The position of the cell penetrating peptide penetratin in SDS micelles determined by NMR

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Abstract Penetratin is a 16 residue peptide, RQI KIWFQ NRRMK WKK-amide, with the ability to penetrate cell membranes and a sequence taken from the homeodomain of the *Drosophila* Antennapedia transcription factor. 600 MHz ^{1}H-nuclear magnetic resonance has been used to study the structure and location of penetratin interacting with a sodium dodecyl sulphate micelle. The positioning of penetratin in the micelle was studied by adding paramagnetic probes (Mn^{2+} ions, 5-doxyl and 12-doxyl stearic acid) to the solvent. The results show that the peptide is a straight helix positioned with its C-terminus deep inside the micelle and its N-terminus near the surface of the micelle. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Penetratin; Homeodomain; Nuclear magnetic resonance; Sodium dodecyl sulfate micelle

1. Introduction

The 60 amino acid long DNA binding domain (homeodomain) of the *Drosophila* Antennapedia transcription factor translocates through biological membranes. A 16 amino acid long peptide, corresponding to residues 43–58 of the homeodomain has the same translocating ability as the whole protein [1,2]. This peptide, Antp(43–58), is named penetratin. The amino acid sequence of penetratin is $R_{43}QL_{45}$ $KIWFQ_{50}$ $NRRMK_{55}$ WKK_{58}, where the numbering of penetratin is from the homeodomain protein. The penetratin peptide has the ability to carry quite big cargoes, such as oligonucleotides, proteins or other peptides, through the biological membranes [3]. The translocation process does not seem to require a receptor and the detailed mechanism is still not understood.

A structural study of penetratin with biotin attached to its N-terminus in a membrane-like environment with negatively charged sodium dodecyl sulphate (SDS) micelles has been conducted previously [4]. It was shown that penetratin interacts with the SDS micelle and adopts an α-helical structure in the micelle environment. Another study using a solvent with negatively charged phospholipid vesicles suggested a similar structure for penetratin without the biotin group [5]. When changing the two tryptophans (residues 48 and 56) of penetratin it was shown that the translocating properties of penetratin were lost [6].

The present study concerns penetratin-NH_2, amidated in its C-terminus, but without biotin in its N-terminus. In SDS micelles penetratin-NH_2 also assumes an α-helical secondary structure, shown here by circular dichroism and nuclear magnetic resonance (NMR) experiments. Using various paramagnetic probes we have determined the position of the peptide relative to the surface and interior of the SDS micelle. The SDS micelle may be considered as a simple mimic of the amphiphilic environment of a phospholipid bilayer. Although the micelle dimensions are comparable with the peptide, which may influence the interaction between the two, the information about positioning should be useful for understanding the mechanism of translocation through membranes for this type of transport peptides.

2. Materials and methods

The penetratin peptide was purchased from Neosystem Lab., Strasbourg. Deuterated SDS was purchased from MSC Isotopes, Canada. The 5- and 12-doxyl stearic acids were from Sigma and the $MnCl_2$ from Merck, Darmstadt. The methanol-d_4, used to dissolve the stearic acids, was purchased from Merck.

2.1. NMR spectroscopy

NMR samples were prepared by dissolving the peptide powder at 2 mM concentration in 300 mM deuterated SDS solution in H_2O/D_2O. Under the conditions used, SDS forms stable micelles with an approximate number of 60 SDS molecules/micelle [7]. The H_2O/D_2O ratio was 90/10. The pH was set to pH 3.4 by adding small amounts of HCl. The sample volume was 600 μl in a 5 mm sample tube.

The NMR spectra were collected using a Varian spectrometer (Varian Unity-600) with a 600 MHz proton frequency in a phase sensitive mode. A triple resonance probe was used. The spectral width was 8000 Hz. The chemical shifts were referenced to 3-trimethylsilyl-d_4-propionic acid (TSPA). Spectra were processed using the vnmr program on a Sun sparc5 work station. All spectra were collected at a temperature of 45°C.

One-dimensional spectra were recorded achieving water suppression by presaturation with 64 transients and 32K data points. Before the Fourier transformation the FID was zero-filled with 32K points. Two-dimensional nuclear Overhauser effect NMR spectroscopy (2D-NOESY) [8] spectra were recorded with a mixing time of 300 ms. The spectra were collected with 256×2048 data points. The number of transients was 32. Before Fourier transformation the data were zero-filled with 2048 points. Two-dimensional total correlated NMR spectroscopy (2D-TOCSY) [9] spectra were recorded with mixing times, t_m, of 30, 60 and 80 ms. The spectra were collected with 256×2048 data points. The number of transients was eight. Before Fourier transformation, the data was zero-filled with 2048 points.

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Abbreviations: SDS, sodium dodecyl sulphate; TSPA, 3-trimethylsilyl-d_4-propionic acid; NOESY, nuclear Overhauser effect NMR spectroscopy; TOCSY, total correlated NMR spectroscopy

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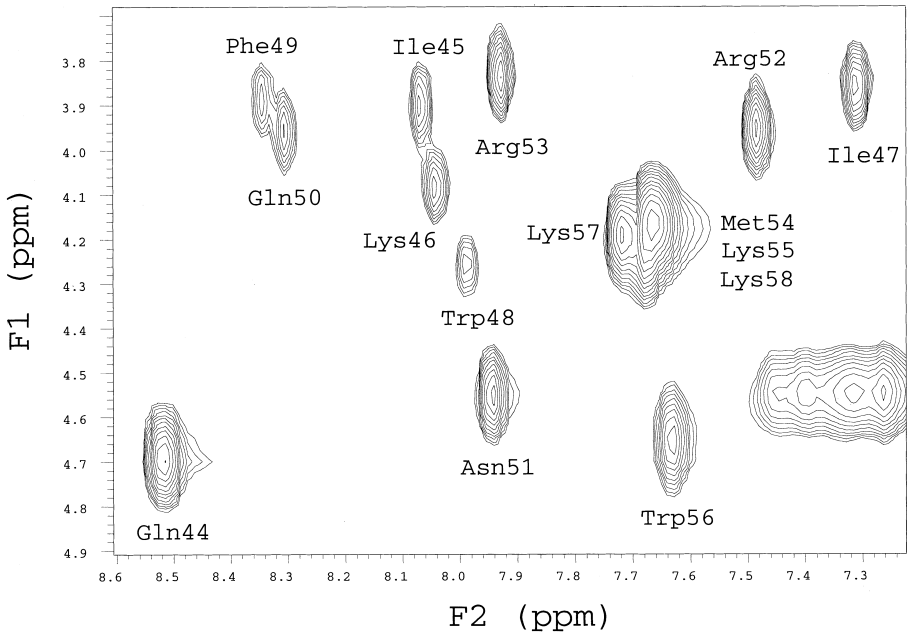

Fig. 1. Partial 600 MHz 2D-NMR TOCSY spectrum, showing the fingerprint region, i.e. H^{α}–H^{N} region, of 2 mM penetratin-NH_2 in 300 mM SDS at 45°C and pH 3.4. The crosspeaks are indicated with the assignments.

2.2. Spin label experiments

The NMR samples were prepared by dissolving penetratin at 2 mM concentration in 300 mM deuterated SDS solution in H_2O/D_2O. Assuming an SDS micelle aggregation number of 60 [7] this corresponds to a micelle concentration of 5 mM. The H_2O/D_2O ratio was 90/10. The 5- and 12-doxyl stearic acids were solubilised in methanol-d_4 and then added to the samples to obtain different concentrations, corresponding to one, two and four spin labels per micelle. The pH was set to pH 3.4 in all experiments.

2.3. Mn^{2+} experiments

The sample was prepared by dissolving penetratin at 2 mM concentration in 300 mM deuterated SDS solution in H_2O/D_2O (ratio 90/10). The $MnCl_2$ was dissolved in H_2O before it was added to the sample. Experiments were performed with different concentrations of $MnCl_2$, from 500 μM to 1.5 mM. The pH was set to pH 3.4 and the temperature was 45°C.

3. Results

3.1. NMR assignments

Fig. 1 shows the fingerprint region of penetratin, i.e. the H^{α}–H^{N} region, from the 2D-TOCSY NMR spectrum, in SDS at 45°C. Assignments were made using standard methods, i.e. 2D-TOCSY and 2D-NOESY. Most crosspeaks are resolved in the fingerprint region but there is an overlap of residues Met^{54}, Lys^{55} and Lys^{58}. The Met^{54} resonances can be assigned by using other parts of the spectra, such as H^{N}–H^{β} and H^{α}–H^{β} crosspeaks. The chemical shift for the H^{α} proton of Phe^{49} is 3.90 ppm and in comparison with the random coil value 4.66 ppm this secondary chemical shift is very large. It is probably due to ring current interactions from the neighbouring residue Trp^{48}. The assignments are presented in Table 1.

3.2. Secondary chemical shifts

The secondary chemical shift ($\Delta\delta$) was calculated as the difference between the random coil chemical shift (δ_{RC}) and the measured ($\delta_{measured}$) as $\Delta\delta = \delta_{RC} - \delta_{measured}$. The random coil value is defined as the chemical shift for a residue, X, in the peptide Gly-Gly-X-Ala [10]. To evaluate the secondary chemical shifts of the H^{α} proton resonances, the average of

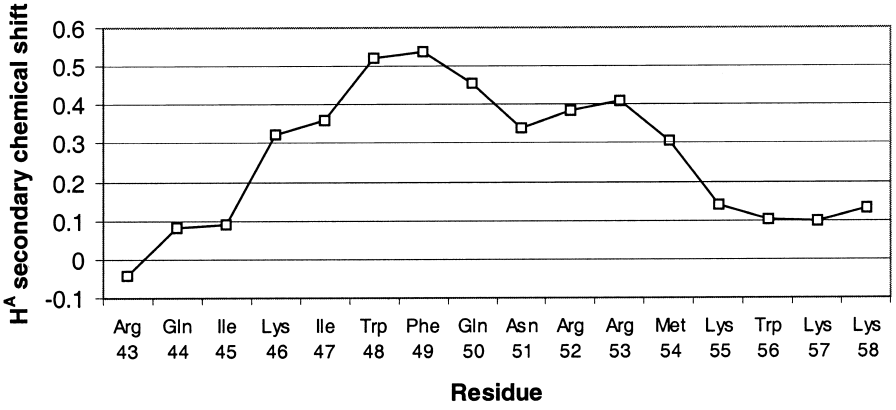

Fig. 2. Secondary chemical shift for H^{α} protons of penetratin-NH_2 in SDS micelles; conditions as described in the legend of Fig. 1. The secondary shifts are calculated as a mean value over three residues [shift($res^{i}-1$)+shift(res^{i})+shift($res^{i}+1$)]/3.

Table 1
Assignment of penetratin-NH_2 resonances (300 mM SDS, pH 3.4, 45°C)

Residue[a]	H^N	H^α	H^β	H(other)
$Arg_{43(1)}$		4.13	2.1	3.19
$Gln_{44(2)}$	8.52	4.70	2.02	2.36/2.16
$Ile_{45(3)}$	8.07	3.90	1.99	1.02
$Lys_{46(4)}$	8.05	4.09	1.92	
$Ile_{47(5)}$	7.31	3.86	1.96	0.92
$Trp_{48(6)}$	7.99	4.26	3.46/3.35	
$Phe_{49(7)}$	8.35	3.90	3.24	
$Gln_{50(8)}$	8.30	3.96	2.22	2.64/2.56/2.46
$Asn_{51(9)}$	7.94	4.56	2.73	
$Arg_{52(10)}$	7.49	3.96	1.45	
$Arg_{53(11)}$	7.93	3.84	1.57	3.08
$Met_{54(12)}$	7.68	4.25	2.56/2.50	
$Lys_{55(13)}$	7.67			
$Trp_{56(14)}$	7.63	4.66	3.32/3.22	
$Lys_{57(15)}$	7.72	4.19	1.77	1.75/1.40
$Lys_{58(16)}$	7.67			

Chemical shifts in ppm relative to TSPA.
[a]The residue numbering is taken from the Antennapedia homeodomain. Original penetratin numbering in parentheses.

the secondary chemical shift ($\Delta\delta$) for the residue itself and the ones on either side were calculated. Secondary chemical shifts of the H^α resonances in peptides or proteins carry information on secondary structure: an upfield (positive) shift of 0.4 ppm

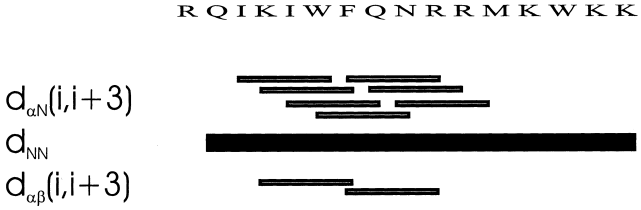

Fig. 3. Amino acid sequence of penetratin and intermediate range NOE connectivities. $d_{\alpha N}(i, i+3)$ and $d_{\alpha\beta}(i, i+3)$. Amide–amide NOE connectivities are also shown.

is characteristic for an α-helix, whereas a downfield (negative) shift of 0.4 ppm is characteristic for a β-sheet [11]. The secondary chemical shifts for the H^α proton of penetratin-NH_2 are presented in Fig. 2.

3.3. NOE connectivities

Fig. 3 shows the intermediate NOE connectivities of $d_{\alpha N}(i, i+3)$ and $d_{\alpha\beta}(i, i+3)$. A large number of $d_{\alpha N}(i, i+3)$ and $d_{\alpha\beta}(i, i+3)$ connectivities are in agreement with an α-helical structure of the peptide, as shown in earlier studies [4]. The H^N–H^N NOE connectivities are also shown.

3.4. Positioning experiments

Fig. 4 shows the 2D-TOCSY spectra from penetratin-NH_2

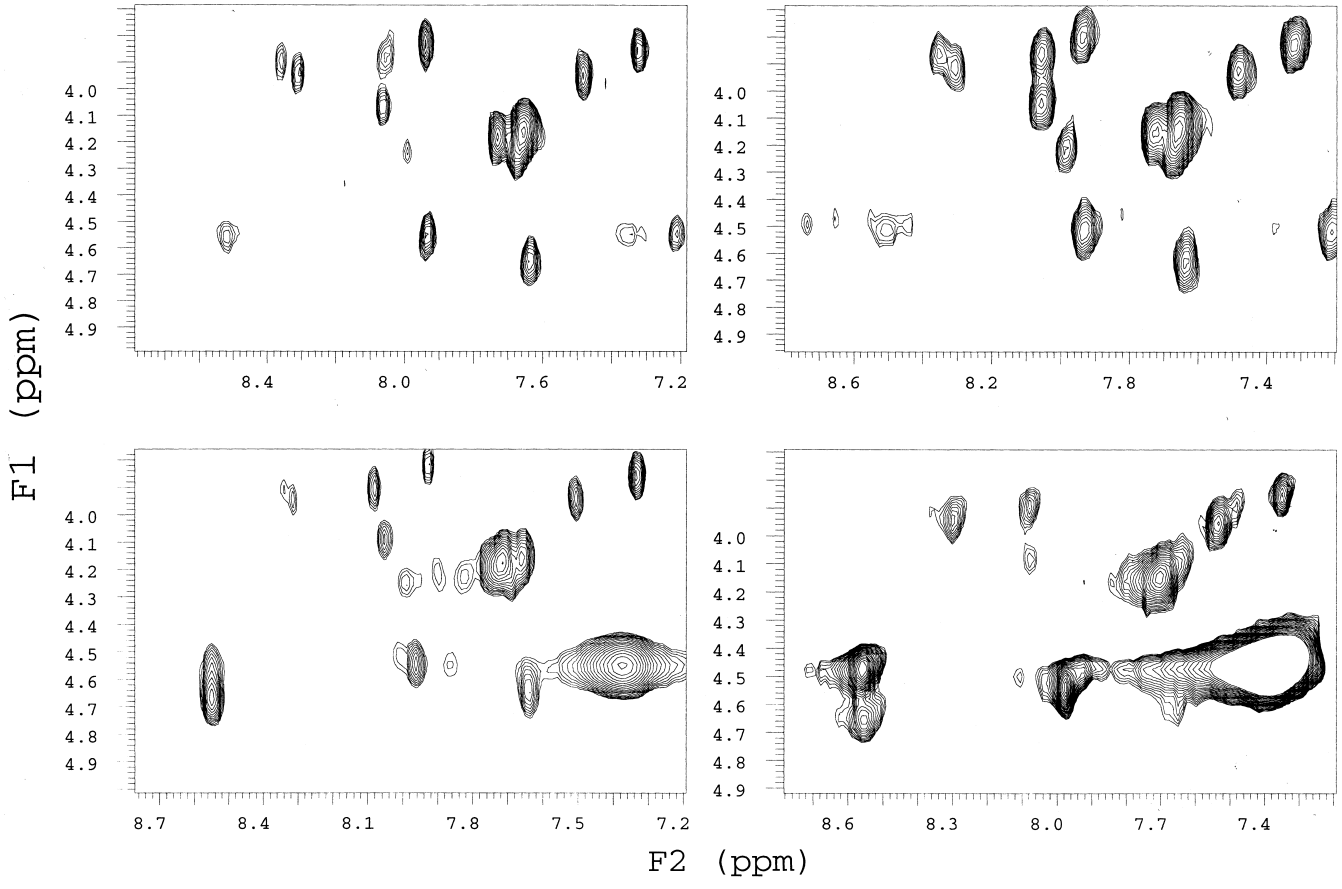

Fig. 4. The left upper panel shows the TOCSY fingerprint region of penetratin-NH_2 in SDS micelles; NMR experiment and conditions are as described in the legend of Fig. 1. The right upper panel shows the fingerprint region of penetratin-NH_2 with 500 μM $MnCl_2$ added. The left lower panel shows the fingerprint region of penetratin-NH_2 with 5-doxyl stearic acid at a concentration of one spin label per micelle added. The right lower panel shows the fingerprint region of penetratin-NH_2 with 12-doxyl stearic acid at a concentration of one spin label per micelle added. All spectra are recorded with 2 mM peptide concentration in 300 mM SDS micelles at 45°C and pH 3.4 on a 600 MHz NMR spectrometer. The two peaks at 4.48 ppm are from the solvent.

in SDS micelles without and with Mn^{2+} ions, 5-doxyl stearic acid and 12-doxyl stearic acid added. To interpret the positioning experiments we measured independently the broadening and the remaining amplitude [12] of the crosspeaks in the fingerprint region. The results are shown in Figs. 5 and 6.

The paramagnetic broadening factor is defined as:

$$\mathrm{BF} = \frac{\Delta\nu_{1/2}(\mathrm{paramag})}{\Delta\nu_{1/2}(0)}$$

where BF is the broadening factor, $\Delta\nu_{1/2}$(paramag) is the linewidth at half height for the crosspeak with paramagnetic agent present and $\Delta\nu_{1/2}(0)$ is the linewidth at half height for the crosspeak with no paramagnetic agent present. The broadening factor is measured in the H^N dimension of the crosspeaks in the fingerprint region (H^N–H^α). There is in general no significant difference if the broadening is instead measured in the H^α dimension.

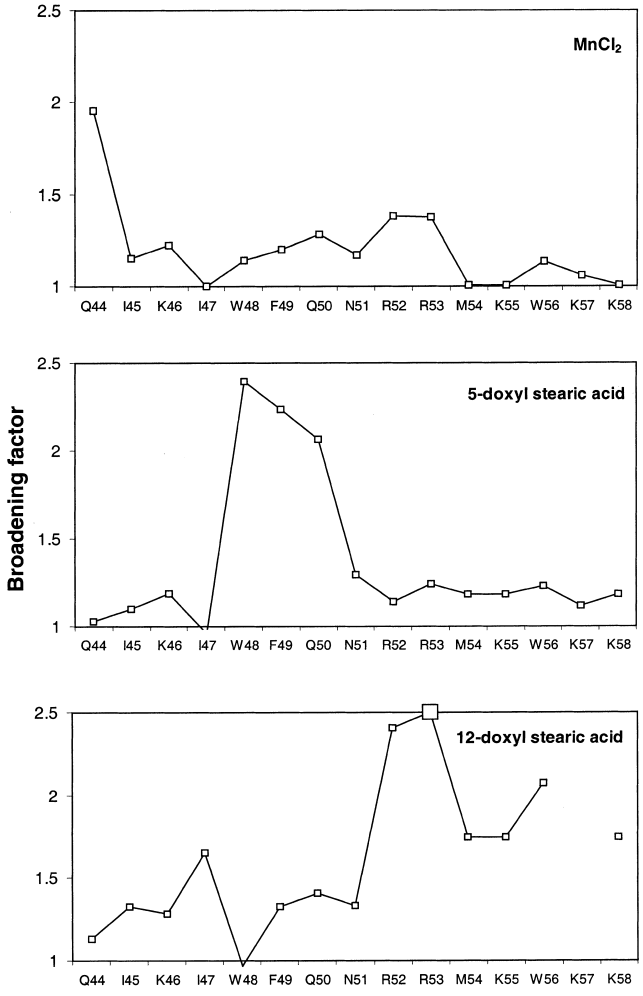

Fig. 5. The broadening factor of penetratin-NH_2 in SDS micelles; NMR experiment and conditions are as described in the legend of Fig. 1. The upper panel shows the broadening factor of the TOCSY crosspeaks of penetratin-NH_2 due to 500 μM $MnCl_2$. The middle panel shows the broadening factor of the crosspeaks of penetratin-NH_2 due to 5-doxyl stearic acid at a concentration corresponding to one spin label per micelle. The lower panel shows the broadening factor of the crosspeaks of penetratin-NH_2 due to 12-doxyl stearic acid at a concentration corresponding to one spin label per micelle.

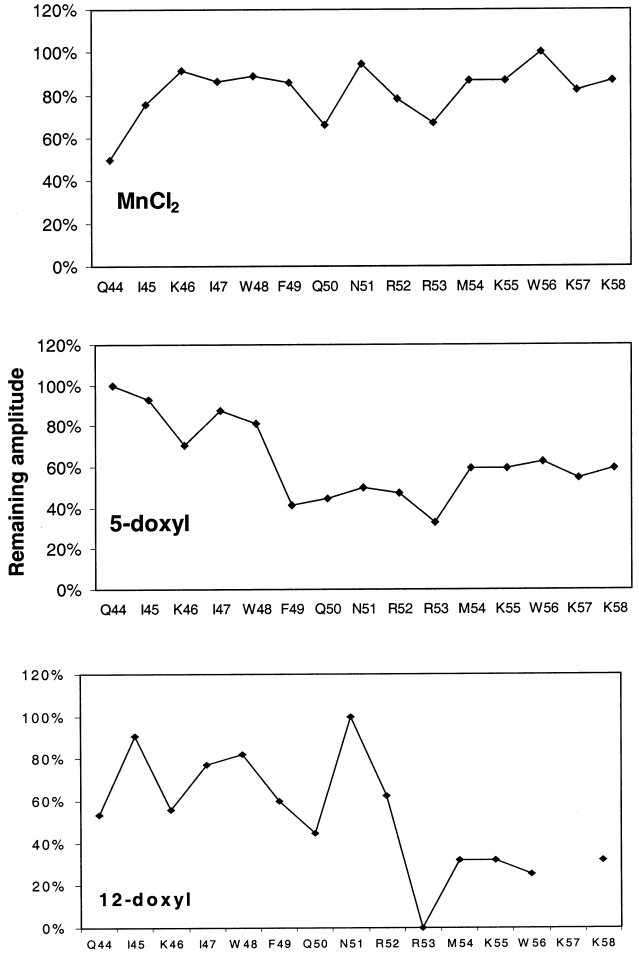

Fig. 6. The remaining amplitude of penetratin-NH_2 in SDS micelles; NMR experiment and conditions are as described in the legend of Fig. 1. The upper panel shows the remaining amplitudes of the TOCSY crosspeaks of penetratin-NH_2 due to 500 μM $MnCl_2$. The middle panel shows the remaining amplitudes of the crosspeaks of penetratin-NH_2 due to 5-doxyl stearic acid at a concentration corresponding to 1 spin label per micelle. The lower panel shows the remaining amplitudes of the crosspeaks of penetratin-NH_2 due to 12-doxyl stearic acid at a concentration corresponding to 1 spin label per micelle.

The remaining amplitude evaluated as a complement to the broadening factor is defined as:

$$\mathrm{RA} = N\frac{A(\mathrm{paramag})}{A(0)}$$

where RA is the remaining amplitude, A(paramag) is the amplitude of the crosspeak measured when the paramagnetic agent is added and $A(0)$ is the amplitude with no paramagnetic agent present. N is a normalising factor, so that the least affected crosspeak has a remaining amplitude of 100%.

The advantage of using the broadening factor instead of the remaining amplitude as a probe of interaction is that the broadening factor is a more direct measurement, closer to the collected data without requiring the normalisation. The advantage of using the remaining amplitude is that this method is not as dependent on having well-resolved crosspeaks with symmetric lineshapes. When both methods agree, the systematic errors should be the smallest.

3.5. Mn^{2+} ions

The upper right panel of Fig. 4 shows a spectrum of penetratin in SDS with Mn^{2+} ions present. It is clearly shown that one residue, Gln^{44}, is most affected and that the rest of the residues are hardly affected at all. This is also shown graphically in Figs. 5 and 6, upper diagrams. The broadening factor for Gln^{44} is much larger and the remaining amplitude lower than for the rest of the residues. In the H^{α}–$H^{sidechain}$ region (data not shown) one can clearly observe that in addition Arg^{43} is more affected then the other residues. When higher concentrations of Mn^{2+} ions are added, residues in the N-terminal part are more affected then in the C-terminal part (data not shown).

3.6. 5-Doxyl stearic acid

In the lower left panel of Fig. 4 is it shown that 5-doxyl stearic acid has the largest effect on two residues, Phe^{49} and Gln^{50}. When analysing the results in more detail (Fig. 5, middle panel), one can see that the broadening effect is large also on residue Trp^{48}. This is not in agreement with the value of the corresponding remaining amplitude (Fig. 6, middle panel). Possible reasons for this discrepancy will be discussed later.

3.7. 12-Doxyl stearic acid

In the lower right panel of Fig. 4 one can see that 12-doxyl stearic acid mostly affects two well-resolved residues, Arg^{53} and Trp^{56}, and some of the unresolved residues Met^{54}, Lys^{55}, Lys^{57} and Lys^{58}. The more detailed analysis (Fig. 5, lower panel) shows a broadening factor that is much larger for these residues than for the other residues. The broadening factor of the residue Lys^{57} is excluded because of uncertainty in measurement due to overlap with the residue Met^{54}, Lys^{55} and Lys^{58}. When 12-doxyl stearic acid is added, the crosspeak from Arg^{53} totally disappears so the broadening appears infinite. Therefore, in the lower panel of Fig. 5, the broadening factor for Arg^{53} is set to maximum of the scale for clarification. The corresponding value of the remaining amplitude is zero (Fig. 6, lower panel).

4. Discussion

The secondary chemical shifts for the H^{α} proton (Fig. 2) and the NOE connectivities (Fig. 3) indicate that the secondary structure is similar to the one determined for biotin–penetratin [4]. The N-terminal and C-terminal parts of the peptide are unstructured and the middle part is in an α-helical conformation. There is however increasing evidence that the induced secondary structure of a transport peptide is not the decisive factor for the transport property [13,14]. Instead, the positioning of the different parts of the peptide in a membrane mimicking environment, as studied here, may be more relevant [12].

The positioning of the peptide relative to the surface and interior of the SDS micelle was studied using paramagnetic probes: Mn^{2+}, 5-doxyl stearic acid and 12-doxyl stearic acid. These paramagnetic probes are expected to cause broadening of resonances from residues outside the micelle (Mn^{2+}), inside but close to the surface (5-doxyl), or deeply buried in the micelle (12-doxyl), respectively.

There should be an inverse relationship between the broadening factor and the remaining amplitude for a given set of spectra. This is also approximately the case for the Mn^{2+} and 12-doxyl stearic acid results (Figs. 5 and 6, upper and lower panels). However, this is not always the case, as is illustrated by the 5-doxyl stearic acid results (Figs. 5 and 6, middle panels). A closer inspection reveals that the broadening factor is

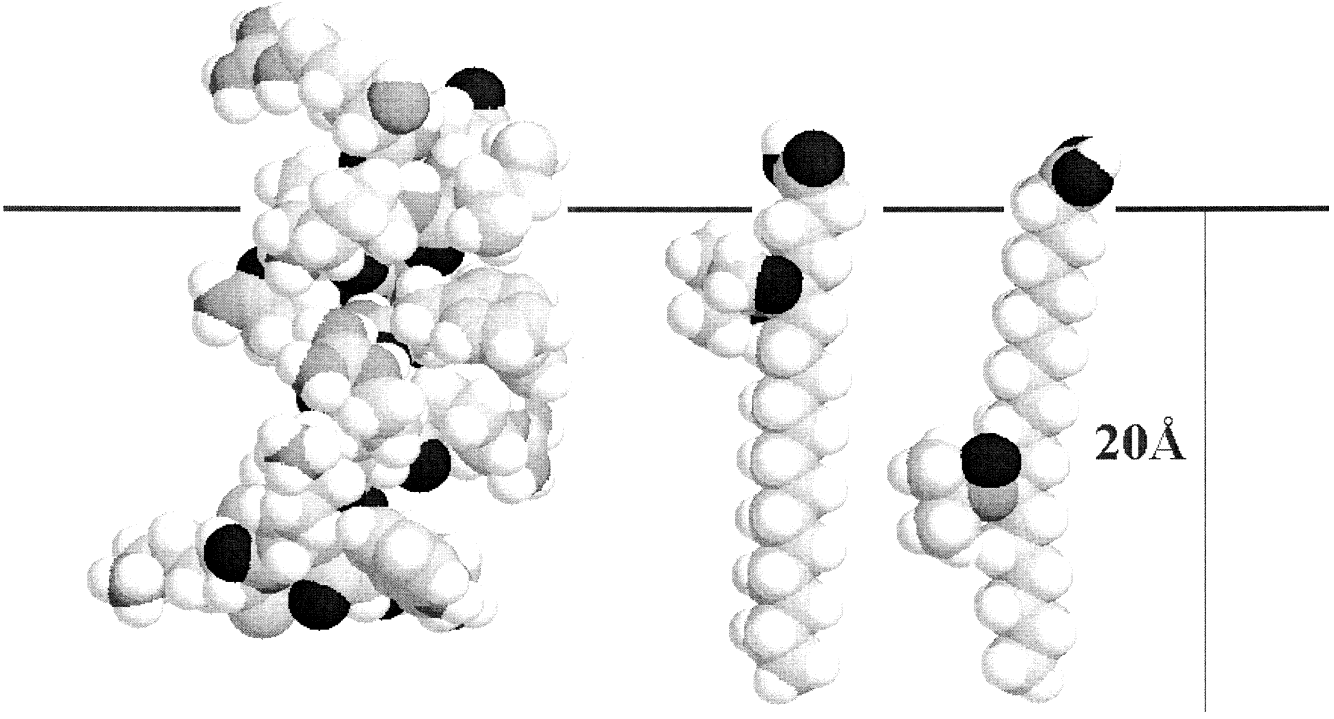

Fig. 7. Schematic picture of the positioning of penetratin in an α-helical secondary structure relative to the surface of an SDS micelle according to the paramagnetic broadening results using Mn^{2+}, 5-doxyl stearic acid and 12-doxyl stearic acid. The geometry of the fatty acids are also indicated, with their lengths of approximately 20 Å. The diameter of a typical SDS micelle should be about 35–40 Å [7].

very large for W48, while its remaining amplitude is not correspondingly small. A possible reason is found by inspecting Fig. 4, lower left panel. The crosspeak has attained an asymmetric shape, possibly due to neighbouring quite strong crosspeaks, and this may affect the broadening factor in an anomalous way. On the other hand, residues 52 and onwards show a small broadening factor, while at the same time also the remaining amplitudes are quite low. Here it is probably the normalisation of the remaining amplitude that is at fault, and the broadening factor should be more reliable. Clearly these observations, indicating likely sources of systematic errors in estimating the paramagnetic effects, suggest that one should be cautious about the detailed interpretation of such effects.

Taken together, the results from the Mn^{2+} ion experiments indicate that the penetratin peptide is located inside the SDS micelle with most of its residues not exposed to the hydrophilic Mn^{2+} ions. Two residues at the N-terminus, Arg^{43} and Gln^{44}, may be located outside the micelle. The experiment with 5-doxyl stearic acid show that the stretch (48–50) is located inside the micelle but near the micellar surface. The 12-doxyl stearic acid shows a great effect on the C-terminus of the peptide and therefore this part is believed to be hidden deep inside the micelle.

Fig. 7 shows a schematic picture of the position of penetratin-NH_2 and the two spin labelled fatty acids used, relative to the SDS micelle surface, in order to illustrate the information paramagnetic broadening experiments. The N-terminus of the peptide is located at the surface, while the middle part is located close to but inside the surface. The C-terminus is located close to the centre of the micelle. In the earlier study on the transport peptide transportan, a similar but not identical situation was observed, where most of the peptide was found to be buried in the SDS micelle, with a central segment and the C-terminus exposed at the surface [12]. The equilibrium position of a transport peptide in an SDS micelle with one or more parts anchored at the polar surface and a major part buried in the hydrophobic interior of the micelle may be an important aspect for understanding the mechanism behind its membrane transport properties.

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