CD spectrum of bacteriorhodopsin

Best evidence against exciton model

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ABSTRACT We summarize the predictions of the exciton model that was originally proposed to explain the observed biphasic band shape of its CD spectrum in the visible region of bacteriorhodopsin (bR). It is shown that to reconcile these predictions with the observed results on the linear dichroism, the retinal isomerization time and, the retinal–retinal distance, the biphasic nature of the observed CD spectrum of bR becomes itself an evidence against the exciton model because of the uncertainty principle.

Reduced bR (RbR), which retains its hexagonal structure, shows a monophasic CD spectrum with relatively small rotational strength as compared to bR. This is shown to disagree with predictions made by the exciton model. The results could best be explained in terms of retinal-protein heterogeneity leading to two or more types of bR in which their retinals suffer opposite sense of intramolecular rotational distortion along their retinal long axis. Such a retinal-protein heterogeneity disappears in reduced bR which is known to have a planar (nondistorted) retinal conjugated system, resulting in a monophasic CD with reduced rotational strength, as observed.

INTRODUCTION

Bacteriorhodopsin (bR) is a membrane-bound protein which produces a trans-membrane electrochemical gradient by pumping the proton across the membrane upon the light absorption (1). bR forms a two-dimensional hexagonal lattice in discrete patches and the protein molecules are arranged in trimers with P_3 symmetry (2, 3).

The CD spectrum of native bacteriorhodopsin has been studied extensively (4–16), to understand its structural properties. The retinal is the only chromophore in bR which absorbs the light at 568 nm and its CD spectrum in the visible region is composed of a negative and a positive band with unequal intensity (4, 14, 15). The study of this biphasic band shape has been the focus of many studies not only because of its importance in illustrating the structural behavior of the retinal in bR and its interaction with its protein environment, but also because of the importance in understanding the origin of this unique biphasic feature (4, 10, 11, 14–16).

The biphasic band shape of retinal CD has been attributed to the presence of exciton coupling between the chromophores in the trimer structure of bR (4, 14–16). The supporting evidence for the exciton model comes from the observed changes in the band shape as bR is solubilized in detergent or as the fraction of retinal to the opsin is decreased in the regeneration of bR (4, 14, 15). These studies demonstrated the transition of the visible CD band from biphasic to a single positive band. This was attributed to the disappearance of the trimer structure (and thus the exciton coupling) destroyed either by detergent solubilization or by the bleaching of retinal in presence of hydroxylamine. The change of the biphasic to monophasic CD band shape in oriented bR film was proposed to agree with the exciton predictions (16).

In the present work, we detail the arguments for and against the exciton model. We show that upon considering the present known isomerization times and the retinal–retinal distance in the bR trimer, the CD spectrum of bR itself becomes an evidence against the exciton model if the uncertainty principle is to be obeyed. We then compare the CD spectrum of bR with its reduced form (RbR). Its absorption maximum is found to shift to 360 nm (17), but does not greatly change the magnitude of the transition moment nor does it destroy the hexagonal structure found in bR (17, 18) upon the retinal reduction. If the exciton model is correct, a shifted biphasic CD spectrum should be observed in RbR as well. However, the CD spectrum of reduced bR is found to be monophasic, which is in agreement with previous results (18). Its reduced rotational strength and monophasic band shape are shown to be consistent with the model which considers heterogeneity, resulting from the existence of different retinals in bR that suffer different degree and signs of intraretinal rotation distortions along the retinal long axis, as the origin of the biphasic visible CD in bR (11).

MATERIALS AND METHODS

The preparation of native bR from *Halobacteria halobium* has been described previously (19). The reduced bR sample was prepared after

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the method described by Schreckenbach et al., (20) with some modifications. Purple membrane suspension of 50–60 μM in 0.1 M NH_4HCO_3 buffer, pH 8.2 was illuminated by light from a 500-watt projector lamp filtered through a Corning glass filter type 3-66 and 3-67 in the presence of 4% (wt/vol) $NaBH_4$. The progress of the extent of the reduction of purple membrane was followed by measuring the absorption of the CD spectra at different times after the light lumination until the sample became totally colorless. The spectra taken for the completely reduced sample in the presence of $NaBH_4$ was similar to that which was separated from the excess reducing reagent.

Absorption and CD spectra of all the samples were taken with a Hewlett-Packard Company (Palo Alto, CA) 8451 diode-array spectrometer and a JASCO Inc. (Easton, MD) J-600 spectropolarimeter, respectively. A 2-mm cell and the shortest distance between the sample cell and PMT detector were employed to reduced possible light scattering in the CD measurement. The baseline correction, curve fitting and calculation of the oscillator and rotational strength from the absorption and CD spectra were performed on a Vax computer.

RESULTS

A. Shape of the CD band and exciton coupling

Figs. 1 and 2 show the absorption and CD spectra, respectively, of native bR recorded at different times during the course of reduction after the addition of the $NaBH_4$ in the presence of light. Gradual reduction of the retinal chromophore in bR by sodium borohydride is reflected by either a decrease in the 568-nm absorption band of retinal chromophore or an increase in the 358-nm absorption band of retinal chromophore or an increase in the 358-nm band of reduced retinyl moiety. Our absorption spectrum of reduced product of bR basically agrees with published results (20). Only a small change in extinction of protein absorption at 280 nm was

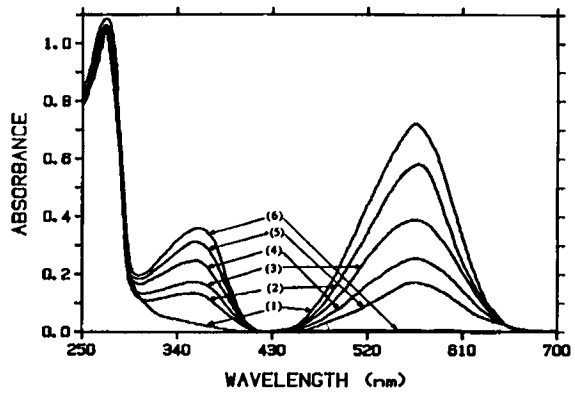

FIGURE 1 Absorption spectra of native bR at different stages of reduction by $NaBH_4$ in 0.1 M NH_4HCO_3 buffer solution in the presence of light (pH = 8.2, T = 20°C). The spectra were recorded at the following times after the addition of $NaBH_4$: (1) 0 min, (2) 129 min, (3) 150 min, (4) 180 min, (5) 210 min, and (6) 300 min.

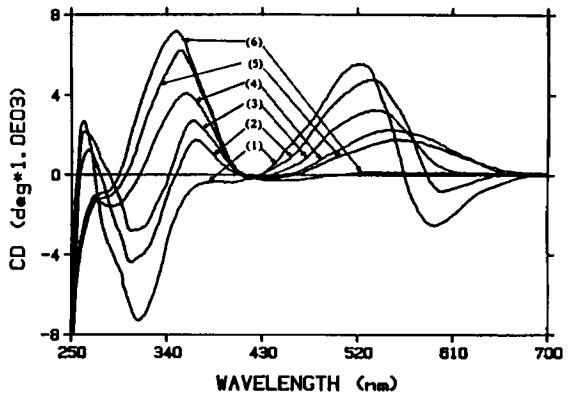

FIGURE 2 Near-UV and visible CD spectra of native bR at different stages of reduction by $NaBH_4$ in 0.1 M NH_4HCO_3 buffer solution in the presence of light (pH = 8.2, T = 20°C). The spectra were recorded at the following times after the addition of $NaBH_4$. (1) 0 min, (2) 129 min, (3) 150 min, (4) 180 min, (5) 210 min, and (6) 300 min.

observed as shown in Fig. 1, but the CD band in this region diminishes as the reduction is completed as shown in Fig. 2. The CD in near UV region is believed to result from the changes in the tertiary structure of the amino acid residues of the protein (5, 7).

In agreement with previous reports (18), reduced chromophore in bR shows only a single positive band at about 354 nm instead of biphasic CD band as expected from the excitonic interaction as the trimeric crystalline structure of the membrane remains unchanged during the reduction treatment (17, 18). Slight shift of the maximum of this band at different reduction times is due to the disappearance of the negative retinal CD band at 317 nm in bR, which is attributed to an electric-dipole forbidden but magnetic-dipole allowed transition (6).

At this point, we cannot exclude the possibility that a small excitonic splitting is present in RbR and thus does not give rise to an observed biphasic CD due to a large cancellation. This could result from the reduced transition moment which can be expected intuitively from the reduced conjugation length along the polyene chain after the reduction of the double bond at the linkage of chromophore to the lysine residue. To estimate the transition dipole moment, we plot the absorption spectra of native bR and its completely reduced form against the wavenumber as shown in Fig. 3. The oscillator strength is calculated from the following equation and the spectra in Fig. 3, assuming the Gaussian band shape for both samples, (21):

$$f_{nk} = 4.32 \times 10^{-9} \int \epsilon(\omega) d\omega. \quad (1)$$

The n and k denote the ground and excited state of the electronic transition and $\epsilon(\omega)$ is the extinction coeffi-

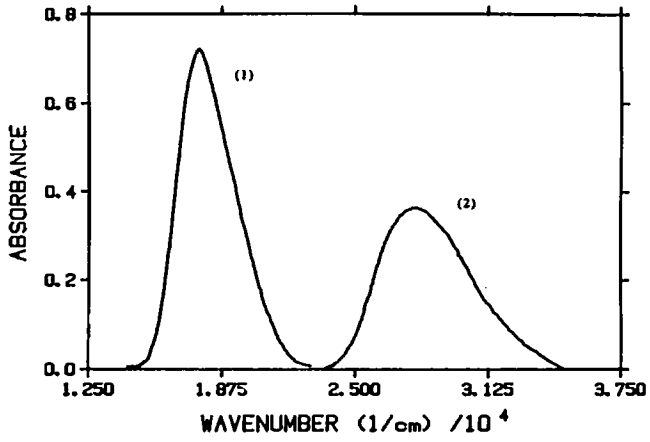

FIGURE 3 Absorption spectra of native bR (1) and complete reduced bR (2) plotted versus the wavenumber in order to calculate the oscillator strength for two samples (see Table 1).

cient at different frequencies ω. $\epsilon(\omega)$ for reduced chromophore at its maximum is found to be 32,000 $cm^{-1}M^{-1}$. The *f* values are calculated to be 0.90 and 0.85 for native and reduced bR, respectively.

The transition moment (μ_{nk}) is obtained from its relationship to the oscillator strength (21) and found to be 10.4 and 8.05 Debye for native and reduced bR samples, respectively. The determined transition moment of bR is in agreement with that previously measured by Ebrey et al. (22).

Using Tinoco's method (23), Ebrey et al. (22) have shown that the excitonic splitting between the doubly degenerate level and the nondegenerate level of the $n \rightarrow k$ transition in the bR is $= 3V_{nk}$, where

$$V_{n,k} = F\,|\mu_{nk}|^2, \qquad (2)$$

μ_{nk} is the transition dipole moment and F includes the terms that depend on the distance between the chromophores in the trimer structure, the orientation and the index of refraction. Because RbR is still crystallined in nature as bR (17, 18), we may assume that the *F* factor is the same for bR and its reduced form. By fitting the CD spectrum of bR, Ebrey et al. (22) found the splitting to be ~600 cm^{-1}. The energy splitting for RbR is then expected to be ~two-thirds that for native bR, i.e., 400 cm^{-1} according to Eq. 2. Thus a biphasic CD band for RbR should also be expected because of relative close splitting energy as compared with native bR if indeed the exciton coupling exists in RbR as well. We have simulated the monomer CD and exciton CD for RbR assuming a 14-nm (~400 cm^{-1}) separation for the exciton coupling using the same method described in the next section for native bR. The experimental CD band width of reduced bR and two-thirds that of rotational strength of native bR (22) are used as the Gaussian band parameters for the simulation shown in Fig. 4. As can be seen, if exciton is the origin of observed band shape in bR, and if the reduction does not greatly change the trimer geometry, the CD of RbR should be given by curve 5 shown in Fig. 4, which shows an obvious biphasic feature. The observed spectrum (curve 6 in Fig. 2) is very different from that predicted by the exciton theory. The lack of observation of biphasic CD band for RbR suggests that either the CD of the retinal chromophore in bR is not due to exciton interaction within its trimeric structure or else the geometric factors in Eq. 2 have changed in going from bR to RbR. A most serious change might be that the retinals in RbR become coplanar with respect to the membrane plane. This would make the transition to the singly degenerate exciton level an electric dipole forbidden and would thus eliminate the exciton CD contribution in RbR.

B. Rotational strength

The asymmetric biphasic retinal CD band in bR was explained as a superposition of a positive band centered at the absorption maximum and an exciton band with equal positive and negative lobes by Heyn et al. (15). The single positive band was attributed to the inherent optical activity of the individual (monomer) retinal molecules within the protein in bR (15, 22). The symmetric biphasic band was attributed to the exciton interaction between the retinal chromophores in bR trimeric cluster structure in the membrane (15, 22). However, whether the resolved symmetric biphasic band is due to the exciton coupling still remains questionable because other alternate explanations may interpret this biphasic

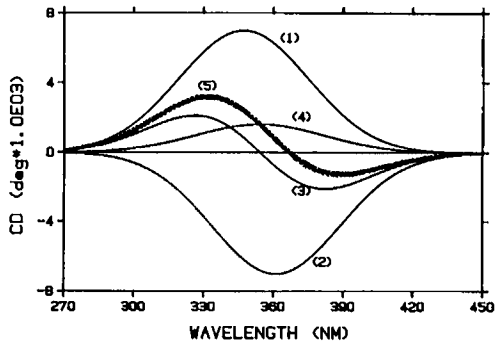

FIGURE 4 Simulated CD spectra for reduced bR predicted from the exciton model. Curves (1) and (2) are the exciton bands; curve (3) is the symmetric band of net addition of (1) and (2); curve (4) is the monomer band; curve (5) (××××) is the predicted asymmetric biphasic band from net result of the sum of curves (1), (2), and (4). The method used is described in the text.

lineshape as well, e.g., a combination of absorption due to two different types of bR with their retinals having opposite circular dichroism signs (11) and their absorption maxima separated by few hundred cm^{-1}.

We fit the retinal CD spectrum of native bR as the sum of a single positive band and two other bands with the opposite signs. We have verified mathematically that the difference in the area between the positive lobe and negative lobe of the asymmetric CD band is equal to that of the single positive band which is assumed to result from the inherent monomeric bR. The value of the difference in the area of the positive and negative bands is then used to simulate a Gaussian band assigned to the monomer CD band centered near its absorption maximum. The shape of the simulated monomeric band is found to be similar to the spectrum of partially reduced bR sample (curve 4 and 5 in Fig. 2). We also simulate two Gaussian bands (with width equal to that of the monomer band) with opposite signs which are added to the simulated monomeric band to give rise to the experimental CD curve. These three simulated CD transitions are shown in Fig. 5 as curves 2, 3, and 4 plotted versus the wavelength. Curve 5 in Fig. 5 shows a symmetric biphasic feature which is a result of the mathematical addition of two Gaussian type bands separated by 20 nm (~ 600 cm^{-1}) which is exciton splitting (15, 22). The above decomposition of the CD spectrum is found to fit both the absorption and the CD profiles according to the requirement discussed by Tinoco (24).

Using numerical integration, a replot of Fig. 5 on a frequency (cm^{-1}) scale and the equation,

$R = k \int \theta(\nu)/\nu d\nu$, we calculated the rotational strength from its decomposed spectra shown by different curves in Fig. 5 for native bR. The results are summarized in Table 1 in cgs unit. Because the biphasic band shape is closely related to the trimeric structure of bR (because it is sensitive to the extent of reconstitution or the detergent solubilization [4, 14, 15]), we therefore, define the calculated rotational strength from the symmetric CD band as the trimer CD as shown in Table 1. For the RbR sample, the CD band at 354 nm plotted versus frequency is presented in Fig. 6 and the calculated rotational strength for this band is also given in Table 1.

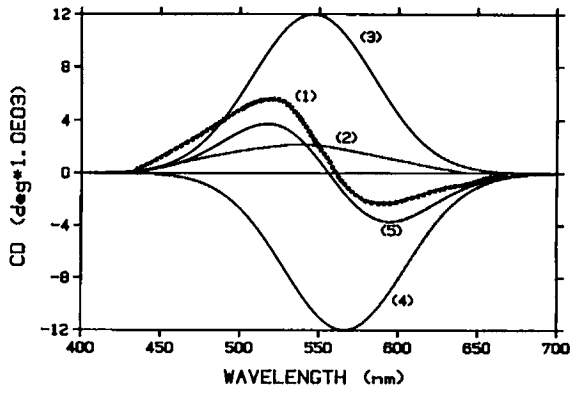

FIGURE 5 Retinal CD spectrum of native bR and simulated monomer and trimer CD bands. The method used is described in the text. Curve (1) (××××) is the experimental CD band; curve (2) is the monomer band; curves (3) and (4) are the trimer bands. Curve (5) is the added result of (3) and (4).

DISCUSSION

A. Changes in the CD upon reducing bR

It has been reported that the chromophore reduction in bR by sodium borohydride in the presence of light only affects the absorption maximum of the chromophore but not the lattice structure of the membrane (17, 18). The transition dipole moments are estimated from the experimentally determined oscillator strength for the retinyl chromophore in both native and reduced bR and they are found to be comparable in magnitude. Using the expected splitting for RbR ($\frac{2}{3} \times 600 = 400$ cm^{-1}) and assuming similar geometry of the retinal within the trimers in bR and RbR, the CD spectrum as predicted by the exciton theory is shown in Fig. 4. This indeed shows a biphasic CD band shape. The fact that the CD spectrum of RbR shows only a distinct positive band at its absorption maximum strongly contradicts the excitonic model predictions given in Fig. 4. This finding should add new evidence to shed doubt on the presence of exciton interaction.

Of course, one might argue that due to changes in geometry, the exciton interaction is reduced to a small extent that its positive and negative subspectra cancel one another. Another possibility is that, if the retinals in the trimer of RbR become coplanar in the membrane plane, this would render the transition to the singly degenerate exciton level to become electric dipole forbidden. This would eliminate the CD exciton contribution to the retinal CD. If either is indeed the case, the CD observed in RbR would result from the inherent individual monomer contribution. Since the transition moment of RbR is smaller than that of bR, and its conjugated system is more planar than that in bR (17), one would expect that the inherent rotational strength of the individual monomer in RbR to be much smaller than that assigned for the monomer in native bR. This prediction is opposite to observations shown in Table 1. The rotational strength observed for RbR is almost three times larger than that assigned to the individual inherent monomer retinal in native bR. Thus, both the CD band shape and the values of the rotation strength

TABLE 1 **Calculated spectroscopic parameters from absorption and CD spectra of native and reduced bR**

	Oscillator* strength (f)	Transition‡ moment (μ) (Debye)	Rotational§ strength (R) (in 10^{-40} cgs)	Total‖ rotational strength (R) (in 10^{-40} cgs)
Native bR	0.90	10.4	29.6 (Inherent monomer band) 129.2 (Positive trimer band) −116.5 (Negative trimer band)	275.3
Reduced bR	0.85	8.05	81.6	81.6

*The f value is calculated using Eq. 1 and spectra in Fig. 3.
‡ Estimated transition dipole moment is obtained from the f value.
§ Rotational strength is calculated using equation, $R = k \int \theta(\nu)/\nu d\nu$, and spectra in Fig. 5 and Fig. 6.
‖Absolute value is taken when the total contribution of rotational strength is calculated.

suggest that the exciton model predictions do not account for the observed changes in these quantities upon reducing bR.

B. CD spectrum of bR; evidence against the exciton model

Recently (11), a number of observations were discussed which could contradict the presence of exciton coupling in bR. These observations are summarized as following: (*a*) If exciton coupling is present, one would expect rapid energy transfer of the excitation between the different retinals in bR which would lead to depolarized retinal absorption for the first daughter formed during the photocycle of polarized excitation. However, the absorption of the K_{610} (25, 26) are found to be highly polarized with respect to the initial polarized excitation. (*b*) The absence of biphasic MCD spectrum for bR (11) indicates the absence of the doubly degenerate state predicted by the exciton model (17). However, MCD

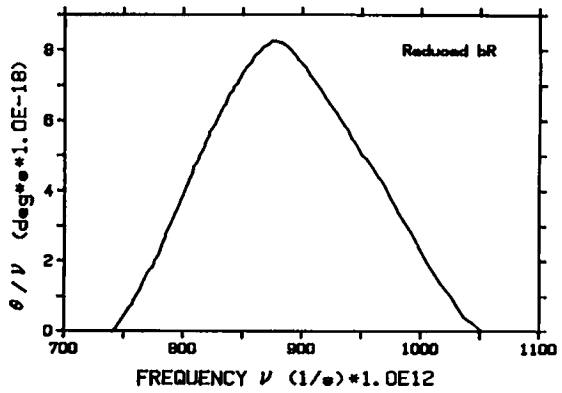

FIGURE 6 Plotting of CD spectrum (θ/ν) versus the frequency ν for completely reduced bR sample. The spectrum is used to calculate the value of the rotational strength (see Table 1).

resulting from exciton coupling can be expected to be vanishing (El-Sayed, M.A., unpublished results) and thus this evidence may not be valid. (*c*) Both the L and M intermediates, which should have different exciton band structure are found to have similar CD spectrum to that of bR, but having the change in the CD phase observed at their corresponding absorption maxima (27). (*d*) The mutagentic substitution of Tyr185 by phenylalanine is found to greatly change the CD as compared to ebR (10). Furthermore, ebR, which has a trimeric structure and is 80% regenerated with retinal, is found to have a nearly monophasic band shape (10).

In addition to the above observations contradicting the exciton model, more observations can be cited which adds to the evidence against it. To fit the CD spectrum, an exciton splitting of 600 cm^{-1} is required to enable the observation of a biphasic spectrum. This splitting is 3V, where V is the excitation exchange energy. Using a dipolar coupling (22, 28), this value of V (200 cm^{-1}) corresponds to an interretinal distance of 12 Å. The observed value is 26 Å (29). If one uses this value to recalculate V, one obtains a value of only 20 cm^{-1}. This gives a splitting of less than 60 cm^{-1}, which is not large enough to give an observed biphasic CD spectrum as shown in Fig. 7.

If the value of 200 cm^{-1} splitting obtained from the application of the exciton model to fit the CD spectrum of bR (22, 28) is used to calculate the excitation transfer time by use of the uncertainty principle, one obtains the transfer time t value:

$$t \cong h/V \cong 25 fs. \quad (3)$$

This time is much shorter than the formation time of the first photochemical intermediate in bR photocycle, which is found to be 400 fs (30, 31). It is thus difficult to explain the observed high anisotropy of the linear dichroism of the retinal absorption in the K intermediate (25, 26)

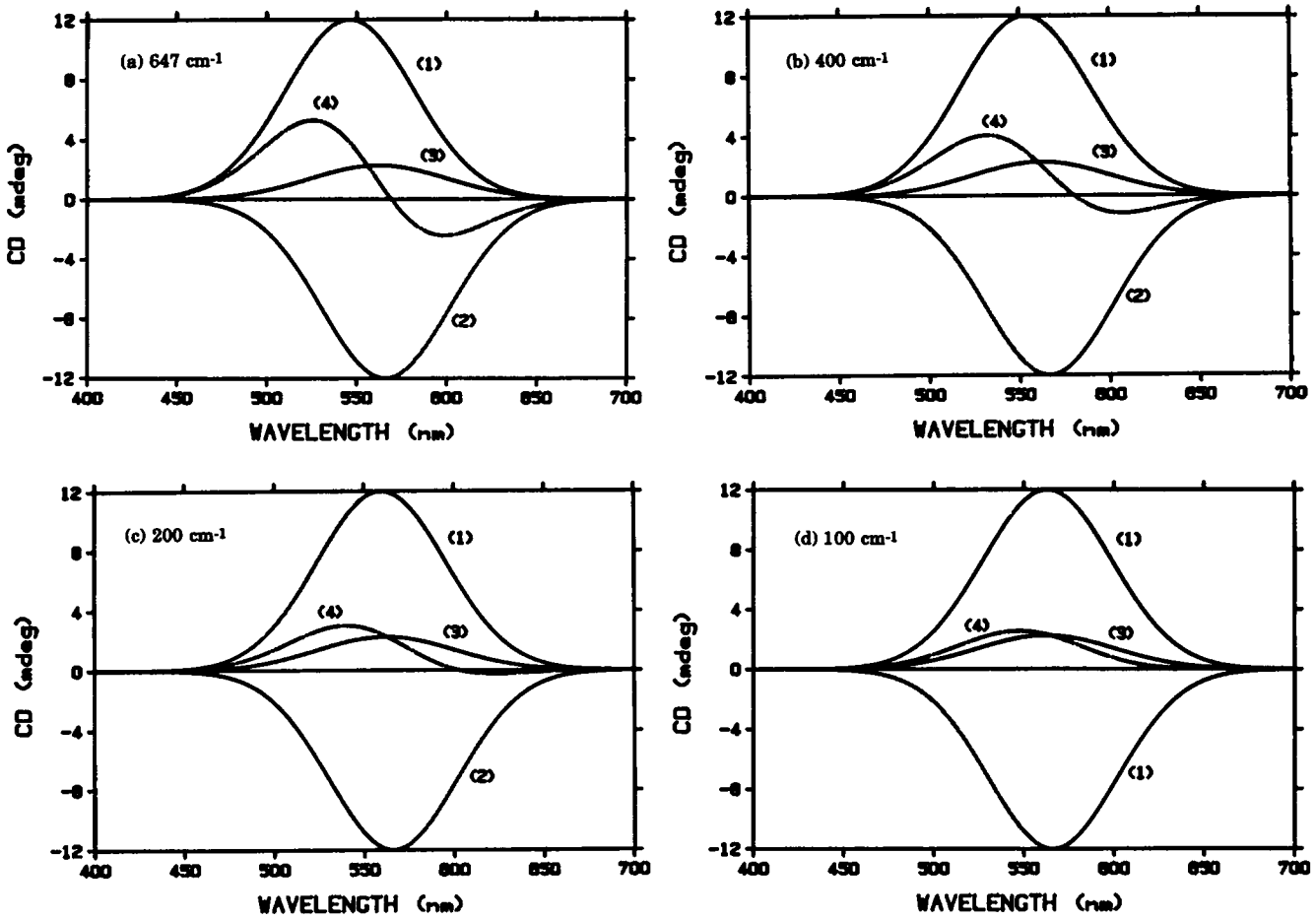

FIGURE 7 Simulated CD spectra for hypothetical bR having different excitonic splittings of 650 (pm bR), 400, 200 and 100 cm^{-1}. Curves (1) and (2) are the positive and negative CD bands resulting from the transition to the split exciton levels; curve (3) is the "inherent retinal CD band," which is the band subtracted from the observed CD spectrum of bR to make the positive and negative bands of equal intensities; curve (4) (-·-·-·-), which is the sum of curves (1), (2) and (3), should correspond to the observed CD spectra of bR if it has the corresponding exciton splitting. It can be seen that the biphasic feature can only be observed if the splitting is $>200\ cm^{-1}$.

because this process is known to result from isomerization process occurring on the subpicosecond time scale. To explain the anisotropy of the absorption of K intermediate, the transfer time has to occur on a time scale of one order of magnitude longer than the isomerization time (0.4 ps), i.e., a few ps time scale. Using the uncertainty principle, these transfer times require exciton splittings of the order of $\sim 1\ cm^{-1}$ which as shown in Fig. 7 can never give rise to a biphasic CD spectrum for bR.

C. Origin of CD in bR

As an alternative mechanism, the biphasic CD spectrum was proposed to result from the retinal-protein heterogeneity giving rise to the presence of two or more different kinds of environments around different retinals in the bR trimer with opposite signs for their optical rotation (11). It is possible that the trimer formation "locks up" the different retinals with different degree and signs of intraretinal rotation distortion around the retinal long axis. The distortion maximizes the mixing between the σ, π^* and π, π^* states and thus renders the transition with nonvanishing electric and magnetic dipole moments and thus strong rotation strength (11). The sign of rotation distortion of the retinal determines the sign of its CD spectrum. In planar retinal, the σ, $\pi^* \leftrightarrow \pi$, π^* mixing vanishes and the visible retinal transition becomes pure π, π^* in character with vanishing magnetic dipole moment. It is thus expected that the rotational strength is greatly reduced and that the biphasic shape of the CD changes to a weak monophasic band shape.

The origin of the rotation strength in the retinal monomer bR can result either from mixing the visible

π, π^* transition with those of the protein or from the dynamical (vibronic) mixing with the retinal σ, π^* transition with out-of-plane or tortional vibration modes. In the above model, it is proposed that when the trimer is formed, the retinals lock up in two or more minima. Could trimer formation do this? Strong evidence for the effect of the trimer formation on the retinal structure in bR pocket is the observation that the rate of light-to-dark adaptation, in which the protein-retinal configuration induces retinal *cis-trans* isomerization, is reduced by a factor of 10 upon trimer formation (22). This suggests the barrier of the *trans*-to-*cis* isomerization of the retinal increases upon trimer formation. In the trimer, the pocket seems to become tighter for the internal motion of the retinal.

The CD of bR can be resolved as shown in Fig. 5 into three different spectra, two having positive CD and one having negative CD. The absolute value of the total rotational strength for bR (Table 1) is found to be 275.3×10^{-40} cgs unit and this is almost 3.5 times larger than that observed of RbR. If indeed distortion of the retinal conjugated system is responsible for the observed optical activity (11), one reaches the conclusion that the retinal system in native bR is more distorted than that in its reduced form. A recent study on polarized infrared vibration spectroscopy by Fahmy et al. (32), indeed suggests distortions along the retinal conjugated system in native bR. Previous studies on the absorption spectrum of RbR by Oesterhelt et al. (17), had concluded that RbR has a retinal conjugated system which is planar.

The intrarotational distortion of the retinal in bR trimers could result from the steric effect due to the β-ionone ring as was proposed for the retinal in rhodopsin (33). Other steric effects can result from the methyl group attached to the retinal system. It is possible that as the trimer is formed, the pocket becomes tighter and the steric effect becomes serious along the six conjugated double bonds. These steric effects can be relieved by intraretinal rotational distortions. This leads to potential minima of the retinal protein in the trimer at intraretinal distortion angles of nonzero value that can be positive or negative. In RbR, the Schiff base linkage ($-C{=}NH^+-$) becomes a single bond ($-C-NH-$) and thus could increase the number of rotational degrees of freedom along an extra flexible single bond without restoring to retinal distortion of the π-electron system. This could allow the relief of the steric strain of the retinal in the pocket of RbR trimer by rotation around the C—N bond. This could explain the planarity of its retinal conjugated system (17), its reduced rotational strength and the absence of a biphasic band shape for its CD spectrum.

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