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# Substance P and antagonists. Surface activity and molecular shapes

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**The molecular properties of substance P (SP) (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met amide) and three of its antagonists were derived by measuring the Gibbs adsorption isotherm, providing information on the surface activity, the molecular shape, and the p$K$ values of the different molecules. The following three antagonists were investigated: [D-Arg$^1$,D-Pro$^2$,D-Trp$^{7,9}$,Leu$^{11}$]SP, ANT I; [D-Arg$^1$,D-Trp$^{7,9}$,Leu$^{11}$]SP, ANT II and [D-Pro$^2$,D-Trp$^{7,9}$]SP, ANT III. SP is only moderately surface active. The amino acid substitutions lead, however, to an increased surface activity of the antagonists. From the concentration dependence of the surface activity it was possible to quantify the packing characteristics of the individual neuropeptides. SP shows cross-sectional areas of $300 \pm 5$ Å$^2$ to $240 \pm 5$ Å$^2$ (pH 5 to 8, 154 mM NaCl) at concentrations below $10^{-5}$ M, i.e., in the physiological concentration range, indicating a folded SP conformation. Upon increasing the packing density to concentrations larger than $10^{-5}$ M the surface area was only half as large ($148 \pm 5$ Å$^2$ to $124 \pm 3$ Å$^2$) suggesting now a relatively extended conformation of the SP molecule with its long molecular axis perpendicular to the air/water interface. In contrast, the three antagonists were characterized by surface areas of $147 \pm 3$ Å$^2$ to $126 \pm 3$ Å$^2$ which were almost independent of concentration. The antagonists thus adopt a relatively extended conformation in the whole concentration range measured. This is further supported by computer modelling which shows that the antagonists are motionally restricted and can adopt neither a bent nor a α-helical conformation. The surface activity of the neuropeptides was dependent on the pH of the solution. At low peptide concentrations (about $10^{-6}$ M) it was possible to resolve and determine the p$K$ values of all individual charged amino acid side chains. The p$K$ values observed for the neuropeptides were about two p$K$ units lower than those of the free amino acids in solution. The p$K$ shifts of the neuropeptides at the air/water interface are explained in terms of the Gouy-Chapman theory. SP and its antagonists bind to lipid bilayers in the order of their surface activity. While the binding of SP is mainly due to electrostatic interactions, hydrophobic peptide–lipid interactions contribute to the binding of the antagonists.**

## Introduction

Substance P (SP), an undecapeptide belonging to the family of tachykinins, has numerous pharmacological effects. One of its most investigated potential roles is that of a neurotransmitter or neuromodulater of nociceptive messages, first suggested in 1953 [1] and now widely accepted [2]. The binding of SP to its membrane-anchored receptor is regarded as an important step in the process of pain transmission. Due to the amphiphilic nature of SP it has been proposed [3,4] that SP penetrates into the lipid phase, where it adopts a partial α-helical conformation, and then diffuses within the membrane to the receptor binding site. A quantitative characterization of SP penetration into neutral and negatively charged lipids has shown that besides penetration of SP into negatively charged lipids, a high accumulation of SP close to the negatively charged membrane surface occurs [5]. It cannot be fully excluded, therefore, that SP might also diffuse on the membrane surface to its receptor site. In both mechanisms the membrane would provide a matrix for fast two-dimensional diffusion.

A number of SP analogues have been synthesized which presumably have antinociceptive effects by in-

Abbreviations: SP, substance P; ANT I, [D-Arg$^1$,D-Pro$^2$,D-Trp$^{7,9}$,Leu$^{11}$]SP; ANT II, [D-Arg$^1$,D-Trp$^{7,9}$,Leu$^{11}$]SP; ANT III, [D-Pro$^2$,D-Trp$^{7,9}$]SP.

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| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| SP | Arg | Pro | Lys | Pro | Gln | Gln | Phe | Phe | Gly | Leu $MetNH_2$ |
| ANT I | D-Arg | D-Pro | Lys | Pro | Gln | Gln | D-Trp | Phe | D-Trp | Leu $LeuNH_2$ |
| ANT II | D-Arg | Pro | Lys | Pro | Gln | Gln | D-Trp | Phe | D-Trp | Leu $LeuNH_2$ |
| ANT III | Arg | D-Pro | Lys | Pro | Gln | Gln | D-Trp | Phe | D-Trp | Leu $MetNH_2$ |

Scheme I.

hibiting the binding of SP to the spinal cord receptors [6]. Three of the most effective potential antagonists are: ANT I [7], ANT II [8] and ANT III [9], the amino acid sequences of which are shown in Scheme I.

Compared to the amino acid sequence of SP the modifications common to all three antagonists consist of an exchange of $Phe^7$ and $Gly^9$ for D-Trp, and stereoisomerisations at the hydrophilic N-terminus.

Knowledge of the physical/chemical difference between SP and its antagonists with respect to their properties in solution at the air/water interface and in the lipid phase can contribute to a better understanding of the characteristics of a biologically active SP molecule. In the present study the properties of SP in solution and at the air/water interface were compared with those of the three antagonists depicted above.

Even though the amino acid replacements in the antagonists seem to be only minor chemical modifications, they lead to distinct differences in the conformation and the average physical/chemical parameters of these molecules as will be reported. With the use of a monolayer method, the following questions have been addressed: (1) How do the surface activities of SP and its antagonists compare with each other? (2) What are the cross-sectional areas of the various peptides at the air/water interface and how do they depend on the concentration and the pH of the solution? (3) Which conformations can be suggested for the peptides when close to a membrane surface? (4) What are the p$K$ values of these peptides?

The surface activities, the area requirements and the p$K$ values bear on the potential membrane interactions.

## Materials and Methods

*Materials.* Substance P (SP) and its antagonists [D-$Arg^1$,D-$Pro^2$,D-$Trp^{7,9}$,$Leu^{11}$]SP (ANT I), [D-$Arg^1$,D-$Trp^{7,9}$,$Leu^{11}$]SP (ANT II = Spantide) and [D-$Pro^2$,D-$Trp^{7,9}$]SP (ANT III), synthesized by Bachem (Bubendorf, Switzerland), were a gift from Merck (Darmstadt, F.R.G.). The purity of the peptides was $98 \pm 1\%$ as determined by HPLC/TFA. All four peptides were acetate salts. The acetate content of the peptide samples amounted to about 20% of the total sample weight. Peptide stock solutions were prepared by dissolving 5 mg peptide in 1 ml of water. The molecular concentrations were calculated taking into account the acetate content. Water used for buffers and solutions was doubly ion-exchanged and glass distilled. 10 mM Tris buffer, adjusted with HCl to the desired pH, was used for pH values in the range of 7 to 11, while 10 mM Mes buffer was employed for the low pH range. All buffers contained 154 mM sodium chloride.

*Monolayer measurements.* The monolayer apparatus (Type RMC 2-T, Mayer Feintechnik, Göttingen, F.R.G.) was designed by Fromherz [10] and consists of a round Teflon trough with a total area of 362 $cm^2$ divided into eight compartments. The surface pressure was measured by the Wilhelmy method, using plates cut from filter paper (Whatman, No. 1). Before each measurement, the trough and the filter paper were thoroughly cleaned with methanol and distilled water. For surface pressure measurements of the peptides as a function of concentration, only one compartment filled with 20 ml of buffer was used. The solution was stirred with a tiny magnet and small increments of a peptide stock solution were added with a microsyringe to obtain the desired concentrations. For each concentration, the surface pressure was monitored for 10–15 min until equilibrium was reached.

*Titration measurements.* pH-titration of the amphiphilic peptides was performed by recording their surface pressure as a function of the pH. The peptide was dissolved in 20 ml buffer with an initial pH close to 7. Increments of 10 $\mu$l of NaOH or HCl (2 M) were then added with a microsyringe. Titration curves were essentially reversible, although slightly higher surface pressures were obtained in the back titration due to an increase in the ionic strength following the repeated addition of NaOH and HCl. The volume change of the solution during titration was small enough not to affect the surface pressure values. The pH was monitored by potentiometric measurements in a reference compartment containing the buffer solution without the peptide, in order to avoid possible disruption of the peptide monolayer by the electrode. As the peptides were present in low concentrations ($10^{-6}$ M to $10^{-4}$ M) in the titration experiments their effect on the pH of the buffered solutions could be neglected. All measurements were performed at ambient temperature ($22 \pm 1$° C).

## Results

*Surface activity of SP and its antagonists*

SP and its antagonists are amphiphilic molecules due to the segregation of charged amino acids at the N-terminus and uncharged amino acids at the C-terminal part of the molecule. Amphiphilic molecules tend to accumulate at the air/water interface, thereby lowering the surface tension of water. The surface pressure, $\pi$, is the difference between the surface tension of pure water, $\gamma_o$, and the surface tension of the surfactant solution, $\gamma$:

$$\pi = \gamma_o - \gamma \quad (1)$$

In Fig. 1, the surface pressures of SP, ANT I, ANT II and ANT III in buffered solutions at pH 7.4 (154 mM sodium chloride) are plotted as a function of the logarithm of their respective concentrations. A linear increase of the surface pressure with the logarithm of the bulk concentration, a typical feature of surfactants, is observed. These curves permit a quantitative comparison of the surface activities of the different amphiphiles and also allow the evaluation of the molecular areas of different peptides at the air/water interface. The deviation from linearity in the case of ANT I will be discussed below.

Fig. 1 demonstrates that the efficiency of these peptides in reducing the surface tension of water differs by orders of magnitude despite the fact that they contain the same number of polar and nonpolar amino acid residues and vary only slightly in their amino acid sequence. In order to achieve, for example, a surface pressure of 8.5 mN/m, the required concentrations are $10^{-4}$ M for SP, $1.3 \cdot 10^{-5}$ M for ANT III and only $2 \cdot 10^{-6}$ M for ANT I and II.

*Surface areas of SP and its antagonists*

From Fig. 1 it is possible to deduce the areas of the individual peptides at the air/water interface by applying the Gibbs adsorption equation (see also Ref. 11):

$$\Gamma = -c/RT(\delta\gamma/\delta c) = -1/RT(\delta\gamma/\delta \ln c) \quad (2)$$

$\Gamma$ is the excess amount of amphiphilic molecules accumulated per unit area at the air/water interface, $c$ is the concentration of peptide in bulk solution, and $\gamma$ is the measured surface tension. As the peptide concentrations are very low in the present study, activity coefficients can be neglected. From the excess surface concentration, $\Gamma$, the area requirement, $A$, of the peptide molecules at the air/water interface can be calculated according to

$$A = 1/\Gamma N_A \quad (3)$$

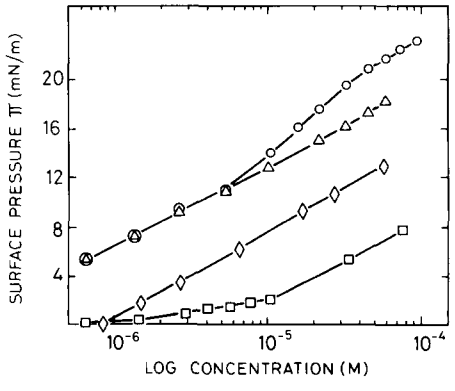


Fig. 1. Surface pressure $\pi$ as a function of the logarithm of concentration of SP (□), ANT III (◇), ANT II (△), ANT I (○) at pH 7.4 (10 mM Tris-HCl, 154 mM NaCl).

TABLE I

*Apparent cross-sectional areas of SP and SP antagonists at pH 7.4 (10 mM Tris-HCl, 154 mM NaCl)*

| Peptide | Area (Å$^2$) |
|---|---|
| SP $c$: $1.5 \cdot 10^{-6}$–$10^{-5}$ M | 240 ± 5 |
| $c > 10^{-5}$ M | 142 ± 3 |
| ANT I | 137 ± 3 |
| ANT II | 140 ± 3 |
| ANT III | 138 ± 3 |

where $N_A$, is the Avogadro number. The surface area, $A$, is thus inversely proportional to the slope of the $\pi$ vs. log $c$ plot.

From the slopes in Fig. 1 the areas of ANT II and ANT III are determined as 140 Å$^2$ and 138 Å$^2$, respectively. (All errors are in the range of ± 5 Å$^2$.) ANT I also gives rise to a linear $\pi$ vs. log $c$ plot at low peptide concentrations, and the corresponding area is 137 Å$^2$. However, at a concentration of $6 \cdot 10^{-6}$ M the slope of the $\pi$ vs. log $c$ plot first increases and then returns gradually to the initial value. The molecular area at very high concentrations is practically identical to that at low concentrations. This parallel shift of the $\pi$ vs. log $c$ curve can be explained by a p$K$ shift of ANT I as will be discussed below. Thus, the areas of the three antagonists at the air/water interface are very similar (Table I).

SP exhibits a behaviour which differs from that of the antagonists. Its $\pi$ vs. log $c$ curve is characterized by two linear parts with distinctly different slopes. The first is in the concentration range of $1.5 \cdot 10^{-6}$ M to $10^{-5}$ M indicating that the SP molecules occupy a constant surface area of 240 Å$^2$. Above the concentration of $10^{-5}$ M a second linear part begins which exhibits a much larger slope. The surface area requirement of SP in this concentration range is only 142 Å$^2$ and corresponds to those of the antagonists. This result suggests that SP may adopt either of two distinct preferential conformations or orientations at the air/water interface depending on the packing density of the molecules. In contrast, the antagonists obviously retain a constant conformation in the whole concentration range measured, even at very low peptide concentrations.

*Surface areas as a function of pH*

Under physiological conditions the bulk pH is rather constant at 7.0–7.4. However, close to a negatively charged membrane surfaces the pH can be much lower due to a local concentration increase of protons. In order to test the influence of pH on the area requirements of SP and ANT II, the surface pressure was measured as a function of concentration at different pH values (pH 5 to pH 8). It is seen in Fig. 2 that the surface pressure increases with increasing pH in the

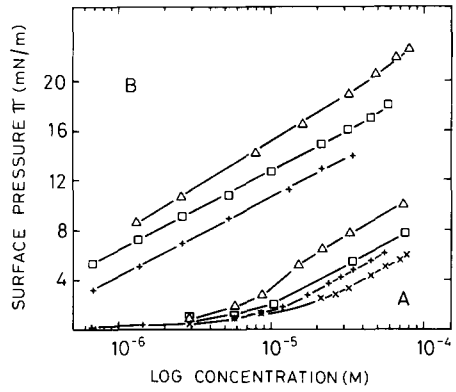


Fig. 2. Surface pressure $\pi$ as a function of the logarithm of peptide concentration A: SP, B: ANT II at different pH values, pH 5 (×), pH 6 (+), pH 7.4 (□), pH 8 (△).

whole concentration range; i.e., the peptides exhibit increasing surface activity at alkaline pH. The slopes of the $\pi$ vs. log $c$ curves also increase with pH indicating a decrease of the area requirements at the air/water interface with decreasing charge at the hydrophilic end of the peptides. The variation of the peptide area as a fuction of the pH is summarized in Fig. 3 for two different concentration ranges. For peptide concentrations $c > 10^{-5}$ M, the surface areas of SP and ANT II are almost equal (150–140 Å$^2$) and constant between pH 5 and pH 6, but decrease abruptly to 125 Å at pH 8 (Fig. 3A). The half-height of these transitions is at pH ≈ 7.5. At low concentrations of SP ($c < 10^{-5}$ M) a similar transition is observed already at lower pH; the half-height of the transition is at pH ≈ 6.7. Under these conditions the area, $A$, changes from $A = 300$ Å$^2$ at pH 5 to $A = 240$ Å$^2$ at pH 8 (Fig. 3B). The pH values at the half-height of the transitions correspond to the respective p$K$ values of the N-terminal aminogroup of arginine.

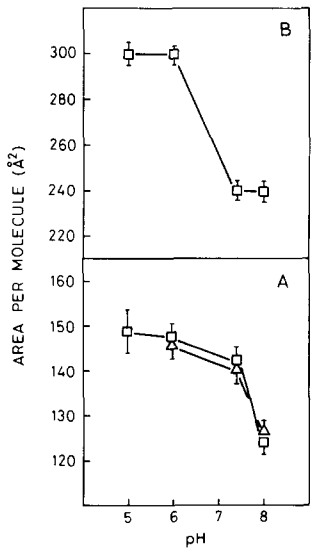


Fig. 3. Area per molecule as a function of the pH of the solution. (A) ANT II (△), SP (□) at concentrations $>10^{-5}$ M; (B) SP (□) at concentrations $1.5 \cdot 10^{-6}$ M to $10^{-5}$ M.

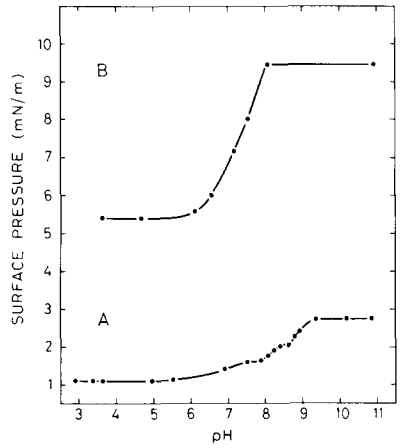


Fig. 4. Titration curves of SP. Surface pressure $\pi$ as a function of the pH at concentrations of $5.94 \cdot 10^{-6}$ M (A), $7.7 \cdot 10^{-5}$ M (B).

*pK measurements*

p$K$ determinations via measurements of the surface pressure have the advantage that very little material is required and that the concentration of the peptide can be very low. This is of special importance for amphiphilic molecules such as SP and its antagonists, which tend to form aggregates at higher concentrations [12].

Figs. 4 and 5 show titration curves for SP and ANT II, respectively, each measured at two different peptide concentrations. The surface pressure $\pi$ increases with pH since the positively charged side-chains become deprotonated, reducing, in turn, the water solubility of the peptide. The highest surface pressure can be expected for the completely deprotonated peptides, which have the strongest tendency to accumulate at the air/water interface.

Inspection of Figs. 4 and 5 reveals several steps in the titration curves. The apparent p$K$ values of the

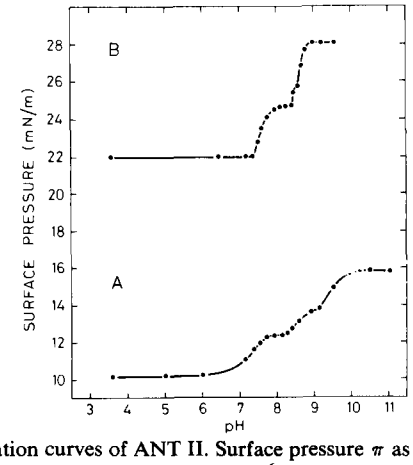


Fig. 5. Titration curves of ANT II. Surface pressure $\pi$ as a function of the pH at a concentration of $5.32 \cdot 10^{-6}$ M (A), $1.12 \cdot 10^{-4}$ M (B).

TABLE II

*pK values of SP and SP antagonists*

The error limit for p$K$ values $\pm 0.1$.

| Compound | Concn. (M) | Arg ($NH_3$) p$K_1$ | Lys ($NH_3$) p$K_2$ | Arg (C=$NH_2$) p$K_3$ |
|---|---|---|---|---|
| Free amino acid [a] | | 9.04 | 10.53 | 12.48 |
| SP | $5.94 \cdot 10^{-6}$ | 6.75 | 8.15 | 8.9 |
| ANT III | $4.96 \cdot 10^{-6}$ | 7.1 | 7.95 | 9.1 |
| ANT I | $5.32 \cdot 10^{-6}$ | 7.25 | 8.1 | 9.3 |
| ANT II | $5.32 \cdot 10^{-6}$ | 7.25 | 8.65 | 9.5 |
| SP | $7.71 \cdot 10^{-5}$ | 7.1 | 7.1 | 7.1 |
| ANT II | $1.12 \cdot 10^{-4}$ | 7.7 | 8.5 | 8.7 |

[a] Lehninger, A.L., Biochemistry, Worth Publishers, Inc.

individual side chains were determined graphically at half-height of the individual titration steps. Surprisingly, and in contrast to earlier measurements [13,14], three p$K$ values can be resolved for SP (Fig. 4A) and the antagonists (Fig. 5A), provided they are measured at very low concentrations ($\approx 5.0 \cdot 10^{-6}$ M).

The corresponding p$K$ values together with their amino acid assignments are given in Table II. At the higher concentration the three individual titration steps move together and eventually merge. In fact, for SP only one apparent p$K$ value can be observed at $7.7 \cdot 10^{-5}$ M (Fig. 4B). This apparent p$K$ of 7.1 corresponds well with the p$K$ of 7.0 measured potentiometrically at $0.89 \cdot 10^{-3}$ M [14]. For ANT II (Fig. 5B) the p$K$ shifts with concentration are less pronounced. Two p$K$ steps can clearly be recognized at both concentrations while the third is at the limit of resolution at high peptide concentration. At low peptide concentration the three p$K$ values are found in the range of 7.2 to 9.5 whereas at high concentration the p$K$ range is 7.7 to 8.7. From Table III, where the differences between the p$K$ values of the free amino acids and those of the peptides in the monolayer are indicated as $\Delta$p$K$, it is seen that the p$K$ values of the guanidino side chains of $Arg^1$ are the most affected by the concentration change, and this is the case for both peptides. The p$K$ of the side chain of $Lys^3$ is shifted from the value of the free amino acid in SP but remains almost constant in ANT II. The shift of the N-terminal amino group is similar for both peptides and is rather small.

TABLE III

*A comparison of the pK shifts calculated on the basis of the Gouy-Chapman theory, $\delta pK_\psi$, with the measured pK shifts, $\Delta pK_{1-3}$, and the mean measured pK shifts $\Delta pK$*

| Compound | $\Delta pK_\psi$ | $\Delta pK_1$ | $\Delta pK_2$ | $\Delta pK_3$ | $\Delta pK$ |
|---|---|---|---|---|---|
| SP $c < 10^{-5}$ M | 1.7 | 2.3 | 2.4 | 3.6 | 2.7 |
| SP $c > 10^{-5}$ M | 2.3 | 1.9 | 3.4 | 5.4 | 3.5 |
| ANT II $c < 10^{-5}$ M | 2.3 | 1.8 | 1.9 | 3.0 | 2.2 |
| ANT II $c > 10^{-5}$ M | 2.3 | 1.34 | 2.0 | 3.8 | 2.3 |

The reproducibility of the measurements with different batches of the acetate salt of SP is within the given margin of error. TFA salts show, however, a different behaviour. At concentrations below $10^{-5}$ M the surface activities and the surface areas were comparable with those of the acetate salt. At concentrations greater than $10^{-5}$ M the surface activities were lower and thus the large area conformation appeared to be retained (Weis and Seelig, unpublished results).

## Discussion

*Surface activity*

The monolayer measurements clearly demonstrate that SP and its antagonists are all surface active molecules. Nevertheless, the tendency of the four peptides to accumulate at the air/water interface and to form peptide monolayers differs largely from molecule to molecule in spite of the similar amino acid sequence and the same number of charged residues. Inspection of Fig. 1 allows a semi-quantitative comparison of the surface activity and yields the following ordering of the peptides according to increasing surface activity:

SP < ANT III < ANT II $\leq$ ANT I

It can be expected that this series also reflects the binding or penetration potential of the four peptides towards bilayer membranes. In previous studies the hydrophobic binding constant of SP to lipid bilayers was found to be in the order of 1 $M^{-1}$ to 1.8 $M^{-1}$ [5,15]. Corresponding measurements with the antagonists lead indeed to distinctly larger hydrophobic binding constants (Seelig, unpublished data).

Since the surface activity critically depends on the balance and interplay of hydrophobic and electrostatic forces, the influence of the various amino acid substitutions of SP on the surface activity will be briefly discussed. Substitution of both $Phe^7$ and $Gly^9$ in SP by D-tryptophan leads to ANT III. According to the hydrophobicity scale of Nozaki and Tanford [16] tryptophan is more hydrophobic than even phenylalanine. The increased hydrophobicity of ANT III would thus explain its enhanced surface activity. In ANT I and ANT II an additional hydrophobic substitution occurs; i.e., $Met^{11}$ is replaced by leucine. On the basis of hydrophobicity considerations one would therefore predict the following order of surface activities: SP < ANT III < ANT I = ANT II.

Since this order is not observed, it is evident that other effects such as electrostatic contributions have to be considered. The average p$K$ values of ANT II are slightly larger than those of ANT I (Table II). At a given pH ANT I thus carries a somewhat smaller elec-

tric charge than its analogue ANT II, which increases its hydrophobicity and consequently its surface activity. The charge difference is reinforced at higher lateral packing density in the monolayer.

*Apparent cross-sectional area and peptide conformation*

The surface areas or apparent cross-sectional areas of all peptides investigated show a strong pH dependence, the surface area being smallest at high pH values. Under these conditions the electric charge is small and electrostatic repulsions can be neglected. The surface area of ANT II at pH 8 (the highest pH measured) was 126 Å$^2$. For comparison, the effective cross-sectional area of ANT II was determined by computer modelling and was found to be about 110 Å$^2$ for an extended ANT II conformation. This rather large cross-sectional area is essentially due to the two proline residues of ANT II which cause distortions of the otherwise extended chain.

At physiological pH (pH 7.4, 154 mM NaCl) the surface areas of all three antagonists are in the range of 137 Å$^2$ to 140 Å$^2$ (Table II). This is only 20% larger than the estimated effective cross-sectional area and allows the following conclusions: (i) all three antagonists adopt a similar average conformation at the air/water interface; (ii) the three peptides are characterized by a relatively extended conformation orienting with the long axis perpendicular to the air/water interface. The formation of a relatively extended conformation is further supported by the fact that the antagonists are sterically hindered in their hydrophobic part and, in contrast to SP (Fig. 6), can therefore not adopt a bent or $\alpha$-helical conformation.

SP exhibits a more complex behaviour than the antagonists. For peptide concentrations larger than $10^{-5}$ M, the surface area is in the range of 148 Å$^2$ to 124 Å$^2$ (pH 5–8) and is thus not much different from those of the antagonists. The small surface area suggests that SP assumes a relatively extended conformation with the chain axis perpendicular to the water surface. Earlier CD measurements have demonstrated the aggregation of SP at higher peptide concentrations, with a concomitant formation of $\beta$-type ordered structures [12]. A similar situation can be envisaged for densely packed SP molecules at the air/water interface.

At concentrations smaller than $10^{-5}$ M, SP adopts a conformation with a larger surface area of 240 Å$^2$ to 300 Å$^2$ depending on the pH. The exact nature of this conformation remains unclear at present. NMR measurements of SP (mM concentration range) in water [17] indicate some folding of SP. A rapid equilibrium between different conformers characterized by different hydrogen bonding situations between the NH protons of Gly$^9$, Leu$^{10}$, Met$^{11}$ and Gln$^{5,6}$ was suggested. A similar folded conformation might be present at the air/water interface at low concentrations. This type of structure would indeed double the average cross section of the molecule at the interface. The formation of a partial $\alpha$-helix might, however, also be envisaged. The cytosolic concentration of SP under physiological conditions is in the order of $10^{-8}$ M. Although the concentration of SP could increase by two to three orders of magnitude close to negatively charged membrane surfaces [5] it probably does not exceed $10^{-5}$ M. The conformation of SP at the membrane/water interface corresponds, therefore, most probably to a folded rather than to an extended state. Tentative molecular models of SP in the extended, bent and the $\alpha$-helical conformation are shown in Fig. 6.

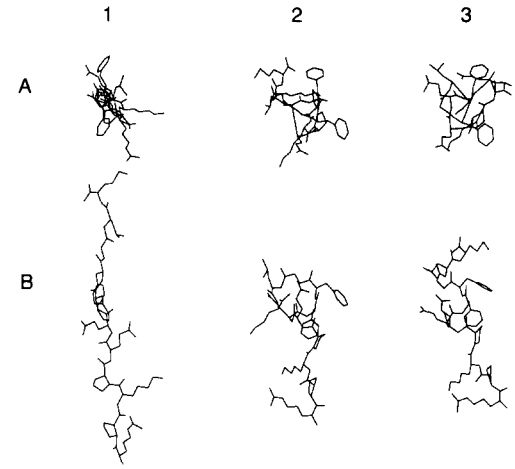


Fig. 6. Top view (A) and side view (B) of SP, with its N-terminus at the bottom of the figure, in the extended (1), bent (2) and $\alpha$-helical (3) conformation. The bent conformation was modelled according to the structure proposed by Chassaing et al. (1986) assuming hydrogen bonds between Gln$^{5,6}$ and Gly$^9$, Leu$^{10}$, Met$^{11}$. For the $\alpha$-helical conformation an $\alpha$-helix was assumed between Pro$^4$ and Met$^{11}$. Contacts involving distances shorter than the sum of Van der Waals radii were avoided in modelling the main chain. Side chains were oriented arbitrarily. (I am indebted to R. Dölz, Biocomputing, Biocenter Basel, who performed the computer modelling.)

*The pK values of SP and its antagonists*

As shown in the present study the p$K$ values of the three conjugate acid/base pairs in SP are about 2 p$K$ units lower than the p$K$ values of the corresponding free amino acids (Table II). The isoelectric point, p$I$, of SP, which is in the range of 10.0 to 10.2 [18,17], is also lower than expected from the p$K$ values of the free amino acids and is in good agreement with monolayer titration measurements performed at very low peptide concentration. Such p$K$ shifts to lower values are not unusual. It is known that the apparent p$K$ of a functional group in a protein may deviate considerably from the p$K$ of the free amino acid. For example, if the positive charges are clustered in one part of the molecule, as is the case for SP and its antagonists, the

neighbouring groups will influence each other unfavorably, reducing the apparent p$K$ values.

When these charged peptides assemble in micelles or monolayers such p$K$ shifts may be enhanced, for two reasons. First, the polarity of the immediate charge environment may change during aggregation. Second, the positive charge at the monolayer surface repels hydrogen ions and facilitates the deionization of the positively charged amino acid side chains.

According to Boltzmann's law the proton concentration near the monolayer $[H^+]_m$ will be given by:

$$[H^+]_m = [H^+]_w \exp(-\psi F/RT) \tag{4}$$

where $[H^+]_w$ is the equilibrium proton concentration in the bulk water phase, $\psi$ is the electric surface potential, $F$ is Faraday's constant, $R$ is the gas constant and $T$ is the temperature. Under these conditions the apparent pH at the positively charged monolayer surface, $pH_m$, is shifted to higher values according to

$$pH_m = pH_w + \psi F/(2.3RT) \tag{5}$$

This pH shift entails an apparent p$K$ shift, $\Delta pK$, of the protonated groups in the monolayer or micelle according to Eqn. 6.

$$\Delta pK = pK_{app} - pK_w = \Delta pK_i - \psi F/(2.3RT) \tag{6}$$

The difference between the apparent p$K$ value, $pK_{app}$, and the p$K$ value in water, $pK_w$, depends on a component, $\Delta pK_i$, due to the change in polarity experienced by a charged group when transferred from the aqueous medium to a monolayer or micelle [19] and a component due to the electrostatic surface potential, $\psi$, which in the following will be called $\Delta pK_\psi$.

The component $\Delta pK_i$ can not be quantified a priori. The surface potential $\psi$ generated by the surface charge density, $\sigma$, at the peptide/water interface may, however, be approximated by means of the Gouy-Chapman theory [20] if $\sigma$ is known from experimental results.

$$\sigma = \left[2000\,\varepsilon_r \varepsilon_0 RT \sum_i c_{i,eq}(e^{-z_i F\psi/RT} - 1)\right]^{1/2} \tag{7}$$

where $\varepsilon_r$ is the dielectric constant of water (at 25 °C), $\varepsilon_0$ is the permittivity of free space, and $c_{i,eq}$ is the concentration of the $i$th electrolyte in the bulk aqueous phase (in moles per liter). The surface charge density, $\sigma$, of a monolayer or micelle is defined, on the other hand, as the ratio of the surface charge $Q$ of the peptide and its surface area $A$, known from the monolayer measurements.

$$\sigma = Q/A \tag{8}$$

The surface charge $Q$ is the product of the elementary charge $e$ and the valency $z$ of the peptide. For SP and the antagonists the valency $z$ is three.

The component $\Delta pK_\psi$, that is, the apparent p$K$ shift due to the surface potential $\psi$, can thus be estimated for SP and its antagonists and can then be compared to the measured apparent p$K$ shifts (Table III). For SP with a surface area of about 300 Å$^2$ at concentrations smaller than $10^{-5}$ M, the surface charge density $\sigma$ is 0.16 Cb/m$^2$ and gives rise to a surface potential of 98 mV under the given experimental conditions (154 mM NaCl). At concentrations greater than $10^{-5}$ M the area decreases to about 150 Å$^2$; as a consequence the surface charge density $\sigma$ doubles and the corresponding surface potential $\psi$ is 137 mV. Due to these surface potentials the p$K$ values of the three acid/base pairs of SP in the monolayer are expected to decrease by 1.7 and 2.3 p$K$ units at concentrations below and above $10^{-5}$ M, respectively. This analysis shows that a concentration dependent p$K$ shift of 0.6 p$K$ units should occur for SP. For the antagonists which retain their surface area over the whole concentration range measured no $\Delta pK_\psi$ is expected, however.

Thus at a first approximation the estimated p$K$ shifts, $\Delta pK_\psi$, of about two p$K$ units agree quite well with the measured p$K$ shifts listed in Table III. This allows the conclusion that the build-up of a positive surface potential is the main reason for a p$K$ decrease in the course of monolayer or micelle formation. A closer inspection of Table III shows, however, that polarity effects and, especially, local electrostatic interactions must play an additional role. This is evidenced by the following: (i) The measured p$K$ shifts of the three acid/base pairs in a given peptide differ from each other, whereby the p$K$ shifts of the guanidino side chain is larger than expected as well for SP as for ANT II. This is most probably due to the fact that the guanidino side group is located between the two other charged groups. (ii) With increasing concentration and increasing packing density of the peptides in the monolayer, the local electrostatic interactions are enhanced and give rise to slightly higher p$K$ shifts than expected on the basis of the Gouy-Chapman theory. The mean measured p$K$ shifts were 0.8 and 0.1 for SP and ANT II, respectively. (iii) The p$K$ shifts vary with the peptide configuration and thus with the relative position of the charged groups to each other.

For ANT II the mean measured p$K$ shifts, $\Delta pK$, are in better agreement with the calculated p$K$ shifts, $\Delta pK_\psi$, than for SP. This seems to indicate that the configuration of SP leads to larger electrostatic interactions than that of ANT II. The same tendency is observed in Table II which shows that the p$K$ values increase in the order of SP < ANT III < ANT I < ANT II. How much the apparent charge of these peptides depends on the N-terminal configuration is best illustrated by a comparison of the p$K$ values of ANT I and ANT II, which differ only in the stereospecificity of Pro$^2$. The p$K$ values of the N-terminal amino groups are equal as seen in Table II. But, the p$K$ values of Lys$^3$ and of the

guanidino side chain of Arg[1] are, respectively, 0.55 and 0.2 p$K$ units higher in ANT II. This small apparent charge difference was also confirmed in lipid binding experiments. The apparent binding constant, including electrostatic and hydrophobic effects, was slightly larger for ANT II than for ANT I, while their hydrophobic binding constants were equal as expected (Seelig, unpublished data).

The fact that potentiometric measurements of SP solutions in the mM concentration range show only one apparent p$K$ value [13] strongly suggests, in light of the present results, that at these concentrations small SP aggregates have already formed. A strong dependence of the apparent p$K$ value on concentration, at levels well below the point at which visible aggregates form, was also observed for local anesthetics containing a tertiary amino group [21,22].

## Conclusions

The difference between SP and its antagonists can be summarized as follows: (1) The antagonists investigated in the present work are more surface active than SP, with their surface activities increasing in the order ANT III < ANT II ≤ ANT I. (2) All three p$K$ values can be resolved for SP and its antagonists at low peptide concentrations ($\approx 10^{-6}$ M). Despite the fact that these peptides carry the same number of charged groups their apparent p$K$ values differ and increase in the order SP < ANT III < ANT I < ANT II. The variations in p$K$ values reflect differences in the N-terminal peptide configurations. (3) SP is present in two distinct conformations or orientations depending on the packing density of the molecules at the air/water interface, in contrast to the antagonists which do not change their conformation in the whole concentration range measured. A comparison of the measured cross-sectional areas with those obtained by computer modelling suggests a relatively extended conformation for the antagonists independent of the concentration and also for SP at concentrations above $10^{-5}$ M. With decreasing pH interchain distances increase and result in greater possibilities for chain movements. At concentrations below $10^{-5}$ M SP appears, however, to adopt a bent conformation.

These results together with earlier lipid binding studies [5] indicate that SP is present in a bent conformation in the aqueous phase close to biological membranes.

A comparison of the present results with biological activity assays [6] shows that the potency of these peptides in antagonizing pain behaviour after intrathecal administration (ANT III < ANT II < ANT I) correlates with their respective surface activites. Their efficiency on smooth muscle preparations (ANT III < ANT I < ANT II) correlates, however, with the apparent charge state of the peptides.

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