Motilin–bicelle interactions: membrane position and translational diffusion

August Andersson, Lena Mäler*

Department of Biochemistry and Biophysics, Arrhenius Laboratory, Stockholm University, 10691 Stockholm, Sweden

Received 6 February 2003; accepted 16 April 2003

First published online 16 May 2003

Edited by Thomas L. James

Abstract The interaction between the peptide hormone motilin and bicelles has been investigated by pulsed field gradient-nuclear magnetic resonance methods and by the use of paramagnetic probes. Diffusion coefficients were measured for motilin, the phospholipids with and without motilin, and for tetramethylsilane. The results show that around 90% of motilin is bound to acidic bicelles and 84% of motilin is bound to neutral bicelles. It is found that the apparent bicelle size is reduced by the presence of motilin. This cannot be explained by changes in 1,2-dihexanoyl-*sn*-glycero-3-phosphatidylcholine solubility. The use of paramagnetic agents to investigate the position of motilin shows that the turn in the N-terminus of motilin is inserted into the bicelle, while the helix most likely resides within the head-group layer.

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Key words: Motilin; Bicelle; Nuclear magnetic resonance; Pulsed field gradient; Diffusion

1. Introduction

Solution state nuclear magnetic resonance (NMR) studies of peptide–membrane interactions generally require the usage of a membrane-mimetic. The isotropic disk-shaped two-component micelles, bicelles, rotate rapidly enough for solution state NMR spectroscopy, but also have a membrane-like bilayer region [1,2]. The latter is a great advantage over micelles, which have been shown to induce curvature in interacting peptides [3] not native to the peptide. The bicelles are versatile and can be formed in a large array of sizes and compositions, making them suitable for a wide range of different types of applications.

In this study the interaction between the 22 amino acids long peptide hormone motilin and acidic bicelles [4] is investigated by solution NMR. Motilin is mainly found in the vertebrate gut where it interacts with the membrane prior to binding to the G protein-coupled motilin receptor [5]. Human motilin has the sequence FVPIFTYGELQRMQEKERNKGQ and the structure has previously been studied in different membrane-mimetic media, such as sodium dodecyl sulfate (SDS) micelles [6] and acidic bicelles [7]. The consensus structure is mainly composed of a C-terminal α-helix, comprising residues Glu9–Arg18, with a hydrophobic stretch forming a turn, involving residues Pro3–Thr6, preceding the helix.

In order to accurately understand the nature of the motilin–membrane interaction it is important to study the position of the peptide within the membrane, as well as the extent of the peptide–membrane binding. In this study we therefore conducted pulsed field gradient (PFG) experiments to measure translational diffusion for motilin in different bicellar systems. Such data can be used to evaluate the extent of the motilin–bicelle interaction and the investigations were made to study effects of charge on motilin–bicelle binding, and motilin-induced changes in bicelle composition. We also investigated the position of the peptide in acidic bicelles ($q = 0.5$, [DMPG]/([DMPC]+[DMPG]) = 0.3) by the use of two paramagnetic probes (Mn^{2+} ions and palmitoyl-stereoyl(5-doxyl)-*sn*-glycero-3-phosphocholine).

2. Materials and methods

2.1. Materials

Commercial motilin was obtained from Neosystem Labs and used as received. Phospholipids, dimyristoyl-*sn*-glycero-3-phosphatidylcholine (DMPC), dimyristoyl-*sn*-glycero-phosphatidyl-3-glycerol (DMPG), dihexanoyl-*sn*-glycero-3-phosphatidylcholine (DHPC) and palmitoyl-stereoyl(5-doxyl)-*sn*-glycero-3-phosphocholine were obtained from Avanti Lipids or Larodan. Deuterated phospholipids $^2H_{54}$-DMPC, $^2H_{54}$-DMPG and $^2H_{22}$-DHPC were used for two-dimensional total correlation spectroscopy (TOCSY) experiments and non-deuterated lipids were used in the diffusion experiments. Samples were prepared according to Andersson and Mäler [7] to produce bicelles with $q = 0.5$ and with a peptide concentration of 3 mM. Negatively charged bicelles were obtained by substituting 30% of the DMPC for DMPG. Diffusion experiments were carried out in 99% ^2H_2O.

2.2. Circular dichroism (CD) spectroscopy

CD spectra were recorded on a Jasco J-720 spectropolarimeter using a 0.05 mm quartz cuvette. Wavelengths ranging from 180 to 250 nm were measured, with a 0.2 nm step resolution and 100 nm/min speed. Spectra were averaged over eight scans. The α-helical content was established from the amplitude at 222 nm as previously described [8].

2.3. NMR spectroscopy

All spectra were recorded using a Varian Inova spectrometer, operating at 600 MHz ^1H frequency, using a triple resonance probe head. All experiments were recorded at 37°C. Translational diffusion experiments were carried out using the modified Stejskal–Tanner spin echo

*Corresponding author. Fax: (46)-8-155597.
E-mail address: lena@dbb.su.se (L. Mäler).

Abbreviations: DHPC, 1,2-dihexanoyl-*sn*-glycero-3-phosphatidylcholine; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine; DMPG, 1,2-dimyristoyl-*sn*-glycero-3-phospho-1-glycerol; PFG, pulsed field gradient; SDS, sodium dodecyl sulfate; TOCSY, total correlation spectroscopy; cmc, critical micelle concentration; TMS, tetramethylsilane

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doi:10.1016/S0014-5793(03)00514-3

Table 1
Translational diffusion constants measured for motilin and the phospholipids in acidic and neutral $q = 0.5$ bicellar solution

Sample	D_{obs} ($\times 10^{-11}$ m^2/s)				
	Motilin[a]	DMPC or DMPC/PG[b]	DHPC[b]	TMS	HDO
Motilin in buffer	21.1 ± 0.2				253 ± 3
Motilin in acidic bicelles	4.7 ± 0.1	2.6 ± 0.1	5.1 ± 0.1	3.3 ± 0.1	235 ± 1
Acidic bicelles		2.2 ± 0.1	4.9 ± 0.1	3.0 ± 0.1	231 ± 2
Motilin in neutral bicelles	6.1 ± 0.1	2.8 ± 0.2	4.9 ± 0.1	3.3 ± 0.1	230 ± 2
Neutral bicelles		2.1 ± 0.1	4.6 ± 0.1	3.0 ± 0.1	234 ± 1

[a]Based on measurements for the aromatic signals.
[b]Based on measurements for the methyl signal of the aliphatic chain of the phospholipid.

experiment with a gradient prepulse [9–11]. The PFG was used with a maximum power of 60 G/cm. Typically a total of 32 transients were recorded in the diffusion experiments to achieve a good signal to noise ratio. The PFG diffusion measurements were carried out using fixed time intervals and 30 linearly incremented power levels, from 1/30 to maximum power. Problems with non-linear gradients were accounted for according to Damberg et al. [12].

The effects of the paramagnetic probes were monitored with TOCSY spectra [13] using a mixing time of 60 ms. The TOCSY experiments were recorded in pure absorption mode using the States method [14], typically as 2048×256 complex points data sets. Data processing included zero filling to 4096 points in both dimensions. Low-power presaturation on the water frequency was used to reduce the water signal.

2.4. Analysis of diffusion measurements

The diffusion data were analyzed by fitting peak integrals to the modified Stejskal–Tanner equation according to Damberg et al. [12]. 1% ^1H_2O was added to all samples in order to estimate viscosity by measuring HDO diffusion. In samples containing mixtures of phospholipids and motilin, specific signals for each molecule were selected to calculate diffusion constants. Tetramethylsilane (TMS) was added to probe the bicelle environment [15]. Diffusion measurements for samples containing different concentrations of DHPC were carried out in order to estimate possible formation of DHPC micelles in the bicelle solution. The measurements on DHPC were carried out below and above the estimated critical micelle concentration (cmc) of ~15 mM [16].

2.5. Spin label experiments

To investigate the position of motilin within the bicelles paramagnetic agents were used. Two probes were utilized, Mn^{2+} ions (250, 500 and 750 μM) and 1 mM palmitoyl-stereoyl(5-doxyl)-*sn*-glycero-3-phosphocholine. The paramagnetic probes were added to samples containing 3 mM motilin in 300 mM bicelle solution. 2D TOCSY experiments with and without the probes were used to monitor the effects of the paramagnetic probes.

3. Results

3.1. Translational diffusion

Translational diffusion coefficients, measured from PFG experiments, were obtained for motilin in buffer, for motilin in the presence of acidic and neutral bicellar solutions, for acidic and neutral bicelle mixtures and for samples containing varying amounts of DHPC (Tables 1 and 2). The aromatic region of the motilin spectrum was used to monitor motilin diffusion but no significant differences in diffusion constants were observed if other parts of the spectrum were used. The methyl group protons of the fatty acid chains of DHPC and DMPC/DMPG, and the TMS signal were used to monitor bicelle diffusion. Small systematic deviations from exponential decay [12] were observed for lipid signals and TMS in the bicelle samples, indicating several states for the bicelles, but not for motilin or water.

The diffusion coefficients of TMS and DHPC are larger than for the long-chained phospholipids in both acidic and neutral bicellar solutions, indicating that these molecular species exist in at least two states. Significant differences are also observed in the diffusion coefficients for motilin and the long-chained phospholipids. The apparent diffusion constant for motilin is almost a factor of two larger than what is seen for DMPC/DMPG in acidic bicelles (Table 1). Another interesting result is that the diffusion of the long-chained phospholipids seems faster in the presence of motilin, indicating that motilin affects the bicelles to apparently become on average smaller.

In order to investigate the influence of the distribution of DHPC in solution and in micelles, experiments on samples containing varying amounts of DHPC were conducted. As seen in Table 2, the measured diffusion constants for DHPC decrease as expected with increasing DHPC concentrations, with a large decrease observed above the cmc. Furthermore, the diffusion coefficient for TMS dissolved in DHPC indicates that TMS induces micelle formation, as the DHPC diffusion becomes slower in the presence of TMS than without TMS.

3.2. Calculating the amount of motilin and DHPC bound to the bicelles

If a two-state exchange process is assumed between bicelle-bound peptide and peptide in solution, it is possible to calculate the amount that is bound by measuring the translational

Table 2
Measured translational diffusion constants for DHPC in solution

Sample	D_{obs} ($\times 10^{-11}$ m^2/s)		
	DHPC[a]	TMS	HDO
5 mM DHPC	56.8 ± 0.7		261 ± 3
10 mM DHPC	54.0 ± 0.4		266 ± 3
20 mM DHPC	42.1 ± 0.2		
20 mM DHPC+0.5 mM TMS	39.5 ± 0.1	36.4 ± 0.1	278 ± 4
40 mM DHPC+0.5 mM TMS	28.8 ± 0.1	22.1 ± 0.1	294 ± 9

[a]Based on measurements for the methyl signal of the aliphatic chain of the phospholipid.

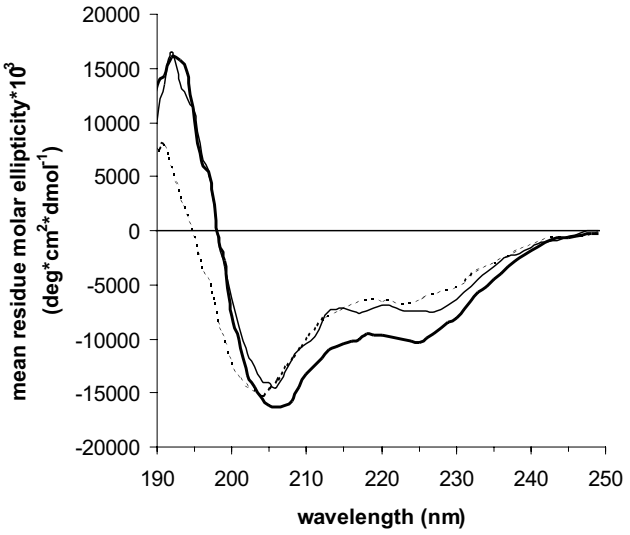

Fig. 1. CD spectra of motilin in buffer (dashed line), DMPC/DHPC bicelles ($q = 0.5$) (thin solid line), and DMPC,DMPG/DHPC bicelles ($q = 0.5$, [DMPG]/([DMPC]+[DMPG]) = 0.3) (thick solid line) at 25°C.

diffusion constants for the different molecular species [17]. By using the diffusion rates of the long-chained phospholipids as indicators for bicelle diffusion and by measuring the diffusion of the free peptide and DHPC, the populations of free and bicelle-bound molecules can be estimated from

$$xD_{\text{bound}} + (1-x)D_{\text{free}}\left(\frac{D_{\text{H}_2\text{O, complex}}}{D_{\text{H}_2\text{O, free}}}\right) = D_{\text{complex}} \quad (1)$$

where x is the amount of molecules bound to the bicelles, D_{bound} is the diffusion constant for DMPC/DMPG, D_{free} is the diffusion constant for the free molecule, i.e. DHPC or motilin, and D_{complex} is the diffusion constant for the molecule in the presence of bicelles. The diffusion coefficients for water, $D_{\text{H}_2\text{O}}$, are introduced to account for differences in viscosity in the different solutions. Calculations were carried out for motilin in the different bicelle samples (Table 3) and they indicate that at least 90% of the peptide is bound to acidic bicelles, whereas the same figure for neutral bicelles is 84%.

The calculations for DHPC are a bit more complex since the diffusion rates vary strongly with the concentration around the cmc. To correctly estimate the amount of bound DHPC in the bicelle samples one has to consider the distribution of DHPC between free, micelle-bound, and bicelle-bound forms. In order to estimate the amount of free DHPC, a simultaneous fit of the diffusion coefficients obtained from the DHPC concentration series and the diffusion coefficients measured for DHPC in the bicelle samples was performed. This yielded an estimate of the amount of free DHPC of ~5% (Table 3) in all bicelle samples, which corresponds to 10 mM.

Table 3
Fraction of bicelle-bound motilin and DHPC in bicelles

Sample	Motilin (% bound)	DHPC (% bound)
Motilin in acidic bicelles	90	94
Acidic bicelles	84	95
Motilin in neutral bicelles		94
Neutral bicelles		95

3.3. CD spectroscopy

CD spectra were recorded for motilin in buffer, neutral and acidic bicellar solutions to investigate the effect of charge on secondary structure (Fig. 1). The CD spectra show that there appear to be subtle differences in the induced secondary structure of motilin in neutral (29% α-helix) and acidic bicelles (38% α-helix). A substantial amount of α-helix is, however, also present for motilin in buffer. These results show that motilin interacts with bicelles in a similar way as it does with neutral and charged phospholipid vesicles [18], indicating that bicelles indeed provide a realistic membrane environment for the peptide.

3.4. Motilin–bicelle positioning studies

The position of motilin with respect to the bicelles was investigated by observing the effects on H^N-H^α cross-peaks in TOCSY spectra after the addition of paramagnetic probes (Fig. 2). Mn^{2+} ions were added to solutions of motilin with and without bicelles and the effect of the paramagnetic ions were much weaker without bicelles. Clear peak broadening effects on the peptide signals were observed in bicellar solution at 250 μM Mn^{2+}, while the effects of 1.5 mM Mn^{2+} on

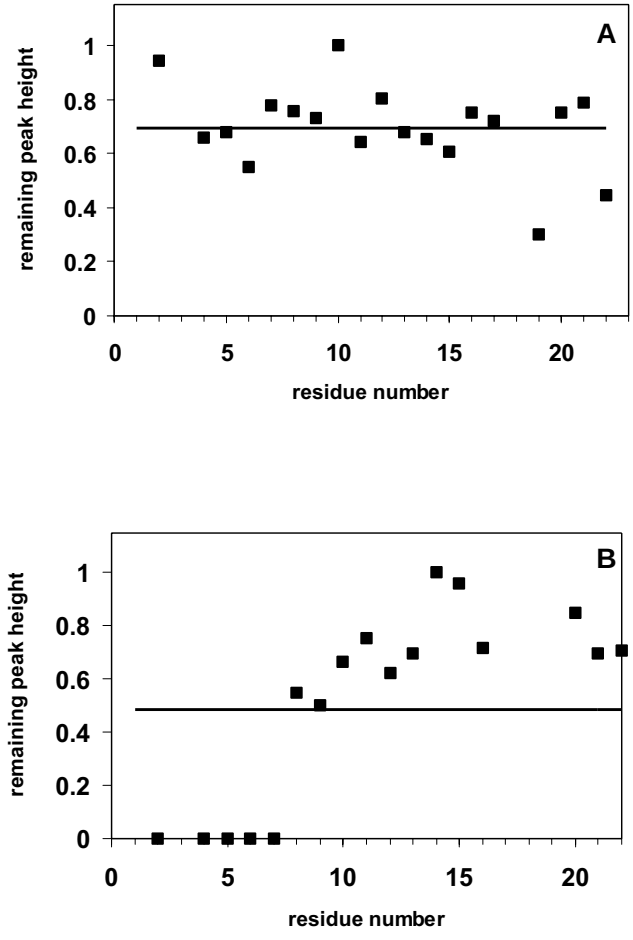

Fig. 2. The relative effects of paramagnetic probes on H^N-H^α TOCSY cross-peaks for motilin in acidic bicelles ($q = 0.5$, [DMPG]/([DMPC]+[DMPG]) = 0.3) are displayed. The values are normalized to give the least affected peak a value of 1. Overlapping peaks and the presence of Pro explain the absence of some residues. The solid lines indicate the average effect on motilin. A: The effect of 250 μM Mn^{2+} on peak height. B: The effect of 1 mM palmitoyl-stereoyl(5-doxyl)-*sn*-glycero-3-phosphocholine on peak height.

motilin in buffer were minimal. This might be explained by electrostatic attractions between the Mn^{2+} ions and the negatively charged bicelles. Mn^{2+} was seen to have no effect on signals from the aliphatic side chains of the phospholipids. The effect of 250 μM Mn^{2+} on the H^N-H^α cross-peaks in the TOCSY spectrum shows that Asn19 and Gln22 were the most affected residues (Fig. 2A). At higher concentrations a more general broadening effect was seen.

Significant effects of 1 mM 5-doxyl-labeled phospholipid were observed on the peptide resonances. The H^N-H^α cross-peaks belonging to residues Phe1–Tyr7, corresponding to the N-terminal turn region, completely vanish (Fig. 2B) while leaving the C-terminal residues more or less unaffected.

4. Discussion

Diffusion experiments were performed to investigate the amount of bicelle-bound and free motilin in the different bicelle solutions. The results for the bicelles themselves show that the diffusion in $q = 0.5$ bicelles ($D_{obs} = 3.0–3.3 \times 10^{-11}$ m^2/s) is around twice as fast as what has previously been seen in $q = 3.2$ isotropic bicelles [15] (1.4×10^{-11} m^2/s). This is reasonable considering the differences in size, although a direct comparison is difficult due to the increasing influence of bicelle shape with increasing q value.

The amount of free DHPC in solution might affect the observed binding coefficients for motilin to both acidic and neutral bicelles, depending on whether it is above or below the cmc. To correctly account for this we measured separately the diffusion constants for DHPC at different concentrations, and the amount of free DHPC in the bicellar solutions was estimated from these data (Table 3). The calculated amount of free DHPC in all bicelle solutions is approximately 10 mM, which is below the cmc [16]. This is in agreement with what has previously been found, although under different experimental conditions [1], and shows that the DHPC solubility is not influenced by bicelle charge, or the addition of motilin.

Turning to the results for motilin, the diffusion experiments show that a large amount of peptide is bound to both the acidic (90%) and the neutral bicelles (84%), although motilin seems to be somewhat more attracted to negatively charged surfaces. Interestingly, much slower overall dynamics and somewhat higher order parameters were found for motilin in acidic bicelles than what was observed in neutral bicelles [7] and clearly, these large differences in dynamics data cannot entirely be explained by different amounts of bicelle-bound peptide. Instead it is likely that motilin interacts differently with the neutral bicelle, in which the peptide is more flexible. In addition, CD shows that there are differences in the induced secondary structure of motilin in neutral and acidic bicelles (Fig. 1). The combined results show that there are dynamic and structural differences for motilin bound to acidic and neutral bicelles, which cannot be explained by large differences in affinity for the two bicellar systems.

Another interesting observation in the diffusion experiments was that motilin seems to reduce the apparent bicelle size. The diffusion of the bicelles is faster when motilin is present. This can clearly not be explained by a change in DHPC solubility, since all bicelle samples were shown to contain the same amount of free DHPC. However, it might be explained if motilin acted as a surfactant molecule, i.e. bound to the bicelle rim, to in effect increase the amount of detergent molecules available for the formation of the bicelle rim, which would theoretically lead to the formation of smaller bicelles. However, this is in contrast to the somewhat weaker interaction between motilin and neutral bicelles suggested both by the present diffusion measurements and CD results and by previous relaxation measurements [7] and investigations in neutral and charged vesicles [18]. The faster diffusion can, however, also be explained by motilin influencing the shape of the bicelle. Finally, a change in distribution of bicelle size upon motilin binding might also influence the DMPC diffusion constant. Considerations concerning the bicelle size distribution can be made in analogy with Håkansson et al. [19], and a larger standard deviation in the distribution is found to give a smaller apparent diffusion coefficient. The present data would suggest that motilin reduces the bicelle size distribution, and from a physical perspective this would imply that motilin stabilizes the bicelle structure. The presence of distributions in bicelle size has previously been discussed by Arnold et al. [20] where it was shown that indeed a range of bicelle sizes is seen. Although it is not clear what is causing the bicelles to apparently become on average smaller, it is interesting that motilin alters the bicelles, an effect most likely related to a change in the shape or distribution of bicelle size.

There are several methods for investigating the position of a peptide relative to a micelle or bicelle, such as amide exchange, observation of peptide-phospholipid nuclear Overhauser effects [21], studies in ordered bicellar systems [22], and the use of paramagnetic probes inserted at different positions within the bicelle [23]. We have chosen to use the latter, and Mn^{2+} ions were used to investigate the acidic bicelle surface, or head-group region, and a phospholipid labeled with a doxyl group in the fifth position in the fatty acid chain was used to probe the bicelle interior. The results, mapped on the motilin structure in acidic bicelles, are shown in Fig. 3. The N-terminal residues of motilin, including a hydrophobic turn in the structure [7], clearly insert into the bicelle interior, as the cross-peaks from residues in this region are greatly re-

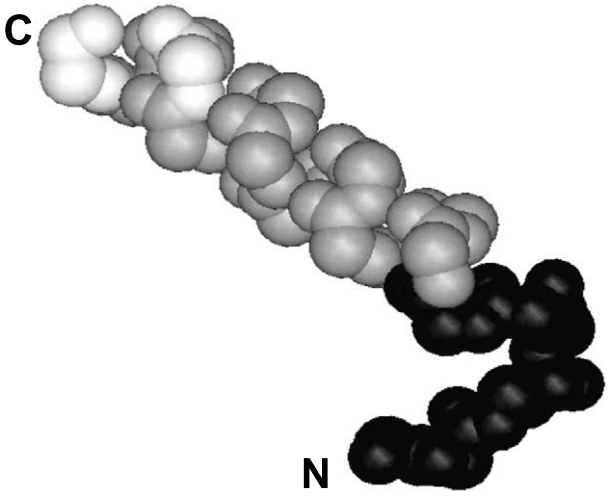

Fig. 3. The effects of paramagnetic probes on H^N-H^α TOCSY cross-peaks mapped onto the motilin backbone structure in acidic bicelles. Black-colored residues are significantly affected by the 5-doxyl spin label and white-colored residues are affected by the Mn^{2+} ions. Gray color indicates that the residues are not affected significantly by either of the paramagnetic probes. The coordinates were obtained from the PDB (accession code 1LBJ).

duced, or even disappear at low concentrations of the 5-doxyl-labeled phospholipid. The combined results from the two probes suggest that the helix in motilin resides close to the surface of the bicelle while the N-terminus inserts into the bicelle interior. This fits very well with the distribution of hydrophobic residues seen in the structure of motilin in acidic bicelles [7]. The N-terminal part has been reported to be involved in the interaction with the receptor [24] and is shown here to be important in the membrane interaction. In similar investigations of motilin in SDS micelles, the effects of the probes were smaller [6], with signals still remaining at higher concentrations. Thus, there appear to be subtle differences between the position of motilin in SDS micelles and that in acidic bicelles, which may be important in the understanding of how motilin interacts with a real biological membrane.

Acknowledgements: We thank Dr Jüri Jarvet for help with translational diffusion measurements and Charlotta Damberg at the Swedish NMR Center, Gothenburg for spectrometer help. This work was supported by the Swedish Research Council.

References

[1] Glover, K.J., Whiles, J.A., Wu, G., Yu, N.-J., Deems, R., Struppe, J.O., Stark, R.E., Komives, E.A. and Vold, R.R. (2001) Biophys. J. 81, 2163–2171.
[2] Luchette, P.A., Vetman, T.N., Prosser, R.S., Hancock, R.E.W., Nieh, M.P., Glinka, C.J., Krueger, S. and Katsaras, J. (2001) Biochim. Biophys. Acta 1513, 83–94.
[3] Chou, J.J., Kaufman, J.D., Stahl, S.J., Wingfield, P.T. and Bax, A. (2002) J. Am. Chem. Soc. 124, 2450–2451.
[4] Struppe, J., Whiles, J.A. and Vold, R.R. (2000) Biophys. J. 78, 281–289.
[5] Itoh, Z. (1997) Peptides 18, 593–608.
[6] Jarvet, J., Zdunek, J., Damberg, P. and Gräslund, A. (1997) Biochemistry 36, 8153–8163.
[7] Andersson, A. and Mäler, L. (2002) J. Biomol. NMR 24, 103–112.
[8] Greenfield, N. and Fasman, G.D. (1969) Biochemistry 8, 4108–4116.
[9] von Meerwall, E. and Kamat, M. (1989) J. Magn. Reson. 83, 309–323.
[10] Callaghan, P.T., Komlosh, M.E. and Nydén, M. (1998) J. Magn. Reson. 133, 177–182.
[11] Stejskal, E.O. and Tanner, J.E. (1965) J. Phys. Chem. 42, 288–292.
[12] Damberg, P., Jarvet, J. and Gräslund, A. (2001) J. Magn. Reson. 148, 343–348.
[13] Braunschweiler, L. and Ernst, R.R. (1985) J. Magn. Reson. 65, 355–360.
[14] States, D.J., Haberkorn, R.A. and Ruben, D.J. (1982) J. Magn. Reson. 48, 286–292.
[15] Gaemers, S. and Bax, A. (2001) J. Am. Chem. Soc. 123, 12343–12346.
[16] Israelachvili, J. (1991) Intermolecular and Surface Forces. Academic Press, San Diego, CA.
[17] Lindman, B., Puyal, M.C., Kamenka, N., Rymdén, R. and Stilbs, P. (1984) J. Phys. Chem. 88, 5048–5057.
[18] Backlund, B.-M., Wikander, G., Peeters, T.L. and Gräslund, A. (1994) Biochim. Biophys. Acta 1190, 337–344.
[19] Håkansson, B., Nydén, M. and Söderman, O. (2000) Colloid Polym. Sci. 278, 399–405.
[20] Arnold, A., Labrot, T., Oda, R. and Dufourc, E.J. (2002) Biophys. J. 83, 2667–2680.
[21] Glover, K.J., Whiles, J.A., Vold, R.R. and Melacini, G. (2002) J. Biomol. NMR 22, 57–64.
[22] Whiles, J.A., Brasseur, R., Glover, K.J., Melacini, G., Komives, E.A. and Vold, R.R. (2001) Biophys. J. 80, 280–293.
[23] Papavoine, C.H.M., Konings, R.N.H. and Hilbers, C.W. (1994) Biochemistry 33, 12990–12997.
[24] Boulanger, Y., Khiat, A., Chen, Y., Gagnon, D., Poitras, P. and St-Pierre, S. (1995) Int. J. Pept. Protein Res. 46, 527–534.