Folding of the Mitochondrial Proton Adenosinetriphosphatase Proteolipid Channel in Phospholipid Vesicles†

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ABSTRACT: The mitochondrial H^+-ATPase proteolipid from *Neurospora crassa* was incorporated into small unilamellar dimyristoylphosphatidylcholine vesicles and its conformation determined by circular dichroism spectroscopy (CD). While the largely α-helical conformation is relatively independent of the method of incorporation into vesicles, i.e., rehydration, detergent dialysis, or detergent dilution, the proteolipid conformation was significantly different in detergent micelles and in organic solvents. Only very slight changes in the CD spectrum were observed upon binding of the H^+-ATPase inhibitor dicyclohexylcarbodiimide to the proteolipid in vesicles, thus suggesting that the inhibitor acts either by blocking the channel or by masking an essential charge group, rather by than causing an overall conformational change in the channel. Additionally, very similar CD spectra were obtained for vesicles with different lipid/protein mole ratios, indicating either that no substantial conformational differences exist between monomer and multimers or that monomers self-associate to form stable complexes during incorporation into vesicles. This study has provided a physical basis for model-building studies of the proteolipid channel structure.

The mitochondrial proton adenosinetriphosphatase (H^+-ATPase) reversibly couples oxidative phosphorylation of ADP to the translocation of protons across the membrane (Kagawa, 1972; Racker & Stoeckenius, 1974). This enzyme complex is composed of a water soluble component, F_1, and an integral membrane component, F_0. The F_1 component exhibits the ATPase (or ATP synthetase) activity. The F_0 component acts as a channel to facilitate the passive translocation of protons through the membrane. The coupled ATPase and proton-translocation activities of the ATPase complexes from thermophilic bacterium PS3 (Sone et al., 1977), *Escherichia coli* (Foster & Fillingame, 1979), *Mycobacterium phlei* (Cohen et al., 1978), spinach chloroplast (Pick & Racker, 1979), and bovine heart mitochondria (Serrano et al., 1976) have been reconstituted in liposomes.

The ATPase activity of the intact F_1-F_0 complex and the proton permeability of F_1-depleted membranes are specifically inhibited by dicyclohexylcarbodiimide (DCCD),[1] which reacts irreversibly with a single acidic residue in one subunit of the F_0 sector (Sebald et al., 1980). This DCCD-binding polypeptide is designated proteolipid because of its solubility in $CHCl_3$/MeOH, even though it does not contain any covalently attached lipids. In *E. coli* mutants that are defective in H^+ translocation, but have functional F_1 ATPase activity, glycine is substituted for the DCCD-reactive acidic residue in the proteolipid (Wachter et al., 1980).

Proteolipid has a molecular weight of ~8000 (by amino acid composition), and 75% of its amino acids are hydrophobic (Sebald et al., 1979b). Radioactive labeling of the subunits of *N. crassa* and *S. cerevisiae* mitochondrial H^+-ATPase complexes suggests the stoichiometry of proteolipid per F_0 may be 6:1, and DCCD titration of one-sixth of the proteolipid molecules results in complete inhibition of ATPase activity (Sebald et al., 1979a). Isolated proteolipids from the mitochondria of yeast, bovine heart, and lettuce chloroplasts have been incorporated into liposomes, resulting in H^+ conductance (Konishi et al., 1979; Celis, 1980; Sigrist-Nelson & Azzi, 1980); this reconstituted H^+-conductance activity is also inhibited by DCCD. However, under similar conditions, other workers have been unable to reconstitute active channels from the *E. coli* (Criddle et al., 1977) or bacterium PS3 (Sone et al., 1978) proteolipid subunits alone. Single channel conductance has been observed in black lipid membranes containing yeast mitochondria proteolipids at pH 2 (Montal et al., 1981).

The conformation of proteolipid within the lipid bilayer and its mechanism of mediating H^+ translocation across the membrane have not been clearly defined. Structural information on this protein will provide a data base for model building and should promote better understanding of the transport process. Proteolipid is an excellent molecule for structural studies of ion channels because of its availability in purified form, stability, and capability of being reconstituted into membranes. In this study, circular dichroism spectroscopy (CD) has been used to obtain information on the secondary structure of purified proteolipid in small unilamellar phospholipid vesicles, detergent micelles, and trifluoroethanol solution.

Materials and Methods

Materials. Proteolipid was prepared from *N. crassa* mitochondria by extraction in $CHCl_3$/MeOH (2:1) (Sebald et al., 1979a). Less than 1 mg of total protein was used in all the experiments herein reported. Dimyristoylphosphatidylcholine (DMPC) and asolectin were obtained from Calbiochem. *n*-Octyl glucoside, trifluoroethanol, tricene, dicyclohexylcarbodiimide, valinomycin, carbonyl cyanide, *m*-chlorophenylhydrazone, and 9-aminoacridine were from Sigma. Spectrum wet cellulose dialysis tubing with a molecular weight cutoff of 25000 was used. [^{14}C]DCCD of specific activity 54 Ci/mol was obtained from CEA, Gif-Sur-Yvette, France.

The proteolipid concentration was determined by the method of Lowry et al. (1951) in the presence of 0.1% $NaDodSO_4$,

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[1] Abbreviations: CD, circular dichroism; DMPC, dimyristoylphosphatidylcholine; DCCD, dicyclohexylcarbodiimide; TFE, trifluoroethanol; $NaDodSO_4$, sodium dodecyl sulfate.

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using a bovine serum albumin standard. Lipid concentrations were determined by a modified Fiske–Subbarow phosphate assay method (1925).

Vesicle Preparations. Method I: Rehydration. Small unilamellar vesicles were prepared, as previously described (Wallace & Blout, 1979), from dried-down solutions which had contained 20 μg of DMPC and 2 μg of proteolipid per μL of $CHCl_3$/MeOH (2:1). The aqueous vesicle suspension (~4 mg/mL DMPC) was sonicated to clarity with a Soniprep 150 ultrasonicator microprobe using 30-s bursts at 37 °C. The resulting suspension was centrifuged at 12800*g* for 6 min. The supernatant contained small unilamellar vesicles. DMPC vesicles were prepared in the same fashion but without proteolipid.

Method II: Detergent Dialysis. A 100-μL 1.5% (w/v) solution of *n*-octyl glucoside and 600 μg of DMPC were added to 60 μg of dried proteolipid. This mixture was sonicated for 15 min a cylindrical ultrasonic tank (Laboratory Supplies) and then dialyzed for 3 days against eight changes of 500 mL of deionized H_2O. The dialyzed sample was then diluted with deionized H_2O, sonicated with the microprobe sonicator, and centrifuged at 12800*g* for 6 min. DMPC vesicles were prepared in the same fashion but without proteolipid.

Method III: Detergent Dilution. Vesicles were prepared according to the procedure of Racker et al. (1979). A 34.8-μg sample of DMPC (0.05 μmol) in 1 mL of 10 mM tricene (pH 8.0) and 10 mM KCl was sonicated to clarity; 20.1 μL of 14.9% *n*-octyl glucoside in the same buffer and 180 μL of the DMPC suspension were added to 102 μg of the dried proteolipid. This mixture was incubated for 10 min at 4 °C and subsequently sonicated for 2 min. The sample was then diluted to 5.0 mL with deionized H_2O and centrifuged at 243000*g* for 90 min at 4 °C. Supernatant was removed, and the pellet was resuspended in 400 μL of deionized H_2O. The suspension was then sonicated to clarity and centrifuged at 12800*g*.

Dialysis. For removal of any proteolipid that was not incorporated into the lipid bilayer, 300 μL of proteolipid–DMPC vesicles, prepared according to method I, was dialyzed overnight, at 21 °C, against 2 L of deionized H_2O. DMPC vesicles without proteolipid were treated in a similar manner. In addition, as a control for conformational changes in the sample that might occur over the dialysis time period, a sample of proteolipid–DMPC vesicles was allowed to stand overnight at 21 °C.

Solution Preparations. I. n-Octyl Glucoside. A 300 μL 1.5% (w/v) *n*-octyl glucoside solution was added to 30 μg of dried proteolipid. The resulting suspension was sonicated and centrifuged at 12800*g* for 6 min.

II. Trifluoroethanol. Precautions were taken to prevent loss of material when proteolipid was transferred to another solvent, so 10 μL of 2 μg of proteolipid/μL in $CHCl_3$/MeOH (2:1) was added to 100 μL of TFE. The solvent was slowly evaporated at 47 °C. A fresh aliquot of TFE was added to the concentrated solution. The solvent was once again evaporated. This process was repeated several times. The proteolipid was dissolved in a final volume of 100 μL of TFE.

III. $NaDodSO_4$ Solubilization. Preformed vesicles were solubilized in 0.1% $NaDodSO_4$. The volume change upon addition was 1% which was corrected for in the calculations of concentration.

DCCD Labeling. [^{14}C]DCCD (7.5×10^{-3} M; 1 μL) in ethanol was added to 100 μL of either proteolipid vesicles ([proteolipid] = 2.11×10^{-5} M; [DMPC] = 3.26×10^{-3} M) or lipid vesicles, prepared by method I, and vortexed. The labeling reaction was carried out at room temperature for 15 min. In these experiments the DCCD/proteolipid mole ratio was 3.6. In other experiments using nonradioactive DCCD, DCCD/proteolipid mole ratios ranging from 5 to 21 were used. As a control for the effect of ethanol on proteolipid conformation, 90 μL of proteolipid-containing vesicles or vesicles alone were treated with 1 μL of EtOH. CD and absorption spectra were determined for both unlabeled and [^{14}C]-DCCD-labeled proteolipid–DMPC vesicles and DMPC vesicles. For determination of the extent of labeling of the proteolipid, [^{14}C]DCCD-labeled proteolipid and free [^{14}C]DCCD were separated by thin-layer chromatography, and the amount of radioactivity present in each fraction was analyzed: 75 μL of [^{14}C]DCCD-labeled proteolipid vesicles (~6×10^5 cpm) were run in parallel with 1 μL of 7.5×10^{-3} M [^{14}C]DCCD on K5 silica gel plates (80 Å, Whatman) developed in chloroform/methanol/water (65:25:3, v/v/v). Scrapings of the plates were taken at 1-cm intervals and placed in scintillation vials; 160 μL of deionized water was then added to completely wet the sorbent and deactivate it. After the vials were allowed to sit at room temperature overnight, 5 mL of scintisol (Isolab) was added to each vial. The vials were vortexed and counted in a Beckman LS100C liquid scintillation counter with a counting time of 5 min. Protein and lipid components were visualized on plates run under similar conditions by using *o*-tolidine and molybdenum blue, respectively, to determine their R_f values in this chromatography system.

Spectroscopy. CD spectra were recorded on a Cary 60 spectropolarimeter fitted with a Model 6001 CD attachment and a variable position detector. The wavelength range scanned was 300–190 nm. The instrument was calibrated with (+)*d*-10-camphorsulfonic acid at 290 nm. In most experiments, measurements were obtained with the photomultiplier tube directly adjacent to the sample cell, resulting in an acceptance angle of ~90°. Measurements were routinely made at 21 °C by using a 0.5-mm path-length cell. Blank runs of DMPC vesicles, 1.5% *n*-octyl glucoside, or TFE were subtracted from the appropriate sample spectra. In general, a scanning speed of 5 nm/min and a time constant of 3 s were used. The spectra reported are the average of three or four scans calculated every 1 nm.

The absorption spectra, from 400 to 190 nm, of proteolipid-containing vesicles and $NaDodSO_4$-solubilized vesicles were measured on a Cary 15 recording spectrophotometer with the CD cell placed closest to the light detector. H_2O and 0.1% $NaDodSO_4$ reference samples were used.

Electron Microscopy. A 5-μL sample of proteolipid-containing vesicles ([proteolipid] = 7.90×10^{-6} M; [DMPC] = 1.69×10^{-3} M) was placed on a Formvar-coated copper grid. The sample was stained with 1% aqueous uranyl acetate. Lipid vesicles ([DMPC] = 1.56×10^{-3} M) were treated in the same manner. Grids were examined in a JEOL 200 electron microscope.

CD Data Analysis. Correction for Optical Artifacts. The measured absorption of a particulate sample is the sum of the absorption of the chromophores, modified by light scattering and absorption flattening effects (D. Mao and B. A. Wallace, unpublished results). The extent of apparent absorption due to scattering, A_s, was measured in a region of the spectrum (i.e., 310–400 nm) where the chromophore absorption was negligible and was extrapolated to other wavelengths according to the following relationship (Leach & Scheraga, 1960): $A_s(\lambda) = k\lambda^{-n}$, where k and n are constants and is the wavelength. Absorption flattening, A_f, was estimated by comparison of the absorption of the particulate sample with a sample containing a homogeneous distribution of chromophores (i.e., vesicles

solubilized with 0.1% $NaDodSO_4$), after correction for scattering effects. For correction of the measured ellipticity for optical artifacts, the effect of differential light scattering was evaluated by varying the detector acceptance angle from 90° to 2° (Schneider & Harmatz, 1976). The differential absorption flattening is assumed to be the same for the absorption of left and right circularly polarized light and can be calculated from the above parameters (D. Mao and B. A. Wallace, unpublished results).

Secondary Structure Estimation. After being corrected for optical effects, data points at 1-nm intervals between 190 and 240 nm were analyzed by both linear (unconstrained) (Magar, 1968) and nonlinear (constrained) (Himmelblau, 1972) least-squares curve fitting procedures to obtain estimates of the amounts of each type of secondary structure present in the samples. The reference data set from a set of 15 water-soluble proteins was provided by J. T. Yang (Chang et al., 1978). The constrained (nonlinear) least-squares fit required that the fraction of each conformation be positive but did not require that the sum of the fractions be unity. The results were normalized to 100% by dividing each fraction by the sum of the fractions to remove variation due to concentration errors. A normalized standard deviation (NRMSD) for each curve fitting was expressed as

$$\text{NMRSD} = \left[\frac{\sum_N (\theta_{\text{exptl}} - \theta_{\text{calcd}})^2}{\sum_N (\theta_{\text{exptl}})^2} \right]^{1/2}$$

where θ_{exptl} and θ_{calcd} were the experimental and calculated mean residue ellipticities and N was the number of data points used. The average number of residues per helical segment was varied between 6 and 30 in order to determine the sensitivity of the results to helix length. Since variation over this range was small, a helix length of 26 was used in the analyses reported since this would be the approximate length of α helix necessary to span a 37 Å thick bilayer.

H^+-Conductance Measurements. Vesicles utilized in activity assays were prepared according to method I or by a cholate/deoxycholate dialysis procedure (similar to method II) (Okamato et al., 1977). Lipid/protein ratios ranging from 500 to 2×10^4 were used. Two methods of analyzing proton transport by proteolipid were utilized. The first examined the dissipation of a preformed proton gradient (Deamer et al. 1972) across the membrane by proteolipid. The proton conductivity was measured as the difference in fluorescence quenching between DCCD-treated and untreated proteolipid-containing vesicles. Lipid visicles without proteolipid served as controls for the action of DCCD on membrane permeability. The second method measured the potassium gradient-driven proton uptake of the vesicles (Sone et al., 1981), with the modification that a 7-min sonication in a cylindrical ultrasonic tank was substituted for the probe sonication in the potassium loading procedure.

Results

Characterization of Specimens. The proteolipid concentration was determined by Lowry assay with bovine serum albumin as a standard. This method may tend to incorrectly estimate the proteolipid concentration because of the low aromatic amino acid content of this protein (Fillingame, 1976). However, errors in concentration could be corrected for by the normalization procedure described in the following section.

During the preparation of vesicles by method I, it was observed that the initial proteolipid–DMPC multilamellar specimen was less opaque than the DMPC sample. The two samples achieved similar clarity after sonication. The recovery of DMPC was generally around 99–100%. The average recovery of proteolipid was near 85%. Therefore, the final protein/lipid mole ratio was somewhat lower than the input value. Electron microscopy of stained vesicles indicated the average diameter of the sonicated vesicles to be ~300 Å.

For method II preparations, the protein/detergent/lipid mole ratio was 1/668/115. Following dialysis, the sample appeared opaque but was clarified upon sonication, during which no foaming was observed. The final lipid/protein mole ratio was 162. The recovery of proteolipid was 60%.

In the detergent, lipid, and protein mixture used in method III preparations, the initial *n*-octyl glucoside concentration was 1.5% and the lipid/protein mole ratio was 709. Firm white pellets were obtained following dilution and ultracentrifugation. Foaming was observed after sonication of the resuspended pellet, indicating that likelihood that some *n*-octyl glucoside was still present in the sample. The final lipid/protein mole ratio was 973, with a proteolipid recovery of 47% and a DMPC recovery of 65%.

Calculation of CD Spectra. Particulate samples such as liposomes can produce optical artifacts that may seriously distort their CD spectra. The measured absorbance of a vesicle sample is the sum of the absorption of the chromophores, modified by light scattering and absorption flattening effects. Light scattering, manifest as additional apparent absorption, is due to the scattering of light in directions outside the acceptance angle of the detector (Schneider & Harmatz, 1976), whereas absorption flattening arises from the nonuniform distribution of the chromophore in the path of the beam (Duysens, 1956). The magnitudes of these effects may be assessed and the spectra appropriately corrected (D. Mao and B. A. Wallace, unpublished results).

Differential scattering was corrected for by a sample–detector geometry that permits collection of light scattered 45° from the incident direction. By use of acceptor half-angles from 45° to 1°, very similar CD spectra were obtained for proteolipid in the small sonicated vesicles. This suggests that virtually all the light scattered near the forward direction was collected by the detector. However, if the scattering envelope of the vesicles was not largely concentrated in the forward direction, then the CD spectra might be somewhat distorted by residual scattering. The absorption flattening corrections have the largest magnitudes in the region of high absorption (i.e., from 190 to 195 nm) but were very small for these vesicles which contain high lipid to protein ratios.

The correction method described is different from the formalism presented by Urry (1972), in that it does not rely on the invarience of the conformation upon dissolution in $NaDodSO_4$ (a condition which is not met for this sample). The method used herein yields very good results in a system of known structure, bacteriorhodopsin (D. Mao and B. A. Wallace, unpublished results). In all, the total distortions due to the optical phenomena for sonicated vesicles are very small, indeed, and together result in correction factors of less than 1% of the measured ellipticities.

The fractional composition of secondary structure types present in a protein was determined from the CD spectra by using both linear and nonlinear least-squares fittings of the experimental spectrum to a reference set of CD spectra obtained from proteins of known secondary structure in the wavelength range 190–240 nm. The analysis assumes the optical activity in this wavelength range is predominantly due to peptide transitions with negligible contribution from the

Table I: Calculated Secondary Structures and Parameters for Some Representative Specimens

sample	method of prepn	lipid/ protein	normalized constrained						unconstrained			
			α^a	β	T	R	NMRSD	sum	α	β	T	R
vesicles	I	214	0.69	0.00	0.15	0.16	0.05	0.80	0.58	−0.11	0.15	0.13
vesicles	I	1197	0.69	0.00	0.12	0.19	0.06	0.74	0.51	0.00	0.09	0.14
vesicles	II	162	0.83	0.00	0.17	0.00	0.05	0.76	0.70	−0.54	0.21	−0.06
vesicles	III	973	0.73	0.00	0.16	0.10	0.05	0.49	0.36	0.00	0.08	0.05
TFE			0.72	0.00	0.00	0.28	0.08	1.00	0.58	0.29	−0.23	0.16
octyl glucoside			0.83	0.13	0.04	0.00	0.11	0.63	0.45	0.06	−0.08	−0.18
$NaDodSO_4$ solubilized		188	0.60	0.18	0.00	0.22	0.06	0.67	0.36	0.28	−0.06	0.13

[a] α, α-helix; β, β sheet; T, β turn; R, random coil.

aromatic amino acids. This condition is met for proteolipid that contains only 8 aromatic (no Trp) amino acids out of a total of 81 amino acids (9.9%). The accuracy of these analyses also depends heavily on the applicability of the reference spectra used (Baker & Eisenberg, 1976). The reference data are derived from a set of 15 aqueous-soluble proteins (Chang et al., 1978). Unless the structural characteristics of proteolipid are well represented in this reference set, the CD spectrum of proteolipid cannot be accurately analyzed in this fashion. The applicability of this reference data set for analyzing membrane protein CD spectra may be assessed by its ability to estimate the structure of bacteriorhodopsin incorporated into small unilamellar DMPC vesicles (D. Mao and B. A. Wallace, unpublished results). The close correspondence between the structure estimated by CD and the structure obtained from electron and X-ray diffraction data (Henderson & Unwin, 1975; Henderson, 1975) and model building (Engelman et al., 1980) suggests the CD reference data set employed may be appropriate for the analyses of membrane protein spectra.

Unconstrained linear least-squares analyses of the experimental data were initially performed. Large negative coefficients would be indications that the analysis had failed. Constrained fits (requiring the fractions to be ≥ 0) are reported here; since they do not produce any negative amounts of secondary structures, the fractional amounts make physical sense.

The extent of agreement between the calculated structure and the measured CD spectrum was reflected in the normalized standard deviation. For soluble proteins an NRMSD of ≤ 0.1 indicates that the calculated structure and the X-ray structure are in excellent agreement. When $0.1 < \text{NMRSD} < 0.2$, the calculated structure is characterized as having secondary structures generally similar to the actual types. NRMSD values greater than 0.2 indicate the calculated structures do not resemble the actual structures (Brahms & Brahms, 1980). NRMSD values in this study ranged from 0.04 to 0.11, indicating that the calculated structures are very consistent with the observed data. However, small NRMSD values are necessary but not sufficient criteria for concluding that the calculated structures resemble the actual structures. The use of reference spectra that do not reflect the structures present in the protein to be analyzed would not result in an accurate determination of secondary structures (Baker & Eisenberg, 1976).

The sum of the fractions of secondary structure was not required to be unity in these calculations. A sum of 1.0 would have been obtained if the reference data set was entirely appropriate and the concentration determination exact. However, such circumstances are rarely attained even for soluble proteins. Constraining the sum to equal one in a nonlinear least-squares fit would tend to distort all the values obtained and be reflected in large NMRSD values.

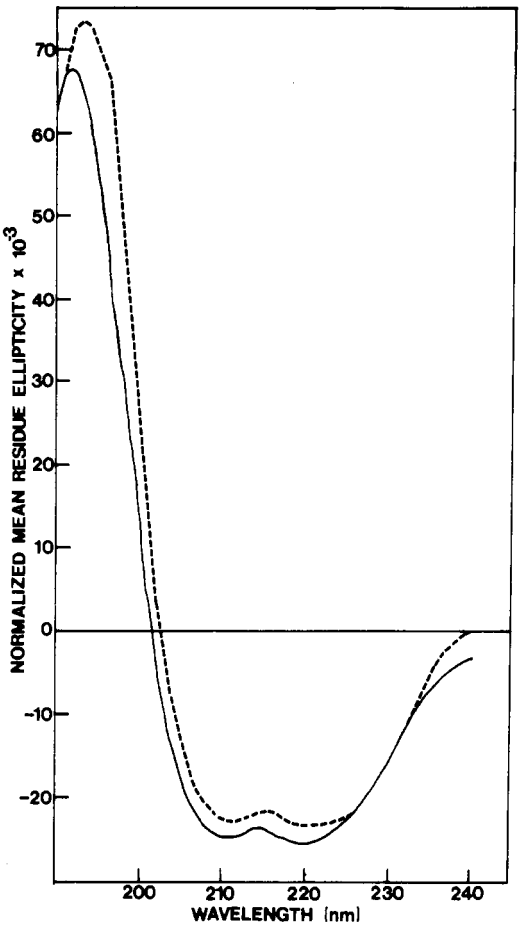

FIGURE 1: Circular dichroism spectrum of proteolipid in (—) trifluoroethanol ([proteolipid] = 1.7×10^{-5} M) and in (---) 1.5% *n*-octyl glucoside ([proteolipid] = 9.6×10^{-6} M) at 23 °C.

That normalization of the values to 100% (by multiplying the fractions by 1/sum) will correct for protein concentration errors can be shown mathematically and experimentally (D. Mao and B. A. Wallace, unpublished data). However, errors caused by using an inappropriate reference data set will remain in the normalized values.

Proteolipid Conformations. Analysis of the CD spectrum of proteolipid in TFE (Figure 1) indicates the structure consists of 72% α helix, 0% β turn and β sheet, and 28% random coil (Table I). In contrast, the CD spectrum of proteolipid in *n*-octyl glucoside (Figure 1) is that of a polypeptide with a substantial α-helix content, but with more β sheet and β turn present. The structure apparently contains 83% α helix, 13%

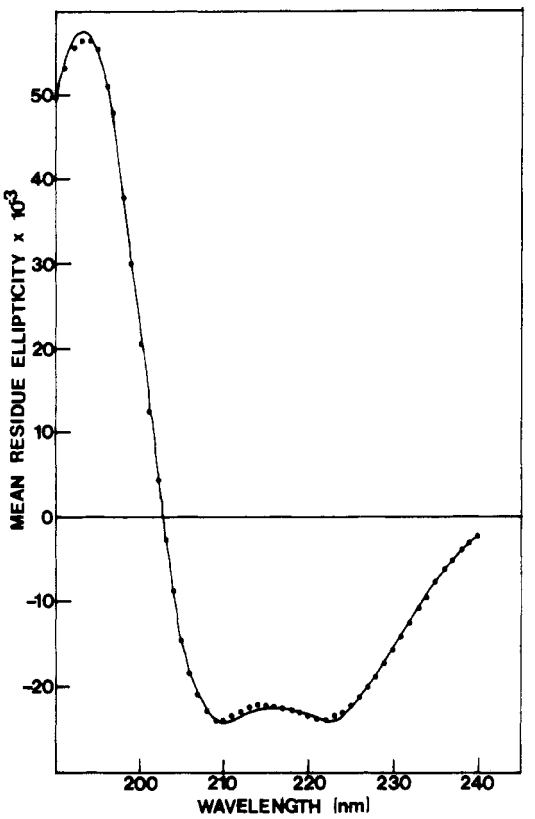

FIGURE 2: CD spectrum of proteolipid vesicles prepared by method I ([proteolipid] = 2.1×10^{-5} M; [DMPC] = 3.3×10^{-3} M; lipid/protein = 155) at 23 °C. (●) Measured CD data corrected for light scattering, absorption flattening, and obscuring effects. The uncorrected spectrum differs in intensity from this by ≤1% at each wavelength. (—) Calculated CD curve for these data.

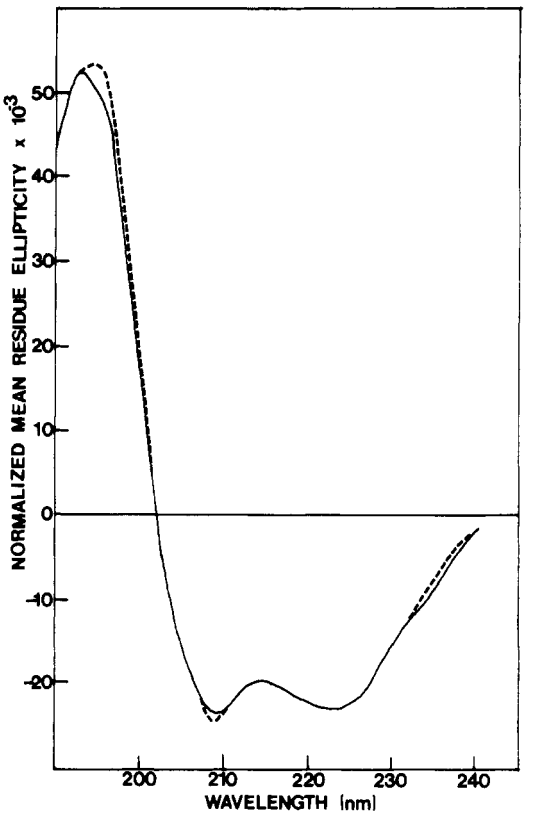

FIGURE 3: Corrected CD spectra of proteolipid vesicles, prepared by method III (---) ([proteolipid] = 1.5×10^{-5} M; [DMPC] = 1.5×10^{-2} M; lipid/protein = 973) and prepared by method I (—) ([proteolipid] = 1.7×10^{-5} M; [DMPC] = 2.2×10^{-2} M; lipid/protein = 1197) at 23 °C.

Table II: Average Structures: Effects of Dialysis and DCCD Labeling[a]

treatment	no. of samples	α helix	β sheet	turns	random coil
none	6	0.75 ± 0.04	0.01 ± 0.03	0.11 ± 0.03	0.13 ± 0.04
dialysis	2	0.72 ± 0.14	0.02 ± 0.02	0.15 ± 0.02	0.11 ± 0.11
equilibrate overnight	1	0.71	0.00	0.13	0.16
DCCD	2	0.74 ± 0.03	0.18 ± 0.07	0.01 ± 0.02	0.07 ± 0.07

[a] Vesicle samples prepared according to method I.

β sheet, 4% β turn, and no random coil (Table I).

The CD spectra of vesicles containing proteolipid prepared using method I (Figures 2 and 3) resemble those of polypeptides with predominantly helical conformations. The proportions of secondary structures calculated for a number of independent samples (Table II) were 75% α helix, 1% β sheet, 11% β turn, and 13% random coil. Proteolipid–DMPC vesicles formed with six different lipid/protein mole ratios (ranging from 155 to 1197) gave nearly identical CD spectra. The lipid concentration was generally kept constant (except in samples with the highest lipid/protein ratios), in order to keep the amount of light scattering by phospholipids invariant. No trend in structural changes with relative amounts of protein and lipid was detected (e.g., see ratios of 214 and 1197 in Table I). Additionally, analysis of the CD spectrum of proteolipid vesicles prepared by the dilution method (Figure 3) indicates a nearly identical secondary structure with that found for method I: 73% α helix, no β sheet, 16% β turn, and 10% random coil (Table I).

Furthermore, the lipid/protein mole ratios in vesicle samples prepared by method I and subsequently dialyzed were equal to those of the undialyzed samples, within experimental error. In one experiment, a sample with a starting lipid to protein ratio of 188 had a ratio of 200 after dialysis; in another experiment, a sample with a starting ratio of 155 was found to have a final ratio of 147. These results are within the accuracy limits of the assays (~5%) and suggest that essentially all the protein partitioned into the lipid phase of the vesicles during incorporation and the partition coefficient of proteolipid in lipid vs. H_2O was greater than 4×10^6. There was more variation in the CD spectra of dialyzed vesicles from different samples than for other experiments in this study and, hence, the higher standard deviations for the calculated fits (Table II). However, the secondary structures of dialyzed and undialyzed proteolipid were identical, within the limits of error of the measurements. These results suggest that the CD spectra obtained for vesicle samples are representative of the membrane-associated conformation and do not have contributions from any aqueous-soluble forms.

The CD spectrum of a sample which had been allowed to sit overnight was measured to determine if the system had reached equilibrium at the time of measurement. It, too, was the same as the CD spectrum of the fresh sample, indicating no detectable conformational change occurs with time.

[^{14}C]DCCD was bound to the proteolipid in vesicles to examine the effect of the proton-translocation inhibitor on the structure of the channel. The extent of labeling was quantitated after separation of the components by thin-layer chromatography. In the developing system used, R_f values

for protein, lipid, and free DCCD were 0.0, 0.18, and 0.89, respectively. Radioactivity (cpm) was plotted vs. distance migrated, and the counts associated with each component were determined. Six percent of the total radioactivity was associated with the protein band; the lipid band contained ~0.1%, whereas most of the counts (>80%) were associated with free DCCD.

In a parallel run without protein present, a small trace of [^{14}C]DCCD contaminant remained at the origin (0.2% of the total radioactivity), and this was subtracted from the total counts attributed to labeled protein at the origin. The stoichiometry of labeling was approximately one DCCD per four proteolipid molecules, even though the starting DCCD/proteolipid mole ratio was approximately 16 times this large. This labeling stoichiometry is similar to the results of others which led to the proposal that labeling of only one proteolipid molecule per oligomeric channel was sufficient to cause inhibition (Sebald et al., 1979a; Friedl et al., 1981).

The CD spectrum of proteolipid in vesicles was affected only slightly by binding of DCCD to the sample. There was a small but reproducible increase in β sheet detected with a concomitant decrease in turn structure (Table II). The difference is slightly greater than the precision level of the experiment (5%) and may possibly represent a small conformational change upon binding of the inhibitor. No net changes were found in the helix and coil components, indicating no substantial refolding of the molecule. However, since in this experiment only one-quarter of the proteolipid molecules were labeled, the actual structural changes per molecule could be as much as 4 times as large as those detected, if the effects were noncooperative (a circumstance mitigated against by the effect of this level of DCCD on the conductance activity of the channel). Additionally, similar results were obtained in experiments that utilized starting DCCD/proteolipid ratios as high as 21/1.

Discussion

Structural studies of the proteolipid channel provide a basis for model building and may promote understanding of the mechanism of proton translocation across membranes. In this paper we have utilized circular dichroism spectroscopy to examine the secondary structure of this molecule in phospholipid bilayers.

To structurally characterize the biologically relevant form of proteolipid, it must be placed in a membrane environment in which proton-translocation activity is retained and for which optical distortions of the CD spectra are minimized, as is the case for small unilamellar phospholipid vesicles. Since protein is most efficiently incorporated at temperatures above the lipid phase transition, vesicles were prepared from dimyristoylphosphatidylcholine which has a phase transition temperature at 23 °C. The bilayer thickness of these lipids above the phase transition temperature is 37 Å (Janiak et al., 1976), and the average diameter of these sonicated DMPC vesicles is ~300 Å.

The validity of the structural information obtained depends on the fidelity of reconstitution of the proteolipid. Purified proteolipids, from various sources, have been incorporated into phospholipid vesicles under conditions similar to the three methods used in this study (Konishi et al., 1979; Sigrest-Nelson & Azzi, 1980; Racker et al., 1979). The CD spectra of vesicles prepared by the rehydration, detergent dialysis, and detergent dilution methods were very similar, so the method of incorporation apparently does not affect the overall conformation. However, the H^+-translocating activity of these specimens was not extensive. In one case (method I incorporation), dissipation of a preformed proton gradient was detected. However, only a modest portion of this activity was DCCD inhibitable, although it was roughly comparable with other reported results (Sigrist-Nelson & Azzi, 1980). The lack of extensive activity may be because the *Neurospora* proteolipid requires the presence of other F_0 subunits, as do the *E. coli* and PS3 proteolipids (Friedl et al., 1981; Sone et al., 1978). Additionally, not every proteolipid molecule in the sample is necessarily in an active conformation, although conductance activity is detected in the specimen. Besides the proton-conducting species, there may exist other structures that do not translocate protons but which do contribute to the spectrum. The observed spectrum is a linear combination of each conformation present, weighted by their concentration in the sample. So, the average conformation estimated by the CD measurements will not necessarily correspond uniquely to the proton-conducting form of the proteolipid.

The major limitations to the accuracy of the proportions of secondary structure calculated from CD data are errors in estimating the protein concentration, the degree of applicability of the CD reference spectra, and corrections to the spectra for light scattering and absorption flattening effects. The precision of the final calculated values is better than ±5%.

Errors in the proteolipid concentrations can be removed by normalization of the structural fractions calculated. Application of this method to myoglobin data which had purposely been improperly scaled demonstrates that this is a consistent way of presenting the estimated structure without the presence of concentration error (D. Mao and B. A. Wallace, unpublished results). However, any uncertainties due to inappropriate reference spectra still persist in the normalized solutions. It was of concern that the use of a reference data set based on water-soluble proteins might not have been appropriate for proteins embedded in the hydrophobic environment of a lipid bilayer. However, the low NMRSD values and good agreement of the CD results for bacteriorhodopsin with those obtained by X-ray diffraction, electron microscopy, and model building (Henderson, 1975; Henderson & Unwin, 1975; Engelman et al., 1980) suggest that the use of this reference data set for membrane proteins may be valid. This may be because the globular proteins used as references generally fold in manners which bury their hydrophobic residues in the interiors of the molecules, environments which may not be so different from the hydrophobic environment of a bilayer. Furthermore, this reference set tends to give particularly accurate secondary structural estimates for proteins with high α-helical contents (Chang et al., 1978; Provencher & Glockner, 1981), a predominant structural motif in proteolipid and other membrane proteins (Wallace, 1982).

The effects of differential light scattering and absorption flattening on the CD spectra of sonicated vesicles are small. For 300 Å diameter vesicles, correction factors are calculated to be on the order of 1% of the measured ellipticities. The validity of this correction method has been demonstrated for a known system, bacteriorhodopsin, incorporated into sonicated DMPC vesicles (D. Mao and B. A. Wallace, unpublished results).

The major conclusion from this work is that proteolipid incorporated into vesicles is predominantly α helical in conformation. The CD results suggest that roughly 75% of the 81 amino acids are involved in helical secondary structures, with 11% in turns and 13% in random coil, which corresponds to approximately 60 amino acids in helical structures and 10 residues each in turns and coillike structures (Table II). β-Sheetlike structures are apparently not found in this molecule.

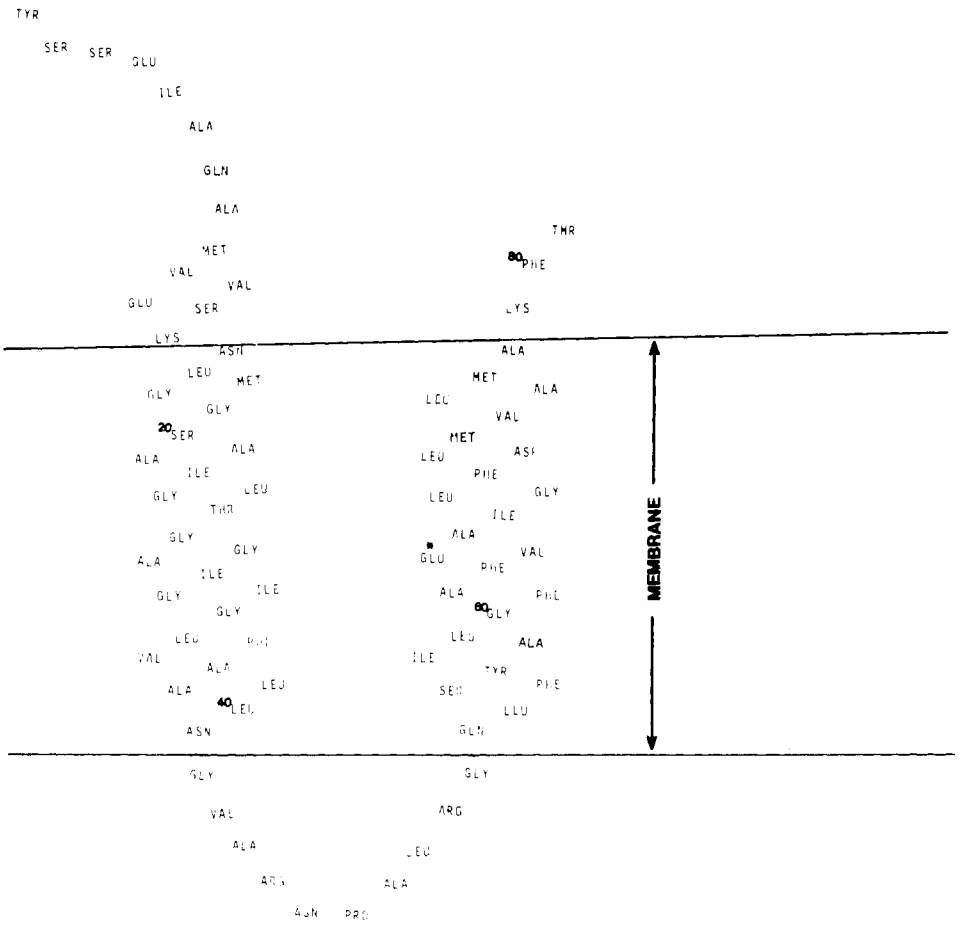

FIGURE 4: Proposed structural model of the proteolipid subunit of the *Neurospora crassa* mitochondrial H^+-ATPase complex in the membrane.

These results provide the basis for building a tentative structural model for the proteolipid (Figure 4), which is consistent with available amino acid sequence, labeling, and genetic mutation information.

Common features in the amino acid sequences of proteolipid from 6 different species are the presence of two extremely hydrophobic segments of ~25 residues separated by a hydrophilic segment (Sebald et al., 1979b). Near the center of the C-terminal hydrophobic segment is the acidic amino acid residue (either Glu or Asp) which reacts with DCCD (Sebald et al., 1980). This same hydrophobic segment is also responsible for the oligomycin sensitivity of the ATPase complex (Sebald et al., 1979b). Studies done with DCCD-resistant *E. coli* mutants indicate resistance to DCCD inhibition can be associated with a single amino acid substitution in the N-terminal hydrophobic segment (Hoppe et al., 1980). These results suggest the molecule may be composed of two hydrophobic segments that span the bilayer connected by the polar segment and hydrophilic N and C termini that are in contact with the aqueous phase or other subunits in the F_1–F_0 ATPase complex (Sebald et al., 1979b). The CD results suggest the extensive hydrophobic segments which span the bilayer may be predominantly helical in nature. Several hydrophilic residues that are placed within the bilayer in the model would be located close enough to the end of the helix so that their charged side chains could reach the aqueous solvent. The positions of the bilayer indicated in the model are consistent with the length of helix necessary to span the membrane.

The number of residues in the two transbilayer hydrophobic segments is ~54, which would account for most of the helical secondary structure detected by CD. However, to account for the ~60 helical residues detected, the helices may extend somewhat beyond the bilayer core into the head-group region. CD evidence suggests the presence of a β turn, which could form the polar linker region joining the two helices; the remaining N- and C-terminal pieces protruding from the bilayer may be composed of random coil. This general picture of helical hydrophobic segments of ~25 residues linked by a relatively polar segment of 12–20 residues is reminiscent of the folding pattern of bacteriorhodopsin (Engelman et al., 1980) and may be generally an energetically favorable motif for membrane protein folding (Wallace, 1982).

It should be noted that in this model the DCCD-reactive Glu-65 is situated in the middle of the lipid bilayer. This is consistent with the result that only hydrophobic carbodiimides (e.g., DCCD), not water-soluble ones, can affect the proton conductance in both mitochondrial and reconstituted proteolipid vesicles (Beechey et al., 1967; Sigrist-Nelson & Azzi, 1980). According to this model, Ile-32, which was found to play a role in DCCD resistance of *E. coli* proteolipid (Hoppe et al., 1980), would be ~6 Å lower along the helix axis than the DCCD-reactive Glu-65, located on the adjacent helix. If there are interactions between the two helices, Ile-32 is in a good position to influence the environment of Glu-65, thus affecting its DCCD-binding capacity. This model has the same topology as the hairpin model proposed by Sebald & Wachter (1978). Alternative models could be proposed in which the helical segments are arranged differently in either their orientation or their disposition relative to the membranes. Details of the folding await chemical labeling studies which can identify more clearly those portions of the molecule exposed to the solvent or buried within the bilayer.

The extent of protein–lipid association and consequences of inhibitor binding have also been examined to understand

the physiologically important aspects of the structure. Dialysis experiments indicate the partition coefficient of proteolipid in DMPC vesicles must be at least 4×10^6 times that in water, suggesting a strong association with the membrane and the likelihood that proteolipid is at least partially embedded within the lipid bilayer. No changes in the proteolipid spectrum were detected upon dialysis, indicating that only a membrane-embedded form of proteolipid is contributing to the CD spectrum of the vesicle sample. Also, no changes in the spectrum were detected with time, suggesting the CD studies were done on systems that had already reached equilibria. The effects of binding the irreversible inhibitor DCCD were examined in order to provide insight into the mechanism of action of the channel. In this study, addition of DCCD was found to cause only slight changes in the CD spectra, indicating that no major net conformational change occurred in the structure of proteolipid. The very small differences suggest that DCCD does not act by causing a substantial alteration in the overall folding of the protein; perhaps it acts either by physically blocking the proton channel or by masking an essential charge group needed to relay the protons across the channel.

It has been proposed that six proteolipid molecules are present in each ATPase complex (Sebald et al., 1979a). For examination of whether the conformation of the molecule in the monomer form is similar to that in the oligomeric state, CD spectra were obtained for vesicles with different lipid to protein mole ratios ranging from 155, for which the average number of monomers per vesicle was greater than 6 (i.e., 16), to 1197, where the average number per vesicle was less than 6 (i.e., 2). No conformational differences were detected, suggesting either that no major structural reorganization occurs upon multimer formation or that proteolipid molecules preferentially form complexes regardless of the lipid to protein ratio.

In organic solvents and detergent solutions, light scattering and absorption flattening effects are minimized because of the relatively small particle size. It was of interest to compare the structure of the protein in lipid vesicles to that in organic solvents and detergent micelles. If the conformation of the proteolipid in these solvents was not grossly changed from its conformation in vesicles, they would be useful in estimating the extent of scattering and flattening. The CD spectrum of proteolipid in TFE indicated an abundance of helix. TFE tends to promote the formation of helical structures because its low capacity to form hydrogen bonds with protein molecules favors intramolecular hydrogen bonding. However, proteolipid in TFE is not entirely helical in conformation, and the amount of helical structure is no more than that of proteolipid in vesicles. While the helical contents in TFE and DMPC vesicles are somewhat similar, other secondary structural features are different. The similarity in these two systems may be merely a coincidence due to the propensity of TFE to promote helix formation. Thus TFE may not be a good model solvent for membrane systems. Detergent micelles, which are an intermediate state in the formation of vesicles by dialysis and dilution, may be more representative of the membrane environment than organic solvents. Nonionic detergents such as *n*-octyl glucoside and Triton X-100 have been used to solubilize integral membrane proteins, often without substantial loss of their biological activity. Ionic detergents such as $NaDodSO_4$ tend to denature membrane proteins. However, some membrane proteins have been shown to be somewhat resistant to the denaturing action of $NaDodSO_4$ at room temperature (Scandella & Kornberg, 1971; Laver, 1963). The effects of different detergents on proteolipid structure were examined by comparing the CD spectra of $NaDodSO_4$-solubilized proteolipid–DMPC vesicles, or proteolipid in *n*-octyl glucoside micelles, with those of the intact vesicles. The spectrum of proteolipid vesicles solubilized in 0.1% $NaDodSO_4$ indicates generally similar helix and random-coil contents to that for vesicles (Table I). A significant amount of β sheet was also detected. Surprisingly, the structure of the proteolipid in the $NaDodSO_4$-solubilized sample seemed to differ no more from the vesicle structure than did the octyl glucoside sample. While octyl glucoside is often considered to be a nondenaturing detergent, slightly more α helix, substantially more β sheet, and less random coil were observed than in the structure of proteolipid in vesicles. Thus, $NaDodSO_4$-solubilized materials were used for estimates of absorption flattening effects.

In summary, the H^+-ATPase proteolipid was incorporated into DMPC vesicles by several different methods. The molecule exhibits a highly α-helical secondary structure, which is largely independent of the method of vesicle preparation. The protein partitions strongly into the bilayer, and no time-dependent structural reorganizations have been observed. Irreversible inhibition of proton translocation by DCCD modification caused only slight net changes in the spectra and is not consistent with any substantial alteration in the molecular conformation. These studies have provided the physical basis for the construction of a tentative structural model for the mitochondrial proton ATPase proteolipid in membranes.

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Binding of Neurohypophyseal Peptides to Neurophysin Dimer Promotes Formation of Compact and Spherical Complexes†

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ABSTRACT: Previous hydrodynamic studies [Rholam, M., & Nicolas, P. (1981) *Biochemistry 20*, 5837-5843] have demonstrated that the dimerization of a neurophysin monomer (prolate ellipsoid with an axial ratio, due to asymmetry, of 5.2) results in a decreased asymmetry (axial ratio, due to asymmetry, of 3.6) as the consequence of a side-by-side association process. By a combination of hydrodynamic measurements, including the use of sedimentation velocity, viscometry, and fluorescence polarization spectroscopy, the influence of hormone binding on the shape and asymmetry properties of the neurophysin dimer was evaluated. The binding of ocytocin, vasopressin, and the tripeptide analogue of the N-terminal sequence of ocytocin, Cys(*S*-Me)-Tyr-Ile-NH_2, results in an increase of $s^0_{20,w}$ and a decrease in both the reduced viscosity and rotational relaxation time of the bis-liganded dimeric species vs. the nonliganded form. The axial ratio (a/b) due to asymmetry of the ligand-bound dimers was found in each case to be equal to, or slightly greater than, 1.0, indicating a compact spherical shape (Stokes radius 21 Å). The profound alteration on molecular dimensions observed upon ligand binding is shown to be the consequence of a ligand-induced conformational change and might explain the intradimeric binding sites positive cooperativity. It is tentatively proposed that the pseudospherical shape of the neurophysin–hormone complexes may enhance the stability of neurophysin and contribute to the prevention of leakage of neuropeptides through the membrane of neurosecretory granules. The data provide a remarkable example of a small protein with a high content in disulfide links and that undergoes conspicuous changes in conformation under the influence of nonapeptide, or tripeptide, ligands.

Physicochemical studies of the neurophysins, hypothalamo-neurohypophyseal proteins associated with the transport and biosynthesis of the neuropeptide hormones ocytocin and vasopressin [for recent reviews see Pickering & Jones (1978), Breslow (1979), and Cohen et al. (1979)], have recently focused on the mechanism of assembly of neurophysin monomers into dimer (Nicolas et al., 1976, 1978b, 1980; Pearlmutter, 1979). Both the shape and asymmetry properties of these species in solution (Rholam & Nicolas, 1981) were studied. Data obtained from a combination of various hydrodynamic techniques (Rholam & Nicolas, 1981) indicated mainly that the single polypeptide chain monomer (M_r 10000) is rigid and highly asymmetrical with an axial ratio close to 5.2. The

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