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Preliminary molecular characterization and crystallization of mitochondrial respiratory complex II from porcine heart

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The mitochondrial respiratory complex II, or succinate:ubiquinone oxidoreductase, is an integral membrane protein complex in both the tricarboxylic acid cycle (Krebs cycle) and aerobic respiration. The gene sequences of each complex II subunit were measured by RT-PCR. N-terminal sequencing work was performed to identify the mitochondrial targeting signal peptide of each subunit. Complex II was extracted from porcine heart and purified by the ammonium sulfate precipitation method. The sample was solubilized by 0.5% (w/v) sugar detergent n-decyl-β-D-maltoside, stabilized by 200 mM sucrose, and crystallized with 5% (w/v) poly(ethylene glycol) 4000. Important factors for the extraction, purification and crystallization of mitochondrial respiratory complex II are discussed.

Mitochondria are cellular organelles of prokaryotic origin that are found in almost all eukaryotic cells. The mitochondrial respiratory system, consisting of five membrane protein complexes (I–V), produces most of the energy in eukaryotic cells by a process called oxidative phosphorylation [1]. Electrons are passed along a series of respiratory enzyme complexes located in the inner mitochondrial membrane, and the energy released by this electron transfer is used to pump protons across the inner membrane.

The electron transport chain comprises five respiratory enzyme complexes arranged in a specific orientation. Complex I (NADH:ubiquinone oxidoreductase), the largest and most complicated of these, is the main point of entry into the respiratory chain for electrons. The mammalian complex I contains nearly 50 different subunits in an unknown stoichiometry [2–5]. Complex II (succinate:ubiquinone oxidoreductase) is a component of the tricarboxylic acid cycle (Krebs cycle), and participates in the electron transport chain by transferring electrons from succinate to the ubiquinone pool. Complex III (cytochrome bc_1 complex) delivers electrons from ubiquinone to cytochrome *c*. It couples this redox reaction to the generation of a proton gradient across the membrane by a mechanism known as the Q cycle [1,6]. Complex IV (cytochrome *c* oxidase complex), as the terminal enzyme of biological oxidation, catalyzes the reduction of O_2 to H_2O at the

Abbreviations
Ip, iron-sulfur protein; Fp, flavoprotein.

site involving heme a_3 and Cu_B by means of protons extracted from the matrix side of the mitochondrial inner membrane and electrons from the cytochrome *c* complex, in a reaction that is coupled with proton pumping [7,8]. The passage of electrons along the electron transport chain in mitochondria generates an electron-chemical proton potential gradient across the inner membrane, and this gradient is harnessed by ATP synthase to make ATP from ADP and P_i. Complex V is the ATP synthase [9,10]. The structures of mammalian complex III [11,12], complex IV [13,14] and complex V [15] have been determined previously.

Mitochondrial complex II [16–19], also known as mitochondrial succinate:ubiquinone oxidoreductase (EC 1.3.5.1), is a key membrane complex in the tricarboxylic acid cycle (Krebs cycle) that catalyzes the oxidation of succinate to fumarate in the mitochondrial matrix as succinate dehydrogenase. Succinate oxidation is coupled to reduction of ubiquinone to ubiquinol at the mitochondrial inner membrane as one part of the electron transport chain. Electrons are transferred from succinate to ubiquinone through the buried prosthetic groups FAD, [2Fe–2S] cluster, [4Fe–4S] cluster [3Fe–4S] cluster and heme, which form an integral part of the complex [18].

The crystal structure of mitochondrial respiratory complex II has been determined at 2.4 Å from porcine heart by our group [20] and at 2.1 Å from chicken heart by Huang *et al.* [21]. It contains four nonidentical subunits: the FAD-binding protein or flavoprotein (Fp, 68 kDa, 622 amino acids), the iron–sulfur protein (Ip, 29 kDa, 252 amino acids), and two membrane-anchor proteins (CybL, 15 kDa, 140 amino acids, and CybS, 11 kDa, 103 amino acids) with a total of six transmembrane helices. Here we provide a detailed report of the preparation, gene sequencing, N-terminal sequencing and crystallization of mitochondrial complex II from porcine heart.

Results and Discussion

The mitochondrial respiratory complex II preparation was extracted and purified from porcine heart. The major purification protocol is multiple fractionated ammonium sulfate precipitation. The final sample used for crystallization tests yielded obvious activity of both complex II and complex III, the purity of complex II being about 60%. However, SDS/PAGE analysis showed the crystal we obtained to be mitochondrial complex II (Fig. 1). Our experience showed that a further purified preparation of complex II could not be crystallized. Instead of further purification (e.g. gel filtration), the mixture was crystallized directly. It may be the case that some indispensable cofactors (i.e. phospholipids) for crystallization were integrated with the four subunits of complex II, and might have been discarded following further purification.

Initial screening using MembFac (Hampton Research, Aliso Viejo, CA, USA) indicated that poly(ethylene glycol) 4000 would be an ideal precipitate for crystallization. After hard screening for detergents and additives, the crystals grew and finally diffracted to 2.4 Å using synchrotron radiation. With preparation and crystallization as described below, good-quality diffracting crystals were obtained with good reproducibility. The mitochondrial complex II from porcine heart was crystallized in an orthorhombic crystal form (space group $P2_12_12_1$) with one complex per asymmetric unit. The crystals, colored red (Fig. 2), grew in the sediment where the concentration of protein was suitable. The addition of detergents played an important role in the crystallization process. After screening with the Detergent Screens kit (Hampton Research), n-decyl-β-D-maltoside was selected as the detergent for crystallization, and n-nonyl-β-D-maltoside as the additive. Another important factor for crystallization of complex II is sucrose. Sucrose was used as a stabilizer for sample storage. During crystallization experiments, complex II would quickly precipitate and could not be crystallized without sucrose. The optimized sucrose concentration for crystallization is about 200 mM. Sucrose serves as an effective amphimictic small molecule to modulate the interaction between membrane protein and detergent.

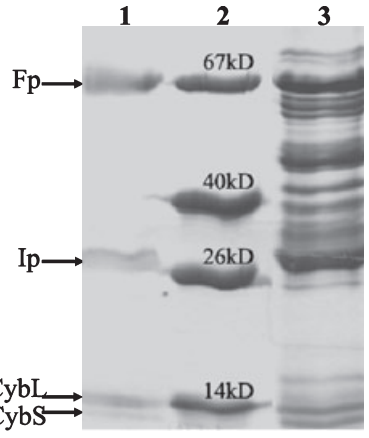

Fig. 1. SDS-PAGE analysis of the crystallization preparation and the crystal of complex II. Lane 1, crystals of complex II. Lane 2, molecular weight markers. Lane 3, crystallization preparation of complex II. The four subunits of complex II are labeled on the left, and the positions of molecular weight standards are indicated on lane 2.

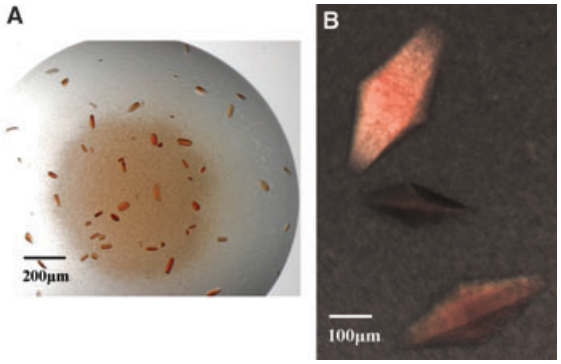

Fig. 2. The complex II crystals. (A) and (B) show crystals in two different wells. The red crystals grew in the sediment where the concentration of protein was suitable.

Table 1. Data collection statistics. SAD, single-wavelength anomalous diffraction.

	SAD	Native
Wavelength (Å)	1.74101	1.0322
Resolution limit (Å)	50.0–3.0 (3.11–3.00)	50.0–2.4 (2.44–2.40)
Total reflections	150 760	546 106
Unique reflections	30 789	60 175
Completeness	85.9 (40.4)	85.1 (40.5)
R_{merge}[a]	10.3 (41.7)	12.5 (50.1)
$<I/\sigma(I)>$	13.0 (2.0)	17.9 (2.9)
Redundancy	4.9 (4.5)	9.1 (3.6)

[a] $R_{merge} = \Sigma_h\Sigma_j | I_{hj} - <I_h> |/\Sigma_h\Sigma_j <I_h>$, where $<I_h>$ is the mean of the observations I_{hj} of reflection h, and I_{hj} is the jth observation of each reflection.

Crystals then appeared in the crystallization mixture and could diffract to 3.5 Å resolution in-house. The typical diffraction of a complex II crystal was anisotropic: it could diffract to 3.5 Å resolution in one direction, whereas it could only diffract to 4.5 Å resolution in another (Fig. 3). One set of single-wavelength anomalous diffraction data ($\lambda = 1.74101$ Å) and another high-resolution native dataset ($\lambda = 1.0322$ Å) were collected at the Advanced Photon Source. The unit cell parameters of the porcine heart complex II crystals are $a = 70.2$ Å, $b = 83.5$ Å, $c = 293.9$ Å, and $\alpha = \beta = \gamma = 90°$, with space group $P2_12_12_1$. It is estimated that there is one molecule per asymmetric unit with a Matthews' coefficient (V_M) of 3.45 $Å^3 \cdot Da^{-1}$ and a solvent content of 64%. The data were processed by HKL2000 [22], and statistics are summarized in Table 1.

As the porcine genome sequence had not been fully released at the time of our study, we measured the accurate mature amino acid sequences of the four complex II subunits by RT-PCR and N-terminal sequencing. The nucleotide sequences of the four porcine complex II subunits were sequenced correctly (Fig. 4), with the exception of the 5′-end and 3′-end primer sequences from human mitochondrial complex II (see Experimental procedures). However, when the recombined T-vector (Fp) was sequenced, we found that the length of the cloned Fp was longer than expected. Further analysis showed that the designed forward primer did not appear in the final PCR product, the reverse primer performing the role instead (supplementary Figs S1 and S2).

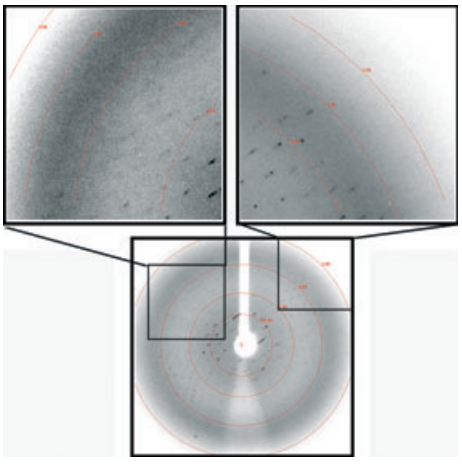

Fig. 3. A typical X-ray diffraction pattern from a native mitochondrial complex II crystal.

N-terminal sequencing experiments were used to identify the mitochondrial targeting signal peptide. A clear signal indicated that the first five residues of the mature Fp N-terminus are SSAKV. Compared with the full measured sequence, the Fp signal peptide was identified as follows: MSGVRAVSRLLRARRLALTWAQPAASPIGARSFHFTVDGNKR. Similarly, the first five residues of the mature Ip N-terminus are AQTAA, and its signal peptide is MAAVVALSLKRWFPATTLGGACLQACRG. Owing to the low transfer efficiency of the two complex II transmembrane subunits, we could not obtain a significant signal from N-terminal sequencing experiments. However, by comparing their full amino acid sequences with the mature sequence of known bovine heart mitochondrial respiratory complex II, we could still identify the signal peptides for porcine CybL and CybS as follows: MAALLLRHVGRHCLRA HLSPQLCIRNAVP (CybL) and MAVLWRLSAACGPRGGGALVLRT SVVRPAHV SAFLQDRHTPGWCGVQHIHLSPSHQ (CybS). Finally, the mature protein sequences of each porcine complex II subunit were obtained, and flavoprotein, iron-sulfur protein, CybL and CybS were shown to contain 622, 252, 140 and 103 residues, respectively.

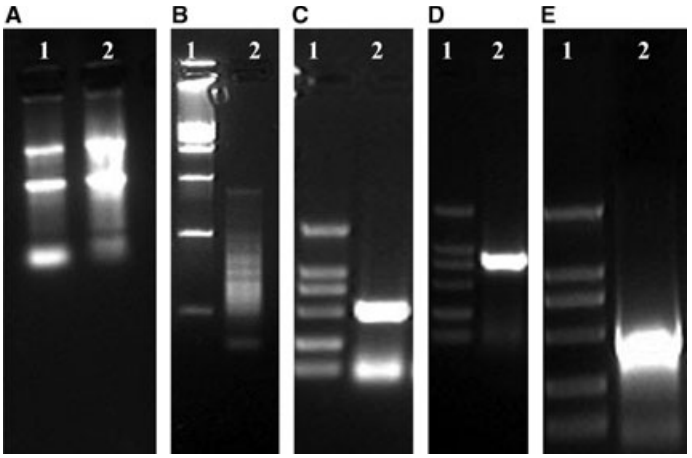

Fig. 4. Sequencing of porcine heart mitochondrial complex II. Total RNA was extracted and purified from the fresh porcine heart, and genes of four complex II subunits were cloned by RT-PCR against the extracted RNA. (A) Lanes 1 and 2, total RNA extract. (B) Lane 1, nucleotide acid marker DL15000 (TaKaRa); Lane 2, FP subunit. (C) Lane 1, nucleotide acid marker DL2000 (TaKaRa); Lane 2, IP subunit. (D) Lane 1, nucleotide acid marker DL2000 (TaKaRa); Lane 2, CybL subunit. (E) Lane 1, nucleotide acid marker DL2000 (TaKaRa); Lane 2, CybS subunit. The FP band is not very clear in the agarose gel, and the longest band was used to run another PCR reaction for amplification.

Experimental procedures

Extraction, purification and crystallization of mitochondrial complex II from porcine heart

The normal procedure for extraction, purification and crystallization has been previously described in brief [20]. A more detailed protocol is given below with specific emphasis.

The extraction and purification protocol comprises four major steps. The first step involved extraction of the porcine heart mitochondrial membrane species from fresh heart muscle by differential centrifugation (3000 ***g***, rotor R12A3, Hitachi Himac CR22G centrifuge; 18 000 ***g***, rotor R20A2, Hitachi Himac CR22G centrifuge; 120 000 ***g***, rotor Ti70: Beckman L8-70M ultracentrifuge [20]). Second, the mitochondrial membranes (20 $mg \cdot mL^{-1}$) were solubilized by a specific concentration 0.9% (w/v) of the detergent sodium cholate. Mitochondrial respiratory complex IV was initially separated from the other complexes. A two-step ammonium sulfate precipitation was used to separate mitochondrial respiratory complex IV (35% saturation) and the remaining complexes (55% saturation). Third, the remainder of the precipitated complexes were resolubilized by a specific concentration, 0.45% (w/v), of sodium cholate and stored overnight. The unresolved contaminants were removed after centrifugation (40 000 ***g***, rotor Ti70, Beckman L8-70M ultracentrifuge [20]). Finally, fractional ammonium sulfate precipitation was used to yield a special fraction with the highest complex II activity at 40–45% saturation. The dialysis method was used to remove ammonium sulfate completely, and 400 mM sucrose was used to stabilize the final sample. The detergent n-decyl-β-D-maltoside (Anatrace, Maumee, OH, USA) at a final concentration of 0.5% (w/v) was used to dissolve the prepared complex II sample for hanging drop crystallization. Finally, complex II was buffered in 25 mM Hepes (pH 7.2), 200 mM sucrose, 100 mM NaCl and 0.5 mM EDTA, and crystallized in 25 mM Hepes (pH 7.2), 5% (w/v) poly(ethylene glycol) 4000, 3% (w/v) 1,6-hexanediol, 100 mM NaCl, and 10 mM $CaCl_2$. Adding n-nonyl-β-D-maltoside (Anatrace) to the crystallization mixture to a final concentration of 1.7 mM produced notably better crystals.

In our experience, the sample/detergent ratio, especially the sodium cholate concentration in different steps, was important for successful extraction and purification. Further differential steps during fractional ammonium sulfate precipitation would yield a more homogeneous sample. The sample from which we could obtain the best crystal appeared at around 43.5% saturation precipitation. The optimal sample concentration for crystallization was 25 $mg \cdot mL^{-1}$.

In addition to X-ray diffraction experiments, several crystals were selected from the crystallization drop, and dissolved in the crystallization buffer, and the sample was then run on an SDS/PAGE gel. Four clear bands appeared in the SDS/PAGE gel, which were consistent with the four subunits of complex II. These four bands were further analyzed by mass fingerprinting spectrometry (data not shown) and confirmed to be the subunits of complex II.

Primer design, reverse transcription and the PCR

During this study, the porcine genome sequence had not been fully released. According to the requirements of structure determination, we had to measure the gene sequences and full amino acid sequences of the porcine complex II subunits. An abundance of gene sequences of mammalian complex II in the GenBank database was helpful for the design of proper primers for our sequencing work. The sequence alignment analysis (see supplementary Figs S1 and S2) against the gene sequences of available mammalian complex II subunits indicated that the homologies of all four mammalian complex II subunits were high enough and that we could directly use the human mitochondrial complex II gene sequences as templates to design the primers for porcine complex II.

We designed four pairs of primers for four subunits, respectively, as follows (F, forward primer; R, reverse primer): F-fp, 5′-ATGTCGGGGGTCCGGGGCCTGTCG CGGC-3′; R-fp, 5′-TCAGTAGGAGCGAATGGCTGGC GGGACG-3′; F-ip, 5′-ATGGCGGCGGTGGTCGCACT CTCCTTGAG-3′; R-ip, 5′-TTAAACTGAAGCTTTCTTC TCCTTATAGG-3′; F-CybL, 5′-ATGGCTGCGCTGTTG CTGAGACACGTTG-3′; R-CybL, 5′-TCACATGGCTGC CAGCCCCATAGAGGAC-3′; F-CybS, 5′-ATGGCGGTT CTCTGGAGGCTGAGTGCCG-3′; R-CybS, 5′-CAGAGC TTCCACAGCATGGCAACAGCT-3′.

Fresh porcine heart from the slaughterhouse was sheared into pieces and immediately immersed in liquid nitrogen. One hundred milligrams of heart tissue was picked up and milled into powder in liquid nitrogen, which was then used to extract and purify total RNA using the Trizol reagent kit (Life Technologies, New York, NY, USA) and the RNeasy kit (Qiagen, Hilden,Germany), respectively.

Purified total RNA was used to run a reverse transcription reaction to synthesize the complementary cDNA by the AMV first-strand cDNA synthesis system (BBI, Toronto, Canada) against the reverse primers of four porcine complex II subunits. PCRs were then run against these complementary cDNAs with respective forward and reverse primers. The products of PCR were analyzed and purified by agarose gel electrophoresis. With the Spin Column DNA Gel Extraction Kit (BBI), the pMD18-T Vector Kit (TaKaRa, Shiga, Japan) and the DNA Ligation Kit (TaKaRa), the RT-PCR products were extracted from the agarose gels and cloned into the T-vector. Finally, genes of the four porcine heart mitochondrial complex II subunits were sequenced commercially from these recombined T-vectors.

N-terminal sequence for signal peptide

Five or six large crystals were selected from the crystallization drop and dissolved in a crystallization buffer containing 1% SDS, which was used to run SDS/PAGE with a gradient-separating gel (10–15%). The resulting gel was soaked in western blot Tris/glycine transfer buffer and assembled into the transfer membrane sandwich against the nitrocellulose membrane. All SDS/PAGE bands in the gel were then transferred onto nitrocellulose membrane by electrophoresis for 2 h at 40 V. The membrane was dyed with Coomassie blue G250, and the four bands corresponding to subunits of porcine complex II were cut out and N-terminal sequenced by the Edman degradation method and amino acid analyzer in the Department of Biology, Peking University.

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Supplementary material

The following supplementary material is available online:

Fig. S1. Multiple sequence alignment of mammalian mitochondrial respiratory complex II gene sequences located at the 5′-end and 3′-end.

Fig. S2. Measured gene sequences and amino acid sequences of porcine mitochondrial respiratory complex II.

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