X-RAY DIFFRACTION STUDY OF MEMBRANE FRAGMENTS RICH IN ACETYLCHOLINE RECEPTOR PROTEIN PREPARED FROM THE ELECTRIC ORGAN OF *TORPEDO MARMORATA*

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1. Introduction

The electric organ of *Torpedo* is a very rich source of cholinergic (nicotinic) receptor protein [1–4]. Its fractionation yields membranes containing up to 30% of their proteins as the cholinergic receptor [4,5]. These fragments presumably originate from subsynaptic areas of the electroplaque plasma membrane and constitute a convenient preparation for the study of the cholinergic receptor protein in its native membrane environment. Binding studies with radioactive [6] or fluorescent [7,8] cholinergic ligands and observations by electron microscopy at high resolution [5,9] have recently been done with these receptor rich-membrane fragments. We present here a first report on the study of the same membrane fragments by X-ray diffraction that gives some evidence for a lattice organization of the receptor protein in the plane of the membrane.

2. Methods

2.1. *Preparation of membrane fragments from Torpedo electric tissue*

Membrane fragments rich in cholinergic receptor protein were prepared by sucrose gradient centrifugation following the method of Cohen et al. [4] from fresh electric tissue of *Torpedo marmorata*. The receptor-rich membrane fragments make a band at 38% (w/v) sucrose. The specific activity of the preparations ranged from 500 to 1500 nmoles of ^{3}H-labelled-α-toxin binding sites per g of protein as estimated by Millipore filtration [10], and the samples contained 0.2–0.5 mg of protein per ml. In the same gradients, a large amount of membrane fragments containing small concentrations of α-toxin binding sites are found at 20% (w/v) sucrose. These fragments are used in control experiments.

2.2. *Preparation of the membrane specimen for X-ray diffraction*

The suspension of membrane fragments in sucrose solution was first diluted 2-fold with distilled water and centrifuged 1 hr at 40 000 *g*. In general the pellet consisted of two distinct layers: an upper translucent layer and a lower fibrous one. In some experiments the upper layer was resuspended by gentle shaking in physiological salt solution (250 mM NaCl, 5 mM KCl, 4 mM $CaCl_2$ and 5 mM Na-phosphate pH 7.0), while the lower one remained at the bottom of the tube and was discarded.

The resuspended upper layer (or total pellet) was further homogenized by a few strokes in a Teflon glass Potter homogeneizer. The concentration of proteins in the homogenized suspension was approx.

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3 mg/ml and 100 g of fresh electric tissue yielded about 1 ml of suspension.

The specimens used for X-ray diffraction were obtained by centrifugation of 0.5 ml of the final membrane suspension for 12 hr at 200 000 *g* in a Beckman analytical ultracentrifuge. The pellet was collected on a mica strip inserted into the centrifuge cell before the centrifugation, following a method described elsewhere [11].

The final concentration of sucrose in the specimen was 5–10% (w/v), and preparations with different water content were obtained by letting the specimen dry in the cold.

The X-ray diffraction pictures were taken with an Elliot rotating anode generator equipped with a mirror monochromator point focusing optic. Exposure times varied from 3–24 hr at 4°C.

3. Results and discussion

Fig. 1 shows the X-ray diffraction pattern given by a membrane specimen oriented with the X-ray beam perpendicular to the sedimentation axis. Because the X-ray beam is much thinner (approx. 0.2 mm) than the membrane pellet (approx. 1 mm along its sedimentation axis), we were able to observe diffraction patterns from different layers of the specimen. A significant inhomogeneity along the length of the sedimentation axis was noticed when both the translucent and fibrous pellets were resuspended for the final sample preparation. However, relatively homogeneous specimens were prepared when only the translucent pellet was used for final sample preparation. Under these conditions we consistently observed sharp, oriented, reflections showing a pronounced arcing around the center of the picture. The orientation was sufficient to distinguish clearly meridional and equatorial reflections (i.e. in directions parallel and perpendicular to the sedimentation axis).

3.1. *Meridional reflections*

Most samples show ten orders of meridional reflections indicating a well ordered lamellar structure. The observed spacing ranged from 300 to 370 Å for water contents varying from 35% to 50% by weight. Such distances are much larger than those found with sarcoplasmic reticulum vesicles oriented by the same

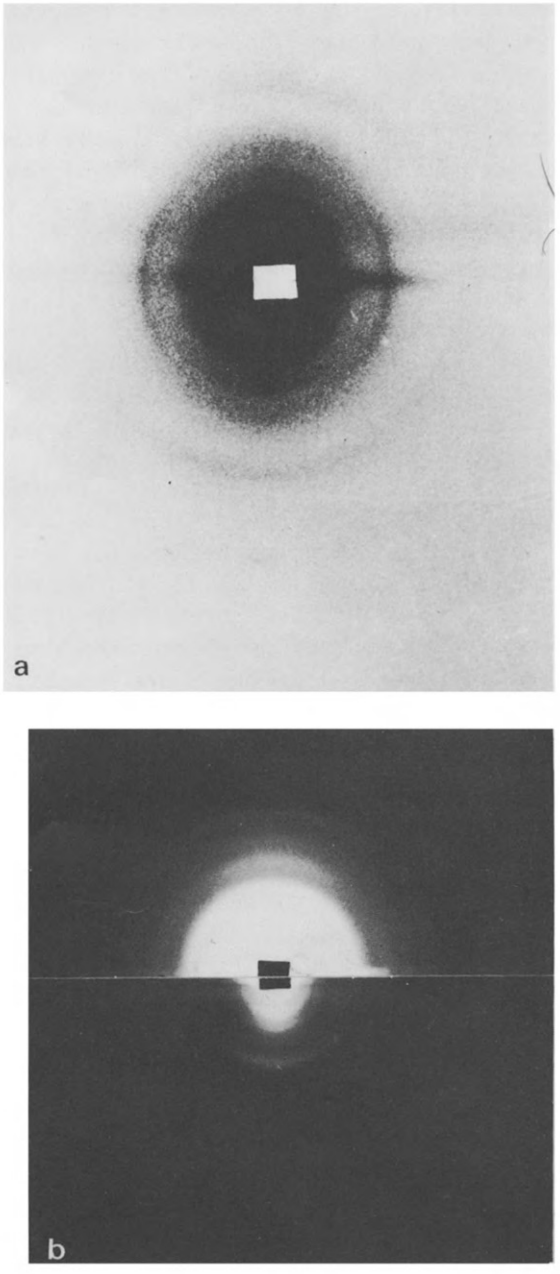

Fig. 1. X-Ray diffraction pattern from oriented subsynaptic membranes: a) The lamellar spacing is 353 A. The first equatorial reflection is clearly seen on this photograph. Lamellar reflections are observed out to the seventh order (out to ten on the original film); b) Same specimen with a spacing of 300 A. The (1,0) reflection is barely seen but the meridional reflections are sharper and better oriented. The two halves of the picture correspond to different time of exposure.

method [11]. Electron micrographs of thin sections of the pellet indicate that the lamellar structure arises from the flattening and stacking of close membrane vesicles (approx. 0.5 μ in diameter) present in the suspension before centrifugation. Occasionally, lamellar spacings of only 150–170 Å were observed. This spacing is the one expected for disrupted vesicles stacked randomly during the centrifugation.

The intensities of the lamellar reflections along the meridian were measured with a densitometer. At present, the phases of the reflections cannot be determined to obtain an electron density profile, but some information about the principal symmetries of the repeating unit was found from the Patterson function calculated from the observed reflection intensities.

The Patterson map is the autocorrelation function of electron density. The peaks in that map are found at distances which correspond to relative distances between the dominant scatterers [12,13]. These relative distances reveal spacings between the membrane layers and distances across a single membrane. Membrane lipids organized into a bilayer give rise to an intense peak in the Patterson map around 35–40 Å, corresponding to the distance between polar heads [12,13]. This feature has been found in the vector maps calculated from the diffraction patterns observed for myelin, retina, and sarcoplasmic reticulum, the biological membranes studied so far at good resolution [11,13]. We have computed the Patterson function to a resolution of 30 Å for four different spacings and the most striking feature of the map is a constant peak at 60 Å instead of 40 Å. This value is too large to be attributed solely to the lipid polar headgroups. One interpretation of such a large distance is that dense sheets of proteins are present on one or both sides of the membrane, and distort the usual bilayer profile. A similar interpretation might also account for an unusually large effective thickness of the membrane (90–100 Å) calculated by weighing the specimen before and after complete drying over P_2O_5.

3.2. *Equatorial reflections*

All the specimens of receptor-rich membrane fragments show a sharp equatorial reflection indicating a lattice organisation of particles within the plane of the membrane (fig. 1a). Membrane fragments from the same origin and prepared in the same manner as those used in the X-ray work show by both negative staining and freeze etching [9] arrays of particles on the membrane surface [5,9]. The surface structure revealed by deep-etching after freeze-fracture presents evidence of an hexagonal lattice organisation. By negative staining this particular structure is seen in about 50% of the membrane fragments. If we assume, for the time being, that the lattice organisation seen by X-ray diffraction and electron microscopy have the same origin, then, one might consider the first equatorial reflection of the X-ray diagram as the (1.0) order of an hexagonal lattice. Granted this assumption, which is further supported by the indications of (1.1) and (2.0) reflections in some of the X-ray pictures, then, one calculates a center-to-center distance of the diffracting repeating units of 91 ± 1 Å in a wet sample. Almost the same value was found on the electron micrographs [9].

Over different degrees of drying the dimensions of the lattice slightly vary. Below 35% of relative water content the array seems to collapse (fig. 2). The corresponding weakening of the equatorial reflections indicates also a disorganization of the lattice. It is then probable that the lattice is highly fragile and this may explain why negative staining pictures often show a disorganized distribution of the particles on the surface of the membrane [9].

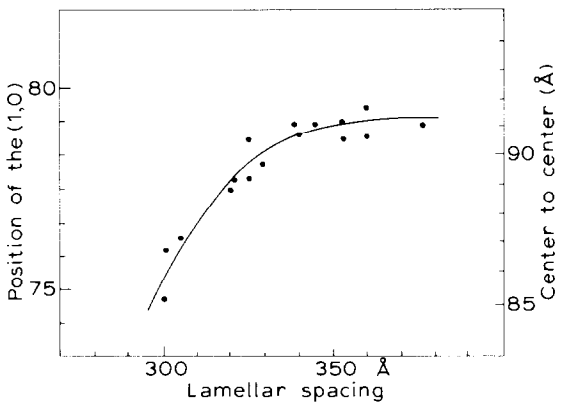

Fig. 2. Measured position of the first equatorial reflection as a function of the lamellar spacing. The scale on the right is the corresponding center to center distance assuming an hexagonal lattice. A sample with a spacing of 380 Å contains about 50% water by weight while 300 Å corresponds to about 35% water.

As already discussed [9] the particles present in the receptor-rich membrane fragments possess the same size, shape and dimensions as the particles present in a preparation of receptor protein purified from *Electrophorus* electric organ. The lattice observed by both X-rays diffraction and electron microscopy therefore likely results from a particular organization of the receptor protein in the plane of the membrane.

No detectable equatorial reflections were found with membrane samples taken from the same initial centrifugation gradient but in regions of lower density (20% sucrose) and containing small amounts of α-toxin binding material. These membrane fragments did not reveal a particulate surface structure by negative staining. Also, with these fragments, sharp, poorly oriented meridional reflections with a spacing of 70–100 Å are observed. Considering the low density of the samples, these reflections might be associated with light membrane fragments or lipo-proteins organized in lamellae.

At much higher diffraction angles, a broad equatorial band appears (not seen on the figures) which corresponds to a spacing of 4.5 Å. Such a band is known to arise from the paraffin chains of the lipids [12]. The width of the band indicates that these chains are in a quasi-liquid state, and their orientation suggests that they are perpendicular to the plane of the membrane.

Experiments are in progress to improve the resolution of the pictures and to determine the phases without ambiguity. In any case the present results show that an unusually large membrane thickness and the presence of an organized lattice of particles characterize the membrane fragments rich in cholinergic receptor protein purified from *Torpedo* electric organ.

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