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Calcium ions inhibit reduction of heme *a* in bovine cytochrome *c* oxidase

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ABSTRACT

The effect of Ca^{2+} on the rate of heme *a* reduction by dithionite and hexaammineruthenium (RuAm) was studied in the cyanide-complexed bovine cytochrome oxidase (CcO). The rate of heme *a* reduction is proportional to RuAm concentration below 300 μM with k_v of $0.53 \times 10^6\ M^{-1}s^{-1}$. Ca^{2+} inhibits the rate of heme *a* reduction by dithionite by ~25%. As the reaction speeds up with increased concentrations of RuAm, the inhibition by Ca^{2+} disappears. The inhibition of heme *a* reduction may contribute to recently described partial inhibition of CcO by Ca^{2+} in the enzymatic assays. The inhibitory effect of Ca^{2+} on heme *a* reduction indicates that ET through heme *a* may be coupled to proton movement in the exit part of the proton channel H.

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1. Introduction

Cytochrome *c* oxidase (CcO) is a terminal component of the respiratory chain of mitochondria and many bacteria. The enzyme gates access of oxidative phosphorylation to oxygen as the final electron acceptor. CcO catalyzes electron transfer (ET) from ferrous cytochrome *c* to oxygen, and free energy of this thermodynamically favourable process is conserved in a form of transmembrane difference of proton electrochemical potential, $\Delta\mu H^+$ (reviewed, [1–3]).

ET in CcO occurs through a series of redox carriers, two copper centers (Cu_A and Cu_B) and two hemes (*a* and a_3):

External electron donor (cyt *c* or an artificial donor) → Cu_A → heme *a* → {heme a_3/Cu_B} → O_2

The exergonic ET to oxygen is coupled to electrogenic translocation of protons across the membrane [4] via a system of proton channels as proposed originally in [5] and studied since then in great detail (reviewed, [1–3,6,7]). Operation of the proton pump may be linked primarily to the steps of heme *a* reduction and oxidation [3,5,7–9].

Besides the redox metals, CcO contains bound non-redox metal ions, and, in particular, there is a special cation binding site (CBS) in subunit I of the enzyme [10–12] located near heme *a* and Cu_A. In CcO from vertebrate mitochondria, the site binds reversibly Ca^{2+} and Na^+ [13–15] as well as some other metal cations, albeit with lower affinity [16]. Ca^{2+} interaction with the mitochondrial CcO was first described long ago [17] and was studied since then in considerable detail with both the mitochondrial CcO [13–15,18] and bacterial mutant oxidase [12,18–20]. However, functional significance of the cation binding remained unknown for a long time, until it was found that Ca^{2+} binding at the CBS may result in 2–3-fold inhibition of CcO activity, the inhibition being prevented by excess Na^+ [16,21,22].

The mechanism of the Ca^{2+}-induced inhibition of CcO is now to be disclosed. Interestingly, the cation binds at the exit part of the transmembrane "proton channel H", associated with heme *a* [3,22]. Therefore, effect of Ca^{2+} on proton movement within the H-channel coupled to redox reactions of heme *a* is a tempting hypothesis [3,16,22].

In order to establish the inhibition mechanism, it is necessary first of all to reveal the steps of intramolecular ET affected by Ca^{2+} binding. It was reported earlier that Ca^{2+} slows down electron transfer from heme *a* to heme a_3 in bovine heart CcO [23]. The inhibition of ET between the hemes *a* and a_3 has been recently confirmed and characterized in more detail by this group using dithionite, ascorbate and different concentrations of ruthenium

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hexaammine (RuAm) complex, $[Ru^{II/III}(NH_3)_6]^{2+/3+}$ [24] as the reductants (paper in preparation). In the course of these experiments, we noticed that under some conditions Ca^{2+} appeared to slow down not only the oxidation, but also the reduction of heme *a*. However, the kinetics of absorption changes in ligand-free CcO is complicated by contribution from the two hemes, *a* and a_3, which renders interpretation of the data less certain. In order to better resolve potential effect of Ca^{2+} on heme *a* reduction, we choose to simplify the experimental model and to explore the reaction with the cyanide complex of CcO, $a^{3+}a_3^{3+}$-CN. In this complex heme a_3 is trapped by cyanide in a ferric state so that ET from heme *a* to heme a_3 is blocked and contribution of heme a_3 spectral changes is eliminated.

In this work the kinetics of heme *a* reduction has been studied in the cyanide-complexed CcO, $a^{3+}a_3^{3+}$-CN, with the aid of a stopped-flow diode array spectrophotometer. Dithionite was used as the electron donor in the absence or in the presence of different concentrations of RuAm. Ca ions are found to bring about modest but distinct partial inhibition of heme *a* reduction by dithionite or RuAm. The inhibition is lifted as the electron input rate increases in the presence of high concentrations of RuAm.

2. Materials and methods

2.1. Chemicals

Sodium dithionite, calcium chloride and bovine liver catalase were from Sigma–Aldrich. Hexaammineruthenium (III) chloride was purchased from Alfa-Division. Dodecyl maltoside (SOL-GRADE) was a product of Anatrace. pH buffers, EGTA and magnesium sulfate were from Amresco.

2.2. Preparation

Cytochrome *c* oxidase was prepared from bovine heart mitochondria by a method of Fowler et al. [25] modified by Dr. A. Musatov (Institute of Experimental Physics Slovak Acad. Sci, Kosice) as described in [16]. The basic medium contained 100 mM HEPES/Tris buffer, pH 8, and 0.1% dodecyl maltoside with other additions as indicated. Care was taken to avoid Na^+ in the solutions (except for using sodium dithionite, $Na_2S_2O_4$, the only dithionite salt available) because Na^+ competes with Ca^{2+} for the CBS in CcO [14,15]. Cyanide complex of CcO was obtained by incubation of the enzyme (24 μM) in the basic buffer with 2 mM KCN in a closed tube for ~20 h at 4 °C. The reaction with cyanide was essentially complete ($\Delta\varepsilon_{434-412}$ ca. 50 $mM^{-1}cm^{-1}$), but a minor shoulder at 444 nm was present indicating partial reduction of heme *a*. Complete oxidation of heme *a* was then achieved by addition of 40–60 μM ferricyanide.

2.3. Measurements

Static spectra were recorded in a CaryBio 300 spectrophotometer (Varian).

The kinetics of CcO reduction was studied in an Applied Photophysics SX-20 stopped-flow spectrophotometer operated in a diode array mode. The instrument allows to record the spectra (190–720 nm range) in a 20 μL cell with 1 cm optical pathway with a minimal interval between the spectra of 1 ms.

Unless indicated otherwise (cf. Fig. 3), one syringe contained 12 μM aerobic oxidized cyanide complex of CcO ($a^{3+}a_3^{3+}$-CN) in the basic buffer with 100 μM EGTA and 1 mM KCN and supplemented where indicated with 0.5 mM $CaCl_2$ or 0.5 mM $MgSO_4$. The second syringe contained 10 mM sodium dithionite in the same 100 mM HEPES/Tris basic buffer, pH 8, with 100 μM EGTA, 8 nM catalase (to remove H_2O_2 formed upon reaction of dithionite with oxygen) and appropriate concentrations of RuAm. Hereafter, final concentrations after mixing are referred throughout the text. These were for the main reactants: CcO, 6 μM; sodium dithionite, 5 mM; catalase, 4 nM; EGTA, 100 μM and, where indicated, Ca^{2+} or Mg^{2+}, 250 μM (i.e., 150 μM excess over EGTA). RuAm concentration was varied between 0 and 3000 μM.

In Figs. 1 and 2 spectral baseline was measured by mixing blank solution in syringe 1 (the basic buffer with 100 μM EGTA), with blank solution in syringe 2 (10 mM sodium dithionite, 100 μM EGTA, and 8 nM catalase in the basic buffer without dodecyl maltoside). To prevent dithionite consumption by oxygen, syringe 2 was bubbled with argon prior to addition of pre-weighed dithionite powder and kept plugged.

In the experiments shown in Fig. 3, the baseline was measured without dithionite in syringe 2. This allowed us to monitor dithionite radical concentration directly in the cell after mixing (the peak at 315 nm) and check its stability.

2.4. Data processing

The spectra were collected in a 190–720 nm nominal range, 1000 scans for the observation period with minimal interval between the points of 1 ms and logarithmic spread of the time points where the observation period exceeded 1 s. To determine the rate constants, global analysis of the spectra/time surface files was made with ProKineticist software provided with the APL SX-20 instrument. The Soret and α-band parts of the files were excised with MATLAB (MathWorks, Inc.) and subjected to global analysis in ProKineticist program separately in order to eliminate influence of the extensive Soret band changes on the visible part data fitting. Alternatively, the kinetic curves at selected wavelengths were extracted from the spectra/time surface files and $\Delta A_{444-480}$ or $\Delta A_{605-630}$ curves were fitted to 1 or 2 exponentials with MATLAB or Origin 7E (Microcal) software. In general, the results obtained by different procedures of analysis were in good agreement. Single exponential approximation was sufficient to fit the kinetics in the α-band, at least for the purposes of this work (e.g., see Supplementary Data Fig. S1A–C). The changes in the Soret revealed sometimes minor contribution of the slower changes.

3. Results

Fig. 1A and B shows typical sets of spectra evolving after mixing the $a^{3+}a_3^{3+}$-CN complex with 5 mM dithionite and 10 μM RuAm (final concentrations). The reduction is complete in less than 2 s. The spectra sets reveal clear isobestic points in the γ- and α-absorption bands of the hemes. In particular, the isobestic point at 437.5 nm persists in the Soret during the entire observation period confirming that heme *a* reduction is the only obvious process observed (Fig. 1A). This is at variance with the data obtained in the absence of cyanide, in which the initial isobestic point at 432 moves to 426 nm at the later stages of the reduction [23]. No evidence for significant reduction of the cyanide complex of heme a_3 was obtained during the observation period of up to 20 s.

Interestingly, the spectral changes in the β-region of the hemes are not homogenous and no unique isosbestic points are retained below the one at ~585 nm (Fig. 1B). Development of the narrow heme peaks at ~520 and ~555 nm is overlaid by bleaching of absorption between the isobestic points at 585 and 463.5 nm with a minimum in the difference spectrum around 480 nm (cf. [26,27]). Identity of this band in the absolute spectra of the oxidized ligand-free and cyanide-complexed CcO [26,28] is not quite certain. According to the data in [29,30], it could belong to the oxidized Cu_A (cf. a difference spectrum of Cu_A obtained for R54M mutant

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