which suggests there is no large change in the interactions between peptide and solvent on going from the ground state to the activated complex for either the acyclic or cyclic peptides.

To determine the source of the rate enhancements for cis−trans isomerization of the Cys-Pro peptide bond in the cyclic peptides, molecular mechanics calculations were carried out on peptides **1** and **2** in both their disulfide and dithiol forms. The structures obtained from Monte Carlo molecular mechanics simulations indicate that the disulfide bond serves to align the prolyl imide nitrogen with the N−H proton of Phe in both the cis and trans isomers. To illustrate, the structures obtained for the cis and trans isomers of **1a** are shown in Figure 2.[16−18] In both the cis and trans isomers, the NH proton of Phe is positioned

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(11) ^{1}H NMR spectra indicate an equilibrium between two conformations for all four peptides. The two conformations were identified as the cis and trans isomers with respect to the conformation across the Cys-Pro peptide bond. Resonances were assigned to the cis and trans isomers by procedures described previously: Larive, C. K.; Rabenstein, D. L. *J. Am. Chem. Soc.* **1992**, *114*, 7331−7337. Assignment of the less abundant isomers to peptides having the cis conformation across the Cys-Pro peptide bond was established by observation of Cys(C_αH)-Pro(C_αH) dipolar cross-peaks in ROESY spectra. Assignment of the more abundant isomers to peptides having the trans conformation was established by observation of Cys(C_αH)-Pro($C_\delta H_2$) dipolar cross-peaks in ROESY spectra.

(12) The trans resonance of a given cis−trans pair of resonances was selectively inverted with the pulse sequence: $90^o_x-\tau-90^o_x-t-90^o\pm x,\pm y$-acquisition, where τ is a fixed delay equal to $1/(2|\upsilon_{cis} - \upsilon_{trans}|)$ and t is a variable delay during which transfer of magnetization occurs by interchange between the cis and trans isomers. The inversion-transfer method used here is described in the following: Robinson, G.; Kuchel, P. W.; Chapman, B. E.; Doddrell, D. M.; Irving, M. G. *J. Magn. Reson.* **1985**, *63*, 314−319.

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(15) The intramolecular hydrogen bond in the proposed mechanism for FKBP-catalyzed Xaa-Pro cis−trans isomerization in proteins is estimated to lower the energy barrier by 1.4 kcal/mol.[3]

(16) The structure of the trans isomer in Figure 2 is quite similar to crystal structures reported for the trans isomers of the disulfide forms of Ac-Cys-Pro-Ser-Cys-NHMe and Ac-Cys-Pro-Val-Cys-NHMe. Falconer, C. M.; Meinwald, Y. C.; Choudhary, I.; Talluri, S.; Milburn, P. J.; Clardy, J.; Scheraga, H. A. *J. Am. Chem. Soc.* **1992**, *114*, 4036−4042.

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Table 1. Equilibrium Constants, Rate Constants, and Activation Parameters for Cis−Trans Isomerization in 90% H_2O/10% D_2O at pH 3.0[a]

peptide	K[b]	k_{ct}/s^{-1}	k_{tc}/s^{-1}	$\Delta H^{\ddagger}_{ct}$ (kcal mol^{-1})	$\Delta S^{\ddagger}_{ct}$ (cal K^{-1} mol^{-1})
1a	9.8 ± 0.2	0.43 ± 0.03	0.042 ± 0.004	18.6 ± 0.7	2 ± 2
1b	7.3 ± 0.3	0.043 ± 0.004[c]	(6.0 ± 0.8) × 10^{-3} [c]	21.3 ± 1.2	6 ± 4
2a	17.7 ± 0.3	0.48 ± 0.17	0.03 ± 0.01	18.2 ± 0.5	1 ± 2
2b	10.4 ± 0.2	0.059 ± 0.006[c]	(7.1 ± 0.8) × 10^{-3} [c]	21.0 ± 0.5	6 ± 2

[a] The rate and equilibrium constants are for 25 °C. [b] K = [trans]/[cis]. [c] The values were extrapolated using the activation parameters obtained from rate data measured at higher temperatures.

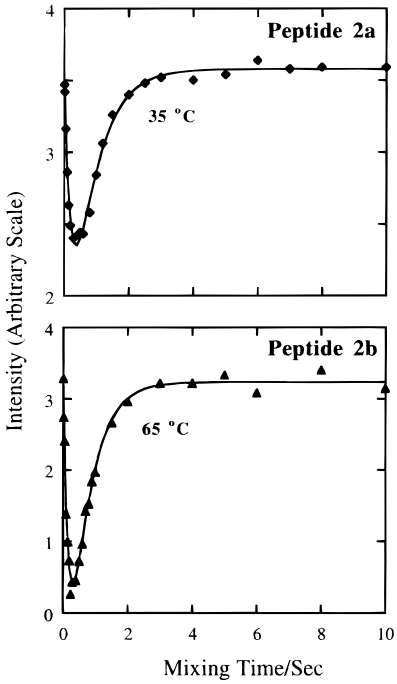

Figure 1. Integrated intensities of the resonances for the Phe N−H and the Thr γ-CH_3 protons of the cis isomers for peptides **2a** and **2b**, respectively, as a function of the mixing time t. The smooth curves were obtained by nonlinear least-squares analysis of the data.

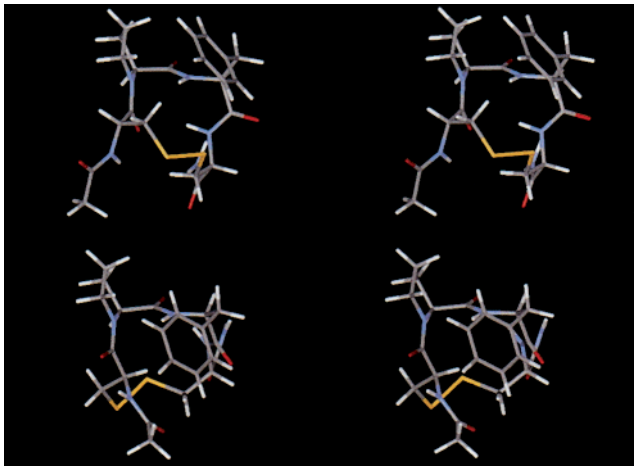

Figure 2. Stereoviews of the structures obtained for the trans (top) and cis (bottom) isomers of **1a** from Monte Carlo molecular mechanics simulations.

so that it can hydrogen bond to the sp^3 lone pair of the pyramidalized proline nitrogen in the transition state. Thus, we propose the rate enhancement is by the same intramolecular catalytic mechanism that has been proposed for FKBP-bound substrate protein.[10,19] However, in the case of peptides **1a** and **2a**, the positioning of the prolyl imide nitrogen adjacent to the NH proton of the next amino acid is a result of constraints imposed on the peptide backbone by the disulfide bond and not by an enzyme. Nevertheless, these results provide experimental evidence in support of the proposal that intramolecular hydrogen bonding lowers the activation energy of the FKBP-catalyzed cis-trans isomerization of the Xaa-prolyl peptide bond in proteins.[10]

Peptides **1** and **2** are being studied as models for the active site of thioltransferase (TTase), an enzyme that catalyzes the formation of enzyme−glutathione mixed disulfides to modulate enzyme activity.[20] TTase is a member of a family of oxidoreductase enzymes, which have in common the Cys-Xaa-Xaa-Cys active site motif.[21] It has been proposed, on the basis of NMR solution structures, that functional differences between the oxidized and reduced forms of *E. coli* thioredoxin, also an oxidoreductase with the Cys-Xaa-Xaa-Cys active site motif, are related to differences in conformational flexibility in and near the active site loop of the oxidized form.[22] The findings reported here suggest this might include an increase in the conformational flexibility of the peptide backbone at the active site when it is in the cyclic disulfide form.

In conclusion, we have found that the rates of cis−trans isomerization by rotation around the Cys-Pro peptide bond are unexpectedly fast for the cyclic peptides **1a** and **2a**. This is the first report of an enhancement of the rate of cis−trans isomerism as a result of conformational constraints imposed by a disulfide bond. We predict that this effect will be present in some other proline-containing peptides with small loops formed by intramolecular disulfide bonds and may be important as a mechanism for increasing the conformational flexibility of the peptide backbone. Further studies are in progress on the kinetics of cis−trans isomerization of other proline-containing peptides, including model peptides for other oxidoreductase enzymes with the Cys-Xaa-Xaa-Cys active site motif as well as cyclic disulfide-containing peptide hormones and toxins.

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Supporting Information Available: NMR spectra which prove the existence of the cis isomer of peptide **1b**, inversion-magnetization-transfer NMR data for **1a** and **1b**, and Eyring plots for **1a**, **1b**, **2a** and **2b** (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(17) The molecular mechanics simulations show that **1b** and **2b** interconvert among an ensemble of random conformations.

(18) The Monte Carlo molecular mechanics simulations were performed using Macromodel V6.5 and the OPLS force field. A 10 000 structure conformational search was performed, with each structure minimized by PRCG. The solvent for simulation was water.

(19) Intramolecular catalysis of cis-to-trans isomerization by an intramolecular N−H···N hydrogen bond to the imide nitrogen has also been demonstrated with a series of acyl−prolyl−amide model compounds. However, the rate of trans-to-cis isomerization is inhibited due to a shift of the hydrogen bond to the oxygen of the amide bond in the trans isomer. The model compounds were studied in organic solvents to mimic the desolvated environment of the FKBP active site and to minimize interferences from the formation of hydrogen bonds to water. (a) Cox, C.; Young, V. G., Jr.; Lectka, T. *J. Am. Chem. Soc.* **1997**, *119*, 2307−2308. (b) Cox, C.; Lectka, T. *J. Am. Chem. Soc.* **1998**, *120*, 10660−10668.

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