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Absorption Flattening in the Circular Dichroism Spectra of Small Membrane Fragments†

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ABSTRACT: The inhomogeneous distribution of chromophore occurring in a particulate suspension can result in a reduction in the apparent molar ellipticity recorded in circular dichroism (CD) spectra. The possibility of such a systematic error has often been a matter of concern when CD spectra of cell membrane proteins are recorded. The recent publication of CD spectra for bacteriorhodopsin in native and sonicated membranes, in detergent-solubilized form, and reconstituted into small unilamellar vesicles [Mao, D., & Wallace, B. A. (1984) *Biochemistry 23*, 2667-2673] gives a unique opportunity to apply the theoretical analysis of Gordon and Holzwarth [Gordon, D. J., & Holzwarth, G. (1971) *Arch. Biochem. Biophys. 142*, 481-488] so as to provide a definitive answer to the question of whether absorption flattening is significant for membrane particles. We show here that the data of Mao and Wallace can be combined with the theoretical analysis of Gordon and Holzwarth to rule out significant absorption flattening effects over the range 200-240 nm for submicrometer-sized membranes. In addition, the results show that absorption flattening can be disregarded even at 190 nm for membranous material in the size range below 100 nm. The demonstration that there are no major flattening effects in the CD spectra of bacteriorhodopsin, particularly in the region of 200-240 nm, means that the experimental spectra are incompatible with the proposal that this transmembrane protein contains seven transmembrane helices.

It has been proposed recently by Mao & Wallace (1984) that incorporation of membrane proteins into small unilamellar vesicles (SUVs) can provide a way to avoid certain errors in estimating secondary structure content from circular dichroism (CD) measurements. The number of protein molecules per vesicle can be made so small that the suspension must approach the homogeneous distribution of absorbers required in the derivation of Beer's law and the absorption flattening effect must theoretically vanish. The introduction of SUVs therefore represents a clever addition to the range of techniques that are available for spectroscopic studies of membrane proteins.

Mao and Wallace have also suggested that absorption flattening causes a significant distortion in the CD spectrum of both native and sonicated purple membrane and that the flattening effect causes a large error in estimating the α-helix content of the constituent protein, bacteriorhodopsin. We show here that this suggestion is actually inconsistent with the experimental data that the authors themselves present (Mao & Wallace, 1984). Furthermore, the suggestion that absorption flattening could cause significant distortions in the CD spectra of submicrometer-sized membrane sheets is inconsistent with both the theoretical analysis and the empirical estimation published by Gordon & Holzwarth (1971).

The accurate measurement of the molar amino acid concentration is essential in order to make an accurate comparison between the molar ellipticity of a specimen of unknown structure and that of the reference structures. Mao and Wallace introduced instead a procedure of spectral renormalization citing, as their justification, the difficulty of determining the concentration of membrane proteins. The renormalization procedure is not an adequate substitute for accurate biochemical determinations, however.

While absorption flattening effects can safely be dismissed when specimens are prepared as SUVs, the spectral curves obtained by Mao and Wallace show that more subtle shifts in spectroscopic wave form can still be produced in such

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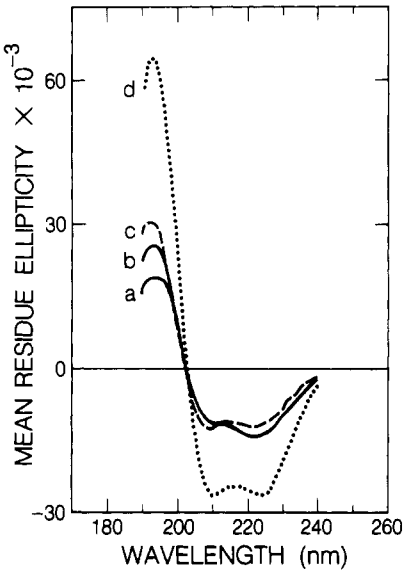

FIGURE 1: CD spectra of bacteriorhodopsin redrawn in simplified form from Mao & Wallace (1984). Curves a and b represent the spectra obtained with native purple membrane and with sonicated purple membrane and bR solubilized in octyl glucoside, respectively; these three spectra are very similar between 200 and 240 nm and are shown here as a single curve, but the curve for native membrane (a) is significantly lower at 190 nm than the average value of the curves for sonicated purple membrane or octyl glucoside solubilized bR (b). Curve c is the spectrum of bR incorporated into SUVs, and it shows small differences from curve b over the whole range of wavelengths. Curve d is the theoretical spectrum calculated by Mao and Wallace for a protein containing 80% α-helix, 10% β-turn, and 10% random-coil.

specimens. These shifts do not alter the estimated helix content by a substantial amount, but they probably do reflect a real change in the chiral structure of nonhelical regions of the peptide chain. The use of SUVs to measure CD spectra may thus be subject to errors in estimating nonhelical components of the secondary structure that are similar to the errors that can occur with the use of detergent-solubilized membrane proteins.

The points mentioned above have significant implications as far as the estimation of the helix content and other secondary structure features of bacteriorhodopsin (bR) are concerned. We point out that the data of Mao and Wallace confirm earlier measurements (Becher & Cassim, 1976; Jap et al., 1983) showing that the mean residue ellipticity of bR in native purple membrane at 221 nm is approximately −15 000 deg cm^2 dmol^{-1}, and therefore, the helix content of this protein is predicted to be only about 50%. This experimental estimate is much lower than the figure of 70–80% required if there were seven transmembrane helices. The data of Mao and Wallace also confirm an earlier observation (Jap et al., 1983) that bR is sensitive to environmentally induced changes in the chiral conformation of nonhelical regions of the peptide chain.

Evaluation of the Absorption Flattening Effect. The CD spectra between 200 and 240 nm presented by Mao and Wallace for native purple membrane, sonicated purple membrane, and bR solubilized in octyl glucoside are nearly identical, as can be seen from the published curves as they are redrawn in Figure 1. The spectrum obtained for bR reconstituted into SUVs shows a shift in CD "intensity" from 221 to 208 nm. There also is hardly any difference in the peak values of the mean residue ellipticity near 193 nm for SUVs, sonicated membranes, and detergent-solubilized bR. The ellipticity of the native membrane patches at 193 nm is about 0.75 times that of the other types of specimen preparation, indicating that absorption flattening is significant for intact purple membrane sheets at the shorter wavelengths.[1] The ratio of "true" solution absorbance to the average number of particles per optical path [cf. Gordon & Holzwarth (1971)] has its most extreme value at 190 nm, and thus, the absorption flattening effect cannot be any greater at longer wavelengths than it is at 193 nm. In addition, it is to be noted that the molar ellipticity of bR in SUVs at 221 nm is actually less than that in native purple membrane, an effect that is opposite to any effect that could be caused by absorption flattening.

The experimental data presented by Mao and Wallace are therefore inconsistent with their suggestion that there is a significant absorption flattening effect in the CD spectra over the range 200–240 nm. The data of Mao and Wallace do show, however, that there can be a significant absorption flattening effect at 193 nm for native membranes. The latter effect can be avoided by the use of sonicated membrane pieces or by the use of detergent-solubilized proteins. While Mao and Wallace suggest that octyl glucoside might cause a change in bR conformation, this suggestion is not consistent with the fact that the CD spectrum of octyl glucoside solubilized bR is nearly identical with that of sonicated membrane sheets.

The theoretical analysis of Gordon and Holzwarth can be used to show quantitatively that the spectra obtained by Mao and Wallace with native and sonicated purple membranes are not consistent with there being a significant amount of absorption flattening at 221 nm. Mao and Wallace calculate that the mean residue ellipticity of bR at 221 nm would be about −28 000 deg cm^2 dmol^{-1} if the helix content were as high as 80%. The measured ellipticity of sonicated purple membrane patches is only −14 000 deg cm^2 dmol^{-1}, however, which would require that the CD be flattened by a factor Q_B (notation of Gordon and Holzwarth) equal to 0.5. This degree of flattening corresponds, for the model of spherical shells, to a value of $A_{sol}/qm = 0.5$, where in the notation of Gordon and Holzwarth A_{sol} is the absorbance of an ideal solution of protein and qm is the average number of absorbing particles per centimeter. The value of qm must increase as $N^{1/3}$ where N is the total number of particles per cubic centimeter. N, in turn, must be inversely proportional to the area of the membrane patches since the total amount of membrane area is conserved upon sonication. taking the size of native membranes to be, conservatively, no smaller than 2500 Å and the size of the sonicated membranes to be 250 Å (as reported by Mao and Wallace), the value of A_{sol}/qm of the native membrane suspension ought to be equal to 2.3. According to Figure 3 of Gordon and Holzwarth, Q_B would then be less than 0.1 for the native membranes, i.e., 5 times smaller than for the sonicated membranes. Experimentally, the molar ellipticity of native purple membranes is hardly distinguishable from that of sonicated membranes at 221 nm, implying that there must be very little absorption flattening in either case at that wavelength.

That membrane patches in the submicrometer size range should have very little absorption flattening is further supported by the fact that Gordon and Holzwarth give an empirical estimate of $Q_B = 0.7$ for red blood cell ghosts at 221 nm. This value corresponds to $A_{sol}/qm = 0.21$. Assuming that the ghosts

[1] A referee has pointed out that the ratio of CD amplitude at 190 nm to that at 223 nm is very low in the native purple membrane spectrum of Mao and Wallace, compared to that of spectra reported by others [see, for example, Jap et al. (1983)]. This fact suggests that the samples used by Mao and Wallace probably suffer from some degree of aggregation. Thus, the absorption flattening that is evident in their spectra at 190 nm, although relatively small in magnitude, is substantially greater than that which can be expected in nonaggregated specimens.

have a size equal to 5 μm, one finds that A_{sol}/qm should be no larger than 0.045 for vesicles that are smaller than 0.5 μm. The corresponding value of Q_B for submicrometer pieces of membrane is only about 0.9.

Errors Inherent in "Renormalization". The value of α-helix content can generally be obtained very accurately by curve fitting, particularly for proteins with substantial α-helix content, if a reference spectrum for the appropriate length of helices is used (Chen et al., 1974; Chang et al., 1978; Siegel et al., 1980). Indeed, the α-helix content can usually be calculated quite accurately just from the molar ellipticity at 221 nm, since β-sheet, random-coil, and β-turn peptide conformations make rather small contributions to the ellipticity at long wavelengths (Chen et al., 1974; Brahms & Brahms, 1980). The estimated α-heix content does not seem to change by more than a few percent if an unconstrained curve fitting is used rather than a constrained fitting (Chang et al., 1978). This is true even when the sum of secondary structures varies from 0.58 to 1.76. The fraction of other conformations cannot be determined accurately from CD spectra, however, presumably because their chirality and thus their CD spectra are not so rigidly determined as is the case for the α-helix.

Renormalization of the fractional sum of secondary structure can frequently cause a large error in the estimated helix content, however, when the sum of (unconstrained) fractions of secondary structure is significantly different from unity. This point is readily seen by inspection of the results shown in Table I of Chang et al. (1978). It has been argued, however, that renormalization of unconstrained fractions is a reasonable procedure when the sum differs by only ±10% from unity, since a ±10% error in the CD "intensity" is within the acceptable range of the various possible experimental errors (Hennessey & Johnson, 1982).

Renormalization can never be used as a justification for not determining the protein concentration accurately, however. The estimation of secondary structure by CD is only as good as the direct measurement of the amino acid concentration of the sample. If errors in protein concentration are suspected of causing the "unconstrained" sum to differ from 1.0 by 20% or more, for example, then the correct procedure should be to redetermine the protein concentration rather than to assume that renormalization will make things right.

The protein content in SUVs was measured by Mao and Wallace by applying the Lowry method in the presence of 0.1% sodium dodecyl sulfate (SDS) using native purple membrane as a standard. Mao and Wallace express reservations that the phospholipids of the SUVs might cause an interference in the Lowry assay, in which case it would have been appropriate to add a similar amount of phospholipid vesicles to the purple membrane standard to correct for the interference. Other methods could also have been used to accurately determine the concentration of bR in SUVs, such as radioactive labeling of a suspension of purple membrane of known concentration, prior to incorporation of bR into SUVs, or measurement of the absorption spectrum in the visible region even though the spectrum of bR in SUVs is distorted relative to that in native membranes (Lozier et al., 1976). We believe, however, that the method of determining protein concentration used by Mao and Wallace for bR in SUVs must have been quite accurate since the value of ellipiticy at 221 nm, where absorption flattening is negligible, is quite close to that of both native and sonicated purple membranes.

Comparison of the Detergent Solubilization Method and the SUV Method. Solubilization of membrane proteins prior to reconstitution into SUVs requires the use of detergents. Detergent solubilization should not cause a major change in the secondary structure of a membrane protein provided that care is taken to use a detergent which retains full functional activity of the protein when measured following reconstitution, or while still in detergent, if possible. In the case of bR, the relatively small size of observed spectroscopic shifts in the visible spectrum (see below) and the lack of bleaching, under careful conditions of detergent solubilization with octyl glucoside or with Triton, suggest to us that there can be very little shift in the tertiary structure, let alone a change in helix content while in these detergents. Absorption flattening must vanish in the case of detergent-solubilized proteins, as it does for SUVs. The recording of CD spectra of detergent-solubilized membrane proteins can therefore be used in many cases. Still, the comparison of spectra obtained with SUVs is clearly a prudent and worthwhile additional step.

Mao and Wallace have argued, contrary to the view we have expressed above, that the helix content of bR solubilized in octyl glucoside may be substantially smaller than that of the native protein. They cite the fact that the visible absorption spectrum is shifted in detergent-solubilized bR as evidence that the protein structure is indeed altered. If their argument is accepted, however, one would have to conclude that the same pitfall must apply to bR in SUVs, since the absorption maximum shifts from 568 nm for bR in native membranes to 555 nm for bR in SUVs (Lozier et al., 1976) and to 553 nm for bR in octyl glucoside (Dencher & Heyn, 1978). However, when one considers that the absorption maximum shifts to 412 nm in the M intermediate of the normal photocycle, it seems most unlikely that the much smaller shifts seen in detergent or in SUVs could correspond to a measurable change in the helix content.

Incorporation of bR into SUVs apparently does not guarantee that the detailed wave form of the CD spectrum will be identical with that of the native membrane. The wave form of bR in SUVs is virtually identical with that reported by Jap et al. (1983) for Triton-solubilized bR and is noticeably different from that of octyl glucoside solubilized bR, that of sonicated purple membrane, or that of papain-cleaved, Triton-solubilized bR (Jap et al., 1983). It seems likely, therefore, that some nonhelical segments of the peptide chain adopt a chiral conformation that is quite similar in the case of Triton-solubilized bR and of SUVs, but which is quite different in the other cases cited.

Implication with Regard to the Secondary Structure of bR. We believe that the arguments presented above show that absorption flattening has no significant effect at 221 nm in any of the spectra presented by Mao and Wallace and that the renormalization procedure used by them with the sample of SUVs is unjustified and erroneous. If these two points are correct, then the data of Mao and Wallace confirm earlier estimates (Becher & Cassim, 1976; Jap et al., 1983) that the helix content of bR represents only about 50% of the peptide chain. If the helix content is as little as 50%, then there can only be a maximum of five transmembrane α-helices as opposed to the seven helices predicted from electron microscopy. We will not repeat here the evidence which has been presented (Jap et al., 1983; Glaeser & Jap, 1984) in support of a structural model that would contain five helices and four strands of β-sheet. We believe that it is only important to stress two points: (1) the experimental CD spectra of bR differ greatly in amplitude from the theoretical spectrum of a protein containing seven helices, each of which is 30 Å or greater in length; and (2) light scattering and absorption flattening do not produce experimentally significant distortions of the CD

spectra of native purple membranes, particularly in the region 200–240 nm, and therefore they are unable to explain the discrepancy between experimental results and the seven-helix model.

Registry No. Octyl glucoside, 29836-26-8.

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Nanosecond Fluorescence Studies of Noncovalent Interaction of Monomeric and Dimeric Intercalators with DNA

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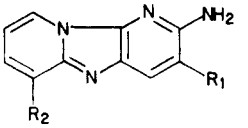
ABSTRACT: The noncovalent interaction of 2-aminodipyrido[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-2) and its derivatives, which are potent mutagens isolated from L-glutamic acid pyrolysate, with calf thymus DNA was studied by steady-state and nanosecond fluorescence spectroscopies. The fluorescence of these compounds exhibits static quenching by noncovalent interaction with DNA. Fluorescence lifetimes of the free and intercalated states of these compounds were determined to be 9–10 and 0.5–1 ns, respectively. The bis-intercalative effect of the dimeric analogue of Glu-P-2, bis(Glu-P-2)spermine (2GP-SP), to DNA was also investigated. This 2GP-SP, which has two Glu-P-2 moieties at each end of spermine, indicates a strong intramolecular interaction exhibiting remarkable quenching of fluorescence spectrum and lifetime (τ = 3.5 ns) in the absence of DNA. In the presence of DNA, however, the 3.5-ns lifetime component of fluorescence disappeared, and a two-exponential decay of fluorescence (t = ~10 and 1.5 ns) was observed at a DNA concentration of more than ~0.001 mM P, while the solution containing a very dilute DNA concentration (≤0.001 mM P) exhibits a three-component decay of fluorescence (1.5, 3.5, and ~10 ns). The potent bis intercalation of two moieties in 2GP-SP with an identical DNA molecule was suggested by the DNA-concentration dependence of these fluorescence lifetimes and their intensity.

The potent mutagens 2-amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-1) and 2-aminodipyrido[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-2), which were isolated from a



Glu-P-2, R_1 = H; R_2 = H
Glu-P-1, R_1 = H; R_2 = CH_3
3-Me-Glu-P-2, R_1 = CH_3; R_2 = H

pyrolysate of L-glutamic acid, (Yamamoto et al., 1978; Takeda et al., 1978), are metabolically active and react covalently with DNA (Hashimoto et al., 1979, 1980). In this case, the intercalation of these compounds with DNA was reported to be essential for the covalent reaction. The physicochemical interaction of these compounds with DNA has been investigated by absorption and fluorescence spectroscopies (Imamura et al., 1980a,b). Further, flow dichromism study suggested that Glu-P-1 and other derivatives are oriented parallel to the planes of base pairs of DNA (Imamura et al., 1980a,b). On the other hand, in some potent antitumor antibiotics such as echinomycin (Dell et al., 1975), luzopeptin (Arnold & Clardy, 1981), and carzinophilin (Terawaki & Greenberg, 1966) containing two heteroaromatic chromophores, bis intercalation of these two chromophores with DNA has been suggested by several kinds of analytical methods. In order to develop potential chemotherapeutic drugs, DNA polyintercalating agents such as bis(acridines) (King et al., 1982) and bis(methidium)sperimine (Dervan & Becker, 1978) have been studied by sedimentation and viscometric analysis and UV, fluorescence, and CD spectroscopies. The underlying principal of these investigations is that bis intercalation may afford the opportunity for improving both nucleotide-sequence selectivity and specificity for DNA. Therefore, dimeric analogues of intercalative Glu-P-2, bis(Glu-P-2)spermine (2GP-SP) and its analogues, were

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