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Peptide Models of Dynorphin A(1-17) Incorporating Minimally Homologous Substitutes for the Potential Amphiphilic β Strand in Residues 7-15†

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ABSTRACT: Two peptide models of dynorphin A(1-17) have been synthesized. These peptides incorporate a minimally homologous substitute sequence for residues 6-17, including alternating lysine and valine residues substituting for the potential amphiphilic β-strand structure in positions 7-15. Model 1 retains Pro^{10} from the native sequence, but model 2 does not. Compression isotherms of peptide monolayers at the air-water interface and CD spectra of peptide films adsorbed from aqueous solution onto siliconized quartz slides were evaluated by comparison to those of idealized amphiphilic α-helical, β-sheet, and disordered peptides. Dynorphin A(1-17) was mostly disordered, whereas β-endorphin was α helical. Dynorphin model 1 had properties similar to those of dynorphin A(1-17) at these interfaces, but model 2 formed strongly amphiphilic β sheets. In binding assays to μ-, δ-, and κ-opioid receptors in guinea pig brain membranes, model 1 reproduced the high potency and selectivity of dynorphin A(1-17) for κ receptors, and model 2 was only 3 times less potent and less selective for these receptors. Both peptide models retained the high, κ-selective agonist activity of dynorphin A(1-17) in guinea pig ileum assays, and like dynorphin A(1-17), model 1 had little activity in the rat vas deferens assay. In view of the minimal homology of the modeled dynorphin structures, these studies support current models of membrane-catalyzed opioid ligand-receptor interactions and suggest a role for the amphiphilic α-helical and β-strand structures in β-endorphin and dynorphin A(1-17), respectively, in this process.

Many biologically active peptides are highly flexible structures. They exist in multiple conformational states in aqueous solution that are profoundly affected by changes in the solution conditions, making it very difficult to identify functional conformations directly with any degree of confidence. Kaiser and Kezdy (1984) have suggested that the conformations adopted by these peptides, when they bind at the interfaces where they act will often be amphiphilic and stabilized by their environment. The identification of peptide segments having the potential to form amphiphilic α helices and β strands is relatively simple (Taylor & Kaiser, 1987), and helical structures of this type, in particular, appear to be very common. Furthermore, the functional significance of these amphiphilic helices has been determined in a number of cases, through the study of peptide models (Kaiser & Kezdy, 1984; Taylor & Kaiser, 1986). This approach involves the synthesis of analogues that are designed to retain the general characteristics of the proposed biologically active conformation in a nonhomologous structure. To the extent that the model peptides are able to reproduce the essential physicochemical and pharmacological properties of the native peptide while homology is minimized, evidence for the functional importance of the proposed conformation is obtained.

The naturally occurring opioid peptides appear to be an ideal system for study using nonhomologous models. These peptides share a common amino-terminal sequence of Tyr-Gly-Gly-Phe-Leu/Met, corresponding to the opioid peptides [Leu^5]- and [Met^5]enkephalin, that has highly specific interactions with its binding sites on μ-, δ-, and κ-opioid receptors (Hollt, 1983; Paterson et al., 1983). In contrast, the carboxy-terminal extensions of this sequence that are found in other opioid peptides appear to interact with receptors in a less specific fashion, judging by the effects of single amino acid residue deletions or substitutions, and yet these sequences have a profound effect on the pharmacological properties (Chavkin

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& Goldstein, 1981; Sanchez-Blazquez et al., 1984; Taylor & Kaiser, 1986). Furthermore, studies by Schwyzer and his colleagues indicate that interactions of the opioid peptides with phospholipid surfaces have important effects on their conformations, and similar interactions on cell surfaces may determine their selective affinities for the different opioid receptors (Erne et al., 1985; Schwyzer, 1986b; Sargent et al., 1988).

We have identified two different types of potential amphiphilic secondary structure in opioid peptide sequences, an amphiphilic α helix in residues 13–29 of β-endorphin (Taylor et al., 1981) and an amphiphilic β strand in residues 7–15 of dynorphin A(1–17) (Taylor & Kaiser, 1986; Vaughn & Taylor, 1989). In the case of β-endorphin, the properties of six peptide models in a variety of physicochemical and pharmacological assays demonstrate that the proposed amphiphilic structure is stabilized at interfaces and is one determinant of the selectivity of β-endorphin for μ and δ receptors over κ receptors, and that its amphiphilic character is required for opioid activity in rat vas deferens (RVD)[1] and analgesic assays, as well as for its resistance to proteolytic degradation (Taylor & Kaiser, 1986, 1989). Dynorphin A(1–17) has the highest affinity and selectivity for κ-opioid receptors among the native opioid peptides (Corbett et al., 1982; Paterson et al., 1983; Garzon et al., 1983) and also appears to derive an increased resistance to proteolytic degradation from its carboxy-terminal sequence (Corbett et al., 1982). The potential amphiphilic β strand in residues 7–15 is characterized by alternating hydrophilic and hydrophobic residues. Five charged residues, four basic and one acidic, occupy the hydrophilic positions, and three strongly hydrophobic residues, plus a proline residue in position 10, occupy the intervening positions (Figure 1). In the peptide backbone conformation defined by the ϕ,φ angles of one strand of a β sheet, these hydrophilic side chains would all lie on one side of that β sheet, and the hydrophobic residues would lie on the other side, giving rise to an amphiphilic structure (Taylor & Kaiser, 1986; Wu et al., 1986; Vaughn & Taylor, 1989). Model building indicates that some distortion of this conformation is required in order to accommodate Pro^{10}, but the amphiphilic character is retained. The functional importance of this proposed conformation has been evaluated through the synthesis and study of two peptides that incorporate minimally homologous models of the carboxy-terminal extension of the [Leu^5]enkephalin structure in dynorphin A residues 6–17. The behaviors of these peptides and dynorphin A(1–17) at model interfaces have been compared to those of idealized amphiphilic model peptides, and their potencies and receptor selectivities in opioid receptor binding assays and guinea pig ileum (GPI) assays have been determined. The results demonstrate that simple model sequences based on the proposed amphiphilic β-strand conformation are functional substitutes for the natural sequence and that Pro^{10} is an important determinant of the behavior of dynorphin A(1–17) at interfaces, including κ-receptor surfaces.

Materials and Methods

Synthetic human β-endorphin, porcine dynorphin A(1–13) amide, and porcine dynorphin A(1–17) were purchased from Peninsula Laboratories, San Carlos, CA, and naloxone was from Sigma Chemical Co., St. Louis, MO. Peptide 1 was resynthesized according to the method described previously (Yokoyama et al., 1980) and purified to apparent homogeneity by sequential chromatography on a Pharmacia chromatofocusing column and semipreparative reversed-phase HPLC column (J. W. Taylor and E. T. Kaiser, unpublished data); peptide 2 was a gift from D. G. Osterman and E. T. Kaiser, The Rockefeller University, and was the crude material obtained from HF cleavage and deprotection (Osterman & Kaiser, 1985); peptide 3 was the HPLC-purified material previously reported (Blanc et al., 1983). Female, Hartley guinea pigs (350–400 g) were purchased from Camm Research, Wayne, NJ, and male Sprague-Dawley rats (250–300 g) were from Charles River Breeding Laboratories. Peptide stock solutions were made up in distilled, deionized H_2O (ddH_2O), except for peptide 2, which was only soluble in solutions made slightly basic (pH 7–8) by addition of NaOH, and DYM 2, which was only soluble in acidic solutions and was dissolved in 10 mM HCl. Except for peptides 1–3, all peptide concentrations were determined by amino acid analysis of the stock solutions, with crystalline L-Ala or α-amino-β-guanidinopropionic acid (Pierce, Rockford, IL) as an internal standard. The peptide content of the monolayers formed by peptides 1–3 (Figure 2) was quantitated in each case by fitting the compression isotherms generated to the data published previously (Fukushima et al., 1979; Osterman, 1985; Taylor, 1983).

Peptide Synthesis. Peptide models DYM 1 and DYM 2 (Figure 1) were synthesized by the solid-phase method (Barany & Merrifield, 1979) on a polystyrene/divinylbenzene copolymer, by use of a Beckman 990 automated synthesizer and the symmetric anhydride coupling protocol of Yamashiro and

A Opioid Peptides and Dynorphin Models

Dynorphin A(1-17) (porcine): H-Tyr-Gly-Gly-Phe-Leu^5-
Dynorphin Model 1: H-Tyr-Gly-Gly-Phe-Leu^5-
Dynorphin Model 2: H-Tyr-Gly-Gly-Phe-Leu^5-

(Dyn A) Arg-Arg-Ile-Arg-Pro^{10}-Lys-Leu-Lys-Trp-Asp^{15}-Asn-Gln-OH
(DYM 1) Lys-Lys-Val-Lys-Pro^{10}-Lys-Val-Lys-Val-Lys^{15}-Ser-Ser-OH
(DYM 2) Lys-Lys-Val-Lys-Val^{10}-Lys-Val-Lys-Val-Lys^{15}-Ser-Ser-OH

β-Endorphin (human): H-Tyr-Gly-Gly-Phe-Met^5-Thr-Ser-Glu-Lys-Ser^{10}-Gln-Thr-Pro-Leu-Val^{15}-Thr-Leu-Phe-Lys-Asn^{20}-Ala-Ile-Ile-Lys-Asn^{25}-Ala-Tyr-Lys-Lys-Gly^{30}-Glu-OH

B Standard Peptides

Peptide 1 (α helix): H-Pro-Lys-Leu-Glu-Glu^5-Leu-Lys-Glu-Lys-Leu^{10}-Lys-Glu-Leu-Leu-Glu^{15}-Lys-Leu-Lys-Glu-Lys^{20}-Leu-Ala-OH

Peptide 2 (β sheet): H-Val-Glu-Val-Orn(Tfa)-Val^5-Glu-Val-Orn(Tfa)-Val-Glu^{10}-Val-Orn(Tfa)-Val-OH

Peptide 3 ("random"): H-Tyr-Gly-Gly-Phe-Met^5-Ser-Gly-Ser-Gly-Ser^{10}-Gly-Ser-Pro-Leu-Gln^{15}-Leu-Lys-Trp-Leu-Gln^{20}-Gln-Lys-Gln-Leu-Leu^{25}-Gln-Leu-Lys-Lys-Leu^{30}-Leu-OH

FIGURE 1: Amino acid sequences of peptides. (A) The native and model dynorphin A sequences have been aligned for comparison, and the altered residues in the models are underlined. (B) The design, synthesis, and many of the properties of peptides 1–3 have been described previously (Fukushima et al., 1979; Blanc et al., 1983; Osterman & Kaiser, 1985). Peptide 2 contains δ-(trifluoroacetyl)-ornithine residues, abbreviated as Orn(Tfa).

[1] Abbreviations: Boc, *tert*-butyloxycarbonyl; CD, circular dichroism; DADLE, [D-Ala^2,D-Leu^5]enkephalin; DAGO, [D-Ala^2,Me-Phe^4,Gly^5]-enkephalinol; ddH_2O, distilled, deionized H_2O; DPDPE, [D-Pen^2,D-Pen^5]enkephalin; DYM, dynorphin A(1–17) model peptide; GPI, guinea pig ileum; HPLC, high-pressure liquid chromatography; RVD, rat vas deferens.

Li (1978). Details of the synthesis and purification of DYM 1 are described elsewhere (Vaughn & Taylor, 1989). The synthesis of DYM 2 was essentially identical. N^{α}-Boc-*O*-benzyl-L-Ser-derivatized Merrifield resin was prepared by the method of Horiki et al. (1978) at a substitution level of 0.44 mmol/g of resin by picric acid titration (Gisin, 1972). The peptide chain was then constructed through coupling of the N^{α}-Boc derivatives of Gly, L-Leu, N^{ϵ}-(2-chlorobenzyloxycarbonyl)-L-Lys, L-Phe, *O*-benzyl-L-Ser, *O*-(2-bromobenzyloxycarbonyl)-L-Tyr, and L-Val (Peninsula Laboratories, San Carlos, CA) in the appropriate sequence and then deprotected and cleaved from the resin by reaction with a mixture consisting of HF/dimethyl sulfide/*p*-cresol (25/65/10) for 4 h at 4 °C (Tam et al., 1986). After evaporation and extraction of the deprotecting reagents, crude DYM 2 was extracted into 10% acetic acid, lyophilized, and then desalted on Sephadex G-15. DYM 2 was purified by reversed-phase HPLC on a semipreparative Altex C_{18} column eluted with a gradient of acetonitrile (29% to 35% in 20 min) in 0.1% H_3PO_4/0.1 M $NaClO_4$, pH 2.5. The material obtained after collection and desalting of the major component was homogeneous by analytical HPLC under isocratic conditions and was used in all of the experiments described here. Amino acid analysis of this material after hydrolysis in 6 N HCl at 115 °C for 26 h: Gly, 2.15 (2); Leu, 0.94 (1); Lys, 6.00 (6); Phe, 1.05 (1); Ser, 1.80 (2); Tyr, 0.98 (1); Val, 4.02 (4). Mass spectral analysis by the time-of-flight, ^{252}Cf fission–fragment–ionization method (Chait et al., 1981) gave a measured MW of 1895.4 from the $(M + H)^+$ peak and 1894.8 from the $(M + 2H)^{2+}$ peak (calculated MW = 1895.3; Δ = 0.1 and −0.5, respectively).

Film Balance Studies. Film balance studies were performed under ambient conditions (22–24 °C) on a Lauda FW-1 film balance interfaced to an Apple IIe for data collection and analysis, with a subphase of 0.16 M KCl in 20 mM NaH_2PO_4/NaOH buffer, pH 7.5. After careful removal of surfactant contaminants by bubbling and aspiration of the surface buffer, peptides were introduced onto the buffer surface in the trough from stock solutions containing about 2 mg/mL peptide, via an acid-washed glass slide dipping into the buffer. Peptide monolayers were allowed to equilibrate in an expanded form at zero surface pressure for 10 min and then were compressed at a constant rate of 0.20 cm^2/s, and their compression isotherms were recorded.

Circular Dichroism Spectra. Circular dichroism (CD) spectra of peptide solutions in the monolayer subphase buffer were recorded on an Aviv Model 62ds spectropolarimeter, using a 0.5-mm path-length cell. CD spectra of surface-bound peptides were studied as films on siliconized quartz glass slides and were recorded on an Aviv Model 60ds spectropolarimeter. The circular slides (22 × 1 mm, Hellma, Jamaica, NY) were cleaned by washing successively with 15% NaOH, detergent, 0.1 M NaOH, and concentrated nitric acid/sulfuric acid (1/3) and then coated with the water-soluble siliconizing agent Prosil-28 (Fisher Scientific) and cured in an oven at 150 °C for 2 h. Peptides were transferred onto these slides from their compressed monolayers on the film balance by dipping and then raising the slides through the monolayers once in each direction at a constant rate of about 2 mm/min. During this process, the slides were maintained in a vertical plane, perpendicular to the direction of compression of the monolayers, and the monolayers were held at constant surface pressure (±0.5 dyn/cm) by frequent manual adjustment of the area. No visible solution adhered to the slides as they emerged. Alternatively, peptides were adsorbed onto the siliconized slides directly from solution by soaking the slides in monolayer buffer containing 20–50 μM peptide for 5 min each and then rinsing them briefly in ddH_2O and allowing them to dry by evaporation under ambient conditions. CD spectra of the surface-adsorbed peptide films were then recorded with sets of four slides for each peptide, as described previously (Vaughn & Taylor, 1989). The data are presented as the average of spectra measured for eight equally spaced orientations of the slides about the light path.

Opioid Receptor Binding and Smooth Muscle Assays. Competitive binding to μ-, δ-, and κ-opioid receptors in guinea pig brain homogenates was assayed exactly as described previously (Taylor & Kaiser, 1989). In assays of μ-receptor binding, [^{3}H][D-Ala^2,Me-Phe^4,Gly^5]enkephalinol ([^{3}H]-DAGO) was used alone at a concentration of 1.0 nM for specific labeling of the μ receptors; in the δ-receptor assays, [^{3}H][D-Pen^2,D-Pen^5]enkephalin ([^{3}H]DPDPE, 1.5 nM) was used in the presence of DAGO (100 nM); and in the κ-receptor assays, [^{3}H]bremazocine (0.2 nM) was used in the presence of both DAGO (200 nM) and DPDPE (200 nM).

Peptides were also assayed for their abilities to inhibit electrically stimulated contractions of isolated segments of whole guinea pig ileum or isolated rat vas deferens in oxygenated modified Krebs Ringer at 37 °C, by established methods (Goldstein & Schulz, 1973) as described previously (Taylor & Kaiser, 1989). In contrast to earlier experiments, care was taken to keep the oxygen bubbling rate in the organ bath as low as possible in order to minimize removal of the peptides to the top of the organ bath as a result of their surfactant properties.

Results

Peptide Model Design. DYM 1 and DYM 2 (Figure 1) were designed to contain nonhomologous models of the extension to the carboxy terminus of [Leu^5]enkephalin found in dynorphin A(1–17) consisting, in this initial study, of natural amino acid residues. The potential amphiphilic β strand in residues 7–15 of the native sequence was replaced with a sequence of alternating Lys and Val residues. Lys was chosen as a positively charged, hydrophilic residue mimicking the predominant characteristic of the hydrophilic side of the proposed amphiphilic structure (four basic residues, one acidic residue, and no uncharged, neutral residues). Lys was chosen over Arg in order to better minimize homology with dynorphin A(1–17). Val was chosen as the hydrophobic component, because there is empirical (Chou & Fasman, 1978) and experimental (Blout et al., 1960; Mutter & Altmann, 1985; Ashida et al., 1986) evidence that this residue tends to stabilize extended peptide backbone conformations of the β-strand type. In addition, the potential conformational importance of Pro^{10} in dynorphin A(1–17) was tested by retaining this residue in DYM 1, and substituting it with Val in DYM 2. Finally, in both models, the only residue connecting the enkephalin structure to the potential amphiphilic β strand, Arg^6, was substituted by Lys, and the neutral, hydrophilic carboxy-terminal residues in positions 16 and 17 were each replaced by Ser.

Peptides 1–3 (Figure 1) were chosen to serve as standards in the assays of opioid peptide conformation at interfaces. These peptides are idealized models of the three types of interface-induced conformation that have been designed in our laboratory to date: α helix, β sheet, and "random coil", respectively. Their design and properties have been described previously (Fukushima et al., 1979; Osterman & Kaiser, 1985; Blanc et al., 1983).

Film Balance Studies. Typical force–area (π-A) isotherms obtained upon compression of the monolayers formed at the

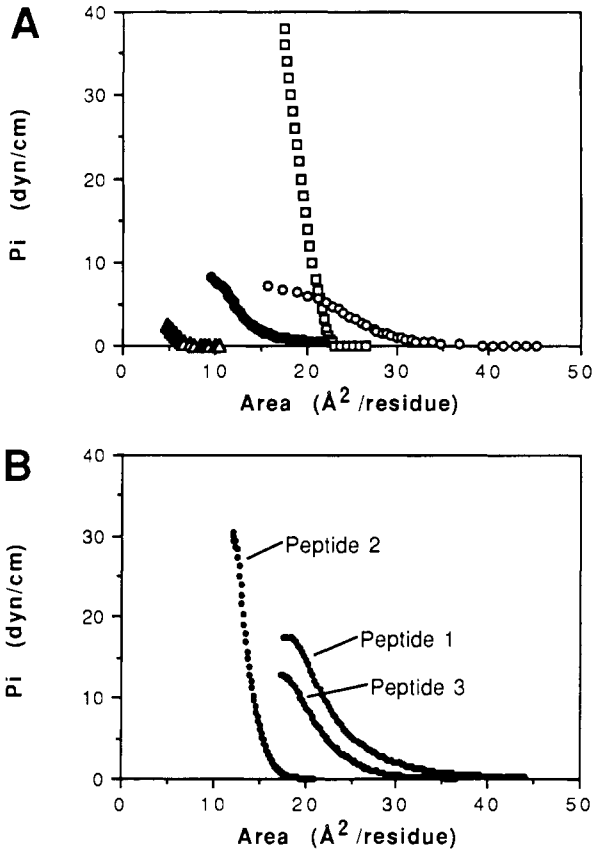

FIGURE 2: Compression isotherms of peptide monolayers at the air–water interface. The monolayers formed by 10–40 μg of peptide introduced to the aqueous buffer surface were slowly compressed at a constant rate, as described in the text, and the surface pressure (dyn/cm) was plotted as a function of the monolayer area (Å²/residue). (A) Compression isotherms of dynorphin A(1–17) (○), DYM 1 (△), DYM 2 (□), and β-endorphin (●) monolayers. The results for DYM 1 were not reproducible for different quantities of peptide introduced to the interface. (B) Compression isotherms of peptides 1–3.

air–water interface by DYM 1, DYM 2, and the native opioid peptides β-endorphin and dynorphin A(1–17) are shown in Figure 2A. Those obtained for DYM 1 occupied a much smaller surface area than expected for a peptide of this size bound to the interface and were only weakly stable upon compression. These characteristics, and the poor reproducibility of the curves, indicate that this peptide model did not transfer efficiently onto the buffer surface and does not adopt a stable conformation at the air–water interface. In contrast, the monolayers formed by DYM 2, which differs from DYM 1 by only one residue, are extremely stable and undergo a sharp transition from the initial expanded, compressible state into a highly incompressible state at about 22 Å²/residue. These characteristics, when compared to the compression isotherms obtained for peptides 1–3 (Figure 2B), indicate that DYM 2 forms amphiphilic β sheets upon compression of its monolayers at the air–water interface. This behavior is also consistent with other studies of idealized amphiphilic β-strand peptides (DeGrado & Lear, 1985). It is, however, the first example of this type of behavior in a peptide where the idealized amphiphilic β-strand sequence is located *within* its primary structure and is bordered on either side by potentially disordered, nonamphiphilic structures.

Dynorphin A(1–17) also forms stable monolayers at the air–water interface, although they collapse at relatively low surface pressures. The π-A curve in Figure 2A indicates that dynorphin A(1–17) has a less compact and much more compressible conformation than DYM 2 at this interface and is similar to the isotherms of β-endorphin (Figure 2A) and peptides 1 and 3 (Figure 2B).

Table I: Analysis of Peptide Monolayer Compression Isotherms

peptide	collapse pressure (dyn/cm)	fit to $\pi(A - A_0[1 - \kappa\pi]) = nRT$: A_0 (Å²/residue)	$\kappa \times 10^2$ (cm/dyn)	n_{true}/n_{fit}
peptide 1	17	23	1.2	1.0
peptide 2	42[a]	13	0.57	4.9
peptide 3	11	23 (38)[b]	1.8	1.8
dynorphin A(1–17)	5	29	4.7	14
DYM 1	<3			
DYM 2	38	21	0.49	6.0
β-endorphin	7	13 (24)[b]	3.1	2.7

[a] Data taken from Osterman (1985). [b] Value calculated by assuming that only residues 13–31 (peptide 3) or 13–29 (β-endorphin) occupy surface area (see text).

The intermediate portions of the compression isotherms in Figure 2 have been fit to eq 1, a semiempirical equation of

$$\pi(A - A_0)[1 - \kappa\pi] = nRT \quad (1)$$

state previously used by Fukushima et al. (1979) to describe the behavior of peptide 1 and the amphiphilic, α-helical serum apolipoprotein A-I in a compressed state at the air–water interface. The values obtained from this fit for the limiting area, A_0, the compressibility constant, κ, and the apparent degree of self-association of the peptide molecules (from n), as well as the collapse pressures for each monolayer, are summarized in Table I. These data support the general observations on the isotherms described above. DYM 2 has the lowest compressibility constant, comparable to that of peptide 2, and dynorphin A(1–17) has the most compressible and the least compact structure, and is apparently highly self-associated. The limiting areas are difficult to interprete, because it is not known how much of the peptide structure is occupying space at the air–water interface and how much lies in the subphase. For example, if only the amphiphilic α-helical segment of the β-endorphin structure occupies surface area, then A_0 may be recalculated as 24 Å²/residue (Taylor, 1986), in agreement with the value for idealized amphiphilic, α-helical peptide 1. If this is correct, then a similar correction should probably be applied to peptide 3, which also has mostly hydrophilic residues in positions 1–12. In this case, A_0 becomes 38 Å²/residue for the disordered conformation that is expected for this model. The parameters calculated for dynorphin A(1–17) can then be seen to be most compatible with a disordered conformation, even if all of its residues are present at the air–water interface. The A_0 value of DYM 2 is compatible with a mixed β-sheet/disordered structure, and an α-helical conformation is discounted on the basis of the very low κ value and the high degree of self-association.

Circular Dichroism Spectra. The CD spectra of dynorphin A(1–17), β-endorphin, DYM 1, and DYM 2 were measured in buffered 0.16 M KCl solution at pH 7.5 (Figure 3) and are all consistent with mostly disordered, random-coil conformations (Greenfield & Fasman, 1969). In the concentration range tested (approximately 5–200 μM), no changes in the spectra of the model peptides were observed, indicating that they are probably monomeric and do not share the tendency to self-associate in aqueous solution that is generally characteristic of amphiphilic β-strand peptides, including peptide 2 (Osterman & Kaiser, 1985; DeGrado & Lear, 1985). This was surprising in the case of DYM 2, on the basis of its behavior at the air–water interface. However, DYM 1 and

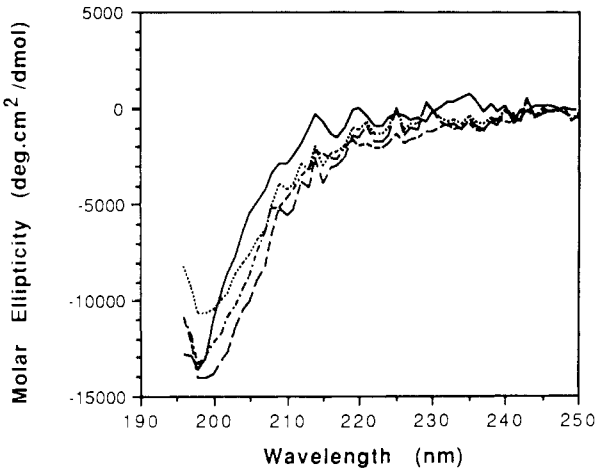

FIGURE 3: Circular dichroism spectra of opioid peptides and models in aqueous solution. Spectra of dynorphin A(1–17) (-·-), DYM 1 (—), DYM 2 (···), and β-endorphin (--) were measured in a 0.5-mm path-length cell at peptide concentrations of 32, 43, 14, and 32 μM, respectively.

DYM 2 were—like many amphiphilic β-strand peptides—insoluble in the millimolar concentration range at neutral pH.

In order to characterize the interface-induced conformations of these peptides further, we attempted to transfer the compressed monolayers they form at the air–water interface onto siliconized quartz glass slides for direct measurement of the CD spectra of the deposited peptide films. This approach has previously been used to study strongly amphiphilic peptide models (DeGrado & Lear, 1985; Bazzi et al., 1987) and hydrophobic signal peptides (Briggs et al., 1986; Cornell et al., 1989) and proved to be quite straightforward for idealized model peptides 1–3. The monolayers formed by peptides 1–3 were compressed to surface pressures of 15, 15, and 11 dyn/cm, respectively, and held at these surface pressures during the transfer. The spectra from sets of four slides prepared in this way were then averaged for eight different orientations of the slides about the light path, in order to eliminate linear dichroism components (Cornell, 1978). These artifacts arise from the compressing force exerted on the monolayers, which tends to align the peptide molecules in a direction parallel to the compressing beam of the film balance. The monolayers are transferred onto the slides with retention of this alignment in one direction in the plane of the surface of the slides. Orientation-dependent components were observed in the spectra of all three idealized models (not shown) but were largest for the β-sheet monolayers of peptide 2, as expected (Cornell, 1978). Unfortunately, it was not possible to quantitate the peptide transferred directly on the basis of the area change of the monolayer, due to the variability in the experiments. Instead, the ellipticity values obtained when the CD spectra were measured were multiplied by the surface areas occupied by each peptide under the conditions of the transfer, in an attempt to account for the different amounts of each peptide coating the slides. This factor was 20 Å^2/residue for peptide 1, 14 Å^2/residue for peptide 2, and 19 Å^2/residue for peptide 3. The final CD spectra obtained for peptides 1–3, transferred as compressed monolayers onto siliconized slides, are shown in Figure 4A.

The CD spectrum of the transferred peptide 1 monolayers is similar to that of polylysine in the α-helical conformation on a planar surface (Stevens et al., 1968) and surface films of another amphiphilic α-helical peptide prepared in the same way (DeGrado & Lear, 1985). The minimum at 222 nm is smaller than that observed for α-helical peptides in solution,

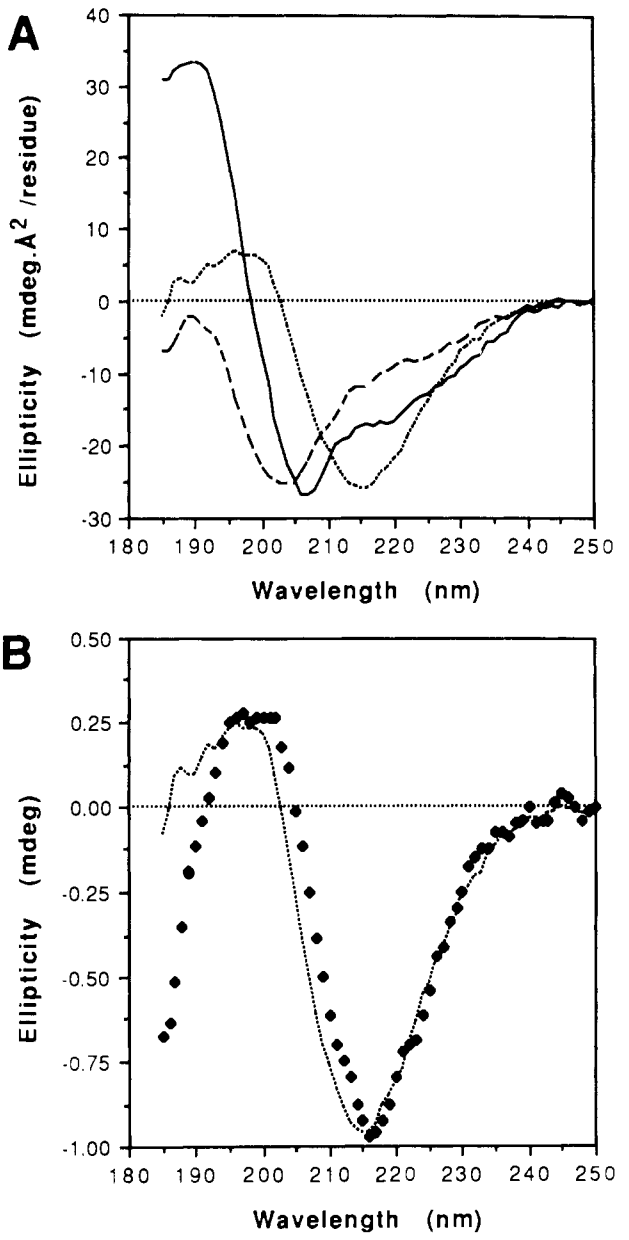

FIGURE 4: Circular dichroism spectra of peptides transferred from the air–water interface onto siliconized slides dipped and raised vertically through their compressed monolayers once in each direction. (A) CD spectra of transferred peptide 1 (—), peptide 2 (···), and peptide 3 (--) monolayers. Peptides 1 and 2 were transferred at a constant surface pressure of 15 dyn/cm, and peptide 3 was transferred at 11 dyn/cm. Ellipticity values were obtained in millidegrees and were multiplied by the areas occupied by each peptide at the surface pressure of the transfer (in Å^2/residue), in order to account for the different quantities of peptide on the slides (see text). (B) CD spectrum of DYM 2 monolayers transferred at 20 dyn/cm. The ellipticity data obtained (◆) are fit to the shape of the CD spectrum of transferred peptide 2 (β sheet) monolayers (···).

because the helices all lie in the plane of the interface. The missing component, present in the CD spectra of helices orientated out of this plane, has been observed in an essentially pure form for bacteriorhodospin oriented perpendicular to the interface in purple membranes (Muccio & Cassim, 1979; Rothschild et al., 1980; Gibson & Cassim, 1989).

The CD spectrum of peptide 2 monolayers transferred onto the slides has a single broad minimum at 215 nm and a low maximum around 198 nm. The position of the minimum at 215 nm is in agreement with the spectrum of films of the β sheet forming peptide described by Bazzi et al. (1987) but differs slightly from the spectra of β-sheet films of DeGrado

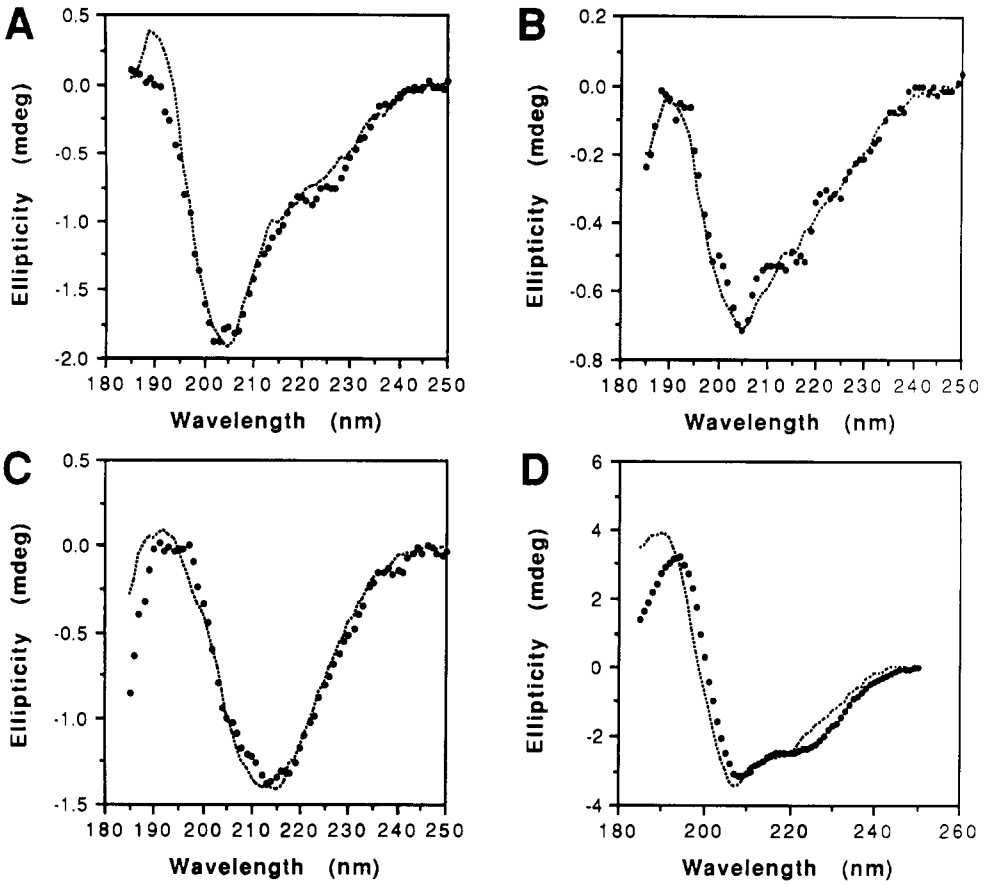

FIGURE 5: Circular dichroism spectra of natural and model opioid peptides adsorbed from solution in aqueous buffer onto siliconized slides. The ellipticity data obtained (●) were examined for their best fit to the shape of mixtures of the standard spectra in Figure 4B (···). (A) Data for dynorphin A(1–17) fit to a mixture of 80% of the random standard and 20% of the α-helix standard. (B) Data for DYM 1 fit to 80% random and 20% β sheet. (C) Data for DYM 2 fit to 60% β sheet and 40% random. (D) Data for β-endorphin fit to 80% α helix and 20% β sheet.

and Lear (1985) or polylysine in the β-sheet conformation on a planar surface (Stevens et al., 1968), which gave broad minima around 219 nm.

The CD spectrum of the peptide 3 films, with a single minimum at 203 nm, is very similar to the spectrum of polylysine in a disordered conformation on a planar surface (Stevens et al., 1968). This result confirms, for the first time, that peptide 3 does indeed adopt a disordered conformation at interfaces, as it was designed to do (Blanc et al., 1983), and demonstrates that it may be difficult to distinguish the behavior of disordered and α-helical peptides on the basis of the compression isotherms of their monolayers at the air–water interface (Figure 2A), assuming that peptide 3 retained its monolayer conformation during the transfer precess. The spectrum of peptide 3 in Figure 4A is somewhat different from the solution spectra of disordered conformations, which have a minimum at 197 nm (Greenfield & Fasman, 1969).

When attempts were made to transfer the compressed monolayers of dynorphin A(1–17), DYM 1, and DYM 2 onto siliconized slides, only the transfer of DYM 2 was successful. This peptide monolayer was transferred at a surface pressure of 20 dyn/cm and gave a CD spectrum that was very similar to that of peptide 2 (Figure 4B), indicating that it consists of β sheets that incorporate most or all of the DYM 2 structure, including the nonamphiphilic segments. A strong slide orientation dependent, linear dichroism component, resulting from alignment of the DYM 2 β strands in the monolayer, was also observed during the measurement of this spectrum (not shown).

The interface-induced conformations of dynorphin A(1–17), DYM 1, and DYM 2 were successfully compared by adsorbing the peptides directly from solution onto the siliconized quartz glass slides in order to measure their CD spectra. This method was ultimately preferred over the transfer of compressed peptide monolayers from the air–water interface, because it is simpler, more often successful for peptides that do not form stable monolayers, and free from possible effects on conformation that might result from the compression forces acting on the monolayers. Peptides were present in the buffered salt solution in greater than 50-fold excess over the quantities required to form a monomolecular film on the surface of the slides and were rapidly adsorbed. The slides were rinsed in ddH_2O, in order to remove excess peptide solution on their surfaces, and then the spectra were measured as before. The resultant spectra, shown in Figure 5, were fit to the standard spectra obtained for peptides 1–3 (Figure 4A) by use of 10% increments of each type of structure, in order to assess the interface-induced conformations. Since the relative amounts of each peptide on the slides were unknown, the standard spectra were adjusted to the shapes of the spectra in Figure 5 without regard for their magnitude. For comparative purposes, β-endorphin was also examined by this method. DYM 1 was calculated to be in a disordered conformation, but with a significant amount of β-sheet structure present. The best fit to the standard spectra was obtained from a mixture of 80% of the peptide 3 spectrum (random) and 20% of the peptide 2 spectrum (β sheet). The DYM 1 spectrum was the weakest of the four studied, in agreement with the instability of DYM 1 at the air–water interface. DYM 2 was predominantly in

Table II: Competitive Binding of Peptide Models to Opioid Receptors in Guinea Pig Brain Membranes

peptide	IC_{50} ± SEM (nM) for radioligand displacement[a]			receptor selectivity
	μ sites	δ sites	κ sites	$(\mu/\delta/\kappa)$[b]
dynorphin A(1–17)	3.16 ± 0.622	13.1 ± 2.9	0.626 ± 0.189	0.20/0.05/1.0
DYM 1	4.82 ± 1.11	39.1 ± 3.2	0.535 ± 0.044	0.11/0.01/1.0
DYM 2	4.97 ± 1.27	12.5 ± 1.8	1.86 ± 0.63	0.37/0.15/1.0
β-endorphin	2.57 ± 0.53	2.64 ± 0.42	313 ± 69	122/119/1.0

[a] Assay conditions are described in the text. [b] Ratio of IC_{50}(κ sites) to each IC_{50} value.

Table III: Opioid Activities of Peptide Models in Guinea Pig Ileum and Rat Vas Deferens Assays

peptide	guinea pig ileum			rat vas deferens
	IC_{50} (nM ± SEM)		K_I (naloxone) (nM)	IC_{50} (nM ± SEM)
	no naloxone	added naloxone[a]		
dynorphin A(1–17)	0.41 ± 0.04	3.14 ± 0.39	15.2	≫10 000
dynorphin A(1–13)-NH_2[b]	0.114 ± 0.019	0.535 ± 0.158	27.1	not tested
DYM 1	3.19 ± 0.44	16.2 ± 2.1	24.4	≫10 000
DYM 2	1.93 ± 0.17	13.2 ± 0.6	17.1	not tested
β-endorphin[b]	30.1 ± 4.9	243 ± 30	2.8	41 ± 2

[a] 100 nM, except for the β-endorphin experiments, when 20 nM naloxone was added. [b] Data taken from Taylor and Kaiser (1986, 1989).

the β-sheet conformation, as it was in the transferred compressed monolayers (Figure 4B), but the ellipticity minimum was shifted to 213 nm and was broader than expected for pure β-sheet structure, indicating that some disordered structure was also present. Significant slide orientation dependent effects were also observed during the measurement of this spectrum, indicating that the β-sheet structures were oriented in a nonrandom fashion in the plane of the slides. This was surprising and suggests that the act of taking the slides in and out of the peptide solutions can also have an orienting effect on the peptide structures. In this case, fitting the data to the standard spectra in Figure 4A indicated a mixture of 60% β sheet and 40% random structure. Dynorphin A(1–17) was mostly disordered but appeared to have a small α-helical component to its surface-bound structure giving rise to a broader minimum at 203 nm with a distinct and reproducible shoulder at 222 nm. However, the shoulder at 222 nm is also consistant with a helical component that is oriented perpendicular to the plane of the slides, as described above. The best fit to the standard spectra indicated 80% random structure and 20% helix. Finally, the conformation of β-endorphin was indicative of a high α-helix content, although the best fit to the spectra in Figure 4A indicated 80% helix and 20% β sheet. This is in agreement with the conformation that we have predicted for residues 13–29 at interfaces on the basis of its amphiphilic character in this conformation (Taylor et al., 1981). However, it also indicates that the helical conformation is propagated throughout most of the peptide structure, despite the helix-breaking Pro in position 13 and the poor amphiphilic nature of the amino-terminal helix that is generated. This result is consistent with the solution conformation of β-endorphin in 60% methanol–40% water determined by proton NMR, which indicated that residues 1–12 and 14–29 all participate in helix formation under these helix-promoting conditions (Lichtarge et al., 1987).

Opioid Receptor Binding. The relative affinities of DYM 1 and DYM 2 for μ-, δ-, and κ-opioid receptors in decerebellate guinea pig brain membranes were compared to those of dynorphin A(1–17) and β-endorphin in competitive binding assays conducted at 4 °C in order to minimize peptide degradation. In these assays, each of the radioligands was employed at a concentration lower than its expected dissociation constant for the receptor in question, so that the radiolabeling of the different receptor types was highly selective and the receptor occupancy by radioligand was very low, as discussed before (Taylor & Kaiser, 1989). The IC_{50} values presented in Table II should, therefore, correspond closely to the dissociation constants for binding of the peptides to each receptor (Cheng & Prusoff, 1973). DYM 1 bound to κ-opioid receptors with an affinity that was essentially identical with that of dynorphin A(1–17) and was more selective for κ receptors over μ and δ receptors than the native peptide. The lowest IC_{50} value obtained for DYM 2 was also in the κ assay, but the replacement of Pro^{10} in DYM 1 by Val resulted in a 3–4-fold reduction in affinity for κ receptors and an increase in affinity for δ receptors of the same magnitude. In contrast to the behavior of these κ-selective peptides, β-endorphin had a high affinity for the μ and δ sites but bound very poorly to κ sites.

Guinea Pig Ileum and Rat Vas Deferens Assays. DYM 1 and DYM 2 rapidly inhibited electrically stimulated contractions of the guinea pig ileum in a dose-dependent manner. The maximal inhibitory effects were typically greater than 90% of the contractions and were completely reversed by addition of the opioid antagonist naloxone. These inhibitory effects were apparently not reversed by proteolysis over periods of 10–20 min, but the effects of DYM 2 could be reversed quite rapidly if the rate of oxygen bubbling in the organ bath was above the minimal level required to keep the tissue viable, presumably as a result of the strong surfactant effects of this peptide. DYM 1 was 8 times less potent in this assay than dynorphin A(1–17), which has a very high potency on the guinea pig ileum, and DYM 2 was about 5 times less potent than the native peptide (Table III).

Since the guinea pig ileum is rich in both κ and μ receptors (Lord et al., 1977), the actions of the dynorphin model peptides on these two types of opioid receptor were distinguished by testing the antagonist potency of naloxone on their inhibitory effects, by use of the single-dose method of Kosterlitz and Watt (1968) to calculate an apparent value for the inhibition constant, K_I. Naloxone binds selectively to μ-opioid receptors, and a value of 2–3 nM for the K_I indicates that these receptors mediate the inhibitory effects, whereas a higher value of 25–30 nM indicates that they are mediated by κ receptors (Leslie, 1987). The results in Table III indicate that, like the potent κ-agonist dynorphin A(1–13) amide, the actions of DYM 1 are mediated by κ receptors, as expected on the basis of the binding assays. DYM 2 also acts selectively on κ receptors in the guinea pig ileum, but like dynorphin A(1–17) its actions may, in part, be mediated by μ receptors. However, the sensitivities of these two peptides to naloxone antagonism were much lower than that of β-endorphin, which acts on μ receptors in this tissue.

In rat vas deferens assays, which are highly selective for β-endorphin among the naturally occurring opioid peptides (Schulz et al., 1979; Sanchez-Blazquez et al., 1984), neither DYM 1 nor dynorphin A(1–17) had any inhibitory effects at concentrations less than 1 μM, and only partial inhiibition of contractions was observed for doses in the 1–10 μM range, although these effects were naloxone reversible (Table III). DYM 2 was not tested because of its low solubility in the Krebs Ringer at micromolar concentrations. None of the κ-selective

peptide products of the preproenkephalin B gene appears to have significant activity in this assay (Sanchez-Blazquez et al., 1984).

Discussion

The two peptide models of dynorphin A(1–17) described in this study, DYM 1 and DYM 2, were designed to explore the functional significance of the potential amphiphilic β-strand structure in residues 7–15 of the native peptide. The opioid peptide β-endorphin was the first amphiphilic peptide hormone to be studied by this approach (Taylor et al., 1981). In that case, the importance of a potential amphiphilic α-helical segment in residues 13–29 was tested through the study of a fully active analogue that incorporated an "idealized" model of the natural amphiphilic structure, another where a *left-handed* helix composed of D-amino acid residues was substituted for the native structure, and other analogues with impaired activities where the segregation of hydrophobic and hydrophilic residues into separate domains on the helix surface was altered and even abolished [reviewed by Taylor and Kaiser (1986)]. After it had been established that completely nonhomologous models that reproduced the essential general characteristics of the native structure were effective substitutes, the models with altered characteristics provided evidence as to their functional significance. The first dynorphin model we describe, DYM 1, incorporates an idealized model for residues 6–17 that includes Pro^{10}—a single residue in the natural amphiphilic segment that might be an important characteristic of that structure, because it must introduce a kink or bend into an idealized β strand. In the second model, DYM 2, the importance of Pro^{10} is investigated through its substitution to create an amphiphilic segment consisting of uniformly repeating lysine and valine residues.

DYM 1 binds poorly to the air–water interface, as does dynorphin A(1–17), and its CD spectrum is similar to that of disordered peptides, both in solution and bound to siliconized glass, as is that of dynorphin A(1–17). In opioid receptor binding assays, it displays the same high affinity for κ receptors as the native peptide and a greater selectivity for these receptors over μ and δ receptors. Neither dynorphin A(1–17) nor DYM 1 has significant potency in RVD assays, and in GPI assays DYM 1 is a potent opioid agonist, although it is less potent than dynorphin A(1–17), and its actions are again more selectively mediated by the κ receptor than are the actions of the native peptide. DYM 1 is, therefore, a successful model of dynorphin A(1–17) in these studies.

The properties of DYM 2 at the air–water interface and adorbed onto siliconized slides contrast markedly with those of DYM 1 and dynorphin A(1–17). DYM 2 is able to self-associate through extensive hydrogen-bond formation in the plane of these interfaces forming highly amphiphilic β sheets. DYM 2 also has a significantly lower affinity for κ receptors in brain membranes than these two peptides, but it has a potency on the GPI that is intermediate between dynorphin A(1–17) and DYM 1, and it remains κ selective in both types of assay. This demonstrates that a simple sequence of alternating lysine and valine residues connected to the [Leu^5]-enkephalin structure via a single basic residue functions as a suitable model for the κ-agonist dynorphin A(1–17) in pharmacological assays but not a planar amphiphilic interfaces.

Clearly, Pro^{10} has the effect of disrupting the potential aggregation of dynorphin A(1–17) at planar interfaces to form amphiphilic β sheets. This might be an important characteristic of the native amphiphilic β-strand structure, since any aggregation to form β sheets would result in a considerable increase in overall amphiphilic character and the hormone would bind very tightly and nonspecifically to surfaces, as does DYM 2. Such behavior, which we have demonstrated for the first time for an amphiphilic β strand that is located internally in a peptide sequence, would be difficult to regulate during the release of a peptide hormone that is packaged at a high concentration in synaptic vesicles and would diminish the ability of that hormone to function at distant sites. In this context, it is interesting that amino acid sequences with the potential to form amphiphilic β strands appear to be very rare in biologically active peptides, whereas amphiphilic α helices, which tend to act as monomers at interfaces (see Table I), are a common feature of hormones and other circulating, biologically active peptides (Kaiser & Kezdy, 1984; Taylor & Kaiser, 1986).

Since the physicochemical properties of an amphiphilic β strand are mostly manifested as a consequence of aggregation into β-sheet structures and this conformation in the non-hydrogen-bonded, isolated form does not have any readily characterized spectroscopic features, it has been difficult to evaluate its functional importance in dynorphin A(1–17). In aqueous solution, dynorphin A(1–17) apears to adopt an extended conformation, on the basis of fluorescence energy transfer experiments (Schiller, 1983). A variety of spectroscopic techniques indicate that dynorphin A(1–13), which does not appear to occur naturally but is a potent κ agonist, also has an extended, unfolded conformation in aqueous solution (Maroun & Mattice, 1981; Rapaka et al., 1987; Renugopalakrishnan et al., 1988). Dynorphin A(1–13) has been shown to bind in aqueous solution to cerebroside sulfate, a lipid implicated in opioid receptor structures, without any observable change in its CD spectrum, and the extended amphiphilic conformation that we propose for the longer native peptide has been involked to explain this binding also (Wu et al., 1986). However, studies of the infrared spectrum of dynorphin A-(1–13) incorporated into or adsorbed from aqueous solution onto multiple phospholipid bilayers deposited on a planar surface have indicated the presence of some helical structure oriented perpendicular to the plane of the surface (Erne et al., 1985). The location of this helical structure at the amino-terminal end of the short dynorphin sequence (residues 1–9) was indicated by photolabeling experiments (Gysin & Schwyzer, 1983) and supported by a theoretical analysis (Schwyzer, 1986a). The strongest evidence we now present for the functional importance of the β-strand conformation in residues 7–15 of the complete native dynorphin A(1–17) sequence is derived from the strategy used to design DYM 1 and DYM 2 so that the potential amphiphilicity and intrinsic stability of the β-strand segment were optimized and homology to the native sequence was minimized, combined with the ability of DYM 1 in particular to reproduce the pharmacological properties of dynorphin A(1–17). The CD spectrum of DYM 1 adsorbed onto siliconized slides indicates that the use of Val as the hydrophobic component has stabilized the β-strand conformation relative to dynorphin in this environment, so that some self-association into β sheets occurs, and it has destabilized the small α-helical component in the native peptide, so that it is absent from the DYM 1 spectrum (Figure 5). This is in agreement with NMR studies of the solution conformation of this model peptide, which identify an extended, β-strand-like conformation for residues 7–17 (Vaughn & Taylor, 1989). The tight binding of DYM 1 to κ receptors (Table II) therefore makes the amphiphilic β-strand conformation of residues 7–15 a likely candidate for interactions with that receptor. It also demonstrates that specific side-chain interactions in this region of the peptide ligand are not required

for either tight κ-receptor binding or high κ-receptor selectivity. Conformations of dynorphin A residues 7–15 other than the proposed amphiphilic β-strand, although they cannot be ruled out, are expected to require stabilization through such specific interactions at the membrane surface, because of their reduced amphiphilic character. It should be noted, however, that a requirement for the proposed amphiphilic conformation in order to produce dynorphin A-like pharmacological properties has not been demonstrated. Indeed, the more potent and κ-selective opioid agonist properties of dynorphin A(1–13) amide suggest that it is not required. Furthermore, it remains possible that an amino-terminal helical structure in dynorphin A is stabilized upon interaction with cell surfaces, as suggested by Schwyzer and his colleagues.

The pharmacological properties of DYM 1 and DYM 2, as well as those of the peptide models of β-endorphin studied earlier (Taylor & Kaiser, 1986, 1989), suggest a general role for amphiphilic secondary structures in determining the different opioid receptor selectivities of the native peptides, dynorphin A(1–17) and β-endorphin. The β strand proposed for dynorphin A residues 7–15 and the α helix proposed for β-endorphin residues 13–29 are both cationic, amphiphilic structures that are expected to bind to the hydrophilic–lipophilic boundary of the phospholipid bilayer surface, which is thought to have a predominant anionic character (Schwyzer, 1986b). Recent experiments (Jacobs & White, 1989) suggest that these structural segments need not penetrate into the lipid compartment of the membrane, in order for the hydrophobic component of their binding to be largely complete, and they are likely to be located at the level of the headgroups. The enkephalin-binding sites on μ-, δ-, and κ-opioid receptors are proposed to be located at different levels relative to this hydrophilic–lipophilic interface (Schwyzer, 1986b). In the case of dynorphin A(1–17), binding of the amphiphilic β strand would constrain the enkephalin segment to lie very deep in the membrane surface near the proposed level of its hydrophobic binding site on κ receptors, because only one basic residue serves to connent these two structures. In contrast, binding of the amphiphilic α helix induced in β-endorphin at the same interface, although it may promote receptor location in general, would not constrain the enkephalin segment to bind to κ sites, because these two structural domains are connected via a neutral, hydrophilic peptide segment that is seven residues in length. Instead, this connecting segment in β-endorphin promotes enkephalin interactions with μ and δ sites, both of which are proposed by Schwyzer to lie in a hydrophilic environment.

An additional role for the proposed amphiphilic secondary structures of dynorphin A(1–17) and β-endorphin in receptor activation is likely. The evidence for such a role is provided by the observation that the most successful models of these opioid peptides bind to the appropriate receptors in guinea pig brain with higher affinities than the native peptides, but have lower potencies in the most stringent smooth muscle assays for these hormones. Thus, the very high agonist potency of dynorphin A(1–17) on κ receptors in the GPI is not fully reproduced by DYM 1, which is about 8 times less potent in this assay, although in binding assays its affinity for these receptors is at least as high as that of dynorphin A(1–17) (Tables II and III). Similarly, two peptide models of β-endorphin that have a higher affinity than the native hormone for μ receptors in the binding assays are about 6 times less potent on the RVD (Taylor & Kaiser, 1986; Taylor & Kaiser, 1989), where opioid agonist activity is thought to be mediated by a limited population of the same receptors and require particularly efficient receptor activation (Smith & Rance, 1983; Miller et al., 1986). Although there are alternative explanations for these moderate discrepancies, a simple explanation that may be consistently applied to all of the model peptide results is that the amphiphilic segments of the native hormones have sequence-specific contacts with their receptors that facilitate receptor activation subsequent to occupation of the enkephalin binding sites. Interactions of this type might lower the activation energy required for signal transduction but might not be observed in receptor binding assays, where ligand affinities for the activated and unactivated forms of the receptor are not distinguished. If the agonist–antagonist character of these opioid peptides is dependent on the cooperative interactions of the enkephalin segment and these amphiphilic structures, the design of new peptide models with enhanced antagonist character might be possible.

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