Conformation and self-association of the peptide hormone substance P: Fourier-transform infrared spectroscopic study

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Fourier-transform i.r. (f.t.i.r.) spectroscopy has been applied to the study of the conformational properties of substance P in aqueous solution. Spectra were obtained in the presence of lipid membranes and Ca^{2+} to assess the role of these factors in induction of the active conformation of the peptide. In aqueous solution substance P was found to be predominantly unstructured at physiological p^2H, where the lack of long-range order is probably related to charge repulsion along the peptide chain. However, substance P aggregated in aqueous solution at $p^2H > 10.0$. Little or no induction of secondary structure was seen on addition of the peptide to negatively charged bilayers, suggesting that interaction with a membrane surface does not play an important role in the stabilization of the active conformation of the peptide. In fact, substance P was found to aggregate in the presence of charged lipids, which would tend to hinder rather than enhance interaction with the receptor. We propose a model for the aggregation of substance P at the bilayer surface, based on our studies of the effect of p^2H and lipid/peptide ratio on spectra. Addition of Ca^{2+} had no effect upon the secondary structure of the peptide or on its interactions with membranes.

INTRODUCTION

Substance P is an undecapeptide hormone which is widely distributed throughout the central and peripheral nervous system. This peptide (with sequence $^+$Arg$^+$-Pro-Lys$^+$-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2) is a member of a family of naturally occurring peptides, the tackykinins. Substance P is known to be involved in various physiological processes, acting as a neurotransmitter or neuromodulator in the transmission of pain and in the regulation of blood flow and gastrointestinal motility [1]. These roles have stimulated interest in the pharmaceutical industry in the design and synthesis of agonists and antagonists.

An obvious first step in this process is the elucidation of the solution and receptor recognized conformation of the native hormone. The determination of the solution structure of small peptide hormones by biophysical techniques is nowadays a relatively simple exercise, and a number of biophysical techniques (c.d., n.m.r., i.r. spectroscopy and light-scattering) have been applied to the elucidation of the solution properties of substance P [1–9]. C.d. and Raman spectroscopy are in agreement that in aqueous solution no preferred conformation can be detected [1,2,4]. When studied by ^{1}H-n.m.r., substance P in aqueous solution was shown to exist in a rapid equilibrium between different conformers characterized by different hydrogen-bonding situations, i.e. it has no preferred conformation [9].

Elucidation of the structure which is recognized by the receptor is a more difficult task. Trapping the peptide in the 'active' conformation presents problems, primarily because the nature of the 'active' peptide is not known. It has been suggested that interaction with the membrane of the target cell or binding of Ca^{2+} may induce the conformation of the hormone which is recognized by the receptor [10–12]. This has led to studies of the conformation of many peptides in membrane mimetic environments such as detergent micelles and 2,2,2-trifluoroethanol (TFE) solution and in the presence of Ca^{2+}, with some evidence for conformational changes under these conditions being presented [1,3,5–7,9].

I.r. spectroscopy is a particularly powerful technique for structural studies of peptides and proteins. Previous i.r. studies on the conformation of substance P have been reported [7,8]. However, these studies were performed on substance P in the solid state [7], at high concentrations [8] or in TFE [7], a solvent recently shown to induce helical structure in proteins intrinsically possessing another conformation [13].

We have repeated and extended these previously made measurements. Here we describe the results of an F.t.i.r. spectroscopic investigation of the conformation of substance P in aqueous solution and in a membrane environment in the presence and absence of Ca^{2+}.

MATERIALS AND METHODS

Substance P and the lipids dimyristoyl phosphatidic acid (DMPA), dimyristoyl phosphatidylglycerol (DMPG) and dimyristoyl phosphatidylcholine (DMPC) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). $CaCl_2$ and SDS were from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). Peptide samples in solution were prepared by dissolution of the peptide in 50 mM Tris/HCl/150 mM NaCl, p^2H 7.6 (Tris-buffered saline, TBS), 25 mM $NaHCO_3$/10.7 mM NaOH, p^2H 10.0 or 50 mM KCl/132 mM NaOH p^2H 13.0 in ^2H_2O (MSD Isotopes, Montreal, Que., Canada) to a final concentration of 10 mg/ml. For samples in the presence of lipid, lipid and peptide were mixed in required proportions (30:1, 20:1 or 10:1 molar ratio) and the samples sonicated in ^2H_2O to yield a final peptide concentration of 10 mg/ml. For experiments in SDS and in the presence of Ca^{2+} the concentrations were 5% (w/v) and 20 mM respectively.

Spectra were recorded either on a Digilab FTS-60 or FTS 40A F.t.i.r. spectrometer continuously purged with dry N_2. For data acquisition, 6 μl of solution was placed between a pair of CaF_2 windows separated by a 50 μm Mylar spacer and mounted in a Harrick demountable cell. For each spectrum, 100 interferograms were collected, signal-averaged and Fourier-transformed to gen-

Abbreviations used: F.t., Fourier transform; TFE, 2,2,2-trifluoroethanol; DMPA, dimyristoyl phosphatidic acid; DMPC, dimyristoyl phosphatidylcholine; DMPG, dimyristoyl phosphatidylglycerol; TBS, Tris-buffered saline.

erate a spectrum with a resolution of 2 cm^{-1}. Buffer spectra were recorded under identical conditions and interactively subtracted. Fourier self-deconvolution was performed as described previously using a half-width of 13.5 cm^{-1} and a resolution enhancement factor (k) of 1.7, and employing software developed in our laboratory [14].

RESULTS AND DISCUSSION

A knowledge of solution conformation is critical in developing an understanding of the relationship between the structure and biological function of peptide hormones. F.t.i.r. spectroscopy is becoming increasingly used to study the conformation of biological molecules. With this technique, information concerning the molecular conformation imposed upon peptides by intra- and inter-molecular forces may be revealed. The i.r. band most diagnostic of protein secondary structure is the amide I band (1600–1700 cm^{-1}) which arises predominantly (80%) from the stretching of the C=O groups of the polypeptide backbone amide groups. Each secondary structure is associated with a distinct hydrogen-bonding pattern, resulting in a characteristic electron density in amide C=O groups, which in turn gives rise to characteristic amide I frequencies [15].

The i.r. spectrum of substance P in TBS, p^2H 7.6, is shown in Figure 1, spectrum a. The amide I band maximum is centred at 1644 cm^{-1}, a position characteristic of peptides with little or no repetitive secondary structure. Tightly folded, strongly-hydrogen-bonded peptides and proteins have amide protons shielded from the solvent, limiting $^1H^+ \rightleftharpoons {}^2H^+$ exchange. As a consequence, on dissolution of the peptide in ^2H_2O, incomplete exchange results and a residual amide II band is observed. The lack of a residual amide II band in these experiments confirms that the peptide exists in a poorly structured conformation. This finding is in agreement with previous c.d. and Raman-spectroscopic studies [1,2,4]. However, a previous i.r. study found an amide I maximum at 1630 cm^{-1}, with a significant number of strongly hydrogen-bonded amide proteins. These spectral features suggest a predominant β-sheet structure. This discrepancy may be related to differences in concentration. The high concentration used in the previous i.r. study is known to induce self-association of the peptide in solution, which may have important structural consequences. It should be pointed out that the physiological concentration of substance P is estimated to be 10 μM [16]. While concentrations in this range can be studied by c.d. spectroscopy, i.r. currently requires higher concentrations (by one to two orders of magnitude). However, as our i.r. results with concentrations at 7.4 mM agree with published c.d. data on substance P in aqueous solution, it appears safe to assume that the supraphysiological concentration used in the present study produce limited, if any, concentration-related artifacts.

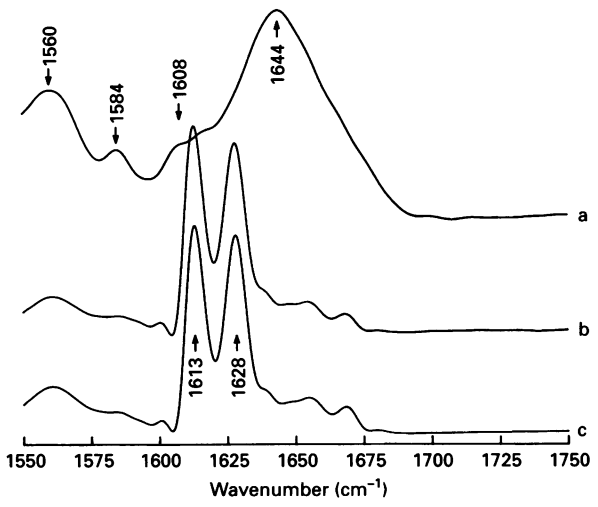

Figure 1 I.r. spectra in the amide I region of substance P in solution at p^2H 7.6 (a), p^2H 10.0 (b) and p^2H 13.0 (c)

The lack of repetitive structure of substance P found in our study may be attributed to the high net positive charges carried by the peptide (charge repulsion preventing adoption of a compact structure) in addition to the presence of two proline residues at positions 2 and 4. The imide groups of proline residues do not allow long-range hydrogen bonds involving amide groups to form, promoting the adoption of a poorly ordered structure. The weak bands at 1608 and 1584 cm^{-1} in Figure 1 originate from amino-acid side-chain vibrations and are assigned to the guanidinium groups of arginine, with absorption from phenylalanine overlapping at 1608 cm^{-1}; the broad band at 1560 cm^{-1} is due to the acetate counterion found in the substance P preparations used.

The lack of repetitive structures in many peptide hormones such as substance P has led to the suggestion that some conformational change must occur to allow recognition of the peptide by the receptor, i.e. the active conformation is not the solution conformation. It has been argued that this may be achieved in two ways, namely through interaction with the cell membrane or through complexation with metal ions. In the first case, interaction with the target cell membrane may induce conformational changes which result in the adoption of a structure which may be recognized by the appropriate receptor [10]. Therefore we have also examined the conformation of substance P in the presence of lipid membranes and detergent micelles. In the presence of zwitterionic lipids such as DMPC, substance P exhibited a spectrum which was identical with that seen in solution (results not shown), indicating that little, if any, interaction with the membrane occurred. This lack of interaction is in agreement with previous c.d. results [3]. As this peptide carries a net positive charge, this is not surprising; any interaction would be expected to be specific for negatively charged membranes.

We repeated this experiment in the presence of the negatively charged lipids (DMPA, DMPG) and in SDS micelles. In the presence of DMPA and DMPG at a lipid/peptide molar ratio of 10:1, these spectra are radically different from the solution spectra of substance P, exhibiting two major amide I bands at 1628 and 1613 cm^{-1} (Figure 2). Amide I bands below 1630 cm^{-1} are characteristic of the formation of intermolecular hydrogen bonds, that is, aggregation. It is important to note that the self-association described here arises from strong hydrogen-bonding between polypeptide chains as opposed to the kind of association caused by exceeding a critical peptide concentration, where the chains are mainly held together by hydrophobic forces. It therefore appears that, on neutralization of the positive charge carried by substance P, the peptide does not adopt a highly folded conformation and does not incorporate into the bilayer, but lies at the surface in an extended conformation with hydrogen-bonding requirements satisfied by the formation of intermolecular hydrogen bonds with its neighbours. This hypothesis is supported by the findings of Williams and Weaver [1], who detected an ordering of the lipid acyl chains of DPPG small unilamellar vesicles on binding substance P. When the peptide

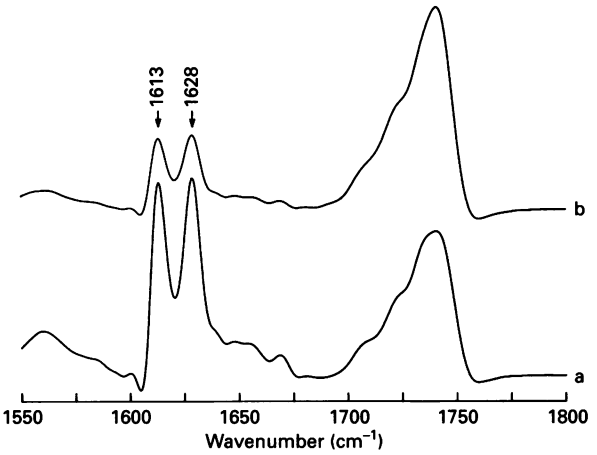

Figure 2 I.r. spectra in the amide I region of substance P in the presence of DMPG at lipid/protein molar ratios of 10:1 (a) and 30:1 (b)

lies at the surface of the negatively charged vesicle, it tends to pull together the lipid headgroups and consequently their acyl chains, so modifying chain packing. A slight ordering of the acyl chains is also inferred from a small, but significant, decrease in the frequency of the CH_2 and CH_3 stretching bands in the i.r. spectra.

The presence of two amide I bands suggests that two types of intermolecular hydrogen bonds are present, the low frequency band corresponding to the stronger hydrogen bonds. We repeated these experiments, varying the lipid/peptide mole ratio to 20:1 and 30:1, and found that the spectra were identical with those recorded with a 10:1 molar ratio, indicating that the appearance of two low frequency amide I bands does not reflect the presence of two types of binding sites.

Two explanations may account for the presence of two types of hydrogen-bonding. Firstly, the presence of positive charges may result in charge repulsion preventing close alignment of parts of the chain, giving rise to relatively weak hydrogen bonds with an absorption at 1628 cm^{-1}. In those regions where there is less charge repulsion, the chains can form stronger hydrogen bonds, and a low frequency (1613 cm^{-1}) band results. Secondly, the bulkiness of the side chains of phenylalanine, glutamine, arginine and lysine may prevent close alignment of regions of the polypeptide chains and also give rise to the two types of hydrogen bonds observed. To determine which of these two was the more plausible explanation, we titrated the side chains of lysine (pK_R = 10.4–11.3; [17]) and of arginine (pK_R = 12; [17]) by recording spectra in solution at p^2H 10.0 and 13.0 (Figure 1, spectra b and c). Although the pK_R values may vary depending upon the environment of the side group, the values should be close to those seen for amino acids in solution. Spectra at p^2H 10.0 exhibited two main amide I bands at 1613 cm^{-1} and 1628 cm^{-1}, indicating aggregation. This aggregation likely arises due to the deprotonation of lysine side chains, decreasing the hydrophilic character of the peptide. This promotes self-association, creating a hydrophobic environment for apolar residues. Rather than interacting with the solvent, the amide C=O group hydrogen-bonding requirements are now satisfied by the formation of intermolecular N–H$\cdots$O=C hydrogen bonds. As discussed above, the presence of two amide I bands below 1630 cm^{-1} suggests two types of hydrogen-bonding of different strengths. The presence of residual charge from the arginine side chains may prevent close alignment of sections of the peptide chains. However, on elevation of p^2H to 13.0, no further change is seen in the spectra, suggesting that deprotonation of arginine does not promote a closer alignment of the chains. Thus we suggest that the presence of two types of intermolecular hydrogen bonds may be related to close alignment of regions of the peptide which do not contain bulky side groups (strong hydrogen bonds, 1613 cm^{-1}) and poor alignment of regions which do contain such groups (weak hydrogen bonds, 1628 cm^{-1}). The ocurrence of two amide I bands below 1630 cm^{-1} arising from peptide aggregation has also been observed for the peptide galanin [18] and some members of the magainin family of peptide antibiotics [19].

The presence of two types of hydrogen bonds indicate that, when substance P molecules aggregate, they may do so with both N-termini aligned and both C-termini aligned. Examination of the amino acid sequence indicates that the six residues in the sequence most likely to give rise to steric hinderance (arginine, lysine and two glutamines) are found in the N-terminal region of the peptide, with two phenylalanine residues in the C-terminal region. We suggest that two N-terminally aligned peptides could produce two strengths of hydrogen-bonding in the following way. Weak hydrogen bonds would form between N-terminal C=O and N–H groups due to steric constraints imposed by the bulky arginine, lysine and glutamine side chains. The relatively smaller side chains of methionine, glycine and leucine would favour closer alignment of the C-termini. In addition, overlap (or stacking) of the phenylalanine residues would allow favourable π–π interactions, further stabilising this aggregate. The peptide does not aggregate with the N-terminus aligned with the C-terminus, since this would result in a single hydrogen-bonding pattern with an amide I frequency intermediate between 1613 cm^{-1} and 1628 cm^{-1}.

Interestingly, SDS had little effect upon the spectrum of substance P, with spectra being identical with those seen in solution. This is in contrast with c.d. and Raman spectra, which suggested a 20–30% helical structure in SDS [1]. The reason for this discrepancy is unclear. The lack of interaction of substance P with SDS is particularly surprising considering the high positive charge carried by the peptide and the pronounced interaction with DMPA and DMPG. However, as has recently been demonstrated, the interaction of positively charged peptides with membranes and micelles appears to be highly dependent upon the charge distribution along the peptide chain and the nature of the headgroup [19].

The aggregation seen for substance P in the presence of negatively charged membranes does not support the concept of induction of an active conformation on interaction with membranes. In addition, most hormones are produced some distance from their eventual site of action. This allows the interaction of the peptide with a large number of membranes which do not contain the appropriate receptor. Clearly, this interaction would not be advantageous, requiring a significantly higher amount of peptide to be released to ensure that some peptide reaches the target cells. The alternative would require a highly specific interaction of the peptide with membranes of specialized composition, favouring interaction of the peptide only with target cells.

A more plausible mechanism for the induction of an active conformation is by complexation with metal ions. It has been suggested that Ca^{2+} may play a role in stabilising the active conformation of many peptide hormones [11,12]. Ca^{2+} binding accompanied by structural changes has been demonstrated for substance P in organic solvents [12] (Ca^{2+} presumably being chelated by amide C=O groups). In addition, this peptide has been suggested to act as a Ca^{2+} ionophore. F.t.i.r. spectra of

substance P in solution in the presence of 20 mM Ca^{2+} revealed no changes in secondary structure, the spectra being identical with those seen in the absence of Ca^{2+}. Furthermore, the i.r. spectra recorded in the presence of SDS, DMPA and DMPG were identical in the presence and absence of Ca^{2+}.

In summary, we find no evidence for stabilization of the proposed active conformations of substance P by Ca^{2+} or phospholipids. We suggest that the selective binding of these hormones to their receptors is due not to the presence of a preferred arrangement of the peptide chains into particular secondary structures, but to the presentation of a preferred primary sequence.

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