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Reaction of *S. cerevisiae* mitochondria with ligands: Kinetics of CO and O_2 binding to flavohemoglobin and cytochrome *c* oxidase

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Kinetic methods used to investigate electron and proton transfer within cytochrome *c* oxidase (Cyt*c*O) are often based on the use of light to dissociate small ligands, such as CO, thereby initiating the reaction. Studies of intact mitochondria using these methods require identification of proteins that may bind CO and determination of the ligand-binding kinetics. In the present study we have investigated the kinetics of CO-ligand binding to *S. cerevisiae* mitochondria and cellular extracts. The data indicate that CO binds to two proteins, Cyt*c*O and a (yeast) flavohemoglobin (yHb). The latter has been shown previously to reside in both the cell cytosol and the mitochondrial matrix. Here, we found that yHb resides also in the intermembrane space and binds CO in its reduced state. As observed previously, we found that the yHb population in the mitochondrial matrix binds CO, but only after removal of the inner membrane. The mitochondrial yHb (in both the intermembrane space and the matrix) recombines with CO with $\tau \cong 270$ ms, which is significantly slower than observed with the cytosolic yHb (main component $\tau \cong 1.3$ ms). The data indicate that the yHb populations in the different cell compartments differ in structure.

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1. Introduction

The electron-transfer chain in the inner membrane of mitochondria maintains a proton electrochemical gradient that is used, for example, to drive formation of ATP and for transport processes. In *Saccharomyces* (*S.*) *cerevisiae* mitochondria, electrons are transferred via the quinone pool to Complex III (ubiquinol-cytochrome *c* reductase, cyt. bc_1), which reduces water-soluble cyt. *c*. Reduced cytochrome *c* is the electron donor to Complex IV (cytochrome *c* oxidase, Cyt*c*O), which catalyzes oxidation of cyt. *c* and reduction of dioxygen to water. Results from extensive studies over the years have generated insights into the function and structure of each of the components of the respiratory chain (for reviews, see [1–3]). However, significantly less is known about interactions between these complexes. For example, recent data from studies of *S. cerevisiae* indicate that Cyt*c*O and cyt. bc_1 interact with each other [4–9] and with the respiratory supercomplex factors 1 and 2 (Rcf1 and Rcf2) [8,10–13], which may have regulatory roles [10–12,14]. Also changes in the lipid environment result in altering specific reaction steps during O_2 reduction by Cyt*c*O [15,16]. Consequently, in order to study mechanistic aspects of the above-referenced interactions, it is important to study intact mitochondria. One approach that has yielded detailed functional information at the molecular level is the flow-flash technique, which allows kinetic investigations of electron and proton-transfer reactions. These studies have almost entirely been done with pure Cyt*c*O in detergent solution or reconstituted in membranes after purification. Because the flow-flash approach is based on photochemical dissociation of the CO ligand, in order to be able to use this technique to study intact mitochondria we need to investigate interactions of CO also with the flavohemoglobin (yHb, see Fig. 1), which is the second CO-binding heme protein in *S. cerevisiae* [17–19]. The expression and ability of the yHb protein to bind CO depends on growth conditions and may depend on the strain used (see below). Therefore, we need to find conditions under which only the Cyt*c*O binds CO or at least conditions under which the CO ligand can be photochemically dissociated only from the Cyt*c*O (to react with O_2).

The *S. cerevisiae* yHb harbors a single B-type heme group, which on the distal side binds small ligands such as NO, O_2 and CO [20]. The protein is a nitric oxide dioxygenase and participates, for example, in detoxification and signaling [21,22]. Results from earlier studies indicate that the yHb protein is localized to the cytosol and the mitochondrial matrix with a relative distribution that is dependent on growth conditions [18]. Yet, the data showed that the yHb remained undetected when using CO-difference spectroscopy in intact mitochondria, presumably because yHb residing inside in the matrix could not be reduced. However, CO binding was observed when the matrix extract fraction was analyzed [18].

Abbreviations: Cyt*c*O, cytochrome *c* oxidase; yHb, the *S. cerevisiae* flavohemoglobin; cyt., cytochrome.

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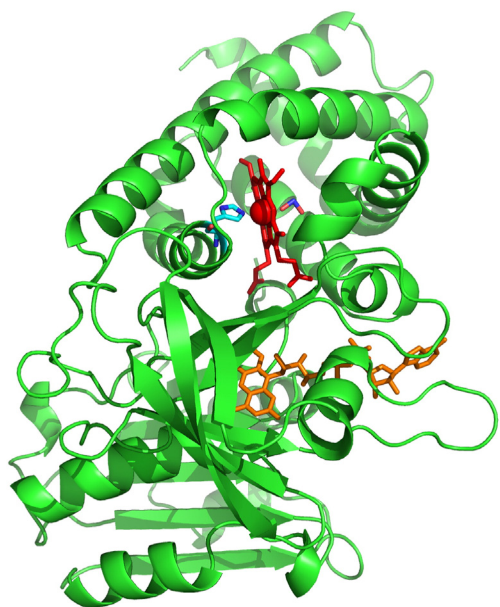

Fig. 1. The structure of the *S. cerevisiae* yHb protein. The yHb protein [20] (PDB code: 4G1V), which consist of a single subunit (green) and two co-factors, a heme (red) and a FAD (orange). The iron (red sphere) of the heme group is ligated to His56 (carbons in cyan and nitrogen in dark blue) and in this structure to an NO_2 molecule (nitrogen dark blue, oxygen pink).

Here, we used time-resolved optical absorption spectroscopy to investigate the kinetics of CO ligand binding in mitochondria from *S. cerevisiae* with the aim to separate contributions from Cyt*c*O and yHb. We confirmed data from earlier studies relating to the localization of the yHb, but found a CO-binding fraction also in the intermembrane space of the mitochondria. We also found that the CO-binding time constants vary depending on the yHb fraction studied and observed that the main part of the mitochondrial yHb binds CO with a time constant that is about a factor of 100 slower than the cytosolic yHb. In addition, we investigated CO ligand binding to Cyt*c*O and the reaction of reduced Cyt*c*O with O_2 in two commonly used *S. cerevisiae* strains, BY4741 and W303. In contrast to earlier reports [23] only minor differences were found in the reactivity between these two strains.

2. Materials and methods

All chemicals were obtained from Sigma Aldrich except for the zymolyase which was obtained from Nacalai Tesque, inc. Kyoto Japan. The bovine mitochondrial Cyt*c*O used to record a reference spectrum was prepared as described in [24].

2.1. Yeast strains and purification of mitochondria

The *S. cerevisiae* strains used were BY4741 and W303, and two variants originating from the W303 strain: yHb removed (*yhb1*Δ) (see, [25]) and a variant (*cox10*Δ, [26]) lacking the Cox10 protein responsible for heme *A* synthesis, resulting in no expression of functional Cyt*c*O.

A pre-culture was prepared by inoculating 100 ml of the medium. After 20 h the culture was scaled up to 3 × 2 l. The cultures were grown at 30 °C over night until an OD600 of 2–3 was reached. The W303 wild-type strain was grown on YPG medium (2% peptone (*w*/*v*), 1% yeast extract (*w*/*v*) and 2% glycerol (*v*/v), the medium pH was adjusted to 5.5). The *cox10*Δ and *yhb1*Δ variants were grown on the same medium but with 2% galactose (*v*/v) instead of glycerol.

Highly purified mitochondria were obtained using the protocol developed by Meisinger *et al.* [27] with the following modifications: DTT was excluded from the "DTT buffer" and the TRIS was not pH-adjusted (pH 10.5), twice the amount of homogenization buffer was used and no BSA was added, no EDTA was added to the "SEM buffer" and the MOPS was exchanged for 20 mM Hepes at pH 7.4, the "EM buffer" was exchanged for 20 mM Hepes at pH 7.4. The mitochondria were kept in a buffer containing 600 mM sorbitol and 20 mM Hepes at pH 7.4 at a concentration of 5–12 mg/ml depending on preparation

2.2. Expression and purification of S. cerevisiae yHb from E. coli

The gene encoding the full length yHb from *S. cerevisiae* was cloned in the *Bam*HI and *Eco*RI restriction sites of the *E. coli* plasmid vector pREST-6A (Invitrogen). The plasmid yHb-pREST-6A was transformed into *E. coli* strain BL21 (DE3) and then inoculated into 10 ml of LB medium with 100 μg/ml Ampicillin. The culture was incubated at 37 °C overnight and transferred into 1 l LB medium with 100 μg/ml Ampicillin. The bacteria were grown to an OD_{600} of 0.5 at 37 °C and the protein expression was induced by 1 mM IPTG for 4 h. The cells were harvested by centrifugation at 5000 × *g* for 15 min. The bacterial pellet was re-suspended in 40 ml of resuspension buffer (50 mM TRIS, pH 8.0; 200 mM NaCl, 1 mM PMSF) and subjected through French Press twice with a pressure of ~1200 Bar. The lysate was collected into a 50 ml tube and 20 μl of benzonase nuclease was added (Novagen; ~300 U). The mixture was placed on an orbital shaker at room temperature for 15 min. The lysate was then centrifuged at 10000 × *g* (4 °C) for 30 min to remove the debris and the supernatant was filtered through a 0.45 μm filter. The 5 ml His tag FF column (GE Healthcare) and the Agilent HPLC purification system were used to purify the recombinant yHb. Briefly, the supernatant was loaded onto the nickel column at the speed of 0.3 ml/min, and four steps of resuspension buffer (with 50 mM, 100 mM, 200 mM and 500 mM imidazole) were injected onto the column to elute yHb. Samples from the eluted fractions were analyzed using SDS-PAGE and the fractions containing yHb were concentrated to 0.5 ml using an Amicon Ultra-15 (cutoff 10 kDa) centrifugal ultrafiltration cartridge. The concentrated yHb fractions obtained from the nickel column were loaded onto the Hiload Superdex 200 10–300 gel filtration column (GE Healthcare), pre-equilibrated with resuspension buffer. The eluted fractions containing yHb were concentrated until a final volume of 0.5 ml was reached and the protein concentration was determined from the absorbance at 280 nm.

2.3. Preparation of mitochondria devoid of the mitochondrial outer membrane, the intermembrane space solution and the matrix fraction

A volume of 1 ml mitochondria with a protein concentration of 5–12 mg/ml, depending on preparation, was centrifuged at 10,000 × *g* for 12 min. The supernatant was discarded and the pellet (typical volume 200 μl) was suspended in buffer (60 mM sorbitol, 20 mM Hepes at pH 7.4) to a final volume of 1500 μl. The lower isotonic pressure resulted in swelling of the mitochondria and thus rupturing of the outer mitochondrial membrane. The suspension was again centrifuged (10,000 × *g*). After centrifugation both the supernatant (solution from the intermembrane space, IMS) and the pellet were saved. The pellet was then re-suspended in the same buffer as above. This procedure was repeated two additional times. Finally, the pellet was re-suspended in buffer to a desired volume and all the IMS fractions were pooled. The IMS fraction was centrifuged (120,000 × *g* for 30 min) to remove membrane fragments from the water-soluble content and the solution was then concentrated from roughly 3.3 ml to 500 μl using a 10,000 kDa cut-off filter (Millipore).

The matrix fraction was obtained from mitochondria devoid of the outer membrane. These mitochondria were treated as described above, but with 6 mM sorbitol buffer instead of 60 mM, which resulted in rupture of the inner mitochondrial membrane and release of the matrix content. The supernatants after three washing steps were pooled, ultra-centrifuged and concentrated using the same procedure as that used to obtain the IMS solution.

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