PHYSICOCHEMICAL STUDIES OF BIOLOGICALLY ACTIVE PEPTIDES BY LOW-TEMPERATURE REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY[a]

DAVID E. HENDERSON* and JILL A. MELLO

Department of Chemistry, Trinity College, Hartford, CT 06106 (U.S.A.)

SUMMARY

The high-performance liquid chromatographic separation of biologically active peptides containing 1-prolyl residues is demonstrated. Rapid isomer interconversion between *cis*- and *trans*-isomeric forms at the prolyl peptide bond is shown to produce classical secondary equilibrium effects in the peak shapes. By operating the column at temperatures in the range − 15°C to 5°C it is possible to obtain normal separations of the various isomeric forms. The use of low column temperatures makes physicochemical studies possible, *e.g.*, the effect of pH on isomer composition in free solution as reported here for morphiceptin.

INTRODUCTION

Low-temperature high-performance liquid chromatography (HPLC) has proved to be an effective method for the study of physicochemical properties. Work to date has demonstrated this for both metal coordination complexes[1] and for rotational *cis–trans*-isomerism of the peptide bond in proline dipeptides[2]. For both of the above chemical systems, isomerization processes with moderate energy barriers result in impared chromatographic performance at room temperature. For chemically reversible systems, the resultant chromatographic behavior is that of a classic secondary equilibrium system, as described by Horváth and co-workers[3,4].

The specific isomerization addressed in this report arises in peptides and proteins containing proline residues. While the peptide bonds at the proline nitrogen are sufficiently rigid to prevent free rotation, the rate of isomerization is typically too fast to permit separation of the isomers by HPLC at room temperature[5]. The resulting chromatograms contain broad peaks that are highly flow-dependent. Subambient temperatures slow the rate of isomerization, enabling the separation of the pure rotational forms by HPLC[2]. This has allowed the use of HPLC for the analysis of isomer concentrations and for physicochemical studies of molecular conformation.

The investigation reported here is part of a long-term project leading to the separation and characterization of pure isomers by physicochemical, spectroscopic and eventually biological methods. The ultimate goal is the elucidation of the structure function relationships of the isomeric forms of biologically active peptides. Low-temperature HPLC allows the analysis of isomeric composition as a function of solvent, temperature, pH, etc. and can provide information about the hydrophobic surface of isomeric forms. The information obtained by HPLC[6] provides an important adjunct to that obtainable from the traditional techniques used for this type of study, most importantly nuclear magnetic resonance (NMR) spectroscopy. The HPLC techniques are more accessible and cheaper than NMR.

A survey of commercially available bioactive peptides indicates the prevalence of peptides containing proline. This amino acid residue is found in peptides of virtually every class of biological activity. The peptides selected for this study were chosen so as to allow a survey of a number of important types of biological activity and to identify classes of peptides for which the active site might depend on one isomeric form of the proline residue. The peptides used in this study, along with their primary structures, are shown in Table I. In the ccourse of this work, a number of important studies by others have come to light which indicate the significance of this type of analysis. The work of Castiglione-Morelli et al.[7] is particularly interesting in that their results correlate well with the findings of this study and demonstrate the potential of low-temperature HPLC as a route to understanding the activity of morphiceptin.

EXPERIMENTAL

The liquid chromatograph consisted of a Constametric-II pump (Laboratory Data Control, Riviera Beach, FL, U.S.A.), a Rheodyne 7126 valve with 10-μl injector loop (Rheodyne, Berkeley, CA, U.S.A.), a Pecosphere-3 × 3 C$_{18}$ 33 × 4.6 mm I.D. (Perkin Elmer, Norwalk, CT, U.S.A.) and a μLC-10 detector (ISCO, Lincoln, NE, U.S.A.). The column was immersed in a constant temperature bath controlled by an Exatrol controller and cooled by a PBC-4 bath cooler (Neslab, Portsmouth, NH,

TABLE I

BIOLOGICALLY ACTIVE PEPTIDES SEPARATED AT LOW TEMPERATURE

Peptide	Sequence
Aliphatic peptides	
Diprotin B	Val–Pro–Leu
Elastin chemotactic fragment	Val–Gly–Val–Ala–Pro–Gly
Arginine peptides	
Bradykinin	Arg–Pro–Pro–Ala–Phe–Ser–Pro–Phe–Arg
Tyrosine peptides	
Morphiceptin	Tyr–Pro–Phe–Pro–NH$_2$
β-Casomorphin	Tyr–Pro–Phe–Pro–Gly–Pro–Ile
Arginine and tyrosine peptides	
Proctolin	Arg–Tyr–Leu–Pro–Thr

U.S.A.). Chromatograms were recorded, using LabTech Notebook (Laboratory Technologies, Wilmington, MA, U.S.A.) with an AT & T 6300 Personal Computer and integrated, using Lotus 123 (Lotus Development, Cambridge, MA, U.S.A.). A Model 90 digital pH/temperature meter and a micro-electrode (Markson Science, Phoenix, AZ, U.S.A.) were used for pH measurements. The pH of THAM buffers was adjusted with phosphoric acid. All mobile phases were prepared from HPLC-grade solvents (Fisher Scientific, Pittsburgh, PA, U.S.A.), which were filtered and then degassed by helium purge. Flow-rates were 1.0 ml/min except where otherwise noted and the chromatograms were monitored at 210 nm.

Ion-pairing reagents were obtained from Eastman Kodak (Rochester, NY, U.S.A.) and from Aldrich (Milwaukee, WI, U.S.A.) Trizma base for buffer preparation and all peptides were obtained from Sigma (St. Louis, MO, U.S.A.). All peptide solutions contained 1 mg/ml of peptide dissolved in the mobile phase unless otherwise noted.

RESULTS AND DISCUSSION

Isomer separations at low temperature

HPLC separations of isomers of four classes of peptides were developed: aliphatic peptides, peptides with a tyrosine residue, peptides with an arginine residue and those with both arginine and tyrosine residues. Aliphatic peptides, Ala–Pro, Leu–Pro, Ala–Pro–Gly, Val–Pro–Leu, Phe–Ala–Pro and Val–Gly–Val–Ala–Pro–Gly, were initially studied using 0.05 M phosphate mobile phases with varying amounts of methanol. All of the peptides produced broad peaks, characteristic of
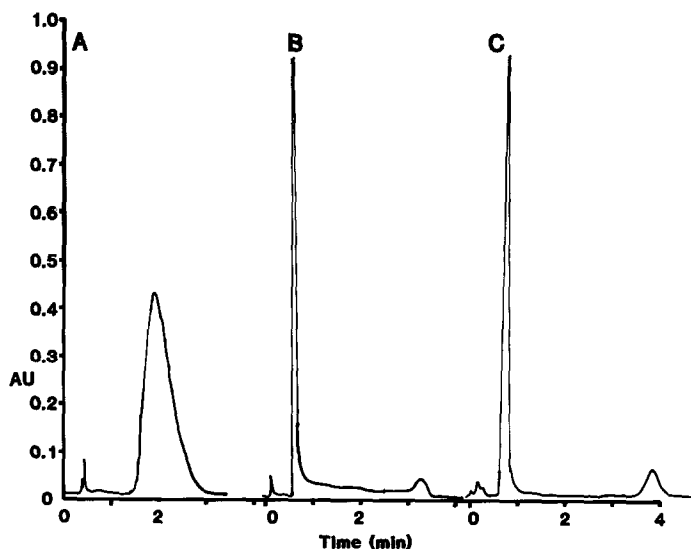

Fig. 1. Separation of Val–Pro–Leu isomers. (A) Room temperature; column Pecosphere 3 × 3 C_{18} 33 × 4.6 mm I.D.; mobile phase methanol–0.05 M phosphate (pH 7.0) (15:85); flow-rate 2.0 ml/min; detection at 210 nm, 0.5 AUFS. (B) Column temperature −0.1°C; mobile phase methanol–phosphate buffer (pH 7.0) (20:80); flow-rate 2.0 ml/min; detector 1.0 AUFS. (C) Column temperature −11°C; flow-rate 1.8 ml/min; detector 1.0 AUFS.
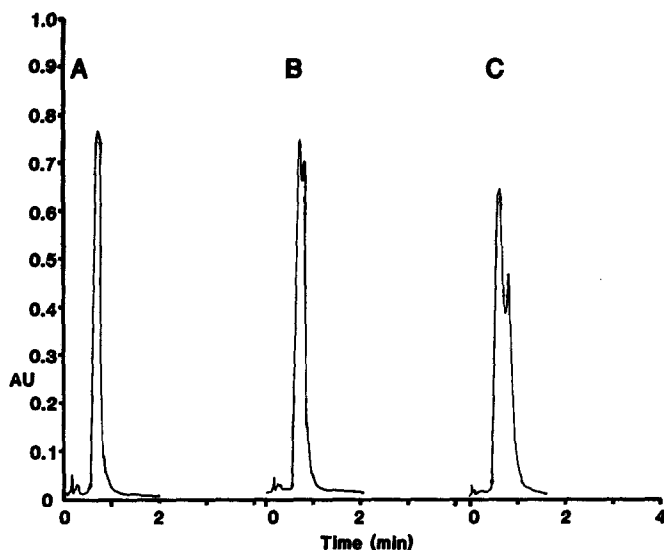
Fig. 2. Chromatograms of proctolin as a function of temperature. Column and detector as in Fig. 1; mobile phase acetonitrile–0.05 M phosphate buffer (pH 7.0)/0.02 M hexanesulfonic acid 20:80; flow-rate 2.0 ml/min; detector 1.0 AUFS. (A) Column temperature 0°C. (B) Column temperature − 5°C. (C) Column temperature, − 10.0°C.

secondary equilibria, at room temperature and two peaks, attributable to the *cis*- and *trans*-isomers, when the temperature was reduced to 0°C. This is typified by the chromatograms for Val–Pro–Leu shown in Fig. 1. The ability to obtain baseline separation depends on the rate of isomerization, flow-rate and retention time. At − 10°C, baseline resolution is obtained even at a flow-rate of 1.0 ml/min (chromatogram not shown), which yields a retention time of about 6.5 min for the second isomer peak. At 0°C a retention time of 3.2 min still produces a noticeable region of sample interconversion between the two peaks, as shown in Fig. 1B.

Separation of polar peptides containing Tyr residues is typified by the separations shown in Fig. 2 for proctolin. This separation required the addition of a alkyl
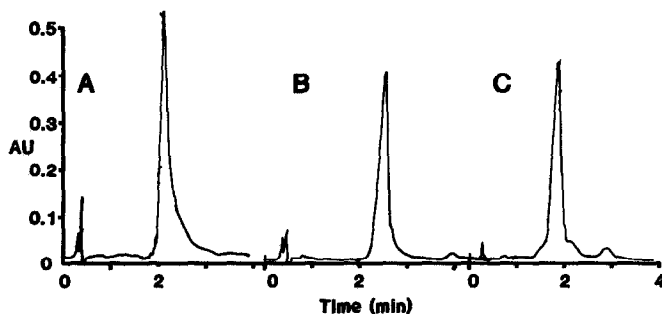

Fig. 3. Separation of bradykinin isomers as a function of temperature. Mobile phase acetonitrile–0.05 M THAM, 0.02 M octanesulfonic acid (pH 7.0) (30:70); flow-rate 1.0 ml/min; 0.5 AUFS; other conditions as in Fig. 1. (A) Column temperature 25°C. (B) Column temperature 0°C. (C) Column temperature − 10°C.
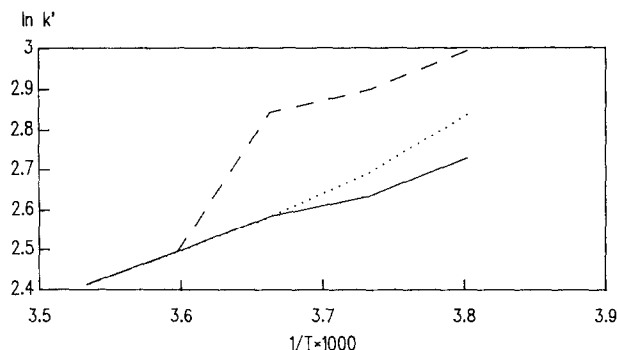
Fig. 4. Van 't Hoff plots for morphiceptin, showing variation of capacity factor (k') as a function of the reciprocal of absolute temperature. Mobile phase acetonitrile–0.05 M THAM, 0.02 M octanesulfonic acid (pH 7.0) (25:75); other conditions as in Fig. 1. ─── = Peak 1; · · · = peak 2; – – – = peak 3.

sulfonate to improve separation of the isomers. The use of acetonitrile instead of methanol as the non-polar component of the mobile phase improved most separations. However, the column became plugged due to salt precipitation when this system was used with phosphate buffers at sub-zero temperatures. The substitution of trishydroxyaminomethane (THAM) for phosphate eliminated this problem.

The separation of peptides containing the Arg residue is demonstrated in Fig. 3 for bradykinin. This peptide contains three proline residues and could occur in eight possible isomeric forms. The chromatograms show the appearance of additional peaks as the column temperature is reduced. It is not clear whether the absence of eight peaks is due to inability to separate the isomers or to the absence of some isomers due to low stability.

The effect of temperature on the capacity factor (k') was studied for morphiceptin and β-casomorphin. A graph of ln k' $vs.$ reciprocal of temperature (Fig. 4) demonstrates the effect of reduced temperature on the resolution obtained for the three isomers of morphiceptin. Note that as the temperature increases no resolution of the isomers is observed due to rapid interconversion. Good separation was attained at − 10.0°C with a mobile phase of 0.05 M THAM (pH 7.0), 0.02 M octanesulfonic
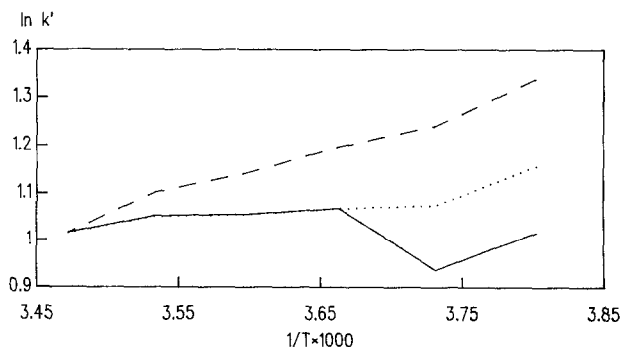

Fig. 5. Van 't Hoff plots for β-casomorphin. Conditions and lines as in Fig. 4.

TABLE II

PERCENT ISOMER AS A FUNCTION OF SOLUTION pH FOR PHE–ALA–PRO

pH	% Isomer	
	Peak 1	Peak 2
3.08	46.9	53.1
4.01	32.6	67.4
5.01	28.8	71.2
5.95	28.5	71.5
6.98	27.2	72.8
7.94	23.7	76.3
8.90	20.9	79.1
9.94	20.4	79.6

acid–acetonitrile (75:25). The results of this study for β-casomorphin are shown in Fig. 5. Figs. 4 and 5 clearly demonstrate the variation in retention as a function of temperature for these two peptides. Such studies can also provide useful information about the hydrophobic character of the individual isomers.

Isomer composition vs. solution pH

Three peptides: Phe–Ala–Pro, bradykinin and morphiceptin, which had been separated most completely were chosen for a study of the effect of pH of the peptide sample on the amount of each isomer. These studies were conducted using the optimal mobile phase and temperature conditions. Eight peptide solutions, identical except for their pH were prepared, covering the pH range from 3.0 to 10.0. All peptide solutions contained *ca.* 0.5 mg/ml. Phe–Ala–Pro solutions were prepared in phosphate buffers, while the other two peptides were prepared with THAM.

Phe–Ala–Pro. Baseline separation of two peaks was obtained for Phe–Ala–Pro at 0°C with a mobile phase of 0.05 M phosphate (pH 7.0)–methanol (70:30). Excellent
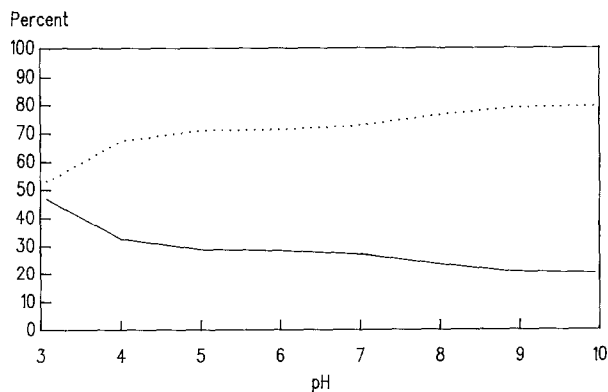

Fig. 6. Composition *cis-* and *trans-*isomers of Phe–Ala–Pro as a function of pH in 0.05 M phosphate solution at room temperature. Mobile phase, methanol–0.05 M phosphate (pH 7.0) (30:70); column temperature 0°C; flow-rate 1.0 ml/min; column and detector as in Fig. 1. Peak 1 is the *trans*-isomer (—), peak 2 the *cis*-isomer (· · ·).

TABLE III

PERCENT ISOMER AS A FUNCTION OF SOLUTION pH FOR MORPHICEPTIN

Peak areas are based on the total area of the three peaks as 100%. A very small leading peak (retention time, 56 s) at low pH which could be the *cis–cis*-isomer is not included.

pH	% Isomer		
	Peak 1	Peak 2	Peak 3
3.06	31.8	47.4	20.8
3.87	32.5	44.6	22.9
5.06	38.5	42.3	19.2
6.02	37.4	45.9	16.7
6.98	35.5	50.0	14.5
7.95	23.3	65.7	11.0
8.92	21.7	67.8	10.6
9.98	20.5	70.2	9.3

separation was obtained for the eight peptide samples. The pH of each sample, covering a range of 3.08 to 9.94, is listed in Table II. A graph of percent isomer *vs.* pH for each Phe–Ala–Pro isomer is shown in Fig. 6. There is a significant shift in isomer composition from pH 3.08 to 9.94. Based on similar behavior for Ala–Pro previously reported, the first peak is assigned to the *trans*-isomer. It has been previously shown for similar aliphatic peptides that the *cis*-isomer predominates at high pH[8]. predominates at high pH[8].

Morphiceptin. The isomeric composition of morphiceptin solutions at various pH values are listed in Table III. A graph of percent isomer *vs.* pH, including all three peaks (Fig. 7) shows the trend of each peak over the entire pH range. Chromatograms for solutions of pH 3.87 and 6.98 are shown in Figs. 8 and 9, respectively. A significant change in the distribution of the area between the first two peaks is obvious from the chromatograms themselves.

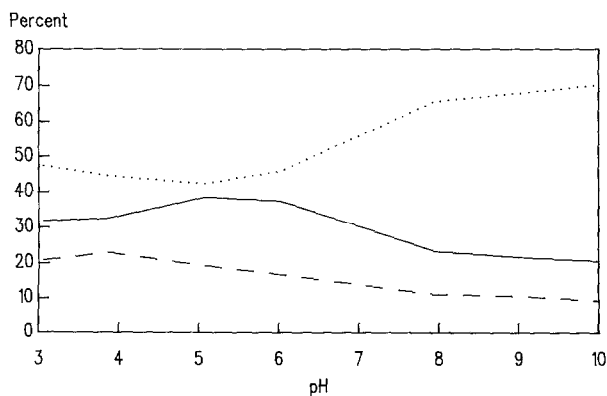The most significant change occurs at the interval between pH 6.98 and pH

Fig. 7. Variation of morphiceptin isomers as a function of pH. Chromatographic conditions as in Fig. 8. Lines as in Fig. 4.
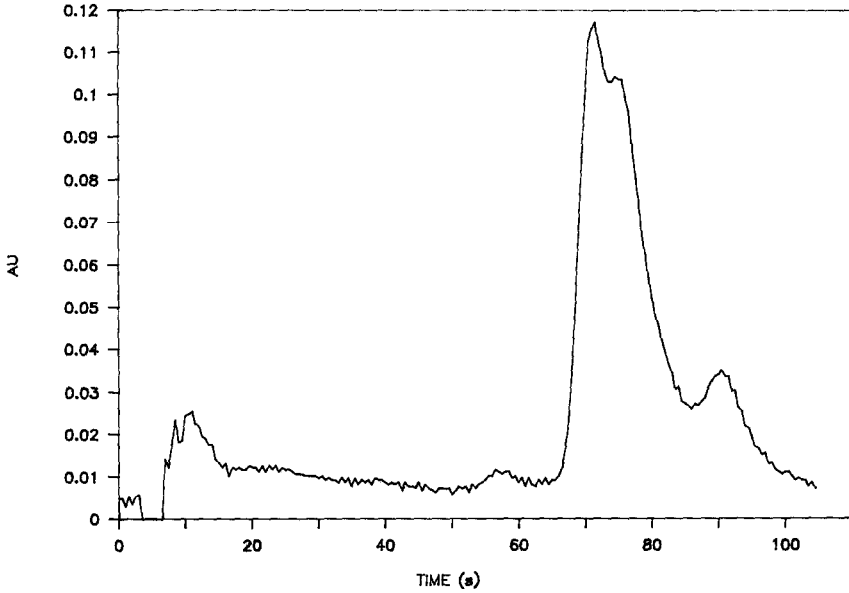
Fig. 8. Chromatogram of morphiceptin solution in 0.05 M THAM at pH 3.87. Column and detector as in Fig. 1. Mobile phase acetonitrile–0.05 M THAM, 0.02 M octanesulfonic acid (pH 7.0) (25:75); flow-rate 1.0 ml/min.
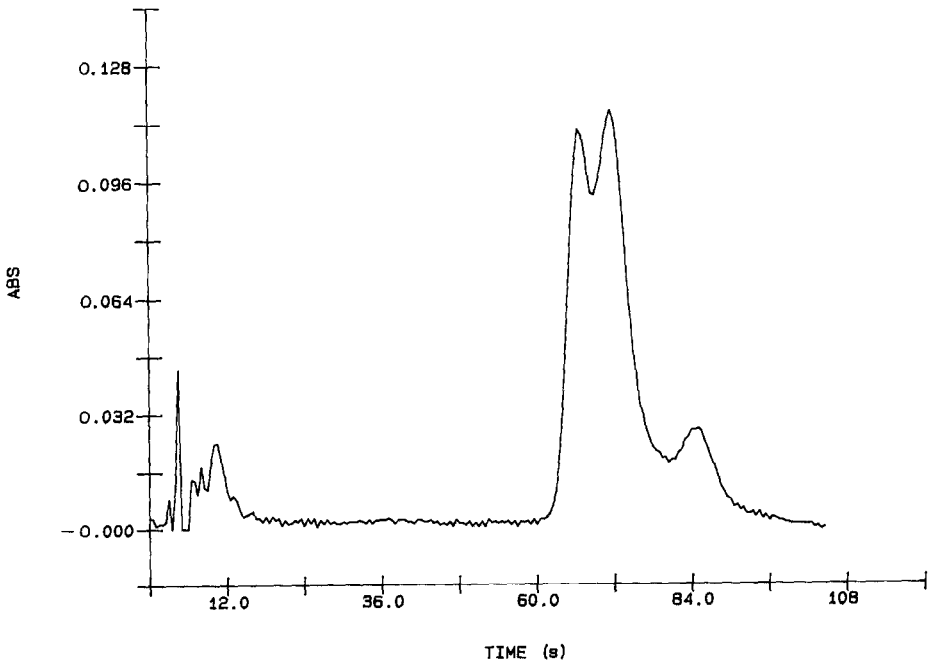

Fig. 9. Chromatogram of morphiceptin solution in 0.05 M THAM at pH 6.98. Chromatographic conditions as in Fig. 8.

7.95, as the first and second peak change by 12.2% and 15.7%, respectively. Thus, a large shift in the conformation of morphiceptin occurs at a biologically important pH range. Recent studies of morphiceptin and its analogues containing other amino acids to restrict conformational flexibility, indicate that the *cis–cis*-form is the biologically active form. This form is not quantitatively measured in the chromatograms, but the small first peak in Fig. 8 may be due to the *cis–cis*-isomer. The isomer ratios for the four peaks in Figs. 8 and 9 imply that the peaks can be assigned to the *cis–cis-*, *cis–trans-*, *trans–trans-* and *trans–cis*-isomers based on a comparison with NMR spectra. The first designation in each pair corresponds to the Tyr–Pro bond and the second to the Phe–Pro bond. The relative amounts of the four isomers reported in dimethyl sulfoxide solution were 7%, 23%, 56% and 14% for the *cis–cis-*, *cis–trans-*, *trans–trans-* and *trans–cis*-conformations respectively[7]. Further study of the peaks and isolation of individual peaks for spectroscopic study will be necessary to confirm the peak assignments.

CONCLUSIONS

There are broad implications of the present study for the entire area of protein and peptide separations. Secondary equilibrium effects due to conformational changes at proline residues can be expected in a large number of proteins and peptides, as the fractions of such molecules which contain at least one prolyl residue is large. The peak-broadening effects of these equilibria may represent a significant contribution to the overall band broadening observed for peptides and proteins. Several solutions to these separation problems are evident. The mobile phase can be selected to provide extreme conditions at which only a single conformation predominates. This will often result in separations which, while of higher efficiency for analytical purposes, have little relevance to the actual structure of the protein. A second alternative is to increase the temperature to accelerate attainment of equilibrium. Again, this approach will often destroy the biological activity and preclude the acquisition of conformational information. Finally, one can reduce temperature to preserve protein structure and attempt to deal with the mutliple peaks produced. This approach produces the greatest amount of information about structural factors. The cost of this approach is a greater demand on the chromatographic column in terms of selectivity, particle diameters and pressure to cope with the high viscosity and slow diffusion rates at low temperatures. Clearly, the optimal approach will depend on the goals of the specific separation.

ACKNOWLEDGEMENTS

REFERENCES

1 D. E. Henderson, S. J. Saltzman, P. C. Uden and Z. Cheng, *Polyhedron*, 7 (1988) 369.
2 D. E. Henderson and Cs. Horváth, *J. Chromatogr.*, 368 (1986) 203.
3 W. R. Melander, H.-J. Lin, J. Jacobson and Cs. Horváth, *J. Phys. Chem.*, 88 (1984) 4527.
4 J. Jacobson, W. R. Melander, G. Valsnys and Cs. Horváth, *J. Phys. Chem.*, 88 (1984) 4536.

5 W. R. Melander, J. Jacobson and Cs. Horváth, *J. Chromatogr.*, 234 (1982) 269.
6 T. Brauman, *J. Chromatogr.*, 373 (1986) 191.
7 M. A. Castiglione-Morelli, B. Hartrodt, K. Neubert, P. A. Temussi and E. Trivellone, *Biochem. Biophys. Res. Commun.*, 152 (1988) 512.
8 C. A. Evans and D. L. Rabenstein, *J. Am. Chem. Soc.*, 96 (1974) 7312.