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SMALL-ANGLE X-RAY SCATTERING FROM MITOCHONDRIA

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Summary

X-ray (CuK_α) scattering curves of rat liver mitochondria are characterized by continuously decreasing intensity from 0.5 to 5 mrad and a broad maximum centered near 20 mrad. The condensed-to-orthodox morphological transition of the inner membranes of intact mitochondria causes a dramatic decrease in scattering at very small angle and a marked shift of the 20 mrad maximum to smaller angle. A similar small-angle scattering maximum is observed with inner mitochondrial membrane fractions prepared by digitonin treatment and osmotic shock/step gradient centrifugation procedures. However, the small-angle X-ray scattering curves of mitochondria after acetone treatment and osmotic lysis/sonication are essentially continuous. These characteristics of mitochondrial X-ray scattering are discussed in terms of known structural features of the organelle.

Introduction

The mitochondrion plays a central role in eukaryote energy metabolism by virtue of its inner membrane oxidative phosphorylation process. Thus, despite the structural complexity of this organelle, it has been the focus of many studies relating biomembrane structure to function. Electron microscopy and light scattering, for example, have revealed that close correlations exist between mitochondrial morphology and respiratory state [1—4]. Such findings have been interpreted by some in terms of direct involvement of cristal membrane conformation in the events of energy transduction [2,5]. Likewise, changes in the fluorescence intensity of the dye 8-anilinonaphthalene-1-sulphonate, when in-

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corporated into mitochondrial membranes, suggest that fundamental, but unspecified, alterations take place in the organization of these membranes upon energization [6,7].

One potentially useful technique has been largely overlooked as a probe of mitochondrial structure, namely small-angle X-ray scattering. In this report, the X-ray scattering characteristics of intact, functional liver mitochondria are presented and discussed in terms of known structural features of mitochondria.

A preliminary report of the findings described below was presented at the 1976 Biophysical Society Meeting [8].

Materials and Methods

Mitochondrial specimen preparation and characteristics

Mitochondria were isolated from the livers of 2—4 month old Sprague Dawley or Charles River rats by the procedure of Hagihara [9] as modified by Parsons et al. [10]. The livers were homogenized in medium containing 0.225 M mannitol, 0.075 M sucrose, 0.1% bovine serum albumin (fraction V, Sigma) and either 1 mM ethylenediaminetetraacetic acid (EDTA) or 0.1 mM ethylenebis(oxyethylenenitrilo)tetraacetic acid (EGTA). Subsequent washes of the material during the differential centrifugation procedures were done in the suspension medium described above, without bovine serum albumin. Isolation media were adjusted to pH 7.2 using solid Tris(hydroxymethyl)aminomethane prior to addition of aliquots of stock solutions of EDTA or EGTA, which were adjusted in advance to pH 7.2 with KOH.

Washed rat liver mitochondria (washed mitochondria) prepared by this procedure routinely displayed state III respiration rates of 100—200 natoms oxygen/min per mg protein during succinate oxidation, with P : O ratios between 1.6 and 1.8 and respiratory control ratios of 5—7. (Parameters measured polarographically as described by Chance and Williams [11] and Estabrook [12] using a Yellow Springs Clark-type oxygen electrode and amplifier. The reaction medium used, medium A, consisted of 0.3 M mannitol, 5 mM $MgCl_2$, 10 mM KCl and 10 mM potassium phosphate buffer, pH 7.2).

Specimens for X-ray scattering experiments were prepared by centrifuging washed mitochondria for 10 min at 12 000 $\times g$ and spreading the resulting suspension in a layer about 1 mm thick between two sheets of "Mylar" (type S, DuPont, 4 μm thick). The unoriented, fully hydrated mitochondrial specimens thus prepared contained between 150 and 200 mg protein per ml, as determined by the biuret method [13].

Divalent cation chelators were included in the usual mitochondrial suspension medium to inhibit endogenous phospholipase activity. Free fatty acids released by the action of such enzymes rapidly uncouple oxidative phosphorylation in dense mitochondrial suspensions [14]. The inclusion of divalent cation chelators was found to increase the half-time of uncoupling in the densely-packed mitochondrial X-ray specimens from 0.5 h to 3 h, an adequate improvement considering the routine length of the X-ray scans (0.5—1.5 h).

Subfractionation of mitochondrial membranes was performed according to procedures referred to in the text. Negative-strain electron microscopy was used to confirm the composition of the various membrane fractions, according

to established criteria [10,15]. Drops of membrane suspension were deposited on farmvar-carbon-coated grids and stained with 1% potassium phosphotungstate, pH 6.8, as previously described [10]. Grids so prepared were examined with a Siemens 1A electron microscope operated at 80 kV.

Small-angle X-ray scattering experiments

The Mylar-enclosed mitochondrial suspensions described above were mounted in stainless steel specimen holders, the temperature of which was maintained and monitored with a thermo-electric unit. The small-angle X-ray scattering from these specimens was recorded with a Siemens diffractometer, consisting of a Kristalloflex-4 generator housing a Cu anode X-ray tube (type AG Cu 4ö/2) run at 30 kV and 30 mA, a Kratky Model 65 slit-collimated camera (focus-to-specimen distance, 190 mm) and a proportional counter detection system (LND type B counter tube, specimen-to-detector distance, 210 mm). Cu K_β radiation was filtered from the incident beam with Ni foil (20 μm thick) and a pulse height analyzer was used to select for Cu K_α scattered radiation.

The detector was generally programmed to step scan through scattering angles (2 θ) from 4 to 40 mrad or 1/400—1/40 $Å^{-1}$ in reciprocal space, r^*, defined as 2 $\sin\theta/\lambda$ with λ equal to the wavelength (1.54 Å) of the Cu K_α photons.

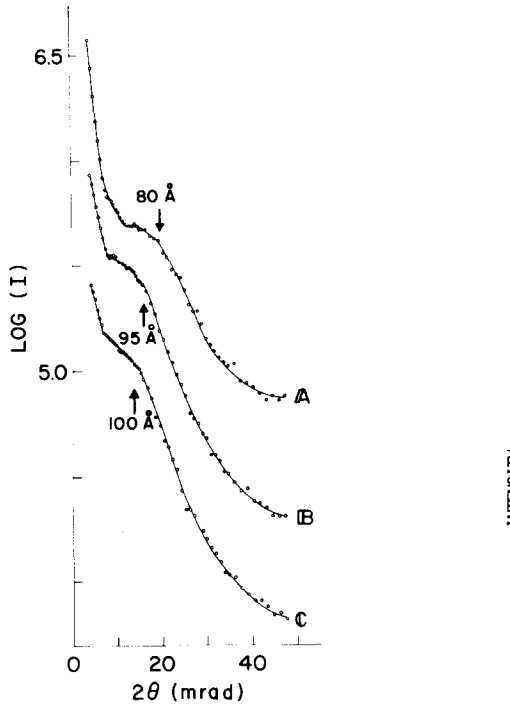
For such experiments, the X-ray beam was collimated to a half-width in the scanning direction of 250 μm at the detector. For scans like those of Fig. 2, in which the X-ray scattering in the region 0.5 to 3 mrad was monitored, the beam was narrowed to a half-width of 65 μm. In all experiments, the beam length at the sample was 25 mm.

All scattering curves presented have been corrected for background and beam geometry ("desmeared") by a computer program based on one obtained from Dr. P.W. Schmidt of the University of Missouri, Columbia. In this desmearing procedure, slit-length collimation corrections were calculated using the infinite-slit approximation [16] and slit-width corrections were made with a weighting function kindly evaluated for the camera geometry used by Dr. R. W. Hendricks of Oak Ridge National Laboratory [17,18]. The positions of maxima in the desmeared X-ray scattering curves (given in the figures in terms of $1/r^*$) were arrived at by subtracting the smoothly varying components of the curves from the maxima and determining the centers of the resulting approximately Gaussian curves.

Results

Scattering from intact mitochondria

Fig. 1 illustrates typical, small-angle X-ray scattering curves obtained from dense, fully hydrated, unoriented washed mitochondria suspensions held at 4°C. These scattering curves are characterized by a single broad maximum which is centered initially near 80 Å but which moves with time to smaller angle (around 100 Å), accompanied by a marked decrease in the initial slope of the scattering curves. This change in initial slope is a consequence of diminished scattering at even smaller angle (Fig. 2), i.e., a decrease of 90% in the integrated scattering intensity in the 0.5—3 mrad region ($1/r^* \approx$ 3000—500 Å).



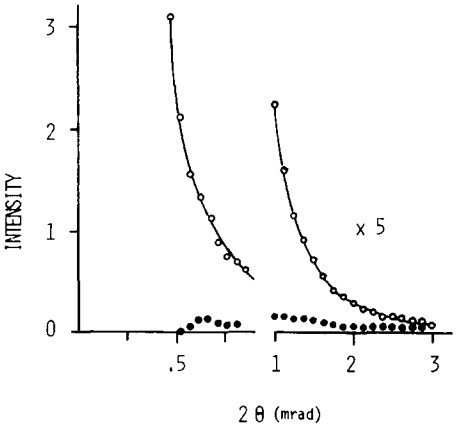


Fig. 1. Small-angle X-ray scattering curves of rat liver mitochondria. Intensity data, *I*, collected from same specimen A, 2 h; B, 8 h; C, 14 h after isolation of the mitochondria. Washed mitochondria used in these and subsequent experiments are suspended in medium held at 4°C prior to and during X-ray exposures, unless otherwise indicated. Successive curves are shifted down by 0.5 log units.

Fig. 2. X-ray scattering from liver mitochondria at very small angle. Open circles, freshly isolated mitochondria in suspension medium at 4°C; closed circles, same specimen held at 35°C for 10 min and returned to 4°C prior to scan.

The kinetics of the scattering changes in Fig. 1 and 2 are temperature-dependent, occurring within 6 h at 4°C, within 1 h at 20°C and within minutes at 35°C. The same scattering changes occur spontaneously when the washed mitochondria are suspended in media which cause a low-scale swelling of the mitochondrial matrix space (0.125 M KCl or 0.15 osM mannitol-sucrose) and can be reversed by suspension of the mitochondria in media which cause matrix contraction (0.4—0.5 M mannitol) [2,19]. These results strongly suggest a correlation between the spontaneous, temperature-dependent changes observed in the X-ray scattering from these specimens and the condensed-to-orthodox transition in mitochondrial morphology. Furthermore, this correlation is strengthened by appropriate changes in light scattering which are simultaneously observed in the Mylar-encased mitochondrial suspensions [4,2].

The spontaneous conversion of densely-packed liver mitochondria to the orthodox conformation is found to occur whether or not the specimens are X-irradiated and appears unrelated to the release of free fatty acids within the suspensions. (The onset of swelling is unaffected by addition of EDTA or EGTA and, at 4°C in the absence of divalent cation chelators, occurs well after

the mitochondria are fully uncoupled.) Nor is this structural transition related to the onset of anaerobiosis in the mitochondrial specimens. Endogenous substrate oxidation rates routinely displayed by these washed liver mitochondria (initial rates as high as 10 natoms oxygen/min per mg protein at room temperature) are sufficient to deplete the O_2 content of the Mylar-encased mitochondrial suspensions within 10 min at 4°C. Normally, therefore, the mitochondrial specimens are already anaerobic by the time the X-ray scattering scans have commenced.

Because of the need for dense mitochondrial suspensions and relatively long (at least 0.5 h) X-ray scattering scans to achieve reasonable counting statistics, anaerobiosis can be avoided in the scattering experiments only by slowing down the endogenous respiration rates. When this is done by pre-incubation of the mitochondria with respiratory inhibitors (1 mM KCN or 10 μg/ml antimycin A), no significant changes are observed either in the initial small-angle

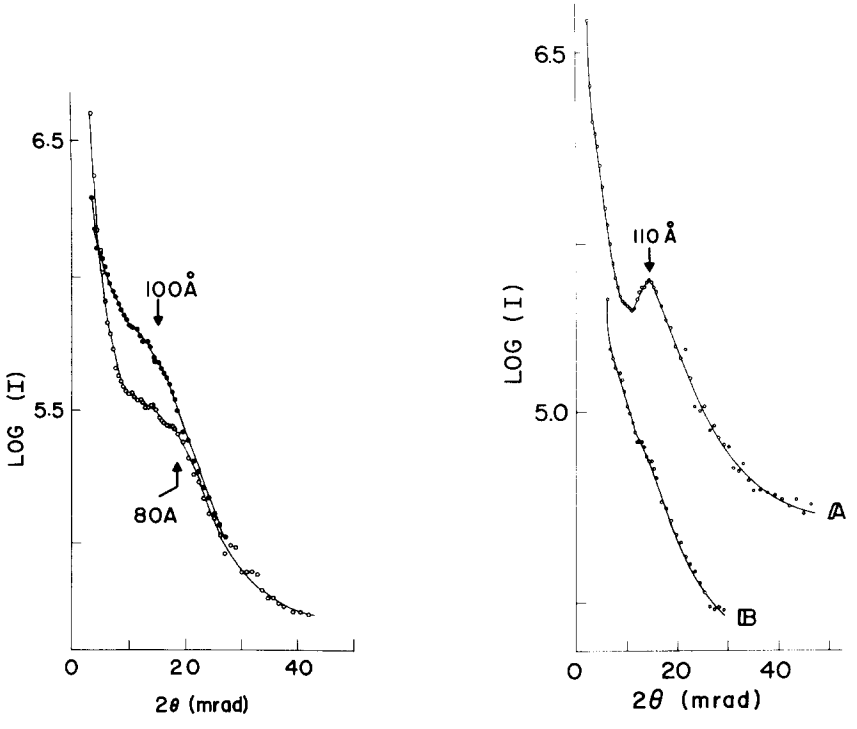


Fig. 3. Small-angle X-ray scattering from "mitoplasts". Open circles, membranes suspended in medium at 4°C; closed circles, same specimen held at 35°C for 10 min and returned to 4°C prior to scan.

Fig. 4. Small-angle X-ray scattering from A, isolated inner mitochondrial membranes and B, swollen and sonicated mitochondria. A, inner membranes isolated by repeated osmotic swelling of washed mitochondria in 80 vols. of 10 mM potassium phosphate buffer (pH 7.2), followed by differential and density gradient centrifugation as described in ref. 10. Final pellet obtained by 10 min centrifugation at 12 000 × *g* in washed mitochondria suspension medium. B, Mitochondria suspended for 5 h in 20 volumes of 1 mM potassium phosphate buffer (pH 7.2) and sonciated for 5 s using Branson W185 sonifier with microtip (12 W output). Specimen prepared by centrifugation of suspension for 30 min at 12 000 × *g* after addition of 4 volumes washed mitochondria suspension medium. Curve B is shifted down by 0.8 log units.

X-ray scattering from the mitochondrial specimens or in the long term scattering changes associated with low-scale mitochondrial swelling at 4°C. When, instead, anaerobiosis is prevented by rapid depletion of endogenous substrate pools (achieved by preincubation of washed mitochondria in medium A with excess ADP + O_2 at room temperature for 10 min), X-ray scattering from the specimens is characteristic of mitochondria in the orthodox state.

Scattering from mitochondrial subfractions

Digitonin treatment of liver mitochondria according to Schnaitman and Greenawalt [20] selectively detaches the outer membranes from mitochondria. The so-called "mitoplasts" that result give rise to essentially the same X-ray scattering maximum as intact mitochondria (Fig. 3), indicating that the maximum arises from structure associated with the mitochondrial inner membrane or matrix. Inner membrane fractions prepared by repeated osmotic lysis and sucrose step gradient centrifugation of washed mitochondria [10,15] display a small-angle X-ray scattering peak that is generally better defined than that obtained with intact mitochondria (Fig. 4A). Electron microscopic observations that inner membrane "ghost" fractions prepared in this way contain much less densely-staining matrix material than intact mitochondria or mitoplasts [10,15,20] strongly suggest that vectors within the cristae themselves are responsible for the scattering maximum.

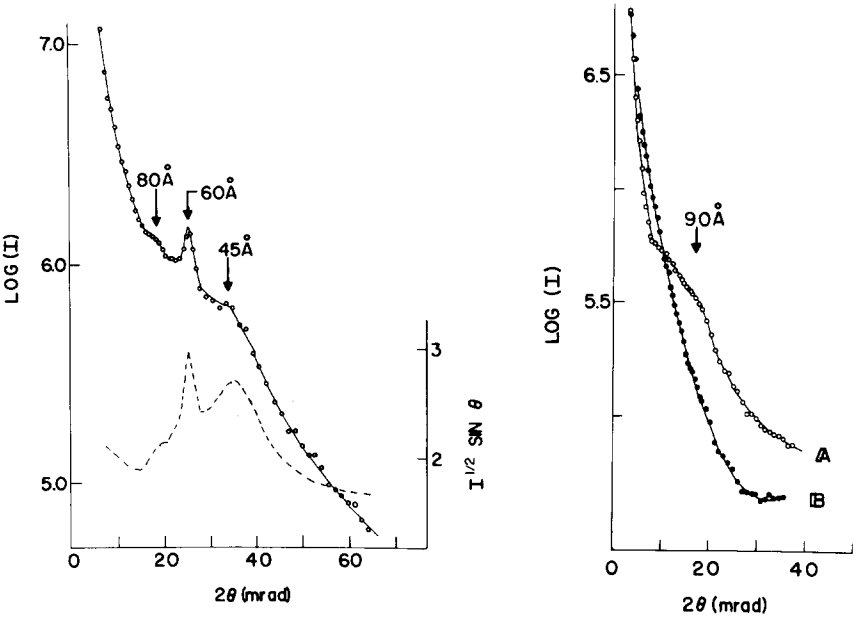


Fig. 5. Small-angle X-ray scattering from dehydrated liver mitochondria. Dense suspension of washed mitochondria dried overnight at 4°C in desiccator. Dashed line is the corrected scattering data plotted as $I^{1/2} \sin\theta$, a format often used for scattering from random membrane suspensions [25].

Fig. 6. Effects of glutaraldehyde on X-ray scattering from mitochondria. Mitochondria fixed for 15 min at room temperature in suspension medium containing A, 1% and B, 3% glutaraldehyde (Polyscience, EM grade) prior to centrifugation (12 000 × *g*, 10 min) as X-ray scattering specimens.

Several physico-chemical procedures were found to totally abolish the mitochondrial small-angle X-ray scattering maximum, including (a) raising the temperature of the suspension for 2 min to 65°C, (b) dehydration (Fig. 5), (c) acetone extraction [21] and (d) extensive hypotonic swelling followed by brief sonication (Fig. 4B).

Scattering after chemical fixation

An obvious drawback of the above experimental procedures is that the specimens of intact mitochondria are in relatively inactive (anaerobic or substrate-depleted or respiration-inhibited) states, a direct result of the need for long X-ray scattering scans and dense mitochondrial suspensions to achieve acceptable counting statistics. The feasibility of using glutaraldehyde, one of the more common mitochondrial fixatives [3,20,22—24], to trap isolated mitochondria in more active metabolic states has been examined. At levels near 1%, glutaraldehyde noticeably diminishes the small-angle X-ray scattering from liver mitochondria; at concentrations of 2% or higher, the maximum near 90 Å is completely abolished (Fig. 6).

Discussion

In thin section electron micrographs of mitochondria in the condensed state, the space bounded by the inner membranes appears densely stained and highly convoluted, enclosing numerous, irregularly shaped, less heavily stained "intracristal spaces" which range in size from several hundred to several thousand Angstroms [1—3,19]. Conversion of mitochondria to the orthodox state involves swelling of the matrix space and deconvolution of the inner membrane. The large decrease in continuous X-ray scattering intensity at 0.5—3 mrad which accompanies the condensed-to-orthodox transition most likely reflects this loss of complexity (i.e., long scattering vectors) in the shape of the inner membrane + matrix.

The data of Fig. 3 and 4A indicate that the 80—100 Å X-ray scattering maximum observed with washed mitochondria suspensions arises from vectors within the inner membrane. This maximum occurs at scattering angles too small to arise from transbilayer phospholipid correlations. Unoriented dispersions of phospholipids and plasma membranes, for example, display prominent X-ray scattering maxima in the 40—50 Å region which have been assigned to such transmembrane vectors [17,25—29]. It is worth noting that mitochondria do display a broad scattering maximum at 45 Å after drying (Fig. 5) suggesting that this treatment induces the formation of extensive bilayer regions (through lipid phase separation) not normally present. Dehydration of mitochondrial cristae also induces a lamellar alignment of these membranes which others have used to separate scattering contributions from transverse (i.e., membrane thickness) and in-plane (i.e., interparticle) correlations [23,30]. The sharp X-ray reflection at $1/r^* = 60$ Å in Fig. 5 is, in fact, indicative of lamellae formation within the dried, unoriented mitochondria specimens. Unfortunately, the other small-angle X-ray scattering changes associated with mitochondrial dehydration (the loss of the 80—100 Å maximum and the appearance of a peak at 45 Å) indicate that drying significantly denatures the struc-

ture of the membranes of intact mitochondria.

The possibility that the small-angle maximum observed with fully hydrated mitochondria might arise from particle interference among protein components of the cristae is suggested by several general considerations. For example, the inner membranes of liver mitochondria are 75% protein by weight [31,32] and the scattering maximum is irreversibly lost at temperatures (60—70°C) which correlate with protein denaturation thermal transitions [33]. More specifically, it is interesting that both acetone and swelling/sonication treatments of mitochondria abolish the 80—100 Å X-ray scattering maximum, since both procedures cause detachment from the cristae of F_1-ATPase [22,34,35], normally present as densely-packed arrays of 80 Å particles [36—38]. However, despite the fact that inner mitochondrial membranes so-treated are otherwise relatively well-defined in negative-stain electron micrographs [22,34,35], the severity of these two procedures prevents a firm correlation between the X-ray scattering maximum in question and the arrays of ATPase subunits.

Assuming that the structures responsible for the observed small-angle X-ray scattering from intact, hydrated mitochondria can be identified, a technical limitation must be overcome, namely the need for dense mitochondrial suspensions to achieve sufficient scattering intensity. While the experimental procedures outlined above are adequate to detect long-term alterations in mitochondrial structure (e.g., the condensed-to-orthodox transition elicited by phosphate acceptor and the X-ray scattering changes induced by uncouplers [39]) they are insufficient to monitor rapid, possibly transient structural changes that might accompany respiratory state transitions in actively respiring mitochondria. Such "real-time" monitoring of small-angle X-ray scattering from dilute suspensions of mitochondria should be feasible, however, with the more intense X-ray sources and more sensitive detectors now becoming available [40].

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