MEMBRANE CRYSTALS OF A SUBUNIT COMPLEX OF MITOCHONDRIAL CYTOCHROME REDUCTASE CONTAINING THE CYTOCHROMES *b* AND c_1

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1. Introduction

Cytochrome reductase (ubiquinol:cytochrome *c* reductase, EC 1.10.2.2.) is a proton-translocating oxidoreductase involved in mitochondrial oxidative phosphorylation [1–3]. The enzyme has M_r ~550 000. It is a dimer and the monomeric unit is comprised of at least 8 different subunits. Three of them carry redox centres. These are the cytochromes *b* and c_1 and the iron–sulphur subunit (M_r ~30 000, 31 000 and 25 000). Five subunits (M_r ~50 000, 45 000, 14 000, 12 000 and 8000) probably do not have redox centres. The enzyme has been isolated from *Neurospora* mitochondria as a monodisperse protein-Triton X-100 complex [4] and membrane crystals have been prepared from the enzyme by adding mixed phospholipid–Triton X-100 micelles and subsequent removal of the detergent [5].

The enzyme in Triton X-100 solution can be cleaved by increasing the ionic strength and a subunit complex which contains the cytochromes *b* and c_1 and the 3 small subunits without redox centres can be isolated in a monodisperse state. We report here that membrane crystals can also be prepared from this cytochrome bc_1 subunit complex, and we compare some structural properties of this complex with those of the whole cytochrome reductase.

2. Materials and methods

Cytochrome reductase was isolated from *Neurospora crassa* mitochondria as in [4]. The enzyme was concentrated by ultrafiltration on a Diaflo XM 300 (Amicon) filter. The cleavage of cytochrome reductase and the isolation of the cytochrome bc_1 complex was done as follows: 3 ml of a solution containing 15 mg/ml cytochrome reductase in 40 mM Tris–acetate (pH 7), 0.05% Triton X-100 was brought to 5 mM ascorbate, 5 mM dithioerythritol, 2% Triton X-100 and 2 M NaCl. After 5 min the solution was subjected to gel filtration on a 1.6 × 100 cm Ultrogel AcA 34 (LKB) column which was equilibrated with 0.2 M NaCl, 50 mM Tris–acetate (pH 7), 2 mM ascorbate, 2 mM dithioerythritol and 0.05% Triton X-100. The cytochrome bc_1 complex eluted as a symmetric, well-resolved peak. The cytochrome bc_1 complex was concentrated by ultrafiltration on a Diaflo PM 30 filter to 3 mg protein/ ml. Membrane crystals were prepared as follows: appropriate amounts of soybean phosphatidylcholine (Carl Roth) and bovine brain phosphatidylserine (Sigma) were diluted with chloroform and dried over a stream of nitrogen at 40°C. The residue was twice dissolved in ether and dried. Phosphatidylcholine (0.4%) and 0.1% phosphatidylserine in 0.5% Triton X-100, 50 mM Tris–acetate (pH 7), 50 mM NaCl, 1.0 mM EDTA, 1 mM ascorbate and 5 μm butylated hydroxytoluene (Sigma) was sonicated for 5 min until a clear solution was obtained. The solution was centrifuged at 100 000 × *g* for 30 min to remove any undispersed phospholipid and metal pieces from the sonicator tip. About 90% of the phospholipid remained in solution as estimated using [^{14}C]-phosphatidylcholine (New England Nuclear). An aliquot of 0.05 ml phospholipid solution was mixed with 0.1 ml protein solution. Triton X-100 was removed by dialysis for 30–40 h at 25°C against 50 mM Tris–acetate (pH 5.5), 50 mM NaCl, 1mM EDTA and 1 mM ascorbate.

The following analytical procedures have been described: electron microscopy and image reconstruction [5]; determination of Triton X-100 binding of protein

and of Stokes radius (r_S) and sedimentation coefficient ($s_{20,w}$) of protein–detergent complexes and calculation of protein M_r [4]; SDS gel electrophoresis of [^{3}H]leucine-labelled protein [6].

3. Results and discussion

Cytochrome reductase in the native mitochondrial inner membrane or in a reconstituted phospholipid bilayer [5] is very stable against high salt concentration. Sonication of mitochondria in the presence of 0.2 M sodium phosphate buffer (pH 7) does not cause dissociation of the enzyme. Cytochrome reductase in a micelle of Triton X-100, however, easily dissociates when the ionic strength is increased. When the cleavage is carried out in the presence of the reductants ascorbate and dithioerythritol 3 different parts are obtained:

(i) A subunit complex which contains the cytochromes *b* and c_1 and the 3 small subunits without redox centres;
(ii) A subunit complex without redox centres which contains the 45 000 and 50 000 M_r subunit;
(iii) An iron–sulphur subunit.

When the mixture of these 3 parts is subjected to gel filtration in the presence of Triton X-100, the cytochrome bc_1 complex elutes as a symmetric peak with a K_{av} of 0.25 [7] well separated from a peak with the K_{av} of 0.36 which contains a mixture of the complex without redox centres and the iron–sulphur subunit (as estimated by SDS gel electrophoresis) and any excess of Triton X-100 micelles (as estimated by [^{3}H] Triton X-100).

Some properties of the cytochrome bc_1 complex in Triton X-100 solution together with corresponding properties of cytochrome reductase are given in table 1. Both preparations are in dimeric states; their M_r values determined from the hydrodynamic properties in detergent solution corrected for the amount of bound detergent are half the M_r values determined from subunit composition. Both preparations bind about the same amount of Triton X-100/mol. Triton X-100 binding/g cytochrome bc_1 complex protein is, however, about twice the Triton X-100 binding/g cytochrome reductase protein. The stoichiometry among the subunits present in the cytochrome bc_1 complex appears to equal the stoichiometry among the corresponding subunits in cytochrome reductase, namely cytochrome c_1 (M_r ~31 000) to cytochrome *b* (M_r ~30 000) to the 14 000, 12 000 and 8000 M_r subunits as 1:2:1:1:1 [4,6], (fig.1).

Membrane crystals are prepared from the cytochrome bc_1 complex by removing Triton X-100 from

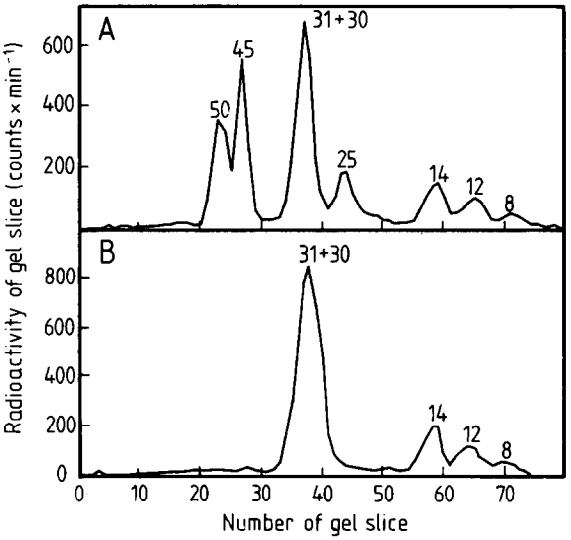

Fig.1. SDS gel electrophoresis of cytochrome reductase (A) and cytochrome bc_1 complex (B). Electrophoresis was performed with preparations from *Neurospora* which was radioactively labelled by in vivo incorporation of [^{3}H]leucine. The numbers refer to apparent M_r values (X 10^{-3}) of the subunits.

Table 1
Properties of cytochrome reductase and cytochrome bc_1 complex

Preparation	Triton X-100 binding		Hydrodynamic properties of protein–Triton X-100 complex		M_r value (X 10^{-3})	
	(g/g protein)	(g/mmol)	r_S (nm)	$s_{20,w}$	From r_S and $s_{20,w}$	From subunit composition
Cytochrome reductase	0.22	120	8.5	19	550	290
Cytochrome bc_1 complex	0.43	115	6.2	12.5	280	125

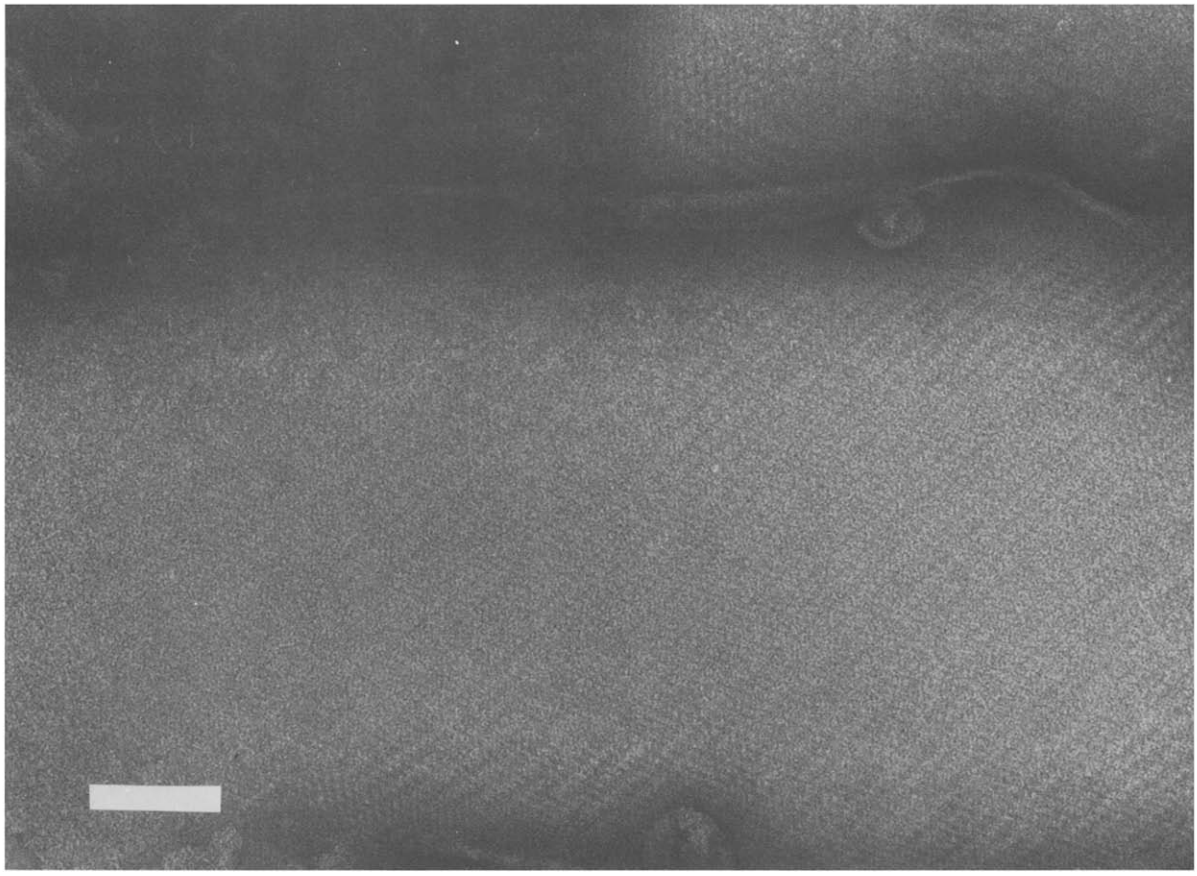

Fig.2. Part of a membrane crystal of the bc_1 cytochrome complex. The scale bar is 100 nm.

a solution containing the protein–Triton X-100 complex and mixed phospholipid–Triton X-100 micelles. Of the Triton X-100 >90% is removed by dialysis for 2 days as measured by [^{3}H]Triton X-100. Electron micrographs of the negatively-stained membranes show sheets of several μm diameter which contain crystalline areas (fig.2). These areas are single layer crystals. The membrane crystals also grow in the form of vesicles or tubes which collapse during fixation and staining and form double layers; moiré patterns arise from the overlap of the lattices on opposite sides of the flattened vesicles.

The diffraction pattern of single layer crystals extends to 2.5 nm (fig.3). The pattern shows the lowest possible symmetry. Only the 2-fold symmetry imposed by Friedel's law is present. Thus these membrane crystals have either p1 or p21 symmetry (nomenclature for 2-sided plane groups according to [8]). These symmetries are distinguished by the phase values of

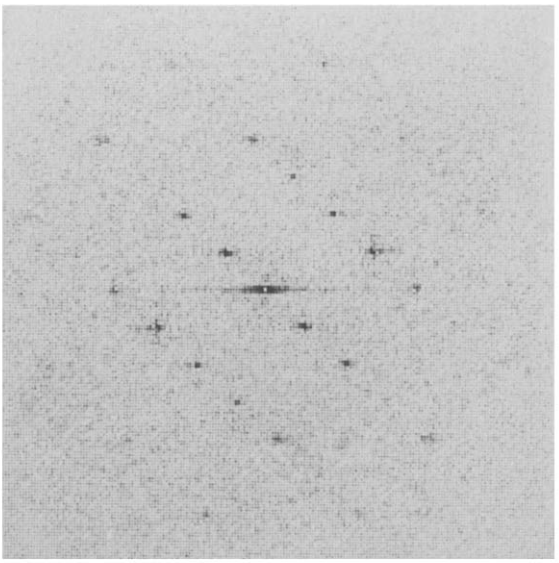

Fig.3. Diffraction pattern of the membrane crystal in fig.2.

the diffraction points. In both plane groups the amplitudes of any pair of diffraction points (h,k) and $(-h,-k)$ are equal, but the additional 2-fold symmetry will restrict the phase values to 0° or 180° for p21 since the projection is centrosymmetric, whereas any phase values between 0° and 360° are possible in pl. The computer-calculated Fourier transform of the image contains both the amplitudes and phases of the diffraction pattern. The average phase deviation from 0° or 180° was 9° for the 13 strongest reflections which is consistent with p21 symmetry. This means that the molecules are related by a 2-fold axis perpendicular to the plane of the membrane and are facing the same way. This is in contrast to the membrane crystals of cytochrome reductase where alternate dimeric molecules face up and down [5,9](fig.4a).

The computer-averaged projected image shows the dimeric state of the cytochrome bc_1 complex (fig.4b). The unit cell dimensions are: $a = 8.1 \pm 0.4$ nm; $b = 14.2 \pm 0.6$ nm; $\gamma = 92° \pm 2°$. The unit cell area is 115 nm^2. This is close to half the area of the unit cell of cytochrome reductase crystals [5] which is 13.7 nm × 17.4 nm = 240 nm^2 (fig.4a). Crystals of the whole enzyme have 2 dimers/unit cell, crystals of the cytochrome bc_1 complex only 1/unit cell.

The dimensions of the cytochrome bc_1 complex in projection are roughly the same as those of cytochrome reductase namely ~10 nm × 7 nm (fig.4a,b). The corresponding r_S and M_r values however differ strongly (table 1), indicating that the cytochrome bc_1 complex is only ~1/2 as big as the cytochrome reductase. A 3-dimensional structure analysis of the cytochrome reductase, based on tilted views of the enzyme showed that the enzyme is elongated, being ~15 nm across the membrane [9]. The dimension of the cytochrome bc_1 complex in the direction perpendicular to the membrane, therefore, can only be ~1/2 of that of the whole enzyme, namely ~7nm. Therefore we assume that in contrast to cytochrome reductase most of the protein of the cytochrome bc_1 complex is embedded in the bilayer. The dimeric cytochrome bc_1 complex is S-shaped in projection. The longest dimension in the plane of the membrane is 13 nm. Each monomer is ~7 nm × 5 nm.

The possibility to split the cytochrome reductase and to obtain membrane crystals of the whole enzyme as well as of a subcomplex increases the chances of achieving high resolution structural information. Since this is our second successful attempt to prepare 2-dimensional crystals from membrane proteins isolated as monodisperse protein–detergent complexes, we are encouraged to propose that similar crystals can be obtained from many other membrane proteins by using the above method. We are currently studying the

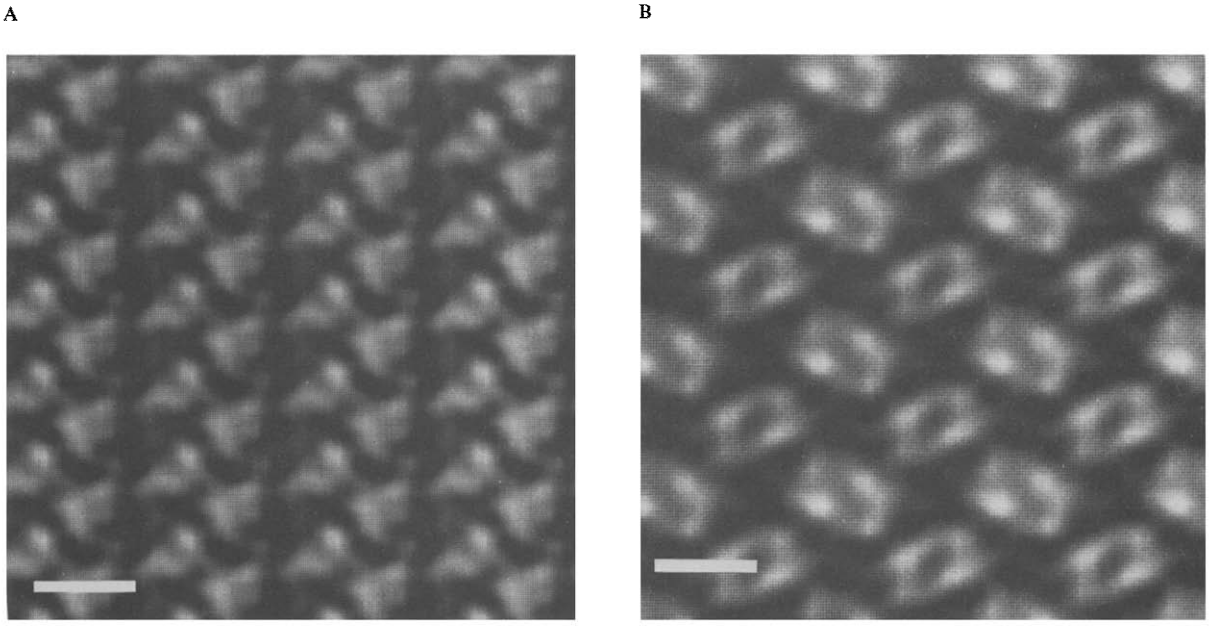

Fig.4. Computer averaged area of: (A) cytochrome reductase; (B) cytochrome bc_1 complex. The scale bars are 10 nm.

3-dimensional structure of the cytochrome bc_1 complex by combining electron micrographs of tilted views.

Acknowledgements

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