AGGREGATION-INDUCED RED SHIFT OF THE COTTON EFFECT OF MITOCHONDRIAL STRUCTURAL PROTEIN*

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The problem of biological membrane structure has recently received new impetus from optical rotatory dispersion (ORD) and circular dichroism (CD) studies. Cotton effects arising from electronic transitions of the peptide bond in the ultraviolet have been reported for the plasma membranes of Ehrlich ascites carcinoma cells,1 human erythrocytes and Bacillus subtilis membranes,2 submitochondrial vesicles of heavy beef heart mitochondria,3 and chloroplast fragments.4 In addition, the ORD spectra of beef spinal cord myelin, rat liver submitochondrial vesicles, and the membranes of Halobacterium cutirubrum and Micrococcus lysodeikticus have been examined.5 The ORD spectra for all of these materials are remarkably similar, but as a group show unique properties which distinguish them from those of other proteins or synthetic polypeptides. They exhibit the form generally characteristic of an α-helix but differ in several important respects. The most striking deviation is a shift of the entire Cotton effect toward longer wavelengths; i.e., the troughs appear at 235–237 μ rather than the usual 233 μ, the peaks are at 200–204 μ instead of 198–199 μ, and the crossovers also occur at comparably higher wavelengths. These experiments were carried out at physiological pH. Detergents such as sodium dodecyl sulfate or lyssolecithin cause the red shift to vanish, and the ORD curves assume the wavelength parameters characteristic of the usual α-helix. A secondary feature is that although in some cases the shape of the Cotton effect is that of a fully helical polypeptide, the rotational amplitude is too low and cannot be accounted for by the usual combinations of α-helix and random coil.

In view of their appearance in a wide variety of membrane systems, these red-shifted curves must represent some common structural feature shared by all. Several interpretations have been offered. Wallach and Zahrle1 suggested hydrophobic interaction of helical protein segments with lipid fatty acid hydrocarbons, Lenard and Singer2 proposed interaction of adjacent protein helices, and Urry, Mednieks, and Bejnarowicz3 interpreted the curves as the sum of lipid and protein rotations.

In this communication we will show that the red shift can be explained in terms of the protein portion of the membrane rather than by the lipid or the combination of lipid with protein. We have found that in the absence of lipid the aggregation of structural protein from mitochondria results in a red shift as well as the decreased rotational amplitude characteristic of membranes. Furthermore, this red-shifted ORD spectrum can be reversibly converted to that typical of an α-helix.

Experimental.—Materials: The isolation of heavy beef heart mitochondria,4 and the preparation of submitochondrial vesicles2 from them was as previously described elsewhere. Mitochondrial structural protein (MSP) was prepared from heavy beef heart mitochondria according to Richardson, Hultin, and Fleischer.8 The phosphorus content was 0.3 μg P/mg protein for the MSP and 18.4 μg P/mg protein for

1292
the sub mitochondrial vesicles. Phosphorus determinations\(^9\) and Folin protein measurements\(^8\) followed established procedures. The Folin calibration curve was run on mitochondria which had been standardized against bovine serum albumin by the biuret procedure.\(^{11}\) Poly-L-glutamic acid (Pilot Chemicals, lot G-83) was determined by a micro-Kjeldahl method.\(^{12}\) For detergent studies, recrystallized sodium dodecyl sulfate and chromatographically homogeneous lysolecithin (Pierce Chemical Co., Rockford, Ill.) were used.

**Optical rotatory dispersion:** For ORD studies, MSP was suspended overnight in cold distilled water at pH 11.8 (KOH). After clarification by centrifugation at 5000 \(\times\) \(g\) for 45 minutes, the protein was diluted 25-fold with distilled water to a concentration of 0.156 mg/ml and stored in the refrigerator at pH 11.5. Daily checks of ORD trough depth and position of this stock solution showed no change during the course of the studies. For each ORD run, a sample of the solution was adjusted in a nitrogen atmosphere to the proper pH with dilute solutions of HCl or KOH; the pH was measured before and after the ORD spectrum was taken and never changed by more than 0.1 pH unit. Reduced mean residue rotations were calculated using a mean residue weight of 115 and refractive indices for water at appropriate wavelengths.\(^{13}\) The dispersion spectra of sub mitochondrial vesicles were taken in 10 mM Tris-HCl containing 1 mM ethylenediaminetetraacetate (EDTA), at pH 8.0.

All spectra were obtained on a Cary 60 spectropolarimeter at room temperature with a 1-cm cell from 350 \(\mu\)m to about 215 \(\mu\)m and a 1-mm cell from about 250 \(\mu\)m to about 190 \(\mu\)m. Occasionally, spectra were taken to below 200 \(\mu\)m in both cells. Baselines were programed with water and were recorded before and after the protein measurements. The effect of light scattering was determined from dispersion curves of polyglutamic acid at pH 4.2 with and without added colloidal silica gel as a scatterer. Scattering increased the noise level but trough position and depth were unchanged. The insensitivity of the Cary 60 to light scattering has been verified by other workers.\(^1\)\(^2\)

**Optical density:** Measurements were made at 340 \(\mu\)m with a Cary 14 spectrophotometer on the MSP solutions directly in the 1-cm ORD cell before and after ORD measurements. Negligible changes in optical densities occurred during the ORD determinations. Scans of optically clear MSP at pH 11 showed no absorption at 340 \(\mu\)m. Exponential scattering curves were obtained from the opalescent solutions at other pH values.

**Results.**—MSP dissolves in alkali or acid (pH \(\geq\) 11 and \(\geq\) 3), and at these extremes of pH the optically clear solutions yield no red shift in the ORD spectra. The trough and peak positions are 232.5 \(\mu\)m and 199 \(\mu\)m, as expected for the \(\alpha\)-helix. The crossover is 219 \(\mu\)m. As the pH is lowered

FIG. 1.—The ORD of mitochondrial structural protein in the disaggregated state at pH 11 and the aggregated state at pH 9.
from 11 or raised from 3 the turbidity increases, and the trough, peak, and crossover red-shift. Solution turbidity as well as the magnitude of the shift progressively increase as neutrality is approached and become greatest at about pH 7.5, when the trough is at 239 m\(\mu\). A decrease in rotational amplitude accompanies the red shift. ORD spectra of MSP at pH 11.0 and 9.0 are shown in Figure 1. The curve for MSP at pH 11 resembles that of an \(\alpha\)-helix with low rotational amplitude. However, the peak-to-trough ratio is considerably less than that of polyglutamic acid so that the curve might be accounted for in terms of the usual considerations of \(\alpha\)-helical and random coil contributions. At pH 9 the spectrum is red-shifted and both peak and trough are reduced in magnitude when compared to the spectrum at high pH.

Similar results are obtained at other pH values, and are summarized in Table 1. The major point to be noted is the simultaneous and progressive effect of pH on the optical density and on the position and amplitude of the Cotton effect. The striking correlation between turbidity, trough position, and trough depth is shown in Figure 2. Similar curves are obtained from peak and crossover positions. As the pH approaches neutrality from either extreme, MSP solutions become increasingly opalescent. In the regions of opalescence (9 \(\gtrsim\) pH \(\gtrsim\) 11 and 3 \(\lessgtr\) pH \(\lessgtr\) 6) the solutions visually resemble virus preparations. The turbidity remains constant for days, and ORD spectra can be taken without difficulty. Within the pH range of \(\sim 6\) to \(\sim 9\), the protein aggregates into visible particles and precipitates. Although optical rotation could not be measured on the flocculant precipitate in water, red-shifted spectra were obtained from the precipitate suspended in 90 per cent glycerol. The insensitivity of the red shift to high glycerol concentrations has been reported. However, even in water, occasionally flocculation did not occur immediately after the pH was adjusted, and we could obtain partial spectra in the region of pH 6 to pH 9. By examining MSP before exposure to extremes of pH, we excluded the possibility that the red shift is an artifact obtained only after first dissolving the protein. Enough protein could be dispersed by sonication into 90 per cent glycerol to produce a spectrum, whose trough appeared at 239–240 m\(\mu\).

In the presence of 0.1 per cent sodium dodecyl sulfate or lysolecithin, MSP solutions remain optically clear and the Cotton effect does not shift to the red over the entire pH range of 3–11. Similarly, the addition of SDS or lysolecithin to flocculated

### Table 1

<table>
<thead>
<tr>
<th>Preparation</th>
<th>pH</th>
<th>(\lambda_{\text{max}})</th>
<th>(\lambda_{\text{min}})</th>
<th>(\lambda_{\text{crossover}})</th>
<th>(\gamma_{\text{max}})</th>
<th>([m']_{\text{min}})</th>
<th>([m']_{\text{max}})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Structural protein</td>
<td>11.0</td>
<td>0.03</td>
<td>232.5</td>
<td>219</td>
<td>199</td>
<td>-4.1</td>
<td>+13.9</td>
</tr>
<tr>
<td>&quot;</td>
<td>10.0</td>
<td>0.06</td>
<td>233.5</td>
<td>221</td>
<td>201</td>
<td>-3.7</td>
<td>+12.9</td>
</tr>
<tr>
<td>&quot;</td>
<td>9.4</td>
<td>0.17</td>
<td>235</td>
<td>223</td>
<td>204</td>
<td>-3.3</td>
<td>+10.0</td>
</tr>
<tr>
<td>&quot;</td>
<td>9.0</td>
<td>0.34</td>
<td>237</td>
<td>225</td>
<td>205</td>
<td>-3.1</td>
<td>+10.1</td>
</tr>
<tr>
<td>&quot;</td>
<td>7.5</td>
<td>0.58</td>
<td>239</td>
<td>226</td>
<td>*</td>
<td>-2.9</td>
<td>*</td>
</tr>
<tr>
<td>&quot;</td>
<td>6.5</td>
<td>0.20</td>
<td>235</td>
<td>*</td>
<td>*</td>
<td>-3.0</td>
<td>*</td>
</tr>
<tr>
<td>&quot;</td>
<td>5.9</td>
<td>0.16</td>
<td>234</td>
<td>223</td>
<td>*</td>
<td>-3.7</td>
<td>*</td>
</tr>
<tr>
<td>&quot;</td>
<td>2.4</td>
<td>0.01</td>
<td>232.5</td>
<td>219</td>
<td>199</td>
<td>-3.8</td>
<td>+11.4</td>
</tr>
<tr>
<td>Structural protein + 0.1% SDS</td>
<td>7.7</td>
<td>—</td>
<td>232.5</td>
<td>220</td>
<td>199</td>
<td>-6.7</td>
<td>+25.2</td>
</tr>
<tr>
<td>Intact mitochondria</td>
<td>8.0</td>
<td>—</td>
<td>237</td>
<td>226</td>
<td>190</td>
<td>-3.4</td>
<td>+13.1</td>
</tr>
<tr>
<td>Mitochondria + 0.1% SDS</td>
<td>8.0</td>
<td>—</td>
<td>233</td>
<td>221</td>
<td>199</td>
<td>-4.1</td>
<td>+14.7</td>
</tr>
<tr>
<td>Polyglutamic acid</td>
<td>4.2</td>
<td>—</td>
<td>233</td>
<td>222</td>
<td>199</td>
<td>-15.5</td>
<td>+68.2</td>
</tr>
</tbody>
</table>

Wavelength precision about 0.5 m\(\mu\); \([m']_{\text{min}}\) are average values for 2–3 measurements at each pH; \([m']_{\text{max}}\) values were measured only once.

* Determination prevented by flocculation and precipitation.
MSP at pH 7.5 produces the same result: the red-shifted Cotton effect reverts to the wavelength parameters shown by lipid-free MSP at pH 11 or pH 3.

Discussion.—The red shift observed in mitochondrial membranes may be accounted for in terms of MSP alone, without invoking lipid-protein interaction. Concurrent changes in ORD parameters and light scattering as measured by absorbance indicate protein-protein interaction with an accompanying aggregation-induced shift of the conventional \( \alpha \)-helical Cotton effect to higher wavelengths. The displacement is greatest over the physiological pH range, and is sufficient to account for the spectral shift observed with mitochondrial membranes. At extremes of pH the protein is disaggregated, perhaps monomolecularly, but as the pH approaches neutrality and the solution becomes opalescent, aggregation and the magnitude of the shift increase. Finally, the protein flocculates and precipitates, but the Cotton effect of the precipitated material is still red-shifted.

Although the mechanisms responsible for aggregation and for the perturbation of the ORD spectra will require elucidation, other materials have been reported which exhibit red-shifted spectra under conditions that promote aggregation. Cassim and Yang\(^4\) reported that the ORD and CD spectra of aggregated helices of poly-L-glutamic acid (PGA) at pH 3.8 are displaced toward the red by about 1 m\(\mu\). The polyornithine–sodium dodecyl sulfate system studied by Gourke and Gibbs\(^5\) may have particular relevance to membranes. Addition of SDS to poly-L-ornithine at pH 7 results in turbidity and a transition from the random coil form of the polymer to an \( \alpha \)-helix with a trough at 235 m\(\mu\) and a peak at 202 m\(\mu\). These systems are poorly understood, but it is likely that similar mechanisms are responsible for perturbation of the spectra of both the synthetic polymers and MSP. The amino acid
sequence of MSP may be such that an interaction between helices, and the resultant red shift, is greater than in the case of synthetic polypeptides.

There are indications that significant differences may occur between red-shifted Cotton effects in the ORD spectra of polypeptides and those of membranes. The PGA system shows increased rotatory strength upon aggregation while aggregation of MSP produces a decrease in rotation. ORD spectra of membranes also appear to exhibit low rotational amplitude based upon total protein content. However, in some particulate systems such as membranes, and especially in intact organelles, calculations of rotation based upon total protein concentration may not be realistic. It must be concluded that, although the red shift is an established property of some aggregated systems, more investigation will be required to define the changes in rotatory strength which accompany the displacements in trough position. Such rotational changes might arise, for example, from changes in polarizability in the neighborhood of the peptide chromophores or from field effects from neighboring helices. We wish to emphasize, however, that the aggregation of MSP results in a decrease in amplitude of the Cotton effect similar to that observed in membranes. The behavior of MSP emphasizes the point made by Cassim and Yang,\textsuperscript{14} that studies of the fine structure of ORD and CD spectra represent a very powerful approach to the investigation of macromolecular interaction.

The presence of considerable $\alpha$-helicity in MSP is significant in the context of current membrane models which have not considered helical arrangement of the protein of the membrane.\textsuperscript{16, 17} Furthermore, the protein helicity is remarkably stable, since the minimum of the Cotton effect is nearly the same at pH's 11.5 and 2.3 and does not change after one week at pH 11.5.

The calculation of percentage helicity from ORD data is difficult. In this case, the difficulty is increased by the uncertainty in experimental values for rotatory amplitude, as pointed out above, as well as by the usual uncertainties in the choice of $[m']_{20}$ for a completely $\alpha$-helical polypeptide.\textsuperscript{18} In fact, if the ORD curve for membranes really is that of a miniaturized $\alpha$-helix, as suggested by some workers,\textsuperscript{1}\textsuperscript{,} they a serious additional uncertainty is introduced into the selection of the proper reference value for trough depth. Calculations based on trough depth assuming a mixture of $\alpha$-helix and random coil\textsuperscript{19} estimate the helical content of both MSP and submitochondrial vesicles at less than one-fourth. If incorrect, this value is likely to be an underestimate. It should be noted that in spite of uncertainties in absolute helical content the helicity in the membrane may be reasonably accounted for in terms of MSP.

The available data are consistent with assigning the red shift in mitochondrial vesicles, as well as in MSP itself, to aggregated structural protein. All of the attributes of the optical behavior of membranes, i.e., the displaced $\alpha$-helical spectrum, the reduced rotatory strength, and the response to detergents, are mimicked by the lipid-free system. The perturbation of the dispersion curves indicate involvement of $\alpha$-helical portions of MSP in the aggregation process, perhaps as side-by-side parallel arrays.\textsuperscript{14} Such aggregates of helices would also be present in membranes. A similar model has been proposed by Lenard and Singer.\textsuperscript{2} One might anticipate that lipid-free structural proteins from other membranes would show similar red shifts upon aggregation. It is possible that the wide range of stability of membrane systems may be explained principally in terms of the requirements for aggregation of
structural proteins. An extreme example would be \textit{H. halobium}, which requires a very high salt concentration for membrane integrity. If these ideas are correct, the addition of detergent to membrane preparations, or simply the dilution of the extreme halophiles to low ionic strength, causes the desaggregation of structural protein. Clearly, detergents exert a profound effect upon membrane protein as well as upon lipid.

Experimental evidence is insufficient to define the location and orientation of the helical protein arrays in the membrane, but the presence of lipid in membranes must impart organization which is absent in the lipid-free system. Lipid-free structural protein exists aggregated at \textit{pH} 7 in a three-dimensional amorphous precipitate, but in membranes phospholipids constrain the aggregation to a plane. The degree of organization which is responsible for the red shift is thus of a simpler level than that of the more-ordered trilaminar structure. Yet there is appreciable organization retained in membranes and constituent proteins in the absence of lipid. The trilaminar arrangement is retained by mitochondrial inner membranes after lipid extraction. The red shift, likewise, is a property of MSP which contains no lipid. Since enzyme function (electron transport) as well as spatial constraints upon aggregation is dependent upon lipid, the role of lipid is on a more subtle level than has been discerned by either electron microscopy or ORD. It is important to note that MSP has recently been converted into membranous vesicles in the presence of mitochondrial phospholipid.

The existence of helical proteins in membranes is not consistent with the usual bilayer model which assumes unfolded polypeptide chains bonded to lipids by polar interaction. The basic features of the bilayer model might be retained by replacing some of the unfolded protein with helices oriented in a two-dimensional array parallel to the membrane surface, but one experiences difficulty in visualizing a conventional bilayer system which takes full advantage of hydrophobic protein-protein interactions. Moreover, binding studies strongly suggest that the interaction between MSP and phospholipid is largely hydrophobic. These considerations would require a more drastic revision of the bilayer model which would allow nonpolar interaction between fatty acid hydrocarbon residues and protein. Such interactions would be favored by aggregated ensembles of helices traversing the membrane with their axes normal to the surface of the membrane. Physiologically uninteresting regions between helices could be occupied by phospholipids, perhaps partly in the bilayer conformation.

Summary.—The ORD spectrum of aggregated lipid-free mitochondrial structural protein shows a red-shifted \(\alpha\)-helical Cotton effect very similar to those obtained from intact mitochondrial vesicles and other membrane systems. At extremes of \textit{pH}, where the protein is disaggregated, the trough and peak positions are 232.5 m\(\mu\) and 199 m\(\mu\), typical for the \(\alpha\)-helix. As the \textit{pH} is lowered from about 11 or raised from about 3, turbidity progressively increases and the trough, crossover, and peak become increasingly shifted toward longer wavelengths. The shift is accompanied by a decrease in rotational amplitude. These aggregation-induced perturbations of the \(\alpha\)-helical spectrum are maximal at about \textit{pH} 7.5, where the trough occurs at 239 m\(\mu\), and the rotatory strength is reduced to about 75 per cent of the value for aggregated protein. The behavior is a reversible function of \textit{pH}. Sodium dodecyl sulfate and lysolecithin prevent both aggregation and the red shift.
All of these phenomena, i.e., the red shift, the decrease in rotatory strength, and the response to detergent, mimic the ORD spectra of membrane preparations. It is reasonable to assign the red shift in mitochondrial vesicles and intact mitochondria, as well as in all other membranes, to aggregated helices of structural protein.

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