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Journal of Chromatography A, 744 (1996) 259–272

JOURNAL OF CHROMATOGRAPHY A

Kinetic study on the *cis–trans* isomerization of peptidyl–proline dipeptides

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Abstract

The equilibrium and kinetic parameters of *cis–trans* interconversion of dipeptides containing peptidyl–proline moiety were investigated using the in-column incubation method with both CZE and HPLC and the ad hoc dissolution method. The use of the latter was possible because the conformational make-up of the solid peptides, and consequently of their ad hoc solution, was sufficiently different from that of the solution at equilibrium. This method with ^{1}H-NMR and CZE analyses yielded very similar results for the *cis–trans* isomerization of Phe–Pro in aqueous sodium borate, pH 8.4, at 10°C with an average value of 0.34 and $6.6 \cdot 10^{-5}$ s^{-1} for the equilibrium and rate constant, respectively. The in-column incubation method is performed by CZE or HPLC so that the conformers are separated in the first half of the column and then incubated in column where they interconvert and reach equilibrium. Subsequent separation in the second half of the column yielded four peaks. Thus by measuring the conformer composition as a function of the reaction time, the rate constant can be evaluated. The forward rate constant of $1.42 \cdot 10^{-4}$ s^{-1} determined by the CZE in-column incubation method for Phe–Pro isomerization at 10°C was twice of the value obtained by the ad hoc dissolution method. It is believed that the inner wall of fused-silica capillaries has a catalytic effect on the isomerization. Computer simulation was also employed to gain further insight on the catalytic activity of the capillary inner wall on such isomerization. Whereas the experimental and simulation profiles of Phe–Pro in aqueous borate buffer, pH 8.4, with a 37 cm long capillary were in excellent agreement, a four times faster interconversion rate had to be used to match the experimental profile obtained with a 57 cm long capillary under otherwise identical conditions. The catalytic effect of the octadecyl silica stationary phase on the isomerization was confirmed by the in-column incubation method with HPLC. The overall rate of the *cis–trans* isomerization of Phe–Pro, which entails the reaction both on the stationary phase and in the mobility phase, was about six times faster at 0°C than the rate measured by NMR in free solution using the mobile phase containing 65% (v/v) sodium phosphate, pH 6.5, and 35% (v/v) methanol. The results presented here serve as a caveat that the effect of the wall in CZE or the stationary phase in HPLC on the reaction cannot be ignored.

Keywords: *Cis–trans* isomerization; Kinetic studies; ; Peptides; Peptidyl–proline; Low temperature NMR; Proline

1. Introduction

Peptides containing peptidyl–proline residues are known to exist at ambient or near ambient temperatures in relatively stable *cis* and *trans* conformations due to the rigidity of the peptidyl–proline bond [1]. The relatively slow interconversion of these conformers has been implicated as the rate-limiting step in

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0021-9673/96/$15.00
PII S0021-9673(96)00241-5

the folding process of several proteins [1]. The isomerization reaction is of interest also in the study of proline specific enzymes such as prolyl *cis–trans* isomerase (PPI), prolidase, aminopeptidase P and HIV-proteinase [1,2]. Moreover, it has been found that in small opioid peptides containing proline moiety, the peptide bond involving the nitrogen of proline is required to be in the *cis* conformation for the receptor binding [3,4]. Nonetheless, the lack of pure peptide conformers of authentic structural identity has been an impediment to explore the conformational aspects of biological activity of such peptides.

The separation of *cis* and *trans* proline dipeptides [5–8] as well as biologically active tetra- and heptapeptides [9] have been carried out by reversed-phase chromatography at sub-ambient temperatures where the rate of interconversion was so low that it did not interfere with the separation. In CZE Meyer et al. [10] have achieved the separation at 25°C of the *cis* and *trans* conformers of a thioxo peptide Ala–Phe–ψ[CS-N]–Pro–Phe–4-nitroanilide where the isomerization was an order of magnitude slower than those with unmodified peptide bonds. Recent work from our laboratory has demonstrated that the *cis–trans* conformers of various dipeptides and oligopeptides can be isolated by preparative RP-HPLC [11] or separated with high resolution by CZE [12] at sufficiently low temperatures. The separation of the isomers by CZE and HPLC are based on the difference in their respective hydrodynamic radii and hydrophobic surface areas. The results were interpreted and the separation was optimized in light of the dimensionless Damköhler number [5–7,11,12].

For kinetic measurements of rapid reactions, relaxation methods first developed by Eigen [13] are often used. For the *cis–trans* isomerization, Lin and Brandts used the pD-jump experiments [14] to investigate the interconversion kinetics of dipeptides. The method is based on the observation that the *cis–trans* ratio of peptidyl–proline dipeptides is strongly pH-dependent so that the *cis* content increases with the pH. The measurement of the kinetic and equilibrium constants of isomerization reactions is conveniently carried out by NMR with complete line shape analysis (CLSA) [15]. However, this method is not applicable to dipeptides because the *cis–trans* interconversion is too slow on the time scale of NMR at temperatures of practical interests. In these cases, the saturation transfer method [15,16] can be used by measuring the rate constant at elevated temperatures and by extrapolating them to ambient temperatures. Alternatively, for various dipeptides containing peptidyl–proline moiety, ^{13}C-NMR has been used for the investigation of the interconversion [17,18].

In this study, the potential of three analytical techniques, ^{1}H-NMR, CZE and RP-HPLC in the investigation of the *cis–trans* isomerization was explored. In particular, two relaxation methods, termed "ad hoc dissolution" and "in-column incubation" were developed for the determination of the kinetic parameters of the *cis–trans* isomerization with five dipeptides in various solvents employed in the separation by CZE and HPLC. In addition, computer simulation of the electrophoretic separation process served as an indirect means to gain further insight in the behavior of such interconverting systems.

2. Experimental

2.1. Chemicals

Tyr–Pro, Val–Pro and Ile–Pro were obtained from Bachem (Philadelphia, PA, USA); Phe–Pro and Ala–Pro were from Sigma (St. Louis, MO, USA). Deuterated solvents were from Cambridge Isotope Laboratories (Boston, MA, USA). Reagent grade phosphoric acid (85%), sodium hydroxide and HPLC grade methanol were purchased from Fisher (Pittsburgh, PA, USA), sodium borate ($Na_2B_4O_7 \cdot 10H_2O$) from Mallinckrodt (Paris, KT, USA), boric acid and glycerol (anhydrous) from J.T.Baker (Phillipsburg, NJ, USA). Buffer solutions for the calibration of the pH meter at pH 10.00 were from Brand-Nu Laboratories (Meriden, CT, USA), at pH 7.00 (potassium phosphate) from J.T.Baker. Deionized water was prepared by the NanoPure purification system from Barnstead (Boston, MA, USA) and used throughout the experiments.

2.2. Instrumentation

2.2.1. ^{1}H-NMR spectroscopy

All measurements were carried out using a Model AM-500 (Bruker, Karlsruhe, Germany) NMR unit

with an ASPECT 3000 computer equipped with a Variable Temperature Unit to carry out measurements at sub-ambient temperatures by using liquid nitrogen as the coolant. The temperature measured within the probe was taken as the incubation temperature at an accuracy of 0.1°C. Only deuterated solvents were used for measurements by ^{1}H-NMR.

2.2.2. Capillary zone electrophoresis

A 2210 Model P/ACE capillary electrophoresis unit (Beckman Instruments, Fullerton, CA, USA) equipped with sample cooling tray was used. For operations at low temperatures, an auxiliary temperature control system was installed as described in a previous paper [12]. UV–Vis detector was used in all experiments with a setting at 214 nm. A PowerMate SX/20 computer (NEC Technologies, Boxborough, MA, USA) with P/ACE, version 3.0 software was used for control of the instrument and data processing. Fused-silica capillaries of 50 μm I.D. were obtained from Quadrex (New Haven, CT, USA) and the length of the column in the experiment varied from 37 to 97 cm. Peptides were dissolved in the buffer at concentration of 1 mg/ml and injected by pressure (0.5 p.s.i.; 1 p.s.i.=6894.76 Pa) for 3–10 s.

2.2.3. Reversed-phase HPLC

Chromatography was carried out by a unit assembled from a ConstaMetric III metering pump, SpectraMonitor variable wavelength detector (LDC/Milton Roy, Riviera Beach, FL, USA) and an injection valve equipped with 20 μl sample loop (Rheodyne, Cotati, CA, USA). Chromatograms were recorded and integrated using Model CI 10 integrator (LDC/Milton Roy). The column effluent was monitored at 280 nm. The columns were jacketed and connected to a Model RTE-4DD refrigerated circulating bath (Neslab, Portsmouth, NH, USA) suitable for operation at temperatures down to −30°C. Columns, 250×4.6 mm, were packed with 5 μm UltraSphere ODS from Beckman.

2.3. Procedures

2.3.1. Evaluation of kinetic parameters

Cis–trans isomerization has been found to be a first order reversible reaction [1],

$$cis \underset{k_r}{\overset{k_f}{\Leftrightarrow}} trans \tag{1}$$

with the respective rate constants k_f and k_r for the forward and reverse reactions. The change in the mole fraction of the *trans* conformer, x, with time, t, is given by

$$\frac{dx}{dt} = k_f(1 - x) - k_r(x) \tag{2}$$

and at equilibrium

$$k_f(1 - x_e) - k_r(x_e) = 0 \tag{3}$$

where x_e is the mole fraction of the *trans* conformer at equilibrium. By combining Eqs. 2 and 3, we obtain

$$\frac{dx}{dt} = \frac{k_f}{x_e}(x_e - x) \tag{4}$$

which yields upon integration

$$x_e \ln\left(\frac{x_e - x_0}{x_e - x}\right) = k_f(t - t_0) \tag{5}$$

where x_0 is the initial mole fraction of the *trans* conformer at the time t_0 of the starting of the experiments.

The equilibrium constant, K, is given by the ratio of either the rate constants or the mole fractions of the two conformers at equilibrium

$$K = \frac{k_f}{k_r} = \frac{x_e}{1 - x_e} \tag{6}$$

The activation energy of the reaction, E_a, is defined by the Arrhenius equation,

$$k = A \exp\left(-\frac{E_a}{RT}\right) \tag{7}$$

where A is the preexponential factor.

For the *cis–trans* isomerization of the dipeptides investigated in this work, the equilibrium constants were evaluated from the mole fraction of the *trans* conformer at equilibrium according to Eq. 6. The forward rate constants were determined based on the slopes of the lines obtained when plotting the LHS of Eq. 5 against $(t - t_0)$. The activation energies, E_a, were then calculated by plotting the logarithmic rate constant against the reciprocal absolute temperature according to Eq. 7.

2.3.2. "Ad hoc dissolution" method with NMR or CZE analysis

The peptide having a conformational make-up significantly different from that in the solution at equilibrium was dissolved in the appropriate solvent thermostated at the experimental temperature.

The concentrations of the *cis–trans* conformers were measured as a function of time by ^{1}H-NMR and CZE and the rate constants were evaluated according to Eq. 5. In NMR studies, a 5 mm I.D. tube was the reaction vessel filled with 500 μl of peptide solution having a concentration of 10 mg/ml. The reaction vessel was placed in the probe thermostated by the VTU of NMR and the temperature of the probe was taken as the temperature of the reaction. The signals used for the evaluation of the conformer compositions are: Phe_γ and Ala_α for Phe–Pro and Ala–Pro; Pro_γ for Val–Pro and Ile–Pro and $Tyr_{\delta ortho}$ for Tyr–Pro. In CZE, microvials with maximum volume of 600 μl were used as the reactors and thermostated in the water bath. The peptide concentration was 1 mg/ml and the temperature of the water bath was taken as the reaction temperature.

2.3.3. "In-column incubation" method with CZE or HPLC analysis

In CZE, the peptide was first introduced into the capillary by pressure (0.5 p.s.i.). Then, the electric field was applied until the peptide conformers migrated to the midpoint between the inlet and the detector window. The voltage was then cut off and the separated bands of the two isomers in the absence of electric field were allowed to undergo interconversion. In the time course of the reaction, each band turned into a mixture of *cis* and *trans* isomers so that upon restoring the electric field and separating the components, an electropherogram with four peaks was obtained. Similarly in HPLC, the peptide conformers were separated in the first half of the column. Upon incubation on the HPLC column, a chromatogram with four peaks was obtained as a result of the separation in the second half of the column. The mole fraction of the conformers were then calculated from their peak heights or peak areas measured by CZE and HPLC. The rate constants were determined from the time course for the ratios during the incubation according to Eq. 5. In CZE, the incubation temperature was the temperature of the coolant measured at the exit of the cartridge. In HPLC, the temperature of the circulating fluid in which the column was jacketed was taken as the incubation temperature.

2.3.4. Computer simulation

For the system under consideration the mass balance equations for the two species take the following form [19]

$$\frac{\partial c_1}{\partial t} = D_1\frac{\partial^2 c_1}{\partial x^2} - (\mu_1 + \mu_{eeo})E\frac{\partial c_1}{\partial x} + k_1c_1 + k_2c_2 \tag{8}$$

$$\frac{\partial c_2}{\partial t} = D_2\frac{\partial^2 c_2}{\partial x^2} - (\mu_2 + \mu_{eeo})E\frac{\partial c_2}{\partial x} + k_1c_1 - k_2c_2 \tag{9}$$

where c, D and μ with subscript 1 and 2 are the concentrations, diffusion coefficients and the electrophoretic mobilities of species 1 and 2, respectively. E is the strength of the applied electric field and μ_{eeo} is the mobility of a neutral marker. In the modelling of the system we assume that the *cis–trans* isomerization is a first order reaction that is unaffected by interaction of the reactant with the tube wall. The two coupled second order partial differential equations were solved [20] for c_1 and c_2 numerically by using a code in FORTRAN7 language on a SPARC Center 1000 machine with a SUN Microsystems Symmetric Multi Processor (SMP).

The finite difference scheme employed in computer calculations called for very small mesh sizes in both time and space because of the very high efficiencies of CZE. The computational time was reduced by using an adaptive grid: very fine where peaks were present and coarse elsewhere. The first order derivative was discretized using the forward finite difference scheme; the numerical diffusion introduced by discretization was taken as the actual diffusion and the second order term was neglected [21]. Thus the system was reduced to one of algebraic equations that were solved simultaneously to yield the concentrations of the two isomers as a function of time at various locations in the capillary.

The following data was required for the simulation: the electrophoretic and the electroendoosmotic

mobilities, the diffusion coefficients, the capillary length and the rate constants. They were obtained from experimental studies on *cis–trans* isomerization in CZE at low temperatures in our laboratory [12]. The results were presented graphically as simulated electropherograms.

3. Results and discussion

3.1. Computer simulation

3.1.1. Effect of the kinetic and equilibrium constants

In order to illustrate the effect of reaction kinetics on the interplay of the *cis–trans* isomerization and electrophoretic separation simulations were performed by varying the forward rate constant, k_f, keeping all other parameters constant. The results are depicted in Fig. 1 and it is seen that when the forward rate constant is small ($k_f \leq 1 \cdot 10^{-5}\ s^{-1}$) the two peaks are well resolved, but with increasing k_f a mixed reaction zone is formed with concomitant deterioration of the separation. Upon further increase in k_f the reaction zone increases and at sufficiently high rate of interconversion the two isomers appear as a single peak. The rationale of using low temperature CZE for separation of these isomers [12] rests with the decreasing rate of interconversion upon lowering the temperature.

Since the interconversion of the *cis–trans* peptide isomers is a first order reaction the equilibrium constant is given by the ratio of the forward and the backward rate constants. The simulations were performed by taking the forward rate constant $5.2 \cdot 10^{-4}\ s^{-1}$, a relatively high value at which the interplay of reaction and separation markedly affects the electrophoretic results. Fig. 2 shows the effect of changing K on the concentration profiles of the two isomers. It is seen that when K is unity, the areas of the two peaks are equal. When $k_f < k_r$ the peak area of the *cis* isomer is greater than that of the *trans* isomer and the situation is reversed when $k_f > k_r$, as seen in Fig. 2. It is noted that K is a weak function of temperature for the reactions under investigation.

3.2. Ad hoc dissolution method

The initial and equilibrium mole fractions of the *trans* conformers of five Xaa–dipeptides are listed in Table 1. It is seen that the mole fractions of the *trans* conformers span a very wide range ($0.965 > x_0 > 0$) initially, but upon reaching equilibrium they are much closer to each other ($0.582 > x_e > 0.245$). As seen, the relative concentrations of the two conformers are significantly different in the solution made ad hoc and after reaching equilibrium for each of the five dipeptides. The large differences have made

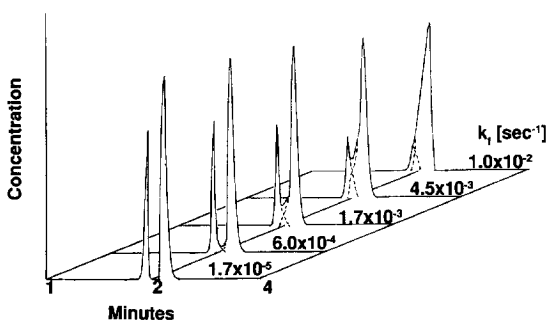

Fig. 1. Simulated electropherograms to illustrate the effect on the concentration profiles of the forward rate constant for the interconversion of *cis* and *trans* peptide isomers in CZE. Conditions; fused-silica capillary 37 cm×50 μm; 100 m*M* sodium borate buffer, pH 8.4; voltage, 30 kV; temperature, 20°C; K, 0.7; D_m, $10^{-4}\ cm^2/s$; mobility of a neutral marker, μ_{eeo} $5 \cdot 10^{-8}\ m^2\ V^{-1}\ s^{-1}$; mobility of the *cis* conformer, μ_{cis}, $1.3 \cdot 10^{-8}\ m^2\ V^{-1}\ s^{-1}$ and mobility of the *trans* conformer, μ_{trans} $0.68 \cdot 10^{-8}\ m^2\ V^{-1}\ s^{-1}$.

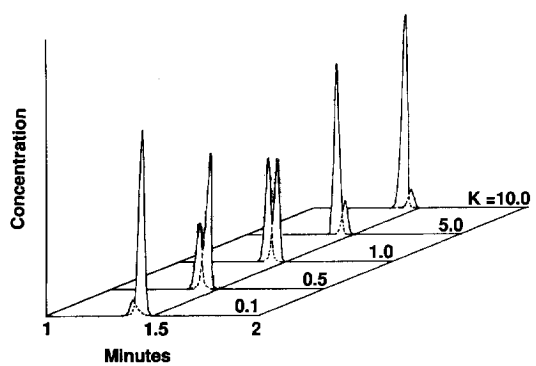

Fig. 2. Simulated electropherograms to illustrate the effect on the concentration profiles of the equilibrium constant for the interconversion of *cis* and *trans* peptide isomers in CZE. Conditions same as in Fig. 1 except the temperature, 30°C; k_f, $5.2 \cdot 10^{-4}\ s^{-1}$; μ_{eeo}, $8 \cdot 10^{-8}\ m^2\ V^{-1}\ s^{-1}$; μ_{cis}, $2.79 \cdot 10^{-8}\ m^2\ V^{-1}\ s^{-1}$ and μ_{trans}, $2.59 \cdot 10^{-8}\ m^2 V^{-1}\ s^{-1}$.

Table 1
The mole fraction of the *cis* and *trans* conformers of Xaa–Pro dipeptides, measured immediately after the ad hoc dissolution and after the composition of the reaction mixture reached a constant (equilibrium) value using the ad hoc dissolution method with ^{1}H-NMR analysis

Xaa	*Cis*		*Trans*	
	Ad hoc solution	At equilibrium	Ad hoc solution	At equilibrium
Ala	0.035	0.420	0.965	0.580
Val	0.876	0.418	0.124	0.582
Ile	0.936	0.496	0.064	0.504
Phe	0.999	0.755	0.001	0.245
Tyr	0.288	0.752	0.712	0.248

Conditions: 100 m*M* sodium borate buffer in ^2H_2O, pH 8.4; temperature, 0°C.

possible the use of the "ad hoc dissolution" method, which is schematically illustrated in Fig. 3, for the determination of the rate constants. The method is based on the observation that in their ad hoc solution the conformational make-up of the dipeptides investigated with the exception of Gly–Pro (not included in the set of five dipeptides) is different from that after reaching equilibrium. After ad hoc dissolution of the solid peptide, the solution is incubated at the temperature of interest and the concentrations of the conformers in appropriate time intervals is determined by either CZE or NMR analysis until each conformer concentration reaches a constant (equilibrium) value. The equilibrium constant and the forward rate constant are calculated according to Eqs. 6 and 5, respectively. From the rate constants measured at different temperatures, the activation energy is evaluated according to Eq. 7.

The data in Table 1 shows that the conformational state of the dipeptides in solid form is different from that in solution at equilibrium although it is retained

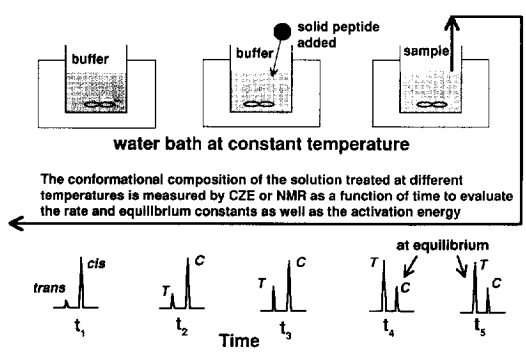

Fig. 3. Schematic illustration of the ad hoc dissolution method with NMR or CZE analysis.

for a while upon going in solution. Analysis immediately after dissolution showed that Tyr–Pro and Ala–Pro were mostly in their *trans* conformation; whereas Phe–Pro, Val–Pro an Ile–Pro were predominantly in their *cis* conformation. An alternative interpretation of the data in Table 1 would be the assumption of widely different dissolution rates for the *cis* and *trans* isomers. However, the observation that the total peak area was conserved in the course of reaction does not lend support to this explanation. Hence, the differences are most likely determined by the history of the solid sample, i.e., the conditions used in its preparation and the composition of the solution from which the peptide was obtained.

3.2.1. ^{1}H-NMR spectroscopy

The ^{1}H-NMR spectra of Ile–Pro obtained at various reaction times measured in 100 m*M* sodium borate, pH 8.4, in ^2H_2O at 0°C are shown in Fig. 4. It is seen that 10 min after preparation of the ad hoc solution, the signal by the *trans* conformer located near 4.20 ppm was relatively small compared to the signal by the *cis* isomer at 4.40 ppm. After 90 min of incubation, the concentration of the *trans* Ile–Pro increased and reached about half of the intensity of its *cis* conformer. It is also seen that at equilibrium the intensities of the signals were commensurable.

Using the ad hoc method under identical conditions, the concentrations of the *trans* conformer of five dipeptides were determined by NMR analysis and plotted against the incubation time according to the integrated rate equation, Eq. 5, and shown in Fig. 5. The concentrations of *cis–trans* conformers of Phe–Pro were measured by NMR in various hydro–organic solvents and plotted according to Eq. 5 and

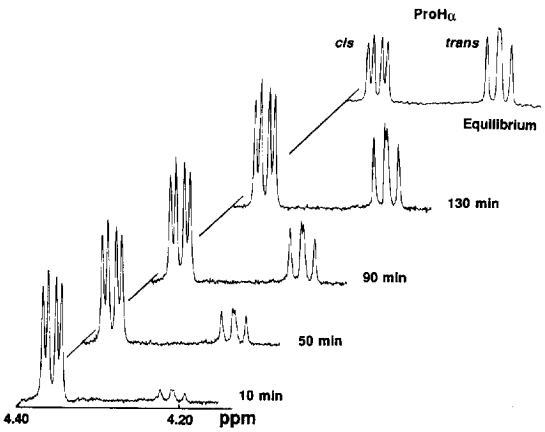

Fig. 4. Typical ^{1}H-NMR spectra of *cis* and *trans* Ile–Pro obtained in the measurement of the interconversion kinetics by the ad hoc dissolution method. The solvent was in 100 m*M* sodium borate in ^2H_2O, pH 8.4, and the mixture was kept at 0°C. NMR spectra were taken after various times shown for each spectrum.

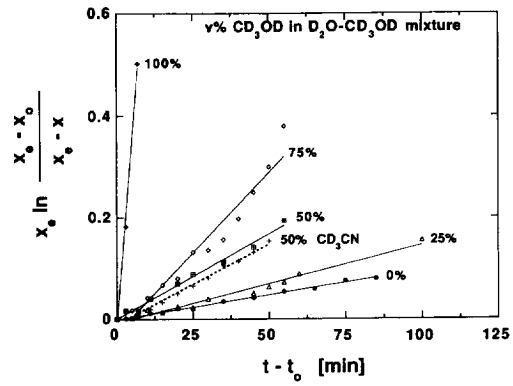

Fig. 6. Plots of the concentration of *trans* Phe–Pro in different hydro–organic solvents measured with the ad hoc dissolution method and ^{1}H-NMR analysis at 0°C against the reaction time according to Eq. 5. (◇) 100% $C^2H_3O^2H$ (CD_3OD); (◇) $C^2H_3O^2H$–^2H_2O (75:25); (⊞) $C^2H_3O^2H$–^2H_2O (50:50); (+) C^2H_3CN–^2H_2O (50:50); (△) $C^2H_3O^2H$–^2H_2O (25:75); (●) 100% ^2H_2O (D_2O).

the results are shown in Fig. 6. Further, the isomerization reaction was also investigated in various sodium borate buffers and the data are plotted in Fig. 7.

The ad hoc dissolution method with ^{1}H-NMR analysis was used also to study the effect of temperature on the interconversion rate constants in three different solvents. The data were plotted according to Eq. 7 and the results are shown in Fig. 8. The linearity of the plots shown in Figs. 5–8 was highly satisfactory with regression coefficients higher than 0.99 in all cases. The data presented here will be used later in the discussion of the kinetics of the *cis–trans* isomerization reaction. It should also be noted that the NMR data were obtained with deuterated solvents. However, they are expected to be

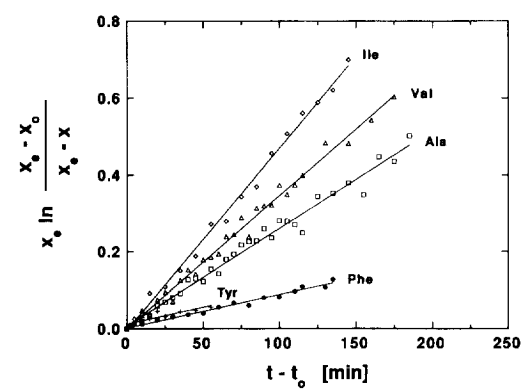

Fig. 5. The concentration of the *trans* conformer of various dipeptides Xaa–Pro having different amino acid residues, Xaa, was determined by the ad hoc dissolution method with ^{1}H-NMR analysis at 0°C and the data were plotted according to the integrated rate equation, Eq. 5. Conditions, 100 m*M* sodium borate in ^2H_2O, pH 8.4 (□) Ala–Pro; (△) Val–Pro; (◇) Ile–Pro; (●) Phe–Pro; (+) Tyr–Pro.

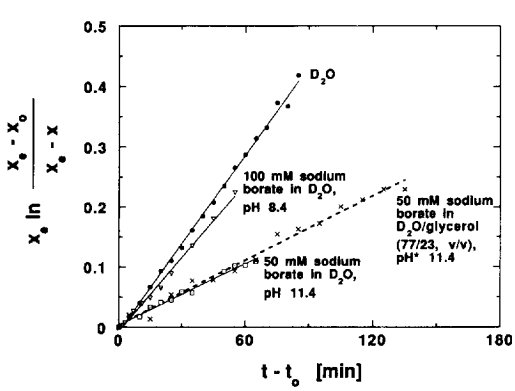

Fig. 7. Plots of the concentration of *trans* Phe–Pro measured by the ad hoc dissolution method with ^{1}H-NMR analysis at 10°C in aqueous buffer at different pH values against the reaction time according to the integrated rate equation, Eq. 5. (●) 100% ^2H_2O; (▽) 100 m*M* sodium borate in ^2H_2O, pH 8.4: (⊡) 50 m*M* sodium borate in ^2H_2O, pH 11.4; (×) 50 m*M* sodium borate in ^2H_2O–glycerol (77:23, v/v), pH* 11.4.

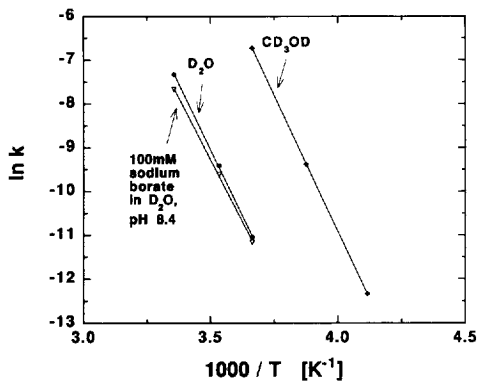

Fig. 8. Arrhenius plots of kinetic data on the *cis–trans* interconversion of Phe–Pro measured by the ad hoc dissolution method with ^{1}H-NMR analysis in various media. (◇) 100% $C^2H_3O^2H$; (▽) 100 m*M* sodium borate in ^2H_2O, pH 8.4; (●) 100% ^2H_2O.

comparable to those with hydrogenated solvents used in CZE and HPLC as according to the literature [22] the isotope effect of deuterated solvents in NMR spectroscopy on the interconversion rate is negligible.

3.2.2. Capillary zone electrophoresis

The ad hoc dissolution method was used to study the isomerization of the dipeptide, Phe–Pro, in neat aqueous sodium borate, pH 8.4, at 10°C. CZE was used as the analytical technique with the same buffer as the BGE and four electropherograms obtained at different incubation times are shown in Fig. 9. It is seen that the peak of the *trans* conformer was undergoing rapid growth during the incubation: it was less than 1% of the total molar concentration two min after dissolution and increased to about 20% after 77 min. Equilibrium concentration of 0.271 in terms of the mole fraction of *trans* Phe–Pro was reached after about two h of incubation.

The *cis–trans* isomerization of Phe–Pro was studied not only at 10°C but also at 4.0 and 1.5°C by employing the ad hoc dissolution method with CZE analysis. The data were plotted according to Eq. (5) and are shown in Fig. 10. The linear regression coefficients of the straight lines are better than 0.99 in all cases. The equilibrium and kinetic parameters investigated using the ad hoc methods with CZE analysis are summarized in Table 2.

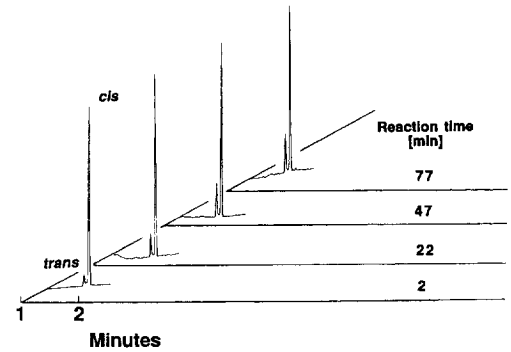

Fig. 9. Typical electropherograms of *cis* and *trans* Phe–Pro in the measurement of the kinetics of their isomerization by the ad hoc dissolution method with CZE analysis. The reaction mixture was in 100 m*M* aqueous sodium borate, pH 8.4, and kept at 10°C at various times shown for each electropherogram. CZE conditions: same buffer and temperature; fused silica 37 cm×50 μm; 30 kV; 15 μA.

3.3. In-column incubation method

The successful separation of the conformers by CZE and HPLC at low temperatures allows the use of another relaxation method to investigate the kinetics of the interconversion of the conformers of peptides with peptidyl–proline moiety. This method is referred to as the in-column incubation method which can be carried out by either CZE or HPLC, and it is schematically illustrated in Fig. 11. After

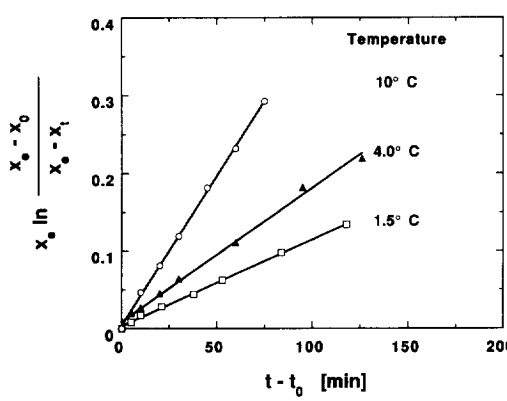

Fig. 10. Plots according to the integrated rate equation Eq. 5 for the *cis–trans* isomerization of dipeptide Phe–Pro in aqueous sodium borate buffer, pH 8.4 at the temperature indicated. The conformer concentration was measured by ad hoc dissolution method with CZE analysis. CZE conditions: fused silica 37 cm×50 μm; in 100 m*M* aqueous sodium borate, pH 8.4; 30 kV. (○) 10°C; (▲) 4.0°C; (□) 1.5°C.

Table 2
Kinetic data for the interconversion of *cis–trans* Phe–Pro in various aqueous buffers of different pH values obtained by the ad hoc dissolution method with CZE analysis (Method A) and the CZE in-column incubation method (Method B)

Method	Buffer	T [°C]	x_e	K	$k_f \times 10^5$ [s^{-1}]	$k_r \times 10^5$ [s^{-1}]	E_a [kcal/mol]	$A \times 10^{-11}$ [s^{-1}]
A	100 m*M* sodium borate in H_2O, pH 8.4	1.5	0.245	0.325	1.86	5.72		
		4.0	0.257	0.346	2.88	8.32	22.2	89.5
		10.0	0.271	0.372	6.42	17.3		
B	100 m*M* sodium borate in H_2O, pH 8.4	1.5	0.245	0.325	4.90	15.1		
		4.5	0.262	0.355	6.46	18.2	19.7	2.15
		10.0	0.271	0.372	14.2	38.2		
B	50 m*M* sodium boratein H_2O–glycerol (77:23,v/v), pH* 11.4	10.0	0.260	0.351	7.95	22.6	–	–

Listed are mole fraction of *trans* conformer at equilibrium, x_e, the equilibrium constant, K, the rate constants for the forward reaction, k_f, and the reverse reaction, k_r. Also listed are the activation energy, E_a, and the preexponential factor A.

migrating in the first half of the column, the isomers are separated. Then the migration is stopped and the conformers are allowed to interconvert during incubation in the middle of the column. Subsequently, the migration process is restarted and the isomers are separated in the second half of the column to yield four peaks. Upon measuring the concentrations of the conformers at different times, the kinetic parameters are evaluated by using Eqs. 5 and 7.

3.3.1. Capillary zone electrophoresis

The series of electropherograms were obtained with Phe–Pro at 10°C in sodium borate containing 23% (v/v) glycerol, pH 11.4, depicted in Fig. 12 using CZE in-column incubation. First, only *cis* and *trans* conformers, c_1 and t_1, were separated and the mole fraction of the *trans* Phe–Pro, t_1, was 0.271 as that at equilibrium. Upon further incubation, each of the two conformers t_1 and c_1 interconverts into its respective *cis* and *trans* conformers, c_2 and t_2. Under the experimental conditions, the *trans* conformer was found to have a smaller mobility due to its larger size than the *cis* conformer [12]. Since the peptide and the electroendoosmotic flow are moving in the opposite directions (counterdirectional CZE), the migration order of these conformers is therefore t_1, c_2, t_2 and c_1. As seen from the electropherograms in Fig. 12, after two h of incubation, the mole fraction of the *trans* conformer, t_1, was only 0.088, much smaller than its initial value of 0.271 as a result of interconversion to its *cis* conformer, c_2.

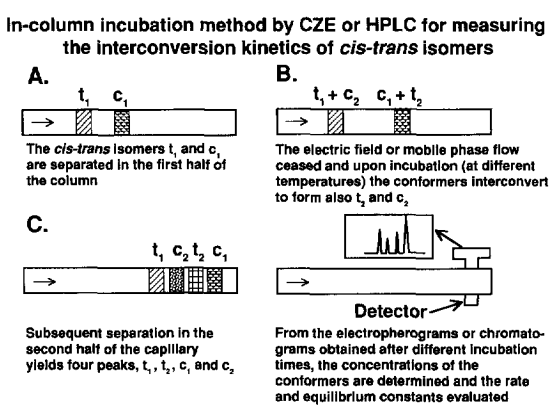

Fig. 11. Schematic illustration of in-column incubation method with CZE or HPLC.

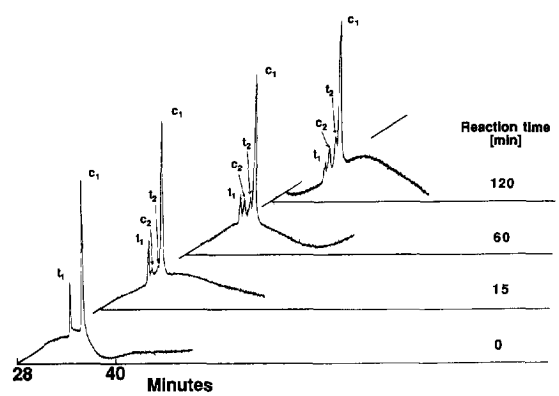

Fig. 12. Typical electropherogram of *cis* and *trans* Phe–Pro in the measurement of the isomerization kinetics by the CZE in-column incubation method obtained at various reaction times at 10°C. Conditions, 50 m*M* sodium borate in H_2O–glycerol (77:23, v/v), pH* 11.4; 10°C; fused silica 67 cm×50 μm; 30 kV; 20 μA.

The concentration of the *trans* Phe–Pro, t_2, in sodium borate containing 23% glycerol, pH* 11.4, were measured using the CZE in-column incubation method at 10°C and plotted against the incubation time as shown in Fig. 13. Also plotted are data measured in the neat aqueous sodium borate buffer, pH 8.4, at temperatures 10, 4.5 and 1.5°C. All four plots are linear with regression coefficients better than 0.99. The equilibrium and kinetic parameters obtained by the CZE in-column incubation methods are also presented in Table 2.

3.3.2. Reversed-phase HPLC

The catalytic effect of the hydrophobic surface of the stationary phase on the on-column isomerization of peptides containing peptidyl–proline moiety in reversed-phase chromatography has long been known [6,7]. This is in agreement with the observation that the rate of interconversion is higher in nonpolar solvents than in hydrogen bonding solvents [23,24]. In order to examine the effect of the stationary phase on the interconversion of the Phe–Pro isomers, we used the in-column incubation method with an octadecyl silica column commonly employed in HPLC. The chromatograms thus obtained have features very similar to those of the electropherograms as shown earlier in Fig. 12 except the much higher column efficiency (plate numbers) in CZE than in HPLC. From the chromatographic data, the equilibrium and overall forward reaction rate constants were found to be 0.27 and $12.7 \cdot 10^{-5}\ s^{-1}$, respectively, in a mobile phase containing 65% (v/v) 50 m*M* sodium phosphate, pH 6.5, and 35% (v/v) methanol at 0°C.

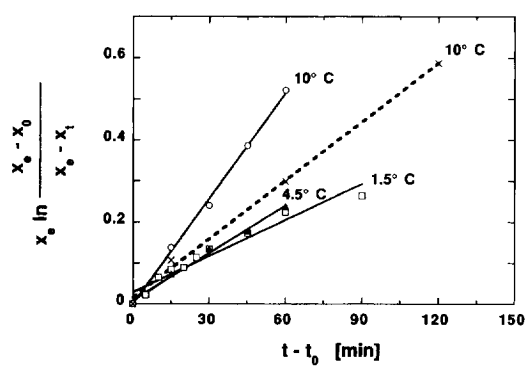

Fig. 13. Plots of the data obtained by the CZE in-column incubation method according to the integrated rate equation Eq. (5) for the interconversion of *cis–trans* Phe–Pro at different temperatures. Solid lines: 100 m*M* sodium borate, pH 8.4; fused silica of 50 μm I.D. of different length at temperatures (○) 10°C, 67 cm; (▲) 4.5°C, 47 cm; (□) 1.5°C, 47 cm. Dashed line: 50 m*M* sodium borate in H_2O–glycerol (77:23, v/v), pH* 11.4; fused silica of 67 cm×50 μm at 10°C.

3.4. Comparison of methods

3.4.1. NMR and CZE

Since in both NMR and CZE techniques, the peptides are in free solution, the equilibrium and kinetic parameters of *cis–trans* isomerization of Phe–Pro measured in the same buffer solution are expected to be the same. Indeed, using the ad hoc dissolution method in aqueous borate, pH 8.4, at 10°C with either CZE or ^{1}H-NMR analysis, the equilibrium and rate constants for the interconversion of Phe–Pro isomers by NMR and CZE are in excellent agreement: the respective equilibrium constants determined by NMR and CZE are 0.31 and 0.37; whereas the rate constants obtained by the two techniques are almost identical with an average value of $6.6 \cdot 10^{-5}\ s^{-1}$ seen from Fig. 14.

However, the forward rate constant measured by the CZE in-column incubation method is about twice as high as the value obtained by the ad hoc dissolution method discussed above. Although there is certain uncertainty about the temperature inside the capillary with Joule heating, the large discrepancy is

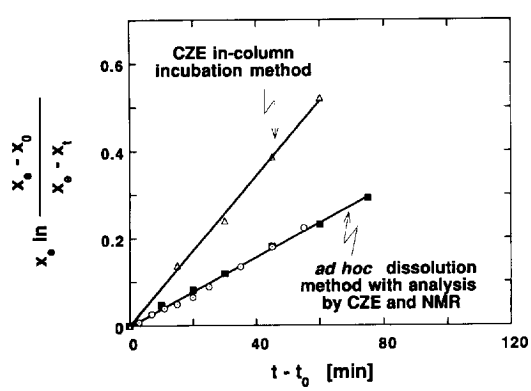

Fig. 14. Forward rate constants of the *cis–trans* isomerization of Phe–Pro in 100 m*M* sodium borate, pH 8.4 at 10°C measured by different methods. (○) ad hoc dissolution with NMR analysis (■) ad hoc dissolution with CZE analysis; (△) CZE in-column incubation.

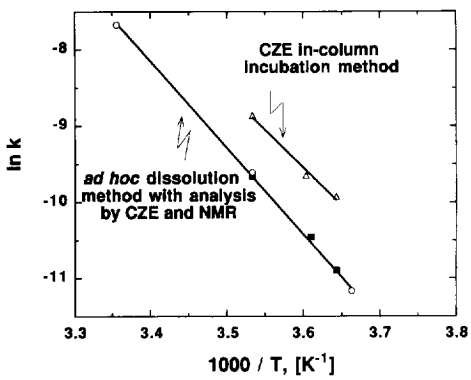

Fig. 15. Arrhenius plots of kinetic data obtained by different methods on the *cis–trans* isomerization of Phe–Pro in 100 m*M* sodium borate, pH 8.4 (○) Ad hoc dissolution with NMR analysis; (■) ad hoc dissolution with CZE analysis; (△) CZE in-column incubation.

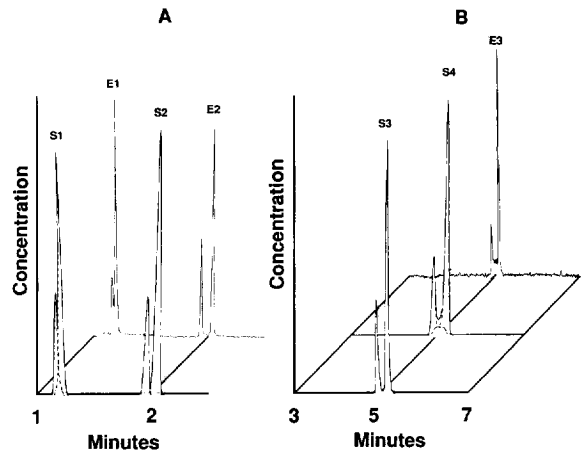

Fig. 16. Comparison of simulated and experimental electropherograms of interconverting *cis* and *trans* Phe–Pro conformers obtained with 37 cm×50 μm (A) and 57 cm×50 μm (B) capillaries. The experimental results (E) were obtained with 100 m*M* sodium borate, pH 8.4 at 30 kV at temperatures: (E1) 25°C, (E2) 5°C and (E3) 5°C. Input data for simulation (units of the rate constants are 10^{-4} s^{-1} and of mobilities 10^{-8} $m^2 V^{-1} s^{-1}$) *K*, 0.37; and D_m, 10^{-5} cm^2/s. The forward rate constants, k_f, are 7.68, 0.73, 0.73 and 3.0 for (S1), (S2), (S3) and (S4), respectively. The mobilities used in the simulation: (S1) μ_{eco}, 6.6; μ_{cis}, 1.5 and μ_{trans}, 1.33; whereas in (S2), (S3) and (S4) the values of μ_{eco}, 4.56; μ_{cis}, 1.08 and μ_{trans}, 0.92 were used. The number of theoretical plates for the simulated profiles is lower by a factor of 3 than in the experimental electropherogram due to limitations on the computational time available.

attributed to the catalytic effect of the capillary wall on the isomerization reaction. As shown in Fig. 15, the activation energy obtained using the CZE in-column incubation method is 19.7 kcal/mole and this compares to 23.1 kcal/mole obtained by the ad hoc dissolution method with both CZE and NMR analysis, (1 cal=4.184 J). This hypothesis is further supported by comparing the experimental to the computer simulation results. The rate constants were evaluated by a procedure similar to that used in the determination of kinetic data on the *cis–trans* isomerization in reversed-phase chromatography [6,7]. Simulations were run with a given set of experimental parameters, but the forward rate constant was varied till the simulated profile matched the experimental one. Further, computer simulations were carried out in order to examine the effect of the capillary length with rate constants obtained by the ad hoc dissolution method with CZE and/or NMR analysis as described above. The results are presented in Fig. 16 where the codes for the *cis* and *trans* Phe–Pro peak pairs are explained in the caption. With a 37 cm long capillary (Fig. 16A), the simulated profiles match perfectly the experimental ones at both 25° (E1 and S1) and 5°C (E2 and S2) when the residence time of the peptide in the capillary is relatively short, i.e., not more than 2 min. With the 57 cm long capillary (Fig. 16B), the residence time increased to 5 min and as a result, an interpeak reaction zone developed even at 5°C with concomitant deterioration of the separation (E3). In contradistinction, the simulation with the rate constant used for S2 shows almost baseline resolution (S3). A four times faster rate constant (S4) was needed in order to match the experimental profile (E3). Further increasing the length to 97 cm resulted in a 6-fold faster rate constant increase in the simulation to match the experimental results obtained under otherwise identical conditions.

3.4.2. NMR and HPLC

For the *cis–trans* isomerization of Phe–Pro in the mobile phase containing 65% (v/v) 50 m*M* sodium phosphate, pH 6.5, and 35% (v/v) methanol at 0°C, measurements by both techniques yielded very similar results for the equilibrium constant: 0.27 and 0.23 by HPLC and NMR, respectively. However, the reaction rate constant of $12.7 \cdot 10^{-5}$ s^{-1}, measured by HPLC, was a factor of 6 times higher than the value

of $2.22 \cdot 10^{-5}$ s^{-1} measured by NMR in free solution. The HPLC in-column incubation method measures the overall rate of reaction that entails the interconversion of the conformers both in the mobile phase and on the stationary phase [6,7]. Our results support the finding of earlier studies that the discrepancy of the rate constants measured by HPLC and in free solution arises from the catalytic effect of the stationary phase.

3.5. Factors effecting the kinetics of interconversion with dipeptides

3.5.1. Effect of the amino acid residue Xaa in five Xaa–Pro dipetides

The ad hoc dissolution method with ^{1}H-NMR spectroscopy was used to examine the effect of the amino acid residue, Xaa, on the *cis–trans* isomerization kinetics of five dipeptides having the general structure Xaa–Pro. As seen from Table 1, the isomeric composition of the dipeptides in solid form or immediately upon dissolution in sodium borate buffer appears to be random largely due to the different history of the solid peptides. On the other hand, the equilibrium mole fractions of the *trans* conformers obtained under the conditions employed here fall into two groups. For the three dipeptides with aliphatic side chain, Ala–Pro, Val–Pro and Ile–Pro, the mole fraction of the *trans* conformer is about 0.5 so that the equilibrium constant is close to unity. On the other hand, for Phe–Pro and Tyr–Pro, two dipeptides with aromatic side chain, the mole fraction of the *trans* form and the equilibrium constant are approximately 0.25 and 0.33, respectively.

The forward rate constants calculated from the slopes of the straight line plots in Fig. 5 are listed in Table 3 together with the reverse rate constants determined from the respective equilibrium constants according to Eq. 6. The forward rate constants with Phe–Pro and Tyr–Pro are about the same and much smaller than with the three dipeptides having aliphatic side chain. A similar observation was reported by Ketzel and Wüthrich [25] in comparing the effect of aliphatic and aromatic side chains on the interconversion.

Table 3
Kinetic data on the interconversion of the *cis–trans* Xaa–Pro dipeptides obtained by the ad hoc dissolution method with ^{1}H-NMR analysis

Xaa	x_e	K	$k_t \times 10^5$ [s^{-1}]	$k_r \times 10^5$ [s^{-1}]
Ala	0.580	1.380	4.26	3.09
Val	0.582	1.391	5.78	4.16
Ile	0.504	1.016	7.93	7.81
Phe	0.245	0.331	1.44	4.35
Tyr	0.248	0.324	1.45	4.48

Conditions: 100 m*M* sodium borate buffer in ^2H_2O, pH 8.4, at 0°C. Listed are the mole fractions of the *trans* conformer at equilibrium, x_e, and the equilibrium constant, K. Also listed are the rate constants for the forward reaction, k_t, and the reverse reaction k_r, that evaluated from plots in Fig. 5.

3.5.2. Effect of solvent

Table 4 shows the effect of various solvents on the kinetic and equilibrium parameters for the isomerization of Phe–Pro, which was the slowest among those of the five dipeptides. The equilibrium constant increases with the concentration of water, e.g., K changes from 0.136 to 0.377 upon increasing the water content of the solution from 25% to 100%. The *cis–trans* equilibrium for such dipeptides particularly in their zwitterionic form [17] is strongly influenced by the solvent: the fraction of the *cis* isomers is often greater in neat organic solvents than in neat aqueous solutions. Meanwhile, the equilibrium constant was found to decrease with increasing pH of the buffer as described in the literature [8,17,18,26]. In our case, upon raising the pH from 8.4 to 11.4, the equilibrium constant decreased from 0.311 to 0.225.

The rate constants calculated from the slopes of the straight line plots in Fig. 6 are listed in Table 4. It is seen that the forward rate constant increases significantly when the concentration of the organic solvent, such as methanol, is increased. When neat methanol was the solvent instead of neat water, the rate of isomerization accelerated by a factor of 75 at 0°C. Moreover, the forward rate constants in water–acetonitrile (50:50) and water–methanol (50:50) mixtures were about the same and double of that in neat water. Nonpolar solvents have an accelerating effect on such isomerization and thus act as a "catalyst" for the interconversion as reported for

Table 4
Kinetic data of the *cis–trans* Phe–Pro in various solvents obtained by the ad hoc dissolution method with ^{1}H-NMR analysis

Solvent	T [°C]	x_c	K	$k_f \times 10^5$ [s^{-1}]	$k_r \times 10^5$ [s^{-1}]	E_a [kcal/mol]	$A \times 10^{-13}$ [s^{-1}]
$C^2H_3O^2H$	0	0.149	0.175	120	686	24.7[a]	6800[a]
^2H_2O–$C^2H_3O^2H$ (25:75)	0	0.120	0.136	10.5	77.2		
^2H_2O–$C^2H_3O^2H$ (50:50)	0	0.137	0.159	5.54	34.8		
^2H_2O–C^2H_3CN (50:50)	0	0.142	0.166	5.20	31.3		
^2H_2O–$C^2H_3O^2H$ (75:25)	0	0.206	0.259	2.53	9.77		
^2H_2O	0	0.274	0.377	1.60	4.24	24.0[b]	25[b]
^2H_2O	10	0.260	0.352	8.16	23.2		
100 m*M* sodium borate in ^2H_2O, pH 8.4	10	0.237	0.311	6.81	21.9	22.5[b]	1.6[b]
50 m*M* sodium borate in ^2H_2O, pH 11.4	10	0.183	0.225	2.82	12.5		
50 m*M* sodium borate in ^2H_2O–glycerol (77:23, v/v) pH* 11.4	10	0.190	0.235	2.98	12.7		

Listed are the mole fractions of the *trans* conformer at equilibrium, x_c, and the equilibrium constant, K. Also listed are the rate constants for the forward reaction, k_f, the reverse reaction, k_r, that were evaluated from the plots in Figs. 6 and 7. The activation energy, E_a and the preexponential factor, A, that were determined from Fig. 8 are listed as well.
[a] Temperature range from −30° to 0°C.
[b] Temperature range from 0° to 25°C.

substituted amides [23] and for a model peptide [24] in the literature. A hypothetical uncharged transition state was postulated [27] for a single peptide bond which favors nonpolar environment. Hence nonpolar solvents tend to accelerate the interconversion around the peptide bond involving the proline nitrogen. On the other hand, water appears to be an "inhibitor" for the isomerization. For example, upon adding 25% (v/v) ^2H_2O to the neat $C^2H_3O^2H$ medium at 0°C, the rate constant decreases 12-fold. In general, the interconversion is slower in aqueous and hydro–organic media than in nonpolar solvents. This is explained by the rigid hydrogen bonds between the water molecules and the peptides to form a "cage" like structure with water molecules making the rotation of the peptide bond slower and thus inhibiting the isomerization. Similar behavior was observed in protein stabilization where the rigid water "cage", first proposed by Frank and Wen [28] and extended by Némethy et al. [29], protects the protein from unfolding at low temperatures. In contradistinction, at high temperatures, the cage becomes loose or melts so the protein unfolds [30–32]. This explains also earlier findings [11] that the separation of interconverting peptide conformers was not successful even at −25°C by normal phase chromatography with a nonpolar mobile phase (hexane–methylene chloride) on silica gel column.

The rate constants for the *cis–trans* isomerization of Phe–Pro in solvents containing sodium borate in neat ^2H_2O or ^2H_2O–glycerol at basic pH values are also listed in Table 4. It is seen that the rate of interconversion decreased with increasing pH of the solution in agreement with the results in earlier studies [5,7]. As above, the rate constants were nearly the same in neat ^2H_2O and in aqueous borate, pH 8.4, upon raising the pH to 11.4, the rate constant was halved. The addition of glycerol to borate buffer, pH 11.4, had no accelerating effect on the interconversion as expected since glycerol is a strongly hydrogen bonding solvent.

3.5.3. *Effect of temperature*

As seen from Table 4, the activation energy, E_a, determined from the Arrhenius plots presented in Fig. 8, was relatively high, ranging from 22.5 kcal/mol in neat aqueous borate buffer, pH 8.4, to 24.7 kcal/mol in deuterated methanol. The high activation energy of the interconversion facilitated the successful separation of the isomers by CZE and HPLC at sub-ambient temperatures. Upon lowering the temperature from 10°C to 0°C, the migration rate of the *cis–trans* isomers decreased only slightly; whereas the rate of the isomerization reaction was reduced by a factor of 5.

As shown in Table 4, the activation energies for

the *cis–trans* interconversion of Phe–Pro are almost the same in both methanol and water; whereas the value of the preexponential factor is 300 times greater in methanol than in water. Thus, the high preexponential factor seems to be responsible for the 75 times faster interconversion rate at 0°C in methanol than water. It is in agreement with the notion that *A* is a measure of the entropy of the system since the smaller *A* value reflects the more orderly environment in the aqueous solution due to strong hydrogen bonding.

Acknowledgments

We thank the Alexander von Humboldt Foundation for financial support in form of a Feodor–Lynen Fellowship for F.K. and the Foundation of German Chemical Industry for providing a Habilitation Scholarship for F.Th. and A.K. is grateful to the Halász Foundation for financial support. This work was supported by grant No. GM 20993 from National Institute of Health, US Public Health Service.

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