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A new crystal form of bovine heart ubiquinol: cytochrome *c* oxidoreductase: determination of space group and unit-cell parameters.

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Abstract

Ubiquinol:cytochrome *c* oxidoreductase, the middle segment of the mitochondrial respiratory chain, is a multi-subunit transmembrane redox enzyme. The purified protein from beef heart mitochondria has been crystallized by three groups in three different forms, but progress toward a structure has been hampered by the limited order (resolution) of the crystals. It has been found that under certain conditions the enzyme crystallizes in a new form suitable for X-ray diffraction studies. These crystals belong to space group $C222_1$ in the orthorhombic system. The cell dimensions are $a = 384$, $b = 118$ and $c = 177$ Å. These new crystals at present diffract to 3.8 Å at best. This is not significantly better than hexagonal [$P6_{1(5)}22$] crystals grown, but the new crystals have the advantage of less spot overlap because of face-centered packing which results in systematic extinctions. More importantly, the availability of the same enzyme in multiple crystal forms may allow phase refinement and extension by the method of molecular replacement.

Ubiquinol:cytochrome *c* oxidoreductase (cytochrome bc_1 complex; E.C. 1.10.2.2) is the middle segment of the mitochondrial respiratory chain, catalyzing electron transport from ubiquinol to cytochrome *c* coupled to translocation of 1 H^+ and 2 charges per electron across the inner mitochondrial membrane (Hinkle, Kumar, Resetar & Harris, 1991). It consists of 11 polypeptides (Schägger, Link, Engel & von Jagow, 1986) with a total molecular mass of 230 kDa. There are four redox centers: two *b* cytochromes, cytochrome c_1 and the Rieske iron–sulfur center. A low-resolution structure for the enzyme from *Neurospora crassa* is available from electron microscopy of two-dimensional crystals (Weiss & Leonard, 1987). A high-resolution structure is available for the lumen-side domain of cytochrome *f* (Martinez, Huang, Szczepaniak, Cramer & Smith, 1994), which is homologous to cytochrome c_1 of cytochrome reductase.

Yu's laboratory has reported three-dimensional crystallization of cytochrome reductase (Yue, Zou, Yu & Yu, 1991). Two other groups independently developed crystallization protocols published in the following year (Kubota *et al.*, 1991; Berry, Huang, Earnest & Jap, 1992). The three groups had crystallized three different preparations of the beef heart enzyme in three different space groups. One of the crystals (Berry *et al.*, 1992) diffracted X-rays to 4.7 Å, and has since been improved to better than 4 Å (unpublished), suitable for calculating an intermediate-resolution structure if phases can be obtained. Here we report that under certain conditions our preparation of the enzyme crystallizes in another form suitable for X-ray diffraction studies, and present some characteristics of these crystals.

Materials and methods*

Protein isolation

The bc_1 complex was isolated from beef heart mitochondria by the procedure described for the potato complex (Berry, Huang & DeRose, 1991). After the DEAE–Sepharose column the peak fractions were concentated by ultrafiltration (Amicon YM-100 membrane) and further purified on a Sepharose CL-6B column in 20 m*M* K-MOPS, 100 m*M* NaCl, 0.5 m*M* EDTA, 0.1 g l^{-1} dodecyl β-D-maltoside. The peak fractions from this column were concentrated by ultrafiltration. The protein was further concentrated and buffer composition adjusted by dialysis against 50 m*M* KP_i, 0.5 m*M* Na–EDTA, and 300 ml l^{-1} glycerol.

Size calibration of the Sepharose columns

Standard proteins cytochrome *c* (m = 12.4 kDa, R_s = 17 Å), bovine serum albumin (66.3, 35), catalase (230, 52), apoferritin (481, 67), and thyroglobulin (669, 82) were chromatographed in the absence of detergent. Void volume of the columns, measured as the elution volume of submitochondrial particles, was 0.36 of column volume on Sepharose CL-6B and 0.38 on CL-4B. The Stokes radius and molecular weight of the bc_1 complex were estimated from calibration curves of R_s *versus* K_{av} and log(m) *versus* K_{av} (not shown).

Crystallization

The protein solution at 0.7–1.0 m*M* was supplemented with 1/15 volume of 1 *M* octyl glucopyranoside. This protein–detergent solution was then mixed with an equal volume of a precipitant containing 100 m*M* KP_i pH 6.7, 3 m*M* NaN_3 and 100 g l^{-1} PEG. Then 10 µl of the protein–precipitant mixture was placed in each well of a nine-well glass plate, which was then placed on a plastic petri dish in a plastic sandwich box containing 50 ml of a reservoir buffer. The reservoir buffer consisted of 100 m*M* KP_i pH 6.7, 3 m*M* NaN_3 and PEG4000 at concentrations of 120, 300 or 350 g l^{-1}. All of the wells were seeded with a homogenate of hexagonal bipyramid crystals. Usually this results in growth of new hexagonal bipyramids in

* Abbreviations used are, DM: dodecyl β-D-maltopyranoside; PEG: polyethylene glycol, average MW 4000; KPi: phosphate buffer with pH adjusted with KOH; K-MOPS: 3-(*N*-morpholino-propane-sulfonate) buffer (pH adjusted with KOH); EDTA, ethylenediamine tetraacetate; R_s, Stokes radius; m, molecular mass; K_{av}, (elution volume minus void volume) divided by (total volume minus void volume) from chromatography on a gel-filtration column. Solutions were prepared on a weight to total volume basis and their concentrations expressed as molar (*M*) or g l^{-1} with the exception of glycerol, which was made as volume per total volume and expressed as ml l^{-1}. Multiplying this by 1.26 gives the concentration in g l^{-1}.

1–2 days. In this experiment we believe the seeding was ineffectual and crystals arose from *de novo* nucleation.

The sandwich box was covered with a tight-fitting lid and sealed with Parafilm to allow equilibration of water between the wells and reservoir.

Results and discussion

Fig. 1(*a*) shows typical crystals of the space group $P6_122$ (or enantiomorph $P6_522$), prepared as described in Berry *et al.* (1992). The crystals are hexagonal bipyramids. The best observed diffraction from such crystals is 3.86 Å (14, −24, −29 reflection; unpublished). Figs. 1(*b*) and 1(*c*) show crystals of a new form that we hve observed occasionally when the concentration of PEG in the reservoir during vapor diffusion was higher than used by Berry *et al.* (1992). The three large crystals in Fig. 1(*b*) are lying flat and show a six-sided profile, with two opposing 90° angles, the other four angles being 135°. The two largest crystals in Fig. 1(*c*) are on edge, with the smallest dimension horizontal. The short edges appear to meet the large faces in 90° angles. Diffraction data discussed below shows that crystals of the type shown in Figs. 1(*b*) and 1(*c*) are orthorhombic.

In the experiment described here either hexagonal or orthorhombic crystals were obtained from the same protein preparation and precipitant solution, depending on the osmolarity of the reservoir solution with which the droplet was allowed to equilibrate by vapor diffusion. The protein/precipitant droplets initially contained 50 g l^{-1} PEG and 150 ml l^{-1} glycerol. Because of the glycerol present in the enzyme solution, vapor diffusion resulted in an increase in volume and dilution of the enzyme–precipitant droplet when the reservoir buffer contained 120 or 300 g l^{-1} PEG. Vapor diffusion caused little or no change in droplet volume when the reservoir contained 350 g l^{-1} PEG.

In a well with low reservoir PEG (Fig. 2), hexagonal rod-shaped crystals had grown by the fourth day (Fig. 2 panel *A*). By day 11 (Fig. 2 panel *B*) they were much larger, and many tiny hexagonal bipyramids had formed. Between the 11th and 16th days (Fig. 2 panel *C*) the hexagonal rods did not grow while the bipyramids grew considerably. No orthorhombic crystals were observed at any time.

Small hexagonal rods developed by day 4 in some of the wells with high reservoir PEG, but not in the well shown in Fig. 3. On day 9 (Fig. 3 panel *A*) small hexagonal rods had formed (some are seen on end so the hexagonal cross-section can be confirmed). Three orthorhombic crystals were also present on the other side of the well (not visible in the figure). The hexagonal rods had not grown significantly larger by day 11 (Fig. 3 panel *B*), but the orthorhombic crystals had grown and many new orthorhombic crystals had appeared. By the 16th day the hexagonal rods had disappeared, while the orthorhombic crystals continued to grow (Fig. 3 panel *C*). Cracks (as seen in Fig. 1*b*) developed in some of the larger orthorhombic crystals between the 11th and 16th days.

The two wells shown in Figs. 2 and 3 are representative of all the wells under the same conditions. Two trays (18 wells) were equilibrated with 350 g l^{-1} PEG in the reservoir, and orthorhombic crystals were observed in all (Figs. 1*b*, 1*c* and 3 show three of these wells). Out of two trays with 120 g l^{-1} PEG, 17 wells (all but 1) had both hexagonal rods and small bipyramids by the 16th day, as in Fig. 2 panel *C*. In some wells with intermediate reservoir osmolarity (300 g l^{-1} PEG, not shown) hexagonal bipyramids and orthorhombic crystals coexisted.

We have seen the orthorhombic crystals in other experiments before and after the one described here, but did not observe whether they were preceded by hexagonal rods. The hexagonal

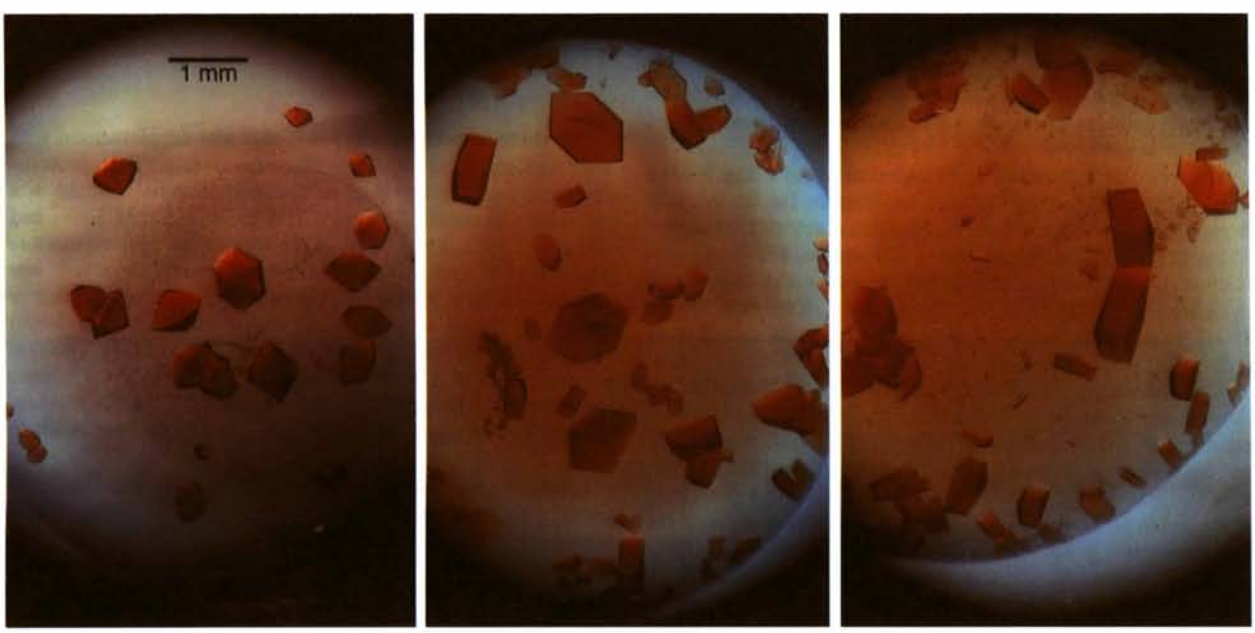

Fig. 1. Two distinct forms of crystals of bovine ubiquinol:cytochrome *c* oxidoreductase. (*a*) Shows typical hexagonal bipyramids, (*b*) and (*c*) show orthorhombic crystals prepared as described in materials and methods with a reservoir buffer containing 350 g l^{-1} PEG400, photographed 16 days after setting up the trays.

rods have often been observed when seeding fails. Diffraction is poor and indicates space group $P6_{1(5)}$ with unit-cell dimensions $a = 134$, $b = 134$ and $c = 752$ Å. These crystals usually do not last long. They seem to grow lengthwise on only one end. The end that is growing tends to get hollow and/or tapered. This can be seen in Fig. 2. The solid ends of the rod-shaped crystals in Fig. 2 panel C are in the same relative positions as in Fig. 2 panel B or panel A. Although the length of these crystals has increased greatly, all the growth has been at the tapered end.

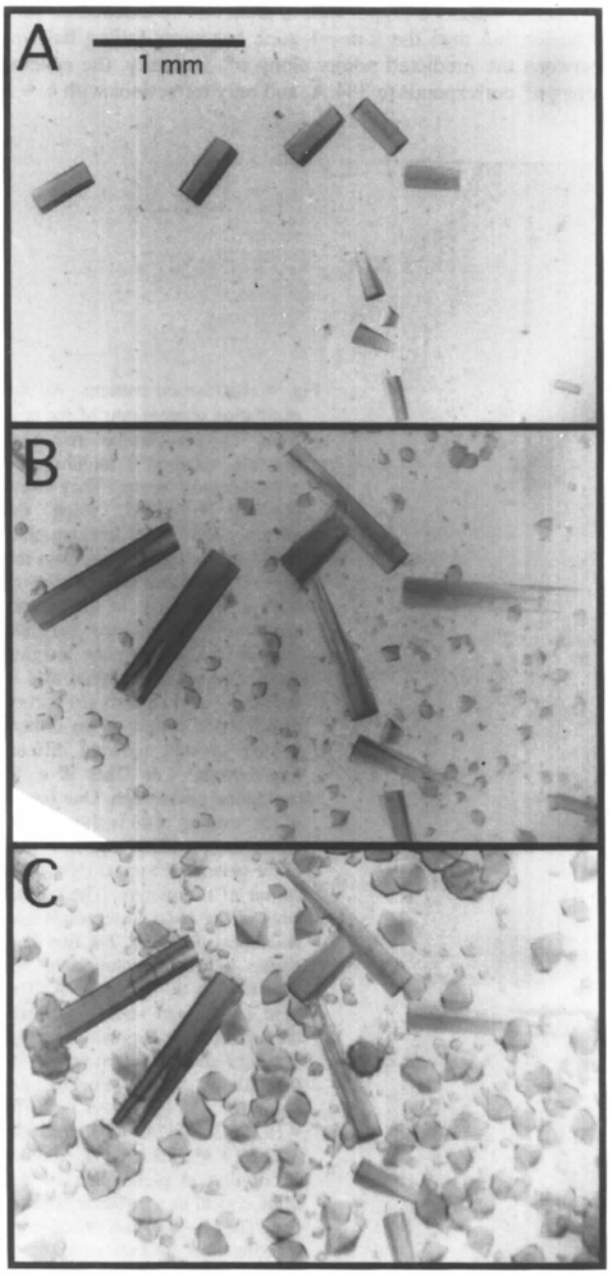

Fig. 2. Stages in the growth of hexagonal bipyramids. A crystallization tray was set up as described in materials and methods, with 120 g l^{-1} PEG in the reservoir. Each well was seeded with homogenized hexagonal bipyramid crystals. One well was photographed at 4 (*A*), 11 (*B*) and 16 (*C*) days and a selected area is shown. Hexagonal rod crystals appeared in *A* and grew between *A* and *B*. Hexagonal bipyramids were also present in *B* and grew between *B* and *C*, while the rods did not.

Crystalline order and space-group assignment

Diffraction of the orthorhombic crystals was tested using the rotation camera on beamline 7-1 at the Stanford Synchrotron Radiation Laboratory. Fig. 4(*a*) shows a pattern from one of the better ordered crystals obtained during a 1° rotation of the crystal. One fourth of the imaging plate is shown, with the center at the upper right corner. Diffraction extends to the edge of the imaging plate, although only the strongest reflections at this resolution are visible. Three reflections with resolution around 3.75 Å are indicated. The circles and squares drawn around the spots show the positions of spots predicted by the

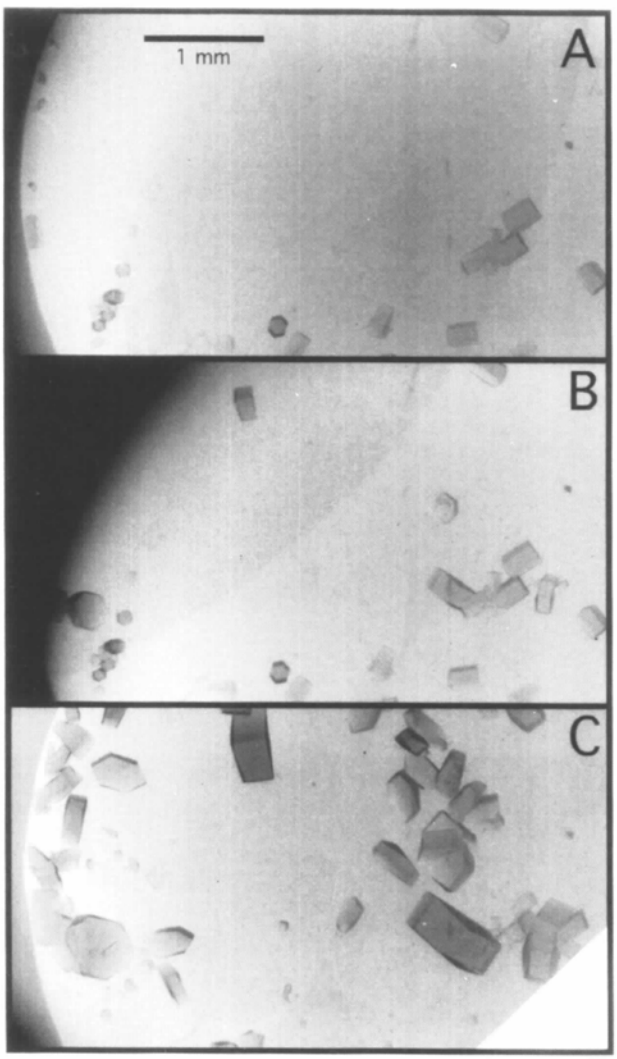

Fig. 3. Stages in the growth of orthorhombic crystals. Conditions were the same as in Fig. 2 except that the reservoir contained 350 g l^{-1} PEG. Photographs were taken at 9 (*A*), 11 (*B*) and 16 (*C*) days. Hexagonal rods were present in *A*, Orthorhombic crystals were also present in *B*, in *C* the hexagonal rods had essentially all dissolved while the orthorhombic crystals grew and became more numerous.

program *DENZO* for this orientation of the crystal and the unit-cell parameters. Squares indicate fully recorded reflections and circles represent partials, assuming 0.4° mosaicity. In addition to diffraction spots there is diffuse scattering, the structural significance of which is not known.

Figs. 4(*b*) and 4(*c*) show diffraction patterns supporting the space-group assignment and unit cell parameters. Fig. 4(*b*) shows the same crystal of Fig. 4(*a*), rotated 6° about the spindle axis to bring the $(h,-h,0)$ and $(0,0,l)$ reflections into the plane. Systematic extinctions along the c^* axis $(0,0,l)$ are seen, with only reflections with even l being present.

Fig. 4(*c*) shows the $(h,0,l)$ plane. This is a 4° oscillation photograph of another crystal, from a position in which the X-ray beam was nearly perpendicular to the large faces of the crystal. The a^* and c^* axes are in the plane, so b^* (and b) are perpendicular to the large faces, *i.e.* the short dimension of the crystal corresponds to the b axis. An apparently square lattice is seen, with spacing corresponding to about 185 Å along a^* or c^*. The $k = 0$ zone (upper central area) can be indexed on such a lattice, but then the $k = -1$ zone has spots falling halfway between the predicted points along a^*. Actually, the spacing along a^* corresponds to 384 Å, and only reflections with $h + k$

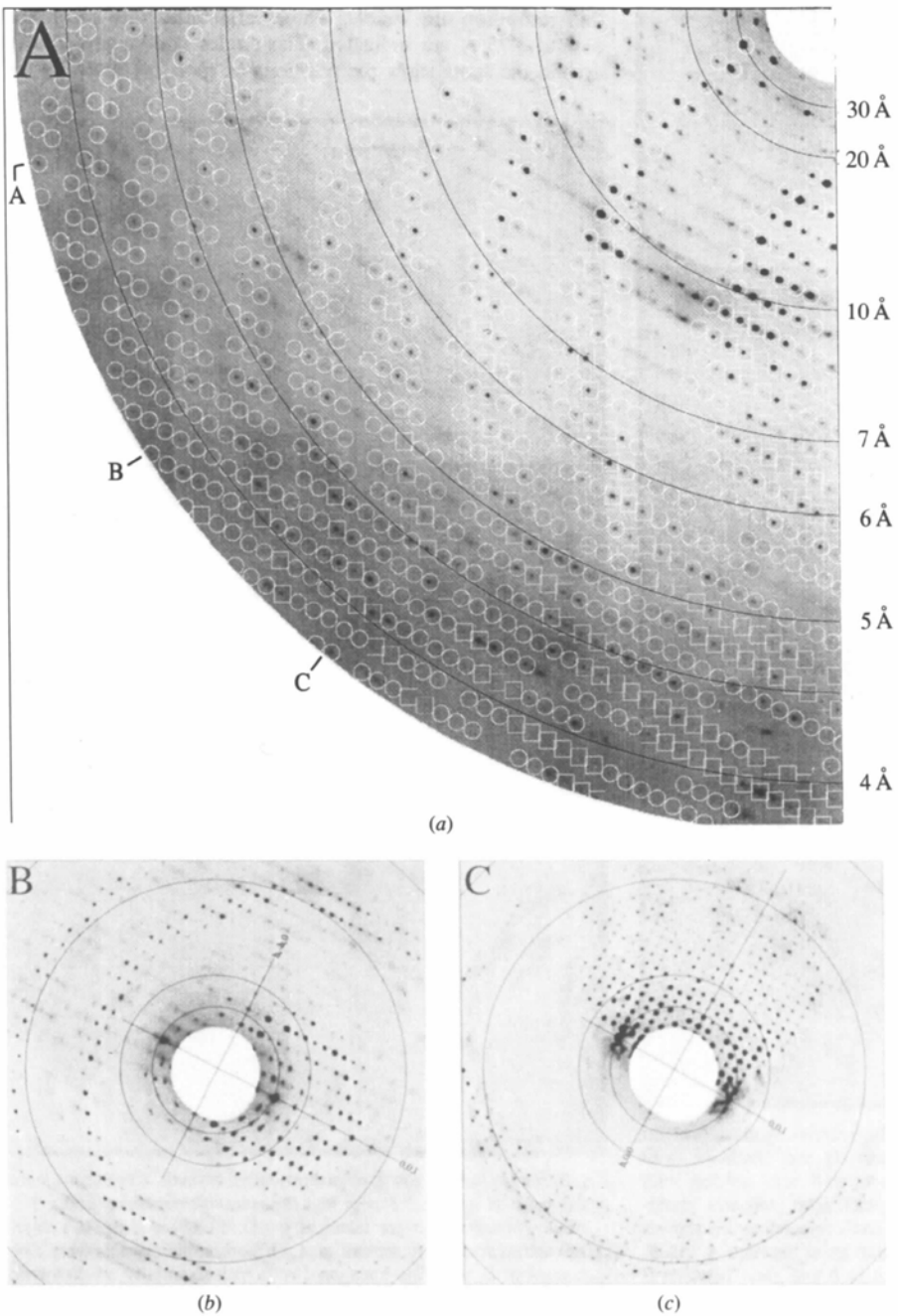

Fig. 4. Diffraction patterns. All are oscillation photographs of the new form of cytochrome reductase crystals, obtained 2 months after crystallization setup. They were obtained at SSRL using the 90 mm radius MAR imaging plate at a distance of 300 mm from the crystal. The X-ray wavelength was 1.08 Å. The width of the images correspond to 90 mm (*a*) and 72 mm (*b* and *c*) on the imaging plate. The spindle rotation axis is horizontal in each case. (*a*) Part of a diffraction pattern of an orthorhombic crystal, showing diffraction beyond 4 Å. This is a 1° oscillation photograph. One fourth of the imaging plate is shown. The direct beam position is at the center of the plate, in the upper right-hand corner of the picture. The curved edge of the plate corresponds to a resolution of 3.72 Å. The fine dark circles indicate resolution of 30, 20, 10, 7, 6, 5, 4.5 and 4 Å. The light circles and squares indicate the predicted positions of spots. The *h*,*k*,*l* and resolution values for the labeled reflections are: (*a*) −4,20,36 (3.772 Å); (*b*) −15,27,23 (3.745 Å); and (*c*) −22,30,9 (3.746 Å). (*b*) Another 1° oscillation photograph of the same crystal in a different orientation. The $(h,-h,0)$ and $(0,0,l)$ rows of reflections are indicated, showing systematic absences along the latter. (*c*) Another crystal from an identical well in the same tray. The crystal is oriented to show the $(h,0,0)$ and $(0,0,l)$ rows of reflections. This 4° rotation pattern was generated by averaging four consecutive non-iverlapping 1° rotation patterns.

even are visible. This indicates face-centered packing. Together with the systematic absences along c^* this identifies the space group as $C222_1$. The unit-cell dimensions are a = 384, b = 118 and c = 177 Å.

Aggregation state of cytochrome reductase solubilized in dodecyl maltoside

The elution volume of the cytochrome reductase complex was 0.60 of the total column volume with Sepharose CL-6B and 0.72 with Sepharose CL-4B, giving K_{av} values of 0.38 and 0.55, respectively.

By comparison with the elution volume of characterized proteins (see *Materials and methods*), our preparation of cytochrome reductase has a stokes radius of about 72 Å, or a molecular mass of 490 kDa. This is consistent with a dimer of the 230 kDa enzyme with some phospholipid and detergent. It has been shown (von Jagow, Schägger, Riccio, Klingenberg & Kolb, 1977; Engel, Schägger & von Jagow, 1980) that the beef heart enzyme prepared by extraction in Triton X-100 and chromatography on hydroxyapatite consists of dimers. The enzyme from *Neurospora* also consists of dimers, and when this preparation crystallizes in two dimensions the enzyme packs as dimers with the monomers related by a twofold axis roughly parallel to the long axis of the monomer and perpendicular to the plane of the two-dimensional membrane crystal (Weiss & Leonard, 1987).

Possible crystal packing arrangement in the orthorhombic crystals

If we assume that the beef enzyme crystallizes as a dimer with the monomers related by a twofold axis, which is common for enzymes that are dimeric in solution and is the case for the *Neurospora* enzyme in two-dimensional crystals (Weiss & Leonard, 1987), then the question arises whether the twofold axis represents crystallographic or non-crystallographic symmetry. The volume of the asymmetric unit of the orthorhombic crystals is 1.0×10^6 Å^3, close to that of the hexagonal crystals (1.28×10^6 Å^3). The volume/mass ratio of the orthorhombic crystals is 4.35 Å^3 Da^{-1} if there is one monomer (230 kDa) per asymmetric unit, or 2.17 if two monomers per asymmetric unit. The hexagonal crystals probably have a single monomer per asymmetric unit (5.56 Å^3 Da^{-1}), based on protein concentration of the crystal (Berry *et al.*, 1992). Unless the orthorhombic crystals are packed much more densely than the hexagonal bc_1 crystals, the asymmetric unit of the orthorhombic crystals contains a single monomer, in which case the twofold axis would have to lie along a crystallographic twofold axis.

The $C222_1$ space group has two distinct twofold rotation axes, one parallel to the a axis and one parallel to the b axis. If the orientation of the monomers in the dimer is the same in the crystal as it is for the *Neurospora* enzyme, the twofold axis is roughly parallel to the long (175 Å) axis of the monomer. In that case there is not room enough to pack the dimer along the b axis, as the unit cell is only 118 Å in the b direction. It would fit with the dimer twofold along the a axis, which is 384 Å.

It may be possible to obtain phases for the low resolution reflections by molecular replacement using a protein envelope based on the electron-microscopy work with the *Neurospora* enzyme. Once some phase information is available from molecular replacement or from isomorphous replacement, the phases may be refined and extended to higher resolution by averaging electron density in equivalent areas of two or more distinct crystal forms, such as our hexagonal and orthorhombic crystals.

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