Inductive Effects on the Energetics of Prolyl Peptide Bond Isomerization: Implications for Collagen Folding and Stability

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Abstract: The hydroxylation of proline residues in collagen increases the stability of the collagen triple helix. Previous X-ray diffraction analyses had demonstrated that the presence of an electron-withdrawing substituent on the pyrrolidine ring of proline residues has significant structural consequences [Panasik, N., Jr.; Eberhardt, E. S.; Edison, A. S.; Powell, D. R.; Raines, R. T. Int. J. Pept. Protein Res. 1994, 44, 262−269]. Here, NMR and FTIR spectroscopy were used to ascertain kinetic and thermodynamic properties of N-acetyl-[β,γ-13C]p-L-proline methyl ester (1); N-acetyl-4(R)-hydroxy-l-proline [13C]methyl ester (2); and N-acetyl-4(R)-fluoro-l-proline methyl ester (3). The pKₐ's of the nitrogen atom in the parent amino acids decrease in the following order: proline (10.8) > 4(R)-hydroxy-l-proline (9.68) > 4(R)-fluoro-l-proline (9.23). In water or dioxane, amide I vibrational modes decrease in the following order: 1 > 2 > 3. At 37 °C in dioxane, the rate constants for amide bond isomerization are greater for 3 than 1. Each of these results is consistent with the traditional picture of amide resonance coupled with an inductive effect that results in a higher bond order in the amide C=O bond and a lower bond order in the amide C=N bond. Further, at 37 °C in water or dioxane equilibrium concentrations of the trans isomer increase in the order: 1 < 2 < 3. Inductive effects may therefore accelerate the folding and enhance the stability of collagen, which has a preponderance of hydroxyproline residues, all with peptide bonds in the trans conformation.

Introduction

Collagen is the principle structural protein in vertebrates.1,2 In vivo, structural collagen consists of three polypeptide chains that form an extended right-handed triple helix.3 Each polypeptide chain contains approximately 300 repeats of the sequence, Gly–Xaa–Yaa, in which Xaa and Yaa are often L-proline (Pro) and 4(R)-hydroxy-l-proline (Hyp) residues, respectively. The hydroxylation of Pro residues is a post-translational modification catalyzed by the enzyme prolyl 4-hydroxylase. Defects in prolyl 4-hydroxylase activity have been associated with the aging process as well as a variety of diseases including arthritis and rheumatism.4,5

The biosynthesis of collagen has been studied extensively.6–8 Collagen strands are synthesized as propeptides in which the pre sequence targets the polypeptide to the Golgi complex and there is removed by a protease. Three procollagen propeptides then become covalently crosslinked through interstrand disulfide bonds within the pro region. The crosslinked chains are subjected to a variety of post-translational modifications before cleavage of the propeptide region and secretion into the extracellular matrix, where the chains fold into a triple helix. Of these modifications, the hydroxylation of proline residues by prolyl 4-hydroxylase is the most prevalent, as Hyp constitutes approximately 10% of all collagen residues.

Numerous in vitro studies with procollagen and model peptides have explored the role of Hyp in the folding and stability of collagen.9 Procollagen polypeptides that are deficient in Hyp can form triple helices, but these triple helices are unstable at room temperature.9,10 Thermal denaturation studies have demonstrated that triple helix stability correlates with both overall Hyp content and Hyp position within the polypeptide.11 Further, studies on model peptides suggest that peptide conformation is significantly affected by the hydroxylation of proline residues.12,13

Several models have been proposed in which Hyp mediates collagen stability by orienting water molecules to form interstrand hydrogen bonds.14,15 In these models, no interstrand hydrogen bonds form directly between the hydroxy group of Hyp residues and any heteroatoms in the adjacent polypeptide.14,15 Numerous in vitro studies with procollagen and model peptides have explored the role of Hyp in the folding and stability of collagen.9 Procollagen polypeptides that are deficient in Hyp can form triple helices, but these triple helices are unstable at room temperature.9,10 Thermal denaturation studies have demonstrated that triple helix stability correlates with both overall Hyp content and Hyp position within the polypeptide.11 Further, studies on model peptides suggest that peptide conformation is significantly affected by the hydroxylation of proline residues.12,13

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contribute greatly to collagen stability. In most peptide bonds, the trans (Z) isomer is greatly favored over the cis (E) isomer. In contrast, the trans isomer of a prolyl peptide bond is only slightly favored over the cis isomer. The interconversion of cis and trans isomers about prolyl peptide bonds has been identified as the rate-limiting step in protein folding pathways, including that of collagen. This attribute of collagen is not surprising because all peptide bonds in triple-helical collagen reside in the trans conformation. Moreover, the enzyme peptidyl-4′-prolyl cis-trans isomerase (PPlase), which catalyzes the cis–trans interconversion of prolyl peptide bonds, accelerates the proper assembly of collagen molecules.

The objective of this study is to determine the energetic consequences for the prolyl peptide bond of having an electron-withdrawing group in the 4(R) position of the pyrrolidine ring. Accordingly, we synthesized derivatives of proline having a hydroxyl or fluoro group at this position. We used these derivatives to determine the effect of electron withdrawal on (i) the pKa of the prolyl nitrogen, (ii) the amide I vibrational mode of a prolyl peptide bond, and (iii) the kinetics and thermodynamics of prolyl peptide bond isomerization. The results have significance for understanding the folding and stability of collagen.

Results

Three proline derivatives were synthesized for this study: N-acetyl-[β,y-13C]-L-proline methyl ester (1; Ac-Pro-OMe); N-acetyl-4(R)-hydroxy-L-proline [13C]methyl ester (2; Ac-Hyp-OMe); and N-acetyl-4(R)-fluoro-L-proline methyl ester (3; Ac-Fp-OMe) (Chart 1). The chirality of 2 is that found in natural collagen. The synthesis of 3 as the methyl ester avoids intramolecular hydrogen bonding, as had been observed in N-acetyl-L-proline and N-acetyl proline N-methylamide. Compounds 1 and 2 were enriched with 13C to improve the precision of data from 13C NMR spectroscopy. Compound 3 was synthesized because fluorine is more electronegative than is an oxygen and should therefore enhance any consequences of electron-withdrawal. Compound 3 was analyzed by 19F NMR spectroscopy.

Table 1. Summary of X-ray Diffraction Analyses

<table>
<thead>
<tr>
<th>compd</th>
<th>pyrrolidine ring pucker</th>
<th>peptide bond isomer</th>
<th>pyramidalization (δ/deg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>C-endo</td>
<td>cis</td>
<td>1.05</td>
</tr>
<tr>
<td>2a</td>
<td>C-exo</td>
<td>trans</td>
<td>−9.24</td>
</tr>
<tr>
<td>2b</td>
<td>C-exo</td>
<td>trans</td>
<td>−1.52</td>
</tr>
<tr>
<td>3a</td>
<td>C-exo</td>
<td>trans</td>
<td>−16.31</td>
</tr>
<tr>
<td>3b</td>
<td>C-exo</td>
<td>trans</td>
<td>−3.54</td>
</tr>
</tbody>
</table>

*From ref 29. 1° The unit cells of crystalline 2 and 3 contain two molecules. In crystalline 2, the hydroxyl group of molecule 2a donates a hydrogen bond to the amide oxygen of molecule 2b. 2° The parameter δ refers to the angle that the amide C=N bond makes with the plane defined by N, Cα, and Cβ of the pyrrolidine ring.

Previously, we used X-ray diffraction analysis to determine the structure of unlabeled derivatives of 1, 2, and 3 (Table 1). These structures indicated that the presence of electron-withdrawing substituents has significant structural consequences on the pyrrolidine ring. First, the peptide bond is cis and the pyrrolidine ring has C3 endo pucker in 1; the peptide bond is trans and the pyrrolidine ring has C3 exo pucker in 2 and 3. Second, the nitrogen becomes increasingly pyramidalized as the electron-withdrawing ability of the substituent is stronger. This increase in pyramidalization is consistent with an increase in the sp3 character of the prolyl nitrogen. Finally, although no difference was detected in bond lengths within the amide group, the C–C bonds adjacent to the electron-withdrawing substituent were shortened significantly.

Inductive Effect on pKₐ. Changes in pKₐ are consistent with an inductive effect. Such effects on the nitrogen of a proline ring are evident in the previously determined pKₐ’s of L-proline (10.64) and 4(R)-hydroxy-L-proline (9.66). The lower pKₐ of Hyp suggests that the hydroxyl group on the pyrrolidine ring is withdrawing electron density from the secondary amino group. Here, the pKₐ’s of the parent amino acids of 1, 2, and 3 were determined by monitoring the pH-dependencies of 1H chemical shifts. The pKₐ’s of 1-proline, 4(R)-hydroxy-L-proline, and 4(R)-fluoro-L-proline are 10.8, 9.68, and 9.23, respectively. This trend is similar to that observed for ethylamine (10.63), ethanolamine (9.50), and 2-fluroethanolamine (8.79) and is consistent with the manifestation of an inductive effect.

Inductive Effect on the Amide I Vibrational Mode. Changes in the frequency of vibrational modes can provide evidence for an inductive effect. The frequency of the amide I vibrational mode, reports on the C=O bond order. In D2O, the amide I vibrational modes of 1, 2, and 3 are maximal at 1608.10, 1613.08, and 1616.02 cm⁻¹, respectively (Figure 2). In dioxane, the amide I vibrational modes of 1, 2, and 3 are maximal at 1658.99, 1660.92, and 1664.78 cm⁻¹, respectively. Thus in both solvents, the C=O bond order appears to increase in the order: 1 < 2 < 3. This apparent


(31) The error in these values is ±0.10, which is the error in measuring pH.


(35) The solvent effect of approximately 50 cm⁻¹ is consistent with those observed for other amides. For references, see: Reichardt, C. Solvents and Solvent Effects in Organic Chemistry; VCH: New York, 1988; pp 313–319.
inductive effect on the amide I vibrational mode is similar to that observed in the C=O stretching frequency of 4-substituted camphors. 39

**Inductive Effect on** $k_{EZ}$ **and** $k_{ZE}$. The traditional picture of amide resonance predicts that an increase in C=O bond order is accompanied by a decrease in C=N bond order. 38 Such a decrease in C=N bond order would facilitate cis→trans isomerization of the amide bond. In contrast, ab initio calculations suggest that little change in C=O bond order accompanies isomerization in the gas phase. 39–41 These calculations do not yet lead to a consensus about the C=O bond order when molecules of solvent are included. 42–45 We had observed that for peptides similar to 1–3 the barrier to isomerization (ΔG$^\ddagger$) correlates with the frequency of the amide I vibrational mode (ν). 26,46 as predicted from the traditional view. To search for an inductive effect on the rate of cis→trans prolyl peptide bond isomerization (eq 1), we measured these rates for 1 and 3 in dioxane 47 and 1 and 2 in water 48 by using inversion transfer NMR spectroscopy.

$$\text{cis} \rightarrow \text{trans}$$

![Figure 1](image)

**Figure 1.** (a) Amide I vibrational modes of 1 ($ν_{max} = 1658.99$ cm$^{-1}$), 2 ($1660.92$ cm$^{-1}$), and 3 ($1664.78$ cm$^{-1}$) in dioxane. (b) Amide I vibrational mode of 1 ($ν_{max} = 1608.10$ cm$^{-1}$), 2 ($1613.08$ cm$^{-1}$), and 3 ($1616.02$ cm$^{-1}$) in D$_2$O.

The effects of temperature on the cis→trans rate constant ($k_{2q}$) and the trans→cis rate constant ($k_{2z}$) are illustrated by Eyring plots in Figure 2. Values for ΔH$^\ddagger$ and ΔS$^\ddagger$ (± SE) were calculated from linear least-squares fits of the data in these plots to eq 2. 49

$$\ln[k(T)/(sK)] = \frac{-\Delta H^\ddagger}{R} \frac{1}{T} + \frac{\Delta S^\ddagger}{R}$$

Figure 2. (a) Eyring plots of the cis-to-trans isomerization of 1 (○) and 3 (●), and the trans-to-cis isomerization of 1 (●) and 3 (●) in dioxane. (b) Eyring plots of the cis-to-trans isomerization of 1 (○) and 2 (□), and the trans-to-cis isomerization of 1 (●) and 2 (■) in 0.10 M sodium phosphate buffer, pH 7.2.
\[
\ln(k/T) = (-\Delta H^\ddagger/RT) + \Delta S^\ddagger/\gamma + \ln(k_0/\hbar)
\] (2)

where \( R \) is the gas constant, \( k_B \) is the Boltzman constant, and \( h \) is Planck’s constant. The values of these activation parameters are listed in Table 2. Because \( k_{EZ} \) and \( k_{ZE} \) are lowered in protic solvents by the formation of hydrogen bonds to the oxygen of the prolyl peptide bond,26,30,46 elevated temperatures were required to detect isomerization in water.

The free energy barriers to isomerization of 1–3 are almost exclusively enthalpic in origin. No significant difference was detected in the values of \( \Delta H^\ddagger \) for 1 and 2. A similar result had been obtained for Gly-Pro and Gly-Hyp in water.\(^{51}\) The \( \Delta H^\ddagger \) values do, however, differ for 1 and 3. In considering proline derivatives 1 and 3 in dioxane, the enthalpic contribution to the barrier associated with \( k_{EZ} \) and \( k_{ZE} \) is reduced by 1.8 ± 1.1 and 3.6 ± 0.5 kcal/mol, respectively. If \( \Delta H^\ddagger \) reflects the amount of bond breaking required for isomerization to occur, then these parameters suggest that the presence of fluorine on the pyrrolidine ring reduces the C–N bond order and hence the barrier to isomerization by withdrawing electron density toward the nitrogen.

**Inductive Effect on \( k_{ZE} \).** The corresponding cis-to-trans and trans-to-cis Eyring plots in Figure 2 are not parallel, indicating that the equilibrium constants are temperature dependent. The effects of temperature on the values of \( k_{ZE} \) (= \( k_{EZ}/k_{ZE} \)) were measured directly by NMR spectroscopy, and the resulting data are illustrated by van’t Hoff plots in Figure 3. Values for \( \Delta H^\ddagger \) and \( \Delta S^\ddagger \) (± SE) were calculated from linear least-squares fits of the data in these plots to eq 3.

\[
\ln k_{ZE} = (-\Delta H^\ddagger/RT) + \Delta S^\ddagger/\gamma
\] (3)

These values are listed in Table 3. This analysis assumes that the enthalpic and entropic differences between the cis and trans isomers are independent of temperature, that is, \( \Delta C_p^\ddagger = 0 \) for the reaction in eq 1.\(^{52}\) The linear van’t Hoff plots for the isomerization of 1–3 (Figure 3) and Ac-Gly-Pro-OMe\(^{27}\) indicate that this assumption is likely to be valid for the reaction in eq 1.

In all conditions studied, the trans isomer of 1–3 is more stable than the cis isomer (Figure 3 and Table 3). A similar result had been observed for Gly-Pro and Gly-Hyp in water at 25 °C.\(^{51}\) Moreover, the values of \( k_{ZE} \) for 1–3 are dependent on temperature such that the trans isomer becomes increasingly favored as the temperature decreases. In other words, \( \Delta H^\ddagger < 0 \) for the reaction in eq 1, as had been observed for Ac-Gly-Pro-OMe\(^{27}\) and calculated with the 6-31G** basis set of the Gaussian 82 ab initio program.\(^{53}\) In addition, the relative stability of the trans isomer is greater in water than dioxane. A similar solvent effect was observed for Ac-Gly-Pro-OMe.\(^{27}\) Finally and perhaps most significantly, the values of \( k_{ZE} \) near physiological temperature increase in the order: 1 < 2 < 3.

**Discussion.**

The presence of electron-withdrawing substituents on the pyrrolidine ring can influence the structure of proline residues.\(^{29}\) Here, such substituents are shown to affect the kinetics and thermodynamics of prolyl peptide bond isomerization.


**Table 2. Activation Parameters for Isomerization of 1–3**

<table>
<thead>
<tr>
<th>compd</th>
<th>solvent</th>
<th>process</th>
<th>( \Delta H^\ddagger ) (kcal/mol)</th>
<th>( \Delta S^\ddagger ) (cal/(mol·K))</th>
<th>( k ) (37 °C) ( ^b ) (s(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>dioxane</td>
<td>cis-to-trans</td>
<td>20.2 ± 1.0</td>
<td>6.2 ± 0.4</td>
<td>0.86 ± 0.44</td>
</tr>
<tr>
<td></td>
<td>water</td>
<td>cis-to-trans</td>
<td>23.5 ± 0.3</td>
<td>14.4 ± 1.0</td>
<td>0.25 ± 0.05</td>
</tr>
<tr>
<td>2</td>
<td>dioxane</td>
<td>cis-to-trans</td>
<td>23.2 ± 1.3</td>
<td>7.1 ± 3.4</td>
<td>0.010 ± 0.008</td>
</tr>
<tr>
<td></td>
<td>water</td>
<td>cis-to-trans</td>
<td>27.7 ± 1.2</td>
<td>22.5 ± 1.2</td>
<td>0.016 ± 0.10</td>
</tr>
<tr>
<td>3</td>
<td>dioxane</td>
<td>cis-to-trans</td>
<td>22.2 ± 0.8</td>
<td>3.8 ± 0.2</td>
<td>0.010 ± 0.004</td>
</tr>
<tr>
<td></td>
<td>water</td>
<td>cis-to-trans</td>
<td>18.4 ± 0.4</td>
<td>1.2 ± 0.1</td>
<td>1.3 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>water</td>
<td>cis-to-trans</td>
<td>19.9 ± 0.4</td>
<td>3.2 ± 0.1</td>
<td>0.31 ± 0.06</td>
</tr>
</tbody>
</table>

\(^a\) Values ± SE were obtained by linear least-squares fitting of the data in Figure 2 to eq 2. \(^b\) Values ± SE were calculated with eq 2.

**Kinetics.** We have shown previously that changes in the free energy of activation (\( \Delta G^\ddagger = \Delta H^\ddagger - T\Delta S^\ddagger \)) for prolyl peptide bond isomerization are proportional to changes in the frequency (\( \nu \)) of the amide I vibrational mode. In other words,

\[
\Delta \Delta G^\ddagger = a_1 \Delta \nu
\] (4)

where \( a_1 \) is an empirical parameter that varies from −0.022 to −0.030 kcal-cn/mol, depending on the peptide and type of solvent.\(^{26,46}\) In all comparisons of 1–3, \( \Delta \nu < 8 \) cm\(^{-1}\) (Figure 1). This small frequency difference corresponds by eq 4 to \( \Delta \Delta G^\ddagger < 0.24 \) kcal/mol or only a 1.5-fold change in rate constant at 37 °C. For 1 and 3, this small difference is apparent in \( \Delta H^\ddagger \), and in \( k_{EZ} \) and \( k_{ZE} \) calculated by extrapolation of the Eyring plots to 37 °C (Table 2), which is the approximate physiological temperature of vertebrates. For 1 and 2, these subtle differences are not detectable by our kinetic assay. Still, X-ray diffraction analyses (Table 1), pK\(_A\) determinations, and amide I vibrational modes (Figure 1) suggest that 2, like 3, has more electron density residing on the nitrogen of the prolyl peptide bond than does 1.
A typical collagen molecule has approximately 30 Hyp residues. The folding of the collagen triple helix is therefore likely to be accelerated by the cumulative effect of electron-withdrawing hydroxyl groups attached to Cα of the pyrrolidine rings. Moreover, our results predict that the incorporation of 4(R)-fluoroproline into collagen or other proteins in which folding is limited by prolyl peptide bond isomerization would lead to a measurable increase in folding rate.

**Thermodynamics.** The value of $K_{ZE}$ for a prolyl peptide bond is mediated by contacts between Cδ of the adjacent residue and Cα or Cβ of the proline residue.18 The value of $K_{ZE}$ for 1–3 increases as the electron-withdrawing ability of the Cβ substituent increases. The origin for this increase may be the shorter Cγ–Cδ bond. The Cγ–Cδ bond length is decreased by (0.013 ± 0.004) Å in 2 and (0.016 ± 0.003) Å in 3.55 In effect, the hydroxyl group or fluorine atom attached to Cγ serves to pull Cδ away from Cγ of the adjacent residue. This structural manifestation of the inductive effect should increase the stability of the trans isomer, thereby increasing $K_{ZE}$.

An alternative explanation for the observed increase in $K_{ZE}$ is based on stereoelectronics and its effect on pyrrolidine ring puckering. Electron-withdrawing groups on Cβ can in theory alter the preferred conformation of the pyrrolidine ring. Specifically, the tendency of molecules to adopt the conformation that has the maximum number of gauche interactions between adjacent polar bonds has been termed the “gauche effect”.54 The gauche effect has been invoked to explain the conformational preferences of double-helical nucleic acids,35,56 but not that of Hyp residues. A gauche effect based on the nitrogen and the hydroxyl group of 2 or fluorine of 3 would impose Cγ-exo pucker on the pyrrolidine rings of 2 and 3. This pucker was indeed observed in crystalline 2 and 3 (but not 1; Table 1),29 and in all of the Hyp residues (but only half of the Pro residues) in crystalline collagen.10 The role of the gauche effect in collagen structure and stability is the object of on-going work in our laboratory.

In water at 37 °C, $K_{ZE}$ is 1.5-fold larger for 2 than 1 (Table 3). Because triple-helical collagen typically contains approximately 3 × 30 Hyp residues, the effect of stabilizing the trans isomer of each Hyp residue has a cumulative effect on collagen stability. This analysis does not exclude a role for water in stabilizing the collagen triple helix.17 It does, however, suggest that the inductive effect contributes to this stability.

**Conclusions.** An electron-withdrawing substituent at the 4(R) position of a pyrrolidine ring has significant structural and energetic consequences. An apparent inductive effect increases pyramidalization of the prolyl nitrogen, lowers the nitrogen pKα, shifts the amide I vibrational mode downfield, and reduces the energetic barrier to isomerization. Furthermore, such a substituent alters the prolyl peptide bond equilibrium constant. These inductive effects may accelerate the folding and enhance the stability of triple-helical collagen, in which all peptide bonds are in the trans conformation.

**Experimental Section**

**Materials.** tert-Butyloxy carbonyl-[βγ-13C]-d,L-proline methyl ester,22 N-acetyl-4(R)-acetoxy-L-proline methyl ester,23 and 3β were synthesized as described previously. l-Proline, 4(R)-hydroxy-L-proline, and all other reagents were from Aldrich Chemical (Milwaukee, WI) and were used without further purification unless indicated otherwise. Solvents for NMR spectroscopy in prepackaged ampules were from Aldrich Chemical and were used without further purification.

**N-Acetyl-[βγ-13C]-d,L-proline Methyl Ester (1).** tert-Butyloxy carbonyl-[βγ-13C]-d,L-proline methyl ester (0.50 g, 2.2 mmol) was added to a solution (10 mL) of dioxane containing HCl (4 N). The resulting solution was stirred at 25 °C for 30 min, then concentrated, dried, and stirred with acetic anhydride (25 mL) at 25 °C for 16 h. The reaction mixture was concentrated under reduced pressure, and the concentrate was dissolved in ethyl acetate. The resulting solution was washed with 1 N HCl (3 × 50 mL), 1 N NaOH (3 × 50 mL), and saturated aqueous NaCl. The organic extract was dried over MgSO4, filtered, and concentrated to a yellow oil. The product was used as such for NMR spectroscopy in prepackaged ampules.

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**N-Acetyl-[βγ-13C]-d,L-proline Methyl Ester (2).** Anhydrous potassium carbonate (0.01 g, 0.08 mmol) was added to a solution of N-acetyl-4(R)-acetoxy-L-proline methyl ester (0.15 g, 0.66 mmol) in 5 g of methanol enriched to 99.98% with 13C. The resulting slurry was stirred at 25 °C for 30 min. The mixture was filtered, and the filtrate was concentrated under reduced pressure. The product was used as such for NMR spectroscopy in prepackaged ampules.

**N-Acetyl-4(R)-Hydroxy-L-proline [13C]Methyl Ester (2).** Anhydrous potassium carbonate (0.01 g, 0.08 mmol) was added to a solution of N-acetyl-4(R)-acetoxy-L-proline methyl ester (0.15 g, 0.66 mmol) in 5 g of methanol enriched to 99.98% with 13C. The resulting slurry was stirred at 25 °C for 30 min. The mixture was filtered, and the filtrate was concentrated under reduced pressure. The product was used as such for NMR spectroscopy in prepackaged ampules.

**NMR Spectroscopy.** Values of pKα, $K_{ZE}$, and $K_{KE}$ were determined by NMR spectroscopy.

**pKα Determinations.** The secondary amine pKα’s of the parent amino acids of 1, 2, and 3 were determined by pH-titration monitored by 1H NMR spectroscopy. Experiments were performed on a Bruker AM500 instrument (498.68 MHz) using a 5 mm 1H probe and a bandpass filter. Solvent suppression was applied to the water signal, and a deuterium oxide insert was used to provide an external lock. Stock solutions (0.40 M; 50 mL) of l-proline, 4(R)-hydroxy-L-proline, and 4(R)-fluoro-L-proline (which was prepared by hydrolysis of tert-butyloxy carbonyl-4(R)-fluoro-L-proline methyl ester) were prepared in 0.1 M sodium phosphate buffer, pH 7.0. An aliquot (0.40 mL) was removed from the stock solution, and the chemical shift difference (Δδ) between the α and β protons (for l-proline and 4(R)-hydroxy-L-proline), or the α and δ protons (for 4(R)-fluoro-L-proline), were measured at 25 °C. The aliquot was returned to the stock solution, and the pH of that solution was decreased by approximately 0.1 units by the addition of an aliquot (0.10 mL) of 1 N KOH. Values of pKα were determined by nonlinear least-squares fits of the Δδ and pH data to the Henderson–Hasselbalch equation.

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**Table 3.** Thermodynamic Parameters for Isomerization of 1–3

<table>
<thead>
<tr>
<th>compd</th>
<th>solvent</th>
<th>$\Delta H^\circ$ a (kcal/mol)</th>
<th>$\Delta S^\circ$ a (cal/(mol·K))</th>
<th>$K_{ZE}$ (37 °C) b</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>dioxane</td>
<td>$-1.04 \pm 0.01$</td>
<td>$-0.84 \pm 0.04$</td>
<td>3.5 ± 0.1</td>
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<tr>
<td>2</td>
<td>water</td>
<td>$-1.04 \pm 0.02$</td>
<td>$-0.46 \pm 0.05$</td>
<td>4.3 ± 0.2</td>
</tr>
<tr>
<td>2</td>
<td>water</td>
<td>$-1.65 \pm 0.12$</td>
<td>$-2.58 \pm 0.38$</td>
<td>4.0 ± 1.1</td>
</tr>
<tr>
<td>3</td>
<td>water</td>
<td>$-2.29 \pm 0.07$</td>
<td>$-4.60 \pm 0.23$</td>
<td>4.1 ± 0.7</td>
</tr>
</tbody>
</table>

a Values ± SE were obtained by linear least-squares fitting the data in Figure 3 to eq 3. b Values ± SE were calculated with eq 3. c Measured directly.

Kinetics. The cis–trans isomerization rate of 1–3 were determined by 13C (for 1 and 2) or 19F (for 3) NMR isomerization transfer NMR spectroscopy.57–59 Experiments were performed on a Bruker AM500 instrument (13C 125.68 MHz) using a 5 mm broadband probe, 1H bandpass filter, and 13C lowpass filter or a Bruker AM400 instrument (19F 376.48 MHz) using a 5 mm 19F probe, 1H bandpass filter, 13F bandstop, and 19F bandpass filter.

Samples of 1, 2, and 3 were prepared at concentrations of 0.10 M and 1.0 mM in dioxane–D2O. Aqueous samples contained 20% (v/v) 2H2O in 0.10 M sodium phosphate buffer, pH 7.2. The rate of prolyl peptide bond isomerization cannot be detected by this method at room temperature. Experiments were therefore conducted at elevated temperatures, 310–360 K. Temperature settings of the spectrometer were calibrated to within 1°C by reference to a glycol standard.

Spectra were obtained using the inversion transfer pulse sequence in eq 5, where Δδ is the difference in the chemical shifts of the cis and trans resonances.

\[ 90^\circ - \frac{1}{2} \Delta \delta - 90^\circ = \tau - 90^\circ \]  

Briefly, the signal for one isomer is placed on a carrier frequency, and the intensity change of the signal for the other isomer is monitored during its recovery from a selective 180° pulse (1/2Δδ). The time-dependence of the change in signal intensity allows for the determination of the isomerization rate.

The time-dependent peak height [M(t)] for each resonance was fit by nonlinear least-squares to eqs 6 and 757 with SIGMA PLOT 4.16 (Jandel Scientific; San Rafael, CA).

\[ M_a(t) = C_{1a} e^{\lambda_a t} + C_{2a} e^{\lambda_a^* t} + M_a^* \]  

\[ M_b(t) = C_{1b} e^{\lambda_b t} + C_{2b} e^{\lambda_b^* t} + M_b^* \]  

In eqs 6 and 7, the “a” subscript refers to the cis resonance, the “b” subscript refers to the trans resonance, and M* refers to the peak height at equilibrium. At each temperature, complementary experiments were performed in which the cis or trans peak was placed on the carrier frequency. Data from both experiments were fit to eqs 6 and 7, with the cis constants denoted as in eqs 5 and 6 and the trans constants denoted as C1a, C2a, C1b, C2b, λ1a, and λ2b. Values of k5a and k5b were then calculated by using eqs 8 and 9.57

\[ k_{5a} = \frac{(C_{1a}^2 + C_{2a}^2)(C_1 + C_2) - (C_{1a}^2 + C_{2a}^2)(C_1 + C_2)}{(C_1^2 + C_2^2)(C_1 + C_2) - (C_{1a}^2 + C_{2a}^2)(C_1 + C_2)} \]  

\[ k_{5b} = \frac{(C_{1a}^2 + C_{2a}^2)(C_1 + C_2) - (C_{1b}^2 + C_{2b}^2)(C_1 + C_2)}{(C_1 + C_2)(C_1^2 + C_2^2) - (C_{1a}^2 + C_{2a}^2)(C_1 + C_2)} \]  

The isomerization rate constants, kEZ and kZE, were calculated by using eqs 10 and 11, where α is the ratio of the cis line width to the trans line width.

\[ k_{EZ} = \frac{(C_{1a} + C_{2a}) + k_{5b}(C_1 + C_2)}{\alpha(C_1 + C_2)} \]  

\[ k_{ZE} = \frac{\alpha(C_{1a} + C_{2a}) + k_{5a}(C_1 + C_2)}{C_1 + C_2} \]  

Thermodynamics. The equilibrium constants for the interconversion of the cis and trans isomers of 1–3 were determined by measuring the peak areas of the 13C (for 1 and 2) or 19F (for 3) resonances for the two isomers. Peak areas were measured with the program FELIX 2.3 (Technologics; San Diego, CA). Experiments were conducted at 300–355 K. Equilibrium constants (kEZ = trans/cis) were calculated directly from the peak areas.

FTIR Spectroscopy. FTIR spectra were recorded on a Nicolet 5PC spectrometer. Experiments were performed at 25°C using NaCl or CaF2 plates, or a ZnSe crystal in a Spectra Tech circle cell. The frequency of amide I vibrational modes was determined to within 2 cm⁻¹.

Samples of 1, 2, and 3 were prepared at concentrations of 0.10 M and 1.0 mM in dioxane (which had been distilled from CaH2) and in D2O. No concentration effects were observed for 1 and 3 in either solvent. A second vibrational mode was present in the amide I region in a 0.10 M solution of 2. This mode was absent in a 1.0 mM solution of 2. Thus, all FTIR spectra in dioxane were recorded on 1.0 mM samples of 1–3.

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