CIRCULAR DICHROISM OF A MEMBRANE PROTEIN OF *NEUROSPORA CRASSA*[1]

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SUMMARY

Membrane protein of a mutant of *Neurospora crassa* has been isolated in aqueous sodium phosphate buffer without employing a chemical agent such as urea, detergent, or organic solvent. The protein moved in a single band on sodium dodecyl sulfate gel electrophoresis and as well on non-SDS gel electrophoresis. The circular dichroism spectrum of the protein exhibited the characteristic low ellipticity but no red shift.

The circular dichroism spectra of proteins in a variety of membranes resemble in general the CD of right-handed α-helical polypeptides but show unique anomalies. The ellipticity at minima was reduced to about one-third and the position of the minima was shifted to longer wavelength (1,2). Ji and Urry (3) found that α-helical poly-L-glutamic acid (PGA) also displayed the membrane type CD upon aggregation, and they suggested that the aggregation dependent light scattering and absorption flattening (4) were responsible for the red shift and low ellipticity.

Several authors have tried to correct the optical artifacts on the theoretical basis of Duysens' absorption flattening and Rayleigh or Mie light scattering (5-10). Experimentally, the distortions on the CD spectra have been partially corrected by sonication (3,11), by treating in the French press (8) and by solubilization in organic solvent or in detergent solution (1,12,13,14). The red shifted minima were always reversed upon the different extent of deaggregation of the membrane suspension. However, the low ellipticity did not increase to the same extent in different experiments.

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Another matter which complicates the interpretation of the optical activity of the membrane proteins is that the CD and ORD spectra represent the average optical activity of heterogeneous proteins in membranes (15-19). To understand the conformation of membrane proteins and to provide an accurate experimental ground on which the distorted CD can be reliably corrected, at present it is essential to know: (1) to what extent optical artifacts distort CD spectra and (2) not only the averaged CD of heterogeneous proteins in membranes but also the CD of each protein species in membranes.

EXPERIMENTAL

The slime mutant of *Neurospora crassa* (a kind gift from V. W. Woodward) was grown by constant shaking in a medium containing 0.5% yeast extract, 0.5% nutrient broth, 2% of Vagels, and 29% sucrose at 3° for 3 days. Cells were harvested by sedimentation at 4,000 x g_{max} for 5 min and resuspended in a grinding medium containing 0.05M Tris, 0.25M sucrose, and 4mM EDTA at pH 8.5. They were then mixed with the same volume of 2mm glass beads, and ground in a jar mill for 2 hours at 5°. The broken cells were filtered through cheese cloth and sedimented at 1,000 x g_{max} for 5 min. They were washed five times in the grinding medium and then washed in 0.05M Tris buffer at pH 7.5 repeatedly until no protein was detected in the supernatant. The membrane preparation was stirred in 0.01M Na_2HPO_4 at pH 10.3 for overnight. The suspension was centrifuged initially at 48,000 x g_{max} for 2 hours to remove rough pellets. The supernatant was subject to a second centrigugation at 314,000 x g_{max} for 7 hours. Membrane proteins remaining in the supernatant were considered soluble. All procedures were carried out at 5°. The soluble proteins were fractionated on an ascending Sephadex G-100 column (2.5 x 50 cm). The column was eluted with 5mM Na_2HPO_4 at pH 10.3, 0.12 ml/min, and the eluant was monitored by absorbance at 285 nm on a Heath Model 701 spectrophotometer. Gel electrophoresis was performed as previously described by Dunker and Rueckert (20).

Protein concentration was determined according to Lowry *et. al.* (21). Poly-L-glutamic acid was purchased from New England Nuclear (Lot #G-153 and MW 38,000).

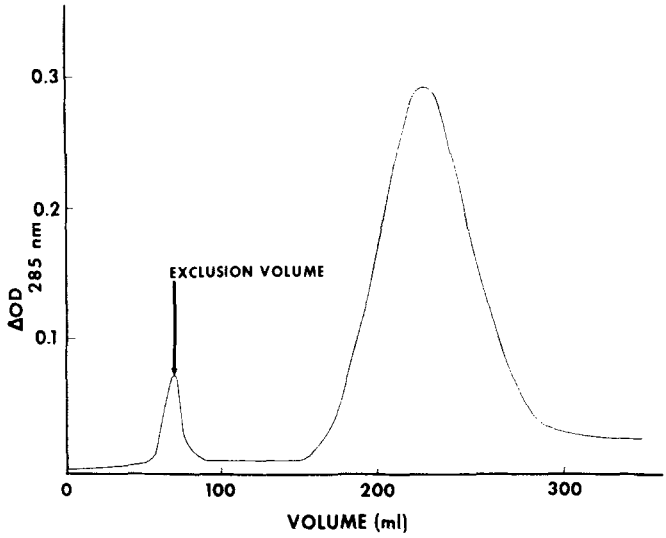
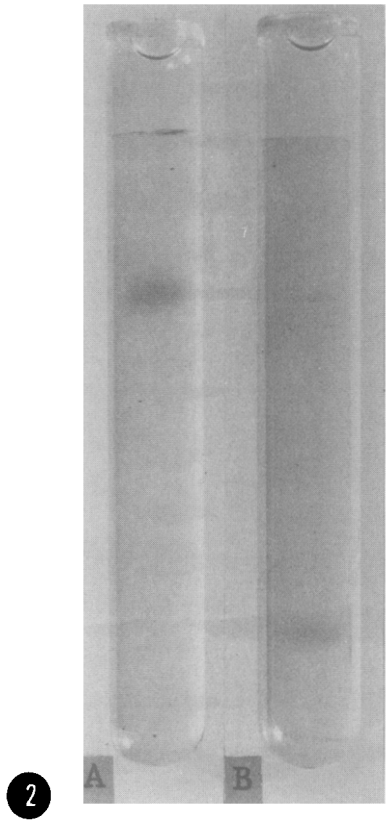


Figure 1. Column fractionation of the soluble membrane proteins. The first peak was eluted at the exclusion volume and contained about 5% of the input proteins. The second peak contained the rest of the input proteins.

CD spectra was measured on a Jasco J-20 CD/ORD spectropolarimeter. A 0.2 mm cell was used. The residue molar ellipticity was estimated as 120 according to the amino acid content (V. W. Woodward, personal communication). An Aminco-Bowman spectrofluorometer was used to determine right angle light scattering. The wavelength of incident and scattered beam was 290 nm where scattered light and fluorescence were well separated.

RESULTS AND DISCUSSION

On the column chromatography, the soluble membrane proteins showed a minor fraction (5%) and a major fraction (95%) as appears in Figure 1. The minor peak was eluted at the exclusion volume which was determined by using blue dextran (MW 2 x 10^6). Since the major protein fraction remained in the supernatant on centrifugation at 315,000 x g_{max} for 7 hours and was eluted after the exclusion volume of the Sephadix G-100 column, it is reasonable to consider that the protein is soluble and that its molecular weight is below 150,000 daltons. The sedimentation coefficient of the soluble protein at the concentration of 2.1 mg/ml was 1.65 and the partial specific volume was 0.75 (Ji and Woodward, manuscript in preparation). Therefore, the molecular weight is near 18,000 daltons.



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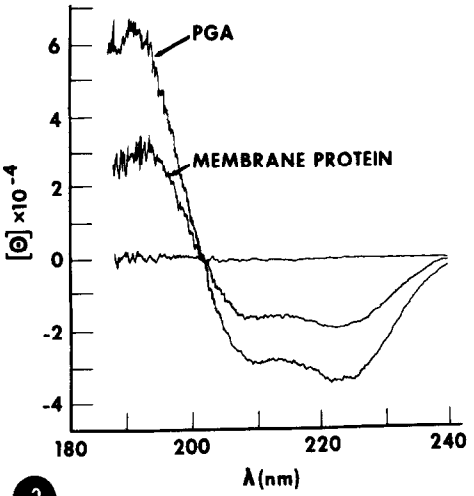

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Figure 2. Polyacrylamide gel electrophoresis (A) and SDS-polyacrylamide gel electrophoresis (B) of the fractionated soluble protein (peak 2 in Figure 1). The gels were electrophoresed downward.

Figure 3. CD spectra of α-helical PGA and the fractionated soluble membrane protein (peak 2 in Figure 1) at pH 7.7. The CD spectrum of the membrane protein was measured in 30 min after the solution was adjusted to pH 7.7. There was no apparent light scattering and absorption flattening in the sample. The CD spectra was obtained at the speed of 4-2 nm/min and at the time constant of 1-4.

Figure 2 shows that the protein in the major fraction exhibited one band in SDS or non-SDS polyacrylamide gel electrophoresis and indicates that the protein is homogeneous in particle size.

Table 1 reveals that right angle light scattering behavior of the major protein fraction at different pH. At pH 9.5 and 10.3 where solubilization, column chromatography, and gel electrophoresis were performed, the intensity of light scattering increased about 2.5-fold compared to that at pH 11.3 where

Table 1. Relative intensity of right angle scattered light of the membrane protein.

pH	Time after pH was adjusted (hrs)	Relative intensity of light scattering
11.3	0-24	1
10.3	0-24	2.5
9.5	0-24	2.5
7.7	0	2.5
7.7	0.5	2.5
7.7	24	30.

sedimentation studies were performed. At pH 7.7 there was no further change in light scattering at least for the initial 30 min which was enough to scan a reasonably good CD spectrum from 250 nm to 190 nm at the speed of 4-2 nm/min and the time constant at 1-4 on the instrument. However, it became visibly turbid and the light scattering increased 30 fold if the solution was kept at pH 7.7 for 24 hours.

Intensity of scattered light is directly proportional to molecular weight of solute when the concentration of solute is kept constant, and assuming the light scattering is isotropic and the virial coefficient of the solute does not change significantly (22). If it is assumed that the 2.5 fold increase in the light scattering at pH 7.7 compared to that at pH 11.3, is totally due to an increase in the molecular weight, the corrected molecular weight becomes 45,000 daltons. This figure is close to that of α-helical PGA employed for measuring α-helical CD spectra. Consequently, the membrane protein in solution at pH 7.7 is expected to scatter light not much more than the α-helical PGA does. In addition, the diameter of the membrane protein at pH 11.3 will be 35 A^{o} versus 48 A^{o} at pH 7.7, assuming that the protein is globular. Since we are interested in measuring CD at around 2000 A^{o}, the light scattering by the membrane protein at pH 7.7 is in Rayleigh scattering. Therefore, the CD spectra of the membrane protein at pH 7.7 should not be distorted by light scattering as the

CD spectra of aggregated membranes were. Furthermore, absorption flattening for such small particles at a concentration less that 1 mg/ml in a cell of 0.2 mm pathlength is negligible (4,8,9).

The CD spectra of the membrane protein in solution at pH 7.7 and a typical α-helical PGA in water at pH 4.5, are shown in Figure 3.

The position of n-π* transition in both of the CD spectra appears at 222nm, but the ellipticity at 222 nm of the membrane protein was 1.67×10^4 (degrees cm^2 $decimole^{-1}$) which is about one half of the ellipticity of α-helical PGA, 3.4×10^4. However, the ellipticity at 192 nm of the membrane protein is less than one half of that of PGA. The data are similar but not identical to those obtained from red blood cell membranes sheared by French press (8) or sonicated (11,14). As the protein solution at pH 7.7 was kept for longer than 24 hours to aggregate, the 222 nm minimum shifted toward longer wavelength and the ellipticity decreased, which is characteristic of the CD spectra of membranes. Therefore, it is concluded that the red shift is a total optical artifact and that the low ellipticity is a partial artifact.

Also, the data indicate that the low ellipticity α-helical CD spectra of membrane proteins do not only represent an average optical activity of the proteins but also the optical activity of some membrane protein.

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