

Detection of *cis*–*trans* Isomers of a Synthetic Peptide Fragment of Erythropoietin

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Abstract

The synthesis of the protected fragment *t*-butoxycarbonyl-alanine-isoleucine-serine(benzyl)-proline (Pro)-Pro-OH derived from the hormone erythropoietin is described. The analysis of the peptide by high-pressure liquid chromatography (HPLC) and thin-layer chromatography (TLC) yields apparently inconsistent results. Although HPLC consistently indicates the presence of only one component, TLC reveals a number of distinct species. Because satisfactory amino acid analysis and fast atom bombardment-mass spectrometry results are obtained, we think it possible that the distinct components arise from the *cis*–*trans* isomerization of the peptide bonds to the prolyl residues. An analysis using capillary electrophoresis under basic conditions identifies four components in the final product. Also, under similar conditions proton nuclear magnetic resonance spectroscopy is able to confirm the presence of *cis* and *trans* isomers. The results from this study demonstrate the usefulness of each of the four techniques in identifying the isomerism of the standard amino acid–Pro bond with respect to the peptide's ionic state.

Introduction

The pioneering studies of Pauling and Corey (1,2) show that the peptide bond is essentially planar. Planarity can be achieved with the peptide bond in either the *cis* or *trans* configuration. In the vast majority of cases examined to date, the peptide bond is found in the *trans* configuration. Except for small cyclic peptides (in which the geometry constrains one or more peptide bonds to be in the *cis* configuration), *cis* peptide bonds are found mainly in an amino acid (Xaa)–proline (Pro) bonds (or Xaa–Yaa, in which Yaa is also an imino acid such as sarcosine) (3,4).

The existence of *cis* and *trans* isomers and the interconversions between them is not purely of theoretical interest. Several important biological processes are affected by this phenomenon. For example, some protein structures involve a *cis* Xaa–Pro bond (5);

the rate-limiting step in some cases of protein folding is a result of the *cis*–*trans* isomerization of a Xaa–Pro bond (6); and there is a wide-spread occurrence of prolyl *cis*–*trans* isomerases (prolyl peptidyl isomerases), certain of which are inhibited by some immuno-suppressant molecules (7,8).

The energy difference between the *cis* and *trans* forms for the peptide bond is quite small (< 8 kJ/mol for most peptide bonds), and the barrier for interconversion between these forms is much lower for Xaa–Pro bonds (54 kJ/mol) than in other cases (84 kJ/mol) (3). Consequently, when *cis* and *trans* isomers exist they are usually in fairly rapid equilibrium and are difficult to separate by any form of chromatography. However, the time needed to acquire a nuclear magnetic resonance (NMR) spectrum is short relative to the rates of the interconversion of *cis* and *trans* isomers. Therefore, NMR spectroscopy (both ¹H and ¹³C) has been widely used to flag the presence of *cis* and *trans* isomers and (in favorable cases) identify which is which (7,8).

In only a few cases has high-performance liquid chromatography (HPLC) been reportedly able to separate *cis* and *trans* isomers. Best results have been obtained at low temperatures (9–11) and also using cyclodextrin columns (12,13). Separations of *cis* and *trans* isomers by capillary electrophoresis (CE) have been reported for thiopeptides (14), in which the energy barrier to interconversion is known to be greater for ordinary peptides at subzero temperatures (15) and ambient temperatures with very rapid analysis times (16). We wish to report a successful separation using CE confirmed by the NMR spectroscopy of the *cis* and *trans* isomers from a pentapeptide under normal operating conditions.

The peptide under study was *t*-butyloxycarbonyl (Boc)-alanine (Ala)-isoleucine (Ile)-serine (Ser)-benzyl (Bzl)-Pro-Pro-OH, which was synthesized as a fragment of Ala-leucine (Leu)-glycine (Gly)-Ala-glutamine (Gln)-lysine (Lys)-glutamic acid (Glu)-Ala-Ile-Ser-Pro-Pro-aspartic acid (Asp)-Ala-Ala-Ser-Ala-Ala-Pro. This sequence had been identified as a possible epitope of erythropoietin, which is an acidic glycoprotein hormone that regulates the proliferation of erythroid progenitor cells and induces their differentiation into mature red blood cells (17).

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Experimental

Materials and methods

Chemicals were of the highest purity available and were used without further purification with the following exceptions. *N,N*-dimethylformamide (Aldrich Chemical Co. Ltd., Gillingham, Dorset, U.K.) was stored over 4-Å molecular sieves for four days. HPLC-grade tetrahydrofuran (Lab-Scan, Stillorgan, Co., Dublin, Ireland) was refluxed for 3 h over calcium hydride, from which it was distilled and subsequently stored. *N*-methylmorpholine (Aldrich Chemical Co. Ltd.) was refluxed over and then distilled from potassium hydroxide. It was finally stored over 3-Å molecular sieves. Pro and HPLC-grade trifluoroacetic acid (TFA) were obtained from Sigma Chemical Co. Ltd. (Poole, Dorset, U.K.). All other HPLC-grade chemicals were purchased from Lab-Scan. Boc amino acids and benzotriazol-1-yl-oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate (BOP) were obtained from Peninsula Laboratories (St. Helens, Merseyside, U.K.).

Thin-layer chromatography (TLC) was performed on precoated plates (silica gel 60, F-254, Merck, Darmstadt, F.R.G., Germany). HPLC was carried out using a Waters Prep liquid chromatograph (LC) 3000 system. Peptide samples were analyzed on a μ -Bondapak (Waters) C₁₈ analytical column. Amino acid analyses were performed using a Waters Pico Tag Amino Acid Analyzer. Peak areas were calculated by a Trivector Trio Computing Integrator. CE was performed on an Applied Biosystems (Cheshire, U.K.) Model 270A CE system.

Fast atom bombardment (FAB)-mass spectrometry (MS) was performed on the final product (P6) using glycerol as a matrix. Spectra were recorded on a VG ZAB-E high-resolution instrument at the EPSRC Spectrometry Service Centre (University of Wales, Wales, U.K.).

¹H-NMR spectra were recorded in a [2H₆]dimethyl sulphoxide (DMSO) solution of both Boc-Ala-Ile-Ser(Bzl)-Pro-Pro-OH and Boc-Ala-Ile-Ser(Bzl)-Pro-Pro-O-Na⁺. The latter was prepared by dissolving the peptide in a stoichiometric amount of dilute

sodium hydroxide (1M) to give a pH of 7.0, followed by freeze drying and redissolving in DMSO. All ¹H-NMR spectra were recorded at 500 MHz on a GE Omega 500 spectrometer. Resonance assignments were made using double quantum filtered phase-sensitive correlation spectrometry (COSY) (2QFCOSY) spectra and phase-sensitive nuclear Overhauser spectroscopy (NOESY) spectra. All 2D spectra were recorded using 512 complex points in both t₁ and t₂ with 32 transients for each flame ionization detector. Following acquisition, both time domain data sets were multiplied by a sine square bell window function that was phase shifted by 60° for COSY spectra and 0° for NOESY spectra. All 2D spectra were zero-filled to 1K before Fourier transformation, thus giving a final matrix of 1K × 1K. The first t₁ value in each 2D spectrum was multiplied by 0.5 after Fourier transformation.

Peptide synthesis

Boc-Ala-Ile-Ser(Bzl)-Pro-Pro-OMe was synthesized in solution by the stepwise addition of Boc-protected amino acids. Initial attempts to form the Pro-Pro bond by the mixed anhydride method gave an impure product, which was in contrast to the findings of Pettit and Das Gupta (18). Such problems have been documented (19). Successful coupling was achieved using the BOP reagent. The methods used for each coupling and yield, amino acid analysis, and details of the TLC analysis are shown in Table I. The methyl ester group was removed by the transesterification method of Lyle et al. (20).

Results

Peptide synthesis

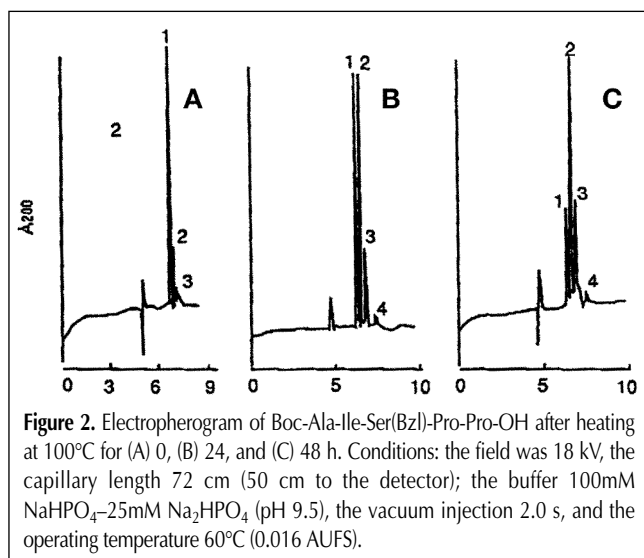
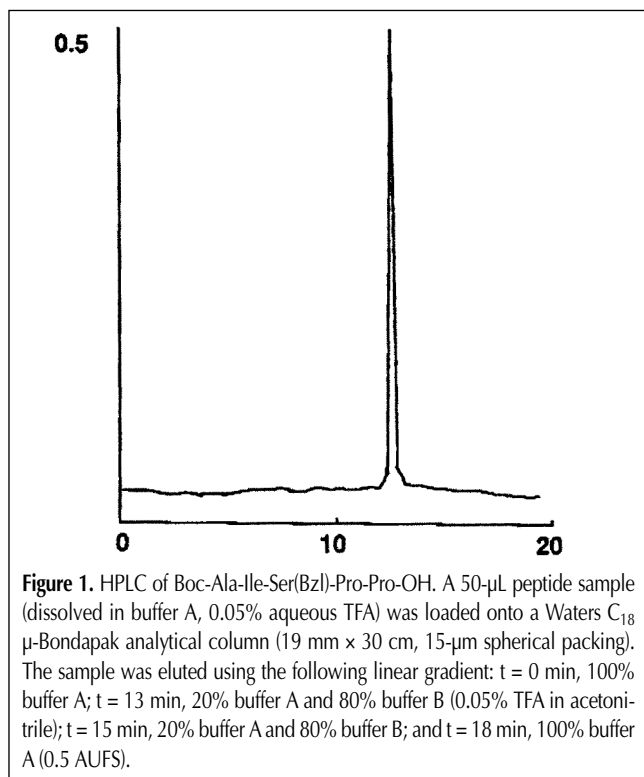
The peptide Boc-Ala-Ile-Ser(Bzl)-Pro-Pro-OH was synthesized as outlined previously. All intermediate peptides gave satisfactory amino acid analyses and were judged to be pure by HPLC. The final product had a satisfactory amino acid analysis, exhibited a

Table I. Results of Peptide Synthesis

Peptide	Coupling method	%Yield	Amino acid analysis				TLC (R _f)			
			Ala	Ile	Ser	Pro	A*	B [†]	C [‡]	D [§]
Boc-Pro-Pro-OMe	BOP + DIPEA** in DMF	65.0					0.74/0.65	0.69/0.61	0.72	0.74
Boc-Ser(OBzl)-Pro-Pro-OMe	BOP + DIPEA in DMF	69.0		1.05	1.05	2.00	0.61		0.87	0.79
Boc-Ile-Ser(OBzl)-Pro-Pro-OMe	mixed anhydride in THF	78.0		1.07	0.88	2.00	0.64/0.49		0.89	0.82
Boc-Ala-Ile-Ser(OBzl)-Pro-Pro-OMe	mixed anhydride in THF	65.0	1.05	1.09	0.90	2.00	0.22		0.81	0.81
Boc-Ala-Ile-Ser(OBzl)-Pro-Pro-OH	transesterification in TEA ^{††} -MeOH-H ₂ O	92.0	1.12	1.08	0.92	2.00	0.81/0.49/0.15	0.70/0.43		

* Chloroform-methanol (95:5).
[†] Chloroform-methanol (90:10).
[‡] Chloroform-methanol (80:20).
[§] Chloroform-methanol (50:50).
^{**} DIPEA, diisopropylethylamine.
^{††} TEA, triethylamine.

molecular ion $[M + Na]^+$ by FAB-MS at 695, and gave one major peak on the HPLC (Figure 1). However, as shown in Table I, several of the intermediate peptides gave more than one spot on the TLC, and the final product showed three spots with one solvent system. It seemed likely that the multiple spots were a result of the *cis-trans* isomers of the peptide bonds to Pro, but further analysis was undertaken to clarify this point. At the dipeptide stage in which two components were detected using TLC, the material corresponding to each spot was isolated by extraction from the TLC plates. The homogeneity of each component was confirmed by further TLC using the solvent systems described in Table I. A solution of each component was allowed to stand at room temperature for approximately one week, after which they were again analyzed by TLC. The same two components were

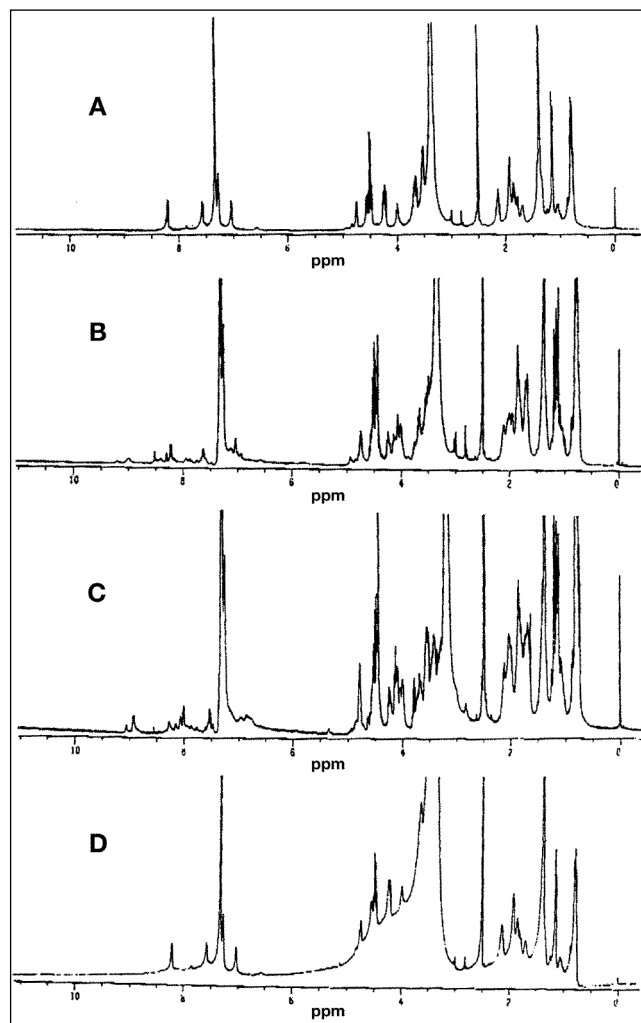


observed for each solution, thus suggesting the interconversion of two conformers resulting from *cis-trans* isomerism.

CE

Because Boc-Ala-Ile-Ser(Bzl)-Pro-Pro-OH was not soluble in water, a solution of the sodium salt was prepared (0.345 mg/mL) and analyzed by CE in the buffer at pH 9.5 at an operating temperature of 60°C. Two aliquots of this solution (which were heated at 100°C for 24 and 48 h, respectively) were also subject to similar analysis. The electropherograms obtained are shown in Figure 2. Figure 2A shows the electrophoretic separation of the unheated sample, exhibiting three significant peaks. Figures 2B and 2C correspond respectively with the two peptide samples that were heated at 100°C for 24 and 48 h and then immediately subjected to CE. It can be seen that a dramatic change in the peak ratio had occurred when compared with Figure 2A.

Percentage peak compositions for each peptide sample were calculated. Sample 1 (unheated) contained three peaks (the major peak having a retention time of 6.48 min). Sample 2 (24 h at 100°C) exhibited four peaks, as did sample 3 (48 h at 100°C). Together with the appearance of four peaks, the electrophero-



grams also showed the change in the peak ratios observed on heating. Peak 1 ($R_t = 6.48$ min) decreased dramatically from 80% to 9%. Peaks 2 ($R_t = 6.68$ min) and 3 ($R_t = 6.97$ min) both increased from 11.5% to 73% and 8.5% to 16%, respectively. Peak 4 ($R_t = 7.58$ min) was not visible in sample 1, but gradually increased in size in samples 2 and 3. When a sample of peptide that had been heated was allowed to cool over several hours, the peak ratio reverted to one characteristic of an unheated sample. This was consistent with the peaks arising from *cis-trans* isomers.

¹H-NMR spectra

The ¹H-NMR spectrum of Boc-Ala-Ile-Ser(Bzl)-Pro-Pro-OH is shown in Figure 3A. The spectrum is quite complex because of overlapping peaks, but the resonances of three amide protons and the Ala methyl group (all doublets) can be clearly distinguished at the δ values of 8.20, 7.56, 7.03, and 1.13 ppm, respectively. Some very minor signals, for example, at δ 7.8 ppm may indicate the presence of traces of *cis-trans* isomers. Complete assignment (Table II) was made using 2QFCSI and NOESY spectra (21), and this confirmed the lack of any significant degree of *cis-trans* isomerism. From the NOESY spectrum, it was possible to identify the conformation of the Ser-Pro-Pro sequence as *trans-trans* because of strong crosspeaks between the Ser α CH and Pro δ protons and the Pro⁴ α CH and Pro⁵ δ protons (21).

The ¹H-NMR spectrum at 25°C of the sodium salt of Boc-Ala-Ile-Ser(Bzl)-Pro-Pro is shown in Figure 3B. There were obvious differences from Figure 3A in all regions. The three amide peaks were still visible but were now joined by at least eight or nine amide doublets of lower intensity. Likewise, the Ala methyl doublet at δ 1.13 ppm now displayed multiple lines, and the α CH region around δ 4.0 ppm had become more complex. Thus, forming the sodium salt had converted a single species into several species. This was consistent with the results of the CE at alkaline pH, in which three or four peaks were seen.

When the sample had been heated before electrophoresis, the proportions of the various peaks were observed to vary in a consistent manner with the period of heating. The effect of heating on the NMR spectrum of the salt is shown in Figure 3C. The

sample was placed in the probe, the probe temperature set at 80°C and the spectrum recorded immediately after this temperature was attained, and the shimming of the magnet was adjusted (a period of approximately 10 min). Again, the spectrum changed quite dramatically (e.g., the Ala NH doublet at δ 7.03 ppm could no longer be distinguished). This was consistent with an increased rate of the interconversion of isomers, leading to a broadening and coalescing of signals. When the sample was cooled, the spectrum reverted towards that of type Figure 3B. The Ala NH doublet at δ 7.03 ppm quickly reappeared, but even after several days the spectrum was not quite identical to Figure 3B. Thus, the rate of interconversion between species slows considerably as the temperature falls. This explains why in the CE experiments samples subjected to heating for different periods gave different results even though all samples were run at the same (lower) temperature.

Figure 3D shows that the differences between the protonated and ionized forms of the peptide were not a result of any permanent change such as racemization or partial deprotection. Figure 3D is identical to Figure 3A except for the water content. This spectrum was obtained after adding a few crystals of oxalic acid to the NMR tube containing a sample of the salt that had previously given a spectrum of the type shown in Figure 3B.

An attempt to assign all the resonances in the spectra of the sodium salt using 2D ¹H-NMR spectra was not totally successful. Three (and in some cases four) sets of resonances could be distin-

Table II. Proton Chemical Shifts* for Boc-Ala-Ile-Ser(OBzl)-Pro-Pro-OH

Residue	NH (ppm)	α CH (ppm)	β CH (ppm)	γ CH (ppm)	δ CH (ppm)	Others (ppm)
Boc						Me (1.36)
Ala	7.03	3.98	1.13			
Ile	7.56	4.23	1.68	1.38, 0.80	0.78	
Ser (OBzl)	8.20	4.47	3.64,		3.50	Ph (7.34), CH ₂ (4.49,4.47)
Pro		4.20	2.14,	1.92	3.70, 1.79	3.53
Pro		4.55	2.14, 1.83	1.92	3.70, 3.53	

* Shifts relative to tetramethylsilane = 0.0 ppm.

Table III. Proton Chemical Shifts* for Boc-Ala-Ile-Ser(OBzl)-Pro-Pro-O-Na⁺

Residue	NH (ppm)	α CH (ppm)	β CH (ppm)	γ CH (ppm)	δ CH (ppm)	Others (ppm)
Boc						Me (1.36)
Ala	7.26, 5.03, 6.93	3.97, 3.97, 3.97	1.2-1.1, 1.2-1.1, 1.2-1.1			
Ile	7.85, 7.60	4.20, 4.23	1.81, 1.68	1.38, 0.80, 1.38, 0.80	0.78, 0.78	
Ser(OBzl)	8.46, 8.25, 7.75	4.91, 1.74, 4.51	3.48, 3.64, 3.50, 3.47			Ph (7.34), CH ₂ (4.49,4.47), CH ₂ (4.45,4.05)
Pro		4.75,	2.06,	2.00, 1.62,	3.30, 3.46,	
				1.13, 1.13,	3.30	
				2.04	2.00, 3.30	
Pro		4.10,	1.86, 1.20,	0.88,	3.50,	1.62
		4.06,	1.83, 1.20,	0.98,	3.47,	
			4.01	2.05, 1.87	3.48	
				1.08		

* Shifts relative to DMSO = 2.50 ppm.

guished for each amino acid (Table III). However, a combination of the inherently small nuclear Overhauser effects (NOEs) were expected for peptides of this size (21). The fact that most species were present in low proportion at the temperature at which the NMR spectrum was recorded and the overlap experienced in some regions meant that the sequence could not be traced out for each isomer. Notwithstanding, one NOE crosspeak was clearly observed between a Ser α CH (δ 4.91 ppm) and a Pro α CH (δ 4.54), and in turn a crosspeak was observed between this proton and another Pro α CH (δ 4.10), thus indicating a *cis-cis* isomer (21).

Discussion

The hypothesis that the multiple spots seen on TLC with the same solvent systems resulting from the existence of *cis-trans* isomers has been confirmed by the CE and NMR studies. It is possible to alter the ratio of the conformers in a reversible manner by heating a solution of the peptide (9–11). With two Pro residues in the peptide a total of four isomers is possible, and this is what was seen with CE. The nature of the differences between the “cold” and “heated” samples can be understood if (in this example) the retention time of an isomer increases with the energy of that isomer. The NMR studies confirmed these findings and also yielded two new pieces of information. The existence of multiple forms of the peptide depended on the state of ionization of the carboxyl group of the peptide. The unionized peptide existed exclusively (within the limits of detection) in one form that was identified as the *trans-trans* isomer from the NOESY spectrum. The ionized form of the peptide exists in three or four forms, and although complete assignments are not possible, the presence of a *cis-cis* isomer was established.

The dependence of the isomer state of a Xaa-Pro bond on the ionization of nearby groups has been known for some time. Of particular relevance to the results presented in this study is the finding of London et al. (22) that Ser-Pro-Pro-OH exists almost completely as the *trans-trans* isomer, but when the carboxyl group is ionized at a higher pH, both peptide bonds exhibit *cis-trans* isomerism. A similar behavior also seems probable in this instance given the reversible nature of the changes.

HPLC was run using buffers containing 0.05% TFA (in this medium the C-terminal carboxyl group would be protonated). Therefore, on the basis of the NMR results presented previously, the *trans-trans* isomer was expected to predominate consistently with the single peak observed. CE was run at alkaline pH so that the carboxyl group would be ionized. Under these conditions, the peptide might be expected to display *cis-trans* isomerism similar to London's peptide, and the presence of multiple peaks was not unexpected. The presence of multiple spots in the TLC results was also expected if the C terminal carboxyl group was ionized on the TLC plate. The silica-gel stationary phase on the TLC plate had a surface containing hydroxyl groups, which may or may not be ionized depending on the exact treatment during preparation.

Other possible causes of this phenomenon such as chemical modification (e.g., loss of Bzl group) or racemization were unlikely. The former was inconsistent with the analytical data and the mild conditions used (samples of the peptide in both the pro-

tonated and salt forms gave identical mass spectra), and neither would be expected to be reversible. Also, the results presented in this study were not contradicting to those of Buoen et al. (23), who reported being able to distinguish *cis-trans* conformational isomers of a 19-residue peptide containing seven Pro residues by HPLC but not by CE. Although CE separates mainly by charge and charge itself would not be affected by the conformational state, in our case the single source of the charge (the C-terminal carboxyl group) was immediately adjacent to the seat of conformational isomerism, and it was not unreasonable that solvation of the carboxyl group should be affected in some way by the conformational state of the peptide. In the peptide of Buoen et al., the Pro residues were distant from the charged groups (N- and C-termini); therefore, the effect of the conformational state on the behavior of the peptide in CE might be expected to be less.

Meyer et al. (14) performed molecular mechanical calculations and demonstrated that *cis* isomers are more compact than the corresponding *trans* isomer and thus should migrate faster, which is contrary to the findings we report. However, their molecules were neutral (separation being driven purely by the electro-osmosis effect). Our molecules were charged, and in order to make a direct comparison it would be necessary to calculate the radii of the solvated molecule, which is a more difficult task. In any case, it cannot be assumed a priori that the most compact isomer will also have the smallest solvation shell. For example, it is well-known that Li^+ (the smallest alkali metal ion) has the largest hydration shell of all the alkali metals. Meyer et al. (14) also suggest that ordinary Pro peptides have *cis-trans* isomerization rates of approximately 10^{-2} s^{-1} , which is too fast for successful separation by CE. Indeed, the separations of *cis-trans* isomers by CE reported to date represent special cases such as thiopeptides (with a measured isomerization rate of approximately 10^{-4} s^{-1}) (14) and ordinary peptides at either subzero temperatures (15) or ambient temperatures but with very rapid analysis times (16). However, Grathwohl and Wüthrich (24) report rates of isomerization as low as 10^{-4} s^{-1} for some Pro peptides, thus the separations we observed by TLC and CE need not be unexpected.

Acknowledgments

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