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SOLUTION AND MEMBRANE STRUCTURE OF ENKEPHALINS AS STUDIED BY INFRARED SPECTROSCOPY[1]

Witold K. Surewicz and Henry H. Mantsch

Division of Chemistry, National Research Council of Canada
Ottawa, Ontario, Canada K1A 0R6

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Summary: The backbone conformation of the two opioid pentapeptides Leu^5-enkephalin and Met^5-enkephalin was studied by the technique of resolution-enhanced infrared spectroscopy. In aqueous solution, the conformation-sensitive amide I bands of the two peptides are identical. The positions of these bands are consistent with the view that in aqueous solution both enkephalins exist as an ensemble of largely unfolded conformers. Interaction of Leu^5- and Met^5-enkephalins with bilayer membranes of ditetradecylphosphatidylcholine results in a substantial refolding of the peptide backbones. The conformation stabilized by the membrane environment is a hydrogen-bonded turn structure. Conformational transitions in enkephalins induced by a lipid environment may play a role in the specific interactions between these hormones and their receptor sites.

The endogenous opioid pentapeptides - enkephalins - are implicated in a wide variety of physiological processes (1). Attempts to delineate the structure-function relationships for enkephalins have resulted in numerous physicochemical studies of the peptide conformation. X-ray diffraction experiments with single crystals of Met^5- and Leu^5-enkephalins established two basic conformations of the crystalline forms of the peptides: a type I' β-turn folded form and a dimeric antiparallel extended structure (for a recent review on enkephalin conformation, see reference (2)). Studies with enkephalins in aqueous and non-aqueous solutions were less conclusive and led to somewhat conflicting models (2).

While the structures determined for opioid pentapeptides in single crystals and in aqueous solution are of obvious intrinsic interest, attempts to establish a conformational basis for the biological action of enkephalins are complicated by the fundamental question whether these structures provide an adequate description of the active conformation of the peptides at the receptor site. Recent hypotheses postulate the functional involvement of a lipid matrix of a target cell membrane in specific hormone-receptor interactions (3-8). The role of lipids could be to facilitate the accumulation of the peptide hormone

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near the receptor site and to induce the specific conformation of the peptide required for its biological activity (3-8). Experimental results on the conformation of membrane-associated enkephalins and other peptide hormones are now urgently needed in order to test these stimulating concepts.

One of the techniques suitable to study the conformation of proteins and peptides in different environments is Fourier-transform infrared (FT-IR) spectroscopy. The technique is of particular value when aided by the methodology of band resolution enhancement (9-12). Earlier infrared spectra of opioid peptides were interpreted as showing differences between the conformations in aqueous solution of Leu^5- and Met^5-enkephalins (13). While the original motivation of the present study was to establish the secondary structure of opioid peptides in a membrane environment, a careful reinvestigation of the solution spectra of enkephalins prompted us also to reassess the previous infrared spectroscopic results for the aqueous forms of the peptides.

MATERIALS AND METHODS

Met^5-enkephalin and Leu^5-enkephalin (acetate salt) were obtained from Bachem; ditetradecylphosphatidylcholine was from Dr. R. Bertchold, Biochemisches Labor, Bern. Enkephalin solutions were obtained by dissolving the appropriate peptide (15 mg/ml) in MES buffer prepared in deuterium oxide and adjusted to p^{2}H 6. Peptide-lipid samples were prepared by adding peptide solution in buffer to a solid lipid so that the lipid-peptide weight ratio of 15:1 was reached. The mixture was vortexed for approximately 5 min during which time the samples were warmed and cooled repeatedly through the lipid transition temperature.

Samples for infrared spectroscopy were assembled between CaF_2 windows separated by a 50 µm teflon spacer. Infrared spectra were recorded at 32°C with a Digilab FTS-60 instrument. For each spectrum, between 250 and 1000 interferograms were co-added and Fourier-transformed to give a resolution of 2 cm^{-1}. Spectra in the 1500 - 1800 cm^{-1} region were corrected for the weak absorption of the buffer. The signal-to-noise ratio was better than 1000:1.

RESULTS AND DISCUSSION

Enkephalins in aqueous solution. Infrared spectra of the aqueous solutions of Leu^5-enkephalin and Met^5-enkephalin are shown in Figs. 1A and 2A, respectively. The conformation-sensitive amide I bands of the two peptides are virtually identical. They extend between approximately 1620 and 1705 cm^{-1}, with a maximum at 1652 cm^{-1} and a distinct shoulder on the high-wavenumber side.

The direct interpretation of the spectra of Figs. 1A and 2A is difficult as the broad amide I band contours of proteins and peptides are usually composed of a number of unresolved components representing backbone amide groups in different conformations (10,14). However, these component bands can be identified by the computational procedure of band narrowing by Fourier self-deconvolution (9-12,14). The deconvolved spectra of both Leu^5- and Met^5-enkephalins (Fig. 1B and 2B) show four component bands in the amide I region: at 1680, 1652, 1643 and 1626 cm^{-1}. Whereas the band at 1643 cm^{-1} can

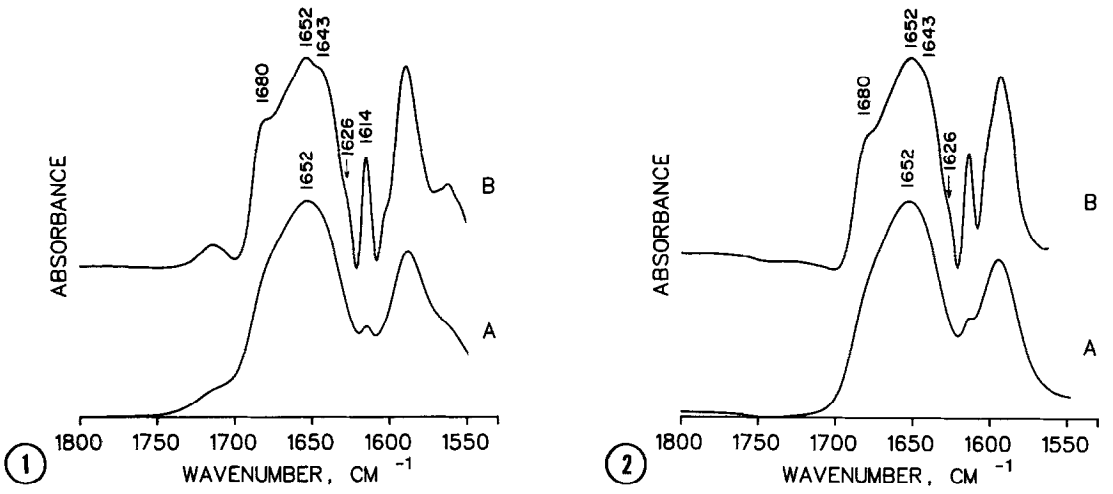

Fig. 1. Infrared spectrum of Leu^5-enkephalin in aqueous solution before (A) and after (B) band narrowing by Fourier self-deconvolution using a 15 cm^{-1} half-width Lorentzian line and a resolution enhancement factor of 2.2.

Fig. 2. Infrared spectrum of Met^5-enkephalin in aqueous solution before (A) and after (B) band narrowing using the parameters given in the legend to Fig. 1.

be assigned to solvated (and deuterium-exchanged) amide gorups of the backbone in a non-ordered conformation (10,14), the origin of a strong band at 1652 cm^{-1} is less clear. In the infrared spectra of polypeptides or proteins, bands around 1655 cm^{-1} are often associated with α-helices (10,14,15); however no helical-structures could be detected in enkephalins by other spectroscopic methods (2) or by theoretical calculations (16). While the 1652 cm^{-1} band in the infrared spectra of enkephalins may represent certain atypical non-periodic structures, no firm conlcusions can be drawn without further normal mode calculations of the vibrational frequencies of enkephalins. The band at 1680 cm^{-1} most likely represents turns (10) and the band at 1626 cm^{-1} is compatible with amide groups in β-strands (10,14). However, the very weak intensity of the latter band indicates that the probability for a β-structure in aqueous solution of enkephalins is very low. The remaining bands in Figs. 1 and 2 are outside the amide I region and represent vibrational modes due to end carboxyl groups (17) and due to the amino acid side chains (18).

Infrared spectra obtained in this study for Leu^5-enkaphalin and Met^5-enkephalin are consistent with the view that in aqueous solution the peptides exist as an ensemble of conformers (2,19-22). In addition to the prevailing non-ordered conformations, the most likely folded conformers appear to be turn-like structures. These conclusions are in apparent contrast with those of a previous infrared spectroscopic study of enkephalins. The Fourier self-deconvolved spectrum of Leu^5-enkephalin reported by Renugopalakrishnan et al. (13) shows four amide I component bands at 1632, 1658, 1663 and 1675 cm^{-1}, whereas that of Met^5-enkephalin has only two bands at 1636 and 1678 cm^{-1}. Accordingly,

the authors concluded that while Leu^5-enkephalin exists in aqueous solution in both β-turn and β-sheet structures, the conformation of Met^5-enkephalin is predominantly that of a β-sheet type. The allegedly different conformational preferences of Met^5- and Leu^5-enkephalins in solution could have far reaching implications for the receptor binding and biological activities of the peptide analogues (13). However, our data argue against the presence of a large proportion of β-sheet structure in an aqueous solution of enkephalins. Moreover, they clearly do not support the notion of substantial differences between the solution conformations of Leu^5- and Met^5-enkephalins.

While a number of factors may contribute to the discrepancy between the results of the present and those of a previous infrared study, a few points stand out. First of all, Renugopalakrishnan et al. (13) used the technique of attenuated total reflection (ATR) infrared spectroscopy. It is very likely therefore that the spectra obtained under these conditions represent mostly enkephalins adsorbed to the ATR plate and not (as assumed by the authors) the free peptides in aqueous solution. Moreover, the low instrumental resolution used in the ATR experiments (8 cm^{-1}) combined with a poor signal-to-noise ratio, greatly increases the potential for artifacts in a band-narrowing analysis of the spectra. Since the random noise is greatly amplified by the resolution-enhancement procedures (23), spectra with a high signal-to-noise ratio are a prerequisite for meaningful deconvolutions. The strong features in a "signal-free" region between 1720 and 1800 cm^{-1}, which are indistinguishable from the real peptide bands (Figs. 1 and 4 of reference (13)), suggest that this crucial condition might have not been fulfilled.

Enkephalins in a lipid environment. Figure 3A shows the infrared spectrum of Leu^5-enkephalin in the presence of a large excess of ditetradecylphosphatidylcholine (DTPC)* (peptide to lipid weight ratio of 1:15). The maximum of the amide I band contour in Fig. 3A is at 1663 cm^{-1}, i.e. at a wavenumber 11 cm^{-1} higher compared to that observed in the spectrum of a lipid-free aqueous solution of the peptide (Fig. 1A). This large shift of the position of the Leu^5-enkephalin amide I band contour indicates substantial conformational changes upon peptide binding and/or incorporation into the lipid bilayers. Fourier self-deconvolution of the spectrum in Fig. 3A reveals amide I component bands at 1680, 1664, 1653 and 1624 cm^{-1} (Fig. 3B). While the bands at 1680, 1653 and 1624 cm^{-1} correspond closely to those found in the spectrum of Leu^5-enkephalin in solution, the prominent band at 1664 cm^{-1} is a feature characteristic only of the lipid-associated peptide. This new band has been

*The use as a model phospholipid of ether-linked DTPC instead of the more conventional ester-linked analogue (dimyristoylphosphatidylcholine) was dictated by the necessity to eliminate the strong lipid carbonyl band. At the lipid to peptide ratio used in this study, the strong lipid band interferes with the much weaker amide I band of the peptide backbone.

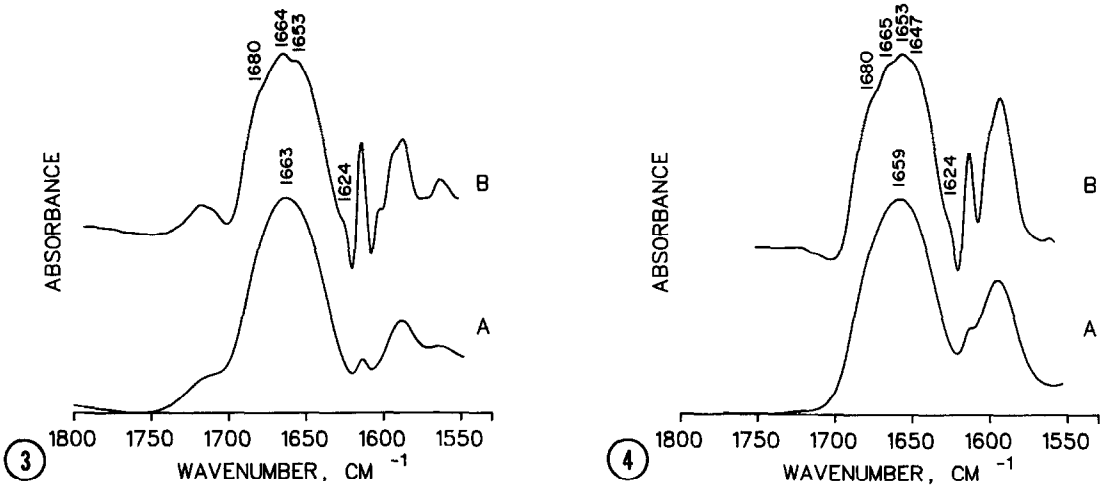

Fig. 3. Infrared spectrum of Leu^5-enkephalin in the presence of ditetradecylphosphatidylcholine bilayers (lipid to peptide weight ratio of 15:1) before (A) and after (B) band narrowing (see legend to Fig. 1).

Fig. 4. Infrared spectrum of Met^5-enkephalin in the presence of ditetradecylphosphatidylcholine bilayers (lipid to peptide weight ratio of 15:1) before (A) and after (B) band narrowing (see legend to Fig. 1).

attributed to amide groups involved in turns (10,14). Another difference between the spectra of lipid-free and lipid-bound Leu^5-enkephalin is the absence in the latter of a component band around 1643 cm^{-1}. This suggests that the new turns observed in the lipid-associated peptide are formed largely at the expense of originally (i.e. in aqueous solution) non-ordered structures.

The changes induced by DTPC in the infrared spectrum of Met^5-enkephalin are qualitatively similar to those described above for Leu^5-enkephalin, although they are somewhat less pronounced. The amide I band contour of Met^5-enkephalin is shifted in the presence of phospholipid only by 7 cm^{-1}, i.e. to 1659 cm^{-1} (Fig. 4A). The deconvolved spectrum of Met^5-enkephalin/DTPC (Fig. 4B) indicates turn structures similar to those observed with Leu^5-enkephalins (band at 1665 cm^{-1}); however, the band at 1647 cm^{-1} suggests that at a similar lipid to peptide ratio, the proportion of non-ordered structures in Met^5-enkephalin is higher than in Leu^5-enkephalin.

Although not all component bands that have been resolved in the amide I region of the infrared spectra of enkephalins can be unambigously assigned to specific conformational entities, the present study provides clear evidence that the interaction of Leu^5- and Met^5-enkephalins with the bilayers of DTPC results in substantial refolding of the peptide backbones. The bilayer-associated enkephalins have a markedly increased tendency to form turns. Moreover, the pattern of hydrogen-bonding in these lipid-induced turns is distinct from that in the turn-like structures in the aqueous solutions of the peptides.

Folding into intramolecularly hydrogen-bonded turn structures has

been previously inferred from proton nuclear magnetic resonance spectra of enkephalins bound to lysophosphatidylcholine micelles (24). However, confident extrapolation of these data to events occurring within the membranes of target cells was hampered by the differences between the properties of the micelles and those of bilayer structures which prevail in biological membranes. The present experimental approach has allowed us for the first time to directly probe the conformation of enkephalins in the environment of lipid bilayers. Our results strongly reinforce the notion (24) that it is a hydrogen-bonded turn structure that is the most likely candidate for a "bioactive" conformation of enkephalins.

The observed lipid-induced conformational changes in enkephalins may have important implications for the recently proposed model of membrane-assisted opioid receptor selectivity (7). According to this model, the selection mechanisms based on peptide-membrane interactions require that the κ- and μ-sites of the opioid receptor are associated intimately with the target cell membrane, whereas the δ-sites protrude into the aqueous compartment, being separated from the membrane surface by not less than the Debye-Hückel length. Such a localization of the δ-sites was based, at least in part, on the assumption that the direct interaction between the δ-selective opioid peptides and the lipid bilayer is very weak. The present experimental results with Met^5- and Leu^5-enkephalins do not, however, corroborate this notion. Enkephalins, while strong δ-receptor agonists, apparently do interact with a bilayer surface; this results in a substantial alteration of the hormone conformation. The folded turn structures induced by the lipid phase of a target cell membrane are likely to play a role in the sequence of events that lead to the specific interaction between enkephalins and their receptor sites.

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