Circular Dichroism of Halorhodopsin: Comparison with Bacteriorhodopsin and Sensory Rhodopsin I†

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ABSTRACT: Circular dichroic (CD) spectra of three related protein pigments from *Halobacterium halobium*, halorhodopsin (HR), bacteriorhodopsin (BR), and sensory rhodopsin I (SR-I), are compared. In native membranes the two light-driven ion pumps, HR and BR, exhibit bilobe circular dichroism spectra characteristic of exciton splitting in the region of retinal absorption, while the phototaxis receptor, SR-I, exhibits a single positive band centered at the SR-I absorbance maximum. This indicates specific aggregation of protein monomers of HR, as previously noted [Sugiyama, Y., & Mukohata, Y. (1984) *J. Biochem.* (*Tokyo*) *96*, 413–420], similar to the well-characterized retinal/retinal exciton interaction in the purple membrane. The absence of this interaction in SR-I indicates SR-I is present in the native membrane as monomers or that interactions between the retinal chromophores are weak due to chromophore orientation or separation. Solubilization of HR and BR with nondenaturing detergents eliminates the exciton coupling, and the resulting CD spectra share similar features in all spectral regions from 250 to 700 nm. Schiff-base deprotonation of both BR and HR yields positive CD bands near 410 nm and shows similar fine structure in both pigments. Removal of detergent restores the HR native spectrum. HR differs from BR in that circular dichroic bands corresponding to both amino acid and retinal environments are much more sensitive to external salt concentration and pH. A theoretical analysis of the exciton spectra of HR and BR that provides a range of interchromophore distances and orientations is performed. By use of a trimer model for BR, chromophore–chromophore separations are found to be in close agreement with neutron and electron diffraction data. The HR data could be fit to either a dimer or trimer model. The dimer fit gives interchromophore distances closer to that of BR than the trimer fit.

Membranes of the archaebacterium *Halobacterium halobium* contain at least four retinal-linked light-activated proteins that perform varied cellular functions. Energy from light absorbed by the retinal chromophores of the proton pump bacteriorhodopsin (BR) and the chloride pump halorhodopsin (HR) drives translocation of these ions across the membrane, allowing maintenance of the electrochemical gradient under conditions of low oxygen (Stoeckenius & Bogomolni, 1982; Lanyi, 1986). The phototaxis receptors sensory rhodopsin I (SR-I) (Spudich & Bogomolni, 1984) and sensory rhodopsin II (SR-II) (Takahashi et al., 1985; Wolff et al., 1986; Spudich et al., 1986) transduce light energy into signals to the flagellar motor and enable cells to accumulate in regions of optimum light.

Comparative studies of the properties of these molecules are needed to understand how they use the same chromophore to mediate diverse phototransduction processes. Of the bacterial rhodopsins, only BR has been characterized extensively with respect to its structure, by both diffraction and spectroscopic methods (Stoeckenius & Bogomolni, 1982). Purification procedures for halorhodopsin (Steiner & Oesterhelt, 1983; Taylor et al., 1983; Sugiyama & Mukohata, 1984; Maeda et al., 1985) permit application of some of these techniques to HR. Mutants producing BR, HR, or SR-I as their predominant pigments (Spudich & Spudich, 1982, 1985; Spudich et al., 1986) facilitate structural studies in the native membrane.

Circular dichroism (CD) spectroscopy provides a sensitive technique for detecting changes in protein structure. Specific features of the BR CD spectrum have been correlated with structural features for aggregated and detergent-solubilized preparations (Heyn et al., 1975; Kreibel & Albrecht, 1976; Becher & Cassim, 1976; Ebrey et al., 1977; Dencher & Heyn, 1978; Mao & Wallace, 1984; Jap et al., 1983). Theoretical analysis of CD effects due to exciton splitting has yielded techniques for determining distances between chromophores in aggregates (Sauer et al., 1966; Tinoco, 1970; Kreibel & Albrecht, 1976; Ebrey et al., 1977; Shepanski & Knox, 1981). CD measurements of HR in the visible region of the spectrum (Sugiyama & Mukohata, 1984) and in the UV (Jap & Kong, 1986) have indicated similarities with BR, the former group reporting aggregation-specific transitions in HR, which are confirmed and extended in this study. We have analyzed HR and BR retinal CD spectra in order to compare interchromophore distances in aggregated forms of the two proteins. Changes in the CD of HR with variations in salt, pH, and illumination are compared to changes in BR under similar conditions, and the HR and SR-I spectra in native membranes are examined.

Materials and Methods

Chemicals. *N*-(2-Hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES),[1] MOPS, Triton X-100, DNase,

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[1] Abbreviations: HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; DNase, deoxyribonuclease I; CCCP, carbonyl cyanide (*m*-chlorophenyl)hydrazone.

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CCCP, and *n*-octyl β-D-glucopyranoside (octyl glucoside) were obtained from Sigma. Phenyl-Sepharose CL-4B was from Pharmacia; Bio-Gel hydroxylapatite was from Bio-Rad Laboratories.

Strains and Pigment Isolation. Bacteriorhodopsin (BR) was isolated from strain S9-P (Stoeckenius et al., 1979) of *Halobacterium halobium* as described (Becher & Cassim, 1975). BR was solubilized by incubation in 0.3% Triton X-100 and 25 mM sodium phosphate buffer, pH 7.0, for 24 h at room temperature in the dark. Halorhodopsin (HR) was purified from the carotenoid deficient BR^- *H. halobium* strain OD2W, derived by isolating a spontaneous white colony from OD2 (Spudich & Spudich, 1982). Purification of HR was by modification of the enrichment steps developed by Steiner and Oesterhelt (1983) and Taylor et al. (1983) as follows. Twelve liters of cells cultured as described (Spudich & Spudich, 1982) in peptone medium to 2×10^9/mL were washed in a basal salt solution (Weber et al., 1982), resuspended in 225 mL of 4 M NaCl and 10 mg of DNase, and dialyzed against 12 L of 50 mM HEPES, pH 7.0, 4 °C, for 18 h. After centrifugation (6000*g*, 20 min, 4 °C) to remove cell debris, membranes in the supernatant were concentrated by centrifugation (200000*g*, 90 min, 4 °C), washed in 200 mL of the buffer, and resuspended in 60 mL of 4 M NaCl. Membranes were solubilized by addition of 115 mL of 4 M NaCl and 10 mM MOPS, pH 7.0 (buffer A), containing 30 mM octyl glucoside, with stirring for 1 h in the dark at 4 °C. After centrifugation (200000*g*, 90 min, 4 °C), the supernatant was applied to a phenyl-Sepharose column (2.5-cm diameter, 100 mL) equilibrated with buffer A (with 30 mM octyl glucoside) and eluted with buffer A (with 15 mM octyl glucoside). Eluted purple fractions were pooled, concentrated, and loaded on a hydroxylapatite column (2.5-cm diameter, 50 mL, equilibrated in buffer A with 15 mM octyl glucoside). Purple fractions eluted from the hydroxylapatite column with this buffer were pooled, concentrated, and loaded onto a second phenyl-Sepharose column (2.5-cm diameter, 60 mL, equilibrated with buffer A with 15 mM octyl glucoside). Absorbance measurements of fractions from this column revealed two elution peaks of HR, differing in the presence of a 410-nm absorbance band in the slower eluting fractions. The ratio of 280- to 580-nm absorbance in the pooled faster eluting fractions was 1.8; after storage for 1 week at 4 °C and dialysis to remove detergent, this ratio was 1.9 (Figure 4). The yield of the faster eluting HR in a typical preparation was 600 μg/L of culture, assuming an extinction coefficient of 50 000 M^{-1} cm^{-1}. HR in these pooled fractions is able to catalyze light-driven electrogenic transport when reconstituted into liposomes as described below.

Cell envelope vesicles were prepared as described (MacDonald & Lanyi, 1979) from strains OD2W and Flx5R. Flx5R is a retinal-lacking strain that after reconstitution with *all-trans*-retinal contains SR-I as the only retinal pigment detectable by flash photolysis (Spudich et al., 1986). Flx5R vesicles prepared from 1.8 L of cells were concentrated to 4 mL in 4 M NaCl. Fifty microliters of 4.3 mM *all-trans*-retinal was sufficient to saturate the SR-I apoprotein and generated an absorbance of 0.4 at 587 nm. In experiments involving membrane vesicles, light-scattering evident in the preparations may distort the spectra with regard to peak maxima and bandwidth.

Reconstitution of HR-Mediated Transport in Liposomes. Asolectin liposomes (20 mg/mL in 1 M NaCl) were prepared in a bath-type sonicator (Heat Systems-Ultrasonics, Inc.) as described (Hasselbacher et al., 1984). Reconstitution of HR into liposomes was based on a previous procedure (Bogomolni et al., 1984). Concentrated HR (0.5 mL, A_{578} = 1.0), pooled from the faster eluting fraction above, and 0.5 mL of the liposomes were dialyzed separately for 24 h against three changes of 0.5 L of 500 mM NaCl. The HR and liposomes were then vortexed together with octyl glucoside from a 500 mM stock solution (2% final concentration), incubated for 1 h on ice in the dark, and dialyzed for 72 h in 200 mL of 0.5 M NaCl with changes every 8 h. HR activity in these liposomes was monitored as light-induced, CCCP-enhanced (15 μM final concentration), passive proton efflux by use of a Beckman/Altex combination pH electrode No. 531167 as described (Spudich & Spudich, 1982). The 1-mL liposome suspension was brought to 2 mL with 0.5 M NaCl, and the sample was placed in a 1-cm path length cuvette, stirred, and thermostated at 37 °C.

Spectroscopic Measurements. Flash spectroscopy of SR-I in vesicle suspensions was as described (Spudich et al., 1986). HR content in vesicle suspensions was quantitated by monitoring generation of its 520-nm-absorbing photointermediate with the same instrument (monitoring wavelength 500 nm, actinic flash through a 600 nm long pass filter, 20 μs/point, 3968 points/sweep). CD spectra were measured on a Jasco J-500C spectropolarimeter. Spectra of purified HR were determined by using a cell with a 1-cm path length. Spectra of envelope vesicles were collected in a 1-mm path length cell moved to within 5 cm of the photomultiplier in order to increase collection of scattered light.

Results

Experimental Results

Purified HR and BR. Purified HR and BR in 4 M NaCl and 10 mM HEPES, pH 7.0, share similarities in CD in all spectral regions (Figure 1a). A bilobe in the 450–650-nm region that has been attributed to exciton interactions between chromophores in BR is evident in both preparations, indicating that under these salt and pH conditions both BR and HR are organized into specific aggregates. The nonconservative bilobe spectrum exhibited by BR has been described as the sum of a split positive band due to the intrinsic optical activity of retinal in its apoprotein environment and a band split into positive and negative components with a crossover at the BR absorbance maximum, caused by exciton coupling between neighboring chromophores (Muccio & Cassim, 1979; Kriebel & Albrecht, 1976; Ebrey et al., 1977). The detergent-solubilized BR preparation (Figure 1b) shows the intrinsic optical activity free from exciton-splitting effects. The superposition of the intrinsic and excitonic bands results in a crossover wavelength red-shifted from the BR absorbance maximum. The HR bilobe, in contrast, is symmetrical about the HR absorbance maximum. An intrinsic, positive HR CD band is evident after elimination of the excitonic coupling between HR chromophores by solubilization of purified HR in 15 mM octyl glucoside (Figure 1b).

Bands in the 250–300-nm regions of the HR and BR CD spectra can be attributed to π–π^* transitions of tryptophan, charged and uncharged tyrosine residues, phenylalanine, and dipole coupling between these amino acid transitions and retinal π–π^* transitions (Becher & Cassim, 1976). Bands for HR and BR are similar in this region for both aggregated and solubilized preparations. One apparent difference in the detergent-solubilized spectra of BR and HR (Figure 1b) is in the sign of a group of bands between 280 and 300 nm. These bands are negative in the spectrum of BR solubilized in 25 mM phosphate and 0.3% Triton X-100 and positive in the

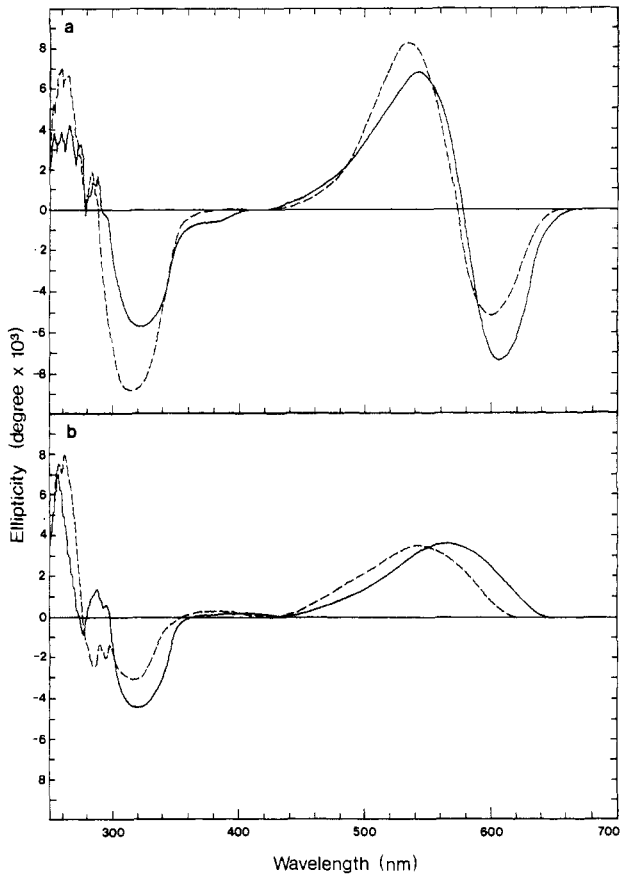

FIGURE 1: (a) Circular dichroism spectra of purified bacteriorhodopsin (---) and halorhodopsin (—) in 4 M NaCl and 10 mM HEPES, pH 7.0. Absorbance of bacteriorhodopsin (at 568 nm) and halorhodopsin (at 578 nm) = 1.0. (b) Circular dichroism spectra of detergent-solubilized purified bacteriorhodopsin (---) (0.3% Triton X-100 and 25 mM phosphate, pH 7.0) and halorhodopsin (—) (1.25% octyl glucoside, 4 m NaCl, and 10 mM MOPS, pH 7.0). Absorbance of BR (at 568 nm) and HR (at 578 nm) = 1.0.

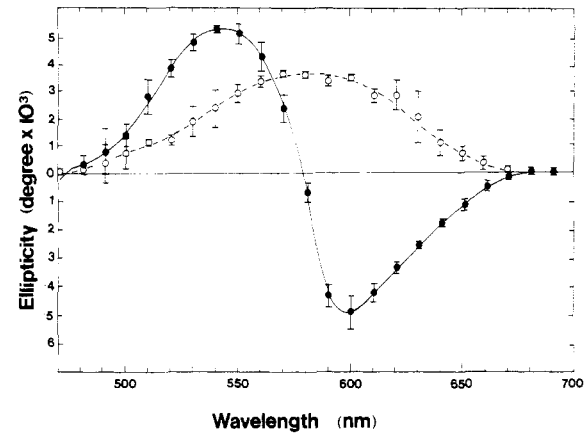

FIGURE 2: Circular dichroism spectra of cell envelope vesicles of halobacterial strains OD2W and Flx5R. Flx5R was reconstituted with *all-trans*-retinal. Envelope vesicles prepared from OD2W cells were concentrated until light-scattering changes between 700 and 750 nm matched light-scattering changes of Flx5R envelope vesicles with no added retinal. Ellipticities of the unreconstituted Flx5R vesicles were then subtracted at 2-nm intervals from reconstituted Flx5R vesicles and the OD2W vesicles to obtain the CD spectra, which were smoothed (3-point averaging). A point every 10 nm from two independent measurements was averaged and plotted. The error bars indicate the range of the two values. Protein concentrations in both preparations were approximately 30 mg/mL; A_{580} after subtraction of the unreconstituted Flx5R blank was 0.9 for OD2W vesicles and 0.4 for Flx5R with retinal. Ratio of HR/SR in the OD2W preparation was approximately 3 as estimated by flash photolysis.

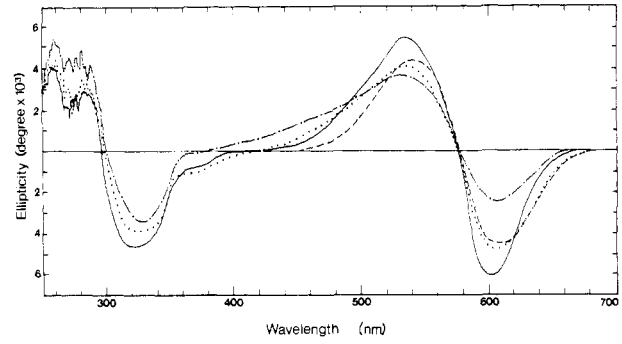

FIGURE 3: Circular dichroism spectra of purified halorhodopsin in varying concentrations of NaCl. HR in 3 m NaCl, pH 7.0 (—); HR in 1 M NaCl, pH 7.0 (···); HR in 100 mM NaCl, pH 7.0 (-·-); HR in 3 M NaCl, pH 7.0, dialyzed from 100 mM NaCl (---). Absorbance at 578 nm of all samples = 1.0.

spectrum of HR solubilized in 4 M NaCl, 10 mM MOPS, and 1.25% octyl glucoside. This change of sign may be dependent on NaCl concentration; these bands are negative in spectra of purple membrane suspended in distilled water (data not shown) and positive when purple membrane is suspended in 4 M NaCl (Figure 1a). One qualitative difference in the HR and BR aggregate CD spectra is the presence of a band appearing as a shoulder on the 320-nm band in the HR spectrum that has no counterpart in the BR spectrum. The origin of this new peak is unknown.

HR and SR-I in Native Membranes. Figure 2 compares the visible CD spectra of cell envelope vesicles of mutant halobacterial strains lacking BR and containing either HR (OD2W) or SR-I (Flx5R) as the major photoactive pigments (monitored by flash photolysis). While HR monomers interact in the membranes, as evidenced by the exciton bands in the spectrum, SR-I shows no evidence of such excitonic coupling. In the figure, SR-I was generated by addition of *all-trans*-retinal to vesicles. No difference in the SR-I content or the general shape of the Flx5R spectrum is observed when retinal is added during growth of the culture (data not shown).

Salt and pH Effects on HR. HR exhibits major changes in the CD spectrum with varying concentrations of NaCl. Decrease of NaCl concentration from 3 to 1 M results in a small decrease in the amplitudes of the exciton and 320-nm bands but no change in the near-UV region (Figure 3). A further reduction in the NaCl concentration to 100 mM affects all regions of the spectrum. In particular, the positive and negative lobes of the exciton peaks become nonconservative, with the positive lobe more pronounced. Dialysis of this sample into 3 M NaCl restores the conservative bilobe spectrum, but the original magnitudes of the bands are not recovered. Further evidence for irreversible structural change after low-salt treatment of purified HR is seen in the far-UV region of the CD spectrum (data not shown). Similarities in the spectra of BR and HR in this region have been observed (Jap & Kong, 1986) and attributed to similarities in protein secondary structure. After incubation of HR in 100 mM NaCl, loss of ellipticity at 222 nm occurs that cannot be restored by resuspension of the protein in 4 M NaCl, indicating that HR, in contrast to BR, is susceptible to denaturation in conditions of low NaCl.

The CD spectrum of BR is relatively insensitive to changes in pH between pH 7 and 9 (Muccio & Cassim, 1979). HR exhibits small but readily detectable pH-dependent changes in all regions of the CD spectrum, indicating that the conformation of HR is more easily modified by the protonation state of residues accessible to the external environment. Absorbance and CD spectra for purified HR (OD_{578} = 0.9)

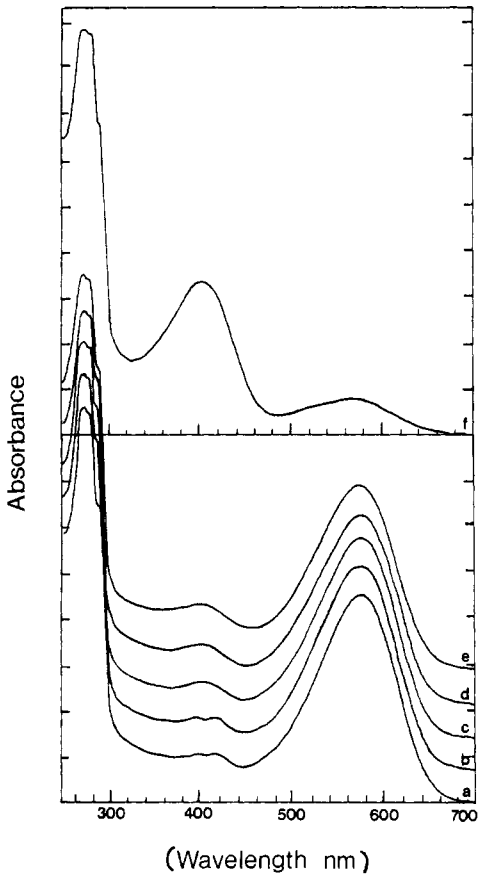

FIGURE 4: Absorbance spectra of purified halorhodopsin in 4 M NaCl after pH was varied by addition of microliter amounts of 1 N NaOH with stirring: (a) pH 7.0; (b) pH 8.0; (c) pH 8.5; (d) pH 8.8; (e) pH 9.0. (f) Purified HR at pH 9.0 was illuminated for 10 min with a xenon arc lamp (Oriel Corp. Model 66030), using an orange (Corning 3-69) filter, IR-absorbing filter, and a 1-cm path length of saturated $CuSO_4$ solution. The sample was protected from room light after bleaching. Absorbance was measured by using a Cary 219 UV-vis spectrophotometer. Each division on the vertical axis equals 0.2 absorbance unit.

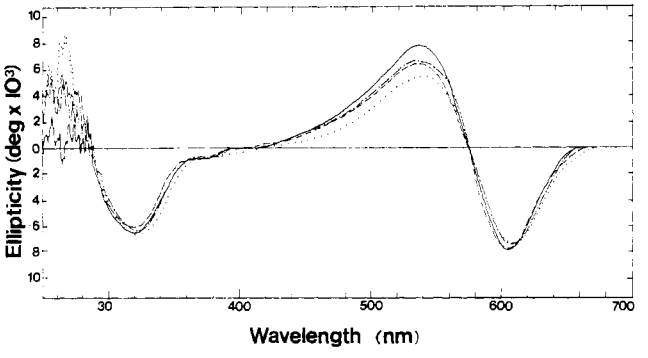

FIGURE 5: Circular dichroism spectra of purified halorhodopsin in 4 M NaCl after pH was varied as in Figure 4: pH 7.0 (—); pH 8.0 (-·-); pH 8.5 (---); pH 8.8 (···).

in 4 M NaCl at various pH values are compared (Figures 4a–e and 5). Absorbance changes with increasing pH are slight and consist mainly of enhancement of a peak in the 400–420-nm region (Figure 4a–e). pH-induced perturbation both of the chromophore's environment and of changes in apoprotein structure are more evident in the CD than in the absorbance spectra. With increasing pH, the exciton-coupled bands become nonconservative, with the negative lobe becoming more pronounced. Changes in the 250–300-nm region are seen, particularly enhancement of a band at approximately 267 nm at pH 8.8. The CD spectrum at pH 9.0 (Figure 6a) shows

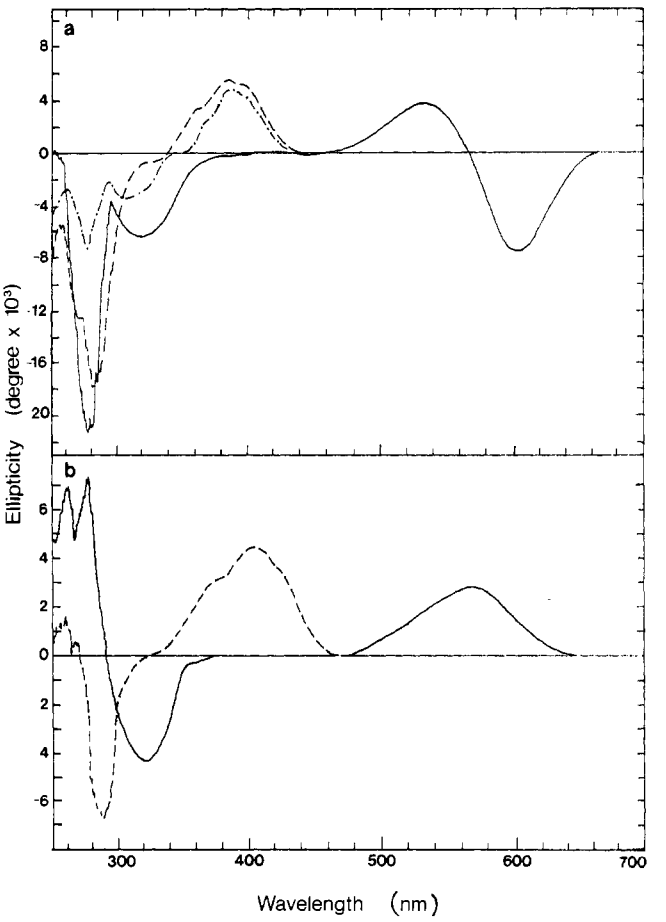

FIGURE 6: (a) Circular dichroism spectra of purified halorhodopsin in 4 M NaCl, pH 9.0, before (—) and after (---) generation of a blue-shifted absorbing species by illumination as described in Figure 4. CD spectrum of purified HR after 18 h of dialysis in 0.5 M phosphate buffer, pH 8.8 (-·-). (b) Circular dichroism spectra of octyl glucoside solubilized, purified HR in 4 M NaCl, pH 9.0, before (—) and after (---) generation of blue-shifted absorbing species by illumination as described in Figure 4.

a major negative band appearing near 280 nm. These effects suggest pH-induced changes in orientation of aromatic amino acid side chains.

Sustained illumination of purified HR at alkaline pH converts HR_{578} to a stable species absorbing near 410 nm (Ogurusu et al., 1981; Lanyi & Schobert, 1983; Figure 4f). The CD spectrum after 10 min of illumination shows a major band near 400 nm and complete loss of ellipticity at wavelengths greater than 440 nm with concomitant disappearance of the 320-nm negative band (Figure 6a). A similar result is obtained with octyl glucoside solubilized HR (Figure 6b).

A species absorbing at 410 nm can be generated in the dark at alkaline pH by suspension of purified HR in chloride-free salt (Lanyi, 1986). The kinetics of recovery of HR_{578} upon lowering the pH differs from this 410-nm species and the species with similar absorption generated by illumination at high chloride concentration (Lanyi, 1986). The CD spectrum of the low-chloride, 410-nm-absorbing material (Figure 6a) is similar to the CD spectrum of the 410-nm species generated by illumination in the region of the main absorbance band, while differences in CD bands between 250 and 300 nm indicate different environments for aromatic amino acid residues in the two 410-nm forms.

Theory

A number of attempts have been made to analyze exciton CD effects in biological systems in terms of chromophore

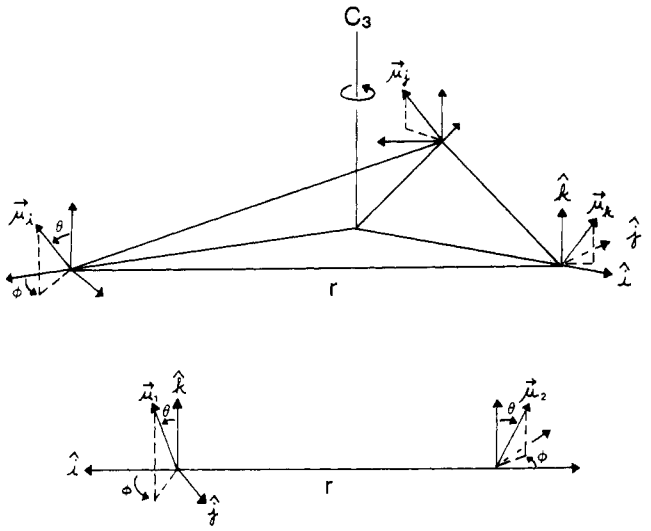

FIGURE 7: Coordinate system and definition of variables for the trimer and dimer exciton models. The electric transition dipole moment vector, μ, is oriented at an angle, θ, normal to the membrane plane, k axis. Note that the angle retinal makes with the membrane plane is commonly reported. This corresponds to $\pi/2 - \theta$. The angle, ϕ, is the angle formed by the i axis with the projection of μ onto the plane of the membrane, $i - j$ plane. The distance between chromphores i and j is given by r. Note that the dimer is antiparallel and the trimer has P_3 symmetry.

separation and orientation (Sauer et al., 1966; Tinoco, 1970; Kriebel & Albrecht, 1976; Ebrey et al., 1977; Shepanski & Knox, 1981). Typically, a combination of absorbance and circular dichroism data is used to determine the parameters of the system. A crucial parameter in such analysis is the dipolar interaction potential V. This is often estimated by using shifts in absorbance peaks or shoulders on absorbance peaks observed upon chromophore aggregation. The circular dichroism spectrum is then used to put constraints on the structural parameters r, θ, and ϕ. See Figure 7 for the definition of these parameters in a dimer and trimer system. These analyses have given large values for V and result in unrealistically short interchromophore separations r (Kriebel & Albrecht, 1976; Ebrey et al., 1977). There are theoretical reasons why these simple models may be inappropriate, and more realistic values of r may be obtained by including interactions between dimers or trimers (Scherz & Parson, 1986). An alternate explanation for the unrealistic parameters obtained with the simple models may be that the experimentally determined exciton energies are inaccurate. In the past, a comparison between monomer absorbance and aggregate absorbance spectra was used to calculate these energies. This practice is avoided in this work as it is difficult to assess absorbance changes due to exciton contributions and those due to conformational changes. Instead, the exciton energy is determined by fitting the CD spectrum to a pair of peaks, each with a Gaussian distribution with respect to energy. The absorbance data are used only to determine the dipolar strength.

The retinal CD spectrum in units of molar ellipticity, $\theta(\lambda)$, was analyzed by fitting the spectrum to the function

$$\theta(\lambda) = A_1 \exp[-(1/\lambda - 1/\lambda_{co} + \Delta)^2/B^2] + A_2 \exp[-(1/\lambda - 1/\lambda_{co} - \Delta)^2/B^2] \quad (1)$$

where the amplitudes, A_1 and A_2, are of opposite signs, λ_{co} is at the crossover wavelength, Δ is the exciton interaction energy, and B is the spectral bandwidth. This function could fit the experimental data to within the noise level by using A_1, A_2, Δ, and B as adjustable parameters and by estimating λ_{co} from the spectrum. A plot showing the residuals for this fitting procedure for the HR and BR spectrum is available in the supplementary material. The rotational strength for each band (R_1 or R_2) is calculated by using all the fitted parameters as described previously (Schellman, 1975). The exciton contribution, $R_\pm$, to the rotational strength of a given band is $R_\pm = \pm(R_1 - R_2)/2$ [cf. Cantor and Schimmel (1980)]. This eliminates any contribution from the intrinsic CD. Comparing the calculated $R_\pm$ values with the intrinsic CD value of 0.5 Debye–Bohr magnetons (Ebrey et al., 1977) for bacteriorhodopsin shows that the intrinsic CD contributes less than 5% to the rotational strength of a given lobe. Thus, it is possible to have a nearly conservative aggregate spectrum even when a significant monomer spectrum exists. Because the curve-fitting functions are Gaussian with respect to energy and not wavelength, it is also possible to have asymmetric spectra that are accurately fit by this method.

Table I: Fitted Parameters from CD Spectra[a]

	R[b]	D[c]	B[d]	Δ[e]	λ_{co}[f]
HR	16 ± 1	93	22.0 ± 0.9	930 ± 37	17360
BR	78 ± 9	96	16.4 ± 1.1	1860 ± 130	17316

[a] All energies (Δ, β, λ_{co}) are in units of cm^{-1}; errors in these parameters were determined from the nonlinear least-squares fitting routine. [b] R, rotational strength in Debye–Bohr magnetons. [c] D, dipolar strength in (Debye)^2 estimated from absorbance spectra. [d] Δ, exciton splitting energy. [e] B, spectral bandwidth. [f] λ_{co}, crossover wavelength.

The Gaussian curve-fitting analysis gives Δ values that are significantly smaller and R values that are significantly larger than those obtained from previous analyses. The R and Δ values are used to establish all possible values for the retinal orientation (θ, ϕ) and retinal–retinal separation, r, for either a dimer or trimer exciton model. In contrast to previous analyses, these values are not uniquely determined. This is a result of not using the absorbance data to determine Δ. Our approach is to determine a map of the geometric variables that is consistent with the experimentally determined R and Δ values. The relationship between the geometric variables and R and Δ for a trimer and dimer model are

trimer model (C_3 symmetry)

$$\pm 2\Delta/3 = (\mu^2/r^3)[1 + 3/4 \sin^2\theta(1 - 4 \sin^2\phi)] \quad (2)$$

$$R_\pm = (3^{1/2}/2\pi)\mu^2(\lambda_{co} \pm \Delta) \sin 2\theta \sin\phi$$

dimer model (antiparallel)

$$\pm\Delta = (\mu^2/r^3)(1 - 2 \sin^2\theta + 3 \sin^2\theta \cos^2\phi) \quad (3)$$

$$R_\pm = (\pi/2)(\lambda_{co} + \Delta)\mu^2 \sin 2\theta \sin\phi$$

See Figure 7 for the definitions of the geometric variables. The transition electric dipole moment is estimated from the integrated absorbance spectrum (Table I). The equations were solved iteratively for r and ϕ by using a Newton method and assuming all possible values for θ. The trimer model has three positive roots for r, two corresponding to the positive equation and one to the negative. Typically, only one root gives physically realistic values. Under the present conditions, the dimer model has a double root for the positive equation. The values of r and sin ϕ are given as a function of θ for halorhodopsin in the dimer (Figure 8c,d) and trimer (Figure 8e,f) model. The corresponding results for bacteriorhodopsin in the trimer model are also given (Figure 8a,b). Interestingly, the bacteriorhodopsin data could not be fit to a dimer model. The bacteriorhodopsin trimer results can be used to assess the validity of this CD analysis. Linear dichroism results place the retinal at an angle, θ, of 71 ± 4° (Heyn et al., 1977). By

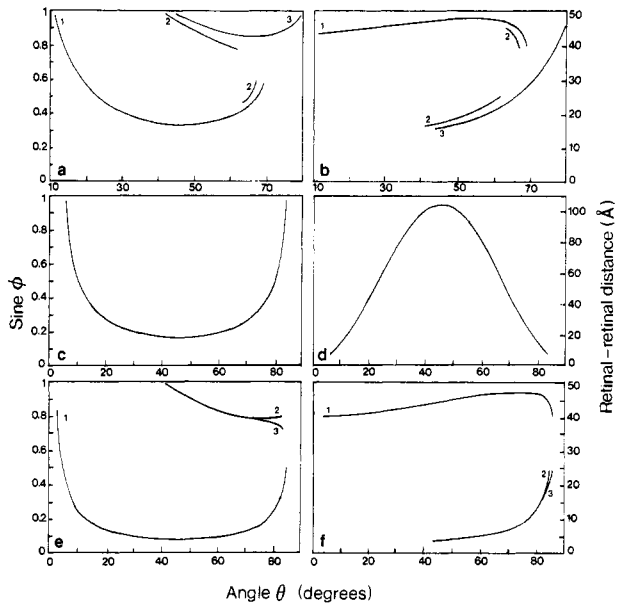

FIGURE 8: Plots of the parameter, r, and sin ϕ as a function of θ for BR in a trimer model (a, b), HR in a dimer model (c, d), and HR in a trimer model (e, f). See Figure 7 for the definitions of r, ϕ, and θ. Plots were generated by using the fitted values for R, B, and Δ obtained from Gaussian fits of the CD spectrum. Integrated absorbance spectra were used to determine μ^2. Equation 3 or 4 was solved iteratively for r and ϕ, assuming values for θ. The separate roots of these solutions are numbered. Regions that contain no values for r and θ result from values that cannot fit the data.

use of this value, Figure 8b gives an interchromophore distance of 31 ± 4 Å for the root numbered 3. Electron diffraction studies (Hayward & Stroud, 1981) give a similar distance if the retinal is placed between helices 6 and 4 and a slightly shorter one (~27 Å) if it is placed between helices 6 and 7. Neutron diffraction data (Seiff et al., 1985) favor a position between helices 6 and 3 and give an intermediate distance. Given the uncertainty in θ, the agreement between the CD analysis and other structural data is quite good. This analysis provides a considerable improvement over the 12-Å (Kriebel & Albrecht, 1976) and 15-Å (Ebrey et al., 1977) distances determined from previous CD analysis. While the point dipole exciton model is admittedly simplistic, it does provide reasonable results in this approach. To adequately test the theoretical limits of simple models, it is first necessary to establish reasonable estimates of the experimental parameters. Since CD is a much more sensitive monitor of exciton interactions than absorbance, it seems more realistic to estimate the interaction energy from the CD spectrum.

The halorhodopsin results for the trimer model are quite similar to the bacteriorhodopsin results; however, there are no roots in the 26–31-Å range. The halorhodopsin data could be fit to a dimer model as well. These results differ from the trimer results in that there are much greater restrictions on the values of θ that give realistic distances. θ would have to be restricted to a range of 10–20° or 70–80° if the retinal is located within a physically realistic region. From these calculations, conformational changes that alter retinal orientation would have a larger effect on the CD of halorhodopsin in a dimer than in a trimer.

Discussion

The CD spectra of purified HR and BR are very similar in all regions, both in 4 M NaCl and after detergent solubilization. The appearance of exciton bands near the absorbance maximum of HR in the absence of detergent indicates that HR, like BR, forms specific aggregates in which retinal–retinal interactions can occur. Detergent solubilization of BR with Triton X-100 and of HR with octyl glucoside eliminates the exciton coupling, and a single, positive band near the absorbance maxima is seen for both proteins. When BR is solubilized with octyl glucoside, both positive and negative lobes of the main absorbance band are reversibly reduced in magnitude (Dencher & Heyn, 1978). Exciton splitting of the main HR absorbance band occurs also in envelope vesicles, demonstrating that these protein–protein interactions occur in the native membranes (Figure 2). CD spectra of SR-I in native membranes show no such interactions, yielding only a positive band centered at the SR-I absorbance maximum. This indicates that SR-I is present in these membranes in monomers or that interactions between the retinal chromophores in SR-I are prohibited by chromophore orientations or distances.

Differences in HR and BR aggregate CD spectra in the retinal region have been interpreted in terms of an exciton point dipole model. The BR results using a trimer model are consistent with previous structural data (Heyn et al., 1977; Hayward & Stroud, 1981; Seiff et al., 1985). The parameters from the BR spectra are inconsistent with the constraints of the dimer model and therefore could not be fit to the model. The HR results could be analyzed with both a dimer and a trimer model. Both HR models give results that are physically possible. On the whole, the dimer model gives results in which the HR retinal orientation and separation are closer to that in BR. For instance, at a retinal separation of 27 Å, θ = 75° and ϕ = 160 or 20°. The BR trimer results at this separation give θ = 68° and ϕ = 118 or 62°. Neutron diffraction (Seiff et al., 1985) and electron diffraction studies (Hayward & Stroud, 1981) on BR give chromophore separation distances between approximately 27 and 31 Å. For the HR trimer model, this range of distances between chromophores is not possible. These results do not prove or disprove either model for HR. They point out that if the separation and orientation of the retinals are similar in BR and HR, then a dimer model would be preferred for HR. Whereas the CD of BR is relatively invariant with change in NaCl concentration, HR shows comparatively large, partially reversible NaCl-induced effects. At 100 mM NaCl, above the dissociation constants for HR chloride binding sites I and II (Lanyi, 1986; Schobert & Lanyi, 1986), the HR bilobe bands are clearly nonconservative, with the positive band dominant. Reequilibration in high NaCl restores the conservative spectrum but not the original band intensities. This result, coupled with the observation that irreversible CD changes occur in the far-UV with protein dialysis in low salt, suggests that changes in protein secondary structure induced by low ionic strength irreversibly affect retinal–retinal interactions in HR aggregates. The CD spectrum of HR is also more sensitive than that of BR to changes in pH between pH values of 7 and 9. As the pH is increased, aromatic amino acid ellipticities increase in magnitude. In addition, the negative lobe of the bilobe becomes relatively larger than the positive lobe with increasing pH. These results indicate that in HR both amino acid and retinal environments are affected by the protonation state of aromatic amino acid residues accessible to the external surface of the protein.

Upon illumination of purified HR preparations with orange light, the disappearance of CD bands near the main HR absorbance region is accompanied by formation of a single positive band with fine structure near 400 nm. This band is very similar in appearance to a CD band produced by stabilizing BR in the M intermediate state at −70 °C (Draheim & Cassim, 1985). An M-like state can also be generated by

dialysis to remove chloride (Lanyi, 1986). We find that this state is also characterized by disappearance of the bilobe bands with concomitant appearance of a positive band near 400 nm. CD spectra of the two M-like states differ in ellipticities between 250 and 300 nm, which is attributable to aromatic amino acid residues.

Because of their similarity in structure and diversity in molecular function, the bacterial rhodopsins present an unusual opportunity to establish structure/function relationships in integral membrane proteins. Comparison by CD and other physical and chemical methods provides a way to identify structural differences, eventually to be correlated with the differing transport specificities of BR and HR and the sensory signaling function of SR-I. Interestingly, the two transport proteins, HR and BR, show a propensity to form specific aggregates. The functional significance of this remains unclear. The SR-I, on the other hand, appears to be monomeric in its native state. This may be a consequence of its sensory signaling rather than ion-transport function. For example, if interactions with other integral membrane proteins are required for sensory transduction, self-aggregation could be a competing interaction. Once SR-I is purified, more detailed comparisons can be made between the structure and function of the bacterial rhodopsins.

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SUPPLEMENTARY MATERIAL AVAILABLE

One plot of residual error vs wavelength for calculated and observed HR (bottom plot) and BR (top plot) CD spectra (1 page). Ordering information is given on any current masthead page.

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