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Role of tryptophan-14 in the interaction of dynorphin A(1–17) with micelles

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Fluorescence spectroscopy has been used to examine the interaction between the opioid peptide dynorphin A(1–17) (dynorphin) and dodecylphosphocholine (DPC) micelles. Fluorescence emission spectra as a function of added lipid indicate insertion of the Trp^{14} side chain into the hydrophobic portion of the micelle, supporting NMR results from this laboratory. A model of interaction with micelles consistent with the fluorescence results and earlier NMR results is proposed. The critical micelle concentration in the presence of peptide was also determined, and is discussed in the context of relevance to both NMR spectroscopy and peptide–lipid interactions. © Munksgaard 1997.

Key words: critical micelle concentration; dodecylphosphocholine; dynorphin A; fluorescence; NMR

There is considerable likelihood that peptide hormones interact with cell membranes, either prior to or as a part of interaction with their receptor (1–4). Until recently, this was a concept that could not be related to experiments employing purified opioid receptors. Cloning of opioid receptors now permits their use in actual determination of active amino acid residues in each receptor type (5–8). Experimental evidence in the form of mutation and ligand binding studies obtained on the cloned opioid receptors shows that certain amino acid residues which are presumed to be in the trans-membrane helices close to the extracellular region are required for opioid peptide activity (9, 10). This implies that the cell membrane plays a role in ligand binding, although its exact role remains to be elucidated.

As membranes are not amenable to study using solution NMR, we and others have used micelles to mimic the lipid environment of the membrane (11, 12). Such a model serves the purpose of reasonably simulating the chemically anisotropic environment of a membrane so that solution NMR studies may be accomplished. The advantage of this approach is that the peptide being studied may interact with the lipid through electrostatic, hydrophobic and other non-bonded interactions, limiting the range of conformations a linear peptide samples in solution. As such interactions almost certainly occur when a peptide encounters a biological membrane, this approach is more informative than examining the range of conformations a linear peptide samples in solution. Micelles have been found to be so useful for solution NMR that a number of membrane associating proteins and peptides have had their micelle bound structures determined (13–17). Accordingly, we have determined the structures of several peptides in the presence of dodecylphosphocholine micelles (18–20).

Although the micelle environment facilitates high-resolution structure determination when the peptide or protein is bound, little is known about where individual residues specifically interact with the lipid micelle and what effect peptide binding has on the critical micelle concentration. Both issues need clarification for a more thorough understanding of the advantages and limitations of the use of micelles in NMR spectroscopy. Fluorescence spectroscopy and other techniques have been used successfully in a number of cases to address the issue of peptide environment in various types of bilayers. For example, there are many informative studies using tryptophan fluorescence in the presence of lipid investigating the role of charge and hydrophobicity in peptide lipid interactions (21–24). More difficult issues being addressed with fluorescence spectroscopy include the depth of insertion and the orientation of tryptophan residues in the membrane (25). Few of these studies, however, have been carried out in micelles, in part because of the obvious differences between micelles and bilayers. Differences in curvature, electrostatics and other properties notwithstanding, micelles have been widely used for solution NMR studies because of their advantageous motional prop-

erties and phase stability. Thus there is a need to understand peptide–micelle interactions in more detail.

Herein, we have used Trp^{14} in dynorphin A(1–17) (dynorphin, **1**) as a fluorescent probe to complement our NMR studies (12, 18, 20).

H-Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-
Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln-OH (**1**)

This seventeen residue peptide has been to shown to be an endogenous ligand for the κ-opioid receptor (26). Dynorphin A(1–13) (the *N*-terminal 13 residues of dynorphin) was shown to exhibit similar activity in opioid binding assays, so that most laboratories have used the shorter peptide (27, 28). It is the 17-residue peptide, however, that is one of the main products of the prodynorphin processing, suggesting that the last four residues play some biological role (29, 30). There are other differences between the 13-mer and the 17-mer. The difference most germane to the work described within is that dynorphin A(1–17) has increased resistance to proteolytic degradation over the 13-mer (31, 32). It is reasonable to speculate that the last four residues of the 17-mer confer some protective role upon the full length peptide. More specifically, Trp^{14} may anchor the peptide to the membrane such that the *C*-terminus is protected. Regardless of the biological significance, it is easily inferred from studies on other peptides and proteins that Trp^{14} would likely mediate dynorphin's interaction with a lipid. Accordingly, the work described within shows that Trp^{14} interacts with dodecylphosphocholine (DPC) micelles using fluorescence emission spectra. This is consistent with NMR studies in this laboratory, which suggested that Trp^{14} is hydrophobically sequestered in the presence of micelles (20). Amide proton exchange experiments showed that the Trp^{14} amide proton, along with the Leu^5, Arg^6, Arg^7 and Ile^8 protons, exchange slowly with water in the presence of micelles. In addition, NOE data indicated the presence of helix from Gly^3 to Arg^9 and a turn from Trp^{14} to Gln^{17}. As no partner hydrogen bond was found for the Trp^{14} amide proton, one may assume from the slow exchange behavior that the Trp^{14} residue is hydrophobically sequestered in the micelle. This assumption, taken together with the complete NMR data (20), suggests a model of interaction with the micelle, schematized in Fig. 1. In this model, the side chains of residues Phe^4, Leu^5, Ile^8 and Trp^{14} are on the same hydrophobic 'face' of the molecule, interacting with the head region of the micelle. Figure 1a shows the orientation of the peptide with respect to the micelle, illustrating the average Gly^3 to Trp^{14} distance. Figure 1b shows the specific amino acid residues that interact with the head region of the micelle. The model is supported by the fluorescence emission spectroscopy data within, in which it is shown that

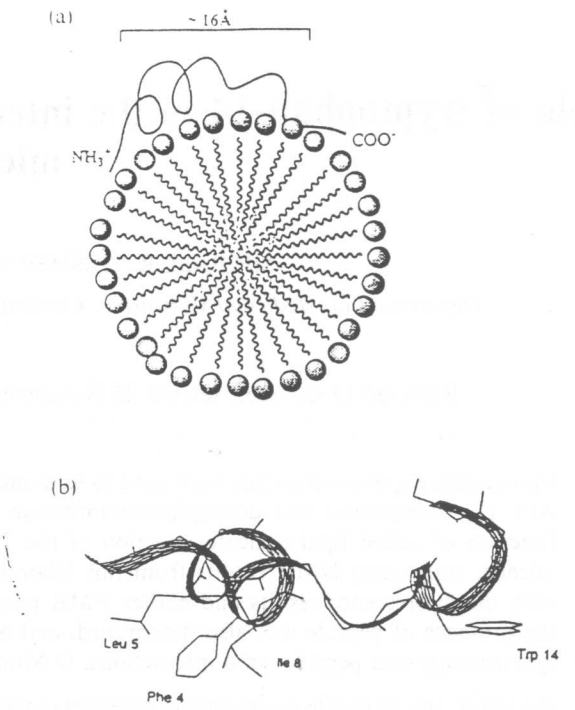

FIGURE 1

Model of interaction of dynorphin A(1–17) with DPC micelles [Tessmer & Kallick (20)]. The peptide interacts with the head region of the lipid with several of its amino acid side chains and part of the peptide backbone (a) in the orientation shown (b) with the specific side chains that interact with the micelle head region highlighted.

Trp^{14} is inserted into the hydrophobic region of the micelle.

In the course of these studies it became apparent that Trp^{14} is also a good probe for measuring the critical micelle concentration (CMC). Critical micelle concentrations have been determined historically by a variety of techniques, most commonly by surface tension measurements. The CMC in the presence of peptide, however, is an important quantity to measure for several reasons. Perhaps the most important reason is that NMR spectroscopists rely on the CMC determined in the absence of peptide, which was shown to be 1.0 mM for DPC (33). As structure determination must be carried out well above the CMC, the effect of peptide on the CMC is of considerable interest. Earlier work in this laboratory showed the effect of high lipid concentration on the aggregation number of the micelles using NMR diffusion measurements (12). In that work the conformation of dynorphin was also investigated over a large range of lipid concentrations. Herein, we examine the effect of low lipid concentrations on the behavior of dynorphin and show the CMC determination using fluorescence spectroscopy by titrating the lipid into the tryptophan containing dynorphin.

EXPERIMENTAL PROCEDURES

Dynorphin A(1–17) was obtained from Star Biochemicals (Torrance, CA) and was 99% pure. Dodecylphosphocholine (DPC) was from Avanti Polar Lipids, Inc. and was used without further purification (Alabaster, AL). Sodium phosphate was from Baker Analytical Reagents (Philipsburg, NJ). All samples were in H_2O with 10 mM phosphate buffer at pH 3.4, identical to the NMR conditions in all respects except solute concentration. Quartz cells from Starna Cells, Inc (Atascadero, CA) were employed for fluorescence measurements. Samples were placed in a constant temperature bath at 298 K and allowed to reach equilibrium for 10 min prior to excitation. The tryptophan residue of dynorphin was excited at 280 nM with an excitation slit width of 2.5 nm. Subsequent fluorescence emission spectra were recorded from 300 to 440 nm with an emission slit width of 2.5 nm at zero lipid added and several different concentrations of lipid. The data were collected on a Perkin-Elmer LS50B spectrofluorimeter. Data were transfered to an Apple computer for processing and plotting. The model of interaction was created with one of the peptide structures generated from DIANA calculations and a model of the micelle (20).

RESULTS AND DISCUSSION

Fluorescence emission spectroscopy was employed to examine the environment of the Trp^{14} side chain indole moiety in dynorphin when the peptide is subjected to increasing amounts of DPC. Figure 2 contains a series of fluorescence emission spectra of dynorphin at micromolar concentrations with increasing amounts of DPC. Earlier studies had confirmed that dynorphin does not oligomerize at any pH from micromolar to well above millimolar concentrations (D. Kallick, unpublished data). Figure 2 shows that the maximum emission wavelength in the absence of added lipid is $\lambda = 350$ nm. At 1.0 mM added lipid the maximum emission wavelength shifts to 345 nm with an increase in intensity, and at 1.5 mM added lipid the maximum emission wavelength shifts to 337 nm with a concomitant increase in intensity. The upper lipid concentration at 2.0 mM represented in Fig. 2 shows that the maximum emission wavelength is about the same as it is at 1.5 mM, 337 nm, but the intensity of the emission increases. The observation of a blue shift in the λ_{max} of fluorescence emission, and an increase in the maximum intensity upon addition of DPC to a solution of dynorphin, both indicate the placement of the Trp^{14} side chain into a less polar environment (34). The contribution of the tyrosine fluorescence emission is expected to be minimal at the wavelengths observed, and the tyrosine emission maximum is relatively insensitive

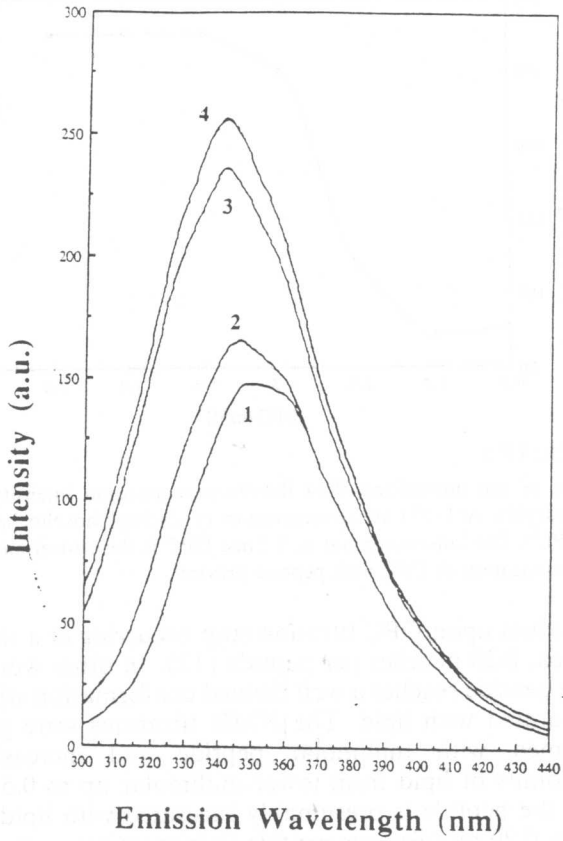

FIGURE 2

Overlay plot of dynorphin A(1–17) fluorescence emission spectra with increasing amounts of dodecylphosphocholine (DPC). The dynorphin A(1–17) concentration was 2.0×10^{-5} M, and the DPC concentrations used were: (1) 0, (2) 1.0, (3) 1.5 and (4) 2.0 mM.

to solvent polarity changes (34). NMR studies support the fluorescence data by the observation of a stabilized Trp^{14} side chain as part of a β-turn at the *C*-terminus of the peptide and a hydrophobically sequestered amide proton in the Trp^{14} residue (20). The model of interaction is shown in Fig. 1, illustrating (a) the orientation of the peptide with respect to the micelles and (b) the hydrophobic amino acid side chains that interact with the head region of the micelle.

Figure 3 is a plot of the normalized fluorescence emission intensity as a function of DPC concentration. An inflection point at 1.2 mM is observed, interpreted as the CMC of the lipid in the presence of dynorphin. Figure 3 also shows that the intensity changes level off at 2 mM lipid. Assuming 50 monomers per micelle from earlier NMR measurements, the concentration of micelles present at 1.2 mM DPC is 21.8 μM (11, 12). The concentration of dynorphin for the studies represented in Fig. 3 was 20 μM, giving an approximate 1 : 1 ratio of peptide to micelle. NMR studies in this laboratory have shown that chemical shift changes in the Trp^{14} and other amino acid

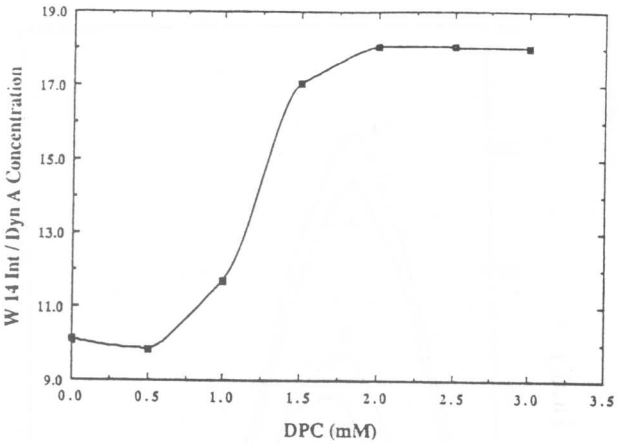

FIGURE 3

Plot of the normalized W14 fluorescence emission intensity of dynorphin A(1–17) vs. concentration of dodecylphosphocholine (DPC). The inflection point at 1.2 mM DPC is the critical micelle concentration of DPC with peptide present.

residues upon DPC titration stop occurring at a ratio of ca. 0.20 micelles per peptide (12). In other words, the peptide reaches a well defined conformation when saturated with lipid. The NMR titrations were performed with millimolar peptide and increasing amounts of lipid from lower millimolar up to 0.5 M. As the peptide is presumably saturated with lipid at only 0.20 micelles per peptide, it is most assuredly so with peptide concentrations of 20 μM used here. This is supported by the fluorescence studies, as no further changes in either fluorescence intensity or emission wavelength are observed at DPC concentrations of up to 9 mM DPC (ca. 180 μM micelle) using 20 μM dynorphin (data not shown).

Dynorphin and other peptides bind stably to DPC such that their conformations may be determined to high resolution well above the CMC (12). Most structural studies on dynorphin have focused on the 13-mer, since its binding at the κ-opioid receptor is similar to that of the full-length peptide (27, 28). Work done to date on the full-length, endogenous ligand (the 17-mer) has been predominantly on model peptides to study the amphipathic nature of residues Arg^7 through Asp^{15} (35, 36). The 13-mer is found *in vivo*, however, in only very low quantities (37, 38). The studies presented here suggest that the insertion of the Trp^{14} indole moiety into a lipid environment may be one of the reasons for the differences between the 13-mer and 17-mer of dynorphin. Indeed, Kaiser and Kezdy suggested protection from proteolytic degradation may be one of the roles of the membrane in binding peptide hormones *in vivo* (2).

As micelles and membranes have significant differences, it is important to relate our results to similar work in membranes. A recent study using similar fluorescence techniques showed that the Trp^{14} in dynorphin A(1–17) also 'bends back' to interact with the lipid environment in phosphatidylserine vesicles, similar to the model of interaction we propose in Fig. 1 (39). Their presumption that the peptide interacts with the lipid in the *N*-terminus was based on the work of Schwyzer and coworkers (1, 3, 4). Because of the similarity in behavior of dynorphin in vesicles and micelles, this supports the contention that micelles are sufficiently similar to membranes in the case of dynorphin–lipid interactions. Interestingly, the Trp^{14} is not required for binding to micelles, as the 13-mer binds to DPC micelles in a conformation that appears to be similar to the 17-mer (Tessmer & Kallick, unpublished data). It is possible that binding of dynorphin in either form is mediated by the hydrophobic face of the helix, but further studies are required to assess the various contributions. Purely speculatively, if these peptides behave similarly at the cell membrane–receptor complex, this supports our hypothesis that the last four residues of dynorphin play a protective role *in vivo*.

A comparison to earlier fluorescence studies on dynorphin in aqueous solution is also instructive (40). Singlet–singlet energy transfer experiments with Tyr^1 as a donor and Trp^{14} as an acceptor indicated that the fluorophores were at least 20 Å apart (40). One would expect the distance in micelles to be somewhat shorter, as the peptide assumes a helical conformation from Gly^3 to about Arg^9, followed by turn as illustrated in Fig. 1a (20). The measured average distance from Gly^3 to Trp^{14} is ca. 16 Å, as shown in Fig. 1a. We also calculated an average distance between the Tyr^1 and Trp^{14} fluorophores of ca. 19.5 Å. This distance is perhaps less reliable than the Gly^3 to Trp^{14} distance because of the less well defined *N*-terminal two residues (20), but indicates that the fluorophores may be closer together in micelles than they are in aqueous solution.

CONCLUSIONS

Fluorescence data presented here support the presumption that dynorphin binds to DPC micelles and increases the CMC slightly over the CMC in the absence of peptide. Comparison of tryptophan's insertion into a more hydrophobic environment in micelles to earlier data in phosphatidylserine vesicles illustrates the similarities of interaction, despite the differences between bilayers and micelles. If dynorphin interacts only with the head region of the lipid as we propose, this is not too surprising. Further studies in this laboratory are elucidating more details of specific peptide–lipid interactions. The similarity observed here between vesicles and micelles is an important result for NMR spectroscopists who rely on micelles to model a membrane. While fluorescence studies cannot give precise information on three-dimensional structure, they are useful for examining peptide–lipid interactions and serve as a complement

to other structural methods. This work also may have important implications for drug delivery, as micelle-solubilized peptides could be a possible vehicle for delivering peptides across cell membranes. Characterization of peptide–lipid interactions is critical for further development in this area.

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