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Capillary zone electrophoresis at subzero temperatures I. Separation of the *cis* and *trans* conformers of small peptides

Stacey Ma, Franka Kálmán, András Kálmán, Frank Thunecke, Csaba Horváth*

Department of Chemical Engineering, Yale University, New Haven, CT 06520, USA

Abstract

The *cis–trans* conformers of two dipeptides, Phe–Pro and Leu–Pro, and two opioid heptapeptides containing one or two proline residues were separated by capillary zone electrophoresis (CZE) in borate buffer at low temperatures down to −17°C. At temperatures near ambient, the relaxation time of the *cis–trans* isomerization is on the time-scale of minutes for the dipeptides and thus commensurate with the migration times in CZE under usual operating conditions. The conformers of both dipeptides could be separated with baseline resolution below 10°C in neat aqueous 100 m*M* sodium borate (pH 8.4). The conformer peaks on the electropherograms were identified by using authentic samples of the *cis* and *trans* forms of Phe–Pro and Leu–Pro that were obtained by reversed-phase HPLC at 0°C, validated by NMR spectroscopy and stored in liquid nitrogen. The interplay of the electrophoretic migration and on-column isomerization reaction in CZE of Phe–Pro under various conditions was analyzed in the light of the Damköhler number (*Da*). The results showed that besides employing low temperature increasing the voltage and/or decreasing the capillary length also reduce the magnitude of *Da* to bring about the separation of interconverting species. In this work the use of low temperature in this work was preferred due to the experimental simplicity. The separation of *cis–trans* conformers of two opioid heptapeptides was carried out by CZE at subzero temperatures with aqueous sodium borate containing 23% (v/v) glycerol at pH* 11.3 as measured with a glass electrode. The two conformers of Tyr–Pro–Phe–Asp–Val–Val–Gly–NH_2 were baseline separated at −12°C and the four conformers of Tyr–Pro–Phe–Gly–Tyr–Pro–Ser–NH_2 due to the presence of two peptidyl–proline bonds in the molecule, were also resolved at −12°C. From the electrophoretic mobilities, the hydrodynamic radii of the *cis–trans* conformers of the dipeptides Phe–Pro and Leu–Pro were estimated. In both cases, the *trans* isomers had 1.3 times greater Stokes radii than the *cis* conformers. This agrees with the observed migration order and molecular modeling results. The hydrodynamic radii of the Phe–Pro conformers were smaller than those of the Leu–Pro isomers despite the lower molecular mass of the latter. The results demonstrate that CZE is suitable for measuring certain molecular properties and suggest that the methods introduced here are applicable to the study of other systems of interconverting conformers.

1. Introduction

Peptides containing proline residues, but at the N-terminus, are known to exist in both the *cis* and *trans* conformations due to the rigidity of the peptidyl–proline bond [1,2]. *Cis* and *trans* proline dipeptides [3–6] were first separated more than 12 years ago by reversed-phase chromatography at subambient temperatures, where the rate of interconversion is slow enough not to

* Corresponding author.

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interfere with the separation. Recently, even subzero temperatures [7,8] were used for the HPLC separation of the *cis–trans* conformers of certain biologically active tetra- and heptapeptides.

In this work we examined the separation of *cis* and *trans* peptide conformers by capillary zone electrophoresis (CZE), a high-performance analytical tool that offers new avenues to the rapid separation of closely related substances. Another advantageous feature of CZE is that the interpretation of the migration data is not encumbered by interactions with a stationary phase as is the case with the retention data in HPLC. This makes the precision instruments for CZE analysis eminently suitable for the measurement of molecular properties and other physico-chemical data.

At ambient temperature, the relaxation time for the *cis–trans* peptide isomerization reactions investigated so far is on the time-scale of minutes. Consequently, the separation of the conformers by CZE requires the use of either very short capillaries and a specially designed instrument or the employment of subambient/subzero temperatures by simple modification of readily available instrumentation. Albeit the significance of temperature control in CZE has been well recognized [9], the technique is almost exclusively practiced at ambient or near-to-ambient temperatures and only very recently was the separation of certain heterocyclic nitrosamines performed at 5°C [10]. No experiments by CZE at subzero temperatures have been reported.

On the other hand, in conventional gel electrophoresis, subzero temperatures as low as −30°C were employed for investigation of interacting biological systems that are rapid on the time-scale of separation. Ligand binding and subunit exchange within hemoglobin [11] were studied by electrofocusing at −5 to −10°C with ethylene glycol in the polyacrylamide gel. Hybrids of human and sickle cell hemoglobin and partially oxidized human carboxyhemoglobin [12] were separated by both gel electrophoresis and isoelectric focusing using aqueous DMSO at −20 to −30°C. Recently, Harrington and Zewart [13] carried out slab gel electrophoresis at temperatures as low as −20°C to enhance the separation efficiency by the application of high voltage without surpassing the permissible current level.

On the basis of our earlier experience with on-column *cis–trans* isomerization and separation in HPLC [3–6], we have chosen to operate our CZE instrument at temperatures sufficiently low to separate the *cis–trans* conformers of two dipeptides and two opioid heptapeptides [14,15]. The separation of the heptapeptide isomers required temperatures as low as −12°C, therefore particular attention was paid to the choice of the antifreeze. The dimensionless Damköhler number was used to analyze the complex interplay of the first-order reversible reaction and the electrophoretic migration behavior of the *cis–trans* conformers and guidelines were established for the optimization of the separation. Authentic samples of both the *cis* and *trans* conformers of Phe–Pro and Leu–Pro, purified by RP-HPLC and validated by NMR spectroscopy, were used for the identification of the conformer peaks in CZE. The mobilities of the conformers were used to estimate their drag coefficients and thus to assess the differences in their three-dimensional molecular structures. The results obtained by this molecular electrophoretic approach are compared with those attained by HPLC measurements.

2. Experimental

2.1. Chemicals

Optically pure dipeptides L-Phe–L-Pro and L-Leu–L-Pro were purchased from Sigma (St. Louis, MO, USA). The opioid heptapeptides Tyr–Aib–Phe–Asp–Val–Val–Gly–NH_2 (Aib = aminoisobutyric acid), Tyr–Pro–Phe–Asp–Val–Val–Gly–NH_2 and Tyr–Pro–Phe–Gly–Tyr–Pro–Ser–NH_2 were donated by Dr. Ralf Schmidt (Laboratory of Chemical Biology and Peptide Research, Clinical Research Institute of Montreal, Montreal, Quebec, Canada).

Analytical-reagent grade phosphoric acid and sodium hydroxide and HPLC-grade methanol

were supplied by Fisher (Pittsburgh, PA, USA), sodium borate ($Na_2B_4O_7 \cdot 10H_2O$) by Mallinckrodt (Paris, KT, USA), boric acid and anhydrous glycerol by J.T. Baker (Pillipsburg, NJ, USA). Potassium hydrogenphthalate (pH 4.01) and potassium phosphate (pH 7.00) buffer standards for calibration of the pH meter were obtained from Baker and potassium carbonate (pH 10.00) buffer standard from Brand-Nu Laboratories (Meriden, CT, USA). Deionized water was prepared using a NanoPure purification system (Barnstead, Boston, MA, USA) and used throughout.

2.2. Capillary zone electrophoresis

CZE experiments were performed by using a P/ACE 2200 capillary electrophoresis unit (Beckman, Fullerton, CA USA). The temperature control unit of the instrument was assisted by an external cooling system, a Model RTE-4DD refrigerated bath (NESLAB, Portsmouth, NH, USA), which accommodated a heat exchanger coil for the coolant fluid. The heat exchanger was placed in the coolant recirculation line between the pump and the cartridge containing the capillary. The bath was filled with ethylene glycol for operation at subzero temperatures. The coolant temperature, measured at the outlet of the cartridge by an Omega thermocouple (Stamford, CT, USA) with a precision of 0.1°C, was taken as the operating temperature. The capillary electrophoresis unit thus modified was kept and operated in a cold room at 7°C. The software for control of the instrument and data processing was the Microsoft Windows version 3.0 of P/ACE. Fused-silica capillaries of 375 μm O.D. and 50 μm I.D. with polyimide cladding were obtained from Quadrex (New Haven, CT, USA). A narrow segment of the outer coating was removed to open a window for UV–Vis detection at 214 nm. In most experiments, the capillary length and the pertinent migration distances were 370 and 300 mm, respectively.

Sodium borate buffer (pH 8.4) was prepared by adjusting the pH of 25 m*M* aqueous sodium borate solution with 100 m*M* boric acid. For operation at subzero temperatures, 77 parts of 50 m*M* aqueous boric acid solution were mixed with 23 parts of glycerol and 1 *M* NaOH was added to the solution to give a glass electrode reading of pH* 11.3 with a calomel reference electrode (Fisher). By addition of glycerol, the freezing point of the medium is depressed to about −20°C [16].

The samples were dissolved in the buffer at a concentration of ca. 1 mg/ml and injected depending on the temperature over a period from 1 to 8 s at 0.5 p.s.i. (1 p.s.i. = 6894.76 Pa). Between runs the capillary was flushed with at least five column volumes of the buffer at an inlet pressure of 20 p.s.i. The reproducibility of peak height in six experiments was found to be better than 3.2% (R.S.D.). The electroendoosmotic flow was measured by acrylamide as the neutral marker with a reproducibility of the migration time better than 0.5% R.S.D. (four experiments).

2.3. HPLC

For preparative chromatography, a unit assembled from an LDC/Milton Roy (Rivera Beach, FL, USA) ConstaMetric III metering pump, Milton Roy SpectraMonitor variable-wavelength detector and an injection valve with a 20-μl sample loop (Rheodyne, Cotati, CA, USA) was employed. Chromatograms were obtained with a LDC/Milton Roy CI 10 integrator. A 250 × 4.6 mm I.D. column packed with 5-μm UltraSpher ODS RP-18 (Beckman) was jacketed and connected to a NESLAB RTE-4DD refrigerated circulating bath suitable for operation at temperatures as low as −30°C. The eluent was a mixture of methanol–50 m*M* monobasic sodium phosphate solution (30:70, v/v). Before use, the pH reading with the glass electrode was adjusted to 6.0 with 1 *M* NaOH and the solution was filtered and degassed. The peptides were dissolved in the eluent at a concentration of 60 mg ml^{-1}. The chromatographic experiments were performed isocratically at a flow-rate of 0.5 ml min^{-1} and at a detection wavelength of 280 nm. The fractions containing pure conformers were collected in liquid nitrogen [8] and then subjected to CZE measurements.

3. Results and discussion

3.1. CZE at subambient/subzero temperatures

The apparent mobility of the migrant molecules, μ_{app}, is the sum of μ_{eeo}, the electroendoosmotic flow coefficient, and μ_{ep}, the electrophoretic mobility of the migrant. Since under our experimental conditions the corresponding velocities have opposite signs, μ_{app} is given by

$$\mu_{app} = \mu_{eeo} - \mu_{ep} \quad (1)$$

The magnitude of μ_{eeo} is determined by the properties of the electrophoretic system as

$$\mu_{eeo} = \frac{\varepsilon\varepsilon_0\zeta}{\eta} \quad (2)$$

where ζ is the zeta potential at the inner wall of the capillary, ε_0 is the permittivity of the vacuum, ε is the dielectric constant and η is the viscosity of the medium. On the other hand, the electrophoretic mobility, μ_{ep}, depends on the migrant's molecular properties and for spherical molecules is given by

$$\mu_{ep} = \frac{ze}{6\pi\eta R_{hd}} \quad (3)$$

where z is the characteristic charge, R_{hd} is the hydrodynamic (Stokes) radius and e is the unit charge.

The current, I, is proportional to the conductivity of the background electrolyte, κ so that

$$I = \kappa E A_c \quad (4)$$

where E is the applied electric field and A_c is the cross-sectional area of the capillary tube. The conductivity depends on the mobility, μ_i, of the individual ions, i, present in the background electrolyte [17] as

$$\kappa = \sum_i z_i F c_i \mu_i \quad (5)$$

where F is the Faraday constant and c_i is the concentration of the ion i.

According to Eqs. 2, 3 and 5, the mobilities of the migrants and background ions are expected to change with temperature mainly owing to changes in the viscosity of the medium. Under the experimental conditions used in this study, the temperature dependence of the viscosity can be described by the Andrade equation [18]:

$$\eta = A \exp\left(\frac{B}{T}\right) \quad (6)$$

where A and B are empirical constants for a given liquid and T is the absolute temperature. If the dependence of the dielectric constant and the zeta potential on T is negligible, according to Eqs. 2 and 6 the electroendoosmotic flow coefficient is related to the temperature by

$$\ln \mu_{eeo} = C_m - \frac{B}{T} \quad (7)$$

where C_m is constant under our experimental conditions. *Mutatis mutandis*, Eq. 7 holds also for logarithm of the electrophoretic mobility, μ_{ep}.

The temperature dependence of the conductivity is given by

$$\ln \kappa = C_k - \frac{B}{T} \quad (8)$$

where C_k is a constant determined by the ionic strength and molecular dimensions of the background ions. According to Eqs. 7 and 8, both the logarithmic conductivity and electrophoretic flow coefficient are linear functions of the reciprocal temperature with slopes representing properties of the electrophoretic medium.

In order to test Eqs. 7 and 8, electroendoosmotic flow coefficient and conductivity data were measured in neat aqueous borate (pH 8.4) and 50 m*M* borate buffer containing 23% (v/v) glycerol (pH* 11.3). The results are plotted in Fig. 1 against the operating temperature in the range from 1 to 50°C (dashed lines) and from −17 to 40°C (solid lines), respectively. Since all plots are straight lines with correlation coefficients better than 0.99, the data strongly support the validity of Eqs. 7 and 8. As expected, the two pairs of lines representing the mobility and conductivity data obtained in the same medium have almost identical slopes. For parameter B in neat aqueous borate buffer, we obtain a mean value of 1802 K, which compares well with the literature value of 1844 K for water [16]. With

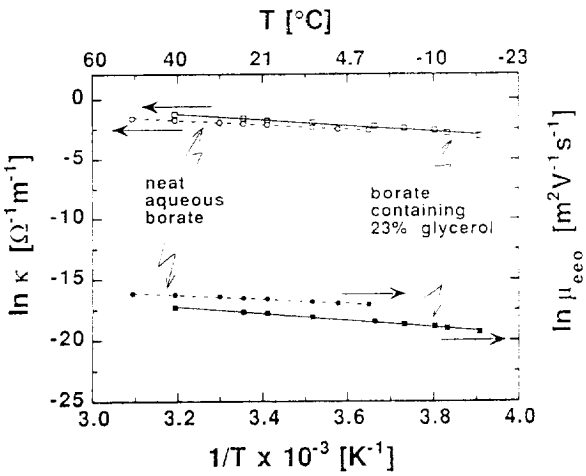

Fig. 1. Plots of the electroendoosmotic flow coefficient, μ_{eeo}, and the conductivity, κ, of the medium against the temperature. (○) κ and (●) μ_{eeo} with neat aqueous 100 mM sodium borate buffer (pH 8.4) in the range 1–50°C; (□) κ and (■) μ_{eeo} with aqueous 50 mM sodium borate containing 23% (v/v) glycerol (pH* 11.3) in the range from −17 to 40°C. Capillary, 37 cm × 50 μm I.D.; voltage, 30 kV; detector setting, 214 nm; marker for electroendoosmotic flow, acrylamide.

the aqueous glycerol mixture, the average value of B is 2610 K and thus significantly higher than that shown above for the neat aqueous buffer. Since at 20°C the respective viscosities of water and the 23% (v/v) water–glycerol mixture are 1.0 and 1.9 CP [19], the findings in this study are consistent with the Lewis–Squires postulate [20] that the sensitivity of the viscosity of liquids to temperature changes depends primarily on the magnitude of the liquid viscosity. Therefore, the higher the viscosity of the liquid, the greater is the change on temperature variation and therefore the larger is B.

The molecular diffusivity, D_m, has a stronger dependence on the temperature than the viscosity does, as seen from the following expression valid for spherical molecules:

$$D_m = \frac{RT}{6\pi\eta R_{hd}} \tag{9}$$

where R is the gas constant. In CZE, when longitudinal molecular diffusion is the only factor contributing to zone dispersion, then the separation efficiency in terms of theoretical plates [21–24] is given by

$$N = \frac{zFV}{2RT} \tag{10}$$

where N is the number of theoretical plates and V is the applied voltage. According to Eq. 10, the separation efficiency increases with decreasing temperature. The enhancement of efficiency at subzero temperatures can be clearly seen in Fig. 2, which depicts two electropherograms of the opioid heptapeptide Tyr–Aib–Phe–Asp–Val–Val–Gly–NH_2 obtained with the glycerol–aqueous borate buffer (pH* 11.3) at −12 and

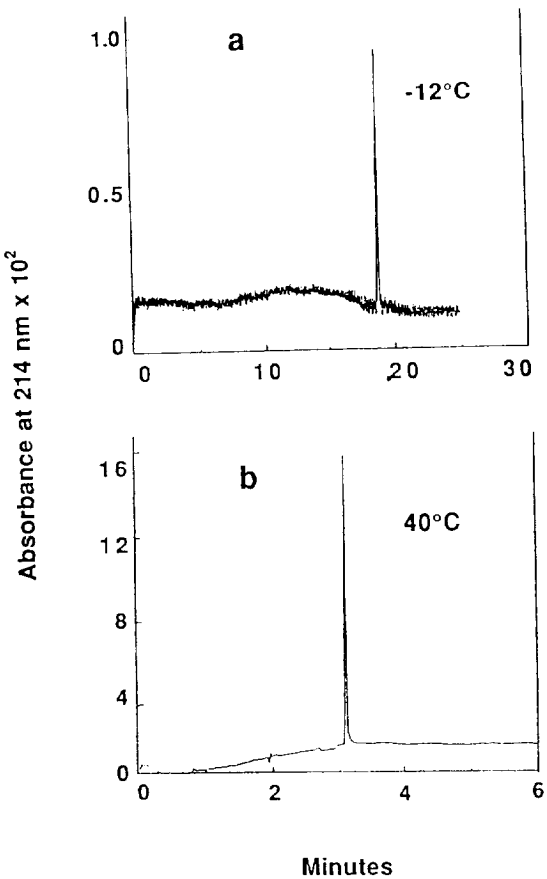

Fig. 2. Electropherograms of Tyr–Aib–Phe–Asp–Val–Val–Gly–NH_2 obtained at −12 and 40°C. Capillary, 37 cm × 50 μm I.D.; buffer, aqueous 50 mM sodium borate containing 23% glycerol (pH* 11.3); voltage, 30 kV; detector setting, 214 nm. (a) Temperature, −12°C; current, 9 μA; (b) temperature, 40°C; current, 47 μA.

40°C. The peak sharpness is nearly the same in the two electropherograms, although the migration time is about six times longer at −12°C than at 40°C.

3.2. Interplay of electrophoretic migration and reaction

The *cis–trans* isomerization of the dipeptides has been treated [2] as a reversible first-order reaction:

$$cis \underset{k_r}{\overset{k_f}{\rightleftharpoons}} trans \tag{11}$$

where k_f is the rate constant for the forward and k_r for the reverse reaction. The equilibrium constant, K_m, is given by

$$K_m = \frac{[trans]}{[cis]} = \frac{k_f}{k_r} \tag{12}$$

and the relaxation time, τ, for the reaction is

$$\tau = \frac{1}{k_f + k_r} = \frac{1}{k_f\left(1 + \frac{1}{K_m}\right)} \tag{13}$$

The Arrhenius equation describes the temperature dependence of the reaction rate as

$$k = A_k \exp\left(-\frac{E_a}{RT}\right) \tag{14}$$

where A_k is the pre-exponential factor and E_a is the activation energy for isomerization.

The interplay of migration and reaction is governed by the dimensionless Damköhler number, *Da*, which is defined as the ratio of the residence time of the migrant in the capillary, t_R, to the relaxation time of the reaction, τ. In capillary electrophoresis, *Da* can be expressed as

$$Da = \frac{t_R}{\tau} = k_f\left(1 + \frac{1}{K_m}\right)\frac{l}{v_{app}} \tag{15}$$

where l is the pertinent migration length of the capillary and v_{app} is the apparent velocity of the migrant. The latter is given by the relationship

$$v_{app} = \mu_{app}E = \mu_{app}\frac{V}{L} \tag{16}$$

where L is the total length of the capillary. By combining Eqs. 1–3, 15 and 16, we obtain

$$Da = k_f\left(1 + \frac{1}{K_m}\right)\frac{lL}{V\left(\varepsilon\varepsilon_0\zeta - \frac{ze}{6\pi R_{hd}}\right)}\cdot\eta \tag{17}$$

As the viscosity and the rate constants are related to temperature by Eqs. 6 and 14, respectively, the *Da* can be further expressed as

$$Da = C\cdot\frac{lL}{V}\exp\left(\frac{B - E_a/R}{T}\right) \tag{18}$$

where

$$C = \left(1 + \frac{1}{K_m}\right)\frac{A_k A}{\varepsilon\varepsilon_0\zeta - \frac{ze}{6\pi R_{hd}}} \tag{19}$$

Examination of Eqs. 18 and 19 shows that the magnitude of *Da* is determined roughly by four factors. The parameter C encompasses the properties of the tube wall, the migrant molecules and some kinetic properties and thus it represents the chemical nature of the system. The other factors are the pertinent tube length, l, and the electric field strength, V/L. The exponential term represents the effect of temperature on the kinetics of the reaction and on the viscosity of the medium.

The enthalpy change for the *cis–trans* isomerization of the dipeptides is close to zero in aqueous media [25,26], thus the equilibrium constant changes very little with temperature. Assuming that the dielectric constant, the zeta potential of the capillary inner wall and the molecular properties of the peptides are also invariant with temperature, the parameter C can be considered constant. According to Eq. 18, in this case the magnitude of the Damköhler number increases exponentially with the reciprocal of temperature and is proportional to the approximate capillary length squared and inversely proportional to the applied voltage. From the results in the literature [3–5] and the above discussion, it follows that the separation of interconverting species is facilitated at low Damköhler numbers. Since the kinetics are determined by the chemical nature of the system, the other avenues to lowering *Da* are based on the somewhat conflict-

ing conditions: the capillary length is short, the temperature is low and the voltage is high.

3.3. Electrophoretic behavior of phenylalanyl–proline and leucyl–proline

The dipeptides Phe–Pro and Leu–Pro were chosen to study the interplay of electrophoretic migration and reversible *cis–trans* isomerization, since the conformers of both peptides are reasonably stable below 15°C. Further, authentic samples of each conformer were available from low-temperature HPLC and subsequently validated by NMR spectroscopy [8]. Throughout this study, the operating conditions, except the temperature and capillary length, were kept constant. Thus, changes in the Damköhler number were brought about by varying one or both of these parameters.

Effect of temperature and capillary length

Electropherograms of Phe–Pro and Leu–Pro obtained at 5, 25 and 40°C with 100 m*M* aqueous sodium borate buffer (pH 8.4) are shown in Fig. 3. As can be seen, the electrophoretic pattern undergoes significant changes within this temperature range. At 5°C, each dipeptide yielded two baseline-separated peaks believed to be the *cis* and *trans* conformers. At 25°C, the resolution was poor and the height ratios of the conformer peaks were different from those at 5°C. Finally at 40°C, each peptide yielded only a single, sharp peak.

In order to investigate the effect of the capillary length on the separation, experiments were carried out at 5°C by using 37, 57 and 97 cm long capillaries of 50 μm I.D. and the electropherograms are depicted in Fig. 4. It can be seen that baseline separation was obtained only with the shortest tube; the electropherograms obtained

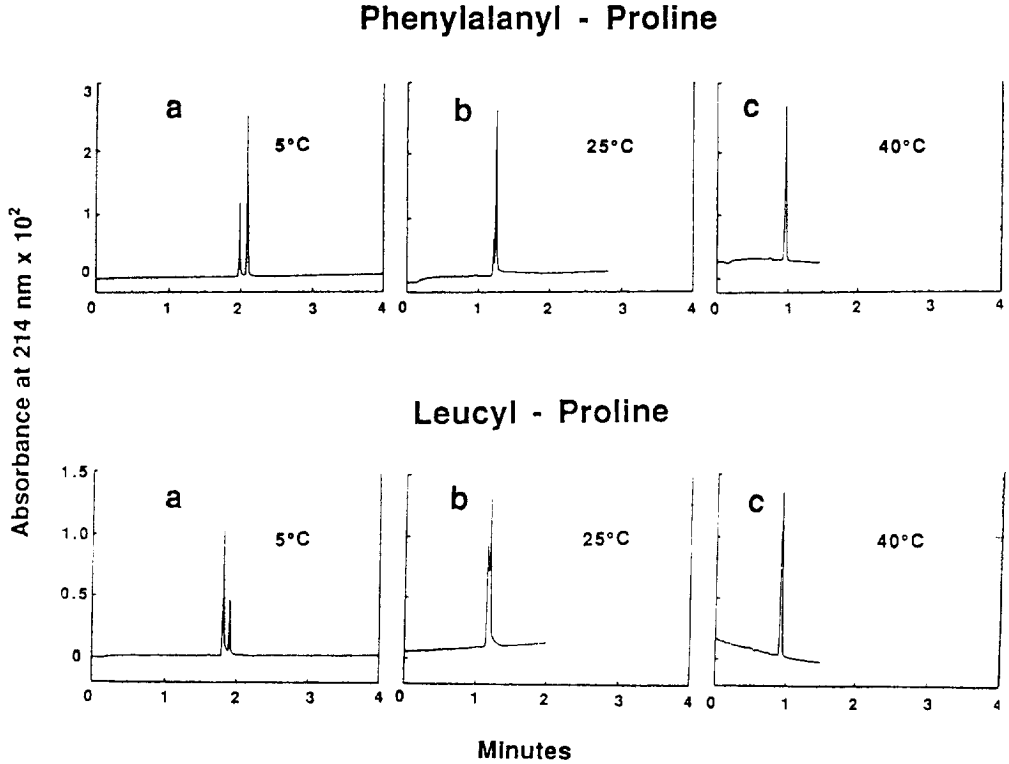

Fig. 3. Effect of temperature on the separation of *cis* and *trans* conformers of Phe–Pro and Leu–Pro. Capillary, 37 cm × 50 μm I.D.; buffer, neat aqueous 100 m*M* sodium borate (pH 8.4); voltage, 30 kV; detector setting, 214 nm. (a) Temperature, 5°C; current, 13 μA; (b) temperature, 25°C; current, 22 μA; (c) temperature, 40°C; current, 29 μA.

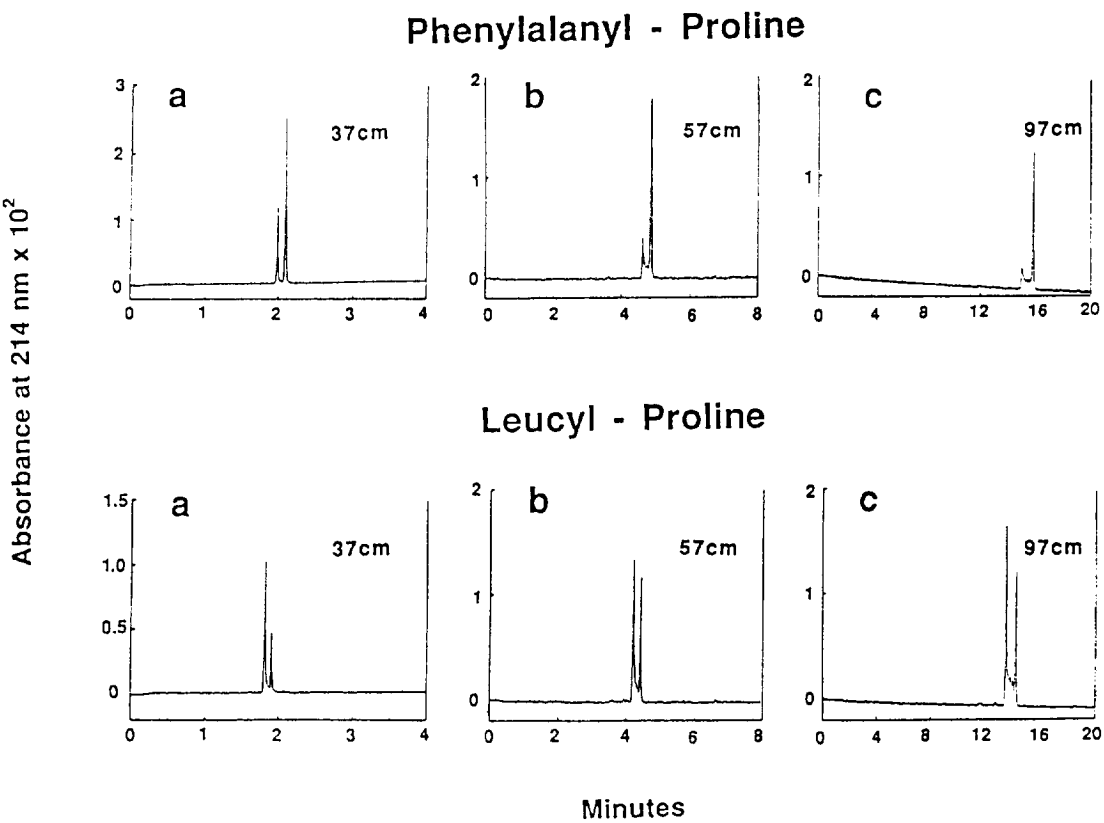

Fig. 4. Separation of the *cis–trans* conformers of Phe–Pro and Leu–Pro with 50 μm I.D. capillaries of different lengths. Buffer, 100 m*M* sodium borate buffer (pH 8.4); voltage, 30 kV; temperature, 5°C; detector setting, 214 nm. (a) Length, 37 cm; current, 13 μA; (b) length, 57 cm; current, 9 μA; (c) length, 97 cm; current, 5 μA.

with the 57 and 97 cm long capillaries show that the migration times were greatly increased and concomitantly the two peaks were not resolved owing to the intervening reaction zone.

In order to identify the conformer peaks, authentic *cis* and *trans* conformers separated by RP-HPLC, collected and kept in liquid nitrogen and validated by NMR spectroscopy, were used since CZE experiments percluded the collection of the isolated conformers in amounts sufficiently large for NMR measurements. Typical chromatograms from the RP-HPLC experiments at 0°C are shown in Fig. 5. The pure conformers isolated in such experiments were subjected to CZE in aqueous sodium borate buffer (pH 8.4) at 11°C. The migration times of the authentic conformers and those of peaks A and B in Fig. 6 are listed in Table 1. Comparison of the migration times in Table 1 shows that the two baseline-resolved peaks A and B obtained in the CZE of the dipeptides are indeed the *trans* and *cis* conformers, respectively.

The electrophoretic behavior depicted in Figs. 3 and 4 is readily explained in the light of the Damköhler number defined in Eq. 15. The measurement of both the equilibrium constant K_m and the forward rate constant k_f for the *cis–trans* interconversion by different methods under conditions employed in HPLC and CZE is described in a forthcoming paper [27]. The following parameters were obtained by the "stop-flow" method for the isomerization of Phe–Pro in the neat aqueous sodium borate buffer: the forward reaction rate constant k_f at 10°C is $1.42 \cdot 10^{-4}$ s^{-1}, the equilibrium constant K_m is 0.37 and the activation energy E_a is 19.4

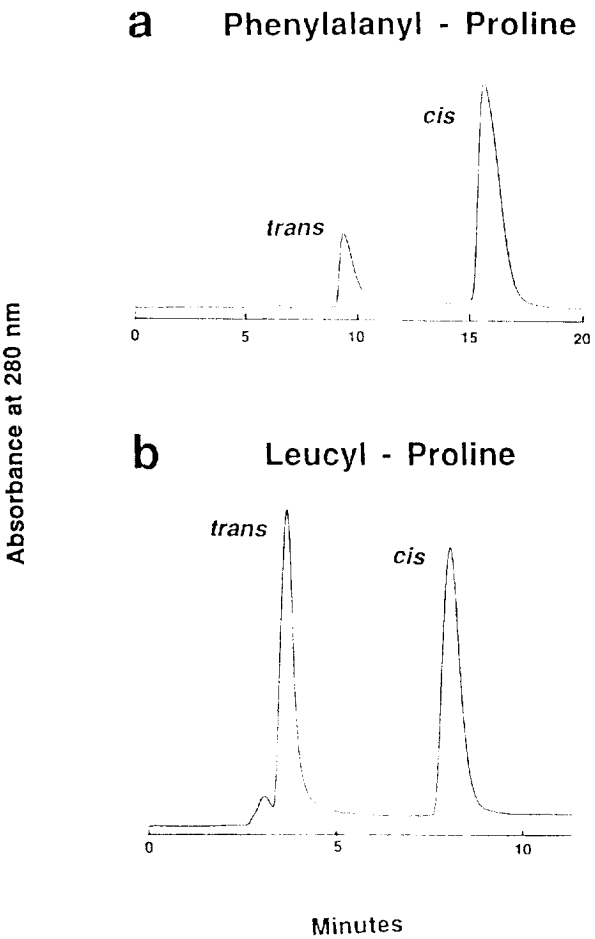

Fig. 5. Chromatographic separation of the *cis–trans* conformers of (a) Phe–Pro and (b) Leu–Pro at 0°C by RP-HPLC. Column, 250 × 4.6 mm I.D., 5-μm UltraSpher ODS; detector setting, 280 nm; flow-rate, 0.5 ml min^{-1}; mobile phase, 50 m*M* phosphate buffer-methanol (70:30, v/v) (pH* 6.00).

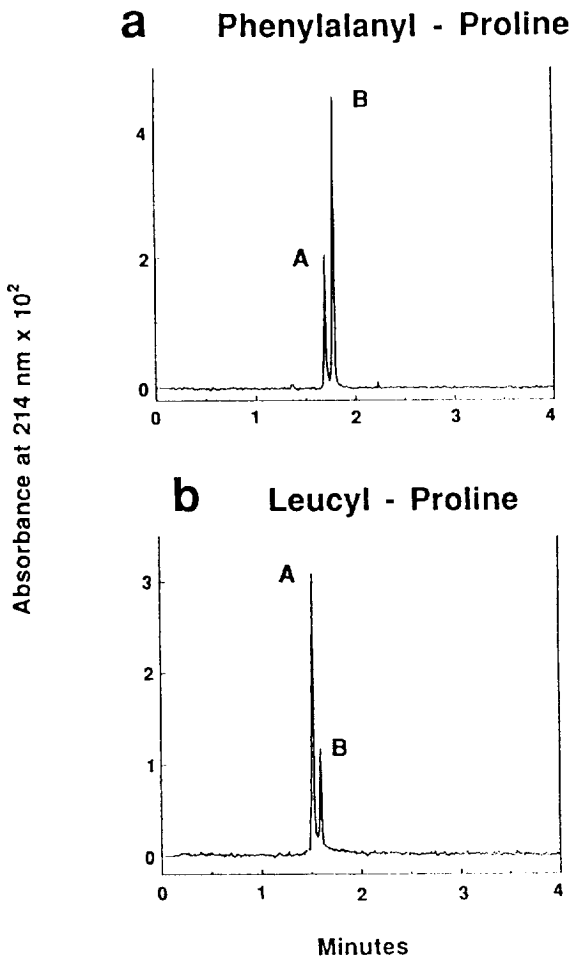

Fig. 6. Electropherograms of (a) Phe–Pro and (b) Leu–Pro showing the peaks of the *cis–trans* isomers identified by using authentic conformers isolated by cryochromatography. Capillary, 37 cm × 50 μm I.D.; buffer, neat aqueous 100 m*M* sodium borate (pH 8.4); voltage, 30 kV; current, 16 μA; temperature, 11°C; detector setting, 214 nm.

kcal mol^{-1}. With the above data and the measured migration times, the Damköhler numbers were calculated by using Eq. 15 and the results are presented in Table 2. As can be seen, the Damköhler number ranges from 0.03 to 1.8 under the conditions investigated and decreases with decrease in temperature and/or the capillary length, as predicted from Eq. 18.

The interference between migration and reaction is conveniently measured by λ, the relative amount of the substance in the reaction zone between the two peaks as illustrated in Fig. 7, which shows the dependence of λ on *Da*. When $Da > 0.5$, the isomerization is rapid on the timescale of migration and the *cis–trans* conformers elute as a single peak. When $Da < 0.1$, the isomerization is sufficiently slow for the interference to be negligible and the two conformers are separated with baseline resolution. In the range $0.1 < Da < 0.5$, there is an intervening plateau region between the peaks with concomitant deterioration of the separation efficiency.

Such *cis–trans* interconversion together with other on-column reactions, e.g., oxidation, ag-

Table 1
Migration times of the authentic *cis* and *trans* conformers and of peaks A and B obtained from each dipeptide by CZE as shown in Fig. 6

Dipeptide	Migration time, t_R (min)			
	Peak A	*trans*	Peak B	*cis*
Phe–Pro	1.669	1.677	1.760	1.750
Leu–Pro	1.545	1.557	1.614	1.598

Capillary, 37 cm × 50 μm I.D.; buffer, 100 m*M* sodium borate (pH 8.4); voltage, 30 kV; current, 16 μA; temperature, 11°C.

glomeration, complexation and different kinds of isomerization, have been observed to interfere with the separation not only in CZE [10] and slab gel electrophoresis [11,12] but also in HPLC [28], where the degree of interference was expressed by the Damköhler number [3]. The critical range of *Da*, where the interference of the on-column reaction with the separation leads to the formation of an inter-peak reaction zone or to additional band broadening at the least, varies with the chromatographic system used and the kinetics of the reaction. However, in general such untoward phenomena are not expected to occur according to the literature [28] when $Da < 0.1$ or $Da > 50$. The critical *Da* range in CZE, however, must be narrower than in HPLC owing to the much higher plate numbers achievable in

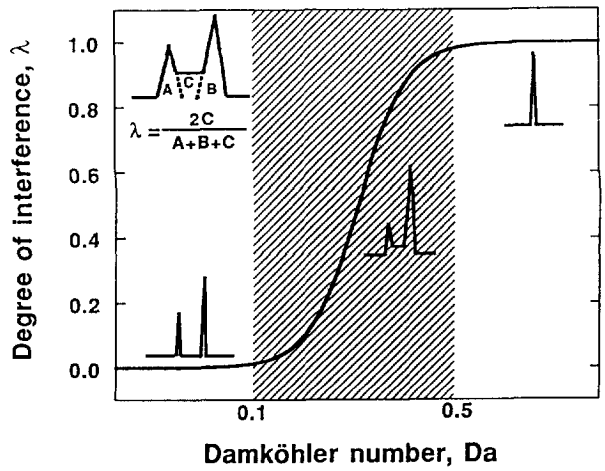

Fig. 7. Schematic illustration of the degree of interference, λ, by on-column *cis–trans* isomerization on the separation of *cis*- and *trans*-Phe–Pro as function of the Damköhler number.

CZE. Indeed, with the *cis–trans* isomerization of Phe–Pro, no interference of the separation by the reaction was observed outside the range $0.1 < Da < 0.5$.

Effect of molecular structure: charge and size

Fig. 6 shows electropherograms of the *cis–trans* conformers of Phe–Pro and Leu–Pro obtained by CZE at 11°C with 100 m*M* sodium borate buffer (pH 8.4) at 810 V/cm. It is intriguing to attempt an interpretation of the observed

Table 2
Damköhler numbers characterizing the interplay of the *cis–trans* isomerization and the electrophoretic migration of Phe–Pro at different temperatures and in capillaries of different lengths under the experimental conditions

Damköhler number, *Da*	Operating temperature, *T* (°C)	Capillary length, *L* (cm)	Migration time, t_R (min)	Forward rate constant, k_f (10^{-4} s^{-1})	Relaxation time, τ (min)
0.034	5	37	2.09	0.73	61.88
0.059	10	37	1.88	1.42	31.69
0.080	5	57	5.00	0.73	61.88
0.096	16	37	1.56	2.77	16.26
0.135	20	37	1.38	4.39	10.25
0.206	25	37	1.21	7.68	5.86
0.256	5	97	15.80	0.73	61.88
0.507	35	37	1.03	22.25	2.02
0.790	40	37	0.96	36.92	1.22
1.823	50	37	0.85	96.97	0.46

Capillary ID, 50 μm; buffer, 100 mM sodium borate, pH 8.4; voltage, 30 kV.

electrophoretic behavior in terms of the molecular properties of the dipeptides [29]. For such an analysis, first the electrophoretic mobilities of the migrants have to be evaluated. It should be noted that under the conditions of our experiments, they are negatively charged and therefore migrate electrophoretically towards the anode against the counteracting electroendoosmotic flow, the coefficient, μ_{eeo}, of which is 4–8 times greater than the μ_{ep} values of the peptides listed in Table 3. As a consequence, the apparently faster migrating sample component in Fig. 6 has the lower electrophoretic mobility.

Our goal is to estimate from the electrophoretic mobilities the Stokes radii of the four dipeptides assuming that the electrophoretic mobility of such small peptides can be expressed by Eq. 3 as a function of their characteristic charge and size. The *cis–trans* conformers of each dipeptides share the same molecular mass and ionogenic functions. However, they have significantly different electrophoretic mobilities, as seen in Table 3. This finding suggests that the drag coefficient of the molecules is strongly affected by the conformation of the peptide.

Similar observations have also been reported on the electrophoretic behavior of the *cis–trans* isomers maleic and fumaric acid [30] and the latter was found to have the lower electrophoretic mobility. By assuming that the characteristic charges of the two isomers are essentially the same, the authors concluded that fumaric acid, the *trans* isomer, has a greater hydrodynamic radius than maleic acid. In our case, we used the Henderson–Hasselbach equation with pK_a values of the amino acids adjusted for microenvironmental effects [31] to estimate the electrostatic charges of the dipeptide conformers, and the results are presented in Table 3. With the z values, which are of course the same for a given *cis–trans* pair, the Stokes radii of each conformer were calculated from their mobilities by Eq. 3 and are also shown in Table 3. For both dipeptides, the *trans* conformers are larger than the *cis* conformers in terms of the hydrodynamic radii, as illustrated schematically in Fig. 8. Thus the slightly smaller size of the *cis* conformers is

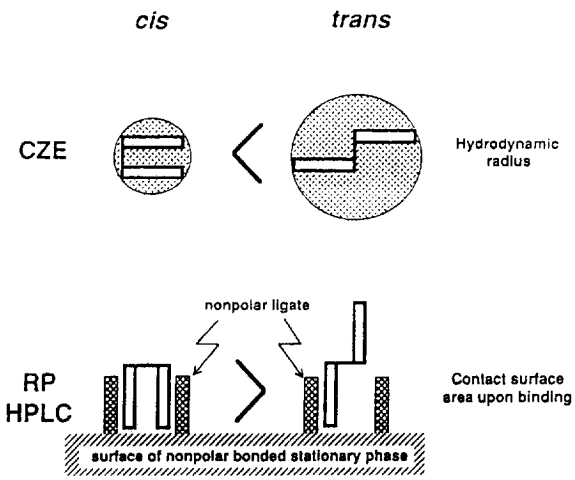

Fig. 8. Illustration of the putative origin of selectivity for the *cis* and *trans* dipeptide conformers in CZE and RP-HPLC. The *trans* isomer has a larger Stokes radius than the *cis* form and therefore has lower electrophoretic mobility in CZE. In HPLC, the *trans* form is less retained because its contact surface with the ligates of the non-polar bonded stationary phase is smaller than that of the *cis* isomer.

Table 3
Properties of the *cis* and *trans* conformers of Phe–Pro and Leu–Pro

Peptides	Molecular mass	Electrophoretic mobility, μ_{ep} (10^{-9} m^2 V^{-1} s^{-1})	μ_{cis}/μ_{trans}	Characteristic charge, z	Stokes radius, R_{hd} (Å)
cis-Phe–Pro	262.3	11.07	1.20	−0.83	4.87
trans-Phe–Pro	262.3	9.24	1.20	−0.83	5.84
cis-Leu–Pro	228.3	7.47	1.34	−0.61	5.31
trans-Leu–Pro	228.3	5.57	1.34	−0.61	7.12

Electrophoretic mobilities were calculated by Eq. 1 from data measured in 100 m*M* sodium borate (pH 8.4) at 11°C and the Stokes radii were calculated by Eq. 3. The charges on the peptides were calculated from the adjusted pK_a values of the amino acid residues [31] by the Henderson–Hasselbach equation.

likely to be responsible for their higher mobility. Higher molecular mass, however, does not necessarily mean a larger hydrodynamic radius, as seen from the properties of the corresponding conformers in Table 3. Despite their higher molecular masses, the Phe–Pro isomers were found to have smaller Stokes radii than the Leu–Pro conformers. Since these results are contingent upon the accuracy of the pK_a values, their further refinement is essential to advance the accuracy of such calculations.

The *cis–trans* conformers of numerous dipeptides were separated by reversed-phase chromatography [3–6] and the selectivity of the chromatographic system was attributed to differences in the hydrophobic surface areas of the conformers. For the two dipeptides in the present study, by careful examination of the appropriate molecular models, the *trans* conformer was found to have the smaller hydrophobic contact areas upon binding. The illustration in Fig. 8 of the hypothetical binding of the *cis* and *trans* dipeptides to the alkyl chains at the surface of non-polar bonded phases reflects the experimental observation that the retention times of the *trans* isomers are shorter than those of the corresponding *cis* conformers.

3.4. Subzero CZE of opioid heptapeptides with one or more proline resides

The *cis–trans* conformers of the dipeptides Phe–Pro and Leu–Pro were successfully separated at temperatures above 0°C, as was shown in Figs. 3, 4 and 6. Comparable separations of isomeric opioid heptapeptides, however, required subzero temperature and the use of glycerol as an "antifreeze" in the sodium borate buffer. This is illustrated in Fig. 9 by the electropherograms of Tyr–Pro–Phe–Asp–Val–Val–Gly–NH_2, which has a single peptidyl–propline bond and therefore is expected to have two

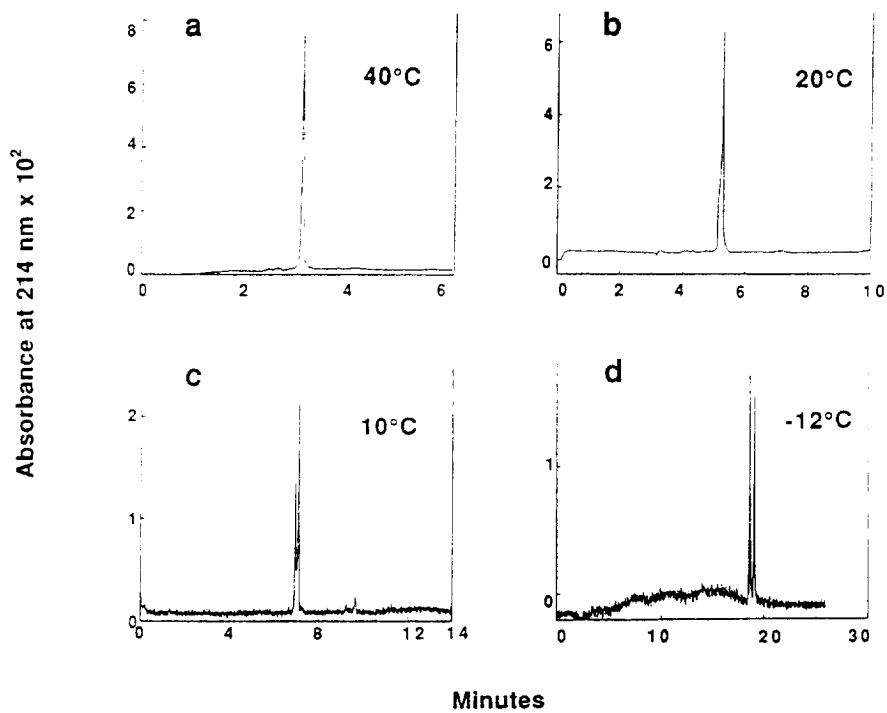

Fig. 9. Electropherograms of Tyr–Pro–Phe–Asp–Val–Val–Gly–NH_2 obtained at various temperatures. Capillary, 37 cm × 50 μm I.D.; buffer, aqueous 50 m*M* sodium borate containing 23% glycerol (pH* 11.3); voltage, 30 kV; detector setting, 214 nm. (a) Temperature, 40°C; current, 47 μA; (b) temperature, 20°C; current, 30 μA; (c) temperature, 10°C; current, 23 μA; (d) temperature, −12°C; current, 9 μA.

relatively stable conformers. A mixture of 77% (v/v) borate buffer and 23% (v/v) glycerol was the electrophoretic medium (the reading of the pH meter with glass electrode was pH* 11.3) and the effect of temperature on the electrophoretic behavior is shown in Fig. 9. The heptapeptide yields only a single sharp peak at 40°C (Fig. 9a), but on lowering the temperature, a shoulder appears at the leading edge of the peak at 20°C (Fig. 9b). The shoulder becomes a sharp peak at the front of the main peak at 10°C, as depicted in Fig. 9c. With further decrease in temperature, this sharp peak increases and at −12°C it becomes taller than the "main" peak and the two are separated with baseline resolution (Fig. 9d).

In order to elucidate the effect of the glycerol content of the electrophoretic medium on the separation of the *cis–trans* peptide isomers at low temperatures, Phe–Pro was also subjected to CZE in the aqueous–organic mixture described above and the electropherograms obtained at four different temperatures are shown in Fig. 10. The peptide yielded only a single peak at 40°C, but on lowering the temperature the two conformers gradually emerged as individual peaks and were separated with baseline resolution at 10°C. In neat aqueous borate, the electrophoretic migration behavior of Phe–Pro was essentially the same as in the medium containing glycerol and, as seen in Fig. 6, the separation of the conformers was also completed on lowering the temperature to 11°C. Comparison of the two cases suggests that the presence of the glycerol has no major effect on the chemical nature of the system. On the other hand, Fig. 10c and d confirm that the resolution improved with further decrease in temperature and reveal that the effect of glycerol is mainly due to imparting to the medium the high viscosity that is responsible for the much improved separation efficiency by virtue of a concomitant decrease in diffusivity and the much longer migration rates.

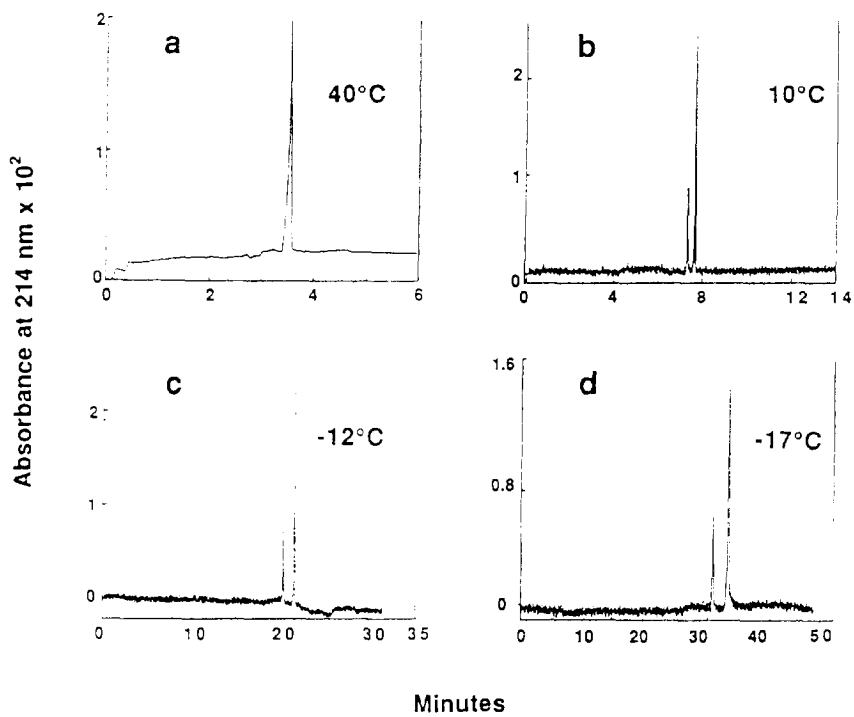

Fig. 10. Electropherograms of Phe–Pro obtained at various temperatures. Capillary, 37 cm × 50 μm I.D.; buffer, aqueous 50 m*M* sodium borate containing 23% glycerol (pH* 11.3); voltage, 30 kV; detector setting 214 nm. (a) Temperature, 40°C; current, 47 μA; (b) temperature, 10°C; current, 23 μA; (c) temperature, −12°C; current, 9 μA. (d) temperature, −17°C; current, 7 μA.

The effect of temperature on the resolution of the *cis–trans* isomers of different peptides in the same electrophoretic medium can be assessed by comparing Figs. 9 and 10, which illustrate the respective electropherograms of the heptapeptide and Phe–Pro obtained in the same medium containing 23% (v/v) glycerol. It is seen that the Phe–Pro isomers could be resolved at 10°C, whereas the separation of the *cis* and *trans* forms of the heptapeptide required −12°C. The temperature, at which the *cis–trans* peptide forms are completely resolved under otherwise fixed conditions, could be used as a practical measure of the difficulty associated with the separation. This temperature measured with a fixed electrophoretic system could be used to rank interconverting peptides according to their kinetics. In turn, by using a suitable peptide standard and different media, the temperature of complete resolution would be a characteristic of media effects.

It is recalled that the Damköhler number is an exponential function of the reciprocal temperature and depends roughly on the capillary length squared. Therefore, reducing the capillary length offers an alternative to lowering the temperature in order to diminish *Da* and thus improve the separation of the conformers. Computer simulations are in progress to shed light on the effect of temperature and capillary length on the separation of such interconverting species over a wide range of conditions.

Up to now, we have examined the CZE of peptides containing a single proline residue. In the following, we report results with the electrophoretic migration behavior of the opioid heptapeptide Tyr–Pro–Phe–Gly–Tyr–Pro–Ser–NH_2, which has two peptidyl–proline bonds and consequently should have, at sufficiently low temperatures, four relatively stable conformers: *cis–cis*, *cis–trans*, *trans–cis* and *trans–trans*. CZE experiments were conducted with the 50 m*M* aqueous sodium borate–glycerol mixture (pH* 11.3). Again, at 40°C a single peak is obtained, as

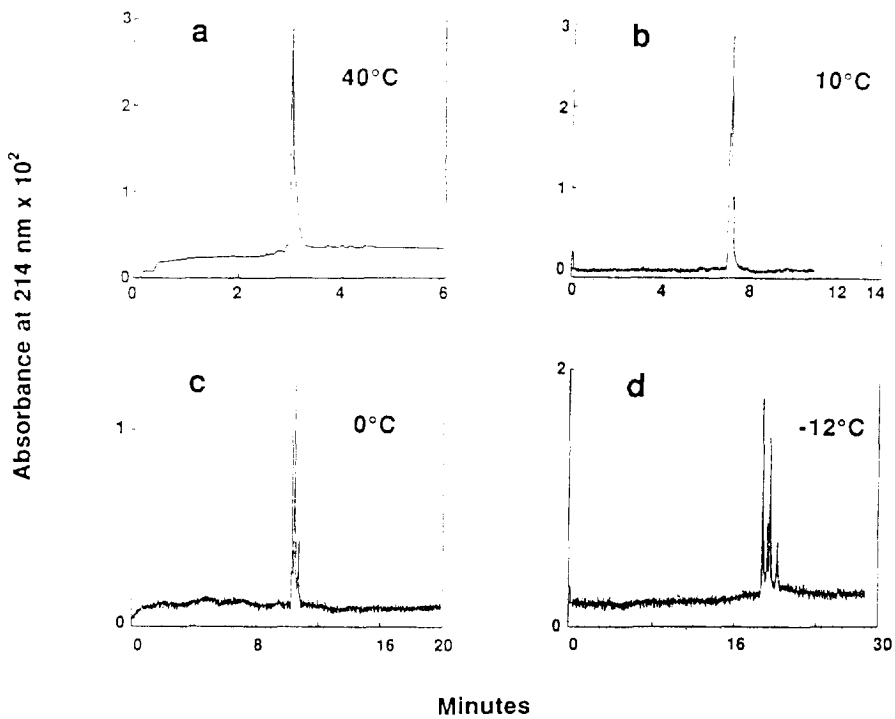

Fig. 11. Electropherograms of Tyr–Pro–Phe–Gly–Tyr–Pro–Ser–NH_2 obtained at various temperatures. Capillary, 37 cm × 50 μm I.D.; buffer, aqueous 50 m*M* sodium borate containing 23% glycerol (pH* 11.3); voltage, 30 kV; detector setting, 214 nm. (a) Temperature, 40°C; current, 47 μA; (b) temperature, 10°C; current, 23 μA; (c) temperature, 0°C; current, 17 μA; (d) temperature, −12°C; current, 9 μA.

shown in Fig. 11a. With decreasing temperature, the peak gradually broadens until several small peaks appear to arise at 10°C as depicted in Fig. 11b. With further decrease in temperature to 0°C, the peaks become more pronounced, as illustrated in Fig. 11c, and at −12°C the four peaks are almost resolved except for the inter-peak reaction zone near the baseline, as seen in Fig. 11d. Further decrease in the temperature to −17°C resulted in a long residence time and concomitantly the resolution of the four conformers deteriorated.

The results demonstrate that CZE can be a highly efficient tool for the separation of *cis–trans* peptide conformers at sufficiently low temperatures, at least when the peptides are small in size and contain no polar amino acid near the proline residue. As an analytical separation method, CZE competes very favorably with HPLC owing to its much higher resolving power, which comes to be even higher at low temperatures, but the latter technique is far superior when large quantities of the pure isomers are to be isolated for further use.

Besides being high-performance techniques for the separation of labile sample components in non-analytical applications, both HPLC and CZE can be used for physico-chemical measurements that provide insight into distinct features of the molecular architecture of the substances under investigation. These approaches are, therefore, appropriately referred to as molecular chromatography and molecular electrophoresis.

The enhanced separation efficiency at subambient/subzero temperatures provides further incentives to use CZE not only for the separation of *cis–trans* conformers of peptides or other reacting samples, but also to carry out analytical CZE at low temperatures in general. This approach is being further pursued in our laboratory to explore the potential of cryoelectrophoresis at temperatures down to −50°C.

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