Biophys. Struct. Mechanism 2, 79—92 (1976)

Biophysics of Structure and Mechanism

Evidence for Chromophore-Chromophore Interactions in the *Purple Membrane* from Reconstitution Experiments of the Chromophore-Free Membrane

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Abstract. We recently presented evidence showing that the visible CD spectrum of the purple membrane from *Halobacterium halobium* consists of two contributions: a broad positive band centered at the absorption maximum due to the interaction of the chromophore with the protein to which it is bound, and an exciton coupling band due to the interaction between chromophores of adjacent bacteriorhodopsin molecules in the hexagonal surface lattice (Heyn *et al.*, 1975). This interpretation receives strong support from the present experiments in which the chromophore-free membrane is reconstituted by the addition of retinal. Since the coupling signal arises from the interaction between *pairs* of neighboring chromophores, its contribution to the spectrum would be expected to be very small in the initial stages of the titration experiment, but increasing quadratically with the percentage reconstitution. The broad positive band, on the other hand, is expected to increase linearly with the percentage reconstitution. On the basis of these considerations a satisfactory explanation of the CD reconstitution experiments could be given. Since it appears to be impossible to explain the titration experiments without the quadratic term, we conclude that chromophore-chromophore interactions play an important role. No significant changes in secondary structure upon reconstitution could be detected consistent with our binding model which neglects cooperativity.

Key words: Circular dichroism — Exciton interaction — Purple membrane — Membrane reconstitution — Binding model.

Abbreviations: CD circular dichroism — UV ultraviolet.

Introduction

The extremely halophilic bacterium *Halobacterium halobium* forms distinct patches in the surface membrane when grown under anaerobic conditions and illumination. These so-called purple membranes contain besides the lipids (which make up about

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25 % of the total mass) only one type of protein. This protein molecule is chemically similar to the visual pigment rhodopsin. It contains the chromophore 13-*cis* or all-*trans* retinal, which is covalently linked to a lysine residue of the protein by a Schiff base and which is responsible for the characteristic purple color. Upon illumination the chromoprotein undergoes a photochemical reaction cycle which includes several intermediates. Because of these similarities with rhodopsin the protein was called bacteriorhodopsin (Oesterhelt and Stoeckenius, 1971). The functions and properties of the purple membrane have been recently reviewed (Oesterhelt, 1975).

In the dark the purple membrane has a strong chromophore absorption band at 560 nm that shifts to 570 nm and increases in oscillator strength upon illumination. These two states of the purple membrane are referred to as the dark and light adapted states, respectively. We recently reported CD spectra of the purple membrane in aqueous solution (Heyn *et al.*, 1975). The most striking feature of these spectra is a pair of bands of opposite sign in the visible spectral region (see Fig. 3, curve e), which contrasts with the broad positive CD band observed for rhodopsin. Since for electronically allowed transitions the CD spectra are proportional to the absorption spectra, these two CD bands should have their counterparts in the absorption spectrum. There is, however, no convincing evidence for two absorption bands at the appropriate wavelengths. An alternative explanation would be that this part of the CD spectrum is essentially an exciton spectrum caused by the interaction of the transition dipole moments of adjacent chromophores. This possibility is favored by the structural properties of the purple membrane patches. The bacteriorhodopsin molecules are tightly packed in a regular hexagonal surface lattice (Blaurock and Stoeckenius, 1971; Henderson, 1975; Blaurock, 1975). The rotational mobility of the protein is probably zero (Razi Naqvi *et al.*, 1973). Electron diffraction studies suggest that the protein molecules are arranged in clusters of three with trigonal symmetry (Henderson and Unwin, 1975).

In our previous paper (Heyn *et al.*, 1975), we presented strong evidence in favor of an interpretation in which the visible CD spectrum is the result of a superposition of a broad positive CD band centered at the absorption maximum (due to the interaction of the chromophore with the protein) and an exciton CD band with zero crossover at the absorption maximum (due to the interaction between chromophores on neighboring proteins). The following observations are in accordance with this interpretation: the CD spectra showed the expected redshift upon light adaptation; the positive and negative CD peaks were separated by about the expected wavelength interval; upon solubilization in Triton X-100 the coupling peaks disappeared with a broad positive band remaining; consistent with the idea that pairs of chromophores are involved, bleaching led first to the disappearance of the negative peak and at higher intensity to a strong decrease of the broad positive band; addition of dimethylsulfoxide resulted in a concerted disappearance of both peaks.

Oesterhelt *et al.* (1974) described a method for the preparation of chromophore-free membrane (apo-membrane). By the successive addition of retinal to this apo-membrane the native membrane can be reconstituted (Oesterhelt and Schuhmann, 1974). It was the purpose of the present investigation to monitor the

reconstitution of the purple membrane using optical activity as a probe. At low percentage reconstitution, one would expect to fill isolated binding sites with retinal. The resulting CD spectrum should show a broad positive band. At a higher level of reconstitution, the probability for an occupied site having a neighboring site occupied becomes appreciable. Under these conditions one would expect to observe the typical exciton bands superimposed upon the positive broad band. Using the simplest model, a quadratic dependency on the single site binding probability is expected for the chromophore-chromophore coupling contribution to the CD spectrum. A reconstitution experiment would thus provide a critical test for the validity of the exciton coupling interpretation. Once this interpretation is firmly established, these coupling signals can be used to gain further information on the system.

Theory

In the first part of this section we will discuss those theoretical aspects of exciton coupling which are needed for a proper understanding of the CD spectra. In the second part we will consider the consequences of the assumption of random non-cooperative binding of retinal to the purple membrane for the reconstitution binding curves.

Exciton interactions and their manifestations in CD spectra have been frequently discussed (Tinoco, 1963; Schellman, 1968). The simplest situation occurs with n identical chromophores each having only one transition (degenerate exciton model). In the presence of an external electromagnetic field at the frequency of this single transition, the originally n-fold degenerate energy level is split into n transitions due to the Coulomb interaction between the transition charges of adjacent chromophores. To lowest order this Coulomb interaction can be approximated by a dipole-dipole coupling between identical transition dipoles of neighboring chromophores. For many systems, however, this description is too crude. Usually several different electronic transitions occur, some of which may be quite close to each other in energy. These different non-degenerate electronic states may interact via their transition charges as well, provided their energy differences are not too large (non-degenerate exciton model). In the case of bacteriorhodopsin, however, the visible band is very far removed in energy from the other electronically allowed transitions, so that the degenerate model provides a good approximation.

For a coupled dimer, the degenerate exciton model leads to a splitting into two bands, which are symmetrically located with respect to the wavelength of the uncoupled chromophore. Each of these bands acquires optical activity in such a way that the rotational strength of one band is the opposite of that of the other (exciton couplet). The wavelength difference between these two split bands depends on the relative geometry of the two transition dipole moments. In almost all situations it is very small with respect to the absorption bandwidth of the uncoupled chromophore. This will certainly be the case for bacteriorhodopsin which has a bandwidth of about 57 nm for the 570 nm band. Thus very small effects are expected in the absorption spectrum. In the CD spectrum, however, even for very small splitting, large effects may be observed since the rotational strengths of the two transitions are of opposite sign. Assuming for the absorption spectrum a

gaussian bandshape with bandwidth Δ and assuming that the band splitting is small with respect to the bandwidth, the positive and negative extrema of the exciton couplet will be $\sqrt{2}\,\Delta$ apart in wavelength. The apparent splitting is thus independent of the transition dipole geometry. The amplitude of the exciton CD spectrum for the dimer decreases with $1/R^2$ where R is the distance between the two chromophores. It is proportional to the square of the extinction coefficient of the transition. Many of the details of the dimer case were recently discussed (Heyn, 1975).

The bacteriorhodopsin molecules of the purple membrane are arranged in a regular hexagonal surface lattice. Little is known about the location of the chromophore within the protein. Since the chromophore is tightly bound to the protein, it can be assumed that the chromophores are hexagonally arranged as well. Motional averaging or ensemble averaging over a large number of different transition dipole moment geometries will lead to a large reduction or vanishing of the exciton CD effects. The recent work of Henderson and Unwin (1975) suggests that the bacteriorhodopsin molecules are arranged in trimer clusters. It seems likely that the interaction between pairs of chromophores within these trimers is considerably larger than between chromophores of different trimers. We will accordingly present the results of the trimer calculation. A more definite calculation can be made as soon as the position of the retinal within the protein is known.

For the trimer we expect that the 570 nm transition is split into three bands. First order perturbation theory leads to one band at

$$\lambda_+ = \lambda_0 - 2\,\frac{\lambda_0^2}{h\,c}\,V_{12} \tag{1}$$

and a doubly degenerate band at

$$\lambda_- = \lambda_0 + \frac{\lambda_0^2}{h\,c}\,V_{12}\,. \tag{2}$$

λ_0 is the wavelength of the chromophore absorption maximum in the absence of coupling, c is the speed of light and h is Planck's constant. V_{12} is the interaction energy between any two transitions in the trimer. All three pair interactions in the trimer are of course identical. The rotational strengths induced by the coupling are given by

$$R_\pm = \mp\,\frac{\pi}{\lambda_0}\,\vec{R}_{12}\cdot\vec{\mu}_1\times\vec{\mu}_2 \tag{3}$$

with R_+ belonging to the band at λ_+ and R_- belonging to the band at λ_-. $\vec{R}_{12}$ is the distance vector between chromophores 1 and 2, in the direction 1 to 2. $\vec{\mu}_1$ and $\vec{\mu}_2$ are the transition dipole moments of chromophores 1 and 2 for the transition at λ_0. Because of the trigonal symmetry we could express $R_\pm$ in terms of the relative geometry of two arbitrarily selected chromophores of the trimer. As in the dimer case the two transitions have equal and opposite rotational strengths. From Eq. (1) and (2) it is clear that the band is no longer split symmetrically with respect to λ_0. The resulting CD spectrum will nevertheless have its new crossover very close to λ_0 because of the small band splitting. For the same reason the distance between the two extrema will again be $\sqrt{2}\,\Delta$. It follows from Eq. (3) that when $\vec{\mu}_1$ and $\vec{\mu}_2$ are coplanar, the CD amplitudes vanish. Hence no exciton CD effects are expected

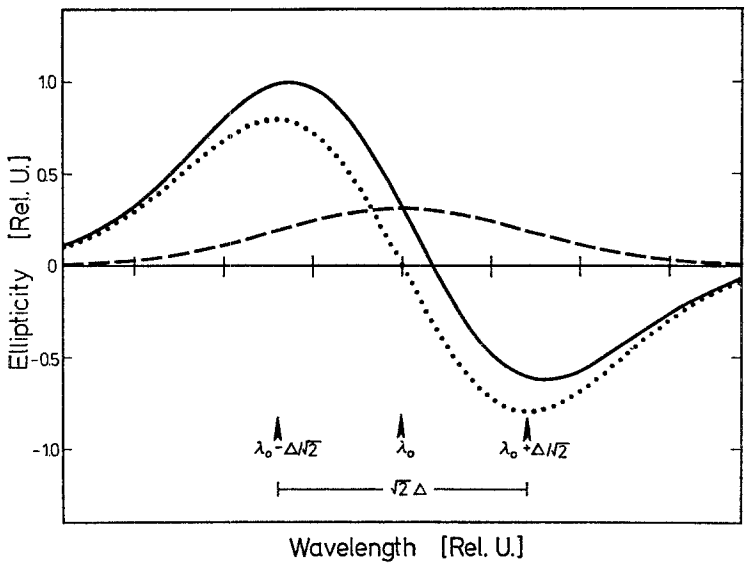

Fig. 1. ———: Simulated CD spectrum obtained by superposition of a symmetric exciton CD spectrum (· · · · · ·) and a positive gaussian monomer band (- - - - - -). λ_0 is the wavelength of the corresponding absorption maximum. Δ is the absorption bandwidth

when the transition dipole moments lie in the plane of the membrane. A second geometry in which the CD amplitudes vanish occurs when the transition dipole moments are coplanar with the trigonal axis of symmetry.

There is no problem in going beyond the trimer to a consideration of the infinite hexagonal lattice in which case a continuous distribution of energies, a true exciton band will be obtained. The CD spectrum will again be proportional to the derivative of the absorption spectrum, with peak to peak distance of $\sqrt{2}\,\Delta$, as long as the bandsplitting is much smaller than the bandwidth (Johnson and Tinoco, 1969). The apparent splitting will thus again be independent of the transition dipole moment geometry.

It is to be expected that even in the absence of chromophore-chromophore coupling, the chromophore bands of bacteriorhodopsin will be optically active. The retinal chromophore is tightly bound to the protein as in rhodopsin and its transition dipoles will interact with the asymmetric environment provided by the protein. Both membrane bound and solubilized rhodopsin are optically active with a positive visible CD band which is approximately proportional to the visible absorption spectrum (Honig and Ebrey, 1974). Membrane bound rhodopsin has of course large rotational and translational freedom and does not form a surface lattice. Fig. 1 shows what happens when an exciton CD spectrum due to the coupling between chromophores of neighboring bacteriorhodopsin molecules is superimposed on a positive CD spectrum due to single bacteriorhodopsin molecules. The crossover of the resulting spectrum is shifted to the red with respect to λ_0, the amplitude of the positive band is now larger than that of the negative band, and

the distance between the two extrema is still approximately $\sqrt{2}\,\Delta$. These three features are indeed observed in the experimental spectra (Heyn *et al.*, 1975). The crossover occurs at about 575 nm. The positive band is larger than the negative band. The two peaks are about 70 nm apart, which is in qualitative agreement with the value of 84 nm predicted using an experimentally determined value for the bandwidth of 57 nm.

In the interpretation of the reconstitution titration experiments we will assume random statistical binding of the retinal chromophores to their binding sites in the hexagonal surface lattice. More complicated models, including for instance cooperativity, can of course also be considered. Since there is at present no clear evidence in favor of such models, we are justified in our attempt to explain the experimental data with the simplest model, containing the smallest number of adjustable parameters. In accordance with the approach used in the first half of this section, we will first consider the trimer as the basic unit. Let p represent the probability that a chromophore binding site is occupied. This quantity will be assumed to be independent of the state of occupancy of its neighbors. Expressed in percentage, p will also be called the percentage reconstitution. The probability that a chromophore binding site is empty is thus $1-p$. Assuming non-cooperative random binding, the probabilities that zero, one, two or three binding sites are occupied can now be easily calculated. We assume that each occupied site will give rise to a monomer spectrum $A_m(\lambda)$, due to the interaction between the chromophore and the protein. In addition each pair of occupied sites will give rise to a dimer spectrum $A_d(\lambda)$. The contribution of the monomer spectrum to the trimer CD amplitude can now be simply obtained by multiplying the probabilities with the specific signals:

$$\{3\,p(1-p)^2+6(1-p)\,p^2+3\,p^3\}\,A_m=3\,pA_m. \tag{4}$$

The contribution of the dimer spectra to the trimer CD amplitude can be similarly obtained:

$$\{3(1-p)\,p^2+3\,p^3\}\,A_d=3\,p^2A_d. \tag{5}$$

We thus obtain for the CD spectrum of each constituent of the trimer

$$pA_m+p^2A_d. \tag{6}$$

It can be shown that, whatever the structure of the lattice is, the CD spectrum will always be of form (6) when pair interactions contribute. We define the normalized CD amplitude A as the amplitude at a given p value, divided by the maximal value at $p=1$:

$$A=\frac{p\,A_m+p^2\,A_d}{A_m+A_d}. \tag{7}$$

Eq. (7) expresses in a clear way what one expects to observe. At low p values only isolated sites will be occupied and the A_m term will dominate. At higher p values some occupied sites will have neighboring sites occupied as well and the p^2 term, due to the coupling between pairs of chromophores, will start to contribute. If no coupling effects occur, the expression for A will of course, in the absence of

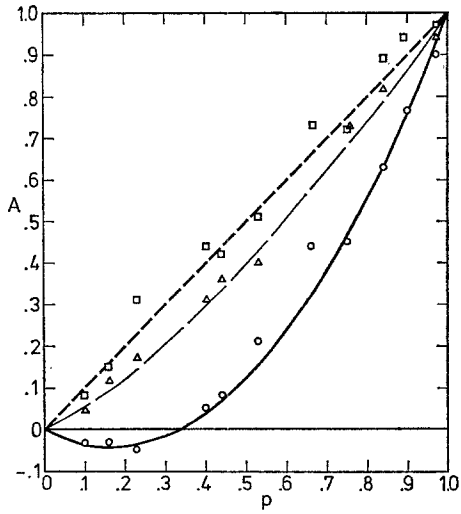

Fig. 2. The relative CD amplitude A plotted versus the fraction p of reconstituted bacteriorhodopsin. The experimental points were taken at 263 nm (□), 535 nm (△), and 605 nm (○). The curves are plotted according to Eq. (8). ------, $A_d/A_m = 0$; – – –, $A_d/A_m \approx 1.5$; ———, $A_d/A_m = -3$

cooperativity, be equal to p. It is thus useful to split eq. (7) into p and a term describing the deviation from it:

$$A = p + p(p-1)\frac{A_d}{A_m + A_d}. \tag{8}$$

The second term in (8), describing this deviation, is factored into two contributions. The first factor $p(p-1)$ is only dependent on p. It is zero at $p=0$ and $p=1$, and has its maximal value at $p=1/2$. It is always negative or zero since $p \leq 1$. The second factor $A_d/(A_m + A_d)$ is independent of p, but strongly dependent on the wavelength. This is due to the fact that A_m is always positive, whereas A_d has the exciton couplet shape and can thus be positive and negative. At λ_0 for instance $A_d = 0$ and $A = p$. At wavelengths at which A_d is negative, the factor $A_d/(A_m + A_d)$ can become very large, both positive and negative. For positive A_d, this factor is positive and between zero and one. In Fig. 2, A is plotted versus p for several values of A_d/A_m. For the same value of $|A_d/A_m|$, the curve with negative A_d gives a much larger deviation from the "non-coupling" straight line p than the curve with positive A_d. For a titration experiment it is of course advantageous to work at a wavelength with a sufficiently large CD amplitude. Thus the most suitable wavelengths are 535 nm and 605 nm. Of these two wavelengths 605 nm is clearly to be preferred, since A_d is negative, $|A_d/A_m| > 1$ and the deviation from the p term of Eq. (8) is largest. Eq. (8) predicts that at 535 nm the deviation from the straight line will be much smaller.

Materials and Methods

Culture of bacteria and isolation of purple membrane. Halobacterium halobium strain R_1 was grown in sterile culture medium (Oesterhelt *et al.*, 1973) in a reci-

procating water bath shaker at 40° C and under white light (1 mW/cm²). Growth of 500 ml shake cultures in 1000 ml Erlenmeyer flasks was for 100 to 125 h at 95 rpm and after that for about 24 h at 80 rpm (stroke 1.5 inch). Preparation of the purple membrane was performed at room temperature with the centrifuges kept at 4° C. The cells were harvested by centrifugation at 12,000 g for 10 min and resuspended in a small quantity of culture medium without peptone; about 0.5 mg DNAase I/l of bacteria culture was then added. The suspension was dialyzed against double distilled water for 20 h at 2° C, exchanging the water two times. The clear lysate was centrifuged at 48,300 g for 50 min. The purple pellet was washed in distilled water and centrifuged at 7,000 g for 10 min. The supernatant containing the purple membrane was gently removed and again centrifuged at 48,300 g for 50 min, whereas the dark pellet was discarded. This procedure was repeated four to six times until no red contaminant could be seen in the supernatant after centrifugation at 48,300 g and no dark sediment was apparent after centrifugation at 7,000 g. The purified purple membrane sediment was resuspended in a minimum of distilled water and layered on a linear sucrose density gradient (24 to 58%, w/v). After about 13 h centrifugation at 183,000 g, the purple bands were collected from the gradient and washed four times in distilled water to remove the sucrose. Under the conditions described, two or three purple bands at different densities could be separated and no red and dark contaminants were seen. Experiments were performed with purple membrane from the main band having a ratio of absorbance at 280 nm to absorbance at 570 nm of about 2.0 (without correction for light scattering). After isolation the purple membrane was stored at 4° C. For CD and absorbance measurements the purple membranes were suspended in 25 mM phosphate buffer (pH 6.88).

Preparation of Chromophore-Free Membrane. The chromophore-free membrane (apo-membrane) was prepared by a method similar to that of Oesterhelt and Schuhmann (1974). Purple membrane suspensions of pH 7.1 containing 1.4 to $1.7 \cdot 10^{-5}$ M bacteriorhodopsin and 1.0 to 1.3 M hydroxylammonium chloride were illuminated at 20° C. The light from a halogen lamp (Schott KL 150) passed through 20 cm water and a cut-off filter (Schott OG 515). The light intensity at the position of the sample was about 10 mW/cm². Decolorization of the samples was monitored in a spectrophotometer at several times and was complete after 14 to 20 h. The membranes were washed four times in distilled water to remove hydroxylamine and retinaloxime and finally diluted in double distilled water to about the protein concentration prior to the washing procedure. Some samples were irradiated for 30 min with a Xenon lamp to destroy the retinaloxime which remained attached to the membrane after washing. All samples were stored at — 80° C. The experiments were performed with aqueous unbuffered suspensions.

Spectroscopic Methods. CD measurements were carried out with a Cary 61. Thermostatable cells with thicknesses ranging from 0.1 mm to 5 cm were used. The optical density was kept below 1.5 at all wavelengths. Absorption spectra were recorded with a Cary 17 or a Cary 118. Molar concentrations and molar ellipticities were calculated using an extinction coefficient of 63,000 M^{-1} cm^{-1} at 568 nm (Oesterhelt and Hess, 1973). Titration experiments were performed by successive addition of 0.5 µl aliquots of concentrated ethanolic all-*trans* retinal solution (2.50 mM or 1.25 mM) to a large volume of apo-membrane suspension (1.2 ml).

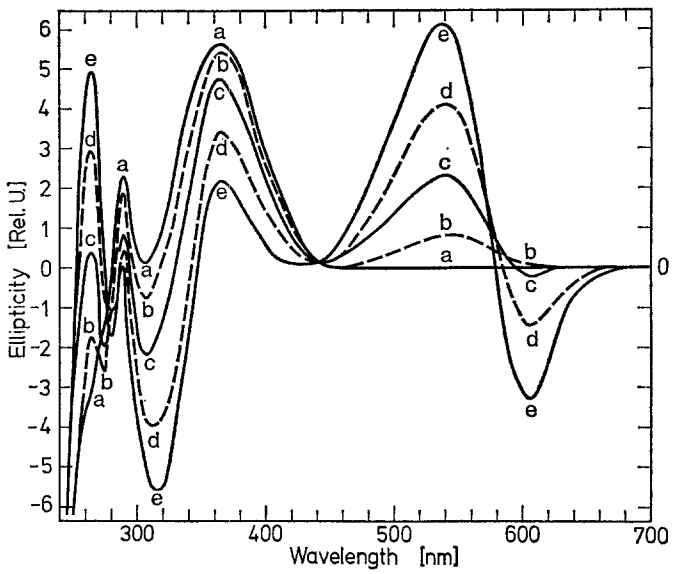

Fig. 3. CD spectra of apo-membrane (curve a), of partially (curves b, c, d) and fully (curve e) reconstituted purple membrane, respectively. From a to e the concentration of added all-*trans* retinal increases. For clarity only a few of the spectra are shown. The 365 nm band is due to retinaloxime that remains attached to the membrane after washing.

Results

Circular Dichroism of the Chromophore-Free Membrane. The CD spectrum of an aqueous suspension of apo-membrane is shown in Fig. 3 (curve a). The band around 365 nm is caused by an appreciable quantity of retinaloxime which remained attached to the membrane after the washing procedure. Apart from this band the apo-membrane shows no optical activity above 300 nm. Below 300 nm, there is negative ellipticity which is part of the long wavelength tail of the protein peptide bands. Superimposed upon this negative background is a positive CD band at 290 nm. Since a similar positive 290 nm band is present in the absorption and CD spectra of the native purple membrane, it will be assigned to the aromatic amino acid residues of the protein. As retinal is added to the apo-membrane, a positive CD band develops at 263 nm, a negative CD band at 318 nm, and a pair of positive and negative bands in the visible region (Fig. 3, curves c, and d). Since these bands are also present in the spectrum of the native membrane, we must assign them to the chromophore. For the 263 nm band, however, we cannot completely exclude the possibility that this is an aromatic amino acid band of the protein whose optical activity is induced by the binding of the chromophore. The CD spectrum of the fully reconstituted purple membrane (Fig. 3, curve e), apart from the 365 nm band, is completely identical to that of the native light adapted purple membrane (Heyn *et al.*, 1975).

Reconstitution Experiments. The purple membrane was reconstituted by successively adding aliquots of 0.5 µl of an ethanolic solution of all-*trans* retinal (2.50 mM and 1.25 mM). At room temperature the reconstitution process was

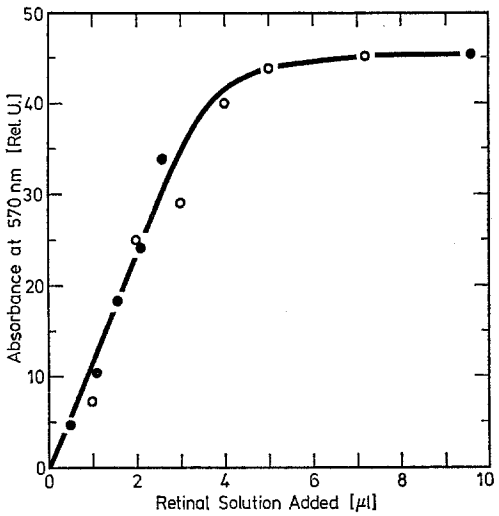

Fig. 4. Amplitude of the absorption difference spectra at 570 nm plotted versus the amount of all-*trans* retinal added to a large volume of unbuffered aqueous apo-membrane suspension. This absorption signal was used to follow the reconstitution. The data of two different experiments are shown (●, ○)

completed within several minutes. The 570 nm absorption band gradually developed with the successive addition of retinal. The total amount of retinal solution added was 9.6 μl and the dilution of the suspension was negligible (1%). The final value of the retinal concentration was about two times that of the protein concentration. The absorption of the reconstituted membrane at 570 nm was somewhat less than that of the native purple membrane before preparation (80%). Most of this loss of concentration can be attributed to the preparation procedure (see Materials and Methods).

After each addition of retinal the CD spectrum was recorded (Fig. 3). Below 30% reconstitution positive CD bands developed around 550 nm and 263 nm and a negative CD band around 318 nm. No negative band was observed at 605 nm. Further titration led to the development of a negative band at 605 nm. The CD spectrum of the fully reconstituted membrane changed in the dark within 1 h to the CD spectrum of the dark adapted purple membrane (blueshift of about 10 nm of the bands in the visible spectral region and a decrease in amplitude). This indicates that the reconstituted membrane behaves similarily to the native membrane.

Using the random binding model, a more precise evaluation is possible. In Fig. 4 the absorption change at 570 nm is plotted versus the amount of added retinal. The curve shows that saturation was reached. The assumption that with excess retinal 100% reconstitution was obtained, is therefore justified. A small error which may enter the quantitative evaluation at this point will be discussed later.

The relative amplitude A at a fixed wavelength was defined as the ratio of the actual to the final CD amplitude. In the same way, p is defined as the ratio of the

actual amplitude to the final amplitude of the 570 nm absorption difference spectrum. The fraction of the reconstituted bacteriorhodopsin is represented by p. From the theory, A should depend on p according to Eq. (8). As long as no chromophore coupling occurs, the experimental points are expected to lie on the diagonal, independent of the wavelength at which A is determined. As we can see from Fig. 2 this is clearly not the case for the experimental points at 605 nm. These points are negative at small p values and then rapidly rise to 1. Fig. 2 (lowest curve) shows that this nonlinear curve is correctly predicted by the random binding theory with $A_d/A_m = -3$. This value for A_d/A_m is in reasonable agreement with the following rough estimate for the monomer and dimer amplitudes from the CD spectra in this wavelength region. The ratio of the CD amplitudes at 535 nm and 605 nm is about -2. Symmetric bands can be obtained by subtraction of a broad monomer band, which has at 535 nm about 25% of the amplitude of the 535 nm positive band, from the asymmetric bands. Thus A_d/A_m equals about -3. The A values at 535 nm are also plotted in Fig. 2. Again, the experimental points lie below the straight line. As expected from the random binding theory, the effect at this wavelength is not so pronounced, since the signs of the CD amplitudes of the monomer and dimer are the same at 535 nm, whereas at 605 nm they are opposite. A reasonable fit at 535 nm was obtained with $A_d/A_m \approx 1.5$.

The main source of error is the assumption that the final value of the reconstitution has been reached. If maximal reconstitution was not obtained, the calculated values for A and p would be too large. A correction, which would reduce both values, results in a shift of the experimental point in a direction approximately tangential to the theoretical curve. The deviation from the diagonal would be barely affected and the qualitative agreement with the theoretical curve would not be altered. Based on the absorption spectrum of the reconstituted membrane, this error should be well below 10%.

The same evaluations were carried out at 310 nm and at 263 nm. The determination of the CD amplitudes at 310 nm was difficult due to the overlapping with the 365 nm retinaloxime band and with the 290 nm protein band. The experimental points at 263 nm were also difficult to determine because of their overlap with the negative protein peptide bands. The 263 nm data are plotted in Fig. 2. No significant deviation from the diagonal occurs. The same holds for the data at 310 nm. Within the framework of the random binding model this implies that no chromophore-chromophore coupling is involved in these two bands.

The reconstitution experiment was repeated with a preparation in which the retinaloxime was completely destroyed by UV irradiation. The results were qualitatively similar to those of the previous experiment. Reconstitution was obtained and a negative band developed at 605 nm.

Conformational Changes Upon Reconstitution. Upon binding of retinal to the chromophore site of bacteriorhodopsin, structural changes in the protein may occur. Therefore the far-UV protein backbone spectra of both the apo-membrane and the reconstituted membrane, were recorded. Since there are more than 200 peptide bonds for each bound retinal, no contribution from the chromophore is expected in the 190 to 250 nm range. Within experimental error, we could not observe a significant change in the far-UV CD spectrum upon the addition of

retinal, which indicates that the secondary structures are identical. This experiment, however, cannot exclude a subtle conformational change.

Discussion

At room temperature, degenerate exciton splitting of the 570 nm absorption band cannot be detected in the absorption spectrum of purple membrane suspensions. CD spectra are a powerful means to investigate such interactions. From the statistical binding model, one expects a quadratic increase of the exciton band with the chromophore concentration, whereas the CD spectrum of the non-interacting chromophores should increase linearly. This allows a clear differentiation between dimer and monomer CD amplitudes. The experimental results presented in Fig. 2 are in good agreement with the predictions of the random binding model. They provide further strong evidence in favor of the exciton interpretation of the CD spectra which was presented in a previous paper (Heyn *et al.*, 1975). There is no need for more sophisticated binding models, although they cannot be excluded by this experiment. On the one hand, no significant conformational change was observed upon reconstitution although such a change might have been expected from a cooperative model. On the other hand, the normal hexagonal lattice of the purple membrane becomes disordered when the retinal is removed since all the diffraction rings in the X-ray pattern get broader (R. Henderson, priv. comm.). Upon reconstitution the hexagonal order reappears (R. Henderson, priv. comm.). The fact, that the exciton CD spectrum, which depends strongly on the exact geometrical arrangement of the transition dipoles, is the same in native and reconstituted membranes, is in complete agreement with this observation. It appears therefore that the long range disorder, which is induced by the removal of retinal, is associated with a very minor change in protein secondary structure. Short range order, extending over several unit cells is still present in the apo-membrane (R. Henderson, priv. comm.). In our spectra we are observing coupling over these distances. The presence of cooperativity could be detected in a careful binding study using either absorbance or CD (at a wavelength where coupling effects are absent) as a signal. A sigmoidal binding curve would be good evidence for cooperativity. The accuracy of our present experiments (Fig. 4) is not sufficient to settle this question.

The random binding model contains as the only adjustable parameter the ratio A_d/A_m. The range of possible values for this parameter is however very limited. From the shape of the CD spectrum and in particular from the ratio of the amplitudes at 535 nm and 605 nm, one concludes that A_d/A_m at 605 nm must lie between -2 and -5. Using a value of -3, a satisfactory fit was obtained.

The reconstitution percentage was directly determined from the absorbance at 570 nm. This procedure is based on the assumption that chromophore-chromophore interactions do not affect the absorption spectrum. Borrowing of oscillator strength from transitions at other wavelengths can be neglected because of the very large differences in energy. Furthermore, splitting due to degenerate coupling was shown to be negligible.

It follows from Eq. (3) that the transition dipole moment of the chromophore cannot be parallel to the plane of the membrane. Earlier experiments on the linear dichroism of oriented purple membrane patches indicated a preferential orienta-

tion of the 570 nm transition dipole moment in the plane of the membrane (Blaurock and Stoeckenius, 1971). However, no quantitative statement was possible (W. Stoeckenius, priv. comm.).

It may be questioned whether the distances between chromophores are small enough to allow the observation of exciton effects. Interchromophore distances, ranging from 12 Å to 45 Å, may be estimated from the 7 Å resolution map (Henderson and Unwin, 1975). Coupling over distances of the order of 20 Å appears to be possible due to the low dielectric constant of membranes and to the high extinction coefficient of the chromophore.

The negative CD band at 318 nm and the positive CD band at 263 nm were assigned to the chromophore. No prominent bands, however, can be distinguished in the absorption spectrum at these wavelengths. This may be due to the fact that light scattering plays a major role in this region. Alternatively this may point to the presence of electronically forbidden transitions. We cannot exclude the possibility that in the visible region as well electronically forbidden transitions lead to some of the CD effects observed.

We note that the amplitude of the exciton couplet is larger than the amplitude of the underlying positive CD band. This implies a rather strong chromophore-chromophore interaction. The exciton energy transfer may possess biological significance in connection with the function of the purple membrane.

Acknowledgement. The authors wish to thank Dr. H. Stieve and Dr. E. Hildebrand for their interest in this work and for valuable discussions. We also wish to thank Dr. D. Dahl for reading the manuscript and E. Brentgens for secretarial help.

N.D. was supported by the Deutsche Forschungsgemeinschaft (SFB 160).

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Received January 23, 1976