Unique biphasic band shape of the visible circular dichroism of bacteriorhodopsin in purple membrane

Excitons, multiple transitions or protein heterogeneity?

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ABSTRACT Over a decade and a half ago, when the first visible membrane suspension circular dichroic (CD) spectrum of the purple membrane (PM) was presented, two mechanisms were proposed to account for the observed biphasic shaped CD band: (*a*) excitonic interactions among the retinals of the sole protein bacteriorhodopsin (bR) in the crystalline structure of the PM, and (*b*) combination of CD bands with opposite rotational strengths due to a retinal-apoprotein heterogeneity of the bR molecules or due to two possible close-lying long-wavelength transitions of the retinal of the bR with opposite rotational strengths. Since that time, an impressive body of experimental and theoretical evidence has been accumulated, mostly consistent with an exciton model but many at serious odds with any heterogeneity or multiple transition model. Recently, a number of articles have appeared reporting analyses of new experimental observations which are proposed to cast serious doubts on the viability of the exciton model, and therefore, may revive the heterogeneity or multiple transition model as an explanation for the unique shape of the CD band of the PM. The intent of this article is to demonstrate that if all observations found in literature baring on this question are considered in toto and in a consistent manner, they can be interpreted without exception by excitons, and furthermore, that there is no plausible evidence available to warrant the revival of the heterogeneity or multiple transition model as an explanation for the unique shape of the biphasic CD band of the PM.

INTRODUCTION

The visible circular dichroism (CD) of the purple membrane (PM) in aqueous suspensions is dominated by a nonconservatively shaped biphasic band centered nearly at the absorbance wavelength maximum (1). This band has been attributed to the π-π^* (NV_1) transition of the retinylidene prosthetic group of the transmembrane chromoprotein bacteriorhodopsin (bR), the sole protein component of the PM (2). Another retinal containing transmembrane protein, visual rhodopsin, in situ in rod outer segment (ROS) membranes, however, exhibits a monophasic CD band centered nearly at the absorbance wavelength maximum in aqueous suspensions (3). To account for this notable difference in the CD band shapes arising from the same electronic transition in these two similar transmembrane protein types, it has been suggested that the appearance of a biphasic band instead of a monophasic one in the PM may be due to excitonic coupling among the retinals of the bR in the PM, to the possibility of two close-lying low wavelength transitions of the retinal of the bR with opposite rotational strengths or to bR heterogeneity in the PM (1, 4, 5). However, for excitons to be generated in a membrane structure, it is essential that the membrane supramolecular structure be well ordered and crystalline in nature. On the other hand, molecular heterogeneity would be more in order with a fluid membrane structure than a highly ordered one since any serious heterogeneity in structure would tend to destabilize crystalline order. The structure of the PM has been shown to be stable, highly ordered, and rigid. It approximates a two-dimensional crystal (6). In contrast, the ROS membrane structure is highly fluid and can best be represented by a fluid-mosaic model (7). Based on the crystalline nature of the PM structure, excitons are probably the most plausible explanation for the biphasic band shape. In fact there is an impressive body of literature supporting the exciton model which is reviewed below. However, recently El-Sayed and co-workers have published a number of papers arguing against the exciton model (8–11). The goal of this article is to demonstrate that most of the experimental observations presented by El-Sayed and co-workers are not actually inconsistent with the exciton model. Furthermore, if all the observations which bear on the question of the excitons, multiple transitions, or protein heterogeneity as the source of this biphasic band of the PM are considered in toto, they can be interpreted without exception, most easily and consistently by excitons. In addition, there is no solid spectral evidence available to warrant the revival of the heterogeneity or the multiple transition model as a reasonable possibility.

Exciton model of the PM

The first theoretical analysis of the biphasic CD band of the PM was based on the application of strong-coupling exciton formalism to the two-dimensional crystalline structure of the PM (12–14). Since the bR molecules are arranged into clusters of three (trimers) with P3 symmetry in a hexagonal lattice, a cyclic-trimeric exciton model was assumed. This model considered only excitonic coupling of the π-π^* (NV_1) transition dipole moments of the retinylidene chromophores within the trimers and neglected completely all other couplings. Optimizational curve fitting computations based on this

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model predicted that the three degenerate π-π^* (NV_1) transitions of the individual retinals at ~571 nm in the trimers of the light-adapted PM should split into three mutually orthogonally polarized excitonic transitions due to this coupling, resulting in two doubly degenerate ones (λ_K^-) polarized in the membrane plane at ~570 nm and a nongenerate one (λ_K^+) polarized parallel to the membrane normal at ~550 nm. The consequence of such a relatively large excitonic band splitting of ~20 nm was an excitonic splitting energy or excitonic band width ($3V_K$) of ~627 cm^{-1} (14). The resonance transition dipole energy (V_K) of 209 cm^{-1} gave an excitonic energy transfer rate of approximately 4×10^{13} s^{-1}. To achieve "best fit" between calculated (based on optimal curve fitting computations) and experimental curves, it was necessary to assume the following parameters: (*a*) distances between retinals to be 10 to 15 Å in the trimers, (*b*) the relative excitonic dipole strengths D_K^+/D_K^- of λ_K^+ and λ_K^- to be ~0.11, (*c*) the excitonic rotational strengths R_K^+ and R_K^- to be ~ ±2.3 Debye magnetons resulting in a conservative biphasic CD band with lobes of opposite polarity, and (*d*) a monomeric non-excitonic CD band, centered at about the absorption maximum, with a rotational strength of ~ +0.05 Debye magnetons (attributable to the induced asymmetry of the retinal in the environment of the apoprotein) to account for the nonconservative nature of the biphasic band.

Consequently, neutron diffraction studies placed the intratrimeric retinylidene distances at 26 Å and the intertrimeric ones at 38 Å (15), while linear dichroic studies set the upper limit of the splitting energy at ~150 cm^{-1} (16). Since V_K is an inverse function of the cube of the retinylidene distance, for a retinylidene distance of 26 Å the value of V_K becomes 20 cm^{-1} and the excitonic splitting energy decreases to 60 cm^{-1} from 600 cm^{-1} which should result in an excitonic band splitting of only ~2 nm. This relatively small band splitting is not sufficient to result in the observed biphasic CD band shape since the positive and negative ellipticity contributions of R^+ and R^- to the CD of the PM would almost entirely be annulled due to overlap (11). The net result of this would be a monophasic CD band centered nearly at the absorbance wavelength maximum similar to the case of the CD band of rhodopsin in the ROS membranes. However, in view of the following evidence (*a*) neutron diffraction studies indicated that intratrimeric retinylidene distances are of similar magnitudes as intertrimeric ones (15) and (*b*) transient dichroism studies have demonstrated that the bR molecules are immobilized in the native PM structure at ambient temperature (17, 18), it is apparent that a trimeric model, which neglects all intertrimeric interactions and involves a single C_3 axis, can not be an appropriate model for the native PM.

The simplest model which includes intertrimeric interactions is a cyclic heptameric model encompassing all 3 different C_3 axes of the PM and 21 chromophoric interactions. Calculations based on this model, using the same computational procedures previously used for the trimeric model, suggested that excitonic coupling will result in the formation of seven excitonic states from the sevenfold degenerate monomeric state of the heptamer (19). Evaluation of V_K, the excitonic resonance energy, requires a knowledge of the orientations of the retinylidene transition moments in the PM. This problem was handled in the following manner. The transition dipole was assumed to be directed along the polyene chain of the retinal since in the light-adapted membrane the retinals are in the all-*trans* configuration. The β-ionone ring of the retinals were placed in positions suggested by the first neutron diffraction study on the location of the retinal in the PM (14). The orientation of the polyene chain in the PM are defined by two angles, the in-plane angle ϕ and the out-of-plane angle θ. θ has been determined by linear dichroism analysis to be ~20° as measured from the plane of the membrane (16). Since the in-plane orientation of the polyene chain was not known, trial values of ϕ were repeated until a fit was achieved between calculated and measured spectra. "Best fit" could be achieved over a relatively wide range of ϕ values.

If, for example, ϕ was taken as 75°, a "best fit" could be achieved between the calculated and the measured visible absorption and CD spectra of the light-adapted PM yielding the following parameters. Based on a retinylidene length of ~15 Å, the interaction distances (assuming point dipoles) were determined from the center of the polyene chains to be ~32 Å. This resulted in the polyene chain describing an angle of ~55° with a line connecting the C_3 axis with the centers of the β-ionone rings. The following V_K, λ_K, D_K and R_K values were obtained for the seven resulting K excitonic states, respectively, (1) 51.35, 567.0, 0.36, −1.73; (2) 38.89, 566.6, 0.90, −1.16; (3) 20.19, 566.0, 3.38, −0.50; (4) −8.01, 565.1, 1.10, −0.22; (5) −17.39, 564.8, 0.46, 0.23; (6) −39.37, 564.4, 0.52, 0.76; (7) −45.66, 563.9, 0.28, 2.62, where the excitonic resonance energy V_K is in cm^{-1}, the wavelength λ_K is in nm, and the dipole strength D_K in units of the dipole strength of the monomeric transition and the rotational strength R_K in Debye magneton. As in the case of the trimeric model to generate the biphasic CD band from the calculated seven excitonic rotational bands it was necessary to include a similar small non-excitonic band. Therefore, the excitonic band splitting is ~3.1 nm and the excitonic splitting energy ($7V_K$) is only 221 cm^{-1}. Since the value of V_K is ~32 cm^{-1}, this should give an excitonic energy transfer rate of $\sim 6 \times 10^{12}$ s^{-1} for this model. This rate is within the order of magnitude of the rate of formation of the first photochemical intermediate in the bR photocycle, which has been determined to be $\sim 3 \times 10^{12}$ s^{-1} (20, 21).

Since the pioneering studies of King et al. (15) in 1980, technically improved neutron diffraction has provided more precise information concerning the projected retinylidene positions in the PM, including now

both the locations of the β-ionone ring as well as the middle of the polyene chain (22–26). Although discrepancies exist between the findings of the various laboratories, a recent high-resolution electron cryo-microscopy analysis of the bR by Henderson et al. (27) seems to be in accord with the findings of Heyn and co-workers (23–26). There are differences between the previously published retinylidene locations of King et al. (15) and the recent ones published by Heyn and co-workers (23–26). However, these differences are not sufficiently large to prevent a reasonable optimizational curve fitting of calculated and measured spectra based on the proposed heptameric exciton model. The computational formalism used for exciton models is not directly dependent on the exact positions of the β-ionic rings but only on the orientation of the polyene chain and their center-to-center distances. The dipole interaction distances, based on a point dipole assumption, would still fall in the range of 26 to 36 Å for inter- and intratrimeric interactions. Furthermore, since the fitting of spectra can be achieved over a fairly wide range of ϕ values, the in-plane projected orientations of the polyene chains suggested by recent studies of the retinylidene locations would present no significant changes in the previous computations of the heptameric model; that is, a fitting of spectra can be achieved with recent values of the retinylidene position parameters as with the previous ones used.

A rigorous exciton model for the PM would be a crystalline one which accounts for the interaction of retinals throughout the two-dimensional structure of the PM rather than bR aggregates of various sizes. The proper way to accomplish this is to treat the cyclic trimer as the fundamental interacting unit instead of the bR monomer as was done in the trimeric and heptameric models. The excitonic state for the infinite PM crystal lattice would be the in-phase combinations of the three exciton states of the trimer with any out-of-phase combinations resulting in cancellations. This will result in three allowed excitonic states for the infinite lattice. The calculations should consider as many neighboring trimers as necessary to achieve convergence (28, 29). Also, computation should be carried out based on the weak-coupling exciton formalism which considers both the electronic and nuclear transitions since an energy transfer rate in the 10^{12} s^{-1} range would classify this excitonic process as a weak coupling type. However, since there is a serious lack of information concerning the fine structure of the PM and the characterization of the electronic and nuclear transitions of the retinal in the bR, computations based on such a model are still very problematic. Initial analysis based on such a model revealed the presence of formidable, as yet, unresolvable computational difficulties (30). Nevertheless, there were indications that such a model could generate the experimental spectral curves. Furthermore, the upper limit of the splitting energy probably will be somewhat lower in a crystalline model based on weak-coupling excitons than the one determined for a heptameric model based on strong-coupling excitons (31).

In summary, two important findings are suggested by these exciton modeling studies assuming reasonable retinylidene distances to fall in the range of 26–38 Å in accord with the neutron diffraction findings: (*a*) excitons localized within trimers cannot account for the biphasic shape of the visible CD band while those delocalized beyond trimers probably can account for this biphasic shape and (*b*) excitonic energy transfer rates are probably within the range of rates of photochemical processes.

Supporting evidence for the exciton model of the PM: membrane studies in suspensions

Clearly from the exciton model analysis discussed above, if the excitonic contributions to the biphasic band are deleted by any perturbation of the PM structure, the biphasic band of PM in aqueous suspension would be transformed into a monophasic one similar to the band observed in ROS membrane CD spectra. Since according to exciton formalism, maintenance of chromophoric order is absolutely essential for achieving excitonic coupling, the loss of the hexagonal lattice structure of the PM would be one of the most important means of bringing about this result. Abolishing the exciton contribution to the CD of bR has been done by three means.

(*a*) It has been observed that when the PM is solubilized by the detergent Triton X-100 the biphasic band shape is transformed into a monophasic one (4, 5). Under these conditions the PM hexagonal lattice structure is complete dissociated and the bR molecules act as independent monomers (32).

(*b*) Since the excitonic interaction energy is inversely proportional to the cube of the dipole interaction distance, if the distance between retinals are increased substantially, then the excitonic coupling will become insignificantly small. When the fraction of bR is decreased by extreme bleaching of the PM with light in the presence of hydroxylamine to ~20% of the original amount or when the fraction of bR of the bleached PM is increased by regeneration with all-*trans* retinal to ~20% of the original amount, the biphasic band shape of the native PM is replaced by a monophasic one (5, 33, 34). With only about a 20% population of bR in the PM in these cases, the distances between retinals are expected to be relatively large. In addition, the hexagonal lattice structure of the PM becomes disordered upon bleaching; however, upon complete reconstitution the hexagonal order is reestablished (33).

(*c*) In a number of different kinds of reconstituted bR-lipid vesicles both band shapes observed in the native and the perturbed PMs are possible depending on a number of conditions. The CD band shape is biphasic when the protein-to-lipid (P/L) ratio is relatively high and temperature is depressed and monophasic when the P/L

ratio is low and the temperature is elevated (35, 36–39). The band shape dynamics with the P/L ratio and temperature also depends on the lipid composition of these vesicles. When the band shape is biphasic, x-ray diffraction patterns from these vesicles seem to be similar to those of native PM patches indicating the presence of a hexagonal lattice structure and the bR molecules are immobile according to transient dichroism measurements (35, 37). However, the diffraction patterns become weaker and are no longer detectable as the biphasic shape is transformed into the monophasic one. In concert with this pattern change, the immobility of the protein is altered to rotational mobility around an axis normal to the plane of the membrane.

However, it has also been noted that even when the PM is subjected to relatively much milder perturbations, which do not observably alter the hexagonal lattice structure of the PM, the transformation of the biphasic band to a monophasic one is still possible. For example, this has been observed when the PM is modified with the solvent dimethyl sulfoxide, resulting in the change of ground state bR_{568} to bR_{460}, or chemical reduction of the chromophore of PM with sodium borohydride and light to bR_{red} (4, 40, 41). In both cases, the x-ray diffraction patterns of the modified membranes have been shown not to be observably different from that of the native one (41–43). The same phenomenon, band shape changes in concert with no apparent hexagonal lattice structure distortion, has also been observed when the ground state bR_{568} of PM is transformed into the photointermediate state M_{412} (44, 45). However, in this case, definite tertiary structure changes of the bR, involving the alteration of the native orientations of the seven bR helical segments with respect to the membrane plane, have been reported, based on oriented far-UV CD (44), neutron diffraction (46), and x-ray diffraction and neutron scattering studies (47).

The retinylidene transition dipole moments are oriented at ~20° to the membrane plane in the native PM (16). According to exciton model analysis, if these transition moments become coplanar in the membrane, the exciton contributions to the biphasic band must vanish since no in-plane excitonic transitions will be allowed (12). However, no retinylidene orientation of this kind has been detected in any of these altered membranes (44, 48). Therefore, the most plausible and consistent explanation for this phenomenon may be in a comparison of the consequences of intertrimeric excitons to those of intratrimeric ones. The bRs are clustered trimerically on the hexagon lattice points of the two-dimensional crystalline structure of the PM. The trimer clusters are separated from neighboring ones by one shell of lipids (49, 50). Molecular rotational diffusion of the trimers at these lattice points around an axis perpendicular to the membrane plane are possible and has been demonstrated for ether treated PM by transient linear dichroism analysis. In the case of the native PM such motions are negligible at ambient temperature (16, 51). No studies of this kind have been done with bR_{460} or bR_{red}. Although a number of studies addressing the question of molecular rotational diffusion changes in the PM during the transition bR_{568} to M_{412} have been published, the results have been contradictory (52–57).

Crystals can undergo various degrees of lattice vibrations (28). In the PM, one mode of lattice vibration might be the in-plane vibrational mode of the trimers at hexagonal lattice points. If lattice vibrations are of sufficient degree, they can significantly interact with excitons. This should result in exciton traps by dampening long range excitonic waves due to destructive interference as a result of averaging over a number of different transition dipole moment geometries. As a consequence, excitons will become localized. In the PM case, this might localize excitons strictly in trimers which as discussed before cannot generate a biphasic CD band shape but only a monophasic one. In crystals, enhancement of lattice vibrations are correlated with lattice looseness. In the PM, it should be correlated with membrane fluidity. Membrane fluidity is a function of the nature of intermolecular interactions in the membrane supramolecular structure. It is likely that solvent treatment, chemical reduction, and bR conformation changes can result in increases in membrane fluidity and thereby enhance lattice vibrations. This can probably account for the monophasic band shape of bR_{460}, bR_{red}, and M_{412} CD. However, the subtle changes in lattice vibrational motions proposed for the PM to dampen long range excitonic waves need not be severe enough to be detected by such methods as x-ray diffraction or transient dichroism. In theory, any molecular motions greater than those resulting from the vibronic and rotational states of the interacting chromophores may be able to decouple excitons (28).

It has been noted that elevation of crystal temperature results in enhanced lattice vibrations. The amplitude of the biphasic band is decreased by temperatures elevated above ambient in the native PM (35). Also, it has been observed that the effects of solvent treatment can be accelerated at a given solvent concentration by elevating the temperature (4).

Additional support for the significance of intertrimeric excitons to the interpretation of the biphasic CD band is realized from studies of the delipidated contracted lattice form of PM. In such modified PM, ~70% of the total PM lipid is removed without significantly altering the trimers. The structure and photodynamics of the contracted lattice PM are similar to that of the native PM except that lattice unit cell dimensions are 59 instead of 62.4 Å. Since the layer of lipids surrounding the trimers have been removed in the contracted lattice PM without significantly altering the arrangement of the bRs in the trimers, intertrimeric excitonic coupling should be enhanced. The rotational strength of the biphasic CD band in the contracted form of the PM is 46% greater

than the one in the native form (58). Notably, the change is identical for the negative and positive lobes of the biphasic band. According to the excitonic interpretation of the biphasic band, this would suggest that the rotational strength of the non-excitonic monomeric CD band is identical in the two forms of the PM but that the rotational strength of the conservative excitonic biphasic CD band is enhanced by 46%.

Additional supporting evidence for the exciton model is advanced from studies of CD spectra of bRs in which the naturally found retinal of bR has been replaced by a variety of analogues with different structures. Variations of structure have been both in the β-ionone ring and the polyene chain section of the retinylidene molecule. Some of the substituted bRs can function as proton translocators while others cannot. The absorption maximum of such substituted bRs can vary over a wide range of wavelengths. However, unfortunately, the visible CD spectra of only a very limited amount of such bRs have been reported. For example, consider the CD of the following substituted bRs:

(*a*) The retinal is substituted with three-dehydroretinal, which has one more double bond in the β-ionic ring than the retinal naturally found in bR (59). This substituted bR exhibits a characteristic biphasic CD band similar to the one observed in native bR. The length of the π-electron conjugation of this modified retinal is longer than the one in native retinal. This results in the absorption maximum being red shifted from 568 to 603 nm. This substituted bR is capable of proton translocation.

(*b*) The retinal is substituted with merocyanine and methylated merocyanine analogues which have symmetric cyanine structures (60). These substituted bRs also exhibit characteristic biphasic CD bands centered at their absorption maxima which are red shifted to 662 nm in both cases. The charge distributions in the ring structure of these chromophores are significantly different than one in the β-ionone ring of natural retinal. Cyanine substituted bRs do not have the ability to translocate protons.

(*c*) The retinal is substituted with *trans*-fixed and *cis*-fixed retinals with fixed 13-ene structure in the polyene trail sections of both analogues (61). This modification makes the tail sections of these chromophores more rigid than that of native retinals. Both substituted bRs exhibit characteristic biphasic CD bands centered at their absorption maxima which are blue shifted to 570 and 547 nm, respectively. Both substituted bRs do not have the ability to translocate protons.

(*d*) The retinal is substituted with *trans*-naphthylretinal which contains naphthylene, a double ring aromatic hydrocarbon, instead of the β-ionone ring of native retinal (62). Although, this analogue has a much more extended π-electron conjugated system than native retinal, the π-electron system seems to be partially separated into two parts, the naphthalene ring and the polyene chain. However, due to conjugation the large double ring head of this retinal is much more planar than the β-ionone ring head of the native retinal. This analogue forms two different substituted bRs, one which absorbs at 503 nm and the other at 442 nm. The 503-nm form is formed when the chromophoric concentration is low (less than one-tenth of bacteriohodopsin concentration) while the 442-nm form requires equimolar concentrations of chromophore and apoprotein. The 503-nm form exhibits a characteristic biphasic CD band centered at the absorption maximum while the 442-nm form exhibits a monophasic band with an extremum at approximately the same wavelength as the absorption maximum. It seems that due to the large head of this retinal, when the retinylidene concentration is increased, the crystalline structure of the PM is disrupted due to a possible conformational change of the bR. Both substituted forms, however, are capable of translocating protons.

(*e*) The retinal is substituted with phenyl- and indene retinals (63). The small single aromatic ring of phenol is less conjugated than the naphthalene ring in naphthylretinal and, therefore, less planar. This substituted bR does not exhibit a characteristic biphasic CD band but a double band with the positive lobe about 12 times larger than the negative one. The large positive band is located approximately at the wavelength of the absorption maximum which is blue shifted to 487 nm. Apparently, similar to the case of naphthylretinal, the π-electron system of this analogue is also partially separated into two parts. The CD spectrum of this bR resembles the M_{412} spectrum which is discussed in detail below. Since there is no oriented CD data for this bR, it is not possible to determine if this very small band is excitonic in origin or not. The bR substituted with the indene retinal, which is more planar than the phenol one, exhibits a more characteristic biphasic band, however, the ratio of the extreme of the positive lobe to the negative one is somewhat larger than the one observed for native bR ($\sim$4 compared with 1.8). Nevertheless, the biphasic band is center approximately at the absorption maximum of this bR at 535 nm. While the phenyl retinal bR is capable of translocating protons, the indene one is not.

It is apparent from the CD data obtained from nine very differently substituted bRs that there seems to be no correlation between the electronic or the spatial structures of the chromophores and the emergence of a biphasic or monophasic CD band. Notably, there is no correlative evidence that the planarity or rigidity of the chromophoric structures in the substituted bRs play any significant role in determining the shape of the CD band.

In summary, it is clear from the discussion of the supporting evidence for the exciton model based on solution studies that the key factor for the biphasic to monophasic band shape change is probably a structural one, enhancement of membrane fluidity of the PM. The enhancement may range from very drastic, which results in

the complete destruction of the hexagonal lattice structure of the PM, to very mild, which results only in a subtle increase in the in-plane lattice vibrations of the trimers.

Supporting evidence for the exciton model of the PM: oriented membrane studies in films

According to exciton model analysis, excitonic transitions must be polarized along three mutually orthogonal coordinates of the PM with two of the coordinates in the plane and a third along the normal to the plane of the membrane. Furthermore, those polarized in the plane should cluster at the higher energies and have positive rotational strengths while those polarized along the normal should cluster at the lower energies and have negative rotational strengths. Therefore, if light is incident along the membrane normal during CD spectral measurements, all optical activity due to excitonic rotatory strengths must vanish due to the unique polarization of these transitions and the sum rule of optical activity (12, 64). This is similar to the model proposed for the excitonic coupling of amide groups in long α-helical polypeptides in which excitonic transitions were calculated to be polarized along the helical axes and in planes perpendicular to it (64). Those polarized along the axes were determined to possess negative rotational strengths and to cluster at lower energies while those polarized in the perpendicular planes were determined to possess positive strengths and to cluster at higher energies. Therefore, if the light is incident along the helix axes during CD spectral measurements all exciton-induced optical activity must vanish. This theoretical expectation has recently received experimental support (65–67).

The CD band shape of hydrated oriented PM films, for which the light is incident along the membranes normal, is monophasic and is located between the wavelength positions of the positive and negative lobes of the biphasic shaped band of the PM suspensions, for which the incident light is randomly oriented in respect to the membrane plane (68). It should be emphasized that this change in band shape is not due to any perturbation of the PM structure but only to changes in the orientations of the membrane planes relative to the incident light. A most important observation is that the addition of glycerol up to 80% by volume to PM suspensions which do not significantly alter the absorption spectrum, but result in changes in the ratio of rotational strengths of the positive and negative lobes of the biphasic band from ~3.4 to 0.8 while the rotational strength of the monophasic film band undergoes polarity changes continuously from positive to negative including zero (69). This results in a 21% decrease and a 89% increase in the ellipticities of the positive and negative lobes of the biphasic band, respectively. Significantly, when the ratio of rotational strengths become one in the suspension CD, the monophasic band vanishes in the oriented spectrum. According to the exciton model analysis, the nonconservative nature of the biphasic band is due to a monomeric non-excitonic band center at about the absorption maximum. Therefore, if this band vanishes, then the biphasic band should become conservative. Similar changes in the suspension and film CD spectra have been observed also when the pH is decreased from 7.0 to 2.4 (70). The film spectra of PM in the reduced ground state bR_{red} or the photointermediate state M_{412} are very similar to the suspension spectra of the PM in these states, exhibiting in both types of spectra a monophasic CD band shape (44, 48). This provides the strongest evidence for the absences of a significant excitonic contribution in the suspension spectra of these states of the PM. However, in both the film and the suspension spectra of the M_{412} state in addition to a very strong positive monophasic band there is a very weak negative band (44). This band cannot, according to theory, be excitonic in nature since it is present in the randomized as well as the oriented spectra of the PM. The rotational strength of the positive band is roughly ten times greater than that of the negative one in the suspension spectrum and twenty times greater in the film spectrum. It should be appreciated that due to a number of inherent experimental difficulties present in the study of the M_{412} state, accurate determination of the characteristics of this small band is not pursuable. The determination of the oscillator strength associated with this band is not possible since the absorption band of this CD band is completely obscured by the relatively massive π-π^* (NV_1) absorption band of the chromophoric retinal. However, a plausible assignment of this negative band may be a possible n-π^* transition of the unprotonated retinal-lysine Schiff-base linkage of the bR in the M_{412} state due to the lone pair electrons of the nonbonding orbital present in the nitrogen of this linkage after deprotonation (44). One may argue that the rotational strength of this band is too large to be associated with a normal n-π^*. It must be realized that n-π^* transitions with relatively large rotational strengths have been observed in other cases. An excellent example of this is the much larger than expected rotational strengths of the n-π^* transitions of the amide groups of helical polypeptides (71, 72).

Alternate explanations for the biphasic band

One alternative explanation suggested for this biphasic band is that it may be attributed to a combination of two close-lying long-wavelength transitions with opposite rotational strengths (5, 9). Such transitions are known to be present in conjugated polyenes (73). They include an electronically allowed, magnetically forbidden ($^1A_g^+ \rightarrow {}^1B_u$) and an electronically forbidden, magnetically allowed ($^1A_g^+ \rightarrow {}^1A_g^-$). Recently, it has been demonstrated by two-photon double resonance spectroscopy of the bR that the retinal of the bR has two low-lying excited singlet states that contribute to the absorption wavelength

maximum. The lower lying state with an absorption maximum at ~568 nm is associated with the allowed $^1B_u^{*+}$-like π,π^* excited singlet state with an oscillator strength of ~0.8 and above this state is an $^1A_g^{*-}$-like state at ~488 with an oscillator strength of ~0.3 (74). These two states with different polarization may give rise to CD bands of opposite signs.

It is difficult to reconcile this model with the experimental evidence discussed in the last two sections. According to the two-transition model, the biphasic band is the resultant of a positive and a negative CD band. The conversion of this biphasic band to a positive monophasic one as a result of a number of perturbations of the PM would imply that the rotational strength responsible for the negative band has been eliminated. It is difficult to correlate this elimination with the changes in the membrane fluidity of the PM discussed in a previous section and suggested to be correlated with this band shape conversion. On the other hand, the evidence obtained from film CD studies can totally rule out the feasibility of this model. Based on the results of the hydrated PM film CD, the transition of the positive band should be polarized nearly in the plane of the membrane while the negative one, nearly perpendicular to it. In glycerol impregnated films the positive band is transformed into a negative one. However, in the PM suspension spectra addition of glycerol up to 80% results in a decrease in the positive lobe and an increase in the negative one without any significant changes in wavelengths and no changes in signs of bands (69). If the two-transition or, for that matter, any two-band model is a plausible explanation for the biphasic band shape, the 80% glycerol suspension spectrum should consist of two negative lobes (which it obviously does not) since the film spectra indicate that sign of positive band changes in glycerol. A similar argument against the two transition or band model can be made based on the low pH results (70).

Another alternative model suggested for the biphasic band is that it may be resultant of two or more bands due to the presence of two or more types of bR with slightly different structural variations of the bR apoprotein in the PM. This can result in bRs with different local protein environments for the chromophoric retinal, possibly resulting in bRs with different absorbance energies and opposite rotational strengths (1, 5, 9–11). Protein heterogeneity with two forms of bR would result in a two-band model and can be disregarded offhand, since as discussed above, this model is inconsistent with experimental results. Heterogeneity with three different forms of bR with absorption maxima at 519, 555, and 591 nm can be shown to fit the biphasic band shape of PM suspensions if the 519 and 555-nm CD bands are assumed to be positive and the 591-nm one to be negative (10).

In order to correlate the observed biphasic to monophasic shape change of this CD band during a number of perturbations of the PM which disrupt the hexagonal lattice with a heterogeneity model, El-Sayed and co-workers proposed that the heterogeneity of the bRs may be due to constraining forces (stearic effects) imposed on the retinal system of the bR molecules by the rigid trimeric structure of the PM (9, 11). However, in cases where the appearance of the monophasic shape is not coupled to disruption of the hexagonal lattice structure of the PM, they proposed that the loss of heterogeneity may be due to configurational changes of the retinal which results in relief from these imposed stearic effects. The evidence discussed above from chromophoric analogue studies argues against this proposal. Therefore, these proposals should be considered to be very speculative due to the lack of any theoretical or experimental evidence for such a molecular mechanism. In addition, the three different forms of the bR necessary for a heterogeneity model would have to possess uniquely different properties in view of experimental results. According to the oriented film spectra of the PM, the 555-nm dipole transition moment should be polarized nearly in the membrane plane, while those of the 519 and 591-nm transition should be polarized nearly along the membrane normal. This would indicate that the retinal transition dipole moments would be oriented ~60° from the membrane plane if the molecular populations of the three forms are similar. Since it has been determined experimentally that this moment is oriented at ~20° from the membrane plane, then the population of the bR molecules in the PM with 555-nm moments should be very much greater than those with 519 or 591-nm moments if the consequences of this model are to be in agreement with experimental evidence. Also, to bring this model in consistency with the results of the studies on the effects of glycerol and low pH on the suspension and film CD spectra of the PM, one would have to conclude that while the 519 and 591-nm transition bR molecules are not significantly affected by these two perturbing agents, the 555-nm one is drastically altered with a change in the sign of its CD. These requirements necessary to fit this model with experimental results do not seem to be reasonable.

Evidence proposed to be contradictory to the exciton model

Recently, a number of observations, which were suggested to be incompatible with excitonic coupling in the PM were presented by El-Sayed and co-workers in a number of publications (8–11). It can be argued that none of these observations actually presents any serious determent to the exciton model. Consider these proposed difficulties:

(*a*) The fluorescence and the absorption of the first photointermediate K_{610} are found to be highly polarized with respect to the initial polarized excitation (8, 75). This suggests that if exciton coupling is present, relatively slow energy transfer of the excitation would be expected between different retinals in the bR. It is argued

that to slow the excitonic energy transfer rate based on a cyclic-trimeric model sufficiently low enough to be consistent with this observation, the excitonic splitting energy would have to be decreased to a value which would not be sufficiently large to generate the observed biphasic CD band shape (11). The weakness of this argument is that to achieve this reduction in energy transfer it is necessary to increase the retinal distances to a value that makes intra- and intertrimeric retinylidene distances comparable. Therefore, intertrimeric exciton coupling cannot exporaneously be ignored as they are in the trimeric model. The cyclic-heptameric model, discussed above, considers both types of excitonic coupling. This model can reduce the transfer rate to $\sim 6 \times 10^{12}\ s^{-1}$ while maintaining the ability to produce the observed biphasic feature. This rate is well within the range of the formation time of the K_{610} intermediate, which has been determined to be $\sim 3 \times 10^{12}\ s^{-1}$. Clearly this observation does not contradict the possibility of excitons in the PM but gives insight into the nature of these excitons.

(*b*) The absence of a biphasic band in the magnetic CD spectrum of the PM suggests either the absence of excitonic interaction in the PM or a small magnetic moment for one of the excitonic states of the PM (9). However, most recently, El-Sayed and co-workers seemed to favor the latter possibility and have concluded that this observation may not be contradictory to the exciton model (11).

(*c*) El-Sayed and co-workers (9–11) suggest that the previously published CD spectra of the photointermediates by Zimànyi et al. (76) are in conflict with excitonic predictions. Their arguments are based on the notion that in the K_{610} and L_{550} intermediate states the trimeric structure is destroyed by a marked shift of the absorption maximum of one of the three retinals as a result of isomerization. The CD spectra of these states as well as the subsequent state M_{412} are, therefore, expected to be significantly different from that of the ground state bR if the excitonic interpretation is valid. They suggest that the observed similarity of the CD of the photointermediate states to that of the ground state bR argues against such an interpretation. This suggestion could be credible if the monomeric contribution to the CD was significant. It has been shown that in a high glycerol environment of the CD of the monomeric bR vanishes (69). The spectra of Zimànyi et al. was measured in 58–65% glycerol. Therefore, no changes in the biphasic shape of the band would be expected due to an isomerization and a wavelength shift of one of the retinal of the trimer at this glycerol concentration. Their suspension and film studies of the L_{550} state and the blue light product of the M_{412} state are in accord with the predictions of the exciton model formalism. The excitonic interpretation of the M_{412} state spectra has been discussed in detail in the two preceding sections.

(*d*) Native bR, wild-type expressed in *E. coli* (ebR) and its constituted mutant with substitutions of Tyr-185 by Phe (Y185F) all are observed in reconstituted lipid vesicles to have different CD spectra (10). El-Sayed and co-workers argue that these results suggest that the local environment of the retinal in bR determines the sign and heterogeneity of the CD spectrum and this is consistent with the protein heterogeneity model rather than the excitonic one as an explanation for the biphasic shape of the CD band (10, 11). However, their arguments rest on a basis of conjecture. The fact that (1:1 weight ratio) reconstituted bR samples probably have lattice structures similar to that of native PM does not ensure that ebR samples reconstituted in the same manner probably also have similar lattice structure (77). The blue-shifted monophasic CD band of ebR is most likely a non-excitonic monomeric band. The blue shifting of the absorption maximum of ebR relative to that of native bR suggests structural differences (78). Structural differences probably can explain the differences in the CD band shapes based on an excitonic interpretation discussed above, since slight changes in membrane flexibility might be correlated with molecular conformational changes. However, in the case of the mutant Y185F there are at least two species of bR in these vesicles with very different absorption maxima and photochemical properties (10). Under these conditions, no significant excitonic coupling is expected. The mutant spectrum provides neither evidence for or against the exciton model. The fact that in this spectrum the non-excitonic retinylidene CD band is negative is not unique. This band is negative in the PM in high glycerol concentrations and in aqueous environments at very low pH (69, 70, 79). The sign of the induced optical activity of the retinal can be changed either by reversing the sign of the net static asymmetrical field imposed by the apoprotein or by altering the screw sense of the physical twist imposed on the retinal by the apoprotein (69, 70, 78). Obviously, the conclusion reached by El-Sayed and coworkers that these spectral results provide support for protein heterogeneity rather than exciton coupling as the source of the biphasic shaped CD band of native PM is definitely not well founded.

(*e*) El-Sayed and co-workers argue that, the change from a biphasic band shape to a monophasic one without elimination of the hexagonal lattice structure of the PM as a result of the conversion of bR_{568} to bR_{red} during the reduction of the PM is inconsistent with predictions of the exciton model. Furthermore, these results can best be explained by protein heterogeneity with two or more types of bR in the native PM which disappears in bR_{red} due to the planar retinylidene conjugated system in this state (17). The discrepancies in their interpretation of this phenomenon and ones discussed in the previous sections in this paper are due to the questionable belief that the retention of crystalline order which is observed at rather limited resolution must mean that the native rigidity of the PM is not altered and, furthermore, that intertrimeric excitonic coupling can be neglected. The pro-

posal that the loss of intertrimeric excitonic coupling in the native PM can be correlated with increased trimeric lattice vibrations (which result from a subtle decrease in the membrane rigidity) as an explanation for the reduction-induced band shape change is supported by well founded theory. On the other hand, the protein heterogeneity model explanation advanced by El-Sayed and coworkers (11) for this shape change, which assumes that the retinal system of the native bR is distorted into two or more types of bR by stearic constraints imposed by the rigid trimeric structure of the PM and relieved during reduction of the PM due to a change in the retinylidene configuration to a more planar form, is speculative and lacks any theoretical support. In fact, the evidence from chromophoric analogue studies strongly argues against this possibility. In addition, El-Sayed and co-workers point out that since the moment of bR_{red} is smaller than the one for bR_{568} and its conjugated system is more planar than that in bR_{568}, the inherent rotational strength of the monomer in bR_{red} should be much smaller than that of the bR_{568}. However, since their calculations of rotational strengths suggest that the one for bR_{red} is about three times larger than the one for bR_{568}, they conclude this contradicts predictions of the exciton model. The problem here is not exciton models per se but their use of an inappropriate cyclic-trimer model to determine the bR_{568} monomeric rotational strength by deconvolution of the bR_{568} biphasic band. The most accurate and direct method for determining relative monomeric rotational strengths is to use oriented film spectra data, not suspension ones, since all oriented spectra are free of exciton contributions according to exciton formalism. Such determinations suggest that the rotation strength of the bR_{red} monomer is three fourths of that of bR_{568} monomer (48). Similarly, the transition moment of M_{412} is also smaller than that of the bR_{568} and its conjugated system is more planar than that of bR_{568}. In this case, the rotational strength of the M_{412} monomer is determined to be about seven-tenths of that of the bR_{568} (44). This demonstrates the consistency and accuracy of rotational strength determinations by this method. There is no data from the reduced PM studies which is contradictory to the predictions of the exciton model.

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