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Optical Activity of Biological Membranes: Scattering Effects and Protein Conformation

Allan S. Schneider, Mary-Jane T. Schneider, and K. Rosenheck

POLYMER DEPARTMENT, WEIZMANN INSTITUTE OF SCIENCE, REHOVOT, ISRAEL

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Abstract. The following question has recently arisen in the literature concerning the interpretation of the optical activity of biological membranes: do the characteristic spectral distortions observed for diverse membrane systems reflect some common and unique aspect of membrane architecture or are they the result of scattering effects owing to the particulate nature of membranous systems? We have confirmed the latter interpretation on the basis of the following experimental observations: (*a*) red blood cell membranes give a normal circular dichroism spectrum when scattering is reduced and (*b*) nonaggregated, nonmembranous helical proteins give distorted membranelike spectra when scattering is introduced. An improved estimate of secondary structure on the basis of undistorted spectra results in about 50 per cent α-helix for red blood cell membrane protein. In addition we conclude that the distortions in optical activity spectra offer no evidence in support of various proposed membrane models.

During recent years there has arisen a literature concerning the optical rotatory dispersion and circular dichroism of biological membranes.[1–9] Optical activity is a sensitive measure of the secondary structure of proteins and offers an attractive nondestructive probe of intact membrane structure. Many different membrane systems have given very similar optical rotatory dispersion and circular dichroism spectra, resembling an α-helical protein, but with characteristic distortions in relative intensity of the 208 nm π-π^* to 222 nm n-π^* troughs, and red shifts in the n-π^* trough and crossover wavelengths. Several interpretations have been offered to account for these distortions in the membrane spectra. It was first proposed that since this unique spectra occurs in diverse membrane systems, it must represent some common aspect of membrane architecture, e.g., protein aggregation with possible alignment of α-helices[1,2] or hydrophobic interaction between protein helices and lipid.[3] On the other hand, there has been the recent proposal of Urry and Ji[8,9] that the distortions in the membrane spectra are the result of scattering artifacts owing to the particulate nature of the system. There is some evidence correlating turbidity with spectral distortions,[2,9] but most of these experiments do not distinguish between effects resulting from scattering and those resulting from aggregation-induced polypeptide interactions. Ji and Urry's[9] results on sonicated mitochondrial membrane fractions are the most convincing. However, since mitochondrial membranes are notably heterogeneous and a spectrum of the total sonicate was not

given, it is uncertain whether their various fractions are representative of the membrane as a whole.

It is the purpose of the present work to provide an experimental test of the alternate interpretations of the characteristic membrane spectra and thus clear up the existing controversy over whether the spectral distortions reflect some common aspect of membrane architecture or result from scattering effects on optical activity. It is important that this point be clarified, since optical activity has been used as evidence in support of certain membrane models and an estimate of the secondary structure of membrane proteins depends on the interpretation of the spectra.

We report the results of a series of experiments designed to test: (*a*) whether the distortions in the circular dichroism spectrum of red blood cell membranes can be removed by reducing the scattering and (*b*) whether the characteristic membrane circular dichroism spectra can be reproduced by making a non-aggregated, *nonmembranous*, helical protein act as a scatterer. Three types of experiments were performed to make the above comparisions of scattering and nonscattering systems:

(*1*) The circular dichroism of intact whole erythrocyte ghosts ($\sim$7μm diameter) was compared with that of sonicated ghosts ($\sim$0.05–0.2 μm diameter).

(*2*) The circular dichroism of hemoglobin in solution was compared with that of hemoglobin concentrated into small scattering "packets" inside partially hemolyzed, sphered red blood cells at different degrees of hemolysis.

(*3*) The circular dichroism of suspensions in silicone oil of aqueous bovine serum albumin droplets ($\sim$10 μm diameter) was compared with the solution circular dichroism of this protein. A similar comparison was also made with hemoglobin.

All conclusions drawn from these experiments concerning spectral distortions in the circular dichroism are equally applicable to the analogous distortions in the optical rotatory dispersion. When we use the term "scattering" we are generally referring to effects due to both scattering and absorption flattening.[10]

Materials and Methods. Erythrocyte ghosts were prepared from human blood by a technique of gradual successive hemolysis in progressively lower hypotonic phosphate buffer, pH 7.4. The basic cycle consisted of centrifuging the cells, aspirating off the supernatant, and resuspending in hypotonic buffer. The cells were initially washed three times in 310 mOsm phosphate buffer, followed by a repetition of the above cycle at the following osmolarities: 200, 150, 100, 80, 60, 40, 30, and 25 mOsm. The resulting ghost suspension appeared milky white and the absorption spectrum in the Soret region indicated a residual hemoglobin peptide content of less than 2% of the membrane protein. Examination in the phase microscope indicated a homogeneous sample of spherical ghosts exhibiting osmotic behavior. Sonication was performed at 4°C in a Raytheon 10-kc sonic oscillator at 0.8 amp. After 5 min of sonication, no particles were visible in the phase-contrast microscope at $\times$600 magnification. Concentration of ghost protein was determined by the method of Lowry[11] with bovine serum albumin as standard. A further reduction in particle size was obtained by centrifuging the sonicated ghosts at 30,000 *g* for 1 hr and keeping the supernatant. Lowry analysis indicated 45% of the ghost protein remained in the supernatant.

For experiments with partially hemolyzed erythrocytes, samples were removed at various stages of hemolysis and washed with phosphate buffer of the next higher tonicity until no hemoglobin was observed in the supernatant. No inhomogeneities in the hemo-

globin distribution in the cells were apparent upon examination with the phase-contrast microscope. Concentration of hemoglobin and ghost protein in the partially hemolyzed cells was estimated by absorption measurements in a Cary 15 spectrophotometer on an aliquot of the stock suspension which had been further hemolyzed by gradual dilution with twice-distilled water. The extinction coefficient of hemoglobin was taken as 131,000/mol heme at the Soret peak[12] and 9,050/mol peptide at the 192 nm peak (or 1,375,000/mol heme). A suspension of ghosts with an absorbance (A) of 1.0 at 192 nm has a concentration of 0.10 mg protein/ml as determined by Lowry[11] analysis.

Suspensions in oil of aqueous protein solutions were prepared from hemoglobin (from second hemolysate) in 50 mOsm phosphate buffer, pH 7.4, ($A_{\text{Soret}} = 1.0$) and bovine serum albumin (Pentex Corp., Kankakee, Ill.) in twice-distilled water ($A_{280} = 1.5$ aqueous phase). The oil was Dow Corning "200" fluid, a dimethyl polysiloxane that does not have appreciable absorption above 200 nm. Two per cent by volume of the aqueous protein solution was mixed with the oil phase and vigorously shaken by hand. Immediately prior to making circular dichroism measurements, the suspensions were sonicated for 30 sec at 4°C in the 10-kc Raytheon sonicator at 0.8 amp. Microscopic examination of the suspensions revealed a distribution of particle sizes centering about 4 μm diameter for the hemoglobin suspension and 10 μm diameter for the bovine serum albumin suspension.

Measurements were made on a Cary 60 spectropolarimeter equipped with a circular dichroism attachment in 1 mm path-length cells. The appropriate solvent reference base lines were run for all samples. In the case of the suspensions in oil, both the silicone oil alone and a suspension of 50 mOsm phosphate buffer (no protein) in silicone oil, prepared as in the aqueous protein suspensions, were run. Both references gave a smooth flat base line, although the aqueous suspension appeared visibly turbid. All absorbances (A) refer to 1 mm path-length cells.

Results. We report here circular dichroism spectra for the three different cases listed in the introduction, each consisting of a comparison of a partially α-helical protein in both a high- and low-scattering situation. We wish to determine whether the characteristic distortions in the circular dichroism spectrum of biological membranes can be clearly identified with optical artifacts or whether they represent the structural or local environmental characteristics of the peptide chains in the membrane.

Figure 1 shows the circular dichroism spectrum of red blood cell membranes in the normal intact state (~7 nm; curve A) and after sonication for 5 min (~0.05–0.2 nm; curve B). The spectrum of the supernatant after 1-hr centrifugation of the sonicate at 30,000 g is also shown (curve C). The scattering of the sonicated membranes as indicated by the absorbance(A) at 350 nm is reduced by a factor of 4 for the total sonicate, and 7 for the supernatant. The characteristic membrane distortions are seen to disappear upon reduction of particle size. Although the possibility of protein structural changes upon

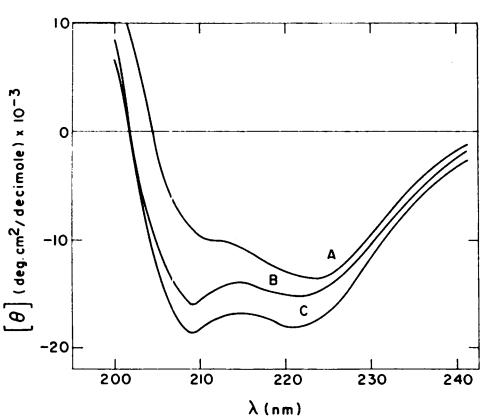

FIG. 1.—Circular dichroism of whole versus sonicated red blood cell membranes: (A) whole membranes; (B) sonicated membranes: total sonicate; (C) sonicated membranes: supernatant after 1 hr centrifugation at 30,000 × g.

sonication cannot be absolutely ruled out, the evidence in the literature (electron micrographs[13,14] and x-ray diffraction[15]) indicates that sonication of red blood cell membranes yields small vesicles of intact membranes. Furthermore, if sonication were inducing helical structural changes, one would expect the sonicated circular dichroism spectra to include a red shift in crossover and a greater increase in 222 trough than 208 trough relative to the whole membranes. The opposite is apparent in Figure 1.

Figure 2 compares the circular dichroism spectrum of hemoglobin in aqueous

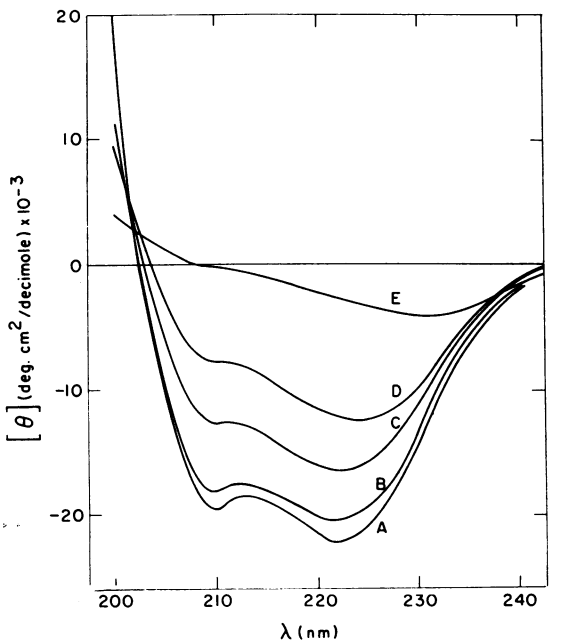

FIG. 2.—Circular dichroism of hemoglobin in aqueous solution versus hemoglobin inside of partially hemolyzed red blood cells.

(*A*) Hemoglobin solution.

(*B*) Same as (*C*) but cells further hemolyzed by dialysis against 25 mOsm P_i buffer, pH 7.4.

(*C–E*) Hemoglobin inside of partially hemolyzed red blood cells.

Ratio of hemoglobin protein/membrane protein: (*B*) ~ 9/1, (*C*) ~ 9/1, (*D*) ~ 16/1, (*E*) ~ 30/1.

solution (curve *A*) with that of hemoglobin in erythrocytes at various stages of hemolysis (curves *C–E*). The former is a molecularly dispersed, perfectly clear solution while the latter cases represent highly scattering (~7 nm) particles of concentrated hemoglobin solution in suspension. The structure of hemoglobin is the same (unaggregated) whether the solution is enclosed in the membrane or homogeneously dispersed in solution, as indicated by the x-ray diffraction studies of Bateman.[16] Several different suspensions are shown, illustrating the effect of different quantities of hemoglobin per particle. With increasing hemoglobin per cell, both scattering and flattening artifacts increase, and the circular dichroism spectrum becomes progressively more distorted, mimicking the distortions in membrane circular dichroism. Curve *D* in Figure 2, in fact, is indistinguishable from a circular dichroism spectrum of a purely membranous system. In this case, the ratio of hemoglobin to membrane protein is about 16/1, and so virtually all of the circular dichroism signal is due to the hemoglobin protein. As further evidence that the membrane itself is not responsible for the distortion, curves *B* and *C* can be compared. Curve *B* shows the same system as *C*, but the cells have been further hemolyzed by dialysis against 25 mOsm buffer. Now virtually all

the hemoglobin is dispersed in solution, and the spectrum is essentially that of the pure hemoglobin solution. The membranes are present and are scattering, but since they represent only a small percentage of the protein present, their effect on the spectrum is small.

Finally, Figure 3*a* shows a reproduction of the characteristic membrane spectral distortions for a nonaggregated, helical protein with no membranous material present at all: a suspension of aqueous bovine serum albumin droplets (~10 μm) in silicone oil; for comparison, Figure 3*b* shows the circular dichroism of the solution of BSA. Similar results are shown in Figure 4 for aqueous hemoglobin

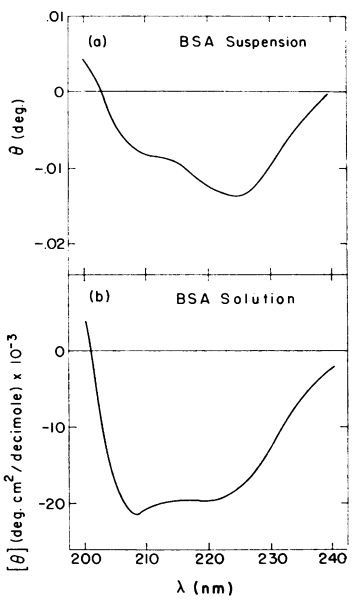

FIG. 3.

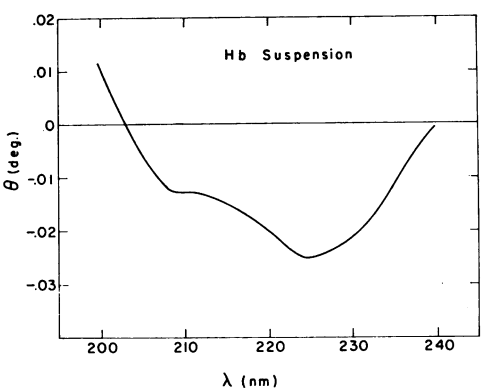

FIG. 4.

FIG. 3.—Circular dichroism of bovine serum albumin (BSA) in suspension versus solution: (*a*) 2% aqueous BSA droplets (~10μ) suspended in silicone oil, (*b*) BSA solution.

FIG. 4.—Circular dichroism of hemoglobin (Hb) suspension. 2% aqueous Hb droplets (~4μ) suspended in silicone oil.

droplets suspended in silicone oil; the circular dichroism of the hemoglobin solution was previously shown in Figure 2, curve *A*. Figures 3*a* and 4 are plotted directly in degrees. The large amount of light lost owing to scattering by the water in oil suspension (independent of circular polarization) makes a conversion to mean residue ellipticities meaningless. The typical membrane-type distortions in the shape of the spectra are apparent in Figures 3*a* and 4: low relative intensity of 210–224 nm trough and red shifts in crossover and trough wavelengths.

Discussion. These experiments demonstrate that the characteristic membrane spectrum is a superposition of a normal α-helical spectrum and an optical artifact. This has been shown for red blood cell membranes, where a normal spectrum was obtained by eliminating scattering, and for globular, nonmembranous helical proteins, where a characteristically distorted spectrum was obtained by introducing scattering. We thus conclude that the distorted helical type spectra characteristic of diverse membrane systems cannot be used as evidence in support of a common and unique membrane architecture. This is not to say

that membrane proteins are not in hydrophobic interaction with lipids or in an aggregated state; it merely says that optical activity gives no evidence on this question for particulate membranous systems.

The various controls that have been cited[1-6] to show that scattering is not responsible for the spectral distortions are inapplicable for reasons adequately discussed by Urry and Ji.[8,9] We can only add, pertaining to controls with higher refractive index media such as glycerol, that such suspending media cannot completely eliminate the scattering effect on optical activity. Part of the reason for this is that circular dichroism scattering effects result from the difference in scattering of left and right circularly polarized light, which in turn depends on the different complex refractive indices of the membrane for these two polarizations. While increasing the refractive index of the medium (e.g., with glycerol) can reduce the absolute scattering for each state of polarization, the difference in refractive index of the membrane for left and right circularly polarized light will always result in a scattering contribution to optical activity. A report is presently in preparation on a theoretical analysis of scattering effects on optical activity, including the contributions mentioned above.

Finally, recognizing that the observed membrane spectra are distorted as a result of scattering, we can use the undistorted spectrum of the sonicated ghosts to obtain a more accurate estimate of the secondary structure of the membrane protein. This will certainly be higher (by about 20%) than previous estimates based on the distorted spectra which were generally in the range of a quarter to a third α-helix. We have analyzed the circular dichroism spectrum of the sonicated ghosts (curves *B* and *C*, Fig. 1) by the method of Greenfield and Fasman,[17] by using their data on polylysine as a reference. Because of variations in the standard reference spectra, the following figures can only be considered to have an accuracy within about 10%. Our estimated average secondary structure of the membrane protein is: $\sim$50% α-helix, 45% random coil, and 0–10% beta.

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