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Isolation of yeast complex IV in native lipid nanodiscs

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ABSTRACT

We used the amphipathic styrene maleic acid (SMA) co-polymer to extract cytochrome *c* oxidase (CytcO) in its native lipid environment from *S. cerevisiae* mitochondria. Native nanodiscs containing one CytcO per disc were purified using affinity chromatography. The longest cross-sections of the native nanodiscs were 11 nm × 14 nm. Based on this size we estimated that each CytcO was surrounded by ~100 phospholipids. The native nanodiscs contained the same major phospholipids as those found in the mitochondrial inner membrane. Even though CytcO forms a supercomplex with cytochrome bc_1 in the mitochondrial membrane, cyt. bc_1 was not found in the native nanodiscs. Yet, the loosely-bound Respiratory SuperComplex factors were found to associate with the isolated CytcO. The native nanodiscs displayed an O_2-reduction activity of ~130 electrons CytcO^{-1} s^{-1} and the kinetics of the reaction of the fully reduced CytcO with O_2 was essentially the same as that observed with CytcO in mitochondrial membranes. The kinetics of CO-ligand binding to the CytcO catalytic site was similar in the native nanodiscs and the mitochondrial membranes. We also found that excess SMA reversibly inhibited the catalytic activity of the mitochondrial CytcO, presumably by interfering with cyt. *c* binding. These data point to the importance of removing excess SMA after extraction of the membrane protein. Taken together, our data shows the high potential of using SMA-extracted CytcO for functional and structural studies.

1. Introduction

Functional studies of membrane proteins typically require isolation of the protein of interest at a sufficiently high concentration. The most widely used approach is solubilization in detergents. In most cases it is possible to find a detergent that fulfils the criteria for a sufficiently pure, structurally intact and, when relevant, active protein. However, different detergents may be optimal for different experimental methods and changes in function may be introduced during the removal of the native lipids. In addition, small and/or loosely bound protein subunits (components) may dissociate along with the native lipids. To restore more native-like conditions, amphipathic polymers (known as "amphipols") may be used to replace the detergent [1]. An even more native-like environment can be re-created by reconstitution of the detergent-purified protein into lipid vesicles [2] or in lipid nanodiscs using a surrounding membrane scaffolding protein [3], which allows functional studies under near-native conditions. However, all these methods involve isolation of the membrane protein of interest using detergent as an intermediate step. Recently, a new technique has emerged that allows extraction and isolation of the membrane protein of interest together with a disc of native lipids [4] using amphipathic styrene maleic acid (SMA) co-polymer (for a recent review, see [5]) without the use of any detergent. This approach has been used to isolate a number of different membrane-bound proteins [6–18], including proton pumps, photosynthetic systems, and ion channels. The procedure has also been used to isolate a secondary transporter from *E. coli* for studies using negative stain electron microscopy [19]. The isolated protein-lipid native nanodiscs (we use here the nomenclature suggested by Dörr et al. [5]) are surrounded by a ring of the SMA co-polymer with a thickness of ~1 nm [12]. The total diameter of the native nanodiscs

Abbreviations: Bis-Tris, 2,2-Bis(hydroxymethyl)-2,2′,2″-nitrilotriethanol; cyt. *c*, cytochrome *c*; CytcO, cytochrome *c* oxidase; DDM, *n*-dodecyl β-D-maltoside; EDTA, ethylenediaminetetraacetic acid; HEPES, 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid; MES, 2-(N-Morpholino)ethanesulfonic acid; PMS, phenazine methosulfate; SDS, sodium dodecyl sulfate; SMA, poly(styrene-*alt*-maleic acid); TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine; Tris, Tris(hydroxymethyl)aminomethane.

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has been found in the range of 10–24 nm, indicating that there is a large degree of variability in the size of the isolated systems and the lipid-protein ratio. In one recent study [8], the SMA co-polymer was shown to disrupt the *Saccharomyces* (*S.*) *cerevisiae* inner mitochondrial membrane, resulting in the formation of native nanodiscs. The extract was shown to display O_2-reduction activity, catalyzed by the Cyt*c*O that was present as one of many components in the mixture of all proteins that were extracted from the native inner mitochondrial membrane.

The mitochondrial Cyt*c*O is a multisubunit membrane-bound enzyme located in the cristae of the inner mitochondrial membrane. The *S. cerevisiae* Cyt*c*O is composed of 11 subunits where subunits I-III form the functionally active core of the enzyme. The Cyt*c*O catalyzes oxidation of water-soluble cyt. *c* and reduction of dioxygen to water in the last step of the respiratory chain. Electrons are donated from the more positive (*p*) side, and protons used in the O_2-reduction are taken up from the more negative (*n*) side, resulting in a charge separation across the membrane. Furthermore, the process is linked to proton pumping across the membrane. The *S. cerevisiae* Cyt*c*O binds four redox-active cofactors. Electrons from cyt. *c* are first donated to copper A (Cu_A) and are then transferred consecutively to heme *a* and the catalytic site, which consists of heme a_3 and copper B (Cu_B). When O_2 reacts with the four electron-reduced Cyt*c*O, electrons are transferred in a number of distinct steps forming intermediates that have been identified and characterized in spectroscopic studies (for review, see [20–22]). First, O_2 binds to the reduced heme a_3 resulting in formation of state **A**. Next, an electron is transferred from heme *a* to the catalytic site, the O-O bond is broken, and protons are transferred internally within the catalytic site resulting in formation of a ferryl state that, for historical reasons, is called peroxy (**P**). Formation of this **P** state triggers proton uptake from the *n*-side solution to the catalytic site forming the ferryl state, **F**, which is also linked to fractional electron transfer from Cu_A to heme *a*. In the final step of the reaction the electron from the Cu_A-heme *a* equilibrium is transferred to the catalytic site where water is formed, leaving the Cyt*c*O in the oxidized state (**O**).

Results from recent studies showed that two additional proteins, called the respiratory supercomplex factors (Rcf) associate with the *S. cerevisiae* Cyt*c*O in the native membranes [23–25]. These factors were suggested to facilitate interactions with the cytochrome (cyt.) bc_1 complex in the cyt. bc_1-Cyt*c*O supercomplex. The Rcfs are presumably loosely bound to the Cyt*c*O and can also associate with the cyt. bc_1 complex. In a recent study we showed that removal of the regulatory Rcf1 protein results in significant changes in the reaction of the reduced Cyt*c*O with O_2 [26] and it is therefore important to make sure that this protein co-purifies with the Cyt*c*O upon isolation of the enzyme. When using "weak" detergents such as e.g. digitonin, the *S. cerevisiae* Cyt*c*O typically co-purifies with the cyt. bc_1 complex in a supercomplex (see above). This supercomplex can be dissociated using detergents such as DDM, but use of this detergent may also results in dissociation of weakly bound components (subunits) such as the Rcf1 polypeptide. Consequently, future studies of the purified Cyt*c*O would benefit from development of new methods to isolate the *S. cerevisiae* Cyt*c*O along with all its regulatory factors, but without cyt. bc_1.

Here, we used the SMA co-polymer to isolate native nanodiscs containing a single Cyt*c*O from *S. cerevisiae* in each disc. The isolated Cyt*c*O contained both Rcf1 and Rcf2, was fully active, and the kinetics of ligand binding and reaction of the reduced Cyt*c*O with O_2 were similar to those observed with intact membranes. Another advantage of the new approach to isolate Cyt*c*O is that the native nanodiscs contained all the major lipid components typically found in the yeast inner mitochondrial membrane. Consequently, the method allows for investigation of Cyt*c*O function in an environment that is similar to that in the native membranes. Our data also show that excess SMA inhibits the Cyt*c*O, which points to the importance of removing excess of the polymer after purification.

2. Results and discussion

2.1. Isolation of the native nanodiscs

The *S. cerevisiae* mitochondria containing His-tagged (7-His on Cox13) Cyt*c*O were treated with 2% SMA (styrene:maleic acid at a ratio of 3:1) and the Cyt*c*O-containing native nanodiscs were isolated via Ni-NTA affinity chromatography as described in Materials and Methods. As shown in **Fig. S1 A** similar SDS-PAGE patterns were observed for the Cyt*c*O-containing native nanodiscs as for the detergent-purified Cyt*c*O. The three bands corresponding to molecular weights of 40 kDa, 33 kDa and 22 kDa [27] represent the core subunits I, II and III, respectively. The remaining bands are also very similar for the two samples. The bands above 55 kDa represent contaminations or only partly dissociated Cyt*c*O complexes, seen both with the SMA native nanodiscs and with the detergent-purified Cyt*c*O. The presence of the loosely-bound Rcf1 and Rcf2 proteins in the Cyt*c*O-native nanodiscs was shown using Western blotting of the SDS-PAGE gels followed by immunostaining with specific *anti*-Rcf1 and Rcf2 antibodies, respectively **(Fig. S1B,C)**. The bright (light) band on the blot area of **Fig. S1B** indicates the position of Rcf1, which is visualized by chemiluminescence. In the case of Rcf2, some cross-reactivity of the *anti*-Rcf2 antibodies was observed for bands with lower molecular weight, where a band corresponding to Rcf2 was positioned at 25 kDa (**Fig. S1C).**

2.2. Size and shape of the native nanodiscs

Negative stain Electron Microscopy (EM) was used to characterize the size and shape of the SMA-Cyt*c*O native nanodiscs (Fig. 1). Fig. 1A shows that the majority of purified SMA-Cyt*c*O native nanodiscs distribute in a monodisperse fashion on the grid, a requirement for accurate analysis of particles by EM classification and reconstruction. A small portion of particles form large aggregates, which might be caused by sporadic adverse effect of the uranyl formate stain, and these were excluded from 2D image analyses. Reference-free 2D classification of the particles shows multiple views of the SMA-Cyt*c*O native nanodiscs (Fig. 1B) that are similar in size and shape to projections of the crystal structure of Cyt*c*O from bovine heart (PDB: 3X2Q) without bound lipids (Fig. 1C). As shown in Fig. 1B and C, the Cyt*c*O accounts for the majority of the mass in the SMA-Cyt*c*O native nanodiscs, with surrounding lipids contributing to a slight increase in particle diameter relative to the forward projections. A histogram of particle diameters (Fig. 1D) shows a bimodal distribution, arising from the disparate dimensions observed for the particles depending on their orientation on the grid. On the basis of the analysis outlined above, we conclude that the native nanodiscs contained one Cyt*c*O per disc and that the approximate dimensions of the discs were 11 nm × 14 nm.

Fig. S2 shows a size-exclusion chromatography (SEC) analysis of the SMA-Cyt*c*O native nanodiscs. From the position of the major cytochrome-containing peak, the size of the particles was estimated to be in the range equivalent to 600–700 kDa, i.e. a relatively broad band. In order to investigate whether this distribution represents a dynamic equilibrium or a distribution of particles with different shapes/molecular weights, we collected fractions around 400 kDa and around 700 kDa, respectively, and re-run these fractions. In both cases we obtained bands corresponding to either 400 kDa or 700 kDa, which shows that there is a distinct distribution of particles in the SEC that is not distinguishable in the EM analysis.

Next, we compared the molecular weight of the native nanodiscs identified in the SEC experiment to the size obtained from the EM analysis. Assuming an average size of 650 kDa (from the SEC experiment) and an average protein (including the lipids) density of 0.8 Da/Å^3, the volume of the isolated particles is ~800 nm^3. Assuming spherical particles they would have a diameter of ~12 nm, which approximately corresponds to the particle size as seen using EM.