Vol. 177, No. 1, 1991 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 31, 1991 Pages 271-278

CONFORMATIONAL ANALYSIS OF THE TETRAPEPTIDE PRO-D-PHE-PRO-GLY IN AQUEOUS SOLUTION

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Received April 18, 1991

Conformational investigations of the tetrapeptide Pro-D-Phe-Pro-Gly in water solution were carried out by ^{1}H and ^{13}C NMR spectroscopy . The internal proline residue allows for the possibility of cis/trans isomerization about the D-Phe-Pro peptide bond resulting in two conformational isomers. The major isomer was identified as the trans isomer. The pH-dependence of the cis/trans equilibrium supports an additional stabilisation of the trans isomer by an intramolecular ionic interaction between the amino- and carboxy-terminus in the zwitterionic state. Based on ^{13}C spin-lattice relaxation times(T_1), different pyrrolidine ring conformations of Pro^1 and Pro^3 could be determined. By combination of several NMR data (vicinal coupling constants $^{3}J_{N\alpha}$, temperature dependence of the NH chemical shifts, differences in the chemical shifts between the ß and γ carbons of the proline residues) and energy minimization calculations, a type II' ß-turn should contribute considerably to the overall structure of the trans isomer.

Proline containing peptides are unique in, at least, two different properties. Firstly, they tend to build up conformational preferences even in short peptides; there are speculations about the role of these thermodynamically favourable interactions to function as "folding seeds"in proteins (1,2) or recognition segments in antibody reactions (3). Secondly, with respect to the prolyl peptide bond, two isomers occur frequently in solution which interconvert slowly on the NMR time scale.
Both isomers, cis and trans, can be utilized as substrates by prolyl cis/trans isomerases, when appropriately substituted in the

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0006-291X/91 $1.50

neighbourhood of proline (4). Prolyl cis/trans isomerases represent the first example of enzymes which can accelerate catalytically the folding of proteins. They are involved in the cyclosporin A (5,6) and FK506 (7) mediated immunosuppression although the mechanism, which connects enzymatic prolyl bond isomerization with immunosuppression, is still unknown.
It is the objective of this paper to study the conformational equilibrium of the tetrapeptide Pro-D-Phe-Pro-Gly at different pH-values, as the prerequisite for monitoring the prolyl cis/trans isomerase activity based on dynamic nmr . Due to the D-amino acid within the peptide chain the conventional prolyl cis/trans isomerase assay by isomer specific proteolysis (4) cannot be utilized.

MATERIALS AND METHODS

The peptide was built up by stepwise synthesis starting from the C-terminal end using the mixed anhydride method.
Purity was checked by analytical TLC, HPLC and by consideration of the 1D NMR spectra. ^{1}H and ^{13}C NMR spectra were obtained on a Bruker WP 200 spectrometer operating at 200,13 and 50,33 MHz, respectively. The proton experiments were carried out at a concentration of 10 mg/ml in 90% H_2O/ 10% D_2O, the ^{13}C data were observed at a concentration of 30 mg/ml. The relaxation times T_1 were measured with the inversion recovery method(8) using a pulse repetition time of 3s and a variable time delay of 0.05-3s. The samples were degassed by repeated freeze-thaw cycles. All spectra were recorded at 298±4 K; for the temperature dependence of the amide proton chemical shifts the temperature was varied between 298 K and 338 K; dioxan was used as an internal standard. Chemical shifts are reported in ppm from internal TMS. In the ^{1}H spectra, the signal of the water protons was suppressed by preirradiation at the appropriate frequency. The 2D NMR spectra were recorded using the standard Bruker software. For the assignment of the ^{1}H NMR spectra,H-H COSY,H-C COSY and homonuclear spin decoupling were used.
The Karplus equations (9,10) used were of the form:
$^{3}J_{N\alpha} = 6{,}4\cos^2\Theta - 1{,}4\cos\Theta + 1{,}9$ (11) with $\Theta = |\Phi + 60°|$ for D-Phe and
$\Sigma^{3}J_{N\alpha} = -10{,}7\cos^2\Phi - 1{,}5\cos\Phi + 15{,}9$ for L-Gly.

RESULTS AND DISCUSSION

Assignment of the ^{13}C signals

In the ^{13}C NMR spectrum of Pro-D-Phe-Pro-Gly two sets of signals are readily recognized which were assigned to the cis/trans isomerism of the D-Phe-Pro imidic bond. The differences in the chemical shifts of the Pro^3 carbons were taken to be indicative (12) for the assignment of the minor resonances to the cis isomer. The ratio of the isomers was determined by integration of well resolved peaks of the inverse-gated ^{13}C spectra. The results obtained correspond to the ratio determined by integration of the amide proton signals at pH 3,5 to 6.

By means of measuring the pH- dependence of the chemical shifts and of carbon relaxation times T_1, it was possible to differentiate the ^{13}C resonances of the two proline residues. The Pro^1 carbons are downfield shifted with increasing pH due to deprotonation at the pK_a-value of 3,6±0,1 and are characterized by longer T_1 relaxation times when compared with the internal proline residue carbons because of their higher mobility (Table 1).

Proline ring conformations

The relaxation times T_1 of relevant carbon atoms are given in Table 1. Assumming that the dipole- dipole relaxation is the predominant mechanism of relaxation and, further, that the overall motion of the proline ring is isotropic (13), the various NT_1 values of the proline ring carbons can be interpreted in terms of ring puckering. The higher NT_1 value of the Pro^1 γ-carbon when compared with the other carbons of the same ring can be explained by the flipping of the γ carbon relative to a plane formed by the remaining ring atoms.

The conformation of the Pro^3-ring, in opposite to Pro^1, is expected to be a half chair form taking the NT_1 values into account, which are equal for the ß and γ carbons.

Table 1
Carbon chemical shifts and NT_1[a] values

Residue		^{13}C chemical shifts (ppm) [trans]	[cis][c]	NT_1(s)[b] [trans]
Pro^1	α	60,15		0,6
	ß	30,21		1,51
	γ	24,08		2,11
	δ	47,04		1,14
	C=O	169,72	168,37	
D-Phe^2	α	53,82	53,34	0,30
	ß	37,18		0,38
	Φ_1	136,18		
	$\Phi_{2,6}$	129,22	129,53	
	$\Phi_{3,5}$	129,79		
	Φ_4	127,83	127,59	
	C=O	171,80		
Pro^3	α	61,27		0,54
	ß	29,72	32,21	1,06
	γ	24,36	22,62	0,96
	δ	48,20		0,88
	C=O	173,81		
Gly^4	α	44,00		1,09
	C=O	176,54		

[a] N number of directly attached protons, T_1 spin lattice relaxation time.
[b] determined for the aliphatic carbon atoms.
[c] atoms without figures have the same value like the trans isomer.

Estimation of torsion angles

The differences in the ^{13}C chemical shifts of the ß and γ carbons of proline, $\Delta\delta_{\beta\gamma}$, are sensitive to both the angle Ψ and the puckering of the proline ring. Using calculations of Giessner-Prettre et al.(14) which describe the correlation between $\Delta\delta_{\beta\gamma}$ and Ψ for trans peptide bonds, a Ψ_3 value of approximately -10° equal to $\Delta\delta_{\beta\gamma}$ =5,17ppm was estimated.

Homonuclear $^3J_{N\alpha}$ coupling constants depend on the torsional angle Φ as descriped by the Karplus equations (9,10). Using the constants from Pardi (11), Φ_2 was calculated to be +156° or +84°. In opposite to the 156° angle, the 84° angle is near to the value of the first corner position of a type II' ß-turn(Φ_{i+1}=60°)(15). The glycine residue is unique due to the ABX spin system of the α methylene protons and the amide proton. Therefore a modified Karplus equation for the sum of the two vicinal coupling constants $\Sigma^3J_{N\alpha 2}$ was used (9). The value of $\Sigma^3J_{N\alpha 2}$ = 11,15 Hz provides solutions for Φ_4 of ± 53° or±138°.

According to the determined pyrrolidine ring conformation of Pro^3, the corresponding Φ_3 angle is restricted to about -75°.

pH- dependence of the cis/trans equilibrium

Because of the identical covalent structures adjacent to the ß and γ carbons of Pro^3 within both isomers, the cis/trans ratio can be determined by measuring the integrals of these characteristic signals (16). Partial signal overlapping of the corresponding carbon resonances of the two prolines limited valuable estimations of this ratio to the ß carbon resonances (Fig.1).

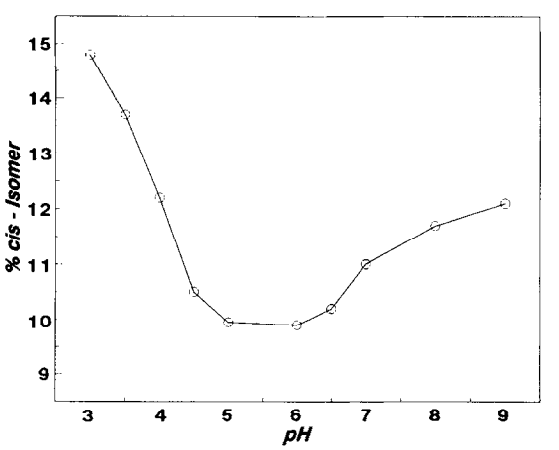

Figure 1.
pH-dependence of the amount of the cis isomer, obtained from the integral intensities of the ^{13}C cis and trans signals of the ß-carbon resonances of Pro^3.

D-amino acids, located N-terminally to proline, usually lower the cis content considerably(17). This is caused by an increase of the conformational space, which is available to the trans peptide in this configuration(18). Accordingly, both the anionic and the cationic form of Pro-D-Phe-Pro-Gly prefer the trans structure at approximately the same level(85%-90%) which was found generally for D-Phe-Pro- peptides. In water, usually the variation of the ionisation state of prolyl peptides does not produce remarkable effects on the conformational equilibrium (19). However, the zwitterionic structure of the tetrapeptide reveals a decreased population of the cis isomer(Fig.1). One explanation therefore could be the additional stabilisation of the trans isomer by an intramolecular salt bridge between the amino- and carboxy-terminus according to the ß-turn conformation (this result is shown below).

Temperature coefficients of amide proton resonances

The temperature dependence of the amide proton chemical shift between 298 K and 338 K was studied in order to evaluate the possibility of hydrogen- bonding in the tetrapeptide. The large negative $\Delta\delta/\Delta T$ values of -6,47 ppb/K for D-Phe^2 and -6,26 ppb/K for Gly^4 indicate the absence of intramolecular hydrogen- bonding in the temperature range studied. But we note, that a 4 → 1 hydrogen bond is not a requirement for turn formation (20).

Theoretical investigations

Simultaneous to the NMR investigations, conformational considerations were accomplished by means of molecular modelling using the program ECEPP83 (21-23).

90 conformations within a range up to 100 kJ/mol above the conformation with the lowest energy were found for Pro-D-Phe-Pro-Gly. Comparing these conformations determined by ECEPP with the dihedral angles obtained from NMR-investigations a structure was found, which shows good correspondence to the NMR-results(Table 2). This structure is 21.9 kJ/mol less stable than the

Table 2
Comparision of the torsions angles obtained by NMR-spectroscopy and by energy minimization calculations

	Ψ_1	ω_1	Φ_2	Ψ_2	ω_2	Φ_3	Ψ_3	ω_3	Φ_4	Ψ_4	energy
					(degrees)						(kJ/mol)
NMR		180	84		180	-75	-10	180	-138		
ECEPP	156	180	80	-133	-172	-75	-7	179	-158	-165	+22.0
MMP2	152	178	84	-131	-178	-76	-4	176	-150	-122	+17.4

theoretically calculated most stable conformation.
Further investigations using the force field method MMP2 (23) led to analogous results.
The relatively large difference between calculated and measured values of Φ_4 indicates high terminal conformational flexibility. The energy difference between the conformations of Φ_4=-158° (theoretical angle) and Φ_4=-138° (experimental angle) is only 3 kJ/mol.

Conformations of trans Pro-D-Phe-Pro-Gly in water
The combination of the NMR investigations and the energy minimization calculations suggests that a significant population of the trans isomer adopts a type II'ß-turn conformation. The $C^1...C^4$ distance of 5 Å lies within the distance criteria of a ß-turn of 7 Å. In Table 3 the characteristic torsion angles of the two central residues of an ideal type II' ß-turn are compared with the appropriate torsion angles of the experimentally determined conformation of Pro-D-Phe-Pro-Gly and were found similar. Further stabilisation of the trans isomer in the ß-turn should result from the interaction of the ionic terminals. The result agrees well with general conformational energy calculations: for D-X_{i+1}-Pro_{i+2} sequences with a trans peptide bond, type II',V' and a γ -turn may occur, whereas for L-X_{i+1}-Pro_{i+2} the turn probability is much lower (24). However, it must be emphasized that the ß-turn conformation is only one of an ensemble of conformations available to the tetrapeptide studied. The supposed ß-turn is very similar to the conformation found in the X-ray structure (data not shown).

CONCLUSIONS

The existence of two main backbone conformations of Pro-D-Phe-Pro-Gly in water, cis and trans with respect to the D-Phe-Pro peptide bond, was observed. The population of the two isomers is dependent on the ionisation state of the terminal residues. The two proline

Table 3
Backbone dihedral angles for an ideal type II' ß-turn and for the experimental determined conformation of Pro-D-Phe-Pro-Gly

	ω_{i+1}	Φ_{i+1}	Ψ_{i+1}	ω_{i+2}	Φ_{i+2}	Ψ_{i+2}
type II' ß-turn[a]	+180	+60	-120	+180	-80	0
Pro-D-Phe-Pro-Gly	-172	+80	-133	+179	-75	-7

[a] Idealized turn geometries are defined in ref. 15.

residues, existing in different ring conformations, restrict the conformational space available for the tetrapeptide. A significant population of the trans isomer probably adopts an ordered structure in water as an type II'ß-turn. In this conformation, there is no stabilisation by hydrogen bonding.
The results extend previously published investigations, which provide evidence for the formation of ß-turns in water even of small linear peptides (25-27). Short- range local interactions specified by the amino acid sequence rather than medium- or long-range interactions are therefore expected to promote the turn conformation. Both amount and conformation of the trans isomer gives an appropriate base to investigate the interaction with prolyl cis/trans isomerases by dynamic nmr.

ACKNOWLEDGMENT

This work was supported by the Bundesministerium für Forschung und Technologie (GN 30K00940).

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