Accelerated Publications

Crystallization of Mitochondrial Ubiquinol–Cytochrome *c* Reductase†

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ABSTRACT: Ubiquinol–cytochrome *c* reductase of beef heart mitochondria was crystallized in the presence of decanoyl-*N*-methylglucamide, heptanetriol, and sodium chloride with poly(ethylene glycol) as precipitant. The largest crystal has dimensions of 4 × 2 × 1 mm. The crystalline enzyme is composed of 10 subunits. It contains 2.5 nmol of ubiquinone, 8.4 nmol of cytochrome *b*, 4.2 nmol of cytochrome c_1, 4.2 nmol of iron–sulfur cluster, and 140 nmol of phospholipid per milligram of protein. Of the last, 36% is with diphosphatidylglycerol. The crystals are very stable in the cold and show full enzymatic activity when redissolved in aqueous solution. Absorption spectra of the redissolved crystals show a Soret to UV ratio of 0.88 and 1.01 in the oxidized and the reduced forms, respectively.

Ubiquinol–cytochrome *c* reductase (commonly known as cytochrome b–c_1 complex or complex III) is a segment of the mitochondrial respiratory chain that catalyzes antimycin-sensitive electron transfer from ubiquinol to cytochrome *c* (Rieske et al., 1986). The reaction is coupled with the translocation of protons across the mitochondrial inner membrane to generate a proton gradient and membrane potential for ATP synthesis. The purified complex contains 7–11 protein subunits depending on the sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)[1] system used (Yu et al., 1974; Bell & Capaldi, 1976; Gellerfors & Nelson, 1977; Schägger et al., 1986; Gonzales-Halphen et al., 1988: Usui et al., 1990). The complex contains two *b* cytochromes (b_{565} and b_{562}), one *c*-type cytochrome (c_1), one high-potential iron–sulfur cluster (2Fe-2S Rieske center), and a ubiquinone. Although the electron-transfer and proton-translocation mechanisms, as well as the spatial arrangement of this complex in the membrane, have been the subjects of intensive investigation (Mitchell, 1976; Wikström et al., 1981; Papa et al., 1982; Hatefi, 1985; Tsai et al., 1987; Gonzales-Halphen et al., 1988), progress in the structural and mechanistic studies of this ubiquitous complex has been slow due to lack of knowledge concerning its three-dimensional structure. Although microscopic crystals of ubiquinol–cytochrome *c* reductase and the ubiquinol–cytochrome *c* reductase–cytochrome *c* complex were obtained in early 1980 (Ozawa & Shimonura, 1980; Ozawa et al., 1983), little progress on the three-dimensional structure has been made since.

Recently we developed a simple method to obtain large crystals of ubiquinol–cytochrome *c* reductase with well-defined shapes. These crystals are very stable and can be made in large quantities. They are composed of only 10 subunits. When the crystals are redissolved, full ubiquinol–cytochrome *c* reductase activity is observed. Although a preliminary X-ray diffraction study of crystals obtained to date indicates only weak to low resolution,[2] it is expected that improved crystals, suitable for X-ray diffraction studies, will be obtained when the crystallization conditions are refined. Herein we report the conditions for crystallization and the properties of crystalline ubiquinol–cytochrome *c* reductase.

Experimental Procedures

Materials. Horse heart cytochrome *c* (type III), bovine serum albumin, sodium deoxycholate, sodium cholate, poly(ethylene glycol)s, and heptanetriol were obtained from Sigma. Other chemicals were of the highest purity commercially available.

The ubiquinone derivative 2,3-dimethoxy-5-methyl-6-geranyl-1,4-benzoquinol (Q_2H_2) was synthesized in our laboratory as described previously (Yu et al., 1985). Decanoyl-*N*-methylglucamide was synthesized according to the reported method (Hildreth, 1982), and the product was recrystallized three times to increase its purity.

Absorption spectra and activity assays were performed in a Cary spectrophotometer (Model 219) at room temperature. Protein (Lowry et al., 1951), cytochrome *b* (Berden & Slater, 1970), cytochrome c_1 (Yu et al., 1972), Q_{10} (Redfearn, 1966), iron–sulfur cluster (McCurley et al., 1990), and phospholipid (Ames & Dubin, 1960) contents were determined by the reported methods. Analytical SDS–PAGE was carried out either according to Laemmli (1970), using 16% acrylamide gel containing 8 M urea, or according to Schägger et al. (1986).

Preparation and Assays of Ubiquinol–Cytochrome c Reductase. Ubiquinol–cytochrome *c* reductase was prepared and assayed by methods reported previously (Yu & Yu, 1980). It was dissolved in 50 mM Tris-HCl buffer, pH 8.0, containing 1 mM EDTA and 0.66 M sucrose to a protein concentration of 22 mg/mL, frozen, and stored at −80 °C until use. Although the dissolving buffer for the purified reductase contains no detergent, a sufficient amount of bound deoxycholate must be present in the protein because at the indicated protein concentration the solution was clear. The purified reductase contains 8.3 nmol of cytochrome *b*, 4.7 nmol of cytochrome

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[1] Abbreviations: Q_2H_2, 2,3-dimethoxy-5-methyl-6-geranyl-1,4-benzoquinol; SDS, sodium dodecyl sulfate; DMG, decanoyl-*N*-methylglucamide; PEG, poly(ethylene glycol).

[2] J. Deisenhofer, A. J. Weaver, K. G. Ravichandran, W.-H. Yue, L. Yu, and C. A. Yu, unpublished observation (1990).

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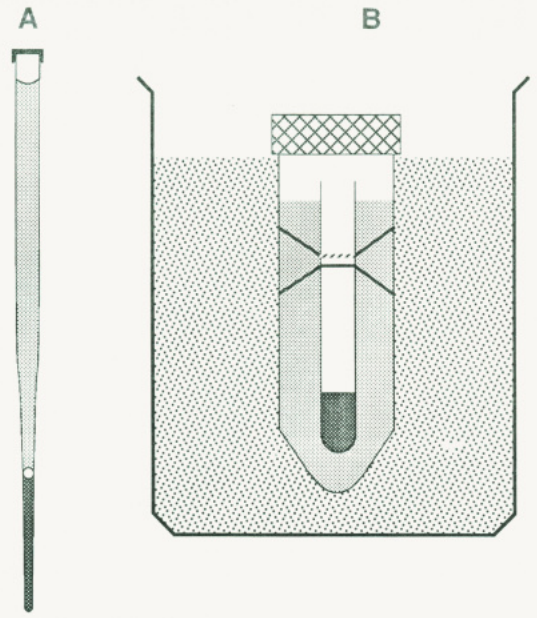

FIGURE 1: Crystallization apparatus: (A) in Pasteur pipet; (B) in test tube.

c_1, 4.1 nmol of iron–sulfur cluster, 3.5 nmol of ubiquinone, and 250 nmol of phospholipid per milligram of protein. The specific activity of the reductase is 11 μmol of cytochrome *c* reduced per minute per nanomole of cytochrome *b* at 23 °C. When the purified reductase was subjected to high-resolution SDS–PAGE, 11 protein bands were observed (Schägger et al., 1986; Gonzales-Halphen et al., 1988).

Results and Discussion

Crystallization of Ubiquinol–Cytochrome c Reductase. Frozen ubiquinol–cytochrome *c* reductase was thawed and mixed with an equal volume of precipitating solution, containing 12% poly(ethylene glycol) 4000 (PEG), 0.5 M sodium chloride, 3.6% heptanetriol, 0.08% DMG, 0.1% sodium azide, 1 mM EDTA, and 50 mM Tris-HCl buffer. The mixture was incubated at 0 °C for 10 min before centrifugation to remove precipitates formed. Fifty-microliter aliquots of clear solution were placed in tip-sealed Pasteur pipets. All air bubbles on top of the solution were removed, and the wall of the pipet above the protein solution was dried before the pipet was filled with the equilibrating (concentrating) mixture. The equilibrating mixture is composed of 1 M sodium chloride, 50 mM Tris-HCl buffer, pH 8, 1 mM EDTA, 0.1% sodium azide, and 22% PEG. Care was taken to ensure that an air space approximately 3 mm in length was formed between the protein solution and the equilibrating solution (see Figure 1A). The pipets were then sealed with parafilm and placed in a specially designed shock-free crystallization chamber. The formation of crystals was checked under a microscope. Good-size crystals formed within 2–4 weeks. During crystallization usually some amorphous precipitates formed, which were mostly removed when mother liquid was drained from the Pasteur pipet.

For preparation of large amounts of crystals, 0.5 mL of protein was mixed with 0.5 mL of precipitating solution; the solution was incubated at 0 °C for 10 min and centrifuged to remove any precipitate formed. The clear protein solution was placed in a 10 × 75 mm culture tube, which was mounted in a 50-mL conical centrifuge tube filled with 40 mL of equilibrating solution in such a way that the level of the equilibrating solution was about 4 mm below the top of test tube. The centrifuge tube was covered with a flat-top screw cap and floated vertically in a water-filled 1-L beaker, which was placed in a Styrofoam box (see Figure 1B). The box was kept in the corner of a cold room (4 °C) for 2 weeks. A large amount of red, thin, square-shaped crystals were formed together with some amorphous precipitates. The latter were removed by a procedure developed for washing crystals of cytochrome $P450_{cam}$ (Yu & Gunsalus, 1970). The crystals and amorphous materials were suspended in the mother liquid by gentle stirring, and the suspension was allowed to settle for a short time (2–3 min). The upper layer was pipetted out and centrifuged in an airfuge for 2 min to remove the amorphous materials. Clear supernatant solution was added to the settled crystals. This process was repeated three times or until no more amorphous materials were presented with the crystals. During the course of our crystallization, various concentrations of protein, poly(ethylene glycol), heptanetriol, and detergents were tried; the conditions given yielded the best results so far.

Properties of Crystalline Ubiquinol–Cytochrome c Reductase. Figure 2 shows crystals of the cytochrome b–c_1 complex at different stages of growth. After a few (5–6) days of incubation (Figure 2A) most of the crystals appear as red, thin octagonal plates that grow into an ellipsoid shape (Figure 2B). A crystal as large as 4 × 2 × 1 mm was obtained. Figure 2C shows crystals grown in the test tube. The dimensions of the thin square crystals are approximately 0.4 × 0.4 × 0.1 mm.

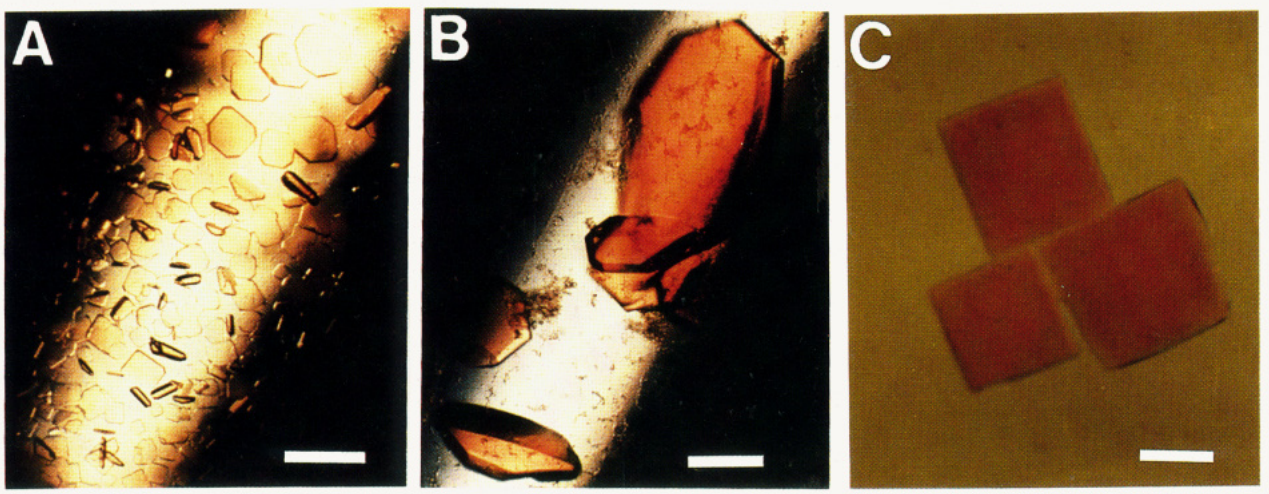

FIGURE 2: Crystals of ubiquinol–cytochrome *c* reductase: (A) early stage in Pasteur pipet; (B) later stage in Pasteur pipet; (C) crystals formed in test tube. The bars represent 1 mm in length in (A) and (B) and 0.2 mm in (C).

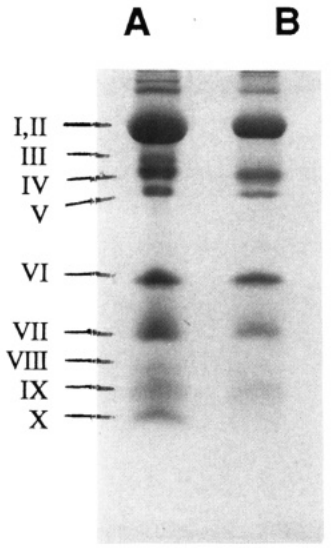

FIGURE 3: SDS–PAGE of crystalline ubiquinol–cytochrome *c* reductase: lane A, crystalline ubquinol–cytochrome *c* reductase; lane B, mother liquid and amorphous precipitates.

Crystals of ubiquinol–cytochrome *c* reductase are very stable; they retain their shape during several months in a cold. These crystals show a high degree of birefringence under polarized light.

Figure 3 compares the SDS–PAGE pattern of the crystalline ubiquinol–cytochrome *c* reductase and the mother liquid. The former shows 10 protein bands corresponding to subunits I–X, and the mother liquid exhibits only 7 protein bands. Cytochrome *b*, "hinge-protein", and band X were missing from the protein complex retained in the mother liquid. The appearance of cytochrome c_1 in the mother liquid suggests that an excess of cytochrome c_1 is present in the purified reductase preparation. This explains why the molar ratio between cytochrome *b* and cytochrome c_1 is often less than 2 in the highly purified preparation. A ratio of 1.7–1.8 is generally obtained. the lower than 2 ratio is often attributed to the partial loss of cytochrome b_{565}, whereas the presence of excess cytochrome c_1 in highly purified ubquinol–cytochrome *c* reductase has not been seriously considered.

Since there are only 10 subunits in the fully active crystal, the smallest (the 11th) subunit with a molecular mass of 6363 daltons may not be an integral part of this complex. Although the amino acid sequence of the smallest protein has been obtained, its function is uncertain. The absence of this protein from crystalline ubquinol–cytochrome *c* reductase strongly suggests that the reported 11th subunit is a contaminant rather than an intrinsic component of the complex.

The presence of excess cytochrome c_1 in the purified complex is also evident from the spectral properties of the crystalline complex. Figure 4 shows the absorption spectra of crystalline ubquinol–cytochrome *c* reductase dissolved in aqueous solution. All the absorption maxima are very similar to those reported for purified ubquinol–cytochrome *c* reductase. However, the ratio of the Soret band to UV is quite different. The Soret to UV ratios are 0.88 and 1.01 for the oxidized and sodium dithionite reduced forms of crystalline ubquinol–cytochrome *c* reductase, respectively. These ratios are 1.03 and 1.33 for the oxidized and reduced purified uncrystallized complex. From the difference spectra of ascorbate-reduced versus oxidized forms and the difference spectra of dithionite-reduced versus ascorbate-reduced forms, with reported molar extinction coefficients, the ratio of cytochromes *b* to c_1 was calculated to be 2:1 in the crystalline complex.

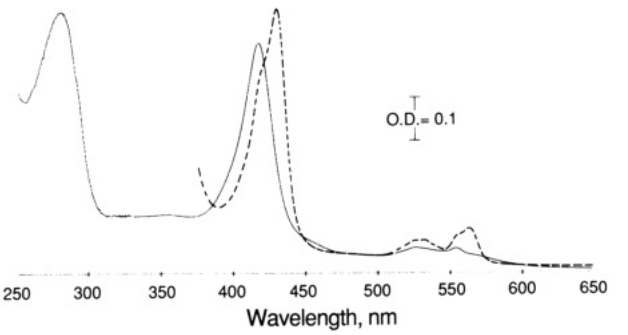

FIGURE 4: Absorption spectra of crystalline ubiquinol–cytochrome *c* reductase. Crystals of ubquinol–cytochrome *c* reductase were dissolved in 50 mM Tris-HCl buffer, pH 8.0, containing 45 mM DMG and 0.2 M ammonium sulfate. The spectra were taken at room temperature. The solid and broken curves represent oxidized and sodium dithionite reduced forms, respectively.

Table I: Comparison of the Phospholipid Composition of Purified and Crystalline Ubiquinol–Cytochrome *c* Reductase

	distribution of phospholipids (%)	
phospholipid	crystals	purified
PC	25.7	34.8
PE	20.5	33.5
DPG	35.6	15.2
others	18.1	16.2

When crystalline ubquinol–cytochrome *c* reductase is redissolved in 50 mM Tris-HCl buffer, pH 8.0, containing 45 mM DMG and 0.2 M ammonium sulfate and assayed, the specific activity, based on cytochrome *b*, is about the same as that observed before crystallization. This suggests that no inactivation of the reductase occurred during crystallization and redissolution.

Chemical analysis shows that the crystalline ubquinol–cytochrome *c* reductase contains 8.4 nmol of cytochrome *b* and 4.2 nmol of cytochrome c_1 per milligram of protein. In addition to the slight difference in the ratios of cytochromes between the crystalline and purified ubquinol–cytochrome *c* reductase, the phospholipid and ubiquinone contents and composition are substantially different. The crystalline complex contains less phospholipid (140 nmol/mg of protein) and ubiquinone (2.5 nmol/mg of protein) than the purified enzyme complex (250 nmol of phospholipid and 3.4 nmol of ubiquinone per milligram of protein). Table I compares the composition of the major phospholipids in crystalline and purified ubiquinol–cytochrome *c* reductase. The crystalline complex has relatively more diphosphatidylglycerol and less phosphatidylcholine and -ethanolamine than does the purified complex.

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Human and *Escherichia coli* Cyclophilins: Sensitivity to Inhibition by the Immunosuppressant Cyclosporin A Correlates with a Specific Tryptophan Residue†

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ABSTRACT: The human T-cell protein cyclophilin shows high affinity for and is the proposed target of the major immunosuppressant drug cyclosporin A (CsA). Cyclophilin also has peptidyl prolyl cis-trans isomerase activity that is inhibited by CsA with an IC_{50} of 6 nM, while by contrast a homologous PPIase from *Escherichia coli* has been found to be much less sensitive to CsA, shown here to be 500-fold less potent at an IC_{50} of 3000 nM. This *E. coli* rotamase lacks the single highly conserved tryptophan residue of eukaryotic cyclophilins, and we show here that mutation of the natural F112 to W112 enhances *E. coli* rotamase susceptibility to CsA inhibition by 23-fold. Correspondingly, the human W121 mutations to F121 or A121 yield cyclophilins with 75- and 200-fold decreased sensitivity to CsA, while k_{cat}/K_m values of rotamase activity in a tetrapeptide assay drop only 2- and 13-fold, respectively. This complementary gain and loss of CsA sensitivity to mutation to or from tryptophan validate the indole side chain as a major determinant in immunosuppressant drug recognition and the separation of PPIase catalytic efficiency from CsA affinity.

The immunosuppressant drug cyclosporin A (CsA),[1] a central therapeutic agent in organ transplant medicine (Borel et al., 1989; Showstack et al., 1989; Starzl et al., 1989), binds with high affinity [IC_{50} value of 5-200 nM (Handschumacher et al., 1984; Harding et al., 1986)] to a cytosolic 18-kDa protein, cyclophilin (CyP), in human T cells (Handschumacher et al., 1984) and blocks T-cell activation by selective blockade of transcriptional activation of T-cell genes for synthesis of cytokines IL-2, IL-4, and GMCSF (Elliott et al., 1984; Kronke et al., 1984) by specifically inhibiting the function of such transcriptional activators as the nuclear factor of activated T cells (Emmel et al., 1989). In 1988 cyclophilin was further identified as a peptidyl prolyl cis-trans isomerase (PPIase) or rotamase with catalytic activity at accelerating rates of interconversion of cis and trans rotamers of Xaa-Pro amide bonds in small peptides and proteins (Fischer et al., 1989; Takahashi et al., 1989). Much effort is underway to determine possible physiologic substrates for this potential "foldase" activity and to determine if the potent inhibition of such enzymatic activity by CsA is the relevant biological readout in immunosuppression.

In addition to cloning, expressing, and purifying wild-type and mutant human T-cell cyclophilins (Liu et al., 1990), we have recently expressed and purified an *Escherichia coli*

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[1] Abbreviations: wtHCyP, wild-type human cyclophilin; wtECyP, wild-type *Escherichia coli* cyclophilin; HCWF, human cyclophilin W121 to F121 mutant; HCWA, human cyclophilin W121 to A121 mutant; ECFW, *Escherichia coli* cyclophilin F112 to W112 mutant; CsA, cyclosporin A.

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