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# CONFORMATIONAL FLEXIBILITY OF THE HORMONAL PEPTIDE BOMBESIN AND ITS INTERACTION WITH LIPIDS

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SUMMARY: The conformational flexibility of the tetradecapeptide hormone bombesin has been studied using circular dichroism and fluorescence of its single tryptophan residue. The spectral changes observed indicate that the peptide changed from a random flexible coil in solution to a helical structure in lysolecithin micelles and dimyristoylphosphatidylserine vesicles. The tryptophan residue in the lipid complexes was located in a hydrophobic environment. The interaction with lipids was shown to involve both hydrophobic and electrostatic forces. 

The tetradecapeptide bombesin has been isolated by Anastasi et al. (1) from amphibian skin. It is a member of a family of compounds that also includes ranatesin, litorin and alytesin. The C-terminal octapeptides are identical in these peptides except that in bombesin and alytesin the amino acid residue at the second last position from the C-terminus is leucine, while in ranatesin and litorin it is phenylalanine (2). The amino acid sequence of bombesin is:

Pyr-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-$NH_2$

The C-terminal octapeptides in amphibian bombesin and porcine bombesin are identical. The Trp and His residues have been shown to be essential for its wide ranging physiological activity (3). It is particularly interesting that recently a high density of receptors for bombesin and bombesin-like peptides have been found in human lung tumours (4) where it has been suggested that bombesin-like peptides play a regulatory function (5). The pharmacological activity as demonstrated by studies performed on synthetic bombesin-like homologs, depended on the C-terminal portion of the peptide and required at least eight amino acid residues (2). It is reasonable to suggest that this dependency is due to the conformational characteristics of the peptide which may play a crucial role in its biological behaviour. This is consistent with the concept that hormonal peptides exist as an ensemble of flexible conformers

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in aqueous solution and which can readily undergo conformational changes required for their activity (6). For example it has been shown that glucagon (7) and β-endorphin (8) change their α-helical content in the presence of lipids.

In this report we present information on the conformation of bombesin in DMPS and in lysolecithin complexes using far UV Circular Dichroism (CD) and the fluorescence emission of the unique tryptophan residue of the peptide.

## MATERIALS AND METHODS

Crystalline bombesin was purchased from Sigma (St.Louis, Mo) and Serva (Heidelberg, FRG) and its purity was assayed by HPLC. The peptide concentration was determined spectrophotometrically assuming an $\varepsilon = 5600$ at 280 nm and a molecular weight of 1620. Lysolecithin and dimyristoylphosphatidylserine (DMPS) were purchased from Avanti Polar Lipids (Birmingham, Ala) and used without further purification. All other reagents used were of analytical grade purity. Lysolecithin was dissolved in buffer at the desired pH, at a concentration higher than 180 μM, the critical micellar concentration. Unilamellar DMPS vesicles were prepared as described previously (9).

Fluorescence measurements were carried out with a Perkin Elmer MPF-44A spectrophotofluorimeter equipped with a DCSU2 correction unit. Fluorescence quantum yields were evaluated using N-acetyltryptophanamide (NATA) as a standard (10). Circular dichroism spectra were obtained using a Jasco 500 A spectropolarimeter equipped with a computer for spectral smoothing. Normally eight spectra were stored and averaged. 0.2 mm cells were used and placed close to the photomultiplier in order to minimize scattering distortions. Absorption measurements were carried out using a Perkin Elmer 576 spectrophotometer. In all experiments the temperature was kept constant at 20° C using thermostated cell holders.

## RESULTS

Fluorescence: The emission spectrum of bombesin in 10 mM cacodylate buffer pH 7, at 20°C had a maximum at 351 nm with a half band width of 57 nm (Fig.1). The addition of 6M GdHCl caused a 30% increase in fluorescence intensity but no shift of the maximum. A red shift in the absorption spectrum in this solution was also observed. The pH behaviour of the fluorescence quantum yield (Fig. 2) showed a sigmoidal profile and a $pK_a$ ca. 6.5. The titrations of bombesin with DMPS at pH 7 and 8.5 are presented in figure 1. In both cases a well defined isosbestic point was evident. The fluorescence maximum shifted to 339 nm and 338 nm respectively. However, while at pH 7 the addition of DMPS caused no change in the intensity of the fluorescence maximum, at pH 8.5 an increase of 35% at 338 nm was observed. For DMPS no change in fluorescence was observed above a lipid to protein ratio (L/P) = 70. At pH 5 the spectral shift was less pronounced, being only 5 nm at L/P = 200. The addition of lysolecithin also caused an increase in the fluorescence intensity (25%) and a spectral shift to 341 nm, but a L/P = 200 was needed to reach saturation.

The fluorescence increase following the interaction with DMPS at pH 8.5 could be used to evaluate the stoichiometry of the binding. The Scatchard plot

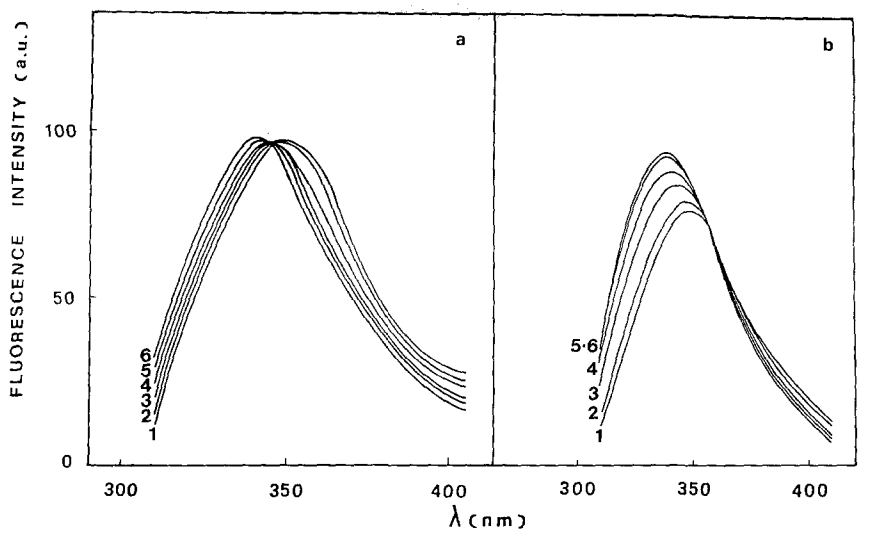
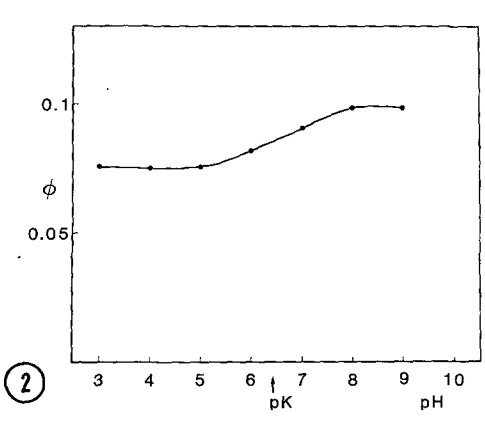



Figure 1. Fluorescence spectra of bombesin ($1.25 \times 10^{-5}$ M) with the progressive addition of DMPS vesicles; spectrum 1, L/P = 0; spectrum 2, L/P = 10; spectrum 3, L/P = 24; spectrum 4, L/P = 37; spectrum 5, L/P = 53; spectrum 6, L/P = 70: a) pH 7; b) pH 8.5.

of the DMPS-bombesin complex is shown in figure 3. Linear regression analysis of this curve provided a binding constant $K_b = 1.3 \times 10^5$ M and the number of DMPS molecules per molecule of bound bombesin was 57. From the binding constant a binding energy $\Delta Go = -6.8$ Kcal/mole could be calculated.

The effect of the ionic strength on the fluorescence spectral shift caused by lipids was tested in order to investigate whether the binding was



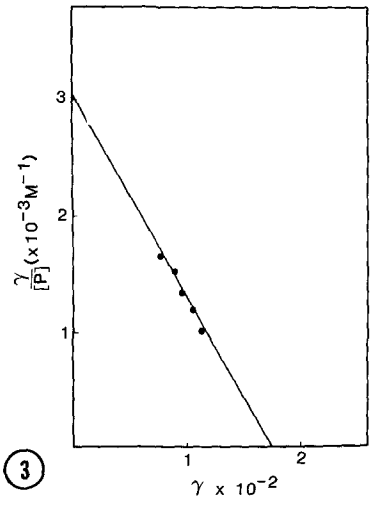




Figure 2. Effect of pH on the fluorescence quantum yield of bombesin.

Figure 3. Scatchard plot of the interaction of bombesin with DMPS vesicles at pH 8.5.

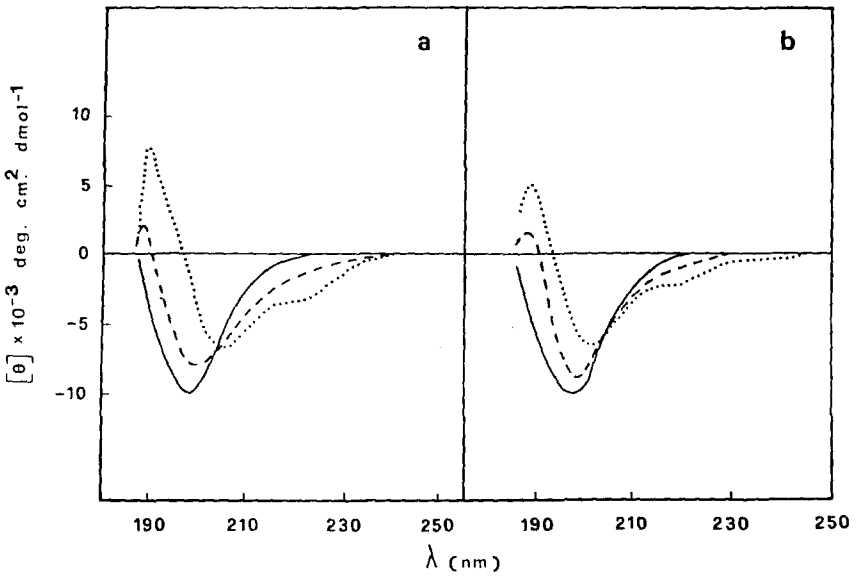



Figure 4. Circular dichroism spectra of bombesin ——— and a) bombesin-DMPS complexes (pH 8.5); L/P = 5 – – – –; L/P = 100 ····· b) bombesin-lysolecithin complexes (pH 7); L/P = 80 – – – –; L/P = 200 ·····

electrostatic in nature. At a saturating DMPS-bombesin ratio, pH 7, the progressive addition of KCl caused a gradual red shift of the fluorescence maximum from 339 nm to 346 nm., ( [KCl] = 300 mM). Further additions of KCl caused the solution to become turbid and the location of a fluorescence maximum could not be determined. On the other hand the addition of KCl up to 0.5 M did not reverse the fluorescence shift caused by the addition of lysolecithin.

Circular dichroism: Far UV CD spectra of bombesin at pH 7 in the absence and presence of several concentrations of DMPS and lysolecithin at different concentrations were therefore recorded and are shown in figure 4. In the absence of lipids the spectrum showed a single negative maximum centered at 200 nm ( $\theta = -10^4$ deg cm$^2$ dmol$^{-1}$), typical of a peptide in a random coil conformation. In the presence of lipids however, the pattern of the spectrum changed dramatically. The overall spectra were red shifted, but showed a positive band with a maximum at 189 nm in both lysolecthin and DMPS. The spectrum of the bombesin lysolecithin complex at L/P = 200, had two negative bands, one at 220-222 nm and one at 203 nm, and one positive peaking at 188 nm. The ellipticities were $-3 \times 10^3$, $-6.4 \times 10^3$ and $5 \times 10^3$ deg cm$^2$ dmol$^{-1}$ respectively. In the presence of DMPS, at L/P = 80, the spectrum also showed three bands one with a positive value peaking at 189 nm,and two negative bands, one at 206 nm and one at 220 nm. The ellipticities of these bands were $7.5 \times 10^3$, $-6.5 \times 10^3$ and $-3.5 \times 10^3$ deg cm$^2$ dmol$^{-1}$ respectively.

DISCUSSION

The fluorescence of bombesin in buffer at all the pH's studied showed a behaviour typical of tryptophan in aqueous solutions (10). The CD pattern in buffer was typical of a polypeptide chain in a random coil conformation. The increase in fluorescence in GdHCl may suggest that bombesin has some secondary structure in aqueous solution. It has been noted that the fluorescence intensity of NATA also increased in GdHCl solutions by a factor of 30 % but this increase can be entirely accounted for by a red shift in the absorption spectrum (11). The sigmoidal pH profile of the quantum efficiency indicated that a ionizable group with a $pK_a$ = 6.5 interacted with the tryptophan. The histidine residue located four residues from the tryptophan would meet this requirement quenching the fluorescence by a proton transfer mechanism (10). This data suggests that bombesin in water is largely in an extended, flexible conformation with very little if any ordered secondary structure. It is interesting to note that both the tryptophan and histidine residues have been shown to be essential for bombesin activity (3).

The blue shift of the fluorescence maximum due to the addition of DMPS (pH 7, pH 8.5) and lysolecithin, demonstrated that bombesin interacted both with charged and neutral lipids. As a result of this interaction with the lipids the tryptophan experienced a more hydrophobic environment and a shift of the emission maximum toward higher energies was consequently observed. The effect of KCl indicated that KCl may compete with the binding of the charged residues of bombesin with DMPS, and suggested that there was an electrostatic interaction in the case of the negatively charged DMPS vesicles. Alternately the salt may alter the structure of these vesicles preventing the penetration of the peptide into the interior of the acyl chain environment. We are unable to distinguish between these alternatives at this time but plan further experiments to clarify this important point. However the lack of any KCl effect on the bombesin-lysolecithin complex, suggested that the interaction force was mainly hydrophobic in this latter case. The fluorescence enhancement in lysolecithin (pH 7) and DMPS (pH 8.5) implied that the tryptophan residue penetrated further into the lipid bilayer in these cases than in DMPS complexes at pH 7 or pH 5. Additionally in the DMPS complexes at pH 8.5 the histidine residue would now be unionized and unable to quench the tryptophan fluorescence. Indeed the state of protonation of the histidine may play an important role in determining the nature of the complex with DMPS. At pH 5 the histidine would be fully protonated so that the peptide bound only to the surface and prevented significant penetration of the peptide into the interior of the lipid bilayer and hence only a small shift of 5 nm to the blue was observed. At pH 7 the histidine would only be partially protonated and both types of binding could be operating, while at pH 8.5 the entire peptide

population would now be able to enter the acyl chain environment. Over this pH range and at 20° C the DMPS vesicles are in a gel state (12).

The amphipathic character of bombesin evaluated with the SOAP method of Kyte and Doolittle (13) showed that the N-terminal portion of the peptide was hydrophilic, while the C-terminal portion was hydrophobic. Hence it is entirely reasonable to suggest that the hydrophobic C-terminus of the peptide enters into the interior of the DMPS vesicles while the positively charged arginine residue near the N-terminus binds to the negatively charged head group at all pH values studied. Additionally at pH 5 the charged histidine would also bind to the surface. In the lysolecithin case, as stated earlier the binding is probably entirely hydrophobic. A similar behaviour has been observed in the interaction of β-Endorphin with cerebroside sulfate (8).

The free energy change of $\Delta G = -6.8$ Kcal/mole calculated for the DMPS-bombesin (pH 8.5) interaction was very similar to the values found for the lipid binding of amphipathic helices such as the apolipoproteins or glucagon (14). These proteins are considered to associate with membranes by forming α-helices whose hydrophobic structure penetrate into the membranes whereas the hydrophilic portion remains in contact with water. The formation of α-helices in bombesin on binding to lipids was supported by the circular dichroism results. The decrease of negative ellipticity at 200 nm and increase of positive ellipticity at 190 nm clearly indicated that the peptide assumed a degree of helical conformation due to the interaction with lipids. The short length of the bombesin chain makes it difficult to predict at what level the helix formation occurs, but it would seem to be reasonable to assign it to the hydrophobic C-terminal portion.

Recalling that it has been demonstrated that the C-terminal octapeptide was essential for the activity of bombesin (6), these conformational changes which we have reported may play an important role in the physiological activity of the hormone. We suggest that when bombesin binds to membranes in vivo the peptide may adopt a rigid structure in an hydrophobic environment, and that the C-terminal segment of the peptide then can interact with the receptor resulting in its biological activity.

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