Kitagawa, T., Kyogoku, Y., & Orii, Y. (1977) *Arch. Biochem. Biophys. 181*, 228–235.
Kitagawa, T., Abe, M., & Ogoshi, H. (1978a) *J. Chem. Phys. 69*, 4516–4525.
Kitagawa, T., Ozaki, Y., & Kyogoku, Y. (1978b) *Adv. Biophys. 11*, 153–192.
Kitagawa, T., Nagai, K., & Tsubaki, M. (1979) *FEBS Lett. 104*, 376–378.
Maxwell, J. C., Volpe, J. A., Barlow, C. H., & Caughey, W. S. (1974) *Biochem. Biophys. Res. Commun. 58*, 166–171.
Nagai, K., Kitagawa, T., & Morimoto, H. (1980) *J. Mol. Biol.* (in press).
Righetti, P., & Drysdale, J. W. (1971) *Biochim. Biophys. Acta 236*, 17–28.
Salmeen, I., Rimai, L., Gill, D., Yamamoto, T., Palmer, G., Hartzell, C. R., & Beinert, H. (1973) *Biochem. Biophys. Res. Commun. 52*, 1100–1107.
Salmeen, I., Rimai, L., & Babcock, G. T. (1978) *Biochemistry 17*, 800–806.
Sono, M., & Asakura, T. (1974) *Biochemistry 13*, 4386–4393.
Sono, M., & Asakura, T. (1975) *J. Biol. Chem. 250*, 5227–5232.
Spiro, T. G. (1975) *Biochim. Biophys. Acta 416*, 169–187.
Spiro, T. G., & Strekas, T. C. (1974) *J. Am. Chem. Soc. 96*, 338–345.
Spiro, T. G., & Gaber, B. P. (1977) *Annu. Rev. Biochem. 46*, 553–572.
Takano, T. (1977a) *J. Mol. Biol. 110*, 537–568.
Takano, T. (1977b) *J. Mol. Biol. 110*, 569–584.
Yamamoto, T., Palmer, G., Gill, D., Salmeen, I. T., & Rimai, L. (1973) *J. Biol. Chem. 248*, 5211–5213.
Yamazaki, I., Yokota, K., & Shikama, K. (1964) *J. Biol. Chem. 239*, 4151–4153.

Interaction of Opioid Peptides with Model Membranes. A Carbon-13 Nuclear Magnetic Study of Enkephalin Binding to Phosphatidylserine†

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ABSTRACT: The binding of enkephalin to phosphatidylserine has been studied, by using ^{13}C NMR, as a model for interactions with components of biological receptors. Chemical shifts, line widths and spin–lattice relaxation times were measured for peptides enriched to 90% in ^{13}C. The pK_a values of the terminal amino and carboxyl groups were determined from the pH dependence of the ^{13}C chemical shifts. Interaction of (2-[2-^{13}C]glycine)methionine-enkephalin, (3-[2-^{13}C]-glycine)methionine-enkephalin, and (3-[2-^{13}C]glycine)methionine-enkephalinamide with phosphatidylserine (PS) was studied as a function of pH. Salt and morphine antagonism to binding was manifest. Binding was shown to be pH dependent, exhibiting a maximum under slightly acidic conditions. Whereas the $-NH_3^+$ group of enkephalin is essential for binding, the data suggest that neither the tyrosyl hydroxyl group nor the COO^- group is involved. Binding affects the ^{13}C spin–lattice relaxation times most strongly; the chemical shifts and line widths of the ^{13}C-enriched material show little perturbation in the presence of PS. The internal flexibility of the peptides is decreased, on binding to model membranes, by 1 order of magnitude. Dissociation constants have been measured as 4×10^{-1} M and 2.6×10^{-3} M for enkephalin and enkephalinamide, at pH 6.3 and 6.4, respectively.

Structure–activity studies on the opioid peptides, in particular the enkephalins, have attempted to relate not only structural but also conformational features of these peptides to the geometry of morphine. Although X-ray (Smith & Griffin, 1978) and solution (Bleich et al., 1977; Jones et al., 1977; Roques et al., 1976; Garbay-Jaurequiberry et al., 1976) studies, as well as theoretical calculations (De Coen et al., 1977; Isogai et al., 1977), may indicate a preferred conformer or set of preferred conformations (Combrisson et al., 1976; Deslauriers et al., 1978; Tancrède et al., 1978; Fischman et al., 1978; Higashijima et al., 1979), such studies do not take into account the nature of substrate–receptor interactions and therefore can provide little insight into the conformation(s) of the receptor-bound peptide. The flexibility of these compounds raises the possibility that the conformation of the bound peptide is completely different from that in the absence of receptor. Structure–activity studies (Gorin et al., 1978) have been used to infer possible peptide conformations at the receptor, but such studies are indirect and therefore less satisfactory than direct observation for the elucidation of receptor-bound peptide conformation. We felt that, by monitoring the bound form of the opioid peptides via NMR spectroscopy, direct indications of peptide conformations on the receptor might be obtained. Thus, we undertook studies of the binding properties of these peptides (Tancrède et al., 1978; Deslauriers et al., 1978; Jarrell et al., 1979).

Phospholipids have been implicated as essential components of the opiate receptor (Abood & Takeda, 1976; Abood et al., 1977a,b), while treatment of brain tissue with lipolytic enzymes greatly inhibits opiate binding (Abood et al., 1978). Lipids have also been shown to bind morphine stereospecifically (Abood & Hoss, 1975). Although the binding of the enkephalins to lipid is generally weaker than to complex biological extracts, it is quite conceivable that lipid is an essential component of the binding site. A systematic study of binding to possible components of the biological receptor is therefore a

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necessary precursor to studies with the intact receptor. We have therefore monitored by ^{13}C NMR the interaction between methionine-enkephalin and lipids (phosphatidylcholine, phosphatidylserine, and cerebroside sulfate). In order to facilitate the study, we enriched the peptides in ^{13}C to 90% at the α-carbons of either the 2-glycyl or 3-glycyl residues.

Three parameters have been measured from our ^{13}C spectra: chemical shifts, spin–lattice relaxation times (T_1), and line widths $(\pi T_2^*)^{-1}$. ^{13}C chemical shifts can provide information on changes in steric strain associated with binding and differences between pK_a values of free and bound species, as well as possible stacking of aromatic residues via ring-current effects. Spin–lattice relaxation times and line widths can provide information on lifetimes and sizes of molecular complexes as well as on the restrictions which occur in the motional characteristics of molecules upon binding.

Materials and Methods

(2-[2-^{13}C]Glycine)methionine-enkephalin and (3[2-^{13}C]-glycine)methionine-enkephalin were obtained as described in Tancrède et al. (1978).

Synthesis of (3-[2-^{13}C]Glycine)methionine-enkephalinamide. (*tert*-Butoxycarbonyl)-(3-[2-^{16}C]glycine)methionine-enkephalin methyl ester (Tancrède et al., 1978) was subjected to ammonolysis in ammonia-saturated methanol for 48 h at ambient temperature. The reaction mixture was concentrated to a residue which was dried in vacuo. Deprotection with trifluoroacetic acid afforded a crude pentapeptide amide which was purified by chromatography on Sephadex G-10 using 0.2 N acetic acid. The product was homogeneous on TLC in two solvent systems and gave the expected ratio of amino acids to ammonia.

Morphine sulfate pentahydrate was purchased from Ingram and Bell, Ltd., Don Mills, Canada. Phosphatidylserine (PS) was obtained from Serdary Research Laboratories, London, Canada, or Lipid Products, South Nutfield, England, and stored in chloroform or chloroform–methanol at −15 °C. Egg lecithin was purchased from Lipid Products. Sulfatides were obtained from Analabs, North Haven, CT. Sonicated lipid dispersions were prepared as described in Tancrède et al. (1978).

The pH values reported in the text are pH meter readings in D_2O and are uncorrected for the deuterium isotope effect at the glass electrode. ^{13}C NMR spectra and spin–lattice relaxation times were measured by using a Varian CFT-20 spectrometer (20 MHz for ^{13}C) as described previously (Tancrède et al., 1978) or a Bruker CXP 300 spectrometer (75 MHz for ^{13}C). Chemical shifts are reported in parts per million downfield from external tetramethylsilane. Dioxane was used as an internal standard for line width measurements.

Results and Discussion

Properties of Methionine-enkephalin in Solution. As a preliminary step to studying the binding properties of enkephalin, we have followed the dynamical behavior of enkephalin in aqueous solution (Tancrède et al., 1978; Deslauriers et al., 1978) and have found no aggregation of the peptide and no pH dependence of the motional characteristics of the various molecular fragments. At low concentration (1 mg/mL) the 2-glycyl residue shows more segmental motion than does the 3-glycyl residue, although this difference disappears at high concentrations (100 mg/mL) and correlates with the increased viscosity of the solution. The overall molecular tumbling can be described as isotropic with a correlation time of ca. (1–3) $\times 10^{-10}$ s rad^{-1}, and internal rotations of side chains (except CH_3 groups) are of the same order of magnitude. At low

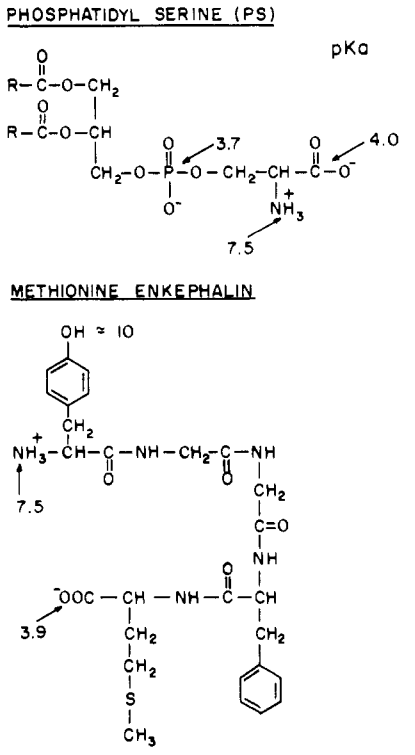

FIGURE 1: Structures and pK_a values of phosphatidylserine (PS) and methionine-enkephalin.

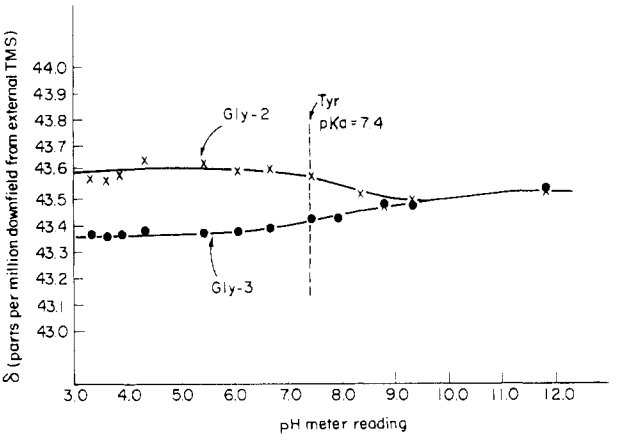

FIGURE 2: pH dependence of ^{13}C chemical shifts of α-carbons of glycine in methionine-enkephalin.

peptide concentrations the segmental motion of the 2-glycyl residue is rapid, with a correlation time of approximately 5 $\times 10^{-11}$ s rad^{-1}.

The pK_a values of the amino (7.5) and carboxyl (3.9) groups of methionine-enkephalin in D_2O have been determined (Figure 1) by following the pH dependence of the ^{13}C chemical shifts of the β-carbon of the tyrosyl residue and the α-carbon of the methionyl residue (Christl & Roberts, 1972). These values are very close to those determined for the same functional groups in PS (Figure 1; Seimija & Ohki, 1973). Although the effects of pH variation on ^{13}C chemical shifts are usually limited to the residue undergoing titration and to its nearest neighbor (Christl & Roberts, 1972; Deslauriers & Smith, 1976), titration of the amino group in methionine-enkephalin is manifest in the shifts of both the 2-glycyl and 3-glycyl α-carbons (Figure 2); this could provide a measure of alterations in pK_a as a result of binding.

Interaction of (2-[2-^{13}C]Glycine)methionine-enkephalin and (3-[2-^{13}C]Glycine)methionine-enkephalin with Phosphati-

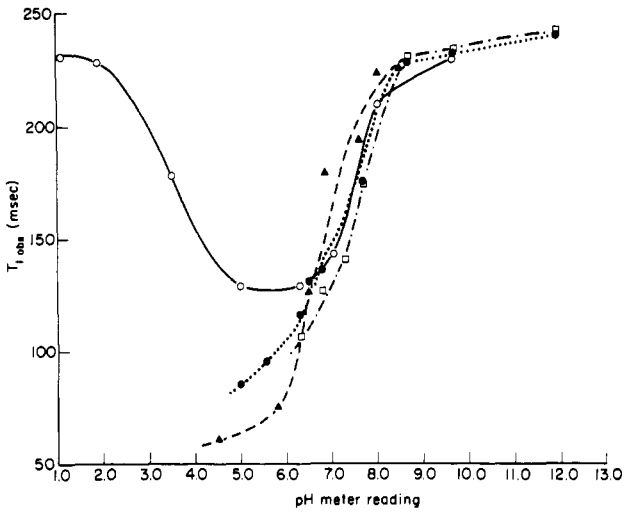

FIGURE 3: pH dependence of T_1 values for the following: (▲) (3-[2-^{13}C]glycine)methionine-enkephalin (1.1 mg) in the presence of PS (78.5 mg); (2-[2-^{13}C]glycine)methionine-enkephalin (1.1 mg) in the presence of PS [85 mg (□) and 75 mg (●)]; (○) (3-[2-^{13}C]-glycine)methionine-enkephalinamide (1.85 mg) in the presence of PS (1.33 mg). Spectra were obtained at 20 MHz; temperature was 30 °C.

dylserine. Acidic lipids have been reported to bind opiates stereospecifically, whereas little binding is observed with neutral lipids (Abood & Hoss, 1975). We have confirmed that egg lecithin causes little perturbation in the ^{13}C spectral characteristics of the enriched enkephalins (Tancrède et al., 1978; Deslauriers et al., 1978). Neither the chemical shifts nor the relaxation times (T_1, T_2*) were affected by the presence of egg lecithin. However, with phosphatidylserine the ^{13}C T_1 values are sensitive to lipid–peptide interaction (Figure 3) with a striking dependence on pH. Under very acidic or very basic conditions, the observed T_1 values for the glycyl residues equal those of free enkephalin. At intermediate pH, these T_1 values decrease to ~80 ms. The pH dependence of the curve can be used to gain information on the nature of the interaction (vide infra). Measurements of ^{13}C chemical shifts and line widths, performed under conditions of maximal binding, show very little change (Jarrell et al., 1979).

Dissociation Constants. Assuming a 1:1 lipid–peptide interaction

$$P + L \rightleftharpoons PL \tag{1}$$

where P is peptide and L is lipid, a dissociation constant K_d can be defined as

$$K_d = \frac{[P_F][L_F]}{[PL]} \tag{2}$$

where $[P_F]$, $[L_F]$, and $[PL]$ are concentrations of free peptide, free lipid, and the peptide–lipid complex, respectively.

Under conditions of fast exchange on the ^{13}C NMR time scale (Dwek, 1973; Swift & Connick, 1962), observed T_1 values can be treated to obtain values for K_d as well as the T_1 value of the bound species ($T_{1,B}$):

$$\frac{1}{T_{1,obsd}} = \frac{1}{T_{1,F}} + \frac{f_B}{T_{1,B}} \tag{3}$$

$$f_B = \frac{[L_T]}{K_d + [L_T] + [P_T]} \tag{4}$$

in which $T_{1,F}$ is the spin–lattice relaxation time of the free species, f_B is the fraction bound (which is assumed to be small relative to $1 - f_B$), and $[L_T]$ and $[P_T]$ are total concentrations of lipid and peptide, respectively.

Equations 3 and 4 may be combined as

$$\frac{T_{1,obsd}T_{1,F}}{T_{1,F} - T_{1,obsd}} = \frac{T_{1,B}(K_d + [P_T])}{[L_T]} + T_{1,B} \tag{5}$$

and a plot of $(T_{1,obsd}T_{1,F})/(T_{1,F} - T_{1,obsd})$ vs. $1/[L_T]$ gives $T_{1,B}$ as the intercept and $T_{1,B}(K_d + [P_T])$ as the slope. Plotting the data obtained for (2-[2-^{13}C]glycine)methionine-enkephalin and (3-[2-^{13}C]glycine)methionine-enkephalin in the presence of phosphatidylserine yields K_d values of 5.4×10^{-1} M and 3.3×10^{-1} M and a $T_{1,B}$ value of 35 ms for both the 2-glycyl and 3-glycyl residues of enkephalin, respectively, at pH 6.3 (Jarrell et al., 1979). These K_d values are much larger than those reported for the PS–morphine interaction [$\sim 10^{-6}$ M; Abood & Hoss (1975)]. Enkephalin interacting with brain membrane preparations shows high- and low-affinity dissociation constants of 5.9×10^{-9} M and 5.9×10^{-7} M, respectively (Birdsall et al., 1976).

Assuming that $T_{1,B}$ remains constant, K_d values can be calculated from the observed T_1 values. The observed pH dependence of K_d (Jarrell et al., 1979) is expected in view of the varying nature of the ionic species present for both phosphatidylserine and enkephalin. However, over the range of binding conditions used (between pH 4.0 and pH 8.0) K_d varied by only a factor of 5 from 3×10^{-1} M to 1.6 M (Jarrell et al., 1979).

Nature of the Peptide–Lipid Interaction. In view of the number of ionizable species in both enkephalin and PS, a variety of interactions between the two molecules are possible at different pH values. The pH dependence of the observed T_1 values (Figure 3) indicates that the principal source of interaction between PS and enkephalin involves a group with a pK value near 7, presumably the α-amino group of enkephalin. This eliminates the tyrosyl moiety as a major contributor to the binding. A number of interactions are possible; *intermolecular* association to PS via the NH_3^+ and COO^- moieties of enkephalin and *intramolecular* interactions between charged groups (effectively charge neutralization) within PS as well as enkephalin.

Enkephalinamide is a useful analogue of modified ionic character; if intramolecular interactions in enkephalin were inhibiting binding via the NH_3^+ group, eliminating the charge neutralization within the peptide should enhance binding to PS. However, if the charged carboxyl function were important, elimination of this group should hamper binding.

Binding of (3-[2-^{13}C]Glycine)methionine-enkephalinamide to Phosphatidylserine. The relaxation times (T_1) of ^{13}C-enriched enkephalinamide, in the absence of lipid, were independent of pH over the range 5.0–9.1. The value of 230 ms is in close agreement with that of (3-[2-^{13}C]glycine)-methionine-enkephalin (240 ms) at a similar concentration (Tancrède et al., 1978) and suggests that the amide has motional characteristics which are similar to those of enkephalin. Enkephalinamide in the presence of PS exhibits a pH dependence of observed T_1 values (20 MHz) (Figure 3) which closely resembles that observed for enkephalin and demonstrates clearly the complete recovery of the T_1 value to that of free enkephalinamide at both high and low pH values.

Binding studies were performed by varying the peptide concentration while maintaining a fixed lipid concentration. A plot of $(T_{1,obsd}T_{1,F})/(T_{1,F} - T_{1,obsd})$ vs. $[P_T]$ (see eq 1–4) yields $T_{1,B}/[L_T]$ as the slope and $T_{1,B}[(K_d/[L_T]) + 1]$ as the intercept, leading to values for $T_{1,B}$ and K_d of 87 ms and 2.6×10^{-3} M, respectively. The K_d value suggests that, at the same pH, enkephalinamide binds more strongly to PS than does enke-

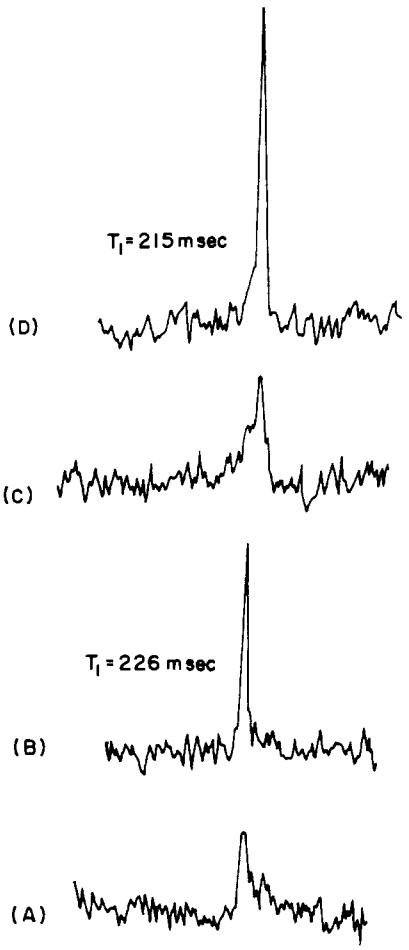

FIGURE 4: Effect of morphine on enkephalin–PS interactions: (A) (2-[2-^{13}C]glycine)methionine-enkephalin (1.20 mg) and PS (29.7 mg); (B) morphine sulfate pentahydrate (5.35 mg) added to sample A; (C) (2-[2-^{13}C]glycine)methionine-enkephalin (1.20 mg) and PS (20.40 mg); (C) (2-[2-^{13}C]glycine)methionine-enkephalin (1.20 mg) and PS (20.40 mg); (D) morphine sulfate pentahydrate (10.1 mg) added to sample C. Spectra were obtained on 1-mL solutions at pH 6.3, 30 °C, and 20 MHz.

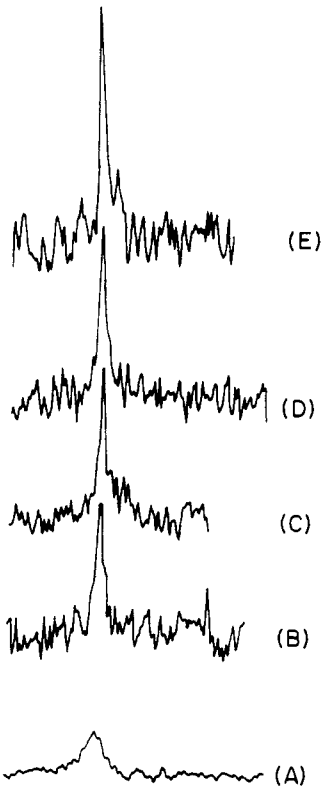

FIGURE 5: Effect of sodium chloride on enkephalinamide–PS interactions: (A) (3-[2-^{13}C]glycine)methionine-enkephalinamide (1.16 mg) and PS (2.83 mg); (A) 3-[2-^{13}C]glycinemethionine enkephalinamide (1.16 mg) and PS (2.83 mg); (B–E) sample A in the presence of 0.05, 0.1, 0.5, and 1.0 M sodium chloride, respectively. Spectra were obtained on 1-mL solutions at pH 6.4, 30 °C and 20 MHz.

phalin, in support of a model in which interaction occurs via the NH_3^+ group of enkephalin rather than by both the NH_3^+ and COO^- groups. The $T_{1,B}$ value (87 ms) is longer than that calculated for enkephalin (35 ms); this may be due to the lack of interaction between the NH_3^+ of PS and the C terminus, allowing more segmental motion within the bound peptide. We must, however, emphasize that such an explanation of the differences in $T_{1,B}$ is highly speculative since 10% errors on measured T_1 values strongly influence $T_{1,B}$ values as well as K_d values (Jarrell et al., 1979).

Correlation Times of Bound Species. Upon binding to a lipid vesicle, enkephalin could be bound as a rigid particle or, alternatively, undergo some degree of internal motion. The rotational correlation time for a unilamellar lipid vesicle is expected to be of the order of 10^{-6} s rad^{-1}, whereas for multilamellar vesicles it is of the order of seconds (Stockton et al., 1976). A $T_{1,B}$ value of 35 ms at 20 MHz corresponds to a rotational correlation time (τ_c) of either 7.6×10^{-10} or 3.0×10^{-8} s rad^{-1}, and bound line widths should be 9 and 20 Hz, respectively [for details of calculations, see Lyerla & Levy (1974)]. Similarly, for enkephalinamide, the observed $T_{1,B}$ value of 87 ms at 20 MHz corresponds to a τ_c of either 2.8×10^{-10} or 7.8×10^{-8} s rad^{-1}, and bound line widths should be 4 and 54 Hz, respectively. Knowing K_d and the line width of free enkephalin, we can calculate, for each of the two possible correlation times of the bound species, an expected "observed" line width for all our experimental situations. Thus, by use of a K_d of 5×10^{-1} M and a line width of 2 Hz for the free species, the expected "observed" line widths for the two possible correlation times of the bound species are calculated to be 3.5 and 5.6 Hz; for methionine-enkephalin (1.2 mg) and PS (75 mg) in 1 mL of solution at pH 6.4, the observed line width is 3.5 Hz. Using a K_d of 2.6×10^{-3} M and a line width of 2 Hz for free enkephalinamide, one calculates, for the two possible τ_c values of the bound peptide, expected line widths of 3 and 10 Hz; the observed line width was 3.6 Hz for enkephalinamide (2.0 mg) and PS (0.6 mg) in 1 mL of deuterium oxide at pH 6.4. Thus, the shorter correlation time would appear to be more consistent with the observed line widths. However, inspection of Figures 4 and 5 suggests that under conditions where binding is relatively strong the complete line shape may not have been observed and, therefore, the measured line widths may not be accurate (vide infra).

In order to obtain additional information on the correlation time and line width of the bound peptide, we conducted measurements at a higher magnetic field. For enkephalinamide with possible τ_c values of 2.8×10^{-10} and 7.8×10^{-8} s rad^{-1}, line widths of 3.9 and 53 Hz, respectively, are expected for the bound species at 7.05 T (^{13}C frequency 75.46 MHz); the corresponding expected observed line widths are 4 and 23 Hz, respectively. A line width of 27 Hz was measured for enkephalinamide (1.52 mg) interacting with PS (2.58 mg) at pH 6.4. The 27-Hz line width obtained at 7.95 T and the observed signal intensities are best explained by the longer correlation time (7.8×10^{-8} s rad^{-1}) for the Gly-3 residue of enkephalinamide. It is unlikely that the correlation time of bound enkephalinamide would differ greatly from that of bound enkephalin. We therefore assign the longer correlation

time (3.0×10^{-8} s rad^{-1}) to bound enkephalin as well. No evidence was obtained for a difference in the chemical shift of the Gly-3 residue in the free and bound form of enkephalinamide. Addition of morphine (morphine/PS molar ratio of ~1.6:1) resulted in a narrowing of the Gly-3 resonance (at 7.05 T) from 27 to 7.5 Hz at pH 6.4.

These results, independently of a correct choice of τ_c, show that both enkephalin and enkephalinamide do not bind as rigid bodies to a lipid vesicle. Rotational correlation times measured for the bound peptides are consistent with the presence of internal or segmental motion which determines the T_1 properties of the bound peptides. Moreover, the rate of segmental motion of the bound peptide has decreased by at least 1 order of magnitude relative to that of the free peptide in solution.

Binding Antagonists. (1) Morphine. Morphine antagonism was verified on the binding of enkephalin and enkephalinamide to PS. The strong binding of morphine to PS (Abood & Hoss, 1975), relative to that of enkephalin, causes reversion of the enkephalin–PS spectrum to that of the free species when only small amounts of morphine are present (Jarrell et al., 1979), as shown in Figure 4.

In the case of enkephalinamide–PS interaction, where the binding constants of enkephalinamide and morphine are more similar, morphine may be viewed as a competitive inhibitor of the peptide binding. In such a case, the K_d value measured in the presence of morphine can be related to the K_d value measured in the absence of morphine. Thus, the K_d determined in the presence of morphine would be $[1 + ([M]/K_{d,M})]$ times that determined in the absence of morphine, where [M] is the concentration of morphine and $K_{d,M}$ is the dissociation constant of morphine binding to PS (Appelton & Sarkar, 1975; Fridovich, 1963). It is interesting to note that even when the morphine concentration exceeds that of PS the spectrum has not reverted to that of the free peptide; 1.2 mg of enkephalinamide in the presence of 5.6 mg of PS and 4.5 mg of morphine sulfate pentahydrate shows a T_1 of 170 ms. By use of the above equation for K_d in the presence of morphine, a $K_{d,M}$ of 7×10^{-4} M can be calculated for the dissociation constant of morphine binding to PS at 6.4. At pH 8.5 a $K_{d,M}$ of $(1–6) \times 10^{-6}$ M has been reported for the morphine–PS interaction (Abood & Hoss, 1975) and at pH 6.4 $K_{d,M}$ is considerably less; based upon the charges of the various species, we estimate an approximately 100-fold decrease to $(1–6) \times 10^{-4}$ M.

(2) Sodium Chloride. High ionic strength has been shown to inhibit binding of opiates with PS (Abood & Hoss, 1975). High concentrations (1 M) of sodium chloride applied under conditions of maximal enkephalin binding caused the spectrum to revert to that of the free peptide. In the case of the enkephalinamide–PS interaction, increasing sodium chloride concentrations resulted in a narrowing of resonances; at 0.5 M sodium chloride the spectrum reverted to that of the free species, as shown in Figure 5.

Binding of Enkephalin and Enkephalinamide to Other Lipids. We have been unable to demonstrate any binding of enkephalin (1.1 mg) to egg lecithin (34.7 mg) at pH 3.0 and 7.0. Similarly enkephalinamide (1.65 mg) showed no binding to egg lecithin (30.8 mg) over a pH range of 5.9–9.1. The absence of peptide–egg lecithin interaction is not surprising since lecithin has no effect on opiate binding to neural membrane preparations (Abood & Takeda, 1976).

We have also been unable to find any evidence for binding of enkephalin (1.2 mg) to an egg lecithin (27.6 mg)–sulfatide (25 mg) mixture at pH 6.3 and 7.1, in spite of the fact that cerebroside sulfate has been reported to interact strongly with opiates (Cho et al., 1976; Loh et al., 1975, 1978). However, our preliminary results suggest that enkephalinamide does bind to cerebroside sulfate with an affinity at least as great as that observed with phosphatidylserine. In addition, it appears that the nature of the enkephalinamide–cerebroside sulfate interaction is similar to that observed for phosphatidylserine.

Conclusion

Using ^{13}C NMR, we have shown that enkephalin and enkephalinamide bind to dispersions of phosphatidylserine. Of the three parameters measured, chemical shifts, line widths and relaxation times, only the last shows enough sensitivity to monitor the interaction accurately. The peptide–PS interaction is pH dependent, with maximum binding occurring under slightly acidic conditions. Neither the tyrosyl hydroxyl group nor the C-terminal carboxyl group is involved in binding; the principal interaction involves the charged $-NH_3^+$ group of enkephalin. The observed stronger binding of enkephalinamide to PS may be related to a lack of *intramolecular* interactions between charged groups (NH_3^+ and COO^-) and thus resembles that of the cationic opiates. Upon binding, the rate of internal motion in enkephalin decreased by 1 order of magnitude although considerable internal rotational freedom is retained for both the 2-glycyl and 3-glycyl residues. Both salt and morphine are antagonistic to peptide binding by PS. The methodology developed herein should provide a means to study intact biological receptors for opiates, such as those of the neuroblastoma glioma hybrid cell line (Sharma et al., 1975).

References

Abood, L. G., & Hoss, W. (1975) *Eur. J. Pharmacol. 32*, 66.

Abood, L. G., & Takeda, F. (1976) *Eur. J. Pharmacol. 39*, 71.

Abood, L. G., Salem, N., Jr., MacNeil, M., Bloom, L., & Abood, M. E. (1977a) *Biochim. Biophys. Acta 468*, 51.

Abood, L. G., Takeda, F., & Salem, N., Jr. (1977b) in *Alcohol and Opiates Neurochemical and Behavioral Mechanism* (Blum, K., Ed.) pp 283–297, Academic Press, New York.

Abood, L. G., Salem, N., MacNeil, M., & Butler, M. (1978) *Biochim. Biophys. Acta 530*, 35.

Appleton, D. W., & Sarkar, B. (1975) *Bioinorg. Chem. 4*, 309.

Birdsall, N. J. M., Hulme, E. C., Bradbury, A. F., Smyth, O. G., & Snell, C. R. (1976) in *Opiates and Endogenous Opioid Peptides*, p 19, Elsevier/North-Holland Biomedical Press, Amsterdam.

Bleich, E. H., Day, A. R., Freer, R. J., & Glasel, J. A. (1977) *Biochem. Biophys. Res. Commun. 74*, 592.

Cho, T. M., Cho, J. S., & Loh, H. H. (1976) *Life Sci. 18*, 231.

Christl, M., & Roberts, J. D. (1972) *J. Am. Chem. Soc. 94*, 4565.

Combrisson, S., Roques, B. P., & Oberlin, R. (1976) *Tetrahedron Lett.*, 3455.

De Coen, J. L., Humblet, C., & Koch, M. H. J. (1977) *FEBS Lett. 73*, 38.

Deslauriers, R., & Smith, I. C. P. (1976) *Top. Carbon-13 NMR Spectrosc. 2*, 1–80.

Deslauriers, R., Tancrède, P., McGregor, W. H., Sarantakis, D., Somorjai, R. L., & Smith, I. C. P. (1978) *Nuclear Magnetic Resonance in Molecular Biology* (Pullman, B., Ed.) pp 285–296, D. Reidel Publishing Co., Dordrecht, Holland.

Dwek, R. A. (1973) *Nuclear Magnetic Resonance in Biochemistry, Monographs on Physical Chemistry* (Harrington, W., & Peacocke, A. R., Eds.) pp 11–47, Clarendon Press, Oxford.

Fischman, A. J., Riemen, M. W., & Cowburn, D. (1978) *FEBS Lett. 94*, 236.
Fridovich, I. (1963) *J. Biol. Chem. 238*, 592.
Garbay-Jaurequiberry, C., Roques, B. P., Oberlin, R., Anteunis, M., & Lala, K. (1976) *Biochem. Biophys. Res. Commun. 71*, 558.
Gorin, F. A., Balasubramanian, T. M., Barry, C. D., & Marshall, G. (1978) *J. Supramol. Struct. 9*, 27.
Higashijima, T., Kobayashi, J., Nagai, U., & Miyazawa, T. (1979) *Eur. J. Biochem. 97*, 43.
Isogai, Y., Némethy, G., & Scheraga, H. H. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 414.
Jarrell, H. C., Tancrède, P., Deslauriers, R., McGregor, W. H., & Smith, I. C. P. (1979) *ACS Symp. Ser. No. 103*, 159-180.
Jones, C. R., Garsky, V., & Gibbons, W. A. (1977) *Biochem. Biophys. Res. Commun. 76*, 619.
Loh, H. H., Cho, T. M., Wu, Y. C., Harris, R. A., & Way, E. L. (1975) *Life Sci. 16*, 1811.
Loh, H. H., Law, P. Y., Ostwald, T., Cho, T. M., & Way, E. L. (1978) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 37*, 147.
Lyerla, J. R., & Levy, G. C. (1974) *Top. Carbon-13 NMR Spectrosc. 1*, 79-148.
Roques, B. P., Garbay-Jaurequiberry, C., Oberlin, R., Anteunis, M., & Lala, K. (1976) *Nature (London) 262*, 778.
Seimija, T., & Ohki, S. (1973) *Biochim. Biophys. Acta 298*, 546.
Sharma, S. K., Nirenberg, M., & Klee, W. A. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 590.
Smith, G. D., & Griffin, J. F. (1978) *Science 199*, 1214.
Stockton, G. W., Polnaszek, C. F., Tulloch, A. P., Hasan, F., & Smith, I. C. P. (1976) *Biochemistry 15*, 954.
Swift, T. J., & Connick, R. E. (1962) *J. Chem. Phys. 37*, 307.
Tancrède, P., Deslauriers, R., McGregor, W. H., Ralston, E., Sarantakis, D., Somorjai, R. L., & Smith, I. C. P. (1978) *Biochemistry 17*, 2905.

Partial Characterization of Undegraded Oat Phytochrome†

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ABSTRACT: We characterized immunoaffinity-purified, undegraded oat (*Avena sativa* L., cv. Garry) phytochrome by several physicochemical techniques. Phytochrome, of greater than 98% purity [Hunt, R. E., & Pratt, L. H. (1979) *Plant Physiol. 64*, 332-336], existed in solution as a dimer of its 118 000-dalton monomers and had a full complement of the typical amino acids with about 35% nonpolar residues, 115 carboxylic acid groups per monomer, and an average of one phosphate per monomer. Although the dimer was not held together by disulfide bridges, each monomer contained three disulfide bonds and 14 reduced cysteines out of a total of 27 cysteine–half-cystine residues. Phytochrome preparations, although very pure, exhibited heterogeneity by discontinuous sodium dodecyl sulfate–polyacrylamide gel electrophoresis, which revealed three closely spaced bands, and by nondenaturing gel electrophoresis at pH 7.0, which revealed four bands. Amino-terminal analysis indicated two residues, Lys and Ala. Manual Edman degradation yielded Leu and Ala after one round and Val and Leu after a second round. These data indicate a possible amino-terminal sequence of NH_3-Lys-Ala-Leu-Val- with some monomers not having Lys.

Phytochrome is the photoreceptor for many light-mediated developmental responses in plants (Smith, 1975). The chromoprotein exists in two photointerconvertible forms, the physiologically inactive, red-absorbing form and the physiologically active, far-red-absorbing form. An understanding of the differences between these two conformations of the chromoprotein is crucial to elucidating its mechanism of action. However, we must first investigate its fundamental physicochemical properties before we will be in a position to probe for these differences.

Physiological responses to light by oats (*Avena sativa* L.) are well documented (Smith, 1975). Nevertheless, undegraded oat phytochrome is not well characterized biochemically because of practical difficulties in obtaining purified preparations in soluble, undegraded form [cf. Pratt (1978) for a discussion]. Most reported characterizations of oat phytochrome are of questionable value since they were performed on a proteolytically degraded, apparently nonphysiological form of the molecule [e.g., see Mumford & Jenner (1966)], which was generated by the action of endogenous proteases, to which phytochrome is highly susceptible (Gardner et al., 1971).

The only undegraded phytochrome that has been significantly characterized is that purified from rye (*Secale cereale* L.; Rice et al., 1973; Rice & Briggs, 1973). However, the physiology of the oat phytochrome system is better documented than that of the rye system, we are able to obtain larger quantities of this relatively scarce pigment (about 0.1% of extractable protein under the best of conditions) from oats than from rye, and there is a large background of information concerning degraded oat phytochrome [see Pratt (1978, 1979) for reviews]. Hence, it is best to begin a characterization of undegraded oat phytochrome rather than to continue with a characterization of rye phytochrome.

Recently, we developed an immunoaffinity purification technique (Hunt & Pratt, 1979) that rapidly yields highly purified undegraded oat phytochrome in quantities sufficient for physicochemical characterization. This phytochrome, except for its reliably higher purity, is identical with conventionally purified phytochrome. We report here a partial

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