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BIOLOGICAL MEMBRANE STRUCTURE

III. THE LATTICE STRUCTURE OF MEMBRANOUS CYTOCHROME OXIDASE

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SUMMARY

Membranous cytochrome oxidase forms a regular two-dimensional array, as visualized by negative staining and electron microscopy. The lattice formed by the spots in the micrographs, which are assumed to be the cytochrome oxidase protein complexes, belongs to the two-dimensional rectangular space group *pg*; the unit cell dimensions are $88 \pm 4 \times 127 \pm 7$ Å, with two protein complexes per unit cell. By using the unit cell area in conjunction with the chemical composition of the membrane and the molecular weights and volumes of the constituents, it was possible to construct a geometrical model for the membrane which quantitatively accounts for the measurements in hand. The lipids are present as lamellar bilayer, and the protein complexes pass all the way through the bilayer.

INTRODUCTION

In the first paper of this series[1], a general model for the geometrical arrangement of the lipids and proteins in biological membranes was presented. In this model, the lipids exist as lamellar bilayer, while the proteins are globular and penetrate deeply into the lipid bilayer. In the second paper[2], a detailed model for the retinal rod outer segment disk membranes was given, based on the available X-ray, electron microscopic, and chemical data. Several reasons were presented for believing that the rhodopsin molecules, which are the principal protein constituent of the retinal rod outer segment disk membrane, are roughly spherical and penetrate approximately half way into the lipid bilayer; the size of a rhodopsin molecule is such that this results in one third to one half of the molecule being in the hydrophobic lipid environment, with the remainder being exposed to the medium. This membrane was described as a two-dimensional liquid crystal, since the rhodopsin molecules are free to move in the plane of the membrane.

The present paper constitutes an attempt to describe the detailed structure of another membrane, that formed by cytochrome oxidase and phospholipid. This mem-

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brane differs in several significant respects from the retinal rod outer segment membrane. The rhodopsin molecules of the disk membrane are monomers, whereas the cytochrome oxidase protein is a complex consisting of several polypeptide chains. The cytochrome oxidase membrane can form a regular two-dimensional array, as opposed to the two-dimensional liquid–crystalline state of the retinal rod outer segment membrane. In the analysis of the retinal rod outer segment membrane structure, X-ray diffraction data played an important part; in the present case measurements made on electron micrographs of negatively stained material will be coupled with chemical analysis and enzymological data to yield a geometrical model.

A preliminary report on this work was given at the New York Academy of Sciences "Conference on Membrane Structure and its Biological Applications", June 2–4, 1971.

METHODS

Beef heart mitochondria were prepared by the method of Crane *et al.*[3], except that 10 mM Tris–HCl buffer (pH 7.8) replaced phosphate as a buffer. Membranous cytochrome oxidase was prepared from these mitochondria by the method of Sun *et al.*[4], but with the following modifications. 10 mM Tris–HCl (pH 7.8) replaced phosphate buffer, and thorough homogenization in a Potter–Elvejhem-type of homogenizer was used in place of sonication. The membranous preparation so obtained was given two additional washings with 0.25 M sucrose – 10 mM Tris–HCl buffer (pH 7.8). The material was stored at –20 °C, at a protein concentration of 20 mg/ml. In one preparation which was abnormally high in lipid content, a second Triton X-100 treatment was introduced, which gave a pellet with normal lipid content and a high lipid fluffy layer; both pellet and fluffy layer contained membranous cytochrome oxidase.

Protein was determined by the method of Lowry *et al.*[5]. The biuret method[6] was not used since it has been shown by Davis and Hatefi[7], and confirmed by us, that this method gives erroneously high results when used in the presence of lipid. Phospholipid was determined by phosphorous analysis according to Chen *et al.*[8]. An average phospholipid molecular weight of 775 was assumed. Heme *a* was estimated by the reduced *versus* oxidized alkaline pyridine hemochromogen difference spectral method, as described by Williams[9], using ε_{mM} (587–620 mn) = 21.7. Cytochrome *c* oxidase activity was determined by the method of Smith[10].

Gel electrophoresis was carried out as described by Weber and Osborn[11]. 5 % acrylamide gels which were 1 % with respect to sodium dodecyl sulfate and contained 5 mM β-mercaptoethanol were employed. Coomassie blue was used to stain for protein.

Electron microscopy was carried out using the Hitachi 11E and Hitachi 11DS microscopes. The magnification power of the instruments was calibrated using a 5000 Å cross lines replica grating supplied by the manufacturer. Specimens were prepared for observation in the following manner. A sample containing 1–2 mg protein per ml, in a solution of 0.25 M sucrose – 10 mM Tris–HCl buffer (pH 7.4) was spread on a carbon-coated grid, and then stained for 20 s by the floating method with either 1 % uranyl acetate (pH 4.0) or 1 % phosphotungstic acid (pH 7.2), as described by Seki *et al.*[12]. In some experiments, fixation at a final concentration of 0.05 % glutaraldehyde preceeded staining. The same results were obtained with and without glutaraldehyde.

For positive staining of material to be thin sectioned, a sample suspension containing 5 mg/ml protein was mixed with an equal volume of 2 % glutaraldehyde in 0.25 M sucrose–0.05 M potassium cacodylate buffer (pH 7.4), and kept for 1 h at 0–4 °C. The mixture was then spun down and washed with the same buffer but without glutaraldehyde. The precipitate was exposed to 1 % OsO_4 for 1 h, followed by staining with 1 % uranyl acetate in 25% ethanol. Dehydration, embedding, and sectioning were carried out according to Allmann *et al.*[13].

All measurements were made directly on the photographic plates using a Nikon two dimensional microcomparator.

RESULTS

A typical cytochrome oxidase preparation contained 26 % phospholipid, 8.4 nmoles heme *a* per mg protein, and had a cytochrome *c* oxidase activity of 9.1 μmoles cytochrome *c* oxidized per min per mg protein. Gel electrophoresis of the material gave three major bands, and three faint bands which were probably impurities. Apparent molecular weights of 11500, 15000, and 29700 were estimated for the three major bands on the gels, using several known proteins with molecular weights in the same range as standards[11]. These molecular weights are in the same range as but not identical to those reported by Keirns *et al.*[14]. The direct and difference spectra of the alkaline pyridine hemochromogen were as expected for heme *a*; no indication of other heme compounds was given by the spectra. On the basis of these analyses, we conclude that our cytochrome oxidase preparation is comparable in purity to highly purified preparations previously reported in the literature[4,14,15].

Electron microscopy

The vesicular cytochrome oxidase membranes which result when prepared as described in Methods display a regular two-dimensional lattice structure on a large proportion of their surfaces (75 % or more in some preparations) when negatively stained and examined by electron microscopy. This is shown in Fig. 1. Reports of similar lattice structures for cytochrome oxidase have appeared in the literature[12,16]. Individual regular domains which extend over distances in excess of 1000 Å were frequently found. The same lattice structure was found regardless of whether the sample was pre-fixed with glutaraldehyde, and with either phosphotungstic acid or uranyl acetate as the stain. The lattice structure could also be detected on an unstained preparation. The best results and highest resolution were given with uranyl acetate, without fixation with glutaraldehyde.

The cytochrome oxidase as prepared was in the oxidized (ferric) state, and this was the form which gave the lattice structure. In some experiments, the preparations were further aerated before examination to insure complete oxidation. Reduction of the oxidase with either sodium dithionite or reduced cytochrome *c* caused a disappearance of the lattice structure. The initial results of a companion study (H. Hayashi, R. A. Capaldi and G. Vanderkooi, unpublished results) indicate that elevated pH and high temperature also lead to the breakdown of lattice structure. We therefore conclude that the structural or conformational integrity of the oxidized cytochrome oxidase protein complex is required in order for the lattice to be stable. The membrane thus displays the cooperativity properties one would expect for a two-dimensional

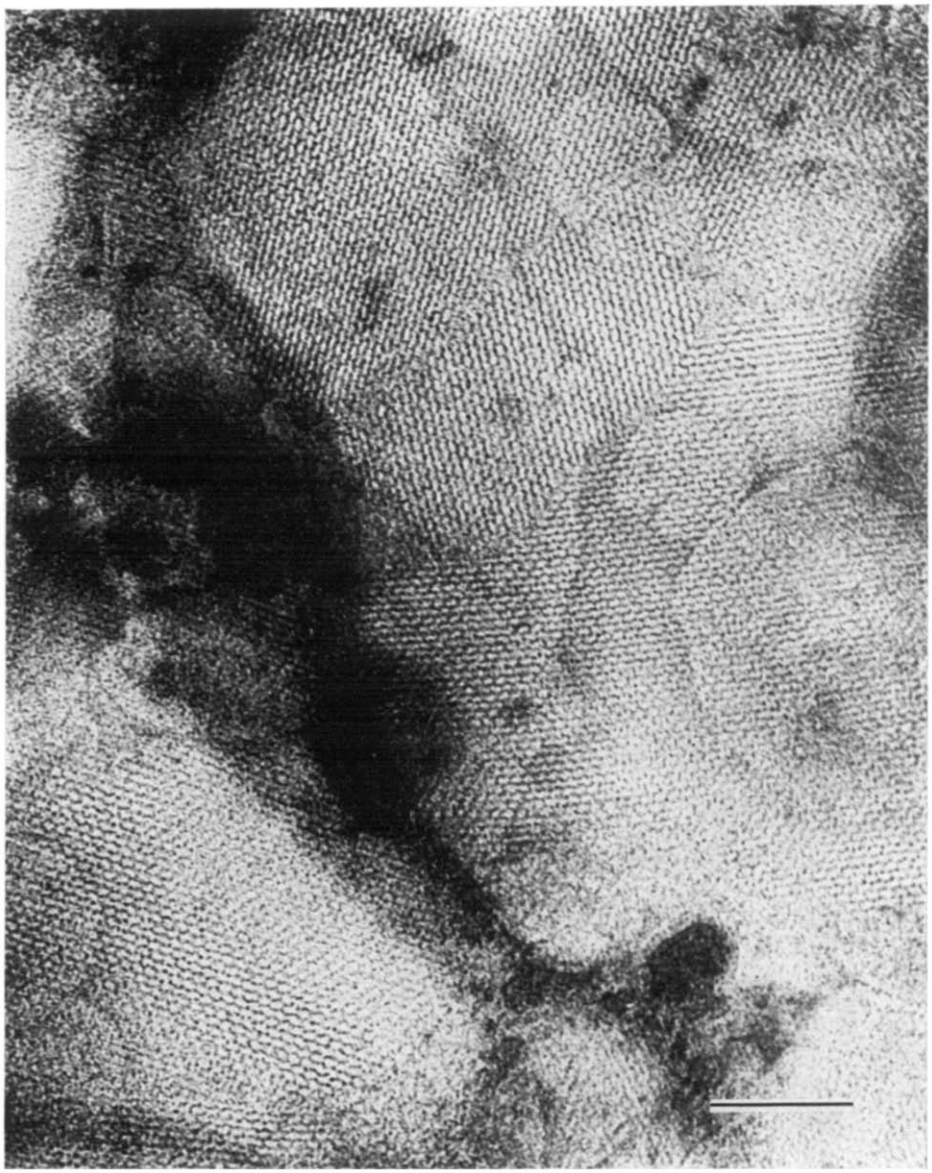

Fig. 1. Membranous cytochrome oxidase negatively stained with uranyl acetate. Several domains of crystalline membrane regions can be seen here. The bar corresponds to 1000 Å.

crystal; if all of the proteins in a given area are not in identical conformations, crystallization does not occur.

Lattice parameters.

The spots on the micrographs form a herringbone pattern. This lattice belongs to the rectangular two-dimensional space group denoted *pg* in ref. 17. There are two asymmetric units per unit cell, which are related by a mirror and glide line of sym-

metry. A 2-fold screw axis in three dimensions would account for the observed two-dimensional surface pattern. (A mirror plane in three dimensions is of course forbidden, on account of the inherent asymmetry of the protein molecules.) Fig. 2 gives a diagramatic illustration of the lattice.

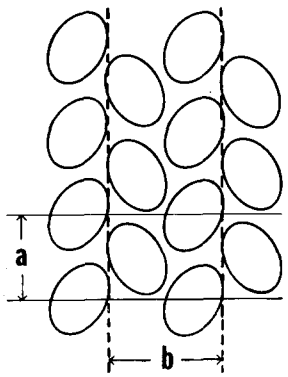

Fig. 2. Diagram of the two-dimensional crystal lattice inferred from micrographs such as the one shown in Fig. 1. The ellipses represent the cytochrome oxidase protein complexes; the dashed lines are the mirror–glide lines of symmetry. The bar corresponds to 1000 Å.

Columns 3 and 4 of Table I give the lattice constants for the two-dimensiona rectangular unit cells, as measured on micrographs from five separate preparations The variability between the lattice constants given by different preparations is not great, but nonetheless appear to be real.

Measurements of individual spot sizes on plates of negatively stained material gave dimensions of $52 \pm 8 \times 60 \pm 8$ Å, and spot areas of 3100 ± 300 Å^2.

Thin section results

Samples of membranous cytochrome oxidase, which were highly crystalline as judged by the negative staining results, were positively stained, embedded, and sectioned. Many membranous vesicles were seen, as well as regions in which several membranes were juxtaposed in a parallel array. The latter regions gave much higher resolution than did the membranes of individual vesicles. This is illustrated in Fig. 3. A double row of spots in Fig. 3 comprises one membrane; this can be determined by following the single membrane loops which appear in the lower part of the figure into the body of the juxtaposed region. A quasi-crystallinity, or inter-membrane correlation of spot positions, is evident in the more clear regions of the juxtaposed membranes; the spots appear to be staggered with respect to each other.

The membrane to membrane spacing in Fig. 3 is 109 ± 6 Å, as measured on the original plate. The dimension of a single spot measured parallel to the membrane orientation is 59 ± 4 Å; the single spot thickness, measured perpendicular to the membrane, is 38 ± 4 Å. The thickness of a double row of spots (one membrane), is 86 ± 5 Å. The spot to spot spacing along the membrane varies between 76 and 94 Å. This spacing is comparable to the spacing between spots seen on the negatively stained surface views.

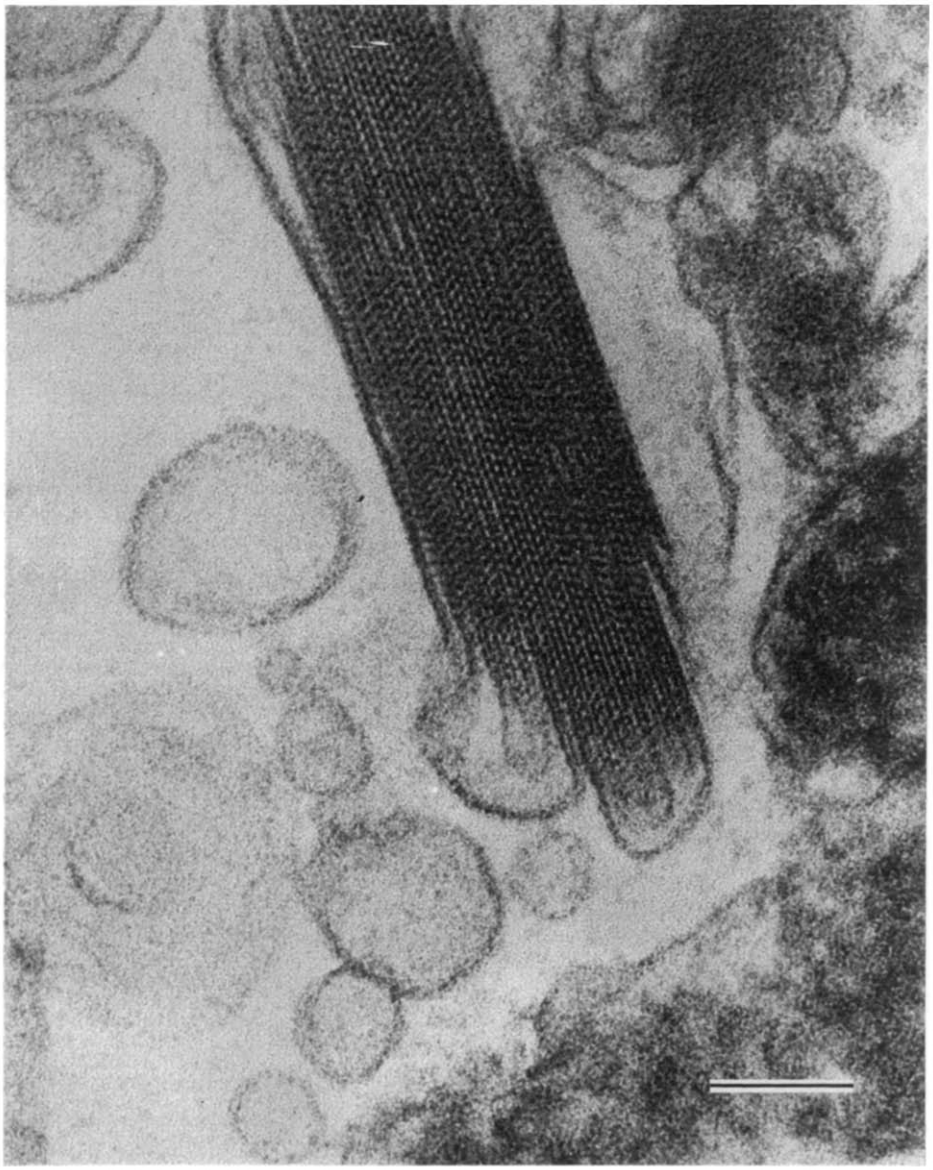

Fig. 3. An OsO_4-stained thin section micrograph of membranous cytochrome oxidase. The bar corresponds to 1000 Å.

Molecular interpretation

It is possible to construct a molecular model for the cytochrome oxidase membrane, using the measurements made on the micrographs in combination with the molecular composition. It will be assumed, on the basis of the work described above, that the spots seen in the negatively stained micrographs are the cytochrome oxidase protein complexes, and that the spaces between the protein complexes are filled with

lipid bilayer. We will show in the following that the use of this assumed molecular arrangement leads to quantitative agreement between the measurements made on the micrographs and measurements calculated from the known sizes and molecular ratios of the component molecules.

The molecular weights of the components must be known before the molecular ratio can be calculated. There is evidence in the literature that cytochrome oxidase normally occurs as a two-heme complex[18–20], and also evidence that this complex is a dimer of equal molecular weight sub-units[21,22]. Consideration of the various reports on molecular weights, as determined directly[14,18,21], or as calculated from the heme *a* content[4,15], led us to adopt a value of 210000 ± 10000 daltons for the two-heme complex. For the phospholipids, the same molecular weight was used as was initially assumed in converting the phosphorus analysis to phospholipid content, *i.e.* 775 daltons, so that any error in this value will be cancelled out. By this manner of treatment a cardiolipin "molecule" is that fraction of the real molecule which contains one phosphorus atom and two fatty acyl groups.

The lipid to protein molecular ratios are given in Column 6 of Table I. Column 7 gives the lipid bilayer area formed by the number of lipid molecules listed in Column 6. These values were calculated using 45 Å^2 monolayer area per molecule. This is the lipid area found in membranes below the thermal phase transition[23], and is also close to the limiting minimum value found by molecular model building and by surface pressure measurements[24]. We have used this low value, rather than the higher (60–70 Å^2) values which have been reported[23] for the *Mycoplasma laidlawii* membranes above the thermal phase transition, since the lipids probably do not have appreciable motional freedom in the present crystalline membrane environment. The lattice dimensions do not significantly change between 4 and 40 °C, which also implies that no area-changing thermal phase transition occurs within this temperature range (H. Hayashi, R.A. Capaldi and G. Vanderkooi, unpublished results).

The area available per protein complex was calculated by subtracting the lipid bilayer area (Column 7, Table I) from the half unit cell area (Column 5); the values are given in Column 8. Comparison of these values (average = 3240 ± 140 Å^2) with the measured spot size on the plates (3100 ± 300 Å^2) shows that they are in remarkably good agreement, considering the approximations and assumptions involved in obtaining these figures.

Finally, if a value is adopted for the molecular volume of a cytochrome oxidase–protein complex, the required thickness of an equivalent solid of constant (given) cross sectional area may be calculated. The protein volume was calculated by summing the amino acid residue volumes, using the volumes per residue given by Waugh[25], and the amino acid composition data of Matsubara *et al.*[26]. A specific volume of 0.75 cm^3/gm was obtained. This gave a molecular volume of 261000 Å^3 for a weight of 210000 daltons. The last column in Table I gives the protein thickness, which was calculated by dividing the volume by the available area figures of Column 8. Several factors will affect this result, among which are the assumed lipid area per molecule, the protein specific volume, and the protein molecular weight. The average thickness value obtained by calculation (81 ± 4 Å) does not greatly differ from the membrane thickness of 86 ± 5 Å which was measured on the thin section micrograph (Fig. 3).

The bottom line of Table I gives the averages over the data of the first four preparations. The mean deviations reported for these average values pertain to the

TABLE I

PROPERTIES OF THE CYTOCHROME OXIDASE MEMBRANE LATTICE

Measurements of lattice dimensions were made on micrograph plates of negatively stained material. The *a* direction is parallel to the symmetry axis, and the *b* direction perpendicular to it. The precision error on individual measurements is less than 2 Å.

Prepn No.	*Lipid content* (%)	*Lattice dimensions* (Å)		*Half unit cell area* ($Å^2$)	*Lipid/protein mole ratio* *	*Lipid bilayer area* ** ($Å^2$)	*Available protein area* *** ($Å^2$)	*Calculated protein thickness*§ (Å)
		a	*b*					
1	27.2	81	132	5350	101 ± 5	2280 ± 110	3070 ± 110	85 ± 7
2	28.5	91	130	5910	108 ± 5	2430 ± 120	3480 ± 110	75 ± 6
3	24.2	89	117	5210	86 ± 4	1950 ± 95	3260 ± 95	80 ± 6
4	26.2	92	115	5290	96 ± 5	2160 ± 100	3130 ± 100	84 ± 7
5§§	42.0	96	127	6100	196 ± 10	4400 ± 220	1700 ± 220	154 ± 7
Average§§§	26.5 ± 1.3	88 ± 4	124 ± 7	5440 ± 240	98 ± 5	2200 ± 150	3240 ± 140	81 ± 4

* Molecules of lipid per half unit cell, assuming a phospholipid molecular weight of 775 and a protein weight of 210000. The error limits in this column and those to the right of it pertain to the protein molecular weight uncertainty; the limits were derived by carrying out the calculations using 200000 and 220000 daltons.

** Bilayer area of lipid, at 45 $Å^2$ per molecule, which can be formed by the number of lipid molecules per half unit cell.

*** Half unit cell area minus lipid bilayer area.

§ Protein volume divided by protein area. Volume ($Å^3$) = ($M_r \times 0.75$)/0.602.

§§ High lipid preparation. Many non-crystalline membranous or lipoidal vesicles were seen in the micrographs of this preparation, in addition to crystalline regions.

§§§ Averaged values for the first 4 sets of data. The mean deviations of the principal values (*i.e.* those calculated using a molecular weight of 210000) are given.

spread of data among the first four preparations, assuming (where applicable) a protein molecular weight of 210000. The data on Prepn 5 were not included in the averaging, for the reasons described in the following paragraph.

Prepn 5 contained much higher lipid than any of the other preparations; it was the fluffy layer obtained from a second Triton X-100 split, as described in Methods. This material showed both highly crystalline regions, and also many small non-crystalline vesicles, when examined electron microscopically. A considerably lower percentage of this material was crystalline than for the preparations with lower lipid content. We conclude that a disproportionation of lipid occurred in this case, with the fraction of lipid present in the crystalline regions being less than that found for the preparation as a whole. The unit cell size for the crystalline regions of this preparation was only slightly larger than found for the other preparations, showing that the lattice does not swell uniformly as a function of the lipid content of the preparation.

The protein thickness calculation was made on the assumption that the protein complex extends all the way through the lipid bilayer. The agreement obtained between the calculations based on molecular composition and volume, and the measurements made on the micrographs, substantiate this model. These data are equally compatible either with the view that cytochrome oxidase is structurally a single well-integrated two-heme complex, or that it is a trans-membrane dimer of two equal subunits. The double row of spots seen in the thin section micrographs would appear to support the latter option, but this conclusion is rather tenuous on account of our lack of concrete information on the staining properties of thin-sectioned material. Fig. 4 illustrates the cross-sectional appearance of the membrane, as derived by the above considerations. Additional support for a trans-membrane cytochrome oxidase complex of this type is given by the immunological experiments of Racker *et al.*[27].

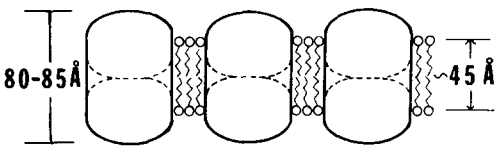

Fig. 4. Cross sectional appearance of the cytochrome oxidase membrane as deduced in the text. The large open figures represent the protein complexes; bilayer lipid fills the regions between the complexes. The dashed lines within the protein complexes indicate the possible mode of dimerization. While it is not apparent from this figure, a complete lattice of protein–protein interactions is assumed to be present. The protein thickness given here is based on the calculated result and the measurements made on the thin section micrograph.

DISCUSSION

The cytochrome oxidase membrane components originate from the inner mitochondrial membrane, and its structure therefore implies certain things about mitochondrial structure. While we do not conceive of the cytochrome oxidase membrane as a mitochondrial membrane prototype, but rather essentially as a synthetic membrane, it does seem reasonable to suppose that the general geometrical relationship of the lipid to the cytochrome oxidase protein in this synthetic membrane will also obtain in the intact mitochondrial membrane. We do not mean to suggest, however,

that the mitochondrial membrane, which contains many proteins or protein complexes, will have a regular lattice structure.

In the repeating unit model of Green and Perdue[28], membranes were described as being built up of lipoprotein repeating units. In terms of this framework, the inner mitochondrial membrane was found to consist of at least four distinct and isolatable complexes, cytochrome oxidase being one of them and called Complex IV (ref. 29). In this model, while the complexes were thought of as building blocks made up of lipid and protein, the geometrical relationship of the lipid to the protein within the complex was not defined. We have now proposed what this relationship is, namely that the protein exists in a compact globular form, and the lipid fills the spaces between the proteins as lamellar bilayer. Protein–protein contacts serve to stabilize the membrane structure.

Certain new insights about membrane structure in general can be obtained by comparing the model which has been developed by Vanderkooi and Sundaralingam[2] for the retinal rod outer segment disk membrane with the present membrane model. These two membrane models have certain common features, as well as differences. In both cases, the lipid is present as bilayer and the proteins are globular. In both cases also, the evidence indicates that the proteins are partly buried in the hydrophobic interior of the lipid bilayer, and partly exposed to the aqueous medium. This placement of the proteins relative to the lipid implies that they have an amphipathic nature–part of the protein surface must be hydrophobic, and part hydrophilic. In the retinal rod outer segment membrane model, there is a double layer of roughly spherical particles which extend from both surfaces of the membrane approximately to the center, while in the cytochrome oxidase model, the protein complex extends all the way through the bilayer. Finally, the retinal rod outer segment membrane is fluid (as indicated by X-ray diffraction[2,31]), whereas the cytochrome oxidase membrane can be crystalline. Thus it may be said that while the same basic organizational pattern is found in the two membranes, their differences in composition and structure give some indication of the range of possibilities which may be found for membrane structures.

NOTE ADDED IN PROOF (Received May 2nd, 1972)

An optical diffraction pattern has been obtained from the micrograph shown in Fig. 1 by Dr. J. Maniloff (Department of Microbiology, University of Rochester, Rochester, N.Y.), which confirmed the lattice structure given in Fig. 2. Missing spots on the diffraction pattern indicate that the lattice actually belongs to the symmetry group *pgg*, which has higher symmetry than the group *pg* inferred from direct examination of the micrographs.

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