ROTATIONAL DIFFUSION AND EXCITON COUPLING OF BACTERIORHODOPSIN IN THE CELL MEMBRANE OF *HALOBACTERIUM HALOBIUM*

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1. Introduction

Bacteriorhodopsin forms a two-dimensional hexagonal crystalline lattice in the purple membrane of *Halobacterium halobium* [1–4]. Rotational motion of bacteriorhodopsin may be investigated by measuring the decay of dichroism of flash-induced absorbance changes; in the rigid lattice of the purple membrane the protein is immobilised [5]. Rotational motion is observed when the lattice is disrupted by organic solvents [6] or when bacteriorhodopsin is incorporated into lipid vesicles [7].

Exciton coupling effects due to the interaction of the retinal chromophores of adjacent bacteriorhodopsin molecules have been observed in the CD spectra of the purple membrane in the 568 nm absorption band [8–10]. The necessary conditions for the occurrence of such effects are satisfied in the crystalline lattice. The results of recent structural investigations suggest that the lattice is formed from trimers [4]. The exciton CD-spectra could likewise be interpreted as arising from the interactions of the three chromophores within a trimer [9], neglecting inter-trimer interactions.

The exciton coupling bands disappear when bacteriorhodopsin is solubilised into micelles containing protein monomers [8,10,11] and when the bacteriorhodopsin molecules within the membrane acquire rotational mobility, either by addition of organic solvents [6] or by incorporation into artificial lipid membranes [7,12]. There appears to be a clear correlation between loss of exciton coupling, observation of rotational diffusion and protein disaggregation. The exciton CD effects in combination with rotational diffusion measurements thus provide useful information concerning the state of aggregation of the bacteriorhodopsin molecules within the membrane.

Bacteriorhodopsin synthesis in the cells depends on the rate of aeration during growth. If bacteriorhodopsin synthesis is not optimal, besides the crystalline lattice of the purple membrane a second bacteriorhodopsin membrane fraction is found. This fraction, which has been termed 'brown' membrane [13], does not show a crystalline lattice of protein molecules [14].

Cells grown in the presence of nicotine ('nicotine' cells) fail to synthesise retinal but continue to produce at a reduced level the protein bacterio-opsin [13]. Such cells contain large amounts of lycopene, since nicotine inhibits the synthesis of retinal by blocking

the cyclisation of lycopene to β-carotene. The plasma membrane is found to contain a membrane fraction similar to the brown membrane but without retinal ('apo-brown' membrane). This fraction is also a differentiated domain which, in addition to bacterio-opsin, contains a cytochrome *b*-type protein absorbing at 418 nm as well as other minor protein species [13]. Addition of all-*trans* retinal to the apo-brown membrane results in reconstitution of the characteristic 568 nm absorption band of bacteriorhodopsin. However, in the reconstituted apo-brown membrane and in the brown membrane it appears that an energy-dependent step is required before bacteriorhodopsin can crystallise to form patches of purple membrane [13].

Here we report CD and rotational diffusion measurements with bacteriorhodopsin in the reconstituted apo-brown membrane. We observe exciton coupling in the CD spectrum which indicates that the protein is not monomeric. However, aggregates of bacteriorhodopsin must be small since fairly rapid rotational diffusion of the protein is detected. We tentatively suggest that bacteriorhodopsin may exist as trimers in the reconstituted apo-brown membrane.

2. Materials and methods

Halobacterium halobium NRL R_1 mutant, strain M_1 [15], was grown and purple membrane isolated as previously described [16]. Retinal free cells (nicotine cells) were prepared by addition of 1 mM nicotine to the growing cell culture. Isolation of the apo-brown membrane fraction and reconstitution of the brown membrane upon addition of retinal was as described previously [17]. Retinal was also removed from bacteriorhodopsin in normal purple membrane-containing cells by exposure to light in the presence of hydroxylamine [18].

Due to the small diameter of isolated membrane fragments (~ 0.5 μm) protein rotation measurements may be complicated by the tumbling of the fragments themselves. We therefore prepared cell vesicles (diameter ~ 1 μm) from the different types of cells. Envelope vesicles prepared from the cells as previously described [16] were dialysed against 80 mM Mg_2SO_4 containing 10 mM Tris–HCl, pH 8.0 centrifuged for 1 h at 100 000 × *g* (until the supernatant is almost colourless) and resuspended in 80 mM Mg_2SO_4.

In the following, cell vesicles are named according to their parent cell types; i.e. nicotine vesicles, NH_2OH vesicles and PM vesicles (vesicles from normal purple membrane-containing cells). Reconstitution of bacteriorhodopsin was achieved by adding all-*trans* retinal in slight excess as a 10^{-3} M solution in isopropanol and the chromophore concentration was determined by difference spectroscopy. Reconstituted vesicles are designated as *R*-nicotine vesicles and *R*-NH_2OH vesicles.

Rotational diffusion of bacteriorhodopsin was measured by observing the decay of dichroism of flash-induced transient absorption changes; the flash photolysis apparatus and general principles of the method are described elsewhere [19]. Spectroscopic properties of bacteriorhodopsin have recently been summarised [11,20]. In the present experiments, we detected ground-state depletion of the 568 nm absorption band of bacteriorhodopsin following excitation by a plane-polarised light-flash of duration 1–2 μs and wavelength 540 nm from a dye laser. Bacteriorhodopsin was light adapted by the 100 W tungsten–halide lamp used as the source of the measuring beam. The results are analysed by calculating the anisotropy parameter $r(t)$ given by

$$r(t) = \frac{A_{\parallel}(t) - A_{\perp}(t)}{A_{\parallel}(t) + 2A_{\perp}(t)} \qquad (1)$$

where $A_{\parallel}(t)$ and $A_{\perp}(t)$ are the absorbance changes at time t after the flash for light polarised parallel and perpendicular to the polarisation of the exciting flash. Measurements were made at room temperature unless otherwise stated.

CD Measurements were carried out with a Cary 61 spectropolarimeter. All measurements were made at 25°C with light-adapted membranes. Absorption spectra were recorded with a Cary 118C spectrophotometer.

3. Results and discussion

3.1. *Rotational diffusion*

3.1.1. *R*-Nicotine vesicles

In fig.1, curve A is the time-dependence of r calculated from transient absorbance changes observed after

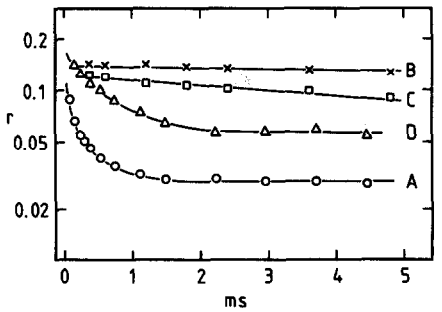

Fig. 1. Time-dependence of r obtained from transient absorption changes measured at 568 nm. (A) R-Nicotine vesicles, (B) PM-Vesicles, (C) R-NH_2OH vesicles, fully reconstituted, (D) R-NH_2OH vesicles, 50% reconstituted.

flash excitation of R-nicotine vesicles. The curve clearly shows two regions; r decreases with time up to about 2 ms but thereafter appears to reach a constant value. In contrast the corresponding curve obtained with PM-vesicles (curve B) is virtually independent of time, in agreement with previous observations that bacteriorhodopsin is immobilised in the purple membrane [5].

The decay of r in curve A cannot be attributed to vesicle tumbling since the vesicles are too large to exhibit appreciable rotational motion on the time scale of the experiment. Hence this decay is clear evidence of rotation of bacteriorhodopsin in the membrane. To proceed further we use the following expression for r, which as shown elsewhere [21], follows from existing theoretical treatments when protein rotation occurs only about a single axis normal to the plane of the membrane:

$$r(t) = A_1 \exp(-D_{\|}t) + A_2 \exp(-4D_{\|}t) + A_3 \qquad (2)$$

where $A_1 = (6/5)(\sin^2\theta\cos^2\theta)$, $A_2 = (3/10)(\sin^4\theta)$, $A_3 = (1/10)(3\cos^2\theta - 1)^2$. Here $D_{\|}$ is the rotational diffusion coefficient about the normal axis and θ is the angle between this axis and the transition moment for absorption (which for all-*trans* retinal coincides with the long axis of the polyene chain). The above equation applies when the same transition moment is used for excitation and measurement. In practice measured values of r are smaller than theoretical expectations due to instrumental factors; this however, has no effect on the form of $r(t)$.

The time-dependence of r observed experimentally with R-nicotine vesicles is in good accord with eq. (2). In particular, r does not fall to zero but reaches a constant time-independent value which may be identified with the coefficient A_3 in the above equation. Hence it is reasonable to conclude that rotation of bacteriorhodopsin is indeed confined to a single axis normal to the plane of the membrane.

In order to evaluate $D_{\|}$ we need to know θ, which in principle can be obtained from the ratio $A_3/(A_1 + A_2 + A_3)$. The assumptions and uncertainties in evaluating θ is this way are discussed in detail elsewhere [12], for present purposes we note that a value of θ of about 75° is consistent with the experimental data. Using this value, we then obtain $D_{\|} = 2300 \pm 500\ s^{-1}$ by fitting the points in curve A to eq. (1). We emphasise that the calculated value of $D_{\|}$ is only weakly dependent on the particular choice of θ, for example changing θ by 5° changes $D_{\|}$ by about 15%.

Further confirmation that we are detecting rotational diffusion of bacteriorhodopsin in the membrane is provided by fig.2 which shows the effect of fixing the vesicles with glutaraldehyde. Rotation is largely abolished although some residual motion remains. Presumably not all bacteriorhodopsin molecules are crosslinked into immobile patches during the fixation. Figure 2 also shows plots of $r(t)$ at different temperatures. As might be expected, rotation becomes faster at higher temperature as can be seen from the more rapid decrease in r.

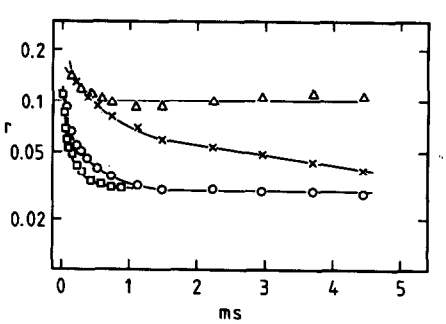

Fig. 2. Time-dependence of r obtained from transient absorption changes measured at 568 nm with R-nicotine vesicles under different conditions. (X) 8°C, (○) 22°C, (□) 40°C, (△) fixed for 30 min in 3% glutaraldehyde, at 22°C.

3.1.2. Brown membrane fragments

Measurements with brown membrane fragments were complicated by the tumbling of the fragments themselves. At long times, r was not independent of time as found with R-nicotine vesicles but exhibited a slow decrease corresponding to a rotational correlation time of about 5 ms. This would be consistent with the tumbling of particles of 4000–5000 Å in diameter. Although no detailed morphological characterisation of the fragments has been made, preliminary electron micrographs indicate that the fragment size is indeed in the above range.

In addition to the slow component, a fast component of rotational motion is also detected in these samples. As with the R-nicotine vesicles, we assign this component to rotation of bacteriorhodopsin in the membrane. We estimate $D_{\parallel} \approx 1200\ s^{-1}$ for the brown membrane fragments; there is considerable uncertainty in this value, however, due to the necessity of subtracting out the slow component from the data. Bearing this in mind, the agreement with the value of $D_{\parallel}$ obtained with R-nicotine vesicles is reasonable.

3.1.3. R-NH_2OH vesicles

For comparison purposes we also made measurements with R-NH_2OH vesicles. Previous investigations showed that the hexagonal lattice is reformed following regeneration of bacteriorhodopsin in NH_2OH bleached purple membrane fragments and whole cells [11,22].

Figure 1 (curve C) shows the time-dependence of r for R-NH_2OH vesicles in which sufficient retinal was added to fully reconstitute bacteriorhodopsin. The difference between curves B and C is significant, in curve C there is a definite decrease in r with time demonstrating the existence of a slow rotational motion in the sample. A possible explanation of this motion is that purple membrane patches formed following addition of retinal are of sufficiently small diameter for us to detect rotation of the patches in the membrane. Support for such an explanation comes from curve D which is the result obtained when less retinal is added so that only about 50% of bacterioopsin is converted to bacteriorhodopsin. In this case faster rotation is observed which could correspond to the formation of still smaller patches.

3.2. Exciton coupling

3.2.1. Exciton coupling effects in cell vesicles

The three preparations, PM-vesicles, R-nicotine vesicles and R-NH_2OH vesicles, all show clear exciton coupling CD-bands in the 568 nm absorption band (fig.3), similar to those observed in purple membrane fragments [8–10]. Due to the large size of the vesicles, considerable light scattering occurred. It was previously observed with purple membrane fragments that light scattering can cause some distortions in the CD spectrum [23]. These artefacts, which consist of an increase in the ratio of the peak heights of the positive 530 nm band and the negative 600 nm band, can be reduced by increasing the refractive index of the aqueous solution or by gentle sonication. Although the ratio of the two peaks is reduced in this way, the CD peak-to-peak amplitude, i.e., the difference in CD at 530 nm and 600 nm, remains approximately the same. To investigate possible artefacts due to light scattering in the present samples, CD spectra were also recorded with vesicles suspended in 35% (w/w) sucrose solution (fig.4). In all cases, the asymmetry of the CD spectrum is considerably reduced, a result which is certainly due to reduction of light scattering in PM-vesicles and most probably in the R-nicotine and R-NH_2OH vesicles, too.

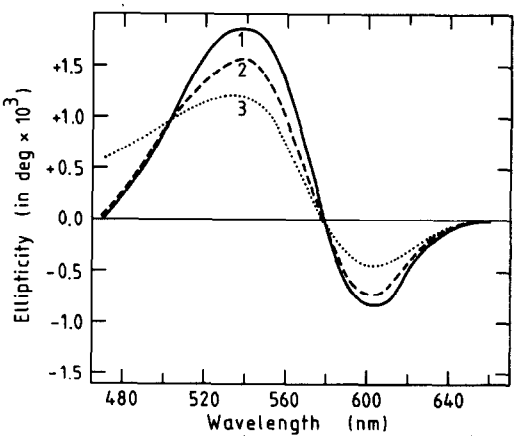

Fig.3. The CD spectra of light adapted suspensions of PM-vesicles (1), R-NH_2OH vesicles (2) and R-nicotine vesicles (3).

Because the amount of light scattering differs in the three preparations, it is most appropriate to compare the various CD spectra using the peak-to-peak amplitudes, i.e., the difference in CD at 530 nm and 600 nm. For the peak-to-peak molar ellipticity $[\theta]$ in degree $cm^2/dmol$ we find 8.2×10^4 for

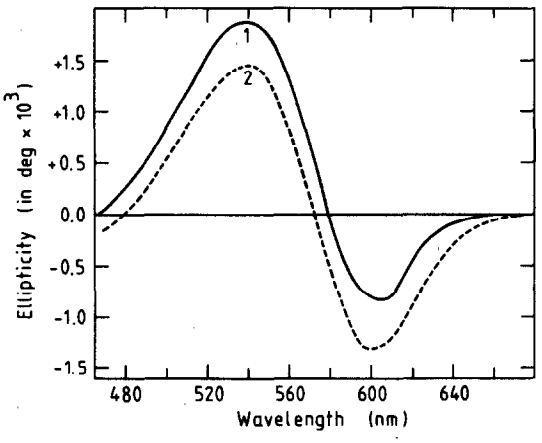

Fig.4. The effect of sucrose on the asymmetry and zero crossover of the CD spectra of PM-vesicles. Curve 1, no sucrose. Curve 2, 35% (w/w) sucrose. Note that the difference between the ellipticity at 530 nm and at 600 nm is not affected by the addition of sucrose.

PM-vesicles, 7.4×10^4 for *R*-NH_2OH vesicles and 5.5×10^4 for *R*-nicotine vesicles. These values may be compared with typical values of between 8 and 10×10^4 for purple membrane fragments [8]. The error in these numbers is considerable, since they depend on the degree of bacteriorhodopsin reconstitution and on the accuracy of the determination of the bound retinal concentration.

It appears therefore that most, if not all of the bacteriorhodopsin is in an aggregated form in all three vesicle preparations. Since the amplitude and shape of the CD spectrum is quite sensitive to the geometrical arrangement of the transition dipole moments of the retinal chromophores of adjacent bacteriorhodopsin molecules, it is likely that the aggregation geometry in the reconstituted vesicles is quite similar to that in the purple membrane.

In addition to the 568 nm bands, the CD spectra of PM-vesicles show a pair of exciton-type bands positive at 385 nm, negative at 435 nm with zero crossover at 410 nm which we attribute to the cytochrome *b*-type protein. We also observe a further weak pair of bands of opposite sign centred around 343 nm; these are most likely due to lycopene. These bands have much higher intensity in the nicotine vesicles which contain large amounts of lycopene.

3.2.2. Exciton coupling in brown membrane fragments

Brown membrane fragments exhibited exciton CD-bands at 568 nm as well as the usual purple membrane bands at 263 nm, 290 nm and 320 nm. They are different from vesicle spectra in the sense that no lycopene bands were observed. This is to be expected since lycopene is not detectable in the brown membrane fragments, occurring exclusively in the red membrane fraction of lower density [13]. The brown membrane fragment preparation appeared to be much enriched with respect to the vesicles in their cytochrome *b* content. Strong exciton-type CD-bands were observed centred at 410 nm, similar to the corresponding bands in vesicles which we attribute to this protein.

3.3. Aggregation state of bacteriorhodopsin in the reconstituted apo-brown membrane

The observation of exciton coupling at 568 nm in the CD spectrum of both *R*-nicotine vesicles and brown membrane fragments is a clear indication that most or all of the bacteriorhodopsin is not in a monomeric form. On the other hand, density gradient analysis of the membrane fragments indicate that the usual purple membrane is not formed, a result confirmed by diffraction measurements [14].

The present rotational measurements demonstrate protein rotation in *R*-nicotine vesicles and brown membrane fragments which is slower than that found for rhodopsin in disc membranes [24] but comparable to or slightly faster than that of band 3 proteins in the human erythrocyte membrane [21]. We can estimate an upper limit for the size of the rotating particle by applying the equation

$$D_{\parallel} = kT/4\pi a^2 h\eta \qquad (3)$$

which applies to a cylinder of radius a embedded to a depth h in a membrane of viscosity η [25]. Taking $h = 45$ Å as the thickness of the membrane and assuming that η is unlikely to be less than 1 P, the present value of $D_{\parallel}$ obtained with *R*-nicotine vesicles implies that the particle radius is less than 200 Å.

Whilst the present data do not in themselves permit a more precise statement about the aggregation state of bacteriorhodopsin in the brown

membrane, it is tempting to speculate that the state may actually be a trimer. This would be in harmony with the observation that in the purple membrane the hexagonal lattice is formed from repeating groups of three molecules [4]. If this speculation were correct, it would imply that it is the crystallisation of trimers which is the energy-requiring step in the formation of the hexagonal lattice. A further implication is that the crystalline lattice is not a prerequisite for the function of bacteriorhodopsin. Elsewhere it is shown that maximal photophosphorylation in intact nicotine cells occurs within 10 min of retinal addition whereas crystallisation takes a period of hours [26].

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