CIRCULAR DICHROIC SPECTRUM OF THE L FORM AND THE BLUE LIGHT PRODUCT OF THE M FORM OF PURPLE MEMBRANE

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ABSTRACT Simultaneously measured low temperature absorption and circular dichroic spectra are presented for different intermediates of the bacteriorhodopsin photocycle in suspension and hydrated film of purple membranes. The data for the L intermediate are in accord with excitonic interpretation of the visible part of the circular dichroic spectrum, suggesting that no large scale structural change of the purple membrane affecting its crystalline structure happens during the L formation. The structure of the membrane, which is disrupted in the M state, is recovered when M is illuminated with blue light at low temperature.

INTRODUCTION

The purple membrane (PM) of *Halobacterium halobium* contains a single protein, bacteriorhodopsin (bR), which constitutes 75% of the membrane's dry weight, the remaining part being lipids. The function of bR is the light-driven translocation of protons across the membrane, which is the first step in the anaerobic ATP synthesis of the bacterium (for a recent review see reference 1). A remarkable feature of PM is its two-dimensional crystalline structure of P3 symmetry with trimers of bR molecules sitting in the points of the hexagonal lattice (2). Each bR molecule contains a retinal chromophore covalently bound to the protein via a protonated Schiff-base (3).

Large scale global structural changes of PM during the bR to M transition (the blue-shifted intermediate of the bR photocycle) involving the partial disruption or alteration of the crystalline structure have been reported using circular dichroic (CD) spectroscopy (4), x-ray diffraction method (5, 6), and Fourier transform infrared difference spectroscopy (7). In the present work we demonstrate that (*a*) the crystalline order of PM resulting in the excitonic contribution to the CD spectrum is still present in an earlier intermediate, L, of the photocycle, and (*b*) the blue light illumination of the M form at low temperature produces intermediate(s) where both the protonation state of the Schiff-base and the crystalline structure of the membrane are recovered.

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MATERIALS AND METHODS

Purple membranes were isolated from *Halobacterium halobium* strain ET 1001 using the standard procedure (8). The solution used for taking the spectra of the L intermediate contained 65% glycerol and 1.7 mM phosphate buffer at pH 7 and for the M studies 58% glycerol, 1.7 M NaCl at pH 10 titrated with 1 M NaOH. The suspension was placed in a 1-mm quartz cell, the absorbance was ~0.5 at 570 nm as measured in a spectrophotometer (Unicam SP 1800; Pye Unicam Ltd., Cambridge, England). Hydrated films were formed by layering several drops of PM suspension (pH 9.4, $OD_{570} \approx 3$), which was previously sonicated, filtered, and degassed onto one of the plates of a two-piece, 0.2-mm quartz cell, dried in a desiccator in gentle vacuum, incubated with a beaker of saturated K_2SO_4 solution (relative humidity 97%) for several days, and sealed just before measurement. The absorbance of the films was between 0.15–0.25 at 570 nm.

The sample was placed in a He cryostat (Displex CSW 202; Air Products and Chemicals, Inc., Allentown, PA) controlled by a home-built temperature controller. Light adaptation was performed at 0°C for 20–30 min by the light of a 500-W projector lamp filtered through $CuSO_4$ solution and a yellow ($\lambda > 500$ nm) filter. Conversion of bR to M was achieved by cooling the sample to 223°K in the light and then lowering the temperature to 200°K in complete darkness. The blue light product of M at 200°K was formed by illuminating the sample for ~20 min using the same projector lamp and $CuSO_4$ solution and a 425-nm interference filter. To form the L intermediate, the sample was cooled after light adaptation to 163°K in the dark, and illuminated at this temperature for ~30 min by the projector, using the $CuSO_4$ solution and a 650-nm interference filter.

Circular dichroic and the corresponding absorption spectra were simultaneously measured in a dichrograph (Jasco J-40C; Japan Spectroscopic Co. Ltd., Tokyo, Japan) by parallel registration of the CD and PMHT (photomultiplier high tension) signals—the latter being a monotonic function of the light intensity behind the sample—using a home-built interface between the dichrograph and a multichannel analyzer (ICA 70, Central Research Institute for Physics, Budapest, Hungary). The baseline correction of the CD spectra and the calculation of the

absorption spectra from the corrected PMHT signals (log I_0/I) were performed in a computer (TPA 1140; Central Research Institute for Physics, Budapest, Hungary).

CD spectra were taken in the 700–310-nm region using a scanning speed of 100 nm/min and a time constant of 0.25 s, and in the 310–250-nm region with 5 nm/min, 4 s. Due to the fast scanning speed in the visible range the back converting of the corresponding photoproduct to the ground state by the measuring light was negligible.

RESULTS AND DISCUSSION

The absorption and the corresponding, simultaneously measured, CD spectra of light-adapted bR (bRLA) in suspension are shown in Fig. 1 (*dashed line*). The shape of the bRLA CD spectrum resembles the spectra previously reported (4, 9–13). The nonconservative biphasic CD band in the visible has been interpreted as a result of the overlapping of a conservative biphasic band arising from an excitonic interaction between the chromophores of neighboring bR molecules and a positive (or negative) band as a result of the interaction of the retinal with its own asymmetric protein environment (9, 13–16). The appearance of the excitonic contribution is intimately linked to the rather rigid, two-dimensional crystalline structure of the PM (17, 18). The interpretation of the near UV part of the CD signal has been recently given in detail by Draheim and Cassim (4, 13), as follows. Part of the negative band ~317 nm is accounted for by a magnetic dipole allowed higher energy $\pi \rightarrow \pi^*$ transition of the retinyl chromophore, whose rotational strength appears as the counterpart of the visible single band arising from the retinal-protein dissymmetric interaction. An electric-magnetic resonance coupling between this transition and an electric dipole allowed $\pi \rightarrow \pi^*$ transition of a nearby aromatic amino acid results in the rest of the 317-nm band and some of the positive near UV bands. The negative bands around 280 nm and 290 nm are most likely associated with $\pi \rightarrow \pi^*$ transitions of tryptophans, and the positive region between 250–270 nm to phenylalanyl residues.

The absorption and CD spectra of the compound formed by illumination of bRLA at 163°K are shown in Fig. 1 (*solid line*). From the position of the absorption maximum at ~545 nm it is concluded that the sample has completely been transformed to the L form (19). To demonstrate whether or not the biphasic visible CD band contains excitonic contribution the spectra of the L intermediate have also been measured in PM films (Fig. 2). From symmetry considerations it follows that if the plane of the PM is oriented perpendicular to the incident light, all excitonic contributions to the CD spectrum must vanish (12). It is evident from Fig. 2 that, as in bRLA, the film CD spectrum in the L form contains a single positive band in the visible. The comparison of the CD spectrum of L in suspension and hydrated film strongly suggests the presence of excitonic bands in the suspension spectrum, showing that the PM still possesses a regular crystalline order in the L intermediate of the photocycle. The nonconservative nature of the visible biphasic band in suspension originates from the overlapping of the excitonic bands with a positive single band caused by the retinal-protein interaction. The reported all-*trans* to 13-*cis* isomerization of the retinal chromophore is most probably reflected by a blue shift of the 317-nm negative CD band during L formation (Figs. 1 and 2). Moreover, there is a significant increase of the amplitude of this band and that of the peak ~260 nm with no appreciable changes in the 280–290 nm bands in the L spectrum as compared with bRLA. However, these changes are not reflected in the corresponding film spectra. Possible explanations of these phenomena can be given as follows. While the aromatic amino acids and their environment responsible for the 280–290-nm peaks do not considerably change during L formation, a new molecular interaction may develop between the retinal chromophore and its protein environment as compared with bRLA, which may be reflected in the optically active transitions being stronger and polarized more out-of-plane, resulting in part of the spectral changes at 310 and 260 nm. The increase of the positive contribution ~570 nm in suspension for L as compared with bRLA must also give rise to the increase of the amplitude of the negative band at 310 nm. Previously it had been shown (13) that in high glycerol concentration

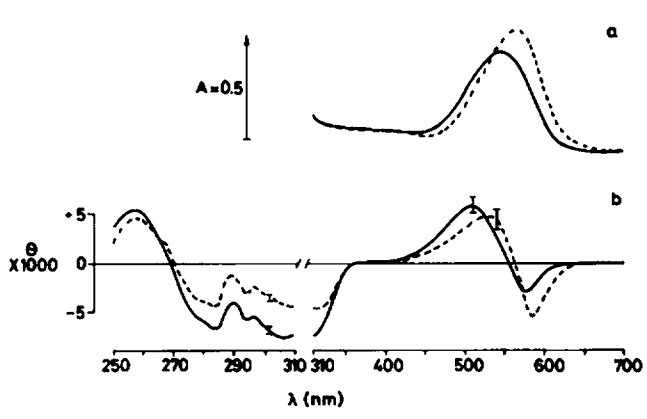

FIGURE 1 Simultaneously measured suspension absorption (*a*) and circular dichroic (*b*) spectra at 163°K of bRLA (*dashed line*) and L (*solid line*) in 65% glycerol.

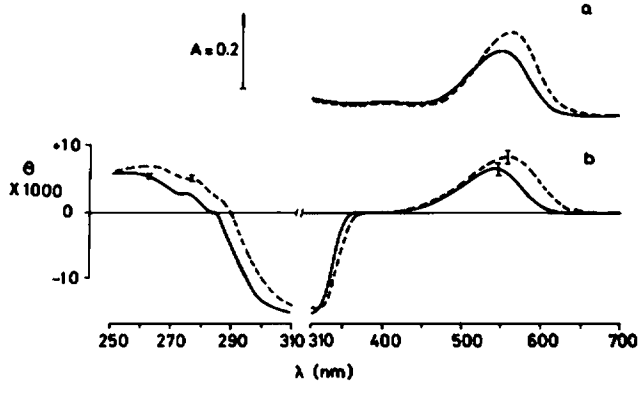

FIGURE 2 Simultaneously measured hydrated film absorption (*a*) and CD (*b*) spectra at 163°K of bRLA (*dashed line*) and L (*solid line*).

the nonexcitonic contribution to the biphasic band system is nearly zero or even negative for bRLA, together with a significant decrease of the 317-nm peak. This effect had been explained by the glycerol significantly altering the dissymmetric environment imposed by the apoprotein on the chromophore in the case of bRLA. However, these effects of glycerol on the structure of the protein do not seem to appear in the case of the L intermediate (Fig. 1). (Since the film studies have been performed in aqueous environment, this can explain why there is less difference between the bRLA and L CD spectra in Fig. 2.) It is worth mentioning that the weak negative CD band at 450 nm reported for the M intermediate (4), which was identified as a probe for the deprotonation state of the Schiff-base, does not appear in the L spectrum, providing independent evidence for the Schiff-base being protonated in L (20).

Fig. 3 shows the absorption and CD spectra of the M intermediate in suspension (*solid line*). There is good agreement between these spectra and those reported by Draheim and Cassim (4), although the fine structure of the CD peak around 410 nm is more resolved in their work due to the better S/N ratio of the point-by-point measurement technique they applied. The most important finding has been that the CD spectrum does not contain any excitonic contribution that was judged by a comparison of the suspension and film CD spectra of M. Our experiments on PM films also support this conclusion (data not shown). The absorption and CD spectra of the blue light product of M at 200°K are shown in Fig. 3 (*dashed line*). The visible part of the spectra is similar to the corresponding spectra of bRLA, although there is an ~5- and 15-nm redshift in the absorption maximum and the zero crossover point of the CD, respectively. Balashov and Litvin (21) have reported a series of intermediates thermally decaying to bRLA after blue light illumination of M. According to the wavelength maxima and the characteristic temperatures where these conformers can be accumulated, the product in our experiment can be assigned to P575 or a mixture of P575 and P585 in their notation. From the absorption spectrum it has been concluded that the Schiff-base becomes reprotonated in these intermediates. Moreover the biphasic CD band appears again (Fig. 3), and a comparison with the corresponding film spectra (data not shown) clearly demonstrated that these bands have an excitonic origin. There is a significant positive nonexcitonic contribution in the visible part of the CD spectrum in contrast to what would be expected for bRLA at this glycerol concentration. This indicates that the dissymmetric protein environment for the retinal is different in this species from that in bRLA. It can be concluded that the photoreactions of M (M→P421 and M→P433, see reference 21) do not only increase the ability of the Schiff-base to accept a proton even at low temperature but also provide the conditions for reestablishing the long-range crystalline order of PM, which is partially disrupted in the M state of the photocycle (4). While the characteristic 317-nm band and the 260-nm positive peak reappear in the CD spectrum of the blue light product, there are significant differences in the 280–295-nm aromatic amino acid region as compared with the spectrum of either the M or the bRLA form. Balashov and Litvin (21) concluded that the small consecutive bathochromic shifts of the absorption maximum of bR after the blue excitation of the M intermediate reflect a continuous conformational change in opsin. These conformational transitions seem to involve one or more aromatic amino acid residues possibly by changing the asymmetric charge environment in the protein, resulting in the observed unique CD spectrum in the 280–295-nm region.

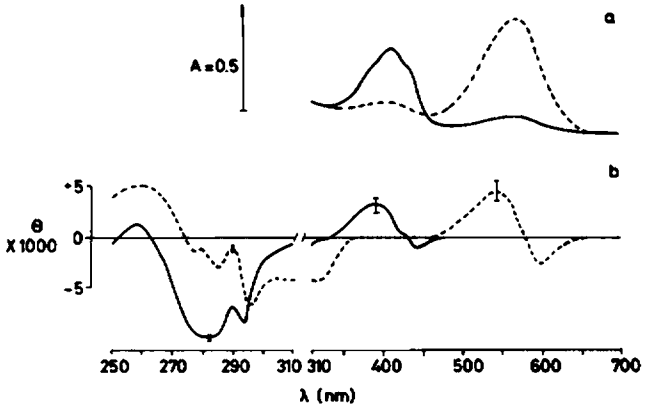

FIGURE 3 Simultaneously measured suspension absorption (*a*) and CD (*b*) spectra at 200°K of M (*solid line*) and the low temperature blue light product of M (*dashed line*) in 58% glycerol.

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