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Biochimica et Biophysica Acta 1236 (1995) 259–265

BBA Biochimica et Biophysica Acta

# Solvent stabilized solution structures of galanin and galanin analogs, studied by circular dichroism spectroscopy

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Received 13 December 1994; revised 7 February 1995; accepted 15 February 1995

**Abstract**

Circular dichroism spectroscopy has been used to study how different solvents stabilize secondary structure in the neuropeptide galanin (rat), two N-terminal fragments of galanin, galanin(1–12) and galanin(1–16), and six other differently charged analogs. Among these analogs, the peptide M40, galanin(1–13)-Pro-Pro-Ala-Leu-Ala-Leu-Ala amide, is a high affinity, receptor subtype specific galanin receptor antagonist. The different solvents include sodium dodecyl sulfate (SDS) micelle solutions, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol (DOPG) vesicle solutions, 100% 1,1,1,3,3,3-hexafluoro-2-propanol (HFP) and 100% 2,2,2-trifluoroethanol (TFE). DOPC vesicles did not change the structure of the peptides as compared to aqueous solvent. The negatively charged DOPG vesicles and SDS micelles induced similar changes towards $\alpha$-helical structures in all peptides. The HFP and TFE solvents have an even stronger tendency to stabilize $\alpha$-helical conformations in these peptides. Since DOPG vesicles can be considered as a model system for negatively charged biological membranes, the solution structures observed in the presence of DOPG or SDS may be the most relevant for the in vivo situation. Correlations between the binding affinity of the peptides to hippocampal galanin receptors and their observed structures in the DOPG solvent were investigated.

*Keywords:* Galanin; Neuropeptide; Circular dichroism; Secondary structure; Solvent stabilization

## 1. Introduction

Galanin [1] is a 29 amino acid long peptide (30 in humans [2]) which has several important functions in the neuro-endocrine system [3,4]. It is a potent inhibitor of insulin release [5] and it has the ability to stimulate the release of growth hormone [6]. Galanin also acts as an inhibitor of the release of acetylcholine in hippocampus [7]. Furthermore, the peptide is believed to have a tonic role in the control of pain threshold, especially after an injury of the peripheral nervous system [8]. It also has the ability to stimulate feeding behavior upon hypothalamic injection [9]. These and other biological functions of galanin are mediated via an identified membrane-bound high-affinity receptor. The receptor is a 54 kDa glycoprotein which is coupled to a pertussis toxin-sensitive G-protein [10].

It has been shown that the N-terminus of galanin is most important for binding to the receptor and that the most crucial residue is the tryptophan in position two [11,12]. The first 15 residues are also absolutely conserved in all species (cow, pig, rat, sheep, chicken, dog and humans) where the amino acid sequences have been determined. Even a short fragment, galanin(1–12), has a good agonist activity in rat ventral hippocampus [13]. Lately, a number of putative chimeric galanin receptor antagonists have been designed (for review see [4]). These chimeric peptides contain the N-terminal part of galanin, galanin(1–13), and in the C-terminus a sequence belonging to another neuropeptide (bradykinin, substance P, spantide, neuropeptide Y) or a non-specific sequence like Pro-Pro-Ala-Leu-Ala-Leu-Ala as in peptide M40. One of these antagonists, M40, which has been shown to block galanin-induced feeding [14,15], is studied in present paper.

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*SSDI* 0005-2736(95)00056-9

To understand the action of galanin and its analogs at a molecular level, the knowledge of the three-dimensional structures involved is important. The solution structure of galanin has been determined in 100% 2,2,2-trifluoroethanol (TFE) [16]. In this solvent, galanin attains a structure consisting of two $\alpha$-helices interrupted by a bend at the only proline residue in position 13. By using circular dichroism spectroscopy we have investigated the secondary structure of rat galanin, two fragments of galanin, galanin(1–12) and galanin(1–16), and six analogs to galanin in water, 100% HFP, 100% TFE or while interacting with phospholipid vesicles or detergent micelles. From the results and the characteristics of the peptide sequences and the solvents, some conclusions are drawn about the nature of the interactions between the peptides and the solvents. An attempt to correlate these results to biological function is also made by investigating the binding affinity of the peptides to hippocampal galanin receptors.

## 2. Materials and methods

### 2.1. Materials

Sodium dodecylsulfate (SDS) was purchased from BDH Limited Poole, UK, 2,2,2-trifluoroethanol (TFE) and sodium chloride (NaCl) from E. Merck, Darmstadt, Germany, and 1,1,1,3,3,3-hexafluoro-2-propanol (HFP) from Sigma, St. Louis, USA. 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol (DOPG) was purchased from Avanti Polar Lipids, USA.

All peptides were synthesized as previously described [17]. Their sequences are summarized in Table 1.

### 2.2. Sample preparation

Stock solutions of 5.0 mg/ml of DOPC and DOPG unilamellar lipid vesicles were produced by sonication as previously described [18]. The sonicator used was a Soniprep 150 (MSE, Scientific Instruments, UK) supplemented with an exponential microtip. The diameter of the DOPC- and DOPG-vesicles has been determined to be approx. 200 Å [19].

Peptide solutions with a concentration of approx. 25 $\mu$M have been titrated with SDS and vesicle solutions, prepared from DOPC and DOPG respectively. In the titration with SDS a 10 mM phosphate buffer (pH 7.0) was used, in all other cases pure water was used. The pH of these samples was varying between 4.4 to 5. The induced conformational changes were monitored by CD-spectroscopy. Spectra of the peptides were recorded in solvents with 0, 1, 2, 5, 10 and 20 mM SDS and with 0, 0.025, 0.05, 0.1, 0.2, 0.5, 1.0 and 2.0 mg/ml of vesicle solution, as well as in 100% HFP and 100% TFE. Several peptides were also studied in a 10 mM phosphate buffer solution (pH 7) with various concentrations of NaCl (0–150 mM) and SDS (0–10 mM), as well as in a 0.1 mg/ml (0.13 mM) DOPG solvent with various concentrations of NaCl (0–150 mM).

### 2.3. Spectroscopic methods

In order to determine the concentration of the peptides, absorbance spectra were acquired on a Beckman DU-7 spectrophotometer or on a Cary 4 spectrophotometer. All peptides studied here have one tryptophan as the main contributor to absorbance in the 280 nm region. The concentration determination and the quantitative evaluation of the CD spectra are dependent on the extinction coefficient of the peptide. For all peptides, except galanin, the extinction coefficient was estimated from the sum of the molar absorbances of the aromatic residues involved [20]. The extinction coefficient for galanin dissolved in water has previously been determined to $\epsilon_{278} = 6620\ M^{-1}\ cm^{-1}$ [21], in agreement to what can be estimated from the aromatic amino acid residues.

CD-spectra were recorded at a temperature of 25° C, using a Jasco-720 spectropolarimeter and 2 mm Hellma quartz cuvettes. The parameters used were a bandwidth of

Table 1
Amino acid sequences of rat galanin, galanin fragments and analogs studied in this paper

| | 1 | | | | 5 | | | | | 10 | | | | | 15 | | | | | 20 | | | | | 25 | | | | 29 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Galanin, rat** | G | W | T | L | N | S | A | G | Y | L | L | G | P | H | A | I | D | N | H | R | S | F | S | D | K | H | G | L | T | -$NH_2$ |
| **Galanin(1-12)** | G | W | T | L | N | S | A | G | Y | L | L | G | | | | | | | | | | | | | | | | | | -$NH_2$ |
| **Galanin(1-16)** | G | W | T | L | N | S | A | G | Y | L | L | G | P | H | A | I | | | | | | | | | | | | | | -$NH_2$ |
| **M40** | G | W | T | L | N | S | A | G | Y | L | L | G | P | P | P | A | L | A | L | A | | | | | | | | | | -$NH_2$ |
| **M132** | G | W | T | L | N | S | A | G | Y | L | L | G | P | P | P | A | L | A | L | Y | | | | | | | | | | -$NH_2$ |
| **M133** | G | W | T | L | N | S | A | G | Y | L | L | G | P | P | P | A | K | A | L | Y | | | | | | | | | | -$NH_2$ |
| **M135** | G | W | T | L | N | S | A | G | Y | L | L | G | P | P | P | A | K | A | L | A | | | | | | | | | | -$NH_2$ |
| **M141** | G | W | T | L | N | S | A | G | Y | L | L | G | P | P | P | A | | | | | | | | | | | | | | -$NH_2$ |
| **M148** | G | W | T | L | N | S | A | G | Y | L | L | G | P | P | P | E | L | E | L | E | | | | | | | | | | -$NH_2$ |
| | 1 | | | | 5 | | | | | 10 | | | | | 15 | | | | | 20 | | | | | | | | | |

1 nm, a 2 s response time, a resolution of 0.5 nm and a scan speed of 10 nm/min. The acquired spectra were the average of three continuous scans, usually measured between 185 and 250 nm, and corrected with respect to baseline and for dilution. The spectra are presented in a noise reduced fashion. Using the assumption that only random coil and $\alpha$-helical secondary structures were present, the mean residue ellipticity at 222 nm was evaluated and used as a measure of the relative helicity. At this wavelength the major contribution to a measured CD-signal of a protein can be attributed to a typical random coil or an $\alpha$-helical structure, with an ellipticity of 3900 and $-38\,000$ deg · cm$^2$/dmol, respectively [22,23].

### 2.4. Binding studies

Binding studies to galanin receptors were carried out in rat ventral hippocampal membranes. Preparation of [$^{125}$I]monoiodo-Tyr$^{26}$-porcine galanin, preparation of the membranes and the displacement of $^{125}$I-porcine galanin by the studied ligands are described in detail in a previous report [24].

## 3. Results

The sequences of rat galanin, its fragments and analogs are presented in Table 1. These peptides were dissolved in different solvents and studied by CD spectroscopy. The relative $\alpha$-helical content of the peptides under varying solvent conditions was evaluated from the mean residue ellipticity at 222 nm, and the data are summarized in Table 2.

Table 2
Estimated amount of $\alpha$-helix in percent of the various peptides in the different solvents

| | $H_2O$ | DOPC | DOPG | SDS | HFP | TFE |
|---|---|---|---|---|---|---|
| Galanin, rat | 14 | 13 | 17 | 16 | 22 | 41 |
| Galanin(1–12) | 9 | 9 | 19 | 14 | 12 | 19 |
| Galanin(1–16) | 11 | 12 | 22 | 14 | 17 | 21 |
| M40 | 11 | 11 | 12 | 14 | 13 | 16 |
| M132 | 11 | 11 | 17 | 14 | 20 | 19 |
| M133 | 10 | 10 | 15 | 14 | 20 | 28 |
| M135 | 10 | 10 | 16 | 15 | 18 | 26 |
| M141 | 9 | 9 | 15 | 12 | 20 | 19 |
| M148 | 12 | 13 | 15 | 15 | 18 | 18 |

This evaluation is dependent on the extinction coefficient for the peptides. It has previously been determined for galanin [21], but are estimated for all other peptides as the sum of the molar absorbance of the aromatic amino acids involved. Quantitative comparisons between the peptides should therefore be done with some caution. The uncertainty is approx. ± 10% of the estimated percentage of $\alpha$-helix, determined from repeated measurements.

### 3.1. Solvent dependent structure stabilization

As an example of how the peptides interact with the different solvents, the CD spectra of galanin dissolved in water, 0.1 mg/ml DOPC, 0.1 mg/ml DOPG, 10 mM SDS/10 mM phosphate buffer, 100% HFP and 100% TFE are shown in Fig. 1. The concentration of DOPG vesicles was chosen so that addition of more vesicle solution did not alter the conformation according to the CD spectra. The peptide/DOPG ratio corresponds to 1:5. In most cases the conformational change occurs already at the lowest concentration of DOPG, at a peptide/DOPG concentration ratio corresponding to 1:1. To ensure that we are above the

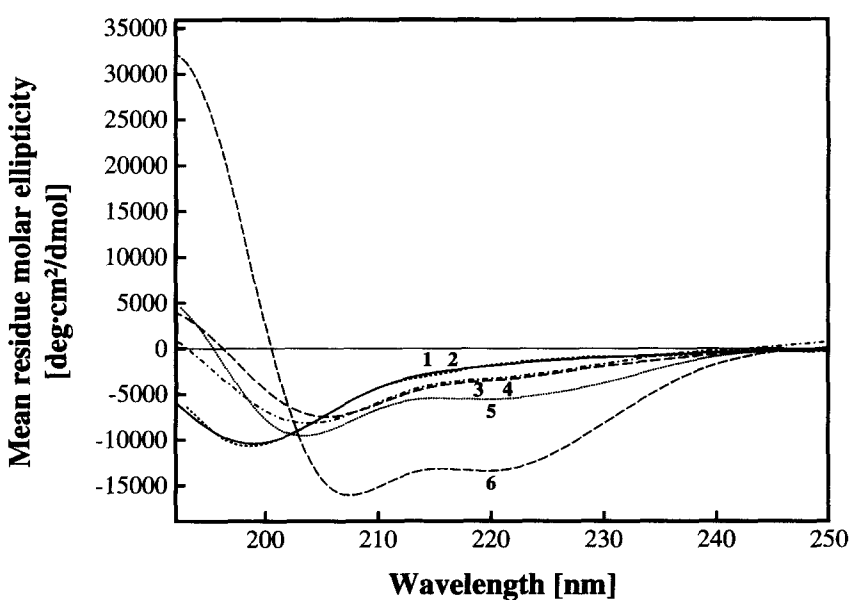


Fig. 1. CD spectra of rat galanin, dissolved in (1) water (—), (2) 0.1 mg/ml DOPC (- - -), (3) 0.1 mg/ml DOPG (-·-·-), (4) 10 mM SDS/10 mM phosphate buffer (– – –), (5) 100% HFP ( · · · ) and (6) 100% TFE (— — —), at a temperature of 25° C.

critical micellar concentration (CMC, at 8.2 mM [25]), we have used 10 mM SDS in the presented CD spectra, which corresponds to a peptide/SDS ratio of 1:400. It should however be noted that the same conformational change is induced by SDS well below the CMC (already at 1–2 mM, equivalent to a molar ratio of 1:40).

A number of observations, related to the nature of the solvent, can be derived from Fig. 1 and Table 2. It is obvious that galanin does not interact with zwitterionic DOPC-vesicles. This observation holds for all studied peptides.

Another observation on galanin as well as all the other peptides is that negatively charged DOPG vesicles and negatively charged SDS micelles both stabilize similar conformational changes. Addition of salt, up to 150 mM NaCl, to the DOPG samples induces conformational changes in the peptides towards a more random coil structure, whereas the peptides in the SDS solvent are unaffected by similar concentrations of NaCl (data not shown).

The fluorinated alcohols (HFP and TFE) have a somewhat different effect on the peptides. In most cases they induce a larger amount of $\alpha$-helix than the other solvents. TFE has the strongest tendency to induce $\alpha$-helix, which is particularly apparent in the case of galanin, but also seen in the cases of M133 and M135 (Table 2, Fig. 2).

We further observed that all the investigated peptides had identical CD spectra in 10 mM phosphate buffer (pH 7.0) and pure water (pH measured as 4.4–5).

### 3.2. Sequence-dependent structure stabilization

Additional more specific observations, connected to the specific amino acid sequence, were also made.

Table 3
Equilibrium binding affinities of galanin and its analogs as determined by displacement of 0.1–0.2 nM $^{125}$I-galanin(1–29), porcine, in rat ventral hippocampal galanin receptors, taken from [a] [10], [b] [13]

| Peptide | $K_D$ [nM] |
|---|---|
| Galanin, rat | 1.0 ± 0.1 [a] |
| Galanin(1–12) [c] | 1400 ± 100 [b] |
| Galanin(1–16) [c] | 3.1 ± 0.7 [b] |
| M40 [d] | 11.7 ± 0.4 |
| M132 | 2.8 ± 0.3 |
| M133 | 3.8 ± 0.4 |
| M135 | 43.7 ± 0.4 |
| M141 | 9.6 ± 0.2 |
| M148 | 6.3 ± 0.1 |

[c] Galanin agonist.
[d] Galanin antagonist.
$K_D$ is the concentration of peptide that causes 50% of $^{125}$I-galanin(1–29) to be displaced from the receptor.

The number of charged residues in the peptides seems to be of importance for the interaction with TFE (and to some extent HFP). This is seen in the spectra of Fig. 2, where the spectrum of M40 is compared with that of M135 as well as M132 to M133. In both pairs the only difference in the amino acid sequence is that the non-charged hydrophobic residue, L-Leu[15], close to the carboxy terminal end, is exchanged to a hydrophilic positively charged L-Lys, with a side-chain of similar size. The exchange results in a clear increase of $\alpha$-helical content of the peptides. Only minor differences in the CD-spectra and hence the secondary structure are seen in the other solvents. Introducing negatively charged amino acids in the C-terminal part, as in M148, does not give any significant differences relative to M40 and M132 (Table 2).

The stabilization effect caused by a single hydrophobic

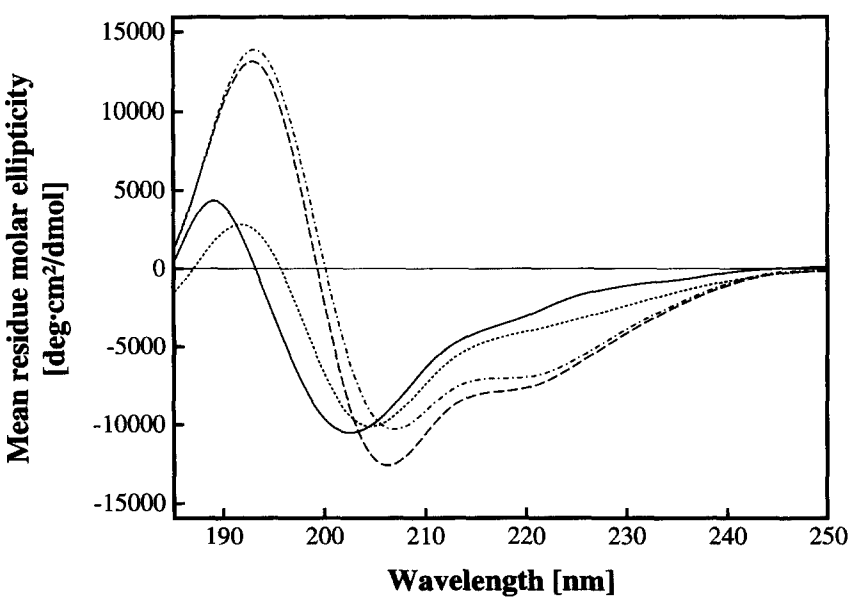


Fig. 2. CD spectra of M40 (—), M132 (- - -), M133 (-·-·-) and M135 (– – –) dissolved in 100% TFE, at a temperature of 25° C.

aromatic residue was studied by comparing the peptides M40 and M132, as well as M135 and M133, where the L-Ala at position 20 is substituted to a L-Tyr. This substitution causes a certain increase of secondary structure of the peptides in the fluorinated alcohols only (Fig. 2, Table 2).

### 3.3. Binding studies

The displacement of $^{125}$I-galanin from rat ventral hippocampal membranes by the peptides M40, M132, M133, M135, M141 and M148 was monitored and the binding affinities are summarized in Table 3, together with previously known constants.

## 4. Discussion

Since the action of galanin and its analogs is mediated via a membrane bound specific receptor, the structures that these peptides attain close to or in contact with membranes should be those which are recognized by the receptor. A phospholipid vesicle is a spherical bilayer which can be considered as a model for a biomembrane and could therefore serve as a reasonable model system for studies of the interaction between a peptide and a biomembrane. Since biological membranes are negatively charged, a possible correlation between the structure of peptides in the DOPG solvent with their binding affinity to galanin receptors has been investigated. From the studies in the SDS, HFP and TFE solvents, it is possible to draw some conclusions about the mechanisms behind solvent induced conformational changes in the peptides.

### 4.1. Electrostatic interactions

The observation that negatively charged vesicles (DOPG), but not electrically neutral ones (DOPC), induce conformational changes of the peptides and that solutions of negatively charged SDS-micelles stabilize similar secondary structures, indicates that the electrostatic forces are important to induce interactions between the peptides and the solvent molecules. FT-IR spectroscopy studies on porcine galanin and galanin analogs also support these observations [21]. It should be pointed out that the structural changes in most cases appear at the lowest concentration of DOPG and SDS. SDS actually induces these changes well below the CMC, acting probably as a monomer. Similar effects of anionic phospholipids on the secondary structures of peptides have previously been observed [19,21,26]. Addition of salt up to 150 mM NaCl should reduce the electrostatic interaction and hence association between the peptide and the SDS or DOPG molecules, resulting in a reduced stabilization and a less ordered peptide structure. This behavior is indeed observed in the DOPG solvent but not in the SDS solvent. The latter did, however, have a higher concentration of the amphiphilic component, 10 mM SDS versus 0.1 mg/ml (0.13 mM) DOPG.

With the peptides investigated here, the effects of introducing single electrically charged hydrophilic residues on the secondary structure could also be studied. Peptides with a positively charged lysine (M133, M135) instead of neutral leucine close to the carboxy terminal (M40, M132), dissolved in TFE, have a tendency to adopt a better defined $\alpha$-helical conformation (Fig. 2). On the other hand, substitutions to negatively charged amino acids in the C-terminus, as in the peptide M148, do not lead to any structural change. Hence, the sign of the charged residue, as well as the position in the sequence, are not surprisingly, important factors. The observations above may be explained by the helix-dipole model, which predicts that helix formation is favored if the pole of the helix dipole and nearby charged residue are of opposite signs [27,28]. In accordance with the NMR-structure of galanin in TFE [16], it is reasonable to believe that the N-terminal part of the peptide has a tendency to form an $\alpha$-helix. The C-terminal portion of this helix dipole has a negative sign and a positively charged residue close by (such as lysine) should therefore increase the $\alpha$-helical content, as observed.

### 4.2. Fluorinated alcohols as solvents

The largest structural changes, compared to the aqueous solution, are observed in the fluorinated alcohols (HFP and TFE) and particularly in TFE. Several studies on solvent induced effects by these fluorinated alcohols have been performed previously, resulting in a number of possible proposed explanations. It has for instance been attributed to a change in the intramolecular hydrogen bond stability, caused by the solvent molecules difference in acidity and basicity [29,30], as well as size [31]. The physical properties of these fluorinated alcohols also alters the electrostatic interactions [30,32,33]. However, the observed structure stabilization of the peptides caused by HFP and TFE as well as the differences between HFP and TFE, could not be explained by one single mechanism, but probably requires a combination of several mechanisms.

### 4.3. Secondary structure versus binding affinity

From a biological point of view the peptide structure in the DOPG-solvent is most relevant in the comparison with the receptor affinities.

The two galanin fragments and M141 are rather similar in sequence (Table 1). A comparison between their structures in the DOPG solvent shows that M141 has the lowest tendency to form an $\alpha$-helical conformation (Table 2), probably due to its proline tail, but still has a reasonable good binding affinity (Table 3). Furthermore, the shorter galanin fragment, galanin(1–12), attains almost as much $\alpha$-helical conformation as galanin(1–16) but binds poorly

to the receptor. These observations suggest that the peptide must have a certain length as well as composition in the C–terminus to be efficiently bound by the receptors.

The introduction of a hydrophobic residue in the peptides M132 and M133 leads to an increase of their binding ability (Table 3). This is correlated with an increased $\alpha$-helical content only in the TFE solvent (Fig. 2), but not, e.g., in the DOPG solvent (Table 2).

One of the studied peptides, M40, has been shown to function as an efficient antagonist to galanin, and is therefore of special interest. The CD-spectra from both galanin and M40 in DOPG-vesicle as well as SDS-micelle solutions suggest that a significant fraction of the peptide bonds are in a disordered state (random coil). However, the spectra show that galanin has a somewhat larger amount of $\alpha$-helical secondary structure than M40. This is possibly due to the fact that the C-terminal end of M40 is too short to attain a well defined secondary structure. The identical N-terminal part (13 amino acids) of galanin and M40, which is most important for receptor interaction, probably attains similar secondary structures in the peptides.

### *4.4. Concluding remarks*

All peptides investigated in this study show stabilized secondary structures while interacting with negatively charged DOPG vesicles or SDS micelles. Since the DOPG-vesicle solvent system should be considered as a reasonable model system mimicking a membrane environment, the SDS-micelle system should be so as well. An even more accentuated stabilization effect is seen in the fluorinated alcohols. A general interpretation of the results is that the electrostatic interactions and possible shielding from water interaction leads to a stabilized secondary structure of the peptides.

The affinity of a peptide for a phospholipid membrane may be related to its biological activity, and may serve as a prerequisite for receptor interactions. We found no clear correlation between extent of stabilized structure of the peptides and their receptor affinities, which shows that receptor recognition as expected is governed by more specific forces between receptor and ligand. However, the observed interactions between peptide and negatively charged membranes suggests a mechanism for accumulation of the peptide at the membrane, which should bring the peptide into the vicinity of the receptor. This may be an important phenomenon governing the probability of peptide–receptor interactions.

## Acknowledgements

We thank L.B.-Å. Johansson for use of the CD spectrometer and E. Vikström for preparing the lipid vesicle solutions. This study was supported by the Swedish Natural Science Research Council, the Magn. Bergwall's Foundation, the Swedish Medical Research Council, NIMH, NUTEK, the Ivar Bendixsons Foundation and Pharmacia Research Foundation.

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