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Biochemistry

Surface properties of an amphiphilic peptide hormone and of its analog: Corticotropin-releasing factor and sauvagine

(amphiphilic α-helix/binding to vesicles by corticotropin-releasing factor and sauvagine/circular dichroic spectrum of corticotropin-releasing factor/peptide monolayer)

S. H. LAU*, J. RIVIER†, W. VALE†, E. T. KAISER‡, AND F. J. KÉZDY*

*Department of Biochemistry, The University of Chicago, Chicago, IL 60637; †Peptide Biology Laboratory, Salk Institute for Biological Studies, San Diego, CA 92138; and ‡Laboratory of Bioorganic Chemistry and Biochemistry, The Rockefeller University, New York, NY 10021

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ABSTRACT **Synthetic corticotropin (adrenocorticotropic hormone)-releasing factor [CRF; for the sequence, see Vale, W., Spiess, J., Rivier, C. & Rivier, J. (1981) *Science* 213, 1394–1397] in aqueous solution exists predominantly as a random coil. At concentrations greater than 1 μM, the peptide shows a tendency to self-aggregate with a concurrent slight increase in the apparent α-helical content as measured by the CD spectrum. The α-helix formed by this molecule is highly amphiphilic—i.e., the hydrophilic and hydrophobic regions are segregated on opposite faces of the helix. As predicted from the potential amphiphilic structure, CRF binds avidly to the surface of single bilayer egg phosphatidylcholine vesicles. This binding appears to obey a simple Langmuir isotherm with the following parameters: $K_d = 1.3 \pm 0.6 \times 10^{-7}$ M and capacity at saturation (N) = 11.0 ± 1.0 mmol of peptide per mol of phospholipid. CRF also readily forms an insoluble monolayer at the air–water interface. The monolayer is composed of monomers of the hormone with molecular areas, $A_0' = 22$ Å^2 per amino acid, suggesting a compact secondary structure. Judged from the collapse pressure (19.0 ± 0.1 dyne/cm; 1 dyne = 10 μN) of the monolayer, the amphiphilicity of CRF approximates that of plasma apolipoproteins, a class of proteins of the most pronounced amphiphilic character. These results suggest that the binding of CRF to the cell membrane is accompanied by the induction of an α-helical secondary structure and it is this predominantly helical form that is the biologically active form of the peptide.**

Corticotropin (adrenocorticotropic hormone)-releasing factor (CRF) is a hormone produced by the hypothalamus; it increases the rate of secretion of corticotropin by the pituitary gland (1, 2). The primary structure of ovine CRF has been determined by Vale *et al.* (3) and is shown in Fig. 1. Vale *et al.* also reported that a considerable portion of CRF is highly homologous to sauvagine, a peptide isolated from the skin of the South American frog *Phylomedusa sauvagei* and to urotensin I (4).

The homology appears to be more conservative toward the amino and carboxyl termini of the molecule, whereas the pentapeptide segment of residues 24–28 in CRF, located toward the center of the chain, seems to be dissimilar. Analysis of the helical potential of CRF by the method of Chou and Fasman (5) indicated that two large sections of the molecule have very pronounced α-helical potential. When the amino acid sequence of the two regions of high helical potential is projected axially using the "Edmundson wheel" (6), the hydrophilic and hydrophobic residues appear to be segregated on opposite sides of the cylindrical helix, as shown in Fig. 1. The hydrophilic and hydrophobic domains on these α-helices are not of equal importance: the hydrophobic domain is almost twice as large as the hydrophilic one. The occurrence of regions of high amphiphilic α-helical potential in CRF is not unique to this hormone: in fact, several peptide hormones of intermediate size (i.e., 10–50 amino acid residues per molecule) possess similar amphiphilic segments. In previous studies, we have presented evidence that amphiphilic secondary structure plays an important role in the action of calcitonin (7) and β-endorphin (8, 9). Similarly, amphiphilic secondary structures appear to play an important role in the function of plasma apolipoproteins (10–13) and of hemolytic peptides such as melittin (14, 15). Thus, if CRF is indeed able to assume an amphiphilic secondary structure readily, then it is quite likely that this amphiphilic structure will be the predominant conformation of the hormone at the cell membrane, its presumed site of action. The purpose of the present paper is to report that CRF and its toxin homolog, sauvagine, do in fact readily assume an amphiphilic secondary structure when exposed to amphiphilic environments—namely, the air–water interface and the surface of unilamellar phospholipid vesicles. These two model environments have been chosen for their accessibility to simple physical chemical experimentation.

EXPERIMENTAL SECTION

Materials. Egg yolk lecithin was purchased from Avanti Biochemicals, and its purity was ascertained by thin layer chromatography on silica gel G from Alltech, using the solvent system chloroform/methanol/water, 70:30:5 (vol/vol/vol).

Fluorescamine [4-phenylspiro-(furan-2(3H),1′-phthalan)-3,3′-dione] was a product of Roche Diagnostics.

The samples of CRF and sauvagine were synthesized by the solid-phase method (3).

Studies of Peptide Monolayers at the Air–Water Interface. All reagents and solvent used in the monolayer studies were of the highest purity available. Glassware was washed with fuming nitric acid. Air was bubbled for 15 min through solutions used for the subphase after which the top portion of the solution was discarded to remove surfactant impurities.

The surface pressure in dyne/cm (π) of the peptide monolayer at the air–water interface was measured as a function of the area of the monolayer using a Lauda film balance and an x–y chart recorder. A peptide solution (30 μg in 100 μl for CRF and 23 μg in 50 μl for sauvagine in 0.01 M Tris·HCl/0.1 M NaCl, pH 7.40) was spread on the surface of the subphase, which consisted of the same buffer. The monolayer was compressed and expanded at a rate of 180 cm^2/min at room temperature (22.4°C).

CD and Ultracentrifugation Studies of the Peptides. CD spectra of CRF in 0.02 M potassium phosphate buffer, pH 7.0/0.16 M KCl were determined using a Cary 60 spectropolar-

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Abbreviations: CRF, corticotropin-releasing factor; apo A-I, apolipoprotein A-I.

A

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                                        11                                  21
CRF     H-Ser-Gln-Glu-Pro-Pro-Ile-Ser-Leu-Asp-Leu-Thr-Phe-His-Leu-Leu-Arg-Glu-Val-Leu-Glu-Met-

                                        10                                  20
Sauvagine       pGlu-Gly-Pro-Pro-Ile-Ser-Ile-Asp-Leu-Ser-Leu-Glu-Leu-Leu-Arg-Lys-Met-Ile-Glu-Ile-


                                        31                                  41
CRF          Thr-Lys-Ala-Asp-Gln-Leu-Ala-Gln-Gln-Ala-His-Ser-Asn-Arg-Lys-Leu-Leu-Asp-Ile-Ala-NH2

                                        30                                  40
Sauvagine    Glu-Lys-Gln-Glu-Lys-Glu-Lys-Gln-Gln-Ala-Ala-Asn-Asn-Arg-Leu-Leu-Leu-Asp-Thr-Ile-NH2
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B

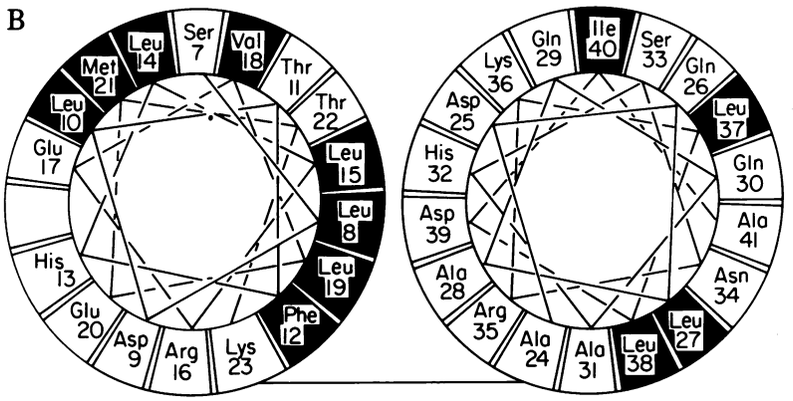

FIG. 1. (*A*) Amino acid sequences of CRF and sauvagine. (*B*) Axial projection of potential α-helical regions of CRF showing the segregation of hydrophobic and hydrophilic residues.

imeter. The concentration dependency of the ellipticity was measured in the same buffer over the 1–113 μM peptide concentration range. The mean residue ellipticity at 222 nm ($[\theta]_{222\,nm}$, deg·cm^2/dmol) was used to estimate the α-helicity according to the equation: % α-helicity = $[(-[\theta]_{222\,nm} + 3{,}000)/(36{,}000 + 3{,}000)] \times 100$ (16). The CD spectrum was measured between 250 and 200 nm at each concentration.

Ultracentrifugation of the peptide was carried out with a Beckman model E analytical ultracentrifuge. Solutions of the peptides at concentrations in the range of 100–300 μg/ml were centrifuged at 36,000 rpm at 24.7°C for 24 hr according to the meniscus-depletion sedimentation equilibrium method. Apparent M_r was calculated by using the equation (17)

$$M_r = \frac{2RT}{\omega^2(1 - \bar{v}\rho)} \frac{d \ln f}{d(r^2)},$$

where R is the gas constant, T is the absolute temperature, f is the interference fringe displacement, r is the distance from the center of rotation, ω is the angular velocity, $\bar{v}$ is the partial specific volume of the peptide, and ρ is the density of the solution.

Unilamellar Phospholipid Vesicles (18). Two milliliters of an ethanolic solution of egg yolk lecithin (33 mg/ml) was injected rapidly using a 50-μl Hamilton syringe into 100 ml of a briskly stirred and N_2 purged 0.16 M KCl solution. The resulting mixture was concentrated to 5 ml by ultrafiltration in a 200-ml Amicon stirred cell using an XM-100A membrane filter. The unilamellar vesicles were purified by passage through a Sepharose CL-4B (Pharmacia) column (2.0 × 90 cm). The effluent of the column was monitored by differential refractometry (Waters Associates differential refractometer, model R 401). The unilamellar vesicles eluted after a peak of multilamellar vesicles, and the appropriate fractions were concentrated again by ultrafiltration. The concentration of the unilamellar vesicles was measured by the UV absorbance using $\varepsilon_{210} = 2{,}000\ M^{-1}\cdot cm^{-1}$ (19). The purity of the vesicle solution was assessed by the ratio of the absorbances at 210 and 300 nm (18). The vesicle solution was stored under nitrogen at 4°C and used within 3 days.

Binding of the Peptides to Unilamellar Vesicles (11). Binding of the peptides to the unilamellar vesicles was carried out under the following conditions: constant concentration of vesicles (0.65 mM lecithin) and various concentrations of peptide (0.30–8.84 μM) in 2 ml of buffer [0.025 M morpholinopropanesulfonic acid (Mops)/0.16 M KCl, pH 7.0] were incubated for 1.5–2.0 hr at room temperature with occasional stirring. At the end of the incubation period, the free peptide was separated from the bound peptide by ultrafiltration, using an XM-100A membrane and a 3-ml Amicon stirred cell. The first 2 drops of the filtrate were discarded and the next 10 drops (300 μl) were collected. The concentration of peptide in both the filtrate and the retentate was determined by the fluorescamine assay. A 50-μl aliquot of the peptide solution was first dried in an oven at 110°C for 1 hr. Alkaline hydrolysis (20) of the dried residue was accomplished at 120°C for 20 min after addition of 0.4 ml of 0.5 M NaOH. After cooling, 0.4 ml of 0.5 M HCl was added, and then 2.0 ml of 0.5 M sodium borate (pH 8.5) was added. Three hundred microliters of a fluorescamine solution (0.2 mg/ml in acetone) was added subsequently to the hydrolysate with vigorous mixing, and the fluorescence intensity was measured using a Turner fluorometer model 111 at $\lambda_{ex} = 390$ nm and $\lambda_{em} = 475$ nm. The relative concentrations of the bound and free peptides were calculated from the relative fluorescence intensities of the filtrate and retentate.

RESULTS

Both CRF and sauvagine formed monolayers at the interface between air and neutral aqueous solutions. These monolayers were stable for at least half an hour as judged by the reproducibility of the isotherms on repeated compression–recompression cycles. This behavior indicates that both peptides form at the interface an amphiphilic structure that will be the preferred state of the peptides. The peptide in the monolayer reaches

a metastable equilibrium with a small amount of peptide dissolved in a thin layer of the subphase located directly below the surface. Slow diffusion of the soluble peptide into the bulk subphase leads ultimately to desorption of the monolayer. The force–area curves (Fig. 2) show a negative second derivative above $\pi = 19.0 \pm 0.1$ and 18.6 ± 0.2 dyne/cm (1 dyne = 10 μN) for CRF and sauvagine, respectively. This point is conventionally designated as the collapse pressure, a measure of the stability of the monolayer. The force–area curves were analyzed at low surface pressure (<0.5 dyne/cm) using the equation $\pi(A - nA_0) = nRT$ (21), where A is the measured surface area of the monolayer, A_0 is the molar excluded area, and n is the number of moles of peptide. The experimental data, plotted as πA vs. π, yielded a straight line from which we calculated a M_r of 5,600 and a molecular area (A_0') of 22 Å^2 per amino acid for CRF. The corresponding values for sauvagine are M_r = 4,200 and A_0' = 17 Å^2 per amino acid. The experimental values of M_r agree with the values calculated from the amino acid sequences—namely, 4,671 for CRF and 4,618 for sauvagine. Therefore, at low surface pressure, both CRF and sauvagine exist predominantly as gaseous monomers at the air–water interface.

The CD spectrum of CRF indicates the presence of a negative peak at ≈220 nm. The α-helicity of CRF in aqueous solution was then estimated from the mean residue ellipticity at 222 nm. With $[\theta] = -4{,}300 \pm 100$ deg·cm^2/dmol at a concentration of 2.26 μM, we calculated 11% helical content. The ellipticity increased when we increased the peptide concentration beyond 1.1 μM, thereby indicating self-association. The apparent increase in ellipticity with increasing peptide concentration might be due to either the formation of an asymmetric oligomer or an increase in the helicity in the oligomer. We feel that the latter is true in this case because CRF has such a low degree of organization in the monomeric state. The concentration dependency of the CD spectra of CRF at 222 nm is shown in Fig. 3. Analysis of this curve indicates that the concentration dependency does not reflect a single stoichiometrically well-defined oligomerization. The data indicate, however, that CRF aggregates in the concentration range studied. The results of the sedimentation studies also indicated that CRF does aggregate above micromolar concentrations: for the first few hours, CRF at a concentration of 59 μM did not sediment to any appreciable extent under our experimental conditions. However, after this initial period, a progressive increase in concentration was observed toward the bottom of the cell and, after 24 hr, the bottom one-third of the cell showed a measurable concentration gradient. From the slope of a ln f vs. r^2 plot, we calculated apparent M_r values in this region. We found a M_r corresponding to that of the tetramer at around one-third of the cell height and, from there, the value of M_r gradually increased

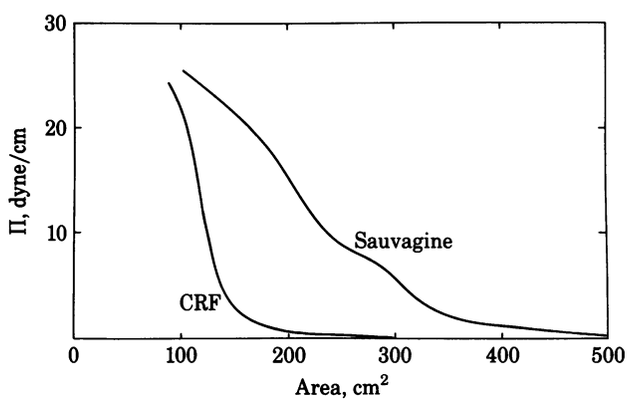

FIG. 2. Force–area curves for CRF and sauvagine.

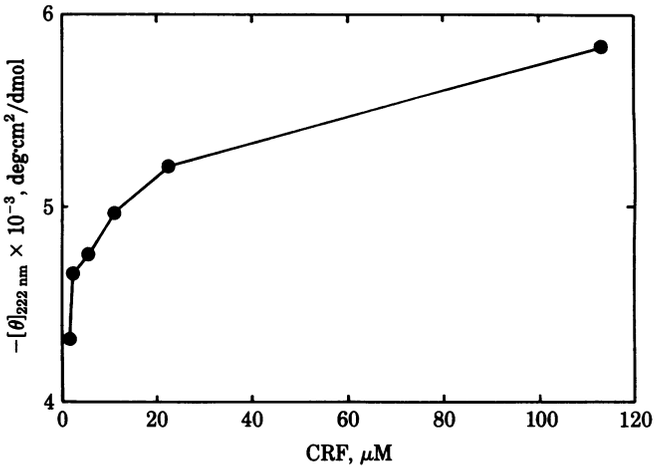

FIG. 3. Concentration dependency of the molar ellipticity of CRF at 222 nm.

to that of an octamer toward the bottom of the cell. Thus, CRF does self-associate at concentrations of the order of 60 μM. In contrast, no such self-association was evident in the sedimentation experiments with sauvagine at comparable concentrations.

The unilamellar egg lecithin vesicles prepared by the injection method undergo slow fusion even when stored at low temperature. Therefore, all binding studies were performed within 3 days of preparation of the vesicles. For measuring the quantity of peptide bound to unilamellar vesicles, we chose the method of sampling the solution by ultrafiltration. To prevent large disturbance of the equilibrium due to concentrating the vesicles by the ultrafiltration procedures, <20% of the solution was passed through the filter for determination of the concentration of unbound peptide. In preliminary experiments, we first ascertained that under our experimental conditions the peptide does not adsorb to any measurable extent to the Amicon XM-100A membrane used.

The studies on the binding of both CRF and sauvagine to pure lecithin vesicles showed strong adsorption of the peptides to the phospholipid bilayer at concentration ranges of 0.3 to 9 μM peptide and 0.65 mM phospholipid. Analytical gel filtra-

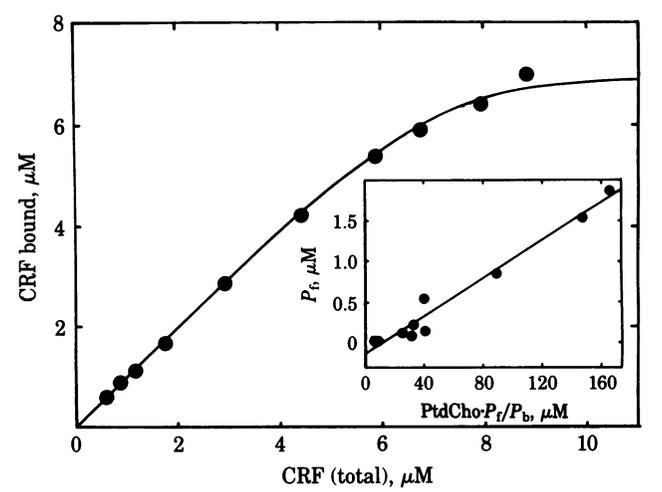

FIG. 4. Plot of CRF bound vs. total CRF in study of the binding of CRF to unilamellar vesicles. The curve shown is calculated from the equation $P_t = P_b\{1 + [(1.3 \times 10^{-7})/(7.1 \times 10^{-6} - P_b)]\}$. (*Inset*) Linearized plot of data from the unilamellar vesicle binding study of CRF.

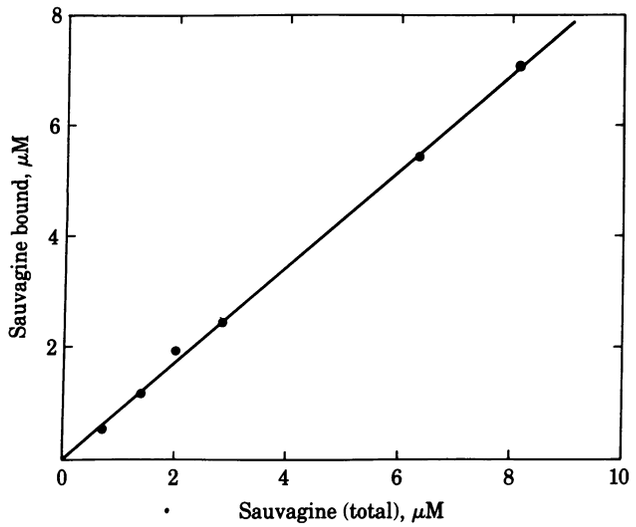

FIG. 5. Plot of sauvagine bound vs. total sauvagine in the study of the binding of sauvagine to unilamellar vesicles.

tion through a Sepharose CL-4B column, equilibrated with the same buffer as the one used in the binding studies, showed that the vesicles did not change their hydrodynamic behavior to any measurable extent. As shown in Figs. 4 and 5, nearly total binding of both peptides occurs as long as the peptide/phospholipid ratio remains below 10 mmol of peptide per mol of phospholipid. In the case of CRF, the curve could be analyzed as a single Langmuir isothermal adsorption using the following equation (11): $P_f = (N \cdot \text{PtdCho} \cdot P_f/P_b) - K_d$, where P_f and P_b are the concentrations of free and bound peptides, respectively, PtdCho is the concentration of egg phosphatidylcholine, N is the asymptote of P_b/PtdCho, and K_d is the dissociation constant. A plot of P_f vs. PtdCho $\times$ P_f/P_b (Fig. 4 *Inset*) yields a straight line, indicating that the data do indeed obey the above equation. The binding parameters K_d and N obtained from this plot are $K_d = 1.3 \pm 0.6 \times 10^{-7}$ M and $N = 11.0 \pm 1.0$ mmol of peptide per mol of phospholipid. In the case of sauvagine, the high affinity of the peptide for the phospholipid surface prevented us from obtaining the binding parameters from the experimental curve.

DISCUSSION

The results presented in this paper indicate that the major portion of the CRF molecule assumes a predominantly amphiphilic α-helical conformation when in an amphiphilic environment. Indeed, *a priori* analysis for α-helical potential according to the method of Chou and Fasman (5), $\langle P_\alpha \rangle = 1.20$, suggests that the CRF molecule should be $\approx$80% helical when the amphiphilicity of the α-helix matches that of the environment. Furthermore, all of the amino acid replacements in sauvagine with respect to CRF (3) are conservative from the point of view of the lipophilicity, with the exception of leucine at the place of lysine (position 36) of CRF. The high collapse pressure of the CRF monolayer at the air–water interface indicates a highly stabilized amphiphilic structure because random oligopeptides of comparable size and with about the same proportion of hydrophobic residues collapse at surface pressures of <10 dyne/cm (22). The compact structure of CRF at the air–water interface is also indicated by the low value of the limiting area per amino acid. It has been shown that proteins that are denatured at the air–water interface are characterized by residue areas as high as 66 Å^2 per amino acid (23). The pronounced potential amphiphilicity of CRF is epitomized in its behavior in the presence of phospholipid surfaces: it binds to single bilayer vesicles of egg lecithin with such high affinity that the dissociation constant is barely measurable by our experimental technique. This high affinity for phospholipid surfaces is the most characteristic property of highly surface-active peptides and proteins such as apolipoprotein A-I (apo A-I) (12, 13) and melittin (14). For instance, the dissociation constant for phospholipid-bound apo A-I is 9.0×10^{-7} M (11) and its 22 amino acid fragments have K_d values ranging upward from $\approx 2.0 \times 10^{-6}$ M (8–12). Thus, CRF has an affinity for phospholipid surfaces that surpasses that of apo A-I, for which the physiological role consists of stabilizing the phospholipid and cholesterol surfaces of lipoproteins. The structural basis for this very high affinity resides in the ratio of hydrophilic vs. hydrophobic residues. Apo A-I consists of amphiphilic α-helices in which the hydrophilic region is about twice as extended as the hydrophobic region. In contrast, melittin, which possesses an unmeasurably high affinity for phospholipid surfaces and even disrupts the vesicular structure of unilamellar phospholipid vesicles is about three-fourths hydrophobic in the helical segment. Thus, CRF with $\approx$60% of the helix hydrophobic is situated between these two extremes of surface active peptides. It is rather well documented that both apo A-I and melittin are in the amphiphilic α-helical conformation when bound to phospholipid surfaces. Thus, the fact that the K_d of CRF is intermediate between those of apo A-I and melittin strongly suggests that, indeed, CRF is also in the amphiphilic α-helical structure when bound to amphiphilic surfaces.

In contrast to melittin, CRF does not appear to provoke the fusion of unilamellar vesicles. We feel that the limited extent of the hydrophobic region in CRF prevents it from penetrating deeply enough into the phospholipid bilayer to cause local disruption of the bilayer continuity. Aqueous solutions of CRF show the characteristic CD spectrum of a predominantly random coil peptide with perhaps 10% helicity. This low helix content is also reflected by the lack of well-defined cooperative oligomerization. In contrast to melittin (24), which is roughly half the size of CRF and produces a well-defined tetramer as the only detectable oligomeric species, CRF appears to undergo a weak random self-association. Thus, under physiological conditions, CRF probably exists in the extracellular medium predominantly as a random coil monomer. One must assume that CRF activates its target cells by adsorbing to the cell membrane and interacting with specific receptors. The hormone–receptor interaction thus occurs in an amphiphilic environment, which we have shown to be conducive to the folding of CRF into a predominantly α-helical conformation. It is tempting to hypothesize that CRF would first adsorb to the membrane and fold into a well-defined secondary structure before interaction with the receptor can occur. The presence of the amphiphilic environment of the membrane should be an essential requisite for expressing the biological activity of CRF. A corollary of this hypothesis is that a major portion of the amino acids in CRF fulfill a purely structural role and they could be replaced by a variety of other amino acid residues of the same hydrophobicity without any effect on the biological activity of the hormone. This indeed is the case for at least two other medium-sized peptide hormones—namely, β-endorphin (8, 9) and calcitonin (7).

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