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Influence of lithium cations on prolyl peptide bonds

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The influence of lithium cations on the *cis*/*trans* isomerization of prolyl peptide bonds was investigated in a quantitative manner in trifluoroethanol (TFE) and acetonitrile, employing NMR techniques. The focus was on various environmental and structural aspects, such as lithium cation and water concentrations, the type of the partner amino acid in the prolyl peptide bond, and the peptide sequence length.

Comparison of the thermodynamic parameters of the isomerization in LiCl/TFE and TFE shows a lithium cation concentration dependence of the *cis*/*trans* ratio, which saturates at cation concentrations >200 mM. A pronounced increase in the *cis* isomer content in the presence of lithium cations occurs with the exception of peptides with Gly-Pro and Asp-Pro moieties. The cation effect appears already at the dipeptide level. The salt concentration can considerably be reduced in solvents with a lower number of nucleophilic centers like acetonitrile. The lithium cation effect decreases with small amounts of water and disappears at a water concentration of about 5%. The isomerization kinetics under the influence of lithium cations suggests a weak cation interaction with the carbonyl oxygen of the peptide bond. Copyright © 2012 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: lithium cation interactions; prolyl peptide bonds; *cis*/*trans* isomerization; NMR spectroscopy; bipolar disorder

Introduction

Lithium occurs in the form of its salts as a trace element in the human organism. However, it is not essential, and an actual biological function is not known so far. Lithium cations play an important role as a mood-stabilizing drug in the treatment of bipolar disorder [1,2]. The underlying mechanisms for the effects of lithium cations on depression and mania are not known yet. Most research concerns the influence on magnesium-activated enzymes as the myo-inositol-phosphatase in the inositol phosphate metabolism and the glycogensynthase-kinase-3, respectively [3–7]. Lithium and magnesium cations share some chemical properties resulting in a competition for magnesium-binding sites. Other authors suggest an interaction of lithium cations with the nitric oxide signaling pathway in the central nervous system [8,9]. Further interesting biological effects of lithium cations concern the circadian rhythm [10–12] and some symptoms of Alzheimer disease [13–15].

Important results on lithium salt effects on the conformation of peptides and various chemical reactions came from the Kessler and Seebach groups [16,17]. A very remarkable finding concerned the change of the *cis*/*trans* equilibrium of peptide bonds, illustrated for a prolyl peptide bond in Figure 1, in solvents like tetrahydrofuran (THF) and trifluoroethanol (TFE). Thus, it could be demonstrated that the conformational equilibrium of the peptide bond between two methylated leucine residues in cyclosporine A is shifted from a *cis* to a *trans* arrangement after addition of an excess of lithium chloride to a THF solution of the peptide [18]. Kofron *et al*. [19,20] found a particularly high influence of lithium chloride on the conformational state of prolyl peptide bonds with a shift from *trans* to *cis* bonds in the peptide Suc-Ala-Ala-Pro-Phe-pNA in TFE and THF. In other solvents, such as ethanol, dimethyl sulfoxide, dimethylformamide, and hexafluoro-2-propanol, no or only very small effects of lithium cations on the *cis*/*trans* equilibrium of prolyl peptide bonds were observed. Small amounts of water prevent the effect. The anion in the lithium salts can be varied without consequences for the effect, whereas comparable effects are absent with the corresponding sodium salts [19]. Secondary amide peptide bonds are also susceptible to lithium cation effects as was shown by the Li^+ influence on the refolding of a proline-free variant of tendamistat and on the increased *cis*/*trans* ratio of the Ala-Tyr bond in the Ala-Ala-Tyr tripeptide [21].

Studies on the folding of 5-hydroxytryptamine type 3 ($5\text{-}HT_3$) receptors demonstrated that the *cis*/*trans* isomerization of a prolyl peptide bond leads to the opening of an ion channel formed by a $5\text{-}HT_3$ receptor hexamer [22,23]. In the light of these results and the general importance of the *cis*/*trans* isomerization of prolyl peptide bonds in protein folding [24–26], it could be postulated that lithium cations may trigger effects based on isomer specificity of biorecognition. This opens up new possibilities for an understanding of the physiological action of lithium cations.

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Figure 1. *Cis/trans* equilibrium of a prolyl peptide bond.

Until now, the extent of the lithium cation influence on the *cis/trans* isomerization of prolyl peptide bonds was not sufficiently investigated with respect to various environmental and structural aspects, such as the cation and water concentrations, the solvent type, the partner amino acid in the prolyl peptide bond, and the peptide sequence length necessary for the occurrence of the effects. In order to provide quantitative data on these aspects, we performed systematic investigations on several peptides employing various NMR techniques to determine thermodynamic and kinetic parameters of isomerization.

Materials and Methods

Peptide Synthesis

The chromogenic peptide Suc-Ala-Ala-Pro-Phe-pNA (**1**) was purchased from Sigma-Aldrich (Deisenhofen, Germany). The peptides of series Ac-Ala-Xaa-Pro-Ala-Lys-NH_2 (**4**) were synthesized by solid-phase peptide synthesis using 0.15 mmol Rink amide resin (Novabiochem, Läufelingen, Switzerland) on a SYRO II multiple peptide synthesizer (MultiSynTech, Witten, Germany). Assembly of the peptides was performed with Fmoc chemistry using a standard protocol with Fmoc amino acids as building blocks and PyBOP (Novabiochem) and *N*-methylmorpholine as coupling reagents in DMF. Piperidine (20%) in DMF was the standard cleavage cocktail used for Fmoc detachment. The resins were treated twice for 10 min. All couplings were performed using a fourfold excess of Fmoc amino acid derivative, PyBOP, and *N*-methylmorpholine in DMF. After detachment of the peptides from the resins and side chain deprotection with TFA/TIS/water (95 : 3 : 2, *v/v/v*) for 2 h at room temperature, the crude peptides were precipitated with diethylether and purified by preparative RP-HPLC on a Gilson 306 system with an SP250-10 Nucleosil 100–7 column (Macherey-Nagel, Düren, Germany) using a water/acetonitrile gradient containing 0.1% TFA in the solvents. The peptides Ac-Ala-Pro-NHEt **2** and Ac-Gly-Pro-NHEt **3** were synthesized by mixed anhydride method in THF/DMF and purified in analogy to the peptides of series **4**. All peptides were lyophilized, and their purity was verified by analytical HPLC using a LiChroCART® column (LiChrospher®100, RP8, 5 μm; 125–4; Merck (Darmstadt, Germany)) with a gradient 5–100% acetonitrile in water (0.1% TFA) and a flow rate of 1 ml/min over 30 min (UV detection at $\lambda = 220$ nm). The molecular masses of the peptides were confirmed by ESI mass spectroscopy. The peptides were stored over P_2O_5 to avoid absorption of moisture.

Peptide Solutions

For NMR analysis, deuterated trifluoroethanol (TFE-d_3) (99% deuteration) was purchased from Euriso-Top (Saarbrücken, Germany) and deuterated acetonitrile (ACN-d_3) (99,5% deuteration) from Carl Roth (Karlsruhe, Germany). Lithium chloride was provided by Sigma-Aldrich (Deisenhofen, Germany). It was dried *in vacuo* at 160 °C for at least 24 h before probe preparation. The peptide solutions were prepared in vials fitted with septa and subsequently transferred into an NMR tube fitted with septa, too. The water content was determined by Karl–Fischer titration employing a 794 Basic Titrino device (Metrohm GmbH, Filderstadt, Germany). Another control of the water content was possible via the signal intensities of the solvent hydroxy resonance in the ^{1}H-NMR spectra.

NMR Measurements

All NMR measurements were performed employing an Avance-700 and a DRX-600 spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany). The signal assignment in the ^{1}H-NMR spectra was achieved by means of several 2D methods like ^{1}H-^{1}H-COSY, ^{1}H-^{13}C-HSQC, and ^{1}H-^{1}H-NOESY, respectively. The scalar coupled spin systems were first identified in the COSY spectra. The ^{1}H-^{13}C-HSQC spectra were recorded to demask anisotropic protons and to resolve resonance overlap. On the basis of the ^{1}H-^{1}H-NOESY, the appropriate signal pairs of the *cis* and *trans* forms could be identified since a significant NOE was observed between the H_δ atom of the proline residue and the H_α atom of the preceding amino acid. Integration of both isomer signals provided the *cis/trans* isomer populations. The software Bruker TopSpin (versions 1.3 and 2.0) was the basis for acquisition, processing, assignment, and integration of the spectra.

The rate constants of the *cis/trans* isomerization in the peptides **1** and **2** were determined by exchange spectroscopy (EXSY) [27,28]. This technique allows the investigation of exchange processes that are slow on the NMR time scale. It has been widely used to determine the rotational barriers of amide bonds on the basis of the magnetization transfer during a chemical exchange like a *cis/trans* isomerization process [29]. For this purpose, NOESY spectra at seven different mixing periods (0.03, 0.5, 0.75, 1.0, 1.5, 2.0, and 3.0 s) were recorded at 300 K. The volume integrals of the cross peaks of the exchanging *ortho* protons of the *para*-nitro moiety in **1** and the H_α protons in peptide **2**, respectively, were determined after normalization of the cross peaks to the diagonal peaks. The rate constants were finally calculated on the basis of the corresponding equations for a two-side exchange process employing the software Mathematica 6 (Wolfram Research, Inc., Champaign, IL, USA). For peptide **1**, the rate constants in the LiCl solution could also be determined by concentration-jump technique [30]. In this case, a shift in the *cis/trans* equilibrium was induced by a jump from a pure TFE solution into the LiCl/TFE mixture. The time dependence of the subsequent intensity changes of the ^{1}H-NMR signals was recorded. The rate constant of this relaxation process was finally determined by non-linear curve fitting. The concentration-jump measurements had to be performed at a lower temperature of 277 K than the EXSY measurements to make the interconversion process slower. A concentration jump into the opposite direction could not be realized. The software Origin 7.5 (OriginalLab Corp., Northhampton, MA, USA) was employed for the non-linear curve fitting to yield the rate constants.

Results and Discussion

From first investigations of the lithium cation influence on the *cis/trans* isomerization of the prolyl peptide bond in Suc-Ala-Ala-Pro-Phe-pNA (**1**) in deuterated TFE (TFE-d_3) [19,20], we know that a considerable increase from about 10–12% to about 60–70% *cis*

isomer occurs in the presence of a high excess of lithium chloride in dry solvents, but a systematic variation of the ion concentration is still missing. Figure 2 shows the change of the *cis* content with varying lithium cation concentration. The peptide concentration was kept at 8 mM, the LiCl concentration varied up to 600 mM. The *cis* content increases continuously from the starting value of 12% in pure TFE and reaches 60% at a LiCl chloride concentration of about 235 mM.

The maximum value of about 68% appears at a LiCl concentration of about 470 mM. Further increase in the salt concentration does not change this value. Figure 3 shows that addition of water to a solution of 8 mM peptide **1** in a 470-mM LiCl/TFE-d_3 solution continuously reduces the *cis* content. A water concentration of more than 5% brings the *cis* population back to the value for pure TFE. Although small amounts of water prevent the lithium cation influence, lithium ions might nonetheless be able to induce physiological effects in membranes and core regions of globular proteins.

The results indicate the need of a considerable molar excess of LiCl to reach the maximum equilibrium shift. It could be argued that the carbonyl oxygens of the tetrapeptide **1** provide five potential cation-binding sites. However, competition between the solvent and the peptide for the lithium cations might be more important. Until now, the effect was only examined in oxygen-containing solvents offering numerous interaction possibilities for the lithium cations, which are even increased in TFE by the fluorine atoms. Possibly, the effect appears already at lower LiCl concentrations in solvents with a lesser number of nucleophilic centers. Unfortunately, the low solubility of peptides and LiCl in hydrocarbon solvents prevents the corresponding NMR investigations. First measurements in a nitrogen-containing solvent like acetonitrile (ACN-d_3), where the peptide **1** and LiCl can still be dissolved in a sufficient amount, could at least confirm the postulated tendency. The results in Table 1 show that the *cis* population is increased to 62% with only a fivefold excess of LiCl. A tenfold excess of LiCl generates the maximum value of 66%.

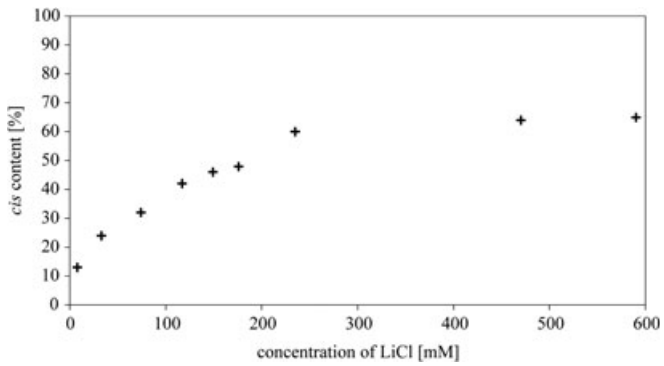

Figure 2. Dependence of the *cis* content of the peptide Suc-Ala-Ala-Pro-Phe-pNA **1** (8 mM) on the LiCl concentration in TFE-d_3.

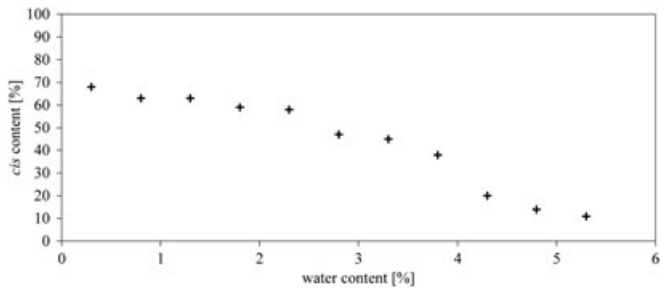

Figure 3. Dependence of the *cis* content of the peptide Suc-Ala-Ala-Pro-Phe-pNA **1** (8 mM in a 470-mM LiCl/TFE-d_3 solution) on the water concentration.

Thus, the described effects on the *cis*/*trans* isomerization could be provoked at much lower cation concentrations in an apolar environment.

Possible binding sites of peptide **1** for lithium cations have been probed by measuring the kinetics of prolyl bond isomerization employing ^{1}H-NMR EXSY and concentration-jump techniques, respectively (see Materials and Methods section). Concentration jumps could only be realized from bulk TFE-d_3 into a LiCl/TFE-d_3 solution. According to EXSY, the obtained values for the rate constants at 300 K in *trans* → *cis* and *cis* → *trans* direction for an 8-mM peptide solution in TFE-d_3 are $k_{t\rightarrow c} = 0.033\ s^{-1}$ and $k_{c\rightarrow t} = 0.24\ s^{-1}$. In a 500-mM LiCl/TFE-$d_3$ solution, these values are $k_{t\rightarrow c} = 0.0092\ s^{-1}$ and $k_{c\rightarrow t} = 0.0045\ s^{-1}$. According to concentration-jump technique, the values for 2 mM peptide in a 300-mM LiCl/TFE-d_3 solution are $k_{t\rightarrow c} = 0.0016\ s^{-1}$ and $k_{c\rightarrow t} = 0.0008\ s^{-1}$. These data are in fair agreement with the EXSY data considering that the concentration-jump measurements had to be performed at a lower temperature of 277 K instead of 300 K to slower the interconversion process.

The data indicate a decrease in the isomerization rates in the presence of LiCl. This finding supports a molecular model where the lithium cations bind to the carbonyl oxygen of the prolyl peptide bond causing an increase in the partial double bond character and consequently an increase in the rotation barrier. The decelerating rate effect is more pronounced for $k_{c\rightarrow t}$ when compared with $k_{t\rightarrow c}$. In contrast, a decreasing rotational barrier is expected for Li^+ ions bound to the nitrogen lone pair [31].

The above studies were performed on Suc-Ala-Ala-Pro-Phe-pNA **1**, which was also subject in comprehensive investigations on the basics of prolyl bond isomerization [19,20,32–35]. To gain access to the influence of the chain length of oligopeptides on their lithium cation-chelating tendencies, the N-terminally acetylated dipeptides Ac-Ala-Pro-NHEt **2** and Ac-Gly-Pro-NHEt **3** were investigated. Interestingly, the simplest congener Ac-L-Pro-NH_2 in LiCl/TFE-d_3 did not show any deviation from the 10% *cis* content obtained in TFE-d_3 solution even at very high LiCl concentrations. Whereas a considerable effect of the lithium cations was found for the Ala-Pro dipeptide **2**, the effect is missing in the Gly-Pro dipeptide **3** (Table 2). This is consistent with earlier investigations on pentapeptides comparing their LiCl/TFE-d_3 solutions with aqueous buffer conditions, where Gly-Pro behaved completely inert against Li^+ effects [36].

Table 1. Change of the *cis* content in a 2.3-mM solution of the peptide Suc-Ala-Ala-Pro-Phe-pNA **1** in deuterated acetonitrile (ACN-d_3) at various LiCl concentrations

LiCl[a]	Molar excess[b]	*Cis* content[c]
0	0	11
2.5	1	12
5.0	2	46
10.0	5	62
25.0	10	66

[a]In millimolars.
[b]Refer to peptide concentration.
[c]In percent.

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Table 2. Population of the *cis* and *trans* forms of the blocked dimers Ac-Ala-Pro-NHEt **2**[a] and Ac-Gly-Pro-NHEt **3**[a] in deuterated TFE (TFE-d_3) and in a 300-mM LiCl/TFE-d_3 solution at 300 K

Peptide	Medium	*Cis*[b]	*Trans*[b]
2	TFE-d_3	14	86
	LiCl/TFE-d_3	50	50
3	TFE-d_3	11	89
	LiCl/TFE-d_3	11	89

[a]5 mM.
[b]In percent.

For dipeptide **2**, the kinetic data of isomerization were also determined. The obtained values for the rate constants at 300 K for a 5-mM peptide solution in TFE-d_3 are $k_{t\rightarrow c} = 0.02\,s^{-1}$ and $k_{c\rightarrow t} = 0.12\,s^{-1}$, respectively. In a 300-mM LiCl/TFE-d_3 solution, these values are $k_{t\rightarrow c} = 0.015\,s^{-1}$ and $k_{c\rightarrow t} = 0.015\,s^{-1}$. They confirm the conclusions drawn from the data for peptide **1** that addition of LiCl decreases the isomerization rate but at a much less Li^+ sensitivity of $k_{c\rightarrow t}$.

The different results for the blocked Ala-Pro and Gly-Pro dipeptides raise the question on the influence of the amino acid preceding the proline residue. Therefore, we performed NMR measurements on 15 representatives of the peptide series Ac-Ala-Xaa-Pro-Ala-Lys-NH_2 **4**, whereby Xaa represented all basic types of proteinogenic amino acids. The same peptide series **4** was already subject in a former study by some of us, which compared the equilibrium data in water with those of a LiCl/TFE solution [35]. Here, we compare the data for bulk TFE-d_3 solvent with the LiCl/TFE-d_3 solution in order to solely extract the lithium cation effect.

Table 3 and Figure 4 illustrate the dominating *cis* isomer enhancing influence of the alanine side chain on the free enthalpies difference $\Delta\Delta G^\circ$ for the prolyl isomerization equilibrium in TFE-d_3 and in a LiCl/TFE-d_3 solution. Most other side chains show this effect on a smaller level. The increase in the *cis*/*trans* ratio is significant but generally smaller than those observed for peptides **1** and **2**. As in the case of the dipeptide Gly-Pro **3**, the corresponding glycine derivative in the peptide series **4** showed no equilibrium shift. Thus, the data for **3** and **4** allow the conclusion that the side chains of the amino acids preceding proline must provide *cis*-stabilizing interaction sites for Li^+. In the case of the aspartate side chain, a small equilibrium shift to the opposite side results, indicating Li^+-mediated stabilization of the *trans* isomer. Using an internally quenched fluorogenic probe, a series of peptide-4-nitroanilides of the type Abz-Ala-Xaa-Pro-Phe-pNA gave a similar side chain/isomer ratio relationship indicating that the N-terminal and C-terminal protecting groups do not affect the cation effects [37].

Table 3. *Cis* populations in 15 representatives of the peptide series Ac-Ala-Xaa-Pro-Ala-Lys-NH_2 **4** in deuterated TFE (TFE-d_3) and after addition of LiCl up to 470 mM and free enthalpy differences of the *cis*/*trans* interconversion determined by NMR spectroscopy at 298 K

	TFE-d_3		LiCl/TFE-d_3		
Amino acid Xaa	% *cis*	$\Delta G^\circ_{t\rightarrow c}$[a]	% *cis*	$\Delta G^\circ_{t\rightarrow c}$[a]	$\Delta\Delta G^\circ$[a]
G	8	6.1	8	6.1	0.0
A	13	4.7	33	1.7	−3.0
L	12	4.9	20	3.4	−1.5
V	13	4.7	18	3.7	−1.0
P	12	4.9	17	3.9	−1.0
S	11	5.2	15	4.3	−0.9
T	10	5.4	14	4.5	−0.9
Q	13	4.7	18	3.7	−1.0
D	13	4.7	10	5.4	0.7
E	16	4.1	26	2.6	−1.5
R	12	4.9	26	2.6	−2.3
K	12	4.9	25	2.7	−2.2
H	14	4.5	18	3.7	−0.8
F	11	5.2	18	3.7	−1.5
M	12	4.9	25	2.7	−2.2

[a]In kilojoules per mole.
[b]$\Delta\Delta G^\circ = \Delta G^\circ(\text{LiCl/TFE}) - \Delta G^\circ(\text{TFE})$.

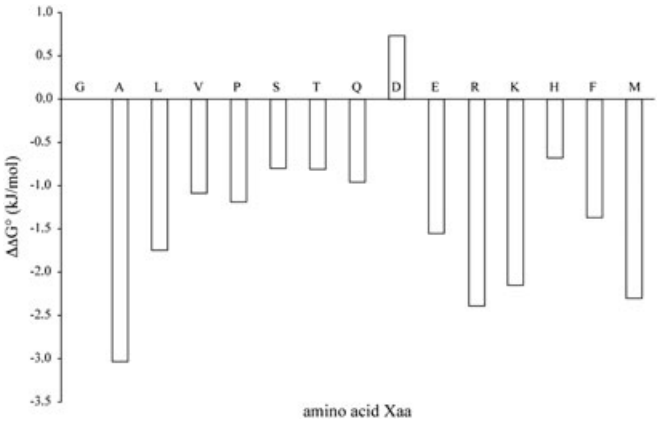

Figure 4. Differences ($\Delta\Delta G^\circ$) between the free enthalpy differences (ΔG°) of the *cis*/*trans* interconversion in pure TFE-d_3 and in a 470-mM LiCl/TFE-d_3 solution in the peptide series Ac-Ala-Xaa-Pro-Ala-Lys-NH_2 **4**.

Conclusions

^{1}H-NMR investigations on a wide variety of oligopeptides identified environmental and structural features allowing lithium cations to affect the conformation of prolyl peptide bonds in non-aqueous solution. In most cases of peptides with varying amino acids preceding proline, the lithium ions shifted the *cis*/*trans* equilibrium to the *cis* conformer. The data for the solvent acetonitrile show that the salt concentration can be considerably reduced in solvents with a lower number of nucleophilic centers, which are otherwise competitive for the lithium cation interaction. Small amounts of water detract the effect, which disappears at a water concentration of about 5%. Dipeptides might represent the minimal chain length for molecules underlying the lithium cation effect.

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