EVIDENCE FOR CHROMOPHORE-CHROMOPHORE (EXCITON) INTERACTION IN THE PURPLE MEMBRANE OF HALOBACTERIUM HALOBIUM

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SUMMARY: The visible circular dichroic spectrum of the purple membrane of H. halobium includes intense positive and negative bands with cross-over near the wavelength of the absorption maximum. We report here results that show that the circular dichroic spectrum has two contributions: a positive band due to retinal bound to the protein, and positive and negative bands due to the interaction of the chromophores of separate proteins. Bleached purple membrane was regenerated by successive additions of retinal. The shape of the circular dichroic spectrum was dependent on the percent of the retinal binding sites occupied. This dependence strongly suggests that the circular dichroic spectrum of the purple membrane has an important contribution from chromophore-chromophore interactions.

The purple-colored membrane of Halobacterium halobium functions as a photocoupler, transporting hydrogen ions across the cell membrane where they are believed to function in ATP synthesis (1,2). Purple membrane contains only a single protein; the chromophore responsible for the color is a retinal bound to this protein via a protonated Schiff base (1,3). This purple membrane protein, also referred to as "bacteriorhodopsin", exists in two relatively stable states: the dark-adapted form (λ_{max} = 559 nm) and a light-adapted form (λ_{max} = 568 nm). The purple membrane protein molecules are found in rigid clusters of three in the membrane with a threefold axis of symmetry perpendicular to the plane of the membrane at the center of each cluster (4,5,6). Becher and Cassim (7,8) have pointed out that the cross-over from a positive to a negative band in the visible CD spectrum (Fig. 1) near the wavelength of the absorption maximum suggests an exciton interaction between the retinal chromophores within a cluster. However, the CD spectrum could also be

Abbreviation: CD: circular dichroism

interpreted as a combination of two close-lying long wavelength transitions with opposite rotatory strengths (9). For example, the transitions proposed by Hudson and Kohler (10) in conjugated polyenes (whose structures are similar to that of the purple membrane chromophore) include an electronically allowed, magnetically forbidden transition ($^1A_g^+ \rightarrow {}^1B_u$) giving a positive CD band, and an electronically forbidden, magnetically allowed transition ($^1A_g^+ \rightarrow {}^1A_g^-$) giving a negative band (9). Alternatively, the CD could be due to two different retinal binding sites in the membrane, giving in one case a positive CD band and in the other, a negative CD band. In this paper, we will disprove the last two hypothesis; rather, our results show the CD spectrum has contributions from two sources--a positive band due to retinal bound to the protein, and a positive and negative band due to the interaction of chromophores from different proteins.

MATERIALS AND METHODS

Cultures of H. halobium were grown and the purple membrane isolated according to the procedures of Becher and Cassim (11), yielding a highly purified preparation as determined by electron microscopy, SDS gel electrophoresis, and absorption spectroscopy.

Purple membrane samples in 0.02 M potassium phosphate buffer (pH 7.00) were light adapted by a three minute exposure to intense (400 watt) light filtered through 3.2 cm of a 2.2% $CuSO_4$ solution and Corning glass filter 3-68, which passes light from 520 nm to 700 nm. Buffered membrane samples were solubilized in Triton X-100 by dropwise addition of a 10% solution of the detergent. Final concentrations of Triton in the samples ranged from 0.2-0.6%. The purple membrane was bleached in 0.3 M hydroxylamine and 0.02 M phosphate buffer (pH 700) by long (c.8-10 hours) exposure to the filtered light described above. The bleached samples were washed free of excess hydroxylamine, placed in buffer, and regenerated by successive additions of 1 µl of 3×10^{-3} M all-trans retinal in ethanol.

The absorption and CD spectra of the samples were recorded before bleaching and throughout the regeneration sequence by a Cary 118c spectrophotometer and a JASCO J-40A spectropolarimeter (time constant, 1 second; sensitivity, 50×10^{-2} millidegrees per cm; recording speed, 20 nm per minute; spectral band width, 4 nm).

RESULTS

All experiments were done using the light-adapted form of the purple membrane (Fig. 1). The visible CD of light-adapted purple membrane (Fig. 1) consists of negative and positive bands at 595 nm and 538 nm respectively with cross-over at 574 nm.

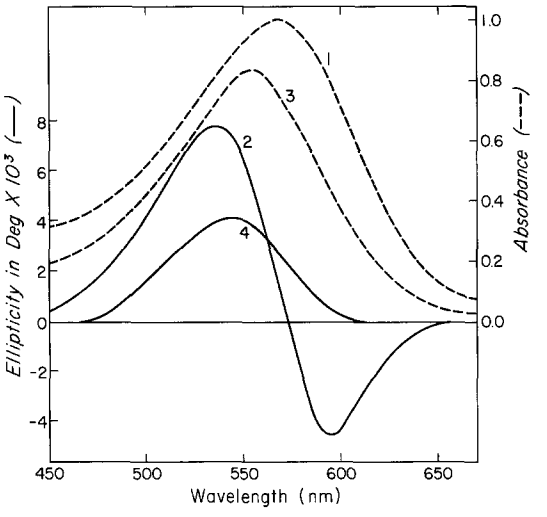
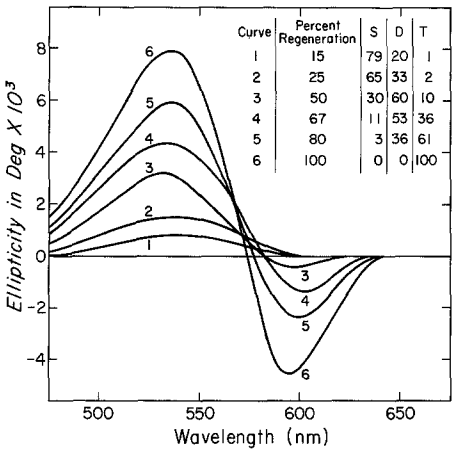


Fig. 1. Absorption and circular dichroic spectra of light-adapted purple membrane before (curves 1 and 2) and after (curves 3 and 4) solubilization in Triton X-100.

Purple membrane was regenerated by additions of all-trans retinal to the bleached apomembrane. The percent of regeneration was determined by the absorption at 568 nm, which increases proportionally with retinal added. The CD spectra associated with regeneration of bleached purple membrane are shown in Fig. 2; each curve corresponds to the percent regeneration of purple membrane shown in the table in the corner of the figure. The bleached membrane has no optical activity in the 450-700 nm wavelength range. With each percent of regeneration, the table also lists the distribution of retinal in the protein clusters which contain three retinal binding sites. Assuming random binding of the retinal molecules, the table lists the percentage of the retinals found as single chromophores in the three site clusters (monomers), as one of two chromophores in a cluster (dimers), and as one of three chromophores in a fully reconstituted cluster (trimers). Although random binding was assumed for these calculations as the most likely possibility, in fact the arguments presented below are not contingent upon this assumption.

The fully regenerated membrane (curve 6) has the same absorption and CD



Curve	Percent Regeneration	S	D	T
1	15	79	20	1
2	25	65	33	2
3	50	30	60	10
4	67	11	53	36
5	80	3	36	61
6	100	0	0	100

Fig. 2. Circular dichroic spectra of purple membrane at successive stages of regeneration.
[Table] Percentages of the chromophore found in monomer (S), dimer (D), and trimer (T) structures as a function of percent regeneration. In calculating these values, random binding of the chromophore was assumed.

spectra as that of the unbleached purple membrane (Fig. 1, curves 1 and 2) indicating that the original state of purple membrane is restored. However, the CD spectra of partially regenerated samples are not proportional reductions of the CD spectra of fully regenerated membrane. Curves 1 and 2, have no apparent negative band and the intensity of their positive CD bands are only 10% and 18% of that of the regenerated positive band although these curves represent 15% and 25% regenerations of the membrane. Similarly, at 50% regeneration, a negative band with only 10% of the intensity of the regenerated band is found, and the positive band is only 30% of the regenerated band. In curves 4 and 5, as the percent of regeneration is increased, the discrepancies between percent regeneration and the intensities of the positive and negative bands relative to fully regenerated membrane decrease.

These results strongly suggest that there is an important contribution to the CD spectrum that is present only when at least two and perhaps all three binding sites in the membrane are occupied. For example, at 15% and 25% regeneration (curves 1 and 2) only negligible amounts of retinal are found as

trimers (occupying fully filled cluster sites), and a minority of retinals are found as dimers. As the percentage of retinals found as dimers and especially trimers, increases, the negative CD band appears and both the positive and negative band intensities increase at escalating rates.

Our results can be most easily explained by a positive CD band associated with each retinal-protein plus a positive-negative pair of bands attributable to retinal-retinal (exciton) interaction between the chromophores in each cluster. For such an interaction, the CD spectrum should vary according to the fraction of retinals occupying clusters with one or two other retinals.

Although a single retinal bound to an apoprotein does have optical activity (approximated by curve 1, which is 79% monomer), it has only a positive CD band. Thus, the CD spectrum of the purple membrane cannot be ascribed to two transitions of opposite rotatory strength in a single chromophore.

An alternative hypothesis for the appearance of the negative CD band at a stoichiometry where all three retinal binding sites are occupied is that the last binding site to be occupied is different than the first two. By hypothesis, one consequence of this different binding site is that the CD of this last retinal-protein to be formed would be negative. Although SDS gel electrophoresis (11) and high resolution electron microscopy (4) suggest only a single type of protein is present, we decided to test this hypothesis in the following way: Solubilization of the membrane by a detergent should leave the CD of the individual molecules relatively unaffected. The addition of Triton (dropwise to a final concentration of 0.4%) to the purple membrane causes a marked decrease in the scattering properties of the preparation indicating the membrane, normally found in round to oval sheets 5,000Å in diameter, is solubilized (Fig. 1, curve 3). Although the absorption band shifts to 552 nm upon Triton addition, there is only about a 5% decline in optical density if the effects of the loss of scattering on the absorption spectrum are taken into account. In contrast to the small absorption change, the CD of the Triton solubilized purple membrane is greatly affected resulting in a single positive

band at 545 nm with 50% of the intensity of the positive CD band of the untreated membrane (Fig. 1, curve 4). This result makes it unlikely that ∿1/3 of the pigment molecules in the purple membrane have a negative CD band, for these should retain their CD bands after the Triton treatment. Rather, it seems more reasonable that the drastic alteration in the CD spectrum is due to the detergent disrupting the clustering of the proteins in the membrane.

We conclude that strong chromophore-chromophore interaction is present in purple membrane. The CD of purple membrane includes a conservative contribution arising from these interactions between the retinals of separate proteins in addition to the intrinsic CD of the purple membrane protein arising from the interaction of retinal with the protein. In addition, since chromophore-chromophore interaction exists in purple membrane: 1) the retinals in each cluster must be relatively close together to allow this interaction and probably are located in a portion of the protein that faces the threefold axis of a cluster, and 2) the retinals cannot all lie in a plane (e.g., of the membrane) since such chromophores have no exciton CD contribution (12).

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