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Binding of *all-trans*-Retinal to the Purple Membrane. Evidence for Cooperativity and Determination of the Extinction Coefficient†

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ABSTRACT: At low bacteriorhodopsin concentration the binding of *all-trans*-retinal to the apomembrane of *Halobacterium halobium*, as monitoried by the absorbance change at 568 nm, occurs in a cooperative manner. The simplest way of analyzing the binding data is based on an all-or-none model and results in a Hill coefficient of 3.0 ± 0.2 and an apparent association constant of 2.8×10^6 M^{-1}. The same sigmoidal binding curve was obtained by using the change in circular dichroism at 365 nm (displacement of retinal oxime) or at 263 nm (retinal-induced change in bacterioopsin). Moreover, the trivial explanation of our results, namely, that the sigmoidal shape is caused by a suitably varying extinction coefficient, could be excluded, since the extinction coefficient was shown to be independent of the degree of binding. The Hill coefficient of close to 3 suggests that protein–protein interactions within bacteriorhodopsin trimers are responsible for the observed cooperative effect. Such an interpretation is consistent with the structure of the reconstituted apomembrane which consists of a hexagonal lattice of bacteriorhodopsin in which the bacteriorhodopsin molecules are arranged in clusters of three. The surprisingly small value of the association constant shows that retinal binding to the apomembrane is not irreversible. This was confirmed by exchange experiments between $retinal_1$ and $retinal_2$ which show that bound $retinal_2$ can be displaced by $retinal_1$ and vice versa. At bacteriorhodopsin concentrations much higher than the reciprocal of the association constant, all the retinal added is bound until all the binding sites are occupied. It is therefore possible to determine the extinction coefficient of the chromophore from the slope of the binding curve. The extinction coefficient obtained in this way is based on a knowledge of the retinal concentration and does not depend on a determination of the protein concentration. The resulting value of 62 700 ± 700 M^{-1} cm^{-1}, at 568 nm, refers to the light-adapted state of the purple membrane at 25 °C in 0.02 M phosphate buffer, pH 6.9, and is corrected for light scattering.

Purple membrane patches consist of a hexagonal array of identical protein units. At each vertex of the unit cell, three bacteriorhodopsin molecules are arranged in a cluster, in such a way that intratrimer distances are smaller than intertrimer distances (Henderson & Unwin, 1975; Unwin & Henderson, 1975). In harmony with this structural evidence, chemical cross-linking with bifunctional reagents produces bacteriorhodopsin trimers in a very high yield (Dellweg & Sumper, 1978). At present, the functional significance of the lattice structure is not yet clear. In view of the structural evidence, it is conceivable that bacteriorhodopsin trimers are the true functional units. Cooperative binding of ligands is a well-known phenomenon for water-soluble oligomeric proteins made up of identical subunits. Little is known about cooperative effects in the binding of ligands to intrinsic membrane proteins which are embedded in the two-dimensional matrix of a membrane. Whereas cooperative binding to linear arrays of

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binding sites is well understood (Schwarz, 1976), the case of binding to two-dimensional arrays of binding sites, such as occurs in membranes, has received little attention. The purple membrane of *Halobacterium halobium* offers a particularly simple system to study such effects. It contains only a single protein, bacteriorhodopsin. The ligand retinal is bound to a lysine residue of the protein via a protonated Schiff base with a 1:1 stoichiometry (Papadopoulos et al., 1978). We thus have a homogeneous two-dimensional array of binding sites. By treatment with hydroxylamine, a colorless chromophore-free apomembrane can be prepared (Oesterhelt & Schuhmann, 1974), which can be regenerated by the addition of external retinal. Titrations of apomembrane patches with *all-trans*-retinal may thus give us information on the apparent binding constant of retinal in its binding site and on the possible existence of cooperative interactions. The minimal number of interacting bacteriorhodopsin molecules may be estimated from the Hill coefficient of the binding curve. Titrations were performed by using both changes in absorbance and changes in circular dichroism at various wavelengths. Since one or more of these specific signal changes (ϵ, $\Delta\epsilon$) may depend on the degree of binding and thus induce an apparent sigmoidal binding curve in the absence of cooperative binding, it is important to exclude this possibility by doing the titrations with various signals. Direct proof that at least one of these specific signals is independent of the degree of binding was obtained for the extinction coefficient of the retinal chromophore at 568 nm. At present, the values for this extinction coefficient range from 55 000 M^{-1} cm^{-1} (Ebrey et al., 1977) to 72 000 M^{-1} cm^{-1} (Englander & Englander, 1977). The most often used value of 63 000 M^{-1} cm^{-1} (Oesterhelt & Hess, 1973) is not corrected for the appreciable contribution from light scattering and although determined at 568 nm refers to the dark-adapted state of the membrane (Oesterhelt, private communication). For rapid spectrophotometric concentration determinations of bacteriorhodopsin, it is important to have available a reliable extinction coefficient which is corrected for light scattering. Furthermore, many spectroscopic experiments, such as the exciton circular dichroism spectra (Heyn et al., 1975), energy transfer experiments with retinal as the acceptor, and linear dichroism spectra of oriented purple membranes (Heyn et al., 1977), can only be evaluated in a quantitative way when the extinction coefficient is accurately known.

Materials and Methods

Preparation of Purple Membrane. Purple membranes were prepared according to standard procedures (Oesterhelt & Stoeckenius, 1974). Purple membranes from *H. halobium* strain R_1L_3 were used in the binding experiments. The experiments on the extinction coefficient were performed with strain R_1M_1 because of its low bacterioruberine content. Purple membrane suspensions were occasionally briefly sonified to reduce light scattering.

Preparation of Bleached Apomembranes. Apomembrane was prepared by illumination in the presence of hydroxylamine, as described previously (Oesterhelt & Schuhmann, 1974; Bauer et al., 1976). Hydroxylamine was removed by dialysis overnight against 4 × 2 L of 0.025 M phosphate buffer, pH 6.9, at 4 °C. Apomembrane was freshly prepared for every titration experiment.

Preparation of Monomers. Bleached bacterioopsin monomers were prepared by solubilization of the apomembrane with Triton X-100 (Packard Instrument Corp.). As compared to the purple membrane, the solubilization of apomembrane is much faster (~0.5 h). Typically, Triton was added to apomembrane in a 4:1 (mg of Triton per mg of bacteriorhodopsin) ratio. Solubilization was initiated by vortexing for 5 min, followed by 10-min centrifugation at 200000*g*. The supernatant (containing about 80% of the protein) was used for regeneration. Maximal regeneration achieved was 69%. At such percentage reconstitution, it is possible to check if regenerated bacteriorhodopsin is indeed monomeric by measuring the CD[1] in the visible absorption band. If solubilization was not complete to the state of monomers or if reaggregation occurred, some exciton component should be observable in the CD (Heyn et al., 1975; Dencher & Heyn, 1978). This was not the case. In harmony with this result, the regenerated monomers absorbed maximally at about 555 nm rather than at 568 nm as expected for aggregated bacteriorhodopsin.

Protein Concentrations. These were determined by the method of Lowry et al. (1950) as modified by Hartree (1972) using bovine serum albumin as the standard. Bacteriorhodopsin concentrations were calculated on the basis of a molecular weight of 26 000.

all-trans-Retinal$_1$ and all-trans-Retinal$_2$. *all-trans*-Retinal$_1$ was obtained from Fluka and used without further purification. Thin-layer chromatography on Merck 5553 plates with hexane–diethyl ether (60:10) as the moving phase showed a single spot. High-pressure liquid chromatography shows that this product contains a trace of 13-*cis*-retinal$_1$ which also binds to the apomembrane and which converts to *all-trans*-retinal$_1$ upon light adaptation. Retinal without a subscript refers to retinal$_1$. *all-trans*-Retinal$_2$ (3-dehydroretinal) was a gift of Dr. D. Oesterhelt and Dr. P. Towner of the University of Würzburg. Its purity was checked by high-pressure liquid chromatography and mass spectrometry. Both retinals were added to apomembrane suspensions in the form of concentrated ethanolic or 2-propanolic solutions (0.5–2 mM). The concentration of *all-trans*-retinal$_1$ in ethanol was determined by absorption measurements at 382 nm, using an extinction coefficient of 42 800 M^{-1} cm^{-1} (Carl, 1972; Sperling & Rafferty, unpublished results), and by weight. Within experimental error, the results of these methods agreed. The agreement was slightly worse with the value of 43 400 M^{-1} cm^{-1} (Robeson et al., 1955). The concentration of *all-trans*-retinal$_2$ in ethanol was determined by absorption measurements at 401 nm, using an extinction coefficient of 41 500 M^{-1} cm^{-1} (von Planta et al., 1962). The retinal solutions were freshly prepared for every titration experiment and kept in the dark.

Titrations. Concentrated *all-trans*-retinal in ethanol was added in the dark to 2.0–2.5 mL of buffered apomembrane suspension in aliquots of 1 or 2 μL with an Agla micrometer syringe (Wellcome Research Laboratories). The syringe was calibrated with distilled water on a 10 γ balance (Sartorius). The final ethanol content of the apomembrane solutions did not exceed 1% (v/v). Dilution corrections were made. During the titration, the apomembrane solution, in 0.025 M phosphate buffer, pH 6.9, remained in the same stoppered cell of path length 1 or 2 cm. After each addition of retinal, the solution was gently mixed by shaking and allowed to stand until equilibrium was reached. The experiments were performed at 20 °C. The kinetics of the regeneration is complicated by the simultaneously occurring light–dark adaptation process which has comparable rate constants. The approach to equilibrium was therefore followed at 532 nm, the isosbestic point for the light–dark adaptation (Dencher et al., 1976). Equilibrium was reached in about 20 min. A slight further

[1] Abbreviation used: CD, circular dichroism.

increase in absorbance at 568 nm occurs overnight, but this is due to rebinding of retinal oxime. No such overnight increase is observed if a difference spectrum between partially reconstituted apomembrane and apomembrane is recorded. In most experiments complete spectra were taken with water as the reference. All solutions were light-adapted immediately before measurement. A cutoff filter (OG 515) prevented possible isomerization of free *all-trans*-retinal during the light adaptation. After passing the absorption maximum, the wavelength was scanned back to just before the maximum, the sample was light-adapted again, and the spectra were rerun to check for possible dark adaptation. Since at low percentages of reconstitution the rate of dark adaptation is quite fast, at these temperatures (Ebrey et al., 1977) such precautions are required.

Absorption and Circular Dichroism Measurements. Absorption measurements were recorded with a Cary 118 spectrophotometer equipped with an attachment to reduce the apparent absorbance due to light scattering. CD measurements were performed with a Cary 61 spectropolarimeter.

Data Analysis. We define the degree of binding ν by the ratio of the observed absorbance increase at 568 nm (ΔA) divided by the increase which should be observed at 100% regeneration ($\Delta A_{100\%}$):

$$\nu = \Delta A / \Delta A_{100\%} \quad (1)$$

$\Delta A_{100\%}$ is not the final absorbance observed at very high concentrations of added retinal, but it equals the absorbance to be expected at 100% regeneration on the basis of the known decrease in absorbance in the bleaching reaction. The well-known end-value problem of spectrophotometric titrations is avoided in this way, and $\Delta A_{100\%}$ does not enter our calculations as an adjustable parameter. It was shown that the extinction coefficient ϵ does not depend on the percentage reconstitution (see Results). We then have for the concentration R_B of bound retinal in a cell of thickness d (centimeters) and at ΔA

$$R_B = \frac{\Delta A}{\epsilon d} \quad (2)$$

The concentration of free retinal R_F can be calculated by subtracting the concentration of bound retinal from that of the total retinal concentration R_T. This requires knowledge of the extinction coefficient ϵ. In the "all-or-none" model of cooperativity, it is assumed that certain clusters of binding sites exist, each comprising n sites, which can only be occupied simultaneously (van Holde, 1971). Intermediates with less than n occupied sites have negligible concentration in this limiting case of extreme cooperativity. In terms of this model, with n interacting bacteriorhodopsin molecules, the degree of binding ν is related to n, R_F, and the association constant K in the following way (van Holde, 1971):

$$\nu = \frac{(KR_F)^n}{1 + (KR_F)^n} \quad (3)$$

K can be interpreted as a mean association constant. The corresponding Hill plot is obtained by transforming eq 3:

$$\log \frac{\nu}{1 - \nu} = n \log K + n \log R_F \quad (4)$$

Both n and K can thus be determined within the framework of this model from the slope and intercept of the Hill plot.

Results

Determination of the Extinction Coefficient of the Light-Adapted Chromophore at 568 nm. Figure 1 shows the change in absorbance at 568 nm of a suspension of apomembranes as a function of the concentration of added *all-trans*-retinal. The data were collected at bacterioopsin concentrations of 14.8 and 17.6 μM (as determined by the Lowry–Hartree method). The protein concentrations were chosen to be much larger than the reciprocal association constant ($K = 2.8 \times 10^6$ M^{-1}; see below). Under these conditions every retinal added binds and the titration curves should consist of two straight-line segments. Indeed, we observe from Figure 1 that the linear parts of the two sets of data coincide, whereas the end value is of course higher for the higher protein concentration. The two horizontal lines in Figure 1 indicate the end values which would be expected when the degree of regeneration is 100%. They were calculated from the known loss in absorbance in the bleaching reaction. Figure 1 shows that 100% reconstitution was reached in the experiment at 14.8 μM bacterioopsin and 98% was reached in the experiment at 17.6 μM bacterioopsin. The two arrows labeled 1 and 2 indicate the protein concentrations as determined by the Lowry–Hartree method. They clearly do not coincide with the breaks in the titration curves, which correspond to the points at which all binding sites are occupied. Since one bacteriorhodopsin binds one retinal (Papadopoulos et al., 1978), these points must occur at the protein concentrations. The data of Figure 1 show that the bacteriorhodopsin concentrations, as determined by the method of Lowry–Hartree, are systematically about 20% too large. For relative concentration determinations the latter method is quite useful however. Indeed, the ratio of the two protein concentrations, 1.19, is quite close to the ratio of the two horizontal lines of Figure 1, 1.21 (expected end values). Since every retinal added is bound under the conditions of the experiment, the extinction coefficient of the chromophore can be determined from the slope of the binding curve of Figure 1. A least-squares fit to all the data in the linear range gives an extinction coefficient of 62 700 ± 700 M^{-1} cm^{-1} at a 95% confidence level. The method employed is independent of a knowledge of the protein concentration and does not depend on the attainment of 100% reconstitution, but it relies instead on an accurate knowledge of the retinal concentration (as described under Materials and Methods). From the fact that the binding curve of Figure 1 is linear over the whole range, we may also conclude that within experimental error ϵ is independent of the degree of binding. The two sets of data of Figure 1 were obtained by

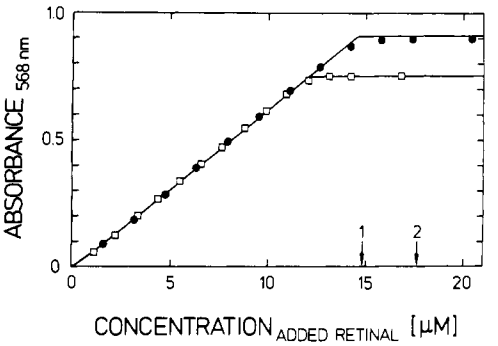

FIGURE 1: Titration of apomembrane with *all-trans*-retinal at high bacteriorhodopsin concentrations. The change in absorbance at 568 nm is plotted as a function of the concentration of the added retinal. The membranes were in the light-adapted state, in 25 mM phosphate buffer, pH 6.9, at 25 °C. (□) Bacteriorhodopsin concentration, 14.8 μM (arrow 1); (●) bacteriorhodopsin concentration, 17.6 μM (arrow 2). The protein concentrations were determined by the method of Lowry–Hartree. The circles represent the average of four measurements; the squares are the average of three measurements. The standard deviation of the error in each point is smaller than the size of the symbols.

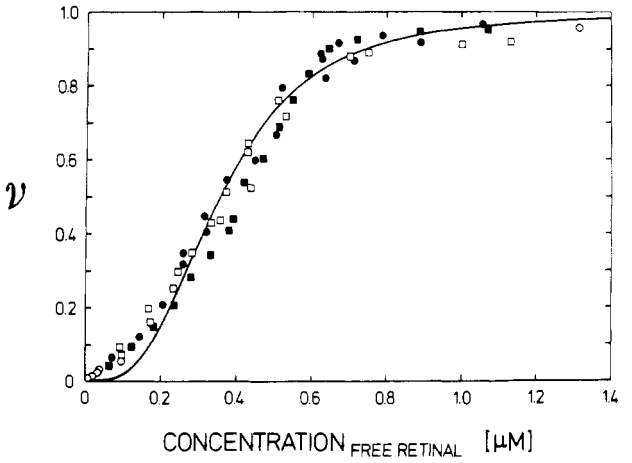

FIGURE 2: Titration of apomembrane with *all-trans*-retinal at low bacteriorhodopsin concentrations. The degree of binding (determined from the change in absorbance at 568 nm by using eq 1) is plotted as a function of the concentration of free retinal. The free retinal concentration was calculated on the basis of eq 2 by using an extinction coefficient of 62 700 M^{-1} cm^{-1}. The titrations were carried out at the following protein concentrations: (●) 0.45; (■) 0.82; (○) 2.32; (□) 2.67 μM. The theoretical curve was calculated on the basis of the all-or-none model (eq 3) with $n = 3.0$ and $K = 2.80 \times 10^6$ M^{-1}.

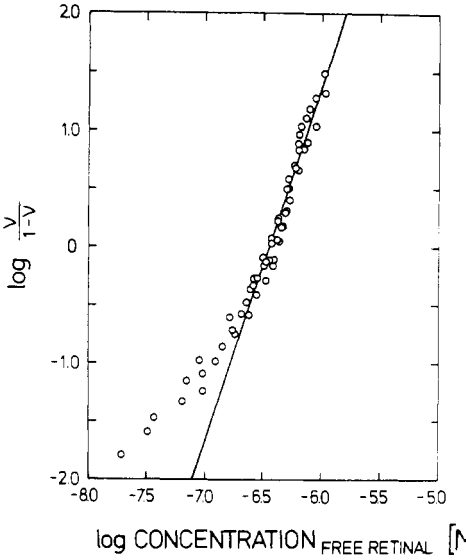

FIGURE 3: Hill plot (eq 4) for the data of Figure 2. The straight line is a least-squares fit to the data, excluding the points at low ν. The slope of this straight line (Hill coefficient) is 3.0 ± 0.2. At low ν the slope is approximately 1.

adding retinal sequentially to a suspension of apomembranes. Single-shot experiments were also performed, in which in one addition of retinal a reconstitution of 70% was reached. The advantage of such experiments is that the total amount of ethanol added could be kept below 0.1% (v/v). The combined result of four single-shot experiments at 70% reconstitution is an extinction coefficient of 61 900 ± 2000 M^{-1} cm^{-1} (the increased error is due to the smaller number of experiments). Thus, we have within experimental error agreement with the much more accurate value from the slope, which is based on a total of 69 data points.

Retinal Binding to the Apomembrane as Monitored by the Change in Absorbance at 568 nm. The results of the spectrophotometric titrations of apomembrane with *all-trans*-retinal at 568 nm are presented in Figures 2 and 3. The absorbance change is measured with respect to the initial absorbance of the apomembrane. The degree of binding, ν, is calculated according to eq 1 by dividing the absorbance change by the absorbance change which would have been reached at 100% reconstitution (calculated from the loss of absorbance due to the bleaching). The free retinal concentration was calculated by using eq 2 with an extinction coefficient of 62 700 M^{-1} cm^{-1} (as determined in the previous section). Figure 2 shows the data from four sets of experiments with protein concentrations varying from 4.5×10^{-7} to 2.67×10^{-6} M. The final absorbance changes at 568 nm for these four protein concentrations were 0.0229, 0.0420, 0.120, and 0.1360. It is clear that, at the low protein concentrations that are required, the final optical densities are quite small. The data shown in Figure 2 have, consequently, considerable error. Nevertheless, it is apparent from Figure 2 that the binding curve is sigmoidal and that the data points at different protein concentrations fall on the same curve. An informative way of plotting the data is in terms of a Hill plot (eq 4), as shown in Figure 3. In the initial binding region at low ν, the Hill plot is linear with a slope of approximately 1. The remainder of the data can be fitted quite well to a straight line with a slope, Hill coefficient, of 3.0 ± 0.2. The association constant determined from the intercept of this straight line with the free ligand axis is equal to 2.80×10^6 M^{-1}. Substituting these values for n and K in eq 3 leads to the theoretical curve of Figure 2. We observe that the all-or-none model gives a good fit to the data except in the low ν region. This is of course to be expected, since we used the Hill coefficient of about 3 over the whole binding range, whereas the Hill plot shows that this coefficient is about 1 at low ν. Alternatively, we may try to fit all the data directly to eq 3. The fit to the low ν data is now slightly better; for the other data it is somewhat worse. The Hill coefficient is of course lower, 2.74, whereas the association constant is changed to 2.75×10^6 M^{-1}. It is a simple matter to construct more complicated models which contain more adjustable parameters and which lead to a better fit. In view of the experimental inaccuracy of our data, however, such an approach is not justified. The all-or-none model, which contains only two parameters, provides an adequate description for our data.

Retinal Binding as Monitored by the Change in CD at 263 nm, at 365 nm, and in the Visible Region. As an alternative and independent method to monitor the binding of *all-trans*-retinal to the apomembrane, we used changes in the CD spectra induced by the binding. This is a very useful method to determine the association constant for the binding of ligands to proteins (Heyn & Weischet, 1975). Since free *all-trans*-retinal is optically inactive, the amount of bound retinal may be determined from the CD if the binding is accompanied by induced optical activity either in the ligand or in the protein. From previous CD studies on the binding of retinal to the apomembrane, it is known that the CD spectrum of the apomembrane displays a strong positive band at 365 nm which is due to membrane-bound retinal oxime (Bauer et al., 1976; Becher & Cassim, 1977). It is likely that the retinal oxime is still close to or in the retinal binding pocket. This is suggested by the strong optical activity at 365 nm, by the observation of significant linear dichroism in this band (Heyn, unpublished results), and by the fact that in time bleached apomembrane slowly turns purple again. Upon reconstitution the 365-nm retinal oxime band decreases in amplitude, consistent with a displacement of the oxime from its binding site. In the near-UV significant changes also occur in the CD spectrum upon retinal binding. A strong band develops at 263 nm which is most likely due to ligand-induced changes in the CD of the aromatic amino acids (Becher & Cassim, 1977; Bauer et al., 1976). The changes in CD at 365 and 263 nm

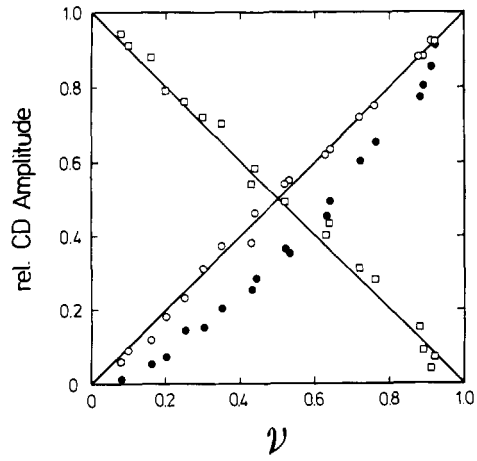

FIGURE 4: Relative change of the CD amplitude at 263 nm (○), at 365 nm (□), and in the visible region (●) as a function of the degree of retinal binding, ν (relative absorbance change at 568 nm). The CD amplitude in the visible region (●) is proportional to the difference in CD at 535 and 605 nm. Whereas the CD amplitudes at 263 nm and in the visible increase with increasing ν, the amplitude of the retinal oxime band at 365 nm decreases with increasing degree of binding.

were used to monitor the binding of retinal to the apomembrane. Titrations were carried out as described under Retinal Binding to the Apomembrane as Monitored by the Change in Absorbance at 568 nm. At each retinal concentration both the absorbance and the CD spectra were measured of the same sample. Within experimental error, these CD binding curves coincided with the binding curve obtained from absorbance measurements (Figure 2). One way to display this agreement is to plot the relative amplitude of the CD changes at these two wavelengths against the degree of regeneration, ν (relative amplitude of the absorbance change at 568 nm). Figure 4 shows that at both wavelengths excellent linear relationships are obtained, implying that all three signals exhibit the same cooperative binding. The data at 263 nm are more accurate than the data at 365 nm which are affected to some extent by the development of a negative band at 320 nm due to bound retinal. In general, the noise in the CD spectra is considerably larger than that in the absorption spectra. The strict proportionality between the increase in absorbance at 568 nm due to retinal binding and the decrease of the 365-nm retinal oxime CD is consistent with the displacement of retinal oxime from a site near or overlapping with the retinal binding site.

The visible region of the CD spectrum has been interpreted in terms of an exciton effect (Heyn et al., 1975; Becher & Ebrey, 1976). The data of Figure 4 in the visible confirm previous experiments (Bauer et al., 1976) in showing that the CD amplitude in this region is not linear in ν. Within the framework of the exciton interpretation, the CD in the visible region is an inappropriate measure for the determination of the retinal association constant, since it is due to interactions between pairs of bound retinal.

Retinal Binding to Bacterioopsin Monomers as Monitored by the Change in Absorbance at 555 nm. It is desirable to compare the binding of retinal to the apomembrane with the case of binding to bacterioopsin monomers, since it is to be expected that in the absence of protein–protein interactions the binding will be hyperbolic with a Hill coefficient of 1. Bleached monomers can in principle be prepared in two ways. One is solubilization of the purple membrane to the stage of monomers, followed by bleaching with hydroxylamine. The resulting product cannot be regenerated. The other way, bleaching followed by solubilization in Triton X-100, was described under Materials and Methods. Unfortunately, the regenerability of these bleached monomers rapidly decreases with time. The kinetics of the loss of regenerability could be described well by a single exponential with a half-life of about 70 min. In view of this problem, no sequential titrations could be performed. Instead, different amounts of *all-trans*-retinal were added simultaneously to a large number of identical bacterioopsin samples. At high bacterioopsin concentrations an extinction coefficient for retinal bound to Triton X-100 solubilized bacterioopsin monomers could be determined from the linear part of the binding curve. Good linear plots were obtained similar to Figure 1. An extinction coefficient of 50 900 M^{-1} cm^{-1} for the light-adapted monomer was determined in this way. It was recently estimated that, after correction for light scattering, the absorbance of Triton X-100 solubilized bacteriorhodopsin is 19% less than that of bacteriorhodopsin in the purple membrane (Dencher & Heyn, 1978). The present value is in good agreement with this observation. Since at most 69% regeneration could be achieved and since the regenerability was strongly time dependent, it was not possible to do a proper titration experiment at low bacterioopsin concentration.

Retinal$_1$–Retinal$_2$ Competition Experiments. These experiments were performed to determine whether retinal$_1$ can displace bound retinal$_2$ and vice versa. If the binding of these ligands is not irreversible, as our results suggest, and if the two association constants do not differ too much, then such displacements ought to be observable in both directions. *all-trans*-Retinal$_2$ has one more double bond in the β-ionone ring than *all-trans*-retinal$_1$, leading to a red shift with respect to *all-trans*-retinal$_1$ in the absorption bands. In the light-adapted state, bound *all-trans*-retinal$_1$ absorbs at 568 nm, whereas bound *all-trans*-retinal$_2$ absorbs at 603 nm (Tokunaga & Ebrey, 1978). Thus, retinal$_1$–retinal$_2$ exchange can in principle be easily observed from a shift in the absorption spectra. Identical apomembrane samples were regenerated with retinal$_1$ or retinal$_2$. Care was taken that 100% regeneration was reached, so that no empty binding sites remained. To the apomembrane fully regenerated with retinal$_1$ a 1.13-fold excess of retinal$_2$ was added; to the apomembrane fully regenerated with retinal$_2$ a 1.13-fold excess of retinal$_1$ was added. The change in absorbance was observed in time. The experiments were performed at room temperature in 0.025 M phosphate buffer, pH 6.9. The exchange process is very slow, taking place on the time scale of weeks. Exchange occurred in both samples. Whereas the blue membrane (apomembrane reconstituted with retinal$_2$) turned clearly purple, the purple membrane (apomembrane reconstituted with retinal$_1$) changed its color only slightly in the blue direction, at least over the period of observation (12 weeks). Had we added equal amounts of retinal$_1$ and retinal$_2$ in both experiments, the final state and color of the system should have been the same if equilibrium had been reached. We conclude from our experiments that the binding of retinal$_1$ is stronger than that of retinal$_2$ but that neither is bound irreversibly.

Discussion

The main experimental finding of this paper, the sigmoidal binding curve for retinal binding to the apomembrane, is based on absorbance measurements at 568 nm and on CD measurements at 365 and 263 nm. These signals monitor different events caused by the binding. The absorbance change at 568 nm is caused by the formation of the protonated Schiff base bond of retinal to an ε-amino group of bacteriorhodopsin which shifts the retinal absorbance from 380 to 568 nm. The change in CD at 365 nm is due to the displacement of retinal oxime by the binding of retinal from a site in which it has large

optical activity or to a change in the chirality of the retinal oxime environment induced by the retinal binding. The actual location of the retinal oxime binding site remains unclear. Although our observations on the CD and linear dichroism of retinal oxime indicate that it occupies the retinal binding site, an allosteric mechanism in which binding of retinal affects the binding and chirality of retinal oxime at some other site cannot be excluded. The change in CD at 263 nm is probably due to a change in the tertiary structure of bacterioopsin, which is induced by the binding and which leads to a change in the optical activity of the aromatic amino acids. All three signals lead to the same cooperative binding curve. The extinction coefficient itself was shown to be independent of the degree of binding, thereby excluding the trivial explanation of our results. The evidence which we presented can therefore be most easily interpreted as cooperative binding of retinal to the apomembrane, due to protein–protein interactions within bacteriorhodopsin trimers. It should be borne in mind, however, that the Hill coefficient of approximately 3 is only suggestive of trimers. The size of the interacting cooperative unit could be larger. From the structural point of view, however, the trimer seems most likely. Recently, some evidence was presented which is consistent with the existence of significant protein–protein interactions. In regeneration experiments it was discovered that the rate of light–dark adaptation depends strongly on the degree of binding (Ebrey et al., 1977). At a low degree of binding the rate constant for this process was 1 order of magnitude faster than that in fully regenerated apomembrane. Similar results were recently also reported for the brown membrane (Peters & Peters, 1978). These experiments demonstrate that the rate constant of this isomerization reaction of retinal depends on the state of occupancy of the array of retinal binding sites, in harmony with the existence of considerable protein–protein interactions. In CD and absorbance experiments on the bleaching of the purple membrane in the presence of hydroxylamine, the tendency was noted for the chromophores to bleach simultaneously in clusters of three (Becher & Cassim, 1977). Furthermore, it appears that the rate constants for the decay of the M intermediate of the photochemical cycle depend on the relative number of protein molecules in that state (Hess et al., 1978). The photocycle kinetics of bacteriorhodopsin thus seems to depend on the state in the photocycle of its nearest neighbors. These kinetic results were also interpreted in the framework of a cooperative trimer model. That cooperative effects are occurring is not so surprising when we recall that the apomembrane shows no long-range protein order, whereas the fully regenerated apomembrane has a hexagonal protein lattice (Henderson, 1977). A crystallization induced by ligand binding seems to occur.

The model we choose to represent the data is to some extent arbitrary. The all-or-none model is the most extreme cooperative model available in which the n bacterioopsin molecules bind retinal simultaneously; no intermediates with less than n occupied sites occur. Real systems never show such extreme cooperativity over the whole binding range. In fact, in general, a slope of 1 is expected in the Hill plot at low ν (van Holde, 1971), as is indeed observed. The model described by eq 3 is of course the limiting case of the more general Adair equation which contains three association constants for the present case. More complicated models, however, are only sensible when more accurate data are available.

It is appropriate to compare the value of our extinction coefficient at 568 nm for the light-adapted state of the purple membrane, 62 700 ± 700 M^{-1} cm^{-1}, to the value most commonly used, 63 000 M^{-1} cm^{-1} (Oesterhelt & Hess, 1973). At first sight these values appear to be almost equal so that except for accuracy not much seems to be gained. There are, however, two important differences. In contrast to our value of 62 700 ± 700 M^{-1} cm^{-1}, the value of 63 000 M^{-1} cm^{-1} (also obtained at 568 nm) refers to the dark-adapted membrane (Oesterhelt, private communication) and includes the contribution from light scattering. Since the extinction coefficient in the dark-adapted state is less than that in the light-adapted state and since light scattering increases the effective extinction coefficient, these two effects compensate to some extent. Nevertheless, an extinction coefficient which includes the appreciable contribution from light scattering (up to 20%) is not a well-defined quantity. Concentrations determined with it will depend on the preparation of the sample (amount of membrane aggregation) and on the extent to which the particular spectrometer used happens to correct for light scattering. A value of 65 000 M^{-1} cm^{-1} at 568 nm and corrected for light scattering was recently determined for retinal binding to the brown membrane in the light-adapted state (Peters & Peters, 1978). Although this value is rather close to ours, it is not directly comparable since the apobrown membrane contains no retinal oxime and since the brown membrane is thought to be noncrystalline [R. Henderson, as quoted in Cherry et al. (1977)].

Several attempts have been made to determine the extinction coefficient by dividing the absorbance of the reconstituted apomembrane by the protein concentration. Even if the protein concentration can be determined accurately, any contribution from protein other than bacteriorhodopsin will lead to an extinction coefficient which is too low. Traces of contaminating proteins at the edges of the purple membrane patches may contribute to this effect. The major contribution will, however, come from bacterioopsin itself, if the reconstitution is not quite complete. The present method does not have these disadvantages.

It was mentioned that single-shot experiments also lead to the same extinction coefficient. If a series of such experiments were done at differing degrees of regeneration and at protein concentrations so low that already some curvature is observable in the binding curves at high ν, an extinction coefficient would be obtained which decreases with increasing percentage reconstitution.

The systematic overestimate in protein concentration according to Lowry–Hartree by about 20% is not surprising, since this method depends on the aromatic amino acid content. It does mean, however, that a number of values which are based on it will have to be revised.

In connection with the CD experiments, it is appropriate to mention that, if the binding really occurred in an all-or-none fashion with $n = 3$ over the whole range and if the exciton CD were entirely due to bacteriorhodopsin trimers, the dependence of the visible CD amplitude on ν in Figure 4 should have been linear. The visible CD spectrum of covalent bacteriorhodopsin trimers, cross-linked with dimethyl 3,3′-dithiobis(propionimidate) (DTBP), has qualitatively the same exciton features as the CD spectrum of the purple membrane (Heyn & Dencher, unpublished results). It is therefore likely that this discrepancy is due to the fact that, at a low degree of binding, the Hill coefficient is actually close to 1, causing a threshold in the appearance of the exciton CD. At higher ν the increase could well be linear. The whole curve would then appear to curve upward, as observed.

We emphasize that the association constant of 2.8×10^6 M^{-1} refers to binding to the apomembrane which contains

retinal oxime. It is conceivable that binding to the brown membrane in vivo occurs with a larger association constant. It is well-known that regeneration is a complex reaction with many intermediates (Schreckenbach et al., 1977, 1978). What we have determined is an overall effective association constant. Its value is surprisingly low. The $retinal_1$–$retinal_2$ exchange experiments confirm, however, that the binding is by no means irreversible. Recent findings on the competitive inhibition of the binding of retinal to the apomembrane by a C_{18}-ketone analogue of retinal are in agreement with this conclusion (Ovchinnikov et al., 1979). Regeneration of bacteriorhodopsin by retinal could be completely blocked by this analogue, and it was concluded that it forms a stronger complex with bacterioopsin than retinal.

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