A RHODOPSIN–LIPID–WATER LAMELLAR SYSTEM: ITS CHARACTERISATION BY X-RAY DIFFRACTION AND ELECTRON MICROSCOPY

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1. Introduction

The study of artificial systems formed by the principal components of biological membranes (proteins, lipids and water) assembled into an ordered system, is one of the possible approaches to the problem of membrane structure and function [1]. A protein particularly suitable for this kind of approach is the visual pigment rhodopsin, located in the disc membranes of the retinal rods of vertebrates, where it constitutes about 80% of the total protein content [2]. It is specifically involved in the early molecular events of the visual excitation although its precise mode of action is not yet understood. Rhodopsin is a typical membrane protein in the sense that it is totally insoluble in water in the absence of lipids or detergents.

We describe in this work a large-scale preparation of highly purified rhodopsin with its associated lipids, and a preliminary study of the artificial rhodopsin–lipid–water system obtained upon elimination of the detergent in which the purified rhodopsin had been solubilised.

2. Methods

2.1. *Preparation of rhodopsin*

All operations were carried out under dim red light at melting ice temperature. In a typical preparation 200 cattle eyes were kept in the dark for at least 2 hr after removal prior to dissection of the retinas. The retinas were homogenised with a Potter homogeniser in isotonic phosphate buffer (pH 7) and sedimented once. A first crude separation of the rod outer segments (r.o.s.) was obtained by flotation on a 36% (w/w) sucrose solution by centrifugation 1 hr at 40,000 *g*. The floats were washed and collected by centrifugation in hypotonic 0.007 M phosphate buffer. The pellets, which now consist of membrane fragments, were homogenised and purified on a continuous sucrose gradient with a small density range: 1.15 g/ml (38% w/w) to 1.11 g/ml (27% w/w). After centrifugation 1 hr at 90,000 *g*, two layers were observed in the gradient, differing in density by only about 0.01 g/ml. The upper layer, which was purple, was collected, washed three times by centrifugation in distilled water and freeze dried. The yield was usually about 1.5 mg per eye.

In order to solubilise the membrane proteins one of two very similar non ionic detergents were used: either Emulphogene B.C. 720 which does not absorb in the region of 280 nm, hence allowing the total protein content of the preparation to be determined spectroscopically; or Triton X-100, that was preferred due to its strong absorption at 280 nm, when the elimination of detergent was to be followed. In both cases the freeze dried product was dissolved in 2% (w/w) detergent solution at a concentration of 10 mg/ml, stirred for 2 hr at room temp. and then

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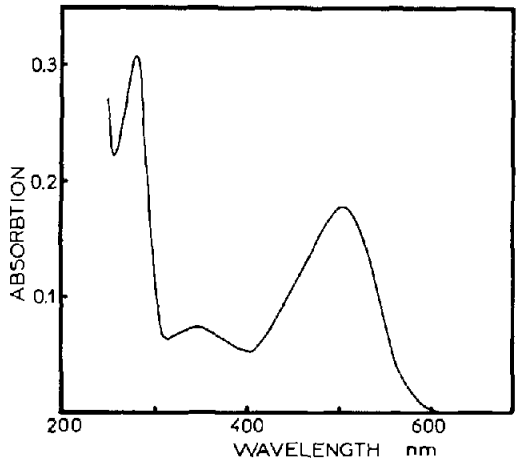

Fig. 1. Absorption spectrum of the rhodopsin–lipid–water pellet obtained after detergent elimination. The detergent used in this case was Triton X-100, and the rhodopsin had not been purified on a column.

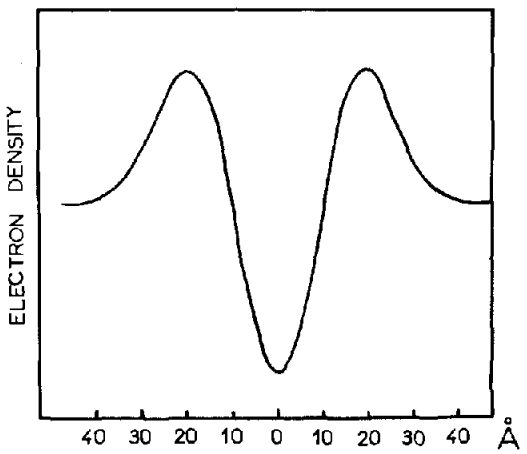

Fig. 2. Electron density distribution through a rhodopsin–lipid–water lamella with 90 Å repeat distance, as obtained by Fourier synthesis from the diffraction intensities.

centrifuged for 1 hr at 40,000 *g*, leaving a white pellet which constituted less than 10% of the product. The clear red supernatant obtained was not sedimentable even after centrifugation for 15 hr at 100,000 *g*. The spectral ratio A_{280}/A_{500} of this solution, with Emulphogene, ranged between 1.75 and 2.03 and the O.D. at 500 nm per mg of freeze-dried product in solution ranged from 0.31 to 0.26 indicating that the rhodopsin was purified to a high degree. Further purification using a column of hydroxyapatite reduced the spectral ratio to 1.6 to 1.7 depending on the initial product. This last step in the purification was seldom deemed worthwhile.

2.2. *Detergent elimination, reconstitution of rhodopsin lamellae*

The detergent rhosopsin solution contains essentially all of the membrane lipids, and careful extraction of the detergent may give rise to an ordered rhodopsin–lipid structure.

Two different extraction procedures have been tried: the first based on the method proposed by Zorn and Futterman [3] involved drying the detergent solution and extracting the dry product with toluene. This method leads to an amorphous product and was abandoned.

In the second method the detergent was extracted from the aqueous rhodopsin–detergent solution by use of toluene containing some lecithin (normally 10 mg lec/ml toluene). As shown below this lecithin in toluene did not interchange notably with the phospholipids associated with rhodopsin but its presence was generally found to be necessary for the complete extraction of the detergent. The detergent solution of rhodopsin was mixed in a homogeniser with the solution of lecithin in toluene in the ratio 2:5. The milky mixture thus obtained was centrifuged (10 min at 12,000 *g*) to produce a separation of the organic (toluene, lecithin and detergent) and aqueous (rhodopsin and lipids) phases. The organic phase was removed and the procedure repeated twice. The amount of detergent extracted was determined by weighing (assuming no lipid exchange) and/or spectrophotometry when the detergent used was Triton X-100; both determinations were generally in good agreement. After three extractions the quantity of detergent that remained in the aqueous phase was negligible (see fig. 1); lecithin was the only phospholipid found in the toluene extracts and it was shown by thin-layer chromatography that no change had occurred in the proportions of the different phospholipids in the

aqueous phase. A small amount of the toluene remained in the aqueous phase giving rise to a fairly solid emulsion. This toluene fraction can be eliminated by careful evaporation in vacuo, yielding an aqueous, detergent free, suspension. This suspension was centrifuged for a few hours at 200,000 *g* after adding sucrose up to 10% w/w, thus eliminating any lipids present that would not be associated with protein. In some cases a small white layer at the top of the sucrose solution was obtained. A transparent pellet was formed, which was washed by dispersion and centrifugation in distilled water. The absorption spectrum of one such pellet is shown in fig. 1. The spectral ratio A_{280}/A_{500} is 1.75, showing that all of the Triton X-100 used in this case has been removed.

For chemical analysis the total lipid and protein fractions in the pellet were separated by chloroform/methanol extraction and their ratio determined by weighing. The lipid phosphorous was measured by the method of Chen [4]. The pellet contained about 60% protein and 40% total lipid (30% phospholipid assuming that the average phosphorous content of the phospholipids was 4%). These values are very near to those determined for the freeze-dried product and the r.o.s. [5].

X-ray diffraction studies were performed, using linear focalisation by the methods described elsewhere [6].

Electron microscopic observations were made with an EM 300 Philips. Freeze-etching technique was applied with the Balzers 360 M apparatus. The samples were directly frozen in Freon 22 (−150°) without cryoprotective agents and stored in liquid nitroegn. The cleaved surfaces were etched at −100° for 60 to 90 sec.

3. Results

3.1. *X-ray diffraction*

The pellet obtained after elimination of the detergent gave rise to two diffuse small angle bands which changed in form with progressive drying. When the water content was less than 70%, sharp equidistant reflections appeared, characteristic of a long range organisation of the smectic type, namely of a structure formed by lamellae, parallel and equidistant, with no other correlation in position and orientation. With further reduction of the water content the reflections sharpened and there was concurrent reduction of the repeat distance. The total observable range of the repeat distance was from 110 Å to 70 Å (water content 70% to 35%). On reduction of the water content below 35% additional reflections appeared, which corresponded to a lipid–water phase. The electron density profile deduced from the diffraction intensities has the general appearence of a lipid bilayer (fig. 2) and does not differ very markedly from the electron density profile of the pure lipids, or of other membranes at the same resolution (30 Å) [7, 8]. It would be difficult to draw accurate conclusions from this type of profile. More interesting information is provided by taking into account the chemical composition of the sample. From a knowledge of the repeat distance, concentrations and specific volumes, the partial thickness of each component may be calculated [6], namely the thickness of a hypothetical planar slab containing the totality of one of the components present in the unit cell [1]. The following partial thicknesses were calculated: rhodopsin 20 ± 2 Å, lipids 18 ± 2 Å. It is interesting to compare the lipid partial thickness in the rhodopsin–lipid–water lamellar phase without rhodopsin. The former value is less than half of that observed in the latter case (45 ± 2 Å). This indicates that a fairly high proportion of the "lipid bilayer" visualised in the electron density profile of rhodopsin–lipid–water lamellar phase is composed of rhodopsin molecules.

In order to assess the effect of the bleaching of rhodopsin on the lamellar structure, the following experiment was carried out. An initial diffraction pattern from an unbleached sample was recorded. Without removing the sample from its position, half of it was illuminated through a cold filter until its colour turned yellow, the other half being masked. Another diffraction pattern was subsequently obtained from the whole sample. The bleached part of the sample displayed different changes depending on the initial repeat distance: i) in samples with an initial repeat distance of 85 Å to 90 Å there was an increase of 5 ± 1% in the repeat distance, whereas the unbleached part remained unchanged, ii) when the repeat distance was about 75 Å the appearance of additional reflections due to lipid lamellae were observed in the bleached part of the sample. It should be noted that 75 Å is a repeat distance close to the

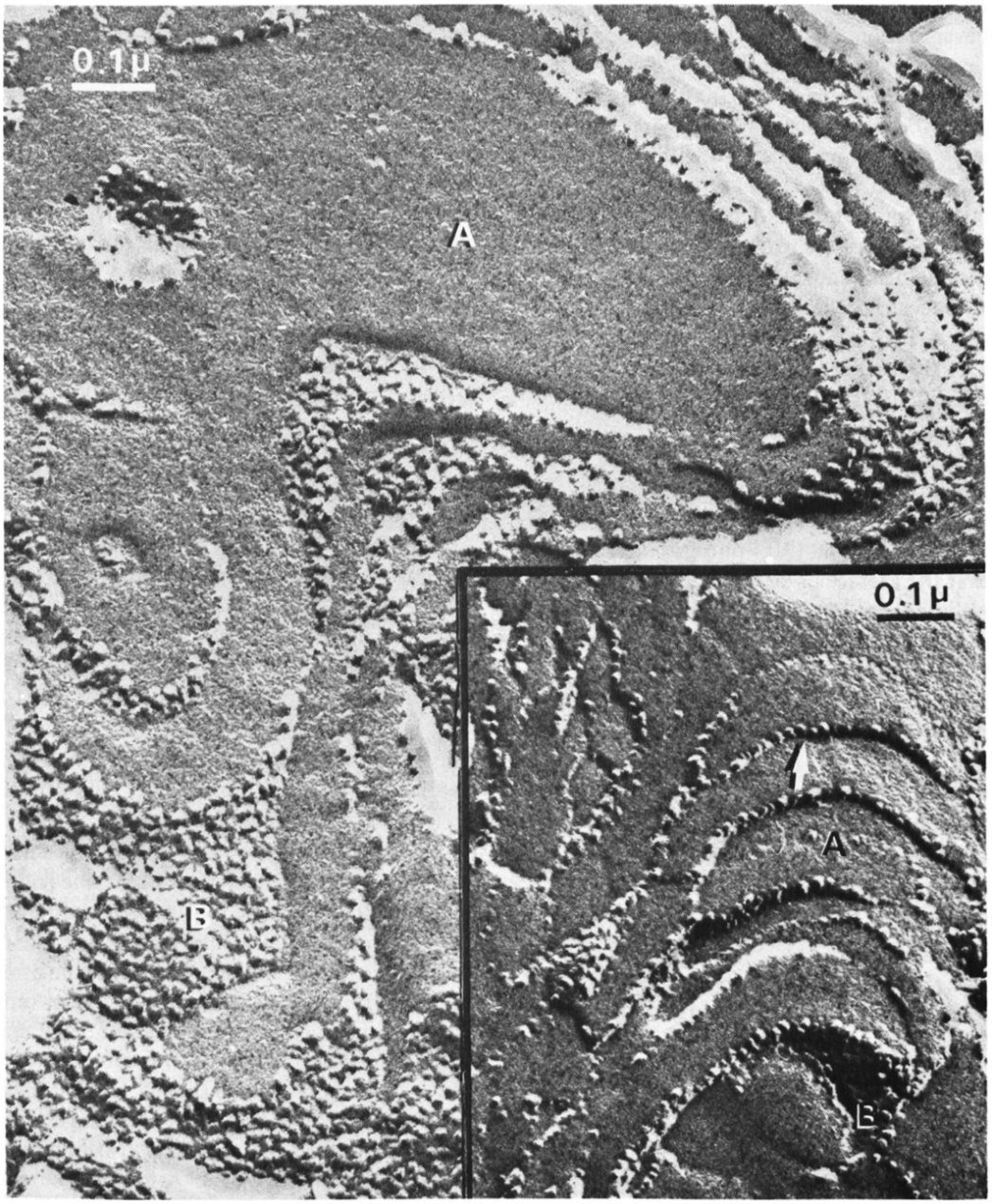

Fig. 3. Freeze-etched rhodopsin–lipid–water lamellar phase. The smooth surfaces (A) are separated by edges containing rows of protruding particles (arrow). In some cases larger surfaces covered by closely packed particles (B) are also seen.

limit below which further drying would produce the same phenomenon.

Both effects confirm that rhodopsin is associated with the lamellar structure and that light induces changes in the organisation and in the protein–lipid interactions.

3.2. *Freeze etching electron microscopy*

Most of the samples used in the freeze etching study has been previously controlled by X-ray diffraction. The water content of the samples was usually about 70%. The replicas showed a stratified arrangement of lamellae (fig. 3). The oblique fracture exposes large flat surfaces which are completely smooth (A). When the cleavage plan jumps, in a stepwise fashion, from one lamella to another, a ridge either convex or concave is usually produced. At the edges a row of protruding subunits are visible (see insert, arrow). Moreover the ridges are continuous with convex or concave surfaces (B) which are between the flat smooth fracture faces.

These surfaces alternating with flat faces area are, on the contrary covered by closely packed (110 to 130 Å) globular subunits. Their fracture nature is not readily apparent and it may be assumed that the exposure of this surface dotted with particles is produced by the etching rather than by the cleavage plane. If this interpretation is correct and assuming that each lamella splits along its inner hydrophobic matrix, the particles exposed by etching would be associated with the external halves of the lamella rather than with its center. To a first approximation we have not yet observed any qualitative difference between the bleached and unbleached samples.

4. Conclusion

A detergent-free rhodopsin–lipid association that maintains the absorption characteristics of unbleached rhodopsin has been obtained taking advantage of a large-scale preparation of highly purified rhodopsin. The structure of this association is lamellar and the first results obtained by X-ray diffraction indicates that the rhodopsin molecules penetrate into the "lipid bilayer". The freeze etching data shows the presence of smooth surfaces and particles on the edges of the lamellae. This indicates that the rhodopsin molecule does not traverse the "bilayer". Whether the particles represent the rhodopsin molecule cannot be established. The exact dimension and shape of the rhodopsin molecule are not well known. However it was postulated by Blasie et al. [9] that the molecule was spherical with a diameter of 40 Å. It was deduced from a recent study by fluorescence [10] that it could be ellipsoidal (major axis 75 Å). The particles visualised by electron microscopy in our system appear to be too large to correspond to the isolated rhodopsin molecules. It is possible that they are rhodopsin-aggregates or rhodopsin–lipid complexes. The structure of the system is modified by the bleaching of rhodopsin. The light induced changes, as shown by X-ray diffraction, are small but unmistakable; we have not yet interpreted them in terms of changes of molecular structure or molecular interactions.

The rhodopsin has so far been studied either in natural membranes or in detergent solutions. The present preparation allows rhodopsin to be studied in a structure not too far from the natural one but which can still be controlled and modified. Further studies on this system are being carried out at the present moment.

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