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X-RAY DIFFRACTION FROM ORIENTED OUTER MITOCHONDRIAL MEMBRANES

DETECTION OF IN-PLANE SUBUNIT STRUCTURE

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SUMMARY

X-ray diffraction patterns from ultracentrifugally oriented specimens of plant outer mitochondrial membranes show five distinct maxima in the equatorial direction. These diffraction maxima arise from in-plane subunits whose dimensions are consistent with those of the features ("pits") seen in electron micrographs of the membranes in negative stain.

INTRODUCTION

The outer mitochondrial membrane has been shown to be permeable to substrate-size molecules but not to molecules as large as cytochrome *c* [1–4]. That the selective permeability of this membrane might be due to the presence of pores is suggested by the appearance of the outer membranes of several types of mitochondria in negative stain. Parsons et al. [5] were the first to observe that the outer membrane surfaces were covered with negative stain-filled "pits" (25–30 Å in diameter) in electron micrographs of plant mitochondria. Similar features have subsequently been detected in electron micrographs of negatively stained mouse brain [6] and *Neurospora crassa* [7] outer mitochondrial membranes.

In this report, X-ray diffraction experiments on isolated plant outer mitochondrial membranes are presented which confirm the existence of an in-plane subunit structure in these membranes, with dimensions consistent with those of the features seen in electron micrographs. In addition, the protein components of the subunits are tentatively identified by experiments on trypsin-treated membranes. Preliminary results of the research described below were presented at the Biochemistry/Biophysics 1974 Meeting (Minneapolis) [8].

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MATERIALS AND METHODS

Outer membranes were isolated from gradient-purified mung bean (*Phaseolus aureus*) hypocotyl and potato (*Solanum tuberosum*) tuber mitochondria as described previously [4, 9]. Outer mitochondrial membrane samples (1–2 mg protein in 3 ml of 10 mM potassium phosphate buffer, pH 7.2) were oriented by centrifugation onto an aluminum strip (20 000–23 000 rev./min, Beckman SW-27 rotor) for periods ranging from 3 to 16 h. These membrane pellets (in the shape of cylinders 1 or 2 mm high and 5 mm in diameter) were mounted in chambers of controlled temperature and humidity and exposed to point-focused X-ray beams incident along or perpendicular to the sedimentation axis. A Searle toroid camera was used for most of these experiments with either a Jarrel Ash Microfocus or Elliot Rotating Anode (type GX6) generator providing the initial Cu K-α (1.54 Å) X-rays. The beam width at the specimen using the toroid camera was about 0.5 mm. Diffraction was recorded on flat X-ray film (Ilford Industrial Grade Type G) positioned approx. 5 cm from the specimen. A lead beam stop was positioned directly in front of the film to block undiffracted X-rays. A Joyce Loebel microdensitometer was used to directly measure the optical density of the diffracted intensity along the film.

For certain experiments, outer mitochondrial membranes (2 mg protein in 20 ml of 0.3 M sucrose and 10 mM potassium phosphate buffer, pH 7.2) were incubated with trypsin (1.4 mg, type XI, Sigma) for 30 min at 25 °C, followed by incubation for 10 min after addition of trypsin inhibitor (3 mg, type I-S, Sigma). The membranes were pelleted (60 000 $\times g$, 90 min) and resuspended in appropriate media for gel electrophoresis experiments (performed as described in the previous report [9]) or for subsequent re-centrifugation as X-ray diffraction specimens.

RESULTS

X-ray diffraction from oriented outer mitochondrial membrane multilayers

Fig. 1 is a contact print of an X-ray film after exposure of a pellet of outer membranes (isolated from mung bean hypocotyl mitochondria) to an X-ray beam incident normal to the sedimentation axis, i.e. parallel to the multilayer planes within the pellet. The beam in this case was aimed about midway into the pellet, giving rise to three distinct regions of background intensity. Little or no intensity is observed in the region directly behind the metal specimen mount, and intermediate intensity is observed in the region defined by the "shadow" of the membrane pellet. The background intensity is overwhelming in this exposure outside the specimen.

Considerable information about the membrane specimen is contained in this single diffraction pattern. The diffuse maximum at wide angle (1/4.5 $Å^{-1}$ for both mung bean and potato outer mitochondrial membranes) is typical of those seen in X-ray diffraction patterns from many types of biological membranes [10–12]. These wide angle reflections are similar to the "short spacings" seen in X-ray diffraction patterns from hydrated phospholipids and are attributed to correlations between fluid hydrocarbon chains alligned more or less perpendicularly to the lamellar direction [13–15]. The sharper maxima also seen at wide angle in the pattern of Fig. 1 are artifacts arising from the aluminum windows of the specimen chamber.

Diffraction maxima are visible in the meridional direction (parallel to the

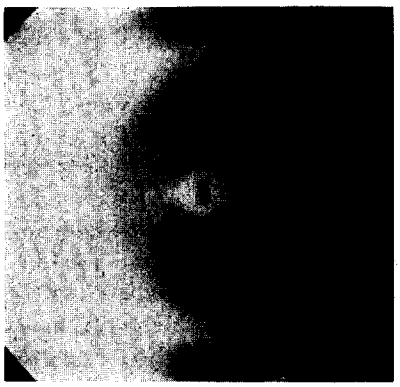

Fig. 1. X-ray diffraction pattern from oriented outer mitochondrial membrane specimen: grazing-incidence diffraction. Outer membranes isolated from mung bean mitochondria were centrifuged for 16 h at 20 000 rev./min in an SW-27 rotor. The pellet was mounted in the specimen chamber (10 °C, 90 % relative humidity) so that the X-ray beam was incident normal to the sedimentation axis (the horizontal direction in this figure). Specimen-to-film distance 49 mm, 24-h exposure.

sedimentation axis) at 1/28 and 1/19 $Å^{-1}$ in the pattern of Fig. 1. Shorter exposures reveal two additional meridional reflections, at 1/80 and 1/42 $Å^{-1}$, which together with the above reflections imply that the membrane specimen has a lamellar structure with a fundamental spacing of about 80 Å.

A more interesting feature of this diffraction pattern is the series of reflections that occur in the equatorial direction (perpendicular to the meridion), four of which are seen in Fig. 1 (at 1/23.5, 1/15.5, 1/10.5 and 1/8.5 $Å^{-1}$). The most intense equatorial reflection occurs at 1/60 $Å^{-1}$ but is visible only in shorter exposures.

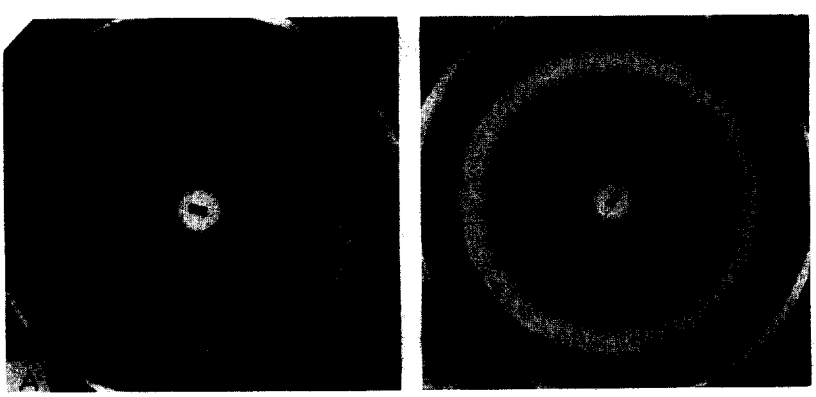

Fig. 2. X-ray diffraction patterns from oriented outer mitochondrial membrane specimens: surface-on diffraction. Outer membranes isolated from (A) mung bean and (B) potato mitochondria centrifuged for 3 h at 23 000 rev./min in an SW-27 rotor. Pellets were mounted in the specimen chamber (25 °C, 75 % relative humidity) so that the X-ray beam was incident along the sedimentation axis. (A) Specimen-to-film distance 50.5 mm, 24.5 h exposure. (B) Specimen-to-film distance 50 mm, 16 h exposure. The membranes of the second specimen were dialyzed overnight against 5 mM EDTA (pH 7), pelleted (60 000 × *g*, 90 min) and resuspended in 10 mM phosphate buffer (pH 7.2) prior to centrifugation onto aluminum strip for the X-ray diffraction experiment.

Similar, though less distinct, low-angle equatorial diffraction maxima have been observed previously with specimens of retinal disk [16], sarcoplasmic reticulum [12] and chromatophore [17] membranes and are attributed to structural features in the plane of the membranes. By rotating the outer mitochondrial membrane specimen so that the X-ray beam is incident along the sedimentation axis of the membrane pellet, these reflections appear as complete rings of diffracted intensity (Fig. 2) as expected for in-plane membrane structure. (When this is done, the X-ray beam also passes through the aluminum foil on which the outer membrane specimen is mounted, giving rise to an additional artifact which nearly coincides with the fourth reflection from these membranes. The aluminum mount on which the foil is positioned has a hole through its center sufficiently large, 1 mm in diameter, to allow free passage of the X-ray beam.)

Nature of the in-plane subunits

In the light of what is know of the composition of the outer membranes of plant mitochondria [9], it is likely that the structures responsible for the observed equatorial diffraction are related to either (or both) of the two predominant classes of protein present in these membranes (bands I and II at apparent molecular weights of 30 000 and 50 000 respectively in sodium dodecyl sulfate-polyacrylamide gradient gel electrophoretograms) or to the polyuronide associated with these membranes.

It was found that overnight dialysis of isolated outer membranes against 5 mM EDTA (a procedure which removes the bulk of the membrane polyuronide [9]) did not diminish but actually increased the intensity of the equatorial reflections relative to the intensity of the 4.5 Å maximum. Initially, the equatorial diffraction from potato outer mitochondrial membranes were observed to be much less intense than those from mung bean outer membranes. After overnight EDTA dialysis, however, the equatorial diffraction from potato membrane specimens was almost equivalent to that from mung bean samples (Fig. 2). Thus polyuronide is not the source of the equatorial reflections and, in fact, reduces the electron density contrast which gives rise to the observed X-ray diffraction.

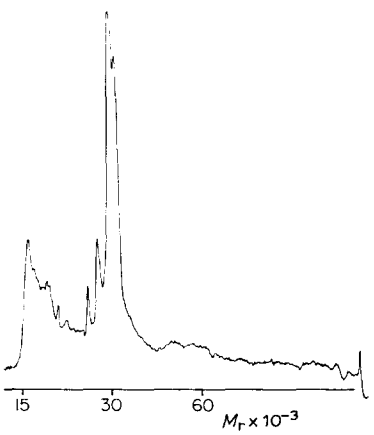

Fig. 3. Sodium dodecyl sulfate-polyacrylamide gradient gel electrophoresis pattern of proteins of mung bean outer mitochondrial membranes after trypsinization.

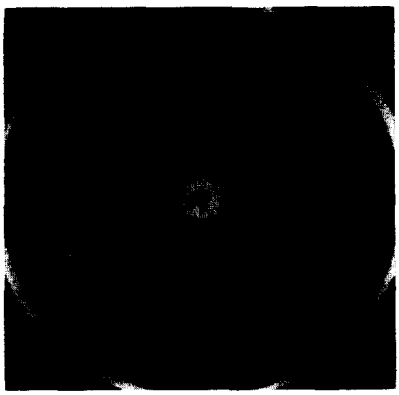

Fig. 4. Surface-on X-ray diffraction pattern from trypsin-treated mung bean outer mitochondrial membrane specimen. Membranes centrifuged for 14 h at 20000 rev./min in an SW-27 rotor. Specimen chamber at 10 °C, 87 % relative humidity. Specimen-to-film distance 49.5 mm, 24.5 h exposure.

Trypsin treatment of isolated plant outer mitochondrial membranes was next employed in an attempt to determine which of the two major classes of membrane protein might constitute the in-plane subunits. As illustrated in the gel electrophoresis pattern of Fig. 3, the high molecular weight protein components (including band II) are no longer present after trypsinization to the extent described in Materials and Methods. On the other hand, the bulk of the band I components are still present, along with low molecular weight degradation products of the protein hydrolysis. (Compare with Figs 2 and 3 of ref. 9.) At the same time, identical trypsin treatment was found to have little or no effect on the equatorial X-ray diffraction patterns of these membranes (Fig. 4), implicating band I proteins as the major constituents of the in-plane subunits responsible for this diffraction.

Radial Patterson function of the equatorial diffracted intensity

Details of the structure of the in-plane subunits responsible for the observed diffraction were obtained by computing its radial Patterson function, $P(r)$ [16, 17].

Diffracted intensities as a function of the distance, d, along the films of Fig. 2A and appropriate shorter exposures were obtained by microdensitometer tracings of the patterns and subtraction of background intensity (provided by similar exposures on the same camera in the absence of a membrane specimen). The diffracted intensity, $I(d)$, can be expressed in terms of radial distance in reciprocal space, r^*, by the expression:

$$I(r^*) = I(2 \sin[0.5 \arctan(d/D)]/\lambda) \qquad (1)$$

where D is the specimen-to-film distance and λ is the wavelength of the incident photons, 1.54 Å. This diffracted intensity arises from a cylindrically symmetrical (because of random radial orientation of the membranes in the pellet) electron density contrast, $\rho(r)$, and is equal to the square of the Fourier transform of $\rho(r)$ into reciprocal space:

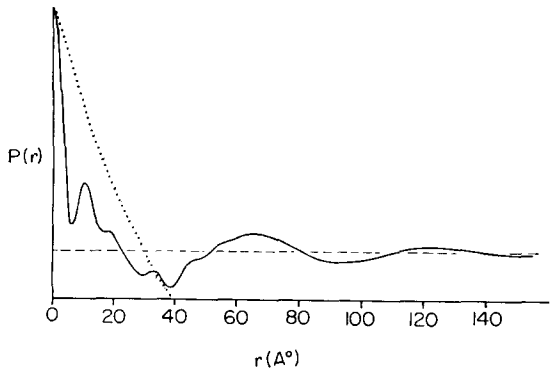

Fig. 5. Radial Patterson function of low-angle, surface-on diffracted intensity from mung bean outer mitochondrial membranes (—) and radial Patterson function of solid cylinder of radius 20 Å [18] (. . . . .).

$$I(r^*) = \left| \int_{-\infty}^{\infty} \rho(r) \exp(2\pi i \boldsymbol{r} \cdot \boldsymbol{r}^*) \mathrm{d}\boldsymbol{r} \right|^2 \tag{2}$$

where $\mathrm{d}\boldsymbol{r}$ is the volume element in real space (in cyclindrical coordinates) and $\boldsymbol{r}$ and $\boldsymbol{r}^*$ are real and reciprocal space vectors, respectively.

The Patterson function of this diffracted intensity is defined as the inverse Fourier transform of $I(r^*)$, which analytically is equal to the self-convolution product (or autocorrelation) of $\rho(r)$:

$$\rho(r) * \rho(-r) = \int_{-\infty}^{\infty} \rho(r')\rho(r'-r)\mathrm{d}r' \tag{3}$$

and which mathematically can be shown equal to:

$$P(r) = 2\pi \int_{0}^{\infty} I(r^*) J_0(2\pi r r^*) r^* \, \mathrm{d}r^* \tag{4}$$

where J_0 is the zero order cylindrical Bessel function.

Fig. 5 is a plot of the radial Patterson function computed on the basis of Eqns 1 and 4 from the low-angle surface-on diffraction patterns from mung bean outer membrane specimens. The form of $P(r)$ is consistent with a planar liquid-like array of subunits, with the autocorrelation of the subunit electron density profile placed at the origin and (at reduced magnitude) at the first and second nearest neighbor distances (65 and 125 Å). This function approaches a minimum at a distance equal to twice the radius of the subunits, here indicating a particle diameter of 40–50 Å. There are also short vectors present in the Patterson at 10, 19 and 33 Å which most likely arise from intraparticle correlations. The presence of these short vectors and the fact that the autocorrelation of $\rho(r)$ at the origin falls off faster than that of a solid cylinder (dotted line, Fig. 5) indicate that the subunit radial electron density is not a simple step function.

DISCUSSION

The X-ray diffraction experiments presented above indicate the presence of a prominent in-plane subunit structure in the outer membranes of plant mitochondria. Furthermore, the details of the in-plane subunit structure obtained by radial Patterson function analysis are consistent with those of the features seen in electron micrographs of these membranes in negative stain. The subunit diameter detected by X-ray diffraction is the same as the closest center-to-center distance observed for the features in electron micrographs (40–50 Å). The 65 Å nearest neighbor distance determined from X-ray diffraction data represents an average separation of adjacent particles, which is expected to be larger than the distance of closest approach for particles in essentially random planar array. Another correlation between the outer mitochondrial membrane structures detected by X-ray diffraction and electron microscopic techniques is provided by estimates of the surface packing of the lipid and protein components of these membranes [19]. The surface density (i.e. number of protein molecules per bilayer surface area) of band I proteins, which appear to give rise to the equatorial X-ray diffraction maxima, is the same as that of the "pits" seen along the surface of the membranes in negative stain ($2 \cdot 10^4/\mu m^2$).

It should be noted that the general shape of the radial Patterson function near the origin (Fig. 5) is consistent with that expected if the in-plane subunits detected were in fact hollow cylinders or pores. However, this is not an unambiguous determination since the radial autocorrelation functions of other cylindrically symmetric shapes (such as cones or "soft" spheres) also fall off more sharply at the origin than that of a solid cylinder. More direct evidence concerning the nature of the in-plane subunits detected in these experiments may be obtained from the cross-sectional electron density profiles of these membranes, which should lead to the localization of the bulk of the membrane protein in the transverse direction.

In similar X-ray diffraction studies on rat liver outer mitochondrial membranes, Thompson et al. failed to detect diffraction maxima normal to the lamellar reflections [10]. Likewise, the pitted structure so evident in electron micrographs of negatively stained outer membranes of plant mitochondria are not seen in electron micrographs of either negatively stained [6] or freeze-fractured [20] liver outer membranes. Whether or not this is due to the absence of equivalent in-plane structures in the latter type of outer mitochondrial membrane is not clear. A factor to be considered in this regard is the more heterogeneous protein composition of liver outer membranes [20] (in comparison with that of plant outer membranes [9]) which might obscure the detection of any single class of in-plane protein structures by the above techniques.

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REFERENCES

1 O'Brien, R. L. and Brierley, G. (1965) J. Biol. Chem. 240, 4527–4531
2 Pfaff, E., Klingenberg, M., Ritt, E. and Vogell, W. (1968) Eur. J. Biochem. 5, 222–232
3 Wojtczak, L. and Zaluska, H. (1969) Biochim. Biophys. Acta 193, 64–72
4 Douce, R., Mannella, C. A. and Bonner, Jr., W. D. (1973) Biochim. Biophys. Acta 292, 105–116
5 Parsons, D. F., Bonner, Jr., W. D. and Verboon, J. G. (1965) Can. J. Bot. 43, 647–655
6 Parsons, D. F., Williams, G. R. and Chance, B. (1966) Ann. N. Y. Acad. Sci. 137, 643–666
7 Stoeckenius, W. (1970) in Membranes of Mitochondria and Chloroplasts (Racker, E., ed.), pp. 53–90, Van Nostrand Reinhold, New York
8 Mannella, C. A. and Bonner Jr., W. D. (1974) Fed. Proc. 333, 1515 (Abstr. No. 1648)
9 Mannella, C. A. and Bonner, Jr., W. D. (1975) Biochim. Biophys. Acta 413, 213–225
10 Thompson, J. E., Coleman, R. and Finean, J. B. (1968) Biochim. Biophys. Acta 150, 405–415
11 Blasie, J. K. (1972) Biophys. J. 12, 191–204
12 Worthington, C. R. and Liu, S. C. (1973) Arch. Biochem. Biophys. 157, 573–579
13 Palmer, K. J. and Schmitt, F. O. (1941) J. Cell. Comp. Physiol. 17, 385–394
14 Palmer, K. J., Schmitt, F. O. and Chargaff, E. (1941) J. Cell. Comp. Physiol. 18, 43–47
15 Dervichian, D. G. (1964) Prog. Biophys. Mol. Biol. 14, 263–342
16 Blasie, J. K. and Worthington, C. R. (1969) J. Mol. Biol. 39, 417–439
17 Cain, J. E. (1974) PhD Thesis, University of Pennsylvania
18 Harget, P. J. and Krimm, S. (1971) Acta Crystal. A27, 586–596
19 Mannella, C. A. (1974) PhD Thesis, University of Pennsylvania
20 Melnick, R. L., Tinberg, H. M., Maguire, J. and Packer, L. (1973) Biochim. Biophys. Acta 311, 230–241