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CIRCULAR DICHROISM OF MITOCHONDRIAL MEMBRANES BEFORE AND AFTER EXTRACTION OF LIPIDS AND SURFACE PROTEINS

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SUMMARY

The circular dichroic spectra of submitochondrial vesicles, membrane-associated proteins, protein-depleted vesicles, and lipid-depleted vesicles were determined. It is possible to remove up to 40% of the protein from the membrane and still retain the trilaminar structure in the residual vesicles. This enables us to assess the secondary structure of the intrinsic membrane proteins and the relative contribution of membrane-associated proteins to the spectrum of the intact membrane. The ellipticity at 222 nm of submitochondrial vesicles was in good agreement with the value calculated from the data on membrane-associated proteins and intrinsic membrane proteins. The spectral data were used to estimate the helical content of submitochondrial vesicles (27%), intrinsic membrane proteins (26–30%), and membrane-associated proteins (15%). The intrinsic membrane proteins of vesicles show remarkable stability in 8 M urea; only 20% of the helical structure is lost. This is in contrast with most globular proteins, as well as membrane-associated proteins, which are largely denatured in concentrated urea. No appreciable change in secondary structure occurs after removal of a large amount of phospholipid (72%) from submitochondrial vesicles.

INTRODUCTION

X-ray crystallography is the primary method for determining molecular structure. However, membranes cannot be ordered into crystals and so only limited information, less than 20 reflections, is obtained even under the most favorable conditions. With this information, and some assumptions, electron density profiles of the membrane can be calculated which allow some speculation on the distribution of lipids and proteins in the membrane[1–4]. There is insufficient information to determine the secondary structure of membrane proteins by *X*-ray diffraction. Instead, the secondary structure of membrane proteins can be determined only indirectly with the use of infrared spectroscopy, ORD or CD[5–12]. These methods are in large part empirical, based on spectra obtained on poly-amino acids of known secondary con-

Abbreviation: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

formation[13]. Such studies indicate that membranes from a variety of sources have a significant amount of ordered secondary structure.

The trilaminar appearance of membranes is well recognized[14]. Recently we have discovered that a large percentage of protein can be extracted from washed membrane vesicles leaving the trilaminar structure intact[15,16]. Special fixation and staining allows visualization of a "surface fuzz" which can be removed by extraction leaving a smooth-surfaced trilaminar membrane[17,18]. The "surface fuzz", designated "membrane-associated protein(s)" or surface protein(s)*, represents a secondary level of membrane organization; the trilaminar membrane, which is comprised of the "intrinsic membrane proteins" and lipid, is considered to be the primary level of membrane organization. The intrinsic and membrane-associated proteins have distinct composition as studied by polyacrylamide gel electrophoresis. The intrinsic membrane proteins include cytochromes $a + a_3$, b and c_1 (refs. 15–17).

It follows from these considerations that previous studies on the secondary structure of membrane proteins reflected both intrinsic membrane proteins and membrane-associated proteins. In mitochondrial membranes, the latter accounts for a significant portion of the protein of mitochondrial membranes, approximately 40 %. The purpose of this study is to evaluate the relative contribution to secondary structure of intrinsic membrane proteins and membrane-associated proteins of mitochondrial vesicles. Beef heart mitochondria were chosen for this study because they are composed mainly of inner membrane (the outer membrane comprises less than 10 % of the total membrane). We have also obtained CD spectra of lipid-depleted membrane vesicles in order to evaluate the effect of lipid on the secondary structure of mitochondrial membrane proteins.

MATERIALS AND METHODS

Sucrose (special enzyme grade) and ultra pure urea were purchased from Mann Research Lab. (New York, N.Y.), crystalline bovine serum albumin from Armour Pharmaceutical Co. (Chicago, Ill.), *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) and dithiothreitol from Calbiochem (Los Angeles, Calif.) and Tris from Sigma (St. Louis, Mo.). Phospholipase A (*Naja Naja*) was obtained from the Miami Serpentarium Lab. (Miami, Fla.) and was further purified as previously described[19]. Sodium dodecyl sulfate was purchased from Fisher (Pittsburgh, Pa.), Triton-X-100 from Rohm and Haas (Philadelphia, Pa.) and Brij 36T from Canamex (Mexico City, Mexico). Deoxycholic acid was obtained from Matheson, Coleman and Bell (Norwood, Ohio) and was recrystallized from ethanol.

Protein was estimated by the method of LOWRY *et al.*[20] and phosphorus by the method of CHEN *et al.*[21]. Absorption spectra were determined with a Cary 15 spectrophotometer using a 2-mm pathlength. Electron microscopy was carried out on embedded thin sections[22].

Mitochondria and derived preparations

A number of preparations derived from beef heart mitochondria were used in

* Membrane-associated protein may be a better term than surface protein, in that some of the protein extracted with dilute acid or urea could come from less tightly bound proteins within the trilaminar structure rather than just from the "surface fuzz".

this study (Fig. 1). Beef heart mitochondria were prepared as previously described[23] using 0.25 M sucrose in 0.01 M HEPES as buffer (pH 7.5). Submitochondrial vesicles were prepared by subjecting the mitochondria to shear in a Parr Bomb (Parr Instrument Co., Moline, Ill.)[24]. Unbroken mitochondria were removed by centrifugation at 20000 rev./min for 10 min in a Spinco No. 50.1 rotor and the vesicles were pelleted at 45000 rev./min for 1 h. A supernatant fraction containing soluble mitochondrial protein was also obtained. The vesicles, after one wash, were resuspended in 0.25 M sucrose–1 mM HEPES, pH 7.5. Vesicles prepared by this method retain optimal respiratory activity from substrate to oxygen.

Vesicles were extracted of phospholipid by treatment with phospholipase A[19]. The amount of phospholipase A and time of incubation were varied to give different degrees of phospholipid degradation (*cf.* Table I). All samples were washed four times with 1 % bovine serum albumin in 0.25 M sucrose to remove the lysophosphatides and fatty acid by-products and once with 0.25 M sucrose to remove bovine serum albumin. A control sample was carried through the same procedure by substituting bovine serum albumin for phospholipase A in the incubation mixture. The adsorption of bovine serum albumin to the washed vesicles was checked using polyacrylamide gel electrophoresis. Densitometry tracings of the gels showed no significant amount of bovine serum albumin (<3 % of the protein); this upper limit is too small to significantly alter the observed CD spectra.

Extraction of submitochondrial vesicles with 8 M urea to remove membrane-associated proteins was performed as previously described[16]. 1 ml of vesicles (28 mg protein/ml) was added to 7 ml of a solution containing 3.84 g urea, 8 mg dithiothreitol and 80 μmoles Tris, pH 8.5. After 30 min at 0–4° the solution was centrifuged at 45000 rev./min for 3 h and the pellet (urea extracted vesicles) was resuspended in

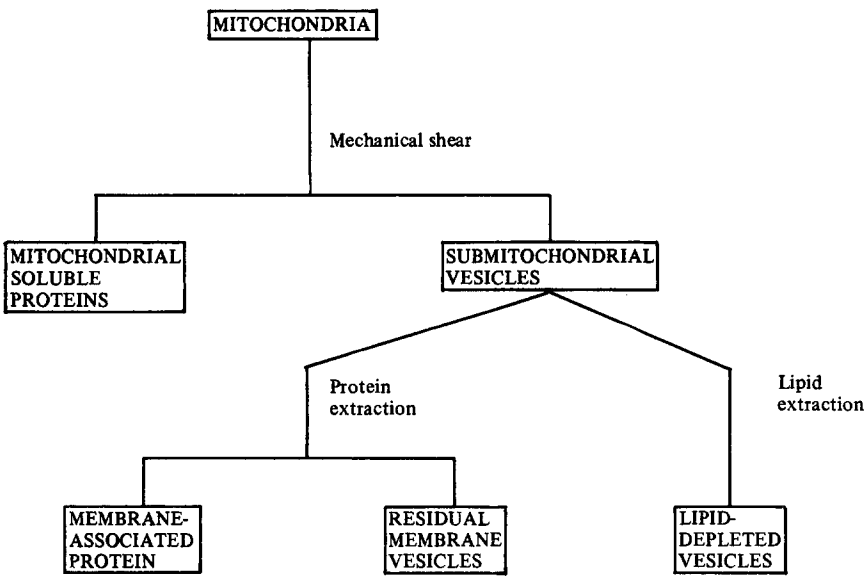


Fig. 1. Flow diagram for the preparation of samples used in this study. All were derived from beef heart mitochondria.

2.5 ml of 0.25 M sucrose–1 mM HEPES, pH 7.5. The supernatant was dialysed against 100 vol. of 10 mM Tris, pH 8.1, and then recentrifuged to remove unsedimented vesicles. The supernate from this centrifugation, designated membrane-associated protein, was used directly for study. Vesicles were also extracted of membrane-associated protein with 1.4 % acetic acid as previously described[15].

CD measurements

The CD spectra were determined using a Cary 60 spectropolarimeter equipped with a Model 6002 CD accessory. The spectra were obtained over the wavelength range of 200–260 nm using a cell of 1 mm pathlength, a full scale of 100 mdegrees, the automatic slit control, and a time constant of 3 sec, although occasionally in the far ultraviolet a time constant of 10 sec was used. Protein concentrations were generally about 0.2 mg/ml, although increasing the concentration to 1 mg/ml had no effect on the spectrum. The absorbance at 220 nm was 0.85 or less for a 1 mm pathlength, and these conditions were such that most of the spectrum was determined with a CD dynode voltage between 250–500 V. At the lower wavelengths the dynode voltage would increase due to increasing absorption. If an appreciable increase was observed, the solutions were generally rescanned at very slow rates. At 220 nm the signal to noise ratio was about 30:1. Unless otherwise indicated the solutions contained 0.25 M sucrose–1 mM HEPES, pH 7.5, and baselines were obtained with the same buffer. Above 205 nm there was no significant ellipticity due to the sucrose. All measurements were made in a thermostatted cell holder at 20°. Molar ellipticities $[\theta]$ were calculated from the relation,

$$[\theta] = \theta/(d \cdot m)$$

where θ is the ellipticity in mdegrees, d is the pathlength in mm, and m is the protein residue molarity which was obtained by dividing the protein concentration in mg/ml by 115, the assumed average residue molecular weight. This equation gives the molar ellipticity in the conventional units of degree·cm²/decimole. Resolution of a CD spectrum into the minimum number of Gaussian curves required for a good fit was

TABLE I

PHOSPHOLIPASE A TREATMENT OF SUBMITOCHONDRIAL VESICLES

The preparation of submitochondrial vesicles, as well as the extraction of phospholipid with phospholipase A, is described under METHODS.

	Phospholipase A treatment				
	Phospholipase A protein (*μg/mg*)	*Time of incubation* (*min*)	*Bound phosphorus* (*μg P/mg*) *	*Phospholipid remaining* (%)	$-[\theta]_{222\ nm}$
Control	0.0	5	22.1	100	10 800
No. 1	0.2	5	19.8	89	11 100
No. 2	1.4	5	13.3	57	11 700
No. 3	10.9	15	7.2	28	11 400

* μg P per mg protein; 1.4 μg P per mg protein of non-lipid phosphorus was subtracted in calculating the percent phospholipid remaining[28].

achieved using the Hewlett–Packard 9100B calculator with the extended memory and plotter. Over the wavelength interval, 212–260 nm, all spectra could be characterized in terms of three Gaussian bands centered at approximately 253, 223, and 205 nm. The band at or near 223 nm was used to estimate the helical content. Details of the procedure used in resolving the spectra are given in the APPENDIX.

RESULTS

CD of mitochondria, submitochondrial vesicles, and mitochondrial soluble proteins

Treatment of submitochondrial vesicles with dilute acid or urea results in the extraction of 40% of the protein with retention of the trilaminar structure of the membrane[15–17]. The trilaminar structure of the submitochondrial vesicles also remains after extraction of lipid[22]. The preparations used in these studies were fully characterized with regard to quantitation and selective nature of the extract as well as the retention of trilaminar structure of the membrane.

The CD spectrum of submitochondrial vesicles is independent of pathlength and concentration as shown in Fig. 2. Protein concentrations of 0.3 and 1.2 mg/ml and pathlengths of 0.1 cm and 1 cm give identical spectra within the limit of sensitivity. In addition, the spectrum obtained for vesicles is not significantly altered by sonication. The ellipticity at 222 nm is similar to that reported by URRY *et al.*[25] for sonicated mitochondria.

A comparison of the CD spectra of intact mitochondria, mitochondrial soluble proteins and submitochondrial vesicles is given in Fig. 3. The spectrum of mitochondria exhibits minima at 208 and 223.5 nm characteristic of the α-helical conformation. The amplitude of the mitochondrial CD spectrum is markedly lower than that of the vesicles, presumably due to optical artifacts[8,12]. The spectrum of submitochondrial vesicles has a well-defined minimum at 222 nm with no indication of a red-shift and a shoulder at about 210 nm while that of the mitochondrial supernate

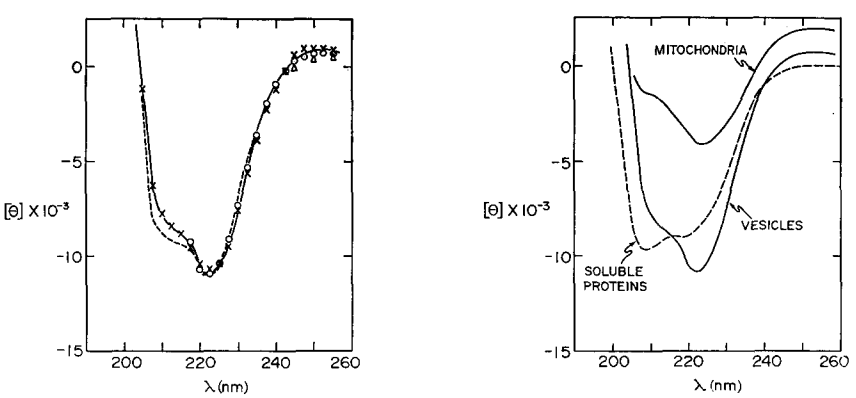


Fig. 2. CD spectrum of submitochondrial vesicles (varying several experimental conditions) before (———) and after (– – – –) sonication. Vesicles were sonicated 5 min at 3 A in a thermostatted chamber at 0–2° with a Branson Sonifier. ○—○, 1.2 mg protein/ml, 1 mm pathlength; ×—×, 0.3 mg protein/ml, 1 mm pathlength; △—△, 0.3 mg protein/ml, 10 mm pathlength. The absorbance of the submitochondrial vesicles at 220 nm was 0.825 (1 mm pathlength).

Fig. 3. CD spectra of mitochondria, submitochondrial vesicles and soluble mitochondrial proteins.

has a well-resolved minimum at 209 nm. It has been our experience that aggregation results in a decreased ellipticity which is accompanied by a small red shift (1–2 nm). In general, the red shift is more pronounced in ORD than in CD[8,26,27]. Both mitochondria and mitochondrial vesicles show positive ellipticity above 245 nm. The positive ellipticity was small except where appreciable light scattering occured, such as for mitochondria, or vesicles under conditions of pronounced aggregation. In such cases, the scattered light could produce an instrumental artifact resulting in a baseline shift.

Effect of lipid extraction

CD spectra of lipid-depleted vesicles are shown in Fig. 4 and their molar ellipticity at 222 nm are summarized in Table I. Three levels of extraction were obtained as judged by the residual phosphorus to protein ratio, corresponding to the removal

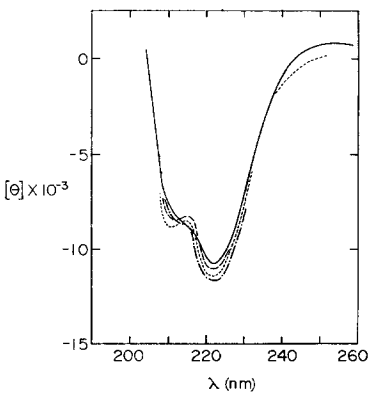


Fig. 4. CD spectra of submitochondrial vesicles and lipid-depleted vesicles. Control ———; Phospholipid-depleted preparation No. 1 – – – –; No. 2 –·—·–; No. 3 ····· of Table I. No significant change in the ultraviolet spectrum, 200–300 nm, was observed after lipase treatment.

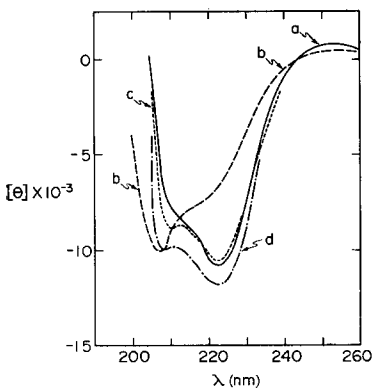
of 11, 43 an 72% of the phospholipid. Removal of up to 72% of the phospholipid produces only minor changes in the CD spectra as compared to control vesicles, although a slight increase in the resolution of the minima at 222 and 210 nm was found. It was necessary to add at least 1 mM HEPES buffer to these samples to avoid flocculation.

Effect of protein extraction

After extraction with acid or urea the residual membrane vesicles give a CD spectrum similar to the original vesicles (Fig. 5) with an ellipticity of −11800 and −10500 at 222 nm for acid and urea vesicles respectively. The spectra also show slightly better resolution of the minima at 210 and 222 nm. The amplitude of the spectrum of vesicles extracted with urea was found to vary with the conditions of extraction. Lowering the concentration of dithiothreitol in the extraction mixture resulted in vesicles which aggregated and gave a diminished spectrum. Gross aggregation was not detected in samples extracted in the presence of dithiothreitol, 1 ml/mg, although it is likely that some aggregation still occurred (see below).

The spectrum of submitochondrial vesicles in 8 M urea (Fig. 6) shows a pronounced decrease in the ellipticity at 222 nm and an increase below 215 nm, suggesting only partial loss of ordered secondary structure of the proteins. The membrane-associated protein exhibitis little ellipticity at 222 nm (−1900) in either 8 M urea or in concentrated guanidine–HCl, suggesting nearly complete denaturation under these conditions. The spectrum of urea-extracted vesicles in 8 M urea shows only a 20% decrease in amplitude at 222 nm. Thus the intrinsic membrane proteins seem to have a very stable ordered secondary structure, whereas the membrane associated proteins are readily unfolded under these conditions.

Table II summarizes the ellipticity at 222 nm of mitochondria, submitochondrial vesicles, and vesicles extracted of membrane-associated protein. Using the percent protein in each fraction and assuming the spectra are additive, the ellipticity of the starting material can be calculated and compared with the experimental results.



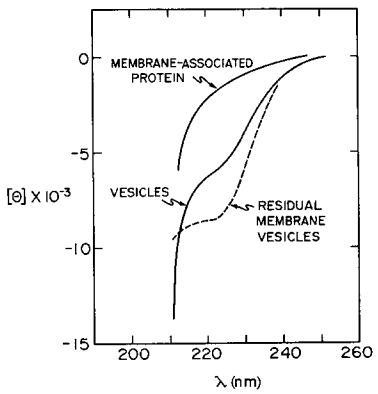


Fig. 5. CD spectra of submitochondrial vesicles (a); membrane-associated protein (b); the residue after urea extraction (c) and acid extraction (d) of submitochondrial vesicles. The spectrum of membrane-associated protein was taken in 10 mM Tris, pH 8.1.

Fig. 6. CD spectra in 8 M urea–10 mM Tris, pH 8.1, of submitochondrial vesicles, membrane associated protein, and the residue after urea extraction of submitochondrial vesicles (residual membrane vesicles).

For whole mitochondria, the observed ellipticity is much lower than the value calculated from the spectra of the vesicles and mitochondrial supernate. This difference is attributed to optical artifacts which greatly reduce the ellipticity of the mitochondrial spectrum[8,12]. On the other hand, the ellipticity of vesicles calculated from the data on membrane associated protein and urea or acid extracted vesicles is in good agreement with the observed value. Since the CD spectra of urea extracted vesicles and membrane-associated protein were obtained using material that had been exposed to 8 M urea in preparation, the good agreement of the calculated and experimental values indicates reversibility of the urea-induced transitions.

The CD spectra of submitochondrial vesicles, residual membrane vesicles, lipid-depleted vesicles, membrane-associated protein and mitochondrial soluble proteins were resolved into Gaussian components, and the band characteristics are given in Table III. These resolved components were obtained to permit a more meaningful comparison of the various CD spectra, to check for the possible occurrence of other bands near 223 nm, and to allow reconstruction of our CD spectra. Due to the lack

TABLE II

MOLAR ELLIPTICITY AT 222 nm OF MITOCHONDRIA, SUBMITOCHONDRIAL VESICLES, AND DERIVED PREPARATIONS

The samples were suspended in 0.25 M sucrose–1 mM HEPES unless stated otherwise.

	Protein distribution (%)	λ*	$-[\theta]_{\lambda}$**	*Calculated*** $-[\theta]_{222\ nm}$
A. *Disruption of mitochondria*				
Mitochondria§	100	223.5	4 200	
Submitochondrial vesicles	80	222.3	10 800	10 400
Mitochondrial soluble proteins	20	*	8 600	
B. *Extraction of vesicles with urea*§§				
Submitochondrial vesicles	100	222.3	10 800	
Residual membrane vesicles	65	222.5	10 600	9 300
Membrane associated protein	35	*	6 900	
C. *Extraction of vesicles with acid*				
Submitochondrial vesicles	100	222.5	10 800	
Residual membrane vesicles	60	222.7	11 800	9 840
Membrane associated protein§§§	40	*	6 900	
D. *Fractions in B in presence of 8 M urea*				
Submitochondrial vesicles	100	*	6 000	
Residual membrane vesicles	65	*	8 600	6 350
Membrane associated protein	35	*	1 900	

* Wavelength of observed ellipticity extremum. In several cases there was no well-defined minimum near 222 nm, and these are indicated by an asterisk.

** When there was no well-defined minimum, the ellipticity at 222 nm was used.

*** The calculated $-[\theta]_{222\ nm}$, is based on the values obtained for the components weighted for their protein content.

§ The measured ellipticity of mitochondria is believed to be low because of optical artifacts (*cf.* text).

§§ The submitochondrial vesicles were extracted with 8 M urea as described in the text. The residue was resuspended in 0.25 M sucrose–10 mM HEPES, pH 7.5, and is designated as residual membrane vesicles. The supernatant from urea extraction was dialyzed *vs.* 10 mM Tris, pH 8.1, and centrifuged to clarity. The optically clear preparation is designated membrane associated protein.

§§§ We have used the ellipticity of the surface protein prepared by urea extraction.

of data below 200 nm, the overlap of various conformation-dependent bands (reflecting mainly the π–π* transitions of the α-helix, β-structure, and nonhelical structure), and the presence of optical artifacts, little physical significance can be attached to these various bands. In particular, the low wavelength band is extremely arbitrary and is included only to allow reconstruction between 212–260 nm of our experimentally determined CD spectra. The band of primary concern is that at 223 nm which is tentatively assigned as the n–π* transition of the α-helix[13].

Effect of detergents

The possibility that aggregation of vesicles after urea extraction causes a decrease in the amplitude of the CD spectrum was investigated using several detergents to disperse the vesicles. Fig. 7 presents representative spectra of urea-extracted vesicles and control vesicles in different concentrations of sodium dodecyl sulfate and Fig. 8 gives the changes in ellipticity of the minima at about 210 and about 222 nm

TABLE III

RESOLUTION OF CD SPECTRA INTO GAUSSIAN COMPONENTS

The maximum ellipticity of the resolved band, $[\theta^\circ]$, the extremum wavelength, λ_0, and the band half-width, $\triangle$, where $[\theta] = [\theta^\circ]/e$, can be used to obtain the rotational strength, $R = 1.234\ ([\theta^\circ] \triangle / \lambda_0) \cdot 10^{-42}$. As discussed in the text and APPENDIX, these values are to be viewed primarily as curve-fitting parameters. Using these data our experimental CD spectra can be calculated with high accuracy over the wavelength interval 212–260 nm.

Preparation	λ_0 (*nm*)	$[\theta^0]$ $\left(\frac{degree \cdot cm^2}{decimole}\right)$	$R \cdot 10^{40}$ (*cgs*)
Submitochondrial vesicles	223	−10 800	−7.2
	206	− 7 000	−3.4
	253	+ 800	+0.43
Residual membrane vesicles (urea extracted)	223	−10 550	−7.6
	204	− 9 300	−4.4
	253	+ 850	+0.62
Residual membrane vesicles (acid extracted)	223	−11 600	−7.7
	204	− 9 050	−5.5
	253	+ 800	+0.47
Lipid-depleted vesicles	223	−11 500	−7.3
	209	− 5 700	−1.6
	253	+ 200	+0.03
Membrane associated protein	219	− 7 200	−3.7
	205	− 7 100	−3.2
	253	+ 450	+0.33
Mitochondrial soluble proteins	221	− 8 600	−6.5
	205	− 7 400	−3.8
	253	0	0

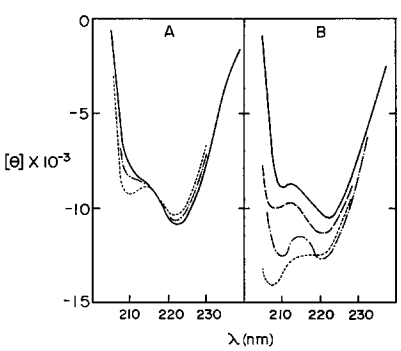


Fig. 7. Effect of sodium dodecyl sulfate on the CD spectra of submitochondrial vesicles (A) and the residue after urea extraction of submitochondrial vesicles (residual membrane vesicles) (B). ———, no sodium dodecyl sulfate; – – – –, 0.03% sodium dodecyl sulfate; –·—·–, 0.05% sodium dodecyl sulfate; ·····, 0.10% sodium dodecyl sulfate.

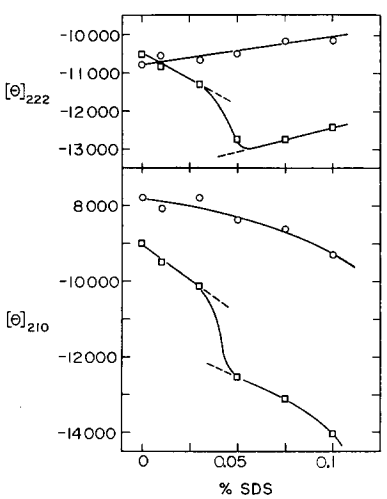


Fig. 8. Molar ellipticity at 222 and 210 nm as a function of sodium dodecyl sulfate (SDS) concentration; ○—○ submitochondrial vesicles; □—□, residue after urea extraction of submitochondrial vesicles (residual membrane vesicles).

as a function of sodium dodecyl sulfate concentration. The ellipticity at 222 nm for control vesicles decreases slightly as the concentration of sodium dodecyl sulfate is increased from 0 to 0.1 % while that of the minimum at 210 nm increases over the same concentration range. Both changes have a nearly linear dependence on sodium dodecyl sulfate concentrations. In contrast, the CD spectrum of urea extracted vesicles exhibits a marked change between 0.01 and 0.05 % sodium dodecyl sulfate which increases the ellipticity of both minima. At higher concentrations of sodium dodecyl sulfate, the change in ellipticity parallels that of the control vesicles. These results suggest that a better value of the ellipticity of urea extracted vesicles can be obtained using sodium dodecyl sulfate to disperse the vesicles. Extrapolation of the data at 0.05–0.10 % sodium dodecyl sulfate to zero gives an ellipticity at 222 nm of approx. −13000 (*cf.* Fig. 8).

Of other detergents tested, deoxycholate gave results qualitatively similar to those found with sodium dodecyl sulfate, but at higher concentrations (0.1–0.3 %). However, at 0.5 % the spectrum for both control and urea extracted vesicles showed a large decrease in ellipticity at 222 nm. Both 0.1 % Triton X-100 and 0.1 % Brij 36T increased the ellipticity of control vesicles at 210 nm without affecting the 222 minimum.

DISCUSSION

Past considerations of membrane arrangement predicted that the proteins of the membrane were largely unfolded[29], or in a β-configuration to form the membrane surface[30]. It now appears that membrane proteins contain 25–40 % α-helix[5–11,25,31]. With our recent understanding of the secondary level of membrane organization[15–17], it could have been argued that the α-helix would not be present in the intrinsic proteins, but would occur only in the membrane-associated proteins. Our studies show that this is clearly not the case. In fact, most of the secondary structure of the mitochondrial inner membrane is present in the intrinsic membrane proteins. Membrane-associated proteins contain about half as much helix as the intrinsic membrane proteins. The secondary structure of the soluble mitochondrial proteins is somewhat greater than that of the membrane-associated proteins and lower than that of the intrinsic membrane proteins.

The secondary structure of the proteins in urea extracted vesicles exhibits remarkable stability. Approx. 80 % of the secondary structure of the intrinsic membrane proteins remains in the presence of 8 M urea (compare $[\theta]_{222\ nm}$, Table II). This is in contrast to most globular proteins which are denatured by concentrated urea. In this regard membrane-associated proteins behave similar to globular proteins. It remains to be seen whether the stability of intrinsic membrane proteins is a property of the individual proteins or is due to their organization in the membrane.

Another interesting observation is that no appreciable change in secondary structure occurs after removal of a substantial amount of lipid (72 %). It is well-documented that phospholipid is required for mitochondrial electron transport[19,28,32], but the precise role is not understood. The requirement of lipid for succinate–cytochrome *c* reductase, as well as cytochrome *c* oxidase, is manifested only after removal of more than 80 % of the lipid[19]. In view of these results it would be interesting to evaluate the role of the remaining phospholipid in the secondary structure of the

membrane proteins. We do know that the trilaminar structure of the mitochondrial inner membrane remains after removal of practically all of the lipid[22].

MYER[33] has investigated the CD of solubilized cytochrome *c* oxidase and found an ellipticity of −12400 at 222 nm. This segment of the electron transfer chain is believed to be a part of the intrinsic membrane proteins and its ellipticity is similar to that of the intrinsic membrane proteins depleted of surface proteins.

GORDON *et al.*[27] have reported changes in the CD spectrum of plasma membranes from Ehrlich ascites cells after treatment with phospholipase A or C. However, the interpretation of these results is complicated by the presence of the breakdown products of phospholipase digestion. These authors show that the addition of lysolecithin to the plasma membranes causes spectral changes similar to that observed after phospholipase A treatment. It would appear that the detergent properties of lysolecithin[34] affect the structure of the proteins as has been shown for other detergents[35,36].

GLASER AND SINGER[31] and GLASER *et al.*[37] have reported on the effect of phospholipase C digestion on red blood cell membranes. They found no significant changes in the CD spectrum after treatment with phospholipase C and breakdown of approx. 65 % of the lipid. This study shows that the secondary structure of the proteins in a different membrane is independent of lipid, although in studies with phospholipase C-treated membranes diglycerides are still present in the membrane.

The CD spectra of homopolypeptides in various conformations are used to provide guidelines for interpreting the more complex spectra of proteins[38,39]. The structure that can be identified most unequivocally using circular dichroism is that of the α-helix which exhibits negative bands near 222 nm, 208 nm, and a positive band near 192 nm, all of which result from the peptide chromophore. Although many difficulties are encountered when using optical activity measurements for the quantitative determination of protein conformation, the technique is quite sensitive for detecting conformation changes involving secondary structure and it is primarily in this context that our results are most meaningful.

CD measurements on membranes present the additional problem of optical artifacts (light scattering, absorption flattening, and differential light scattering) which reduce the ellipticity of the proteins. GLASER AND SINGER[31] have shown that the ellipticity of erythrocyte membranes at 222 nm is less sensitive to these artifacts than at 208 nm. We confirm this with the submitochondrial vesicles since we invariably find that normal dispersing methods and agents, *e.g.* sonication and addition of detergents, affect the 210 nm band but have negligible effects on the 222 nm band.

The data presented in Table III on the resolved bands are most accurate for the transition at 223 nm which we assign to the n–π^* band of the α-helix[13]. Significantly, this single Gaussian curve will describe the CD data between 220–235 nm with a high degree of accuracy. This suggests that there are no other bands of high rotational strength within a few nanometers of 223 nm, since the addition of two Gaussian curves will not produce a Gaussian curve. Clearly, if two bands corresponding to different conformations, *i.e.* the n–π^* transition of the α-helix and the β-structure, occur at the same value of λ_0 with similar band half-widths, the present method could not resolve these. However, for model compounds these minima occur at 221 and 215 nm, respectively[39]. The band characteristics for the transition at 204–209 nm are subject to considerable error since our data do not extend below 200 nm

where strong transitions associated with various conformations occur. The positive ellipticity with a maximum at 253 nm may reflect instrumental artifacts, although the heme moiety, aromatic amino acids, and disulfides contribute in this region as well[38]. We have also resolved the CD data of GREENFIELD AND FASMAN[39] on aqueous solutions of poly-L-lysine in various conformations. The α-helix gave Gaussian bands at 221, 206, and 191 nm with respective rotational strengths of $-25.1 \cdot 10^{-40}$, $-9.3 \cdot 10^{-40}$ and $+44.8 \cdot 10^{-40}$. The resolved n–π^* band of our various mitochondrial preparations is within $\pm$ 2 nm of these model compound data.

The problem of determining percent helicity from optical activity measurements has been discussed by others[39]. Clearly, one cannot expect the model compounds to exhibit identical spectra to globular proteins which contain rigid structures and short, often imperfect, helices. Nevertheless, CD spectra can be used both qualitatively for determining the presence of secondary structure and semi-quantitatively to estimate the amount. Homopolypeptides in the random coil conformation generally give an ellipticity at 222 nm of about + 4000[39], while denatured proteins often yield slightly negative values[40,41]. The α-helix generally exhibits negative ellipticities of 30000–35000 at 222 nm[39]. Using these various limits one can estimate a percent helicity for the proteins of submitochondrial vesicles of 25–40 %. Using the measured ellipticity of the membrane-associated protein in 8 M urea ($[\theta]_{222\ nm} = -1900$) as a typical value for nonhelical membrane proteins, and a value of -35000 for the α-helix, we estimate 27 % helix for the submitochondrial vesicles, 15 % helix for the membrane-associated protein, 26–30 % helix for the intrinsic membrane proteins, and 20 % helix for the mitochondrial soluble proteins. The rotational strength of a given transition can also be used to estimate the percent helix, and in principle this is considerably more accurate than using the ellipticity at a given wavelength. With a rotational strength of $-25.1 \cdot 10^{-40}$ for the n–π^* transition of the α-helix and a value of 0 for the nonhelical conformation, the percent helix for the vesicles and soluble proteins is essentially the same as that found using the 222 nm ellipticity values*.

Based on infrared spectroscopy, GRAHAM AND WALLACH[42] have concluded that β-structure is present in mitochondrial membranes under certain metabolic conditions. In model compounds the CD band of β-structure generally occurs at 215 nm[39], although there are reports suggesting that both red and blue shifts occur[43]. We invariably find both 208–210 and 222 nm CD extrema, indicative of the α-helix, with no indication of a 215 nm band. Moreover, we emphasize there is no indication of a 215 nm band following curve resolution. This suggests that either the amount of β-structure is small or the membrane environment is such that a sizeable wavelength shift is induced and either the 222 nm band or lower wavelength bands reflect both α- and β-conformations. The greater ellipticity at 222 nm, relative to 210 nm, could be explained by such a red shift. However, this could be due to optical artifacts which diminish the ellipticity at 210 nm[8,31]. Clearly, other experimental techniques are required to evaluate the extent of β-structure.

The CD spectrum of urea-extracted vesicles was improved by the addition of sodium dodecyl sulfate to the buffer, presumably by dispersing the vesicles. However, since sodium dodecyl sulfate is capable of both dissolving membrane proteins[44] and

* There seems to be a small positive band at 217 nm in nonhelical poly-L-lysine[39] for which we estimate a rotational strength of $+2.7 \cdot 10^{-40}$. Assuming this value pertains to the n–π^* transition of the nonhelical structure, the percent helix is somewhat higher than that reported above.

causing changes in secondary and tertiary structure[35,36], it is important to determine the effect of sodium dodecyl sulfate on submitochondrial vesicles. Sodium dodecyl sulfate causes only small changes in the ellipticity of vesicles as compared to the increase in ellipticity of urea-extracted vesicles. Even if solubilization or unfolding occurs at higher sodium dodecyl sulfate concentrations, little change in ordered secondary structure is seen. Thus, the data for urea-extracted vesicles in sodium dodecyl sulfate give a reasonable estimate of the helical content of the intrinsic membrane protein.

APPENDIX

Resolution of a CD spectrum into constituent gaussian curves

One way to resolve a CD spectrum is to determine the minimum number of Gaussian bands that will give a satisfactory fit[45]. A Gaussian band is of the form,

$$[\theta] = [\theta^\circ] \exp [-(\lambda - \lambda_0)^2/\Delta^2]$$

where $[\theta^\circ]$ denotes the maximum ellipticity of the resolved band, which may be positive or negative, λ_0 is the wavelength where this extremum occurs, and Δ is the band half-width defined as the wavelength interval from λ_0 to the wavelength where $[\theta]=[\theta]/e$. These parameters are determined for each band with the criterion that a summation over all resolved bands will accurately fit the observed CD spectrum.

For each of the vesicle preparations, well-defined extrema were obtained at 223 and 253 nm. The positive 253 nm band was resolved without difficulty for these spectra since there was no obvious overlap with other bands at the extremum wavelength, *i.e.* $\lambda_0=253$ nm, and the observed maximum value of $[\theta]$ could be equated to $[\theta^\circ]$. The band half-width was obtained by fitting the spectrum above 253 nm. The value of λ_0 for the negative 223 nm band was taken as the wavelength where the well-defined minimum ellipticity was observed. The value of $[\theta^\circ]$ at 223 nm was found to be within 0.5 % of the observed ellipticity at the minimum. The half-width of this band was obtained by fitting the spectrum above 223 nm. Since $[\theta^\circ]/e$ is approx. -4000, corresponding to a wavelength of about 235 nm, Δ can be obtained directly from the experimental spectrum.

Below approx. 215 nm, the CD spectrum of most proteins is quite complicated since the π–π^* transition associated with the various conformations overlap considerably. In addition to this difficulty, optical artifacts tend to distort the spectrum significantly in this region. Thus, any resolution below 220 nm is extremely arbitrary. We have found that one additional negative band at about 205 nm, in conjunction with the two higher wavelength bands, permits an excellent fit to the experimentally determined spectra of the various vesicles between 212–260 nm. Trial and error methods were used to generate values of λ_0, $[\theta^\circ]$ and Δ for this low wavelength band. Whereas λ_0 is near that expected for the π–π^* component parallel to the α-helix axis, little significance can be attached to the rotational strength in view of the problems given above. To fit the data below 212 nm required the introduction of a positive band at 200 nm with a rotational strength of about $+ (4.5 \pm 1.3) \cdot 10^{-40}$ for the various vesicle preparations. However, the highly subjective nature of these latter data do not warrent their inclusion in Table III. Both the membrane-associated proteins and

the mitochondrial solube proteins exhibited shoulders in the range 219–221 nm, rather than well-defined minima. Consequently the band parameters were obtained using trial and error methods to fit the spectrum between 221–235 nm for the membrane-associated proteins and between 223–260 nm for the mitochondrial soluble proteins. A good fit over these wavelength intervals was obtained with $[\theta^\circ]$ values of about 98–99 % of the measured ellipticity at 220 nm.

The membrane-associated proteins exhibit a well-defined minimum at 207 nm. Using the parameters established above for the 219 nm band, we were able to fit the observed CD spectrum over the wavelength interval 200–235 nm by the introduction of one additional negative band with the characteristic parameters given in Table III. The parameters of the 253 nm band were determined as described above for the vesicles.

The mitochondrial soluble proteins have a well-defined minimum at 209 nm. The spectrum could be described over the wavelength range 210–260 nm using the 221 nm band discussed above, and one additional negative band centered at 205 nm. To fit the data between 200–210 nm required, the introduction of a positive band characterized by the following parameters, $\lambda_0 = 196$ nm, $[\theta^\circ] = +12000$, and $\Delta = 6$ nm.

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