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CIRCULAR DICHROISM OF HUMAN ERYTHROCYTE MEMBRANES SOLUBILIZED BY *N*-PENTANOL

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SUMMARY

1. The circular dichroism (CD) spectrum of *n*-pentanol-solubilized human erythrocyte membranes, a preparation which consists of lipoproteins having relatively low turbidity, does not show the red-shift characteristic of CD spectra of intact membranes. The double minima indicating α-helix are observed, but are blue-shifted from the usual α-helix minima extrema.

2. Solution of the lipoproteins in 70% 2-chloroethanol, an α-helix promoting solvent, results in a red-shift of the 219 nm minimum to 221 nm, as well as a large increase in ellipticity. These data suggest a significant amount of β-structure was present in the pentanol-treated erythrocyte membranes, which may have been generated by the pentanol treatment.

3. Aggregation of the lipoproteins by $CaCl_2$ and $MgCl_2$ results in a turbid preparation with its CD spectrum showing a distinct red-shift and sharply decreased amplitude similar to the features of the CD spectra of the original membrane preparation, indicating that the light scattering and absorption flattening effects due to the particulate nature of the membranes do exert a strong influence on their CD spectra, and in particular may obscure sizable contributions of β-structure.

INTRODUCTION

Membranes exhibit optical rotatory dispersion (ORD) and circular dichroism (CD) spectra in the far ultraviolet region which reflect secondary structure of the proteins. A comparison of the spectra of soluble proteins and membrane stroma showed that the membranes are characterized by a red-shift of the extrema as well as a low amplitude and broadening of the signals. A number of explanations for these features have been offered[1-8].

The unusual features of the ORD and CD spectra of membranes have in general been thought to arise from either (a) some specific state of the macromolecules within the membranes, or (b) the effect of the particulate nature of the membranes, which, by causing light scattering and Duysen's absorption flattening, results in distortions of the spectra.

It has been suggested that the sources of the characteristic features of membrane ORD and CD spectra include the presence of adjacent interacting α-helices[1], hydrophobic interaction between the α-helices of the proteins with lipids[2], lipid–protein

interactions or protein–protein interactions responsive to lipid environment[3], and the direct contribution of lipids[4].

Study of the effects of states of aggregation of mitochondrial protein on ORD and CD spectra resulted in the conclusion that aggregated helices of membrane protein were responsible for the red-shift[5], although the mechanism by which aggregation produces the red-shift was not clear. Urry and Ji[6] proposed that light scattering and absorption flattening inherent in particulate systems could result in the low amplitude of membrane CD spectra. These authors[7] correlated light scattering and absorption flattening with red-shift and decrease in amplitude of CD spectra of particulate systems. The CD spectrum of erythrocyte membranes has also been shown[8] to increase in amplitude and to blue-shift when the particle size was decreased by sonication.

There now seems to be general agreement that optical artifacts do contribute distortions of the CD spectra of membranes, and a variety of approaches has been made to correct these artifacts produced[9–12].

An optically clear lipoprotein preparation can be obtained from erythrocyte membranes, by treatment with *n*-pentanol[13]. The pentanol treatment of the membrane gives a preparation containing about 80 % of both proteins and lipids of the original membranes with an amino acid composition representative of the original membrane proteins. Sedimentation of this type of preparation gives two apparently homogeneous lipoprotein fractions in a sucrose gradient. The solubility and low turbidity of this preparation offer the opportunity for directly obtaining CD spectra of membrane lipoproteins with little distortion due to light scattering and absorption flattening. Although the extent to which the states of the proteins in the solubilized lipoprotein units reflect those in the intact membrane remains to be established, the fact that the composition of these homogeneous lipoproteins is representative of the intact membrane suggests some relevance of the solubilized lipoprotein units to native membrane structure.

Further, several membrane enzymes remain active in the pentanol-solubilized membranes[14], indicating that the treated membrane proteins retain much of their structure. NMR studies have also shown[14,15] that the lipids and proteins in the pentanol-solubilized preparations and in the ultrasonically dispersed membrane preparations are similar with respect to mobility. It was the object of the present investigation to compare CD spectra of the pentanol-treated erythrocyte membranes with the characteristic CD spectra of untreated membranes.

METHODS

Human erythrocyte membranes were prepared from freshly drawn A-positive blood by the technique of Dodge *et al.*[16]. The membranes were solubilized by the n-pentanol treatment of Zwaal and Van Deenen[13]. Protein analysis was by the biuret method with bovine serum albumin as a standard.

Circular dichroism spectra were obtained with a Cary 60 spectropolarimeter equipped with a Cary 60.01 CD unit. Baselines were run before and after sample spectra, which were run at least three times. The maximum time constant and slow scan spectra (less than 5 nm/min) were utilized. Mean residue ellipticities were calculated on the basis of a mean residue weight of 110.

RESULTS AND DISCUSSION

The CD spectrum from 250 to 185 nm of the *n*-pentanol-solubilized erythrocyte membranes in distilled water (Table I) shows the double minima suggesting α-helix. Clearly, there is no red-shift of the classic α-helix minimum at 221–222 nm[17] to the approx. 224 nm position characteristic of membrane CD spectra. There is, on the other hand, a blue-shift of the minimum to 219 nm, suggesting that there are conformations other than the classic α-helix and random coil in these lipoproteins. Contributions from appreciable amounts of β-structure with a minimum in this region expected to be about 217 nm with a mean residue ellipticity about half that of the α-helix[17] at 221–222 nm, could produce the observed shift and are compatible with evidence for presence of some β-structure in membranes[18–20].

TABLE I

ELLIPTICITY OF PENTANOL-SOLUBILIZED ERYTHROCYTE MEMBRANES

Reported λ_{max}, λ_{min} and corresponding ellipticities taken from spectra for Preparation B in distilled water. Triplicate determinations were used with baselines obtained before and after each spectra.

λ_{max} and λ_{min}	*Mean residue ellipticity** (*degrees·cm²·dmole⁻¹*)
λ_{min} = 219 nm	−10 100
λ_{min} = 209 nm	− 9 740
λ_{max} = 192 nm	−19 100

* Mean residue ellipticities based on a mean amino acid residue weight of 110.

The CD spectrum of pentanol-solubilized membranes in distilled water was treated by the method of Greenfield and Fasman[21]. These calculations also indicate that there is a significant amount of β-structure. The lipoproteins were calculated by this method to contain 24% α-helix, 40% random and 36% β-structure. While these values cannot be assumed to reflect quantitatively the secondary structure, they do indicate that the CD spectrum cannot be accounted for fully as a mixture of classical α-helix and random coil.

The mean residue ellipticity of the 219 nm minimum of −10 100 degrees·cm²·dmole⁻¹ is almost twice that observed[11] for erythrocyte membranes after freezing, suggesting that the apparent damping of the amplitude of the CD spectra of membranes is not a feature of the CD spectrum of soluble membrane lipoproteins.

The CD maximum observed for these lipoproteins is at 192 nm, with a mean residue ellipticity of 19 100 degrees·cm²·dmole⁻¹ (Table I). The position of this maximum is about that expected for α-helix[17], *i.e.*, 190–192 nm which due to the relative ellipticities of α-helix and β-structure in this region would be expected to dominate the spectrum more in the region of the maximum than in the region of the minima for mixtures of α-helix and β-structure. The interpretation of the CD spectrum at this maximum is complicated by the potentially large contribution at the 196–197 minimum of unordered structure[17], which has negligible effect in the wavelength region of the minima of α-helix and β-structure.

The spectral region expected to reflect the orientation of aromatic residues (about 300 to 250 nm) has received little attention in the studies of membranes. The absence of appreciable Cotton effect from 240 to 300 nm for erythrocyte and *Bacillus subtilis* membranes has been reported[1] although irregularity in the 250 to 300 nm region of the ORD spectrum of plasma membranes from Ehrlich ascites carcinoma microsomes has been attributed to oriented aromatic chromophores[2].

Diffuse positive and negative signals are observed from about 295 to 255 nm in the CD spectrum of the *n*-pentanol-solubilized erythrocyte membranes. Although the signal-to-noise ratio was too low to allow the reliable assignment of extrema positions and ellipticities to these signals, there does appear to be some orientation of the aromatic sidechains of the proteins. The apparent multiplicity of signals observed suggests that the oriented aromatic chromophores in these solubilized membranes are in a variety of orientations and environments. This perhaps is also responsible for the low ellipticities of the individual extrema.

TABLE II

COMPARISON OF CD SPECTRA* OF PENTANOL-SOLUBILIZED ERYTHROCYTE MEMBRANES AND THE Ca^{2+} AND Mg^{2+} AGGREGATED FORM

Sample	λ_{min} (*nm*)	*Mean residue ellipticity*** (*degrees·cm²·dmole⁻¹*)
Pentanol-solubilized erythrocyte membranes, $3.8 \cdot 10^{-2}$ mg protein/ml, in distilled water	219	−10 900
	208 (poorly defined)	−10 200
Pentanol-solubilized erythrocyte membranes, $3.8 \cdot 10^{-2}$ mg protein/ml, in approx. 10^{-2} M $CaCl_2$ and $MgCl_2$	223	− 5 820
	212 (poorly defined)	− 4 180

* For Preparation A, from spectra obtained in triplicate with a baseline before and after the spectra. Instrumental settings and pathlengths are identical for the two spectra.

** Mean residue ellipticities based on a mean amino acid residue weight of 110.

Aggregation of the soluble lipoproteins to an insoluble turbid form was effected by the addition of $CaCl_2$ and $MgCl_2$ to the preparation. Aggregation was obvious on inspection and was also followed spectrophotometrically at 350 nm as a function of Ca^{2+} concentration. The CD spectra of the corresponding preparations are compared. Mean residue ellipticities at the minima are given in Table II, and the baseline-corrected spectra are shown in Fig. 1. The only difference in composition in the two preparations was the presence of 10^{-2} M $CaCl_2$ and $MgCl_2$ in the aggregated sample; protein concentrations, path lengths, and instrumental settings were identical for the two spectra. The maintenance of identical concentrations and pathlengths satisfies[7] criteria for a valid test of light scattering effect, based on variation of particle size only. However, the possibility that the divalent cations caused conformational changes in the proteins cannot be excluded.

The influence of the Ca^{2+} and Mg^{2+} induced aggregation on the CD spectrum (Table II) was striking. The aggregated sample showed a 4-nm red shift from 219 nm

to 223 nm and a decrease of almost 50% in the mean residue ellipticity. Both the red-shift and the sharply decreased amplitude are the effects anticipated if the particulate nature of membranes is responsible for the unusual features of membrane CD and ORD spectra. These results clearly indicate that the unique features of the

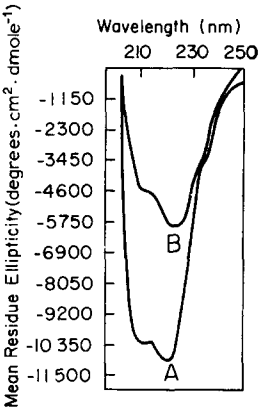

Fig. 1. Baseline-corrected CD spectra of pentanol-solubilized membranes, in solution in water (A) and aggregated in 10^{-2} M Mg^{2+} and Ca^{2+} (B), as described in Table II.

CD and ORD spectra of particulate membrane preparations as has been previously proposed[7,8] are for the most part attributable to the light scattering and absorption flattening effects of these preparations. The possibility of contributions from proteins in conformations other than the classic α-helix, β-structure, or random structure or in unusual environments such as suggested by Gordon *et al.*[3] is not excluded by this observation.

The possible contributions of β-structure to membrane proteins have been considered to be fairly small although possibly significant for plasma membranes[2]. Mitochondrial membranes appear to have a larger amount of β-structure[18] than erythrocyte membranes and *Mycoplasma laidlawii* membranes appear to have even more[19].

2-Chloroethanol is well established as a solvent which favors formation of α-helix[22]. The apparent contribution of β-structure to the CD spectrum of the lipoproteins in aqueous solution has been further studied by observing the effect of 2-chloroethanol on the CD spectrum. If the conformation of the proteins in the lipoproteins studied is a mixture of α-helix and random coil only, solubilization of the lipoproteins in 2-chloroethanol will convert some random structure to α-helix to produce an increase in ellipticity but this should not result in a shift in the position of the minimum associated with the N–π transition since this transition for α-helix is insensitive to environment[4]. On the other hand, a significant amount of β-structure with a minimum at 217 nm would be converted to α-helix on solution in 2-chloroethanol with a shift to the red as well as an increase in ellipticity. It should be noted that this shift is in the opposite direction to the blue-shift observed[1] on solution of particulate membranes in 2-chloroethanol.

Results of solubilization of the lipoproteins in 70% 2-chloroethanol are presented in Table III and Fig. 2. There is a 2 nm red-shift of the 219 nm minimum

TABLE III

COMPARISON OF CD SPECTRA* OF PENTANOL-SOLUBILIZED ERYTHROCYTE MEMBRANES IN DISTILLED WATER AND IN 70% 2-CHLOROETHANOL

Sample	*Solvent*	λ_{min} (*nm*)	*Mean residue ellipticity*** (*degrees·cm²·dmole⁻¹*)
Pentanol-solubilized erythrocyte membranes, $7.2 \cdot 10^{-1}$ mg protein/ml	Distilled water	219	−11 200
		210	−10 900
Pentanol-solubilized erythrocyte membranes, $7.2 \cdot 10^{-1}$ mg protein/ml	70% 2-chloroethanol	221	−19 800
		209	−20 500

* For Preparation B, from spectra obtained at least in triplicate, with baselines at least in triplicate, uncorrected for refractive index. Instrumental settings and pathlengths are identical for the two spectra.

** Mean residue ellipticities based on amino acid mean residue weight of 110.

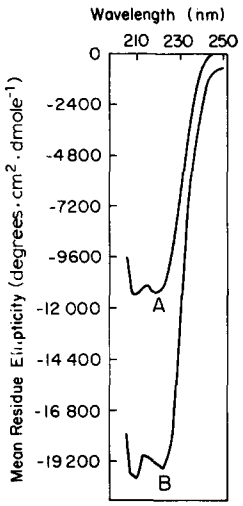

Fig. 2. Baseline-corrected CD spectra of pentanol-solubilized membranes, in water (A) and in 70% 2-chloroethanol (B), as described in Table III.

to 221 nm, as well as a large increase in mean residue ellipticity. These results do suggest the presence of a significant amount of β-structure, or at least some conformation other than α-helix and random coil in these pentanol-solubilized erythrocyte membranes.

The CD spectra of the pentanol-solubilized erythrocyte membranes indicate that a somewhat higher contribution of β-structure may be present in erythrocyte membranes than has in general been indicated for various plasma membranes[2,20] although the estimate is in line with corresponding estimates for *Mycoplasma laidlawii* membranes[19].

The data with respect to β-structure do indicate that there is a significant amount of β-structure in the lipoproteins obtained by pentanol-solubilization of human erythrocyte membranes, although to what extent this β-structure may have

been induced by the pentanol treatment has not been established. The data also indicate that distortions of the CD spectra of these lipoproteins elicited by aggregation to a particulate form completely obscure the sizable contribution of β-structure, although as previously noted, the cations Ca^{2+} and Mg^{2+} may also produce some conformational changes. This evidence suggests that consideration should possibly be given to β-structure when correction for the distortions due to the particulate nature of membranes is attempted, rather than treating the membrane spectra as distorted spectra of mixtures of α-helix and random coil only.

Evidence for β-structure cannot without additional information be interpreted as suggesting the nature of the tertiary structure of proteins in membranes, as it is becoming evident that large amounts of β-structure may be accommodated in globular proteins, such as lysozyme, as well as in extended configurations. Furthermore, it is possible for a protein to have a high degree of helical order and yet not be in a compact globular form, forming instead an extended helical rod. The CD and ORD spectra in the 250 to 185 nm region only yield direct information on secondary structure only. Protein–sodium dodecyl sulfate complexes, which may serve in some respects as a model for protein–phospholipid complexes in the membrane, have a high degree of secondary order but are extended rather than globular. For this reason caution should probably be exercised in interpreting the degree of tertiary order from CD and ORD.

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