# 3*S*-Fluoroproline as a probe to monitor proline isomerization during protein folding by $^{19}$F-NMR†

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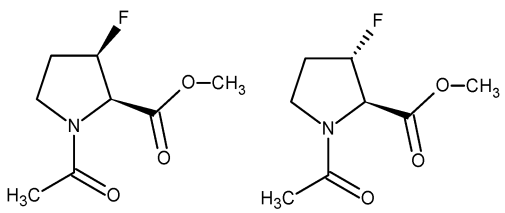
**Variable-temperature inversion transfer NMR is used to determine the kinetic and thermodynamic parameters of *cis–trans* isomerization of *N*-Ac-(3*R*) and (3*S*)-fluoroproline-OMe.**

Understanding how proteins change in structure from an unfolded to a folded state is complicated, in many cases, by the *cis* to *trans* isomerization of prolyl peptide bonds.[1] While dependent upon the residues surrounding a particular proline,[2] it is assumed that in the unfolded state, proline will isomerize to equilibrium values approximated by model peptides (typically ~80% *trans*, 20% *cis*).[3] Although this assumption is valid for some proteins, model peptides cannot *a priori* predict the population of *cis–trans* isomers in the unfolded state. The ability to directly measure this population in a protein would have distinct advantages, in particular in monitoring how this population changes during the folding process.[4]

The idea to monitor directly the population of proline *cis–trans* isomers in the unfolded state is not new, and was pioneered initially by Torchia and co-workers, through the biosynthetic incorporation of [4-$^{13}$C] proline into collagen, and later applied to the study of staphylococcal nuclease.[5] Although methods and instrumentation (cryogenic probes) have been developed to improve sensitivity, carbon remains one of the least sensitive nuclei for detection and thus has limited its use for monitoring processes which occur in real time.

As an alternative to carbon detection of proline *cis–trans* isomerization, we report here the kinetics and thermodynamics of isomerization of the simple compounds Ac-(3*R*) and (3*S*)-fluoroproline-OMe (compounds **1** and **2**, respectively)[6] in water–$D_2O$ solution using $^{19}$F-NMR.[7] Studies on these model compounds will provide a basis for future studies in which biosynthetic incorporation of 3*S* and 3*R* fluoroproline will be used for monitoring *cis–trans* isomerization of proline during protein folding.




**1** **2**

The use of $^{19}$F-NMR is unique for protein folding studies since a 1-D $^{19}$F spectrum provides residue-specific information on the folding of proteins, and the development of stopped-flow NMR methods has allowed time-dependent folding information to be obtained with dead times less than 2 seconds.[8] The $^{19}$F signal to noise ratio is as good as hydrogen, which is approximately 8 times as sensitive as carbon.[9] Fluorine is only slightly larger than hydrogen (0.15 Å), and in most cases is non-structurally perturbing.[10] Finally, London and co-workers have shown that the peptide [*p*-fluoro-Phe]bradykinin, which contains a -Pro-*p*-FPhe- peptide bond, exhibited well resolved *cis* and *trans* resonances that could be used to monitor the catalysis of *cis–trans* isomerization by the enzyme cyclophilin.[11]

The Ac-(3*S*) and (3*R*)-fluoroproline-OMe analogs also exhibit clearly resolvable *cis* (*E*) and *trans* (*Z*) resonances—for **2** the resonances are separated by 0.8 ppm, while for **1** the resonances are separated by nearly 2 ppm. This large difference in chemical shift allowed us to monitor the kinetics of *cis–trans* isomerization using inversion-transfer NMR.[12] Eyring analysis of **1** and **2** is shown in Fig. 1, and the activation parameters derived from this plot are summarized in Table 1. The calculated equilibrium constant from the kinetic data is consistent with the observed ratio of integrated signal intensities of *cis* and *trans* isomers—at 37 °C for **1**, $K_{Z/E}$ is 8.2 ± 0.2 (90% *trans*, 10% *cis*) and for **2** $K_{Z/E}$ is 4.12 ± 0.04 (80% *trans*, 20% *cis*). In addition, the rate constants for **2** in water are similar to that reported for Ac-proline-OMe (37 °C) in sodium phosphate buffer, pH 7.4.[13a]

The data in Table 1 indicate that the transition state enthalpic differences between compounds **1** and **2** are small, suggesting that water-mediated hydrogen bonding to the prolyl peptide bond has not been perturbed. However, the entropic barrier of **1** is approximately twofold greater than **2**, with values that are negative, which is unusual for proline and other amides,[13] and for proteins in general.[14] One of the reviewers has aptly pointed out that a similar trend is also observed when comparing the Ac-(4*R*) and (4*S*)-fluoroproline-OMe derivatives,[13a,15] suggesting that a fluorine in the *syn* configuration of the pyrrolidine ring may sterically hinder the barrier to rotation around the C–N bond.

Raines and co-workers first showed that synthesis of a collagen-like peptide with the sequence H-(Pro-(4*R*)-fluoroproline-Gly)$_{10}$-OH resulted in a triple-helical collagen with greatly enhanced thermal stability, which was attributed

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† Electronic supplementary information (ESI) available: $^{19}$F-NMR spectra of **1** and **2** at 35 °C in 90% $H_2O$/10% $D_2O$, synthesis of (3*S*)-fluoro-L-proline and *N*-acetyl-(3*S*)-3-fluoro-L-proline methyl ester, and determination of the kinetic and thermodynamic parameters. See DOI: 10.1039/b821952d

 

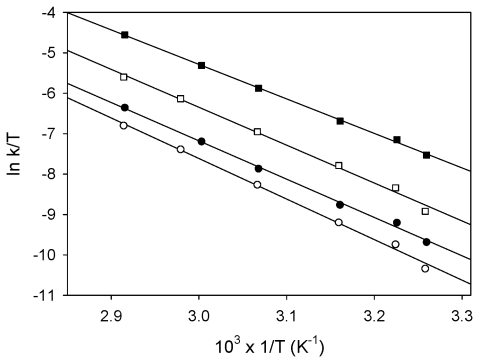


**Fig. 1** Eyring analysis of the temperature dependence of isomerization for **1** (closed symbols) and **2** (open symbols). Data were analyzed according to ref. 15. The symbols (●) (○) correspond to *trans* (*Z*) to *cis* (*E*) isomerization, whereas the symbols, (■) (□) correspond to *cis* to *trans* isomerization. Linear least squares fits of the data (-) are shown.

**Table 1** Eyring parameters for compounds **1** and **2**

| | | $E_a$[a] | $\Delta H^{\ddagger}$[a] | $\Delta S^{\ddagger}$[a] | $k$[b]/s$^{-1}$ |
|---|---|---|---|---|---|
| **1** | *E* to *Z* | 81.3 (2.1)[c] | 78.8 (2.0) | −20.1 (0.7) | 0.028 (0.002) |
| | *Z* to *E* | 73.5 (1.1) | 70.9 (1.1) | −28.7 (0.6) | 0.229 (0.008) |
| **2** | *E* to *Z* | 85.9 (2.7) | 83.4 (2.6) | −10.8 (0.5) | 0.016 (0.001) |
| | *Z* to *E* | 80.5 (2.9) | 77.9 (2.8) | −16.5 (0.8) | 0.065 (0.005) |

[a] All thermodynamic parameters are in kJ mol$^{-1}$ except entropy, which is in J mol$^{-1}$ K$^{-1}$. [b] Data recorded at 37 °C. [c] Error is in parentheses.

to a stereoelectronic inductive effect of the fluorine.[16] The inductive effect changed the kinetics and equilibrium values of *cis* and *trans* isomers of Ac-Pro-OMe, from ~80% *trans*, 20% *cis* to 90% *trans*, 10% *cis*. Independently, Renner and co-workers showed for the first time the ability to biosynthetically incorporate fluoroproline analogs into barstar C40A/C82A/P27A, which has only one *cis* proline (Pro 48), in *Escherichia coli*. Incorporation of (4*R*)-fluoroproline decreased the stability of the protein, whereas incorporation of (4*S*)-fluoroproline increased the thermal stability, since (4*S*)-fluoroproline favors the *cis* isomer.[15] Incorporation of the difluoro analog (4,4-$F_2$), which exhibits a *cis–trans* isomer ratio similar to proline, leads to a protein in which the stability was unchanged.

However, because of the overlap of the signals from the *cis* and *trans* isomers for the 4-fluoroproline derivatives, obtaining isomerization rates or equilibrium values from simple 1-D $^{19}$F-NMR experiments could not be achieved, and more complicated two-dimensional $^{19}$F-NMR methods were required.[15] Based on our study using the small model compounds **1** and **2**, the *cis* and *trans* isomers are easily distinguishable, and may provide a useful alternative for measuring the ratio of *cis* and *trans* isomers in the unfolded state and real-time kinetics of *cis–trans* proline isomerization by $^{19}$F-NMR.

Raines and co-workers have shown that hydroxylation at the 3-position of Ac-(3*S*)-hydroxyproline-OMe has little influence on the kinetics of *cis–trans* isomerization, and thus it is perhaps not surprising that Ac-(3*S*)-fluoroproline-OMe displays similar kinetic isomerization parameters as both natural and 3-hydroxy-substituted prolines.[17] The study presented here indicates that biosynthetic incorporation of (3*S*)-fluoroproline into proteins would have little impact on the natural population of *cis* and trans isomers. Indeed, the biosynthetic incorporation of (3*S*)-fluoroproline, which was reported recently in an elegant study by Conticello and co-workers,[18] and more recently into the protein ribonuclease T1 (Carl Frieden, personal communication), should display similar kinetics of *cis–trans* isomerization as that of the wild-type protein, and if there are effects on the structure, activity or folding kinetics, it will be most likely due to other mechanisms.[10]

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7 NMR experiments were carried out on a Varian INOVA400 spectrometer with a tunable inverse detection probe. Samples were at a concentration of ~0.2 mg ml$^{-1}$ in 90% $H_2O$/10% $D_2O$. Temperature was corrected using the change in chemical shift of methanol methyl and hydroxyl resonances. The data at 35 °C (ESI†) were referenced to neat TFA which was present in a coaxial insert in the NMR tube.

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