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Received 14 September 1998; accepted 3 August 1999

Thermodynamic Origin of *cis*/*trans* Isomers of a Proline-Containing β-Turn Model Dipeptide in Aqueous Solution: A Combined Variable Temperature ^{1}H-NMR, Two-Dimensional ^{1}H,^{1}H Gradient Enhanced Nuclear Overhauser Effect Spectroscopy (NOESY), One-Dimensional Steady-State Intermolecular ^{13}C,^{1}H NOE, and Molecular Dynamics Study

Abstract: *The* cis/trans *conformational equilibrium of the two Ac–Pro isomers of the β-turn model dipeptide [^{13}C]–Ac–L-Pro–D-Ala–NHMe, 98% ^{13}C enriched at the acetyl carbonyl atom, was investigated by the use of variable temperature gradient enhanced ^{1}H-nmr, two-dimensional (2D) ^{1}H,^{1}H nuclear Overhauser effect spectroscopy (NOESY), ^{13}C,^{1}H one-dimensional steady-state intermolecular NOE, and molecular dynamics calculations. The temperature dependence of the* cis/trans *Ala(NH) protons are in the region expected for random-coil peptides in H_2O ($\Delta\delta/\Delta T = -9.0$ and -8.9 ppb for the* cis *and* trans *isomers, respectively). The* trans *NH(CH_3) proton indicates smaller temperature dependence ($\Delta\delta/\Delta T \sim -4.8$ ppb) than that of the* cis *isomer (-7.5 ppb). 2D ^{1}H,^{1}H NOESY experiments at 273 K demonstrate significant NOEs between ProH$^\alpha$—AlaNH and AlaNH—NH(R) for the* trans *isomer. The experimental NOE data, coupled with computational analysis, can be interpreted by assuming that the* trans *isomer most likely adopts an ensemble of folded conformations. The C–CONH(CH_3) fragment exhibits significant conformational flexibility; however, a low-energy conformer resembles closely the βII-turn folded conformations of the x-ray structure of the related model peptide* trans-*BuCO–L-Pro–Me–D-Ala–NHMe. On the contrary, the* cis *isomer adopts open conformations. Steady-state intermolecular solute–solvent*

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Contract grant sponsor: NATO and Research Committee of the University of Ioannina
Contract grant number: 960146 (NATO)
Biopolymers, Vol. 53, 72–83 (2000)

CCC 0006-3525/00/010072-12

(H_2O) $^{13}C,^{1}H$ NOE indicates that the water accessibility of the acetyl carbonyl carbons is nearly the same for both isomers. This is consistent with rapid fluctuations of the conformational ensemble and the absence of a highly shielded acetyl oxygen from the bulk solvent. Variable temperature ^{1}H-nmr studies of the cis/trans *conformational equilibrium indicate that the* trans *form is enthalpically favored ($\Delta H° = -5.14$ kJ mole^{-1}) and entropically ($\Delta S° = -5.47$ J · K^{-1} · mole^{-1}) disfavored relative to the* cis *form. This demonstrates that, in the absence of strongly stabilizing sequence-specific interresidue interactions involving side chains and/or charged terminal groups, the thermodynamic difference of the* cis/trans *isomers is due to the combined effect of intramolecular and intermolecular (hydration) induced conformational changes.* © 2000 John Wiley & Sons, Inc. Biopoly 53: 72–83, 2000

Keywords: *hydration;* cis/trans *isomerism; gradient enhanced ^{1}H-nmr; ^{13}C-nmr; NOE; molecular dynamics; enthalpy; entropy*

INTRODUCTION

Peptide bond isomerization is important in many processes that require alternation of peptide and protein structure. Among them are the transport of peptides through membranes, oligomerization, folding, and catalytic activity of peptides and proteins.[1–3] Therefore, numerous studies have been reported on the hindered internal rotation of peptide bonds and a variety of statistical, theoretical, x-ray, and spectroscopic techniques has been applied.[1–8] The presence of a cyclic side chain and/or alkyl substitution of the peptide bond, as in proline and sarcosine, is of particular interest because of the resulting increase in population of the *cis* isomer about the X–Pro bond.[4–6] Furthermore, the proline φ-angle is highly constrained by the five-membered pyrrolidine ring and contributes a conformational rigidifying effect to proline-containing peptides.[8]

Intra- and intermolecular (hydration) effects might be the primary factors that contribute to the difference in stability between *cis* and *trans* peptides in aqueous solution. Peptide bonds interact strongly with water[7,9]; therefore, even a modest difference between the free enthalpies of hydration of the *cis* and *trans* isomers would have a significant bearing on peptide structure. For example, if the *trans* configuration was associated with a stronger dipole than the *cis* configuration, then, the *trans* isomer would be expected to be relatively favored in water; conversely, the *cis* configuration might be relatively favored in less polar surroundings such as the interior of a globular protein. The difference in enthalpy for the *cis* and *trans* isomers of X–Pro bonds in aqueous solution has been reported to be zero for model peptides[10–12]; however, other investigators reported a variety of values up to 1.3 kcal/mole.[13–16] Radzicka et al.[17] found that the *cis/trans* equilibrium of simple amides is almost completely insensitive to the solvent environment. Thus, although $\Delta G°$ of solvation of the peptide bond is approximately −10.0 kcal/mol, $\Delta G°$ of solvation of the *cis* and *trans* isomers appear to differ by less than 0.1 kcal/mol. It was concluded that the transfer of a peptide bond from the interior of a protein to an exposed position on its surface would not, in itself, be expected to change its intrinsic preference for the *cis* or *trans* configuration.

Nuclear magnetic resonance is a primary experimental technique in investigating *cis/trans* peptide bonds with emphasis to the effects of solvents, concentration, temperature, pH, intra- and intermolecular hydrogen bonds, and determination of rates and barriers to conformational isomerization.[4,5,13–18] Detailed information, however, on the thermodynamic origin of the *cis/trans* peptide bonds is rather limited and the results are often contradictory. More recently, there have been several developments both in sensitivity and in multipulse sequences, with particular emphasis to gradient methodology, for investigating the conformation of peptides in aqueous solution and the interaction of water with molecules of biological interest via two-dimensional and three-dimensional homonuclear nuclear Overhauser effect (NOE) and rotating frame NOE (ROE) type dipolar cross-relaxation processes between water protons and polypeptide protons.[19–21] However, to our knowledge, only one report has so far been published in investigating the interaction of water with the side chains of the *cis/trans* isomers of a peptide that forms an unusually highly populated type VI β-turn.[22] Heteronuclear $^{13}C,^{1}H$ Overhauser effect spectroscopy[23–26] can provide a valuable probe for investigating hydration of the peptide carbonyl carbon. However, this method has only been explored in the case of simple amides.[27]

In this paper we report the application of variable temperature gradient enhanced ^{1}H-nmr, two-dimensional (2D), $^{1}H,^{1}H$ gradient enhanced NOE spectroscopy (NOESY), steady-state one-dimensional (1D) selective intermolecular $^{13}C,^{1}H$ NOE experiments, and NOE distance constraints molecular dynamics

calculations of the blocked model dipeptide [^{13}C]–Ac–L-Pro–D-Ala–NHMe, 98% ^{13}C enriched at the acetyl carbonyl atom, in aqueous solution (Ac is the amino-terminal blocking group —$COCH_3$ and —$NHCH_3$ is the carboxy-terminal blocking group). By carrying out variable temperature nmr experiments in water, we investigated the thermodynamic origin of the peptide *cis/trans* conformational equilibrium and provide the first direct experimental evidence that the *trans* isomer of the above proline-containing β-turn model dipeptide is enthalpically favored and entropically disfavored relative to the *cis* form. This model dipeptide, was chosen because it is one of the simplest molecules compatible with β-turn folding containing an intramolecular hydrogen bond of the $(i + 3) \rightarrow i$ type[28–30] in a variety of organic solvents; it does not experience a conformational transition (βI to βII folding mode) when changing from the solute to the crystalline state[31,32] and backbone conformational preferences should be representative of the amino acids in the absence of side-chain specific interactions.

MATERIALS AND METHODS

Peptide Synthesis

The blocked dipeptide [^{13}C]–Ac–L-Pro–D-Ala–NHMe has been prepared by classical procedures.[28,29,32] The acetyl group (CH_3CO) was introduced on proline by the coupling reaction of CH_3COOH (98% ^{13}C enriched) and HCl–L-Pro-D-Ala-$NHCH_3$.

NMR Measurements

^{1}H-nmr spectra of [^{13}C]–Ac–L-Pro–D-Ala–NHMe in 90% H_2O/10% D_2O, concentration 15 m*M*, were recorded on a Bruker AMX-600 instrument equipped with a 5 mm Z-gradient probe. Chemical shifts were measured with reference to internal 3-(trimethyl silyl)3,3,2,2-tetra-deuteropropionic acid Na salt ($\delta = 0.000$). Temperature calibration was achieved by the use of 4% methanol in methanol-d_4. Variable temperature 2D gradient enhanced NOESY experiments[33] were performed on a Bruker AMX-400 instrument equipped with a Z-gradient probe, spectral width 4274 Hz, acquisition time 0.23 s, relaxation delay 1 s. NOE intensities were measured by integration of the volume of the respective cross peaks in the 2D spectra and were used to obtain upper limit distance constraints.

Conventional ^{13}C and 1D ^{13}C-^{1}H NOE difference spectra were recorded on a Bruker AMX-600 instrument equipped with a 5 mm multinuclear probe. In seeking the observation of a differential interaction between water and the *cis* or the *trans* carbonyl group, it is important to minimize magnetization transfer between the two isomers by the use of low temperature (273 K) and to choose the appropriate combination of selective, low-power decoupling followed by a high-power broadband composite decoupling during the acquisition of free induction decays.[25–27] The T_1 of the H_2O resonance was found to be 1.4 s. The ^{13}C longitudinal relaxation times of the *cis/trans* carbonyl carbons were 2.6 s at 150.91 MHz. Selective saturation of the H_2O resonance was achieved by continuous wave preirradiation of the solvent protons for 2.5–6 s with a decoupling band width $\gamma B_2 \sim 5$–12 Hz, which avoids the partial excitation of the Cα protons and minimizes small frequency shifts of the solute signals due to demagnetization effects.[34] NOE factors η were calculated from peak-height ratios after exponential multiplication and Fourier transformation. Thus $\eta = \{[I]_{NOE}/[I]_B\} - 1$, where the subscripts NOE and B refer to the NOE spectrum and the unperturbed spectrum, respectively.

Molecular Modeling

Theoretical calculations were performed using a Silicon Graphics O2 workstation and Quanta version of MSI. The structures of the *cis* and *trans* isomers of Ac–L-Pro–D-Ala–NHMe were first minimized using a combination of steepest descent and conjugate gradient algorithms. The minimizations were performed using a dielectric constant $\varepsilon = 81$ and solvation box (8 Å).[35,36] The resulting minimized structures were imposed to adopt the experimental NOEs by applying distance constraints in combination with conjugate gradient minimization algorithm. The minimization procedure was completed when no improvement in the model could be achieved (NOE distance constraints remained constant). To further explore the low energy conformers of the *cis* and *trans* isomer, molecular dynamics was applied at 1000 K using 1.2 and 1 ps time frame for heating, equilibration, and simulation steps.[37] One hundred structures from the simulated ones were minimized using iteration steps and a conjugate gradient algorithm. The one hundred structures generated three clusters using dihedral criterion of 40° (rms min 0.617° and rms max 87.935). From the three clusters the three lowest energy structures for each cluster were considered to best represent the NOE data.

RESULTS AND DISCUSSION

The *cis/trans* interconversion of amides and peptides indicates a relatively high activation barrier ($\Delta G^{\#} \sim 20$ kcal/mole[2,5,18,38]). This process, therefore, is usually slow on the nmr time scale and the two species can be observed separately if a sufficient spectral resolution is available. The slow rate of the *cis/trans* isomerization of X–Pro peptide bonds is an essential factor in the experiments discussed below.

The assignments of proton resonances of the *cis/trans* isomers of Ac-L-Pro-D-Ala-NHMe were determined from the 2D correlated spectroscopy and 2D

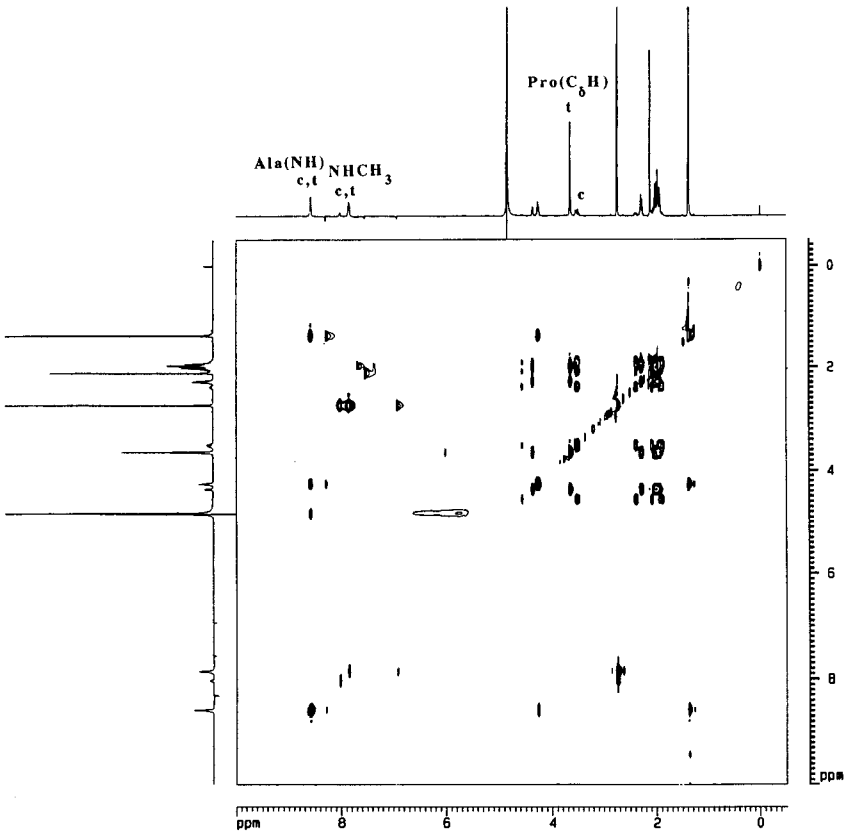


FIGURE 1 2D TOCSY-WATERGATE spectrum of [^{13}C]–Ac–L-Pro–D-Ala–NHMe in 90% H_2O/10% D_2O, concentration 15 m*M* at 297 K on a Bruker AMX-600 instrument equipped with a 5 mm Z-gradient probe. Spin–locking pulse 70 ms. Spectral acquisition parameters: 256 increments, number of scans 48, spectral width in F_1 and F_2 9615 Hz, 0.21 s acquisition time, 3 s relaxation delay, 4 dummy scans, a shifted squared sine-bell function was used in both dimensions. "c" and "t" denote the *cis* and *trans* isomers, respectively.

total COSY (TOCSY) ^{1}H,^{1}H gradient enhanced spectra. A number of relayed connectivities can be observed in the 2D TOCSY spectrum (Figure 1), which allows the unambiguous identification of the spin systems of the amino acids L-Pro and D-Ala and the terminal —NH(CH_3) group. The Ala NH proton shows the expected cross-peak connectivities to both $C^\alpha H$ and CH_3 protons; however, the resonances of the *cis* and *trans* isomers are strongly overlapped. The shieldings of the *cis/trans* amide protons of the —NH(CH_3) terminal group are significantly different, however, the *cis/trans* —CH_3 protons are strongly overlapped.

Temperature Dependence of Amide Proton Resonances

The temperature dependence of amide proton resonances can be used to identify backbone hydrogen bonding.[5,39] The temperature coefficients of amide proton resonances are expected to be -6 to -10 parts per 10^9 (ppb) per K for random-coil peptides in H_2O. The range from -2 to -4 ppb/K suggests strong solvent shielding and -4 to -6 ppb/K denotes moderate shielding. Due to spectral overlap, low intensity and quadrupolar broadening due to ^{14}N nucleus, the NH resonances from the *cis* form of the peptide could be resolved only over a limited temperature range. Temperature coefficients were calculated by a linear least-squares fit to up to 14 experimental data points for each resonance (Figure 2). The Ala NH proton resonances show the characteristic temperature dependence expected for random coil peptides, where amide protons are completely exposed to solvent ($\Delta\delta/\Delta T = -9.0$ and -8.9 ppb for the *cis* and *trans* isomers, respectively). On the contrary, the *trans* NH(CH_3) proton indicates a smaller temperature dependence ($\Delta\delta/\Delta T = -4.8$ ppb) than that of the *cis* isomer ($= -7.5$ ppb).

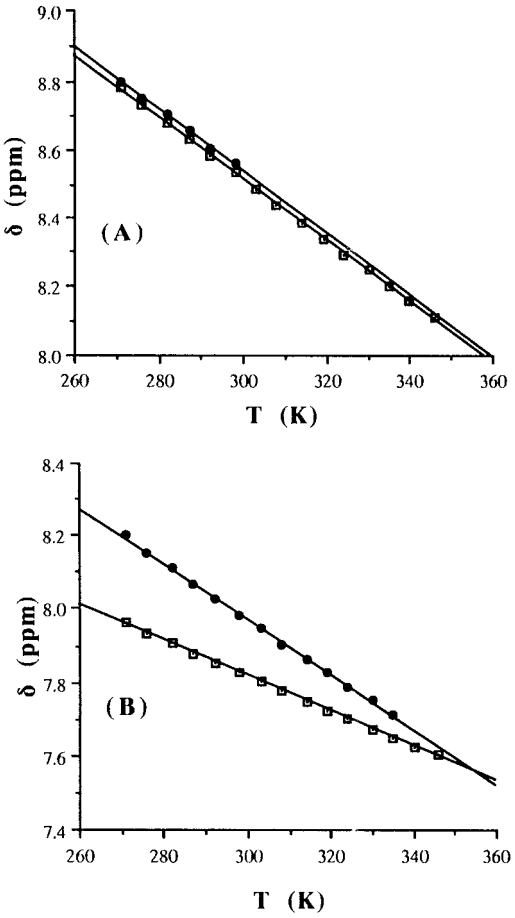


FIGURE 2 Temperature dependences of the *trans* (⊡) and *cis* (●) amide proton nmr chemical shifts of [^{13}C]–Ac–L-Pro–D-Ala–NHMe in 90% H_2O/10% D_2O, concentration 15 m*M*, on a Bruker AMX-600 instrument equipped with a 5 mm Z-gradient probe. (A) Ala NH and (B) $NHCH_3$ protons.

The model protected dipeptides with the general formula RCO–L-Pro–X–NHiPr (R = Ac, iPr, and tBu) have been investigated in detail with a variety of theoretical, x-ray, and spectroscopic techniques. McDonald and Still[8] investigated by the use of a new set of molecular mechanics parameters the conformational free energies of model dipeptides and concluded that β-turns are present in small amounts in most dipeptides. The calculations predict the most populated hydrogen bonding pattern in $CHCl_3$ to be a γ-turn across the proline. A γ-turn across the X residue in the Ac–Pro–X–NHMe sequence becomes increasingly favored as X becomes more bulky. These conclusions, however, are at variance with detailed ir, nmr, and x-ray structural investigations of Marraud and collaborators.[28,29,31,32] These authors suggested that the conformational properties of model dipeptides in solution can be interpreted in terms of three, slowly interconverting on the ir time scale, conformers: (a) An open conformer with free NH and CO bonds; (b) a γ-folded conformer characterized by a bifurcated $i \leftarrow (i + 2) \rightarrow (i + 3)$ hydrogen bond; and (c) a β-turn type II folded conformer characterized by a $(i + 3) \rightarrow i$ intramolecular hydrogen bond, which has been observed also in the crystalline state. The γ-folded conformers are converted in weakly acidic chlorinated (CH_2Cl_2) or weakly aprotic (CH_3CN) solvents into the β-folded form, which is then converted into the open form in a stronger aprotic solvent such as $(CH_3)_2SO$.

The high temperature coefficient of the AlaNH proton demonstrates that the population of the γ-folded conformation across the proline or a γ-turn with a bifurcated $i \leftarrow (i + 2) \rightarrow (i + 3)$ hydrogen bond is unlikely to be significant in aqueous solution. On the basis, however, of the reduced temperature coefficient and the significant shielding of the $NH(CH_3)$ proton of the *trans* isomer, compared to that of the *cis* isomer, it can be concluded that this amide proton is partially shielded from the solvent. It is therefore likely that the *trans* isomer exists in a folded conformation (see discussion below).

Variable Temperature 2D Grandient Enhanced ^{1}H,^{1}H NOESY–NOE Distance Constraints Molecular Dynamics

The gradient enhanced 2D NOESY spectra at 298 and 273 K indicate the presence of several intraresidue and sequential NOE cross peaks especially at low temperature. Seven interresidue and nine intraresidue NOEs were observed for the *trans* isomer and only one interresidue and two intraresidue NOEs for the *cis* isomer (strongly overlapped *cis* and *trans* NOE cross peaks were not considered in the structure analysis). At 298 and 273 K a strong NOE cross peak between ProC^{α}H and AlaNH is observed for the *trans* isomer (Figure 3). At 298 K, a medium size AlaNH—$NH(CH_3)$ cross peak is observed for the *trans* isomer, which becomes rather strong at 273 K. The respective cross peak for the *cis* isomer is beyond recognition even at 273 K (Figure 3).

NOESY spectra are capable for providing information on short proton–proton distances in the range from the van der Waals distance of 2.0 Å to approximately 4.4 Å.[18,40] Wüthrich et al.[18,40] provided a detailed description of proton–proton distances in standard tight turns of type I and II for L-Pro–L-X sequences. For a typical hydrogen bonded β-II turn conformation the following interproton distances are short enough to yield significant NOE interactions: ProC^{α}H–AlaNH $\approx$ 2.1 Å, ProC^{α}H–AlaC^{α}H $\approx$ 2.3 Å,

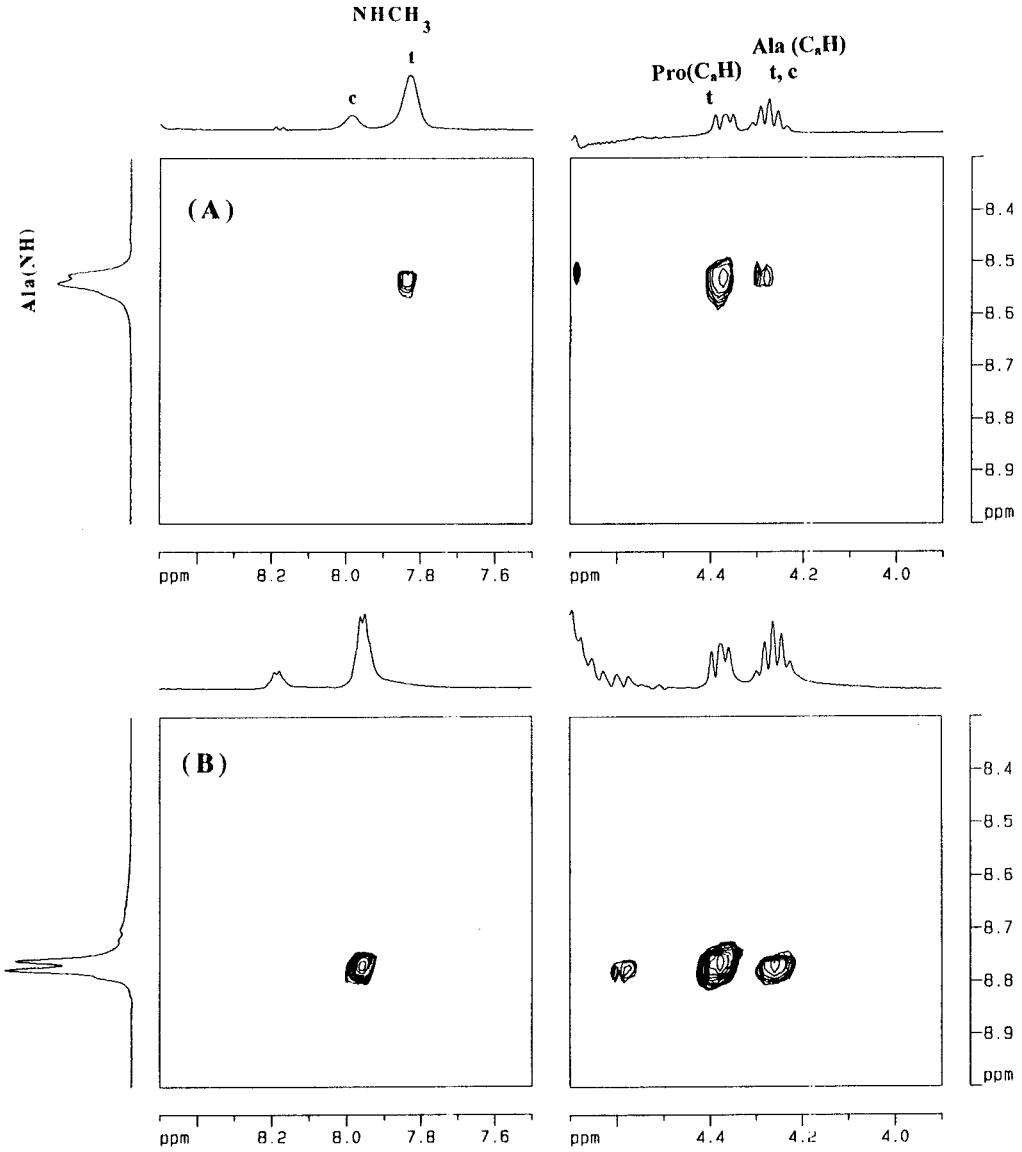
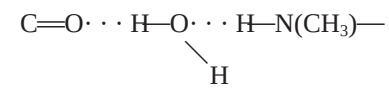


FIGURE 3 Selected region of the gradient enhanced $^1H,^1H$ NOESY spectra of [^{13}C]–Ac–L-Pro–D-Ala–NHMe in 90% H_2O/10% D_2O, concentration 15 m*M* on a Bruker AMX-400 equipped with a 5 mm Z-gradient probe. (A) 298 K, mixing time 750 ms, 176 transients were acquired for each of 256 increments. (B) 273 K, mixing time 500 ms, 336 transients were acquired for each of 256 increments.

AlaNH–NH(CH_3) ≈ 2.4 Å, ProC^{α}H–NH(CH_3) ≈ 3.3 Å. Proton–proton distances for L-Pro–D-X sequences can be obtained from the x-ray structural data of the related model peptide *trans*-Bu–CO–L-Pro–Me–D-Ala–NHMe (*t*-BuCO is the tertiobutyl CO group), which crystallizes in both anhydrous and monohydrated states.[32] Table I lists the most significant proton–proton distances (Å) of *trans-t*-BuCO–L-Pro–Me–D-Ala–NHMe in both the anhydrous and monohydrated state. In the anhydrous structure the *t*-butanoyl oxygen is directly hydrogen bonded to the —NH(CH_3) proton [(i + 3) → i hydrogen bond with N(H) · · · O(C) = 2.97 Å]. In the hydrated form this hydrogen bond is mediated by a water molecule



resulting in a slight expansion of the hydrogen bonded βII-turn ring N(H) · · · O(W) = 2.815 Å, O(W) · · · O(C)

Table I Significant Proton–Proton Distances (Å) of *trans-t*-BuCO–L-Pro–Me–D-Ala–NHMe and *trans*-Ac–L-Pro–D-Ala–NHMe

	trans-t-BuCO–L-Pro–Me–D-Ala–NHMe		*trans*-Ac–L-Pro–D-Ala–NHMe	
Proton Pair	Anhydrous State[a]	Monohydrated State[a]	Model (A)[b]	Model (B)[b]
ProC^{α}H, AlaNH	2.3	2.5	2.1	2.1
ProC^{α}H, AlaC^{α}H	4.3	4.4	4.4	4.4
ProC^{α}H, NH(Me)	3.4	3.8	3.8	4.0
ProC^{β}H, AlaC^{β}H	4.4	3.3	5.3	5.4
AlaC^{α}H, AlaNH	2.9	3.0	2.9	2.7
AlaC^{α}H, NH(Me)	3.0	2.8	3.5	3.2
AlaNH, NH(Me)	2.5	2.6	2.4	3.0

[a] The x-ray structural data from Refs. 31 and 32.
[b] Low-energy conformers derived from NOE distance constraints molecular dynamics (Figure 4).

= 2.752 Å). The molecule of water is also bonded to a neighboring peptide molecule with an intermolecular hydrogen bond O(W) · · · O(C) = 2.812 Å. Introduction of the water molecule results in some significant conformational changes especially as concerns the dihedral angles ψ_1, defined by the atoms N^1—$C^{\alpha,1}$—C'^1—N^2, and φ_2, defined by the atoms C'^1—N^2—$C^{\alpha,2}$—$C^{1,2}$, and the hydrogen-bonding scheme. The dihedral angle ψ_1 increases from 136° in the anhydrous state to 164.3° in the monohydrated state and the angle φ_2 increases from 97°, in the anhydrous state, to 138.6° in the monohydrated state. This results in a significant increase in the distance between the N(H) and O(C) atoms from 2.97 Å, in the anhydrous structure, to 5.00 Å in the monohydrated state; however, the general folded shape of the molecule is retained.

Although the conformational averaging that usually occurs in peptides hinders a rigorous interpretation of NOE intensities in terms of a unique structure, it is useful to calculate a limited number of structures compatible with NOE constraints, which helps us to visualize the conformational properties of the ensemble. Figure 4 illustrates stereoviews of two low-energy conformers of *trans*-Ac–L-Pro–D-Ala–NHMe derived from NOE distance constraints molecular dynamics, and Table I lists the most significant proton–proton distances (Å). For model (A), the N(H) · · · O(C) distance is 3.2 Å, and thus comparable with the x-ray distance of 2.97 Å in the anhydrous state. However, the (N)H · · · O(C) distance of 2.6 Å is significantly longer than the respective (N)H · · · O(C) hydrogen-bond distances in the x-ray structures of several model dipeptides *t*-BuCO–L-Pro–X–NHMe (X═L-, or D-Leu, Val, Cys, Met, Phe, and Tyr), which indicate a relatively narrow distribution with most probable H · · · O bond lengths in the region of 2.08–1.89 Å.[29] This is due to the fact that in the low-energy conformer of Figure 4(A), the N—H · · · O angle is strongly bend (~ 141°), which implies a relatively weak direct $i + 3 \rightarrow i$ intramolecular NH · · · OC hydrogen bond interaction. For model (B), a rotation by 45° is observed for the Ala$C\alpha$–CONH(CH_3) bond, which results in a significant increase of the N(H) · · · O(C) distance from 3.2 Å in model (A) to 4.2 Å. Evidently the C—CONH(CH_3) fragment exhibits significant conformational flexibility; however, the general folded shape of the molecule is retained in model (B).

The experimental 2D-NOESY data coupled with computational analysis, therefore, are in good agreement with the x-ray structural data of the related model peptide *t*-BuCO–L-Pro–Me-D-Ala-NHMe and demonstrate that the *trans*-isomer in aqueous solution accommodates an ensemble of folded conformations. The formation of βII-turns is also consistent with the $^3J_{N\alpha} \sim 8.8$ Hz for the residue D-Ala. As pointed out by Marraud et al.,[29] βI- and βII-turns of the L-D sequence could be distinguished by low (4.5 Hz) and high (9.0 Hz) values of $^3J_{N\alpha}$ of residue $i + 2$. Interestingly "reverse turns" are frequently observed in proteins, and eight classes of β-turns, four of which are without $i + 3 \rightarrow i$ interaction, have been listed.[41]

Although there are several recent reports that proline-containing sequences of short linear peptides can have significant secondary structure in water,[22,42–44] to the best of our knowledge, this is the first reported case of highly populated reverse turn conformations of a model dipeptide in aqueous solution. Interestingly, extensive molecular dynamics simulations of Tobias et al.[45] of the conformational equilibria of two

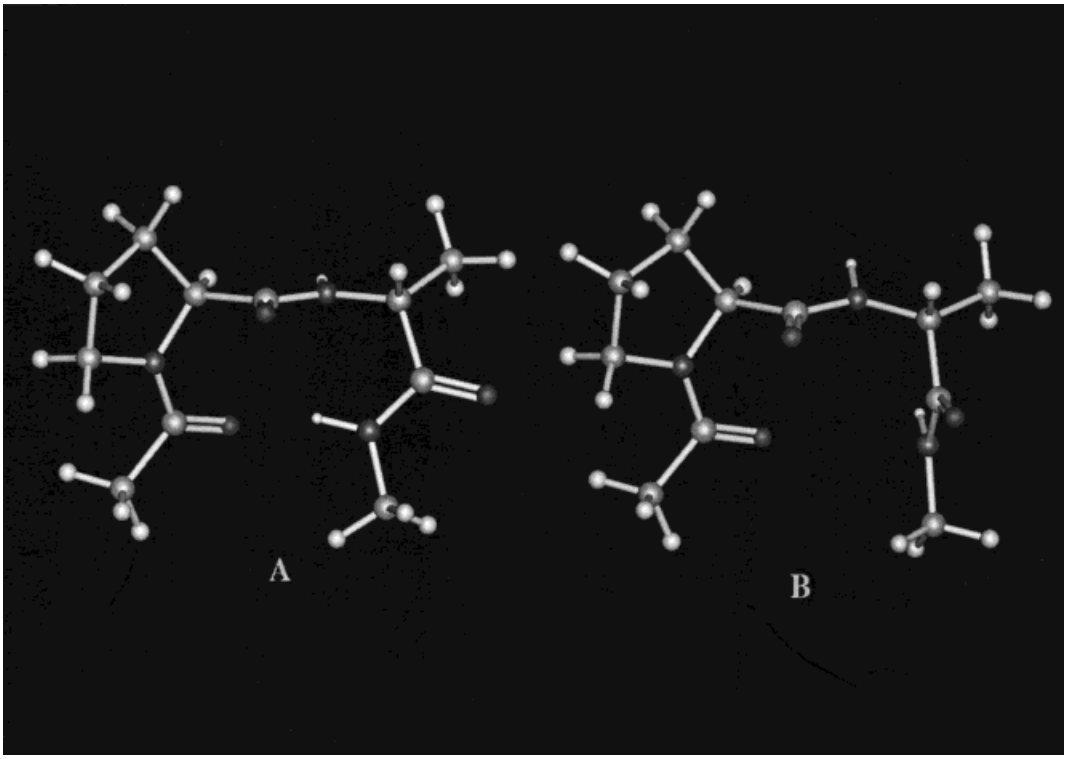


FIGURE 4 Stereoviews of two low energy conformers of the *trans*-Ac–L-Pro–D-Ala–NHMe derived from NOE distance constraints molecular dynamics.

protected dipeptides, Ac–Ala–Ala–NHMe and *trans*-Ac–Pro–Ala–NHMe, in water indicated that reverse turns with and without a direct intramolecular $i + 3 \rightarrow i$ hydrogen bond are unstable by several kcal/mol with respect to the extended conformations.

1D Steady-State Intermolecular $^{13}C,^{1}H$ NOE

The ^{13}C-nmr spectrum of the acetyl carbonyl resonances of the model dipeptide (Figure 5) indicates two resonances that can be attributed to the *cis/trans* isomerization of the Ac–Pro amide bond. On the basis of the presence of the major *trans* conformer ($\delta = 175.6$ ppm), the smaller high frequency component ($\delta = 175.9$ ppm) can be attributed to the *cis* isomer, and its intensity corresponds to a population of 16.3%, in agreement with integration data from ^{1}H-nmr. Figure 5B shows the $^{13}C,^{1}H$ NOE difference spectrum. Hydration of both isomers is clearly demonstrated by the significant NOE peaks between the water protons and the acetyl carbonyl carbon of the blocked dipeptide. The ratio of the integrated NOE peak intensities of the two resonances ($\eta = 0.024$ for the *cis* and $\eta = 0.022$ for the *trans* isomer) are not significantly different compared with the conventional 1D ^{13}C-nmr spectrum. This indicates that the water clustering around the acetyl carbonyl carbons is nearly the same for both isomers provided that the observation of equal NOE at the two CO signals is not due to exchange averaging a differential effect. Exchange phenomena, however, at 273 K seem to play a negligible role since 1D steady-state homonuclear ^{1}H-^{1}H NOE difference spectra, with selective irradiation of the *trans*-proline Cα proton, demonstrate significant NOEs to the *trans*-Cβ protons of Pro and the *trans* NH of D-Ala (Figure 6) but negligible migration of magnetization through *cis/trans* exchange to protons of the *cis*-isomer for a variety of selective low-power irradiation times (0.5–6 s).

The above results are at variance with $^{13}C,^{1}H$ HOESY experiments of the *cis/trans* isomers of the sterically hindered *tert*-butylformamide in H_2O/D_2O (85%/15%).[27] These studies indicated that the NOE cross peaks from the water proton resonance to the CO carbons of the two isomers are significantly different compared to the conventional 1D ^{13}C-nmr spectrum. Thus the *trans* amide CO NOE peak is smaller compared to the *cis*-isomer, while the respective integrals are reversed in the conventional 1D ^{13}C-nmr spectrum. This was interpreted by assuming that the water clustering of the water molecules

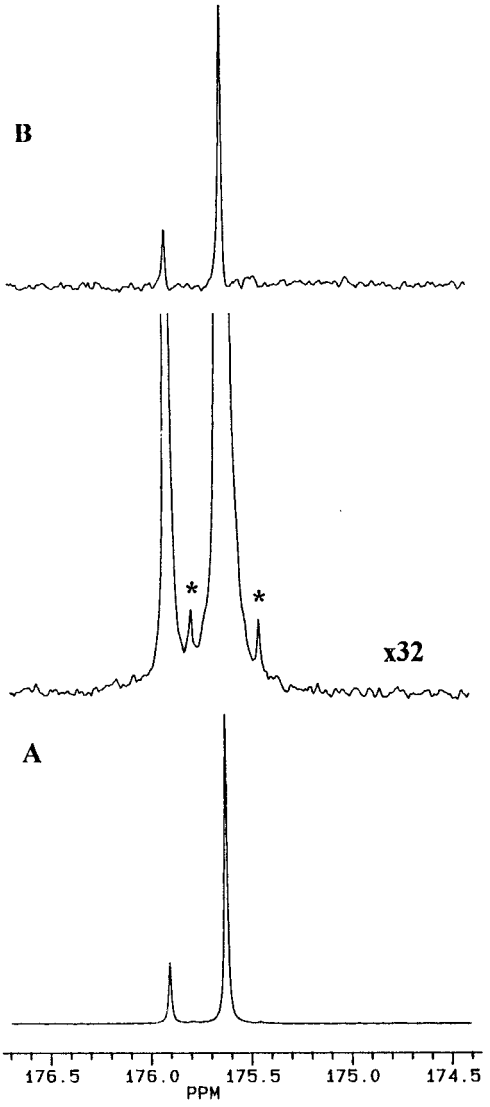


FIGURE 5 (A) Conventional ^{13}C-nmr spectrum (150.91 MHz) of [^{13}C]–Ac–L-Pro–D-Ala–NHMe in 90% H_2O/10% D_2O, concentration 15 m*M* at 273 K, 5 mm sample tube, on a Bruker AMX-600 instrument. Spectral acquisition parameters: 2.41 s acquisition time, 27.17 KHz spectral width, 6496 scans. The asterisks mark the position of the ^{13}C satellites due to one bond ^{13}C-^{13}C coupling after vertical expansion (×32). (B) 1D ^{13}C-^{1}H NOE difference experiment, 6496 scans, 6 s selective low-power irradiation of the water resonance.

around the *trans* amide CO group is significantly reduced, compared to the *cis* CO group, due to the presence of the bulky *tert*-butyl group.

It could be concluded that the hydration state of the acetyl carbons of the model dipeptide is essentially the same for both isomers. This result is not at variance with the previous discussion that the *trans* isomer mostly likely adopts an ensemble of folded low-energy conformations. Although the various fast interchanging conformational substates, such as folded and unfolded states, are expected to have variable hydration properties, the experimental data reflect the ensemble average and do not allow unambiguous determination of the solvation of individual conformational substates. Uniform fast time scale hydration has been observed for oxytocin, via NOESY and ROESY type dipolar cross-relaxation processes between water protons and polypeptide protons, irrespective of the sequence position and hydrophobic character of the amino acids.[19] In this context, similar hydration characteristics have been observed for a ribonuclease C-peptide analogue that exhibits a high population, approximately 60% by CD spectroscopy, of helical conformers in aqueous solution.[46]

Our data, therefore, are consistent with rapid fluctuations of the conformational ensemble and the carbonyl group CH_3CO— of the *trans* isomer of the model dipeptide is in close proximity to water although it adopts significant population of secondary structure.

Analysis of Thermodynamic Quantities Relating to *cis/trans* Equilibrium

The *cis/trans* equilibrium of an X–Pro peptide group can be described either by the equilibrium constant K_{eq}

$$K_{eq} = \frac{[trans]}{[cis]} \tag{1}$$

or by the free enthalpy ΔG°

$$\Delta G^\circ = -RT \ln K_{eq} \tag{2}$$

The thermodynamic parameters of the *cis/trans* isomerism in water can be obtained from variable temperature experiments, provided that the *cis/trans* forms are present simultaneously and the relative concentrations of the two species can be determined accurately. Once the equilibrium constant for this process has been determined at several temperatures, then, according to the Van't Hoff equation,

$$\ln K_{eq} = -\frac{\Delta H^\circ}{RT} + \frac{\Delta S^\circ}{R} \tag{3}$$

a plot of $\ln K_{eq}$ vs $1/T$ provides ΔH° and ΔS°. Because of the limited sensitivity of ^{1}H-nmr, applications of this probe are, in practice, confined in the range of free enthalpies between approximately 2

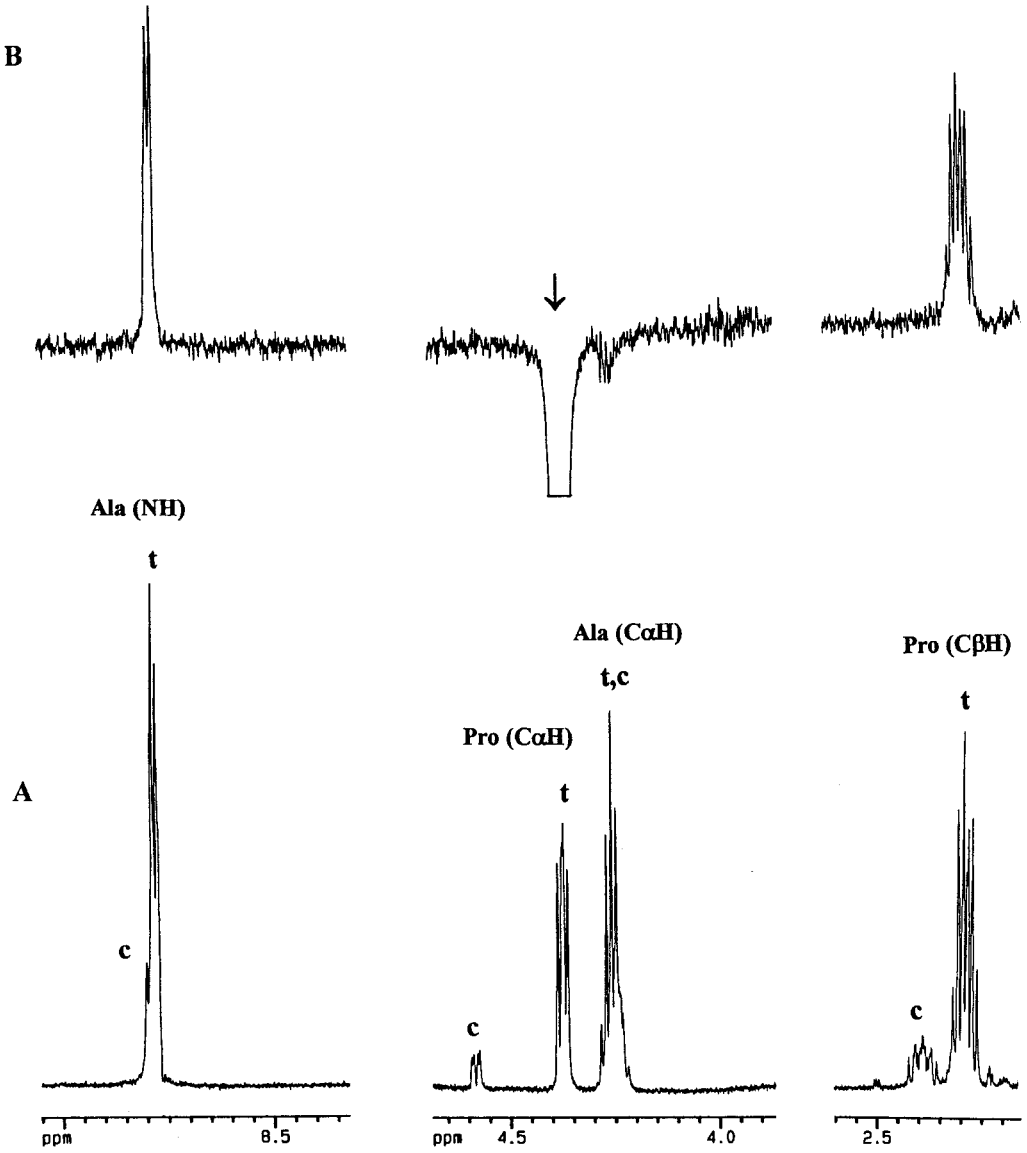


FIGURE 6 (A) Selected regions of a conventional ^{1}H-nmr spectrum of [^{13}C]Ac-L-Pro-D-Ala-NHMe in 90% H_2O/10% D_2O, concentration 15 m*M* at 273 K, on a Bruker AMX-600 instrument equipped with a 5 mm Z-gradient probe. Spectral acquisition parameters: 1.70 s acquisition time, 2 s relaxation delay, 8 scans. (B) One-dimensional steady-state NOE difference spectrum resulting from selective irradiation (marked by the arrow) of the *trans*-proline Cα proton for 3 s, 80 scans. The "c" and "t" denote the *cis* and *trans* isomers, respectively.

kcal/mol $> \Delta G^\circ > -2$ kcal/mole. One of the attractive features of this technique is that, within this range, small differences in ΔG° of the order of several tenths of a kcal/mole are readily discernible.

The *cis/trans* equilibrium of the Ac–L-Pro bond is most readily measured by the relative intensities of the $C_\delta H$ proton resonances of proline. For $C_\delta H$ the relative chemical shifts between *cis* and *trans* proline are of the order of 0.15 ppm, and hence the resonances of the isomeric forms are well separated at 600 MHz. Figure 7 shows a representative van't Hoff plot. The relation can be expressed as

$$\ln K_{eq} = -0.65784 + 0.61827\left(\frac{1}{T}\right) \qquad (4)$$

The line represents the best fit to Eqs. (2) and (3) with a correlation coefficient of 0.991, $\Delta H^\circ = -5.14$ kJ/mole and $\Delta S^\circ = -5.47\ \mathrm{J \cdot K^{-1} \cdot mole^{-1}}$. It can be

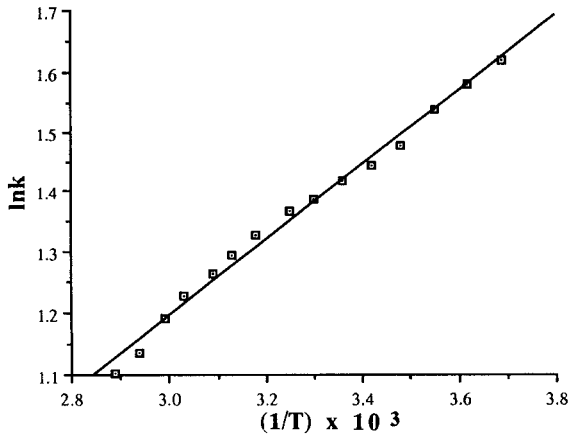


FIGURE 7 Representative van't Hoff plot based on the *cis/trans* equilibrium nmr integration data of the δ-CH proline protons of [^{13}C]–Ac–L-Pro–D-Ala–NHMe in 90% H_2O/10% D_2O, concentration 15 m*M*. The solid line represents the best fit to Eq. (3).

concluded, first, that the *trans* form is enthalpically favored and entropically disfavored relative to the *cis* form. Second, the difference in ΔG° for the Ac–Pro bond originates primarily from enthalpic differences.

Cheng and Bovey[15] investigated by the use of ^{13}C-nmr acetyl-L-proline and glycyl-L-proline, and concluded that the *trans* conformer is favored by enthalpy (the magnitude of the apparent enthalpy is in agreement with our results) but disfavored by entropy, Table II. The latter was attributed to greater solvent immobilization by the *trans* isomer. Higashijima et al.[14] investigated N-acetyl–L-proline N-methylamide in various solvents by the use of ^{1}H-nmr. In D_2O (concentration ≈ 88 m*M*) the fraction of the *cis* isomer was found to decrease only slightly on lowering the temperature. The apparent enthalpy and entropy changes for the conversion of the *trans* isomer to the *cis* were found to be 0.3 kcal · mole^{-1} (1.26 kJ · mole^{-1}) and −4.6 J · deg^{-1} · mole^{-1} respectively (Table II). The sign of ΔS° is not in agreement with our results, and the magnitude of ΔH° is appreciably different.

Recently, Eberhardt et al.[16] investigated the thermodynamic origin of prolyl peptide bond isomers of the racemic dipeptide Ac–Gly–[β, δ-^{13}C] Pro–OMe in aqueous buffer and in toluene. It was concluded that the difference in ΔG° for the X–Pro isomers originates almost entirely from enthalpic differences. Further, the similarity of the enthalpies determined in aqueous buffer ($\Delta H^\circ = -1.27$ kcal/mole) and in toluene ($\Delta H^\circ = -1.27$ kcal/mole) suggests that the enthalpic forces that differentiate the *cis* and *trans* isomers of prolyl peptide bonds are similar in protic and aprotic environments. Differences in entropy, though small, favor the *cis* isomer in both aqueous buffer and toluene. This entropic preference was found to be less in aqueous buffer [$\Delta S^\circ = -0.25$ cal · mole^{-1} K^{-1}] than in toluene [$\Delta S^\circ = -0.71$ cal · mole^{-1} K^{-1}]. This was interpreted by assuming a lower solvent accessibility of the amide CO group in the *trans* isomer, which diminishes the ability of this group to restrict H_2O molecules through hydrogen bonding.

Our result that the difference in ΔG° for the Ac–L-Pro bond originates primarily from enthalpic differences, is in agreement with that of Eberhardt et al.[16] However, the contribution of entropy was found to be appreciably larger. This can be attributed to the significant folding of the *trans* isomer, and thus reduced disorder or configurational freedom.

In conclusion variable temperature ^{1}H-nmr studies of the *cis/trans* conformational equilibrium of the β-turn model dipeptide [^{13}C]–Ac–L-Pro–D-Ala–NHMe indicate that the *trans* form is enthalpically favored and entropically disfavored relative to the *cis* form. The *trans* isomer most likely adopts an ensemble of low-energy folded conformations, whereas the *cis* isomer adopts more open conformations. This demonstrates that in the absence of strongly stabilizing sequence-specific interresidue interactions involving side chains and/or charged terminal groups the influences of solvent water and the conformational properties of the *cis/trans* isomers are not identical.

Table II Apparent Enthalpy (ΔH°) and Entropy (ΔS°) Changes for the Conversion of the *cis* to the *trans* Isomer for Model Peptides

Peptide	Solvent	ΔH° (kJ · mole^{-1})	ΔS° (J · K^{-1} · mole^{-1})
Gly–L-Pro[15]	D_2O	−4.2	−9.7
Ac–L-Pro–NHMe[14]	D_2O	−1.26	4.6
Ac–Gly–[β,δ-^{13}C]Pro–OMe[16]	80% H_2O/20% D_2O	−5.33	−1.05
Ac–L-Pro–D-Ala–NHMe	90% H_2O/10% D_2O	−5.14	−5.47

Financial support from NATO (Collaborative Research Grant, 960146) and the Research Committee of the University of Ioannina is gratefully acknowledged. The nmr spectra were obtained on the ULIRS Bruker AMX-600 facility located at QMW and the NMR Centre of the University of Ioannina (Bruker AMX-400). Thanks are also due to the referees who made important suggestions incorporated in the revised manuscript.

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