THE CIRCULAR DICHROISM OF BACTERIORHODOPSIN: ASYMMETRY AND LIGHT-SCATTERING DISTORTIONS

Malka BRITH-LINDNER and Kurt ROSENHECK
Department of Membrane Research, Weizmann Institute of Science, Rehovot, Israel

Received 16 February 1977

1. Introduction

The location of the binding site for retinal in bacteriorhodopsin and the orientation of the retinal chromophore with respect to the membrane components are still not known. CD can, in principle, give information on intermolecular arrangement and site-symmetry. The CD-spectrum of bacteriorhodopsin in the visible spectral region is composed of negative and positive components of unequal strength [1]. This asymmetry in the CD-spectrum of purple membrane suspensions in water has been attributed [1] to a superposition of a symmetric pair of negative and positive bands arising from exciton-interaction between neighbouring chromophores in a regular array, and another, positive band that is due to the interaction between each single chromophore and the protein site to which it is bound.

In the analysis of the CD-spectra of suspensions of large particles distorting effects due to light-scattering, on band shape, as well as intensity, should be taken into consideration [2–4]. It is shown in this work both experimentally and theoretically that the asymmetry in the CD-spectrum of the purple membrane in water suspensions can be attributed to such scattering distortions.

2. Materials and methods

Halobacterium halobium M-1 was grown as described by Danon and Stoeckenius [5]. The following procedure was used in order to obtain the purple membrane patches. The cells were concentrated and resuspended in basal salt to which DNAase and RNAase were added (each 50 μg/ml cell-suspension). The suspension was dialyzed against water overnight and subsequently centrifuged at 30 000 rev./min for 30 min. The purple pellet was resuspended in water and the washing procedure was repeated several times. Fractionation of the membrane fragments was performed on linear sucrose-gradients (25–50% sucrose with 60% solution at the bottom of the tubes). The centrifugation was run for 20 h at 25 000 rev./min using a Beckman SW 27 rotor. The purple band was collected, dialyzed against water and washed by repeated centrifugations.

Sub-bacterial vesicles of *H. halobium* M-1 were prepared by sonication according to MacDonald and Lanyi [6] with minor modifications and tested for integrity [7]. The Branson Model B-12 Sonifier (80 W at 4°C for several min) was used for sonication of the purple membrane patches.

The CD- and ORD-spectra of light adapted suspensions of purple membrane and sub-bacterial vesicles were measured on the Cary 60 spectropolarimeter with 6002 CD-attachment. Refractive indices were measured on the Abbe-3L Refractometer (Bausch and Lomb).

3. Results and discussion

The CD-spectra of light adapted suspensions of purple membrane in water, basal salt, sucrose/water and glycerol/water solutions were measured in the 275–650 nm region. Two of these spectra, in the visible range only, are compared in fig.1. The CD-spectrum is composed of two bands of opposite signs. It is observed that the ratio of the ellipticity of the

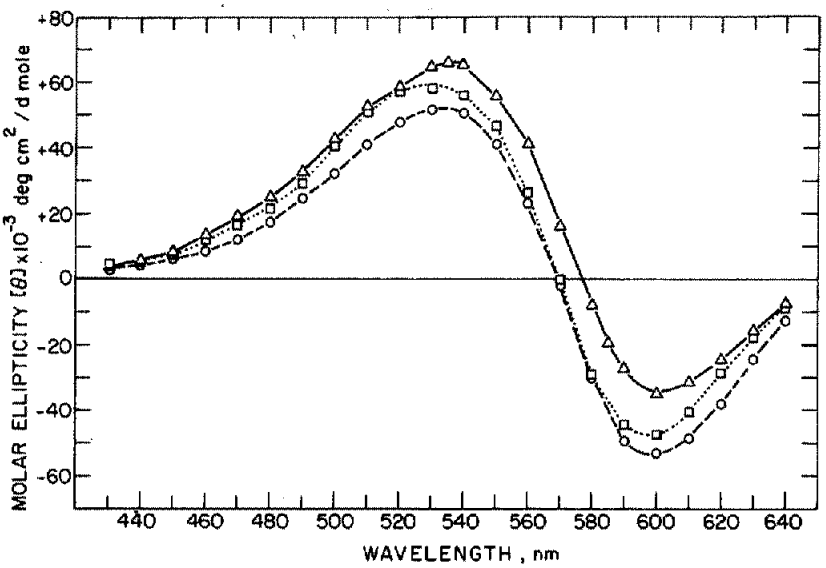

Fig.1. CD-Spectra of light adapted suspensions of purple membrane patches. (Δ) In water, (○) in 60% v/v glycerol/water, (□) in water after 3 min sonication. Molar ellipticities are based on spectrophotometrically determined chromophore concentrations. The extinction at 567 nm was taken as 63 000 M^{-1} cm^{-1} [11]. Scattering background was subtracted from the absorption spectra.

negative band to that of the positive band is considerably different in the two suspension media. The asymmetry is quite pronounced in water, the positive band at shorter wavelength being much more intense than the negative one. In 60% v/v glycerol/water the spectrum is apparently symmetric. The cross-over of the CD also shifts in the different media. It lies at 576 nm in water and at 569 nm in 60% glycerol/water. It should be noted that the wavelength of the absorption maximum is independent of the solvent.

In table 1 the ratio of the maximum $\Delta\epsilon$-values of the negative and positive bands are compared for the different suspension media and correlated with the refractive index. The ratios of the integrated values of $\Delta\epsilon\ (\lambda)/\lambda$ of the negative to positive bands are also compared (designated in the table as R_{neg} and R_{pos}). It can be concluded that the smaller the refractive index of the media, compared to the refractive index of the membrane particles, the more asymmetric the CD-spectrum becomes. The refractive index of the membrane is estimated to be $\geqslant 1.5$. It can also be visually observed that the turbidity of the suspensions decreases as the refractive index of the medium is increased by addition of either sucrose or glycerol. The turbidity is also a function of particle size and decreases upon sonication of the membrane suspension. As can be seen in fig.1, there is less asymmetry in the CD of sonicated membrane suspensions. Thus, there

Table 1
Correlation of the magnitude of the asymmetry of the visible CD with the refractive index of the suspension medium

Medium	$(\Delta\epsilon)_{neg}/(\Delta\epsilon)_{pos}$	R_{neg}/R_{pos}	n (λ = 589.3 nm)
Water	0.54	0.27	1.332
Basal salt	0.80	0.43	1.372
Sucrose/water 50% w/w	0.93	0.59	1.417
Glycerol/water 60% v/v	1.03	0.73	1.422

is a direct correlation between light-scattering of the suspensions and the asymmetry of their CD-spectra.

Scattering distorts the CD-spectrum of turbid suspensions of particles having optically active entities by virtue of two effects. One is the result of different amounts of scatter of left and right circular polarizations, due to different refractive indices for these polarization states. The other is the absorption flattening effect [8]. Due to the flattening effect the transmittance of the suspension will increase and the absorbance and CD will decrease accordingly. The scattering and absorption contributions add to the normal intrinsic optical activity. Schneider [9] has outlined a method in which the actual CD of the scattering suspension can be calculated from the intrinsic optical activity of the suspended particles, knowing the dispersion of the real and imaginary parts of the particle refractive index. The explicit functional dependence of the suspension CD on the particle parameters, such as refractive indices, particle size, etc. are determined by the scattering function suitable for the particle. In this procedure a best fit is sought between the experimental CD and that calculated for the scattering suspension, by varying the input CD. The input CD giving the best fit is taken to be the corrected intrinsic CD-spectrum of the membrane particles, free of turbidity effects. This method has been applied recently to obtain corrected spectra for the membranes of chromaffin granules [3] and red blood cells [4].

We have undertaken to calculate the CD of the scattering suspension of sub-bacterial vesicles of *H. halobium* in order to verify theoretically if the asymmetry in the visible CD-spectrum can be attributed to scattering effects. The sub-bacterial vesicles were chosen in order to be able to apply a coated sphere model [3]. It may be noted that sub-bacterial vesicle suspensions appear much more turbid than equivalent purple membrane suspensions. This is due to light-scattering from proteins, other than bacteriorhodopsin, that are also present in the membrane. However, these proteins do not adsorb, nor have optical activity in the visible spectral region and, therefore their scattering does not introduce additional distortions. As trial input of (intrinsic) CD and ORD we used the spectra of purple membrane patches suspended in 60% v/v glycerol water in which scattering is reduced to a minimum. The real and imaginary parts of the particle refractive index were estimated as described in previous work [3]. The resulting best fit calculated CD-spectrum is compared in fig.2 to the experimental spectrum of the sub-bacterial vesicles.

The calculated and experimental spectra coincide practically over the whole spectral range, except for

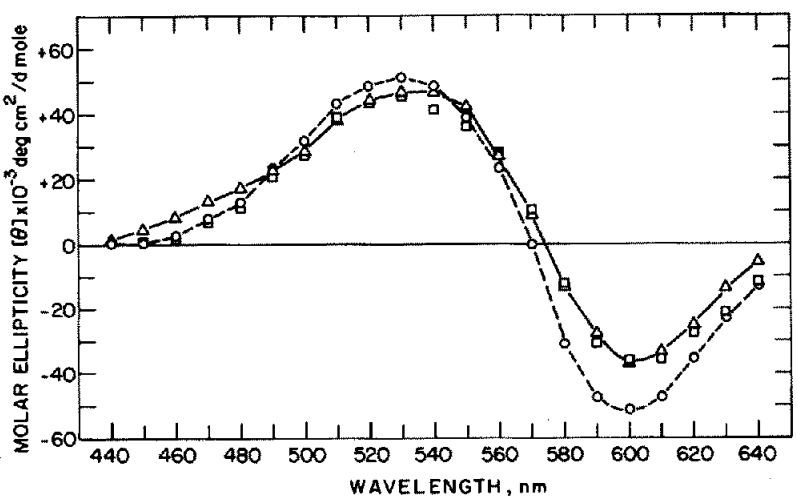

Fig.2. Comparison of the visible CD-spectrum of sub-bacterial vesicles to that calculated for scattering spherical particles of the following characteristics. Particle radius 0.25 μm, membrane thickness 4 nm, membrane real refractive index at selected wavelengths: n_{480} 1.498, n_{530} 1.522, n_{570} 1.547, n_{600} 1.546, n_{640} 1.518. (Δ) Experimental spectrum of sub-bacterial vesicles, (□) calculated spectrum of spherical particles, (○) input spectrum.

the 440–480 nm region, where a slight discrepancy is apparent. Also shown in fig.2 is the input CD-spectrum which is almost symmetric.

In view of the results obtained in this work we tend to attribute the asymmetry in the CD-spectrum of the purple membrane suspension in water to distortions arising from a light-scattering effect. As briefly mentioned before, this asymmetry was interpreted [1] as a superposition of a broad, positive, symmetric band centered at the absorption maximum (567 nm) and a band system consisting of oppositely signed lobes with equal amplitudes, symmetric with respect to the absorption maximum. In the light of our results any contribution of the former kind is very small, at the most, in the intact purple membrane, when it comprises its full complement of retinal moieties. A single positive band appears only during the early reconstitution steps of the retinal-depleted purple membrane [12] and may thus be attributed to the interactions within the monomeric pigment.

The CD can thus be interpreted as being essentially due to exciton-interaction between the chromophores in the hexagonal array of protein trimers [10], which constitutes the bulk of the purple membrane. It therefore appears reasonable that this spatial symmetry is retained also by the retinal moieties of the light-absorbing centers.

References

[1] Heyn, M. P., Bauer, P. J. and Dencher, N. A. (1975) Biochem. Biophys. Res. Commun. 67, 897–903.
[2] Schneider, A. S., Schneider, M. J. T. and Rosenheck, K. (1970) Proc. Natl. Acad. Sci. USA 66, 793–798.
[3] Rosenheck, K. and Schneider, A. S. (1973) Proc. Natl. Acad. Sci. USA 70, 3458–3462.
[4] Gitter-Amir, A., Rosenheck, K. and Schneider, A. S. (1976) Biochemistry 15, 3131–3137.
[5] Danon, A. and Stoeckenius, W. (1974) Proc. Natl. Acad. Sci. USA 71, 1234–1238.
[6] MacDonald, R. E. and Lanyi, J. E. (1975) Biochemistry 14, 2882–2889.
[7] Eisenbach, M., Bakker, E. P., Korenstein, R. and Caplan, S. R. (1976) FEBS Lett. 71, 228–232.
[8] Duysens, L. N. M. (1956) Biochim. Biophys. Acta 19, 1.
[9] Schneider, A. S. (1973) Methods Enzymol. 27D, 751–767.
[10] Henderson, R. (1975) J. Mol. Biol. 93, 123–138.
[11] Oesterhelt, D. and Hess, B. (1973) Eur. J. Biochem. 37, 316–325.
[12] Bauer, P. J., Dencher, N. A. and Heyn, M. P. (1976) Biophys. Struct. Mech. 2, 79–92.