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# Cation Binding Site of cytochrome *c* oxidase: Progress report ☆

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ABSTRACT

Cytochrome *c* oxidase from bovine heart binds $Ca^{2+}$ reversibly at a specific Cation Binding Site located near the outer face of the mitochondrial membrane. $Ca^{2+}$ shifts the absorption spectrum of heme *a*, which allowed earlier the determination of the kinetic and equilibrium characteristics of the binding, and, as shown recently, the binding of calcium to the site inhibits cytochrome oxidase activity at low turnover rates of the enzyme [Vygodina, T., Kirichenko, A., Konstantinov, A.A (2013). Direct Regulation of Cytochrome *c* Oxidase by Calcium Ions. PloS ONE 8, e74436]. This paper summarizes further progress in the studies of the Cation Binding Site in this group presenting the results to be reported at 18th EBEC Meeting in Lisbon, 2014. The paper revises specificity of the bovine oxidase Cation Binding Site for different cations, describes dependence of the $Ca^{2+}$-induced inhibition on turnover rate of the enzyme and reports very high affinity binding of calcium with the "slow" form of cytochrome oxidase. This article is part of a Special Issue entitled: 18th European Bioenergetic Conference. Guest Editors: Manuela Pereira and Miguel Teixeira.



## 1. Introduction

Cytochrome *c* oxidase (CcO) is a terminal enzyme of the respiratory chain of mitochondria and many bacteria. The enzyme locates in the coupling membrane and gates access of the respiratory chain to oxygen as electron acceptor in the aerobic living cells, catalyzing overall reaction:

$$4\ \text{cyt}\ c^{2+} + O_2 + 8H^+_{\text{inside}} = 4\ \text{cyt}\ c^{3+} + 2H_2O + 4H^+_{\text{outside}}.$$

Physiological importance of the reaction stimulated extensive studies of the structure as well as of the electron transfer and energy transduction mechanisms of the enzyme (reviewed, [1–5]). Molecular mechanism of CcO has become a paradigm in the studies of the redox-coupled proton pumping by membrane-bound redox enzymes.

The enzyme contains 4 transition metal centers – two hemes A and two copper centers – that convey electrons from the donor, cytochrome *c*, to the acceptor, molecular oxygen. The sequence of electron transfer can be described by a simplified scheme:

$$\text{cyt}\ c \rightarrow Cu_A \rightarrow \text{heme}\ a \rightarrow \text{heme}\ a_3\ /\ Cu_B \rightarrow O_2.$$

Besides the redox centers, CcO from mitochondria and bacterial oxidases of A-type [6] contains a special Cation Binding Site (CBS) which can bind $Ca^{2+}$ or $Na^+$ [7]. The site is located in subunit I very close to the outer surface of the protein (Fig. 1A). Binding of $Ca^{2+}$ to this site brings about a red shift of absorption spectrum of heme *a* as first described by Wikstrom and coworkers [8,9], long before the CBS itself was revealed in the crystal structure of the bacterial and bovine oxidases [7,10]. Binding of $Ca^{2+}$ at the CBS can be easily followed spectroscopically, and characteristics of the binding were explored in considerable detail [9,11–17]. However, despite the progress in the studies of the properties of the CBS, functional significance of calcium and sodium binding with the site remained unknown for a long time.

Recently we were able to show that $Ca^{2+}$ binding with bovine CcO brings about inhibition of the enzyme at low turnover rates and high redox potential of cytochrome *c* [18], which are turnover conditions close to those in the respiring mitochondria. No inhibition could be observed at high turnover rates of CcO, i.e. under the conditions of common assays of cytochrome oxidase activity. This explained why the effect was not observed earlier.

The mechanism of the inhibition induced by $Ca^{2+}$ remains yet to be established. Most interestingly, $Ca^{2+}$ bound in the CBS interacts with several core residues forming the exit of a putative transmembrane $H^+$ transfer pathway described by Yoshikawa and co-workers in the

*Abbreviations:* BAPTA, 1,2-bis(2-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; CBS, Cation Binding Site; CcO, cytochrome *c* oxidase; EGTA, ethylene-bis(oxyethylenenitrilo)tetraacetic acid; HEDTA, N-(2-hydroxyethyl)ethylenediamine-*N*,*N*′,*N*′-triacetic acid; TN, turnover number; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine

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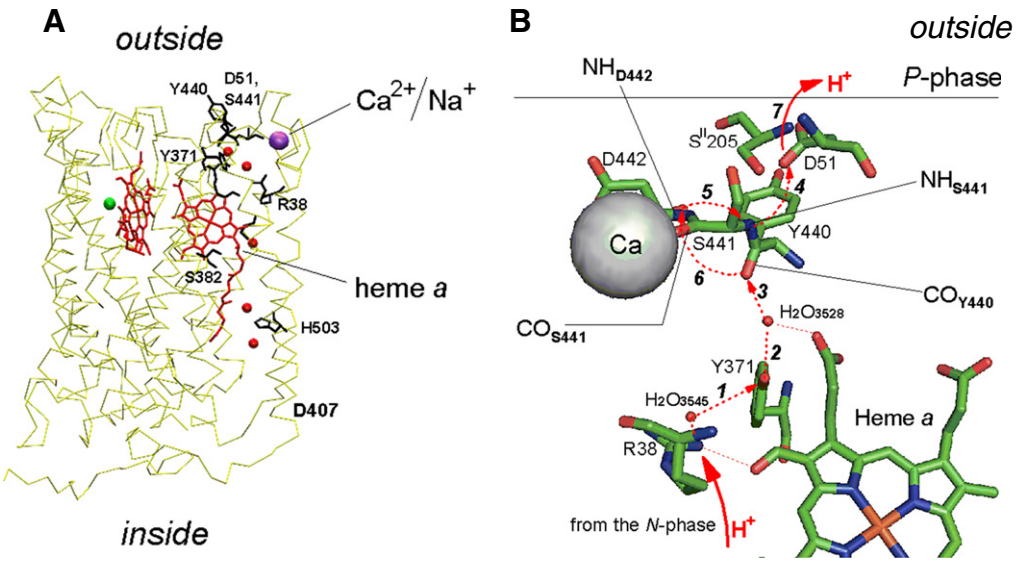


**Fig. 1.** The Cation Binding Site in subunit I of cytochrome oxidase (adapted from [19]). (A). *Location of the CBS in subunit I of bovine enzyme*. Based on the structure with PDB entry code: 1V55. Components of the hypothetical H-proton pathway are depicted as orange spheres (fixed water molecules) and black sticks (amino acid residues, A-propionate and carbonyl groups of heme *a*). Enlarged picture of the exit part of the H-channel is shown in panel B. (B). *Interaction of $Ca^{2+}$ with the exit of proton channel H in bovine heart oxidase*. Structure of the exit part of the H-channel is shown based on the oxidized COX crystal structure (PDB entry code: 1V54). The groups shown belong to subunit I except for $S^{II}205$ from subunit II. A hypothetical scenario for proton transfer has been combined from Refs. [4,53–55]. Proton transfer steps are depicted by red arrows, and their sequence is indicated by the numbers (see Ref. [19] for a detailed description of the scenario). $Ca^{2+}$ binding at the CBS is expected to inhibit the proton transfer via the exit part of the H-channel [19].

bovine CcO and denoted as "H-channel" [4,20] (Fig. 1B). Therefore, the inhibitory effect of calcium on CcO provides a strong argument in favor of functional significance of the H-channel, whatever the function of the channel may be (e.g., see [17] for discussion). As pointed out in [19], $Ca^{2+}$ bound at the site is expected to inhibit the critical step of $H^+$ transfer from the protonated Tyr440 carbonyl function to Ser441 imide function within the exit part of the H-channel that was postulated by Yoshikawa and coworkers [4,20] (Fig.1B).

In view of the evidence for modulation of CcO activity by metal cations, the inhibitory effect of $Ca^{2+}$ being counteracted by $Na^+$ [19], involvement of the Cation Binding Site of CcO in direct regulation of mitochondrial respiration and oxidative phosphorylation by intracellular cations becomes a topical issue. Accordingly, further detailed studies of the cation binding at CBS are of obvious significance. The paper summarizes recent progress of the studies in this direction in our group that will be presented at the 14th EBEC Meeting in Lisbon.

## 2. Materials and methods

Sodium dithionite, $CaCl_2$, choline chloride (C-1879, >99%), cytochrome *c* type III, TMPD and calcium buffers (EGTA, BAPTA and HEDTA) were from Sigma-Aldrich. pH-buffers, sodium chloride and magnesium sulfate were purchased from Amresco. Dodecylmaltoside "SOL-GRADE" was from Anatrace.

Bovine heart CcO was isolated by a method of Fowler et al. [21] as modified in this laboratory following an advice from Dr. Andrey Musatov (University of Texas Health Science Center, San Antonio, TX). The procedure follows initially the protocol of Fowler et a.l [21] including the steps of "red/green splitting", sedimentation of crude CcO and washing the sedimented enzyme with the isolation buffer. Subsequent steps of salt fractionation with KCl in deoxycholate followed by dialysis are omitted. Instead, the washed crude oxidase is solubilized in 100 mM K-phosphate buffer pH 7.5 with 1 mM EDTA and purified by stepwise salting out the impurities with ammonium sulfate in the presence of cholate. To this end, protein concentration is adjusted to 20 mg/ml. Cholate is added to 1 mg per 1 mg of protein and then saturated ammonium sulfate (pH adjusted to 8 immediately before the addition) up to 35% of saturation. After 30 min incubation, the brownish sediment is spun down (10 min × 18 000 rpm in JA-20 rotor) and discarded. The $(NH_4)_2SO_4$ fractionation procedure is then repeated several times increasing the ammonium sulfate concentration stepwise from 37.5% to 45% of saturation in ~1% steps until precipitation of CcO begins. Then CcO is precipitated by adding slight excess of ammonium sulfate. The sediment of the enzyme is solubilized in 25 mM HEPES pH 8 (with no detergent added) and is quickly frozen in small portions in liquid nitrogen.

Concentration of the enzyme was determined from the absorption difference spectra (reduced *minus* oxidized) using Δε value of 27 $mM^{-1}$ $cm^{-1}$ for 605/630 nm wavelength pair. Most of the experiments were performed in a basic buffer containing 100 mM HEPES–Tris, pH 7.5–8, 0.1% of dodecylmaltoside (DM) and 0.1 mM EGTA (if not indicated otherwise). Other additions are indicated in the legends in figures.

Concentrations of free $Ca^{2+}$ at known concentrations of added $Ca^{2+}$ and calcium buffers (EGTA, HEDTA, BAPTA) at given values of pH, temperature and ionic strength were calculated with a software WinMAXC, v.2.05 or WinMAXC Standard [22].

Spectrophotometric measurements were made in a Varian Cary300Bio or an SLM-Aminco 2000C dual wavelength/split beam instrument in 10 mm optical pathway cells at 25 °C. Turnover numbers for CcO (e.g., 10 $s^{-1}$) are expressed in electrons per second per cytochrome oxidase monomer. Oxygen consumption was measured with a covered Clark-type electrode in an Oxytherm (Hansatech) instrument in a thermostatted cell at 25 °C. The data were processed with an OriginPro 7e software (Microcal).

## 3. Results and discussion

### 3.1. Ionic specificity of the Cation Binding Site in cytochrome c oxidase

The ionic specificity of the Cation Binding Site of CcO was studied originally by Wikstrom and co-workers [9]. $Ca^{2+}$ and $H^+$ were the only two cations reported to bind to COX inducing red shift of heme *a* absorption spectrum. "The specificity of $Ca^{2+}$ and $H^+$ in causing this effect is quite unique… Cations tested in this respect included $La^{3+}$, $Eu^{3+}$, $Mg^{2+}$, $Sr^{2+}$, $Zn^{2+}$, $Ni^{2+}$, $Ba^{2+}$, $Li^+$, $K^+$, and $Na^+$, none of which

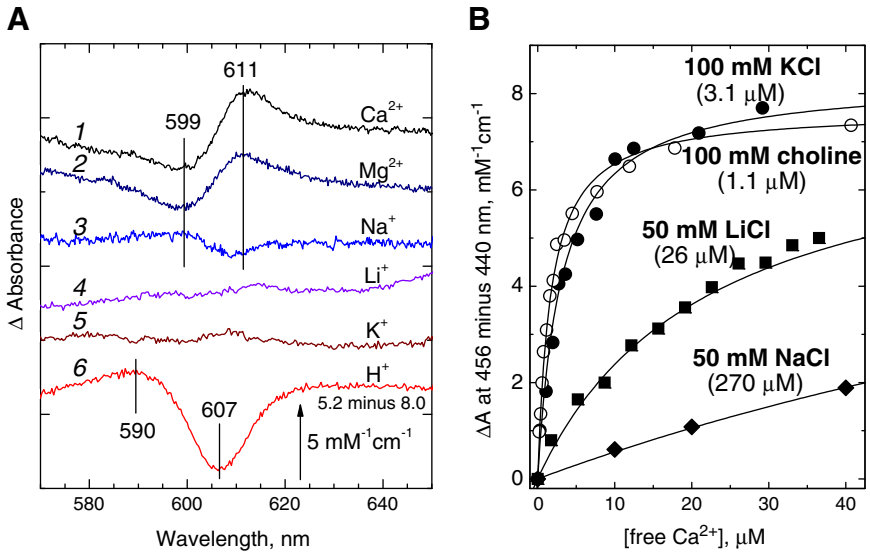


**Fig. 2.** Ionic specificity of the cation-binding site in CcO. Bovine CcO (1.5–2 μM) was inhibited by 4 mM KCN and reduced by 5 mM ascorbate/0.1 mM TMPD in the basic buffer (100 mM HEPES/Tris pH 8.0, 0.1% dodecyl-maltoside with 100 μM EGTA). Conditions for pH-jump experiment are indicated separately below. (A) *Difference spectra induced by cations*. The spectra have been recorded in 3 min after addition of $CaCl_2$ (200 μM), $MgCl_2$ (22 mM), NaCl (100 mM), LiCl (200 mM), KCl (200 mM) or calibrated amount of acetic acid to reach pH 5.2. In the case of the pH-jump, the pH-buffer concentration was reduced to 25 mM and the medium was supplemented additionally with 30 mM $K_2SO_4$ to compensate for a decrease in the ionic strength and with 1 mM BAPTA to ensure removal of free calcium at acidic pH. (B) *Effect of monovalent cations on $Ca^{2+}$ binding with CcO*. The $Ca^{2+}$-induced red shift of heme *a* was titrated with $Ca^{2+}$ in the presence of various monovalent cations at the concentrations indicated. Amplitude of the difference spectra induced by $Ca^{2+}$ was measured in the Soret band. The apparent $K_d$ values for $Ca^{2+}$ in the presence of the monovalent cations are given in brackets and correspond to the curves drawn through the points.

**Table 1**
Characteristics of cation binding with CcO.

| Cation | Spectral effect | Kd |
|---|---|---|
| $Ca^{2+}$ | Red shift of heme *a* | ~ 1 uM ("fast" CcO), 15–30 nM ("slow" CcO) at pH 8 |
| $Mg^{2+}$ | Red shift of heme *a* | 7.2 mM |
| $Na^{+}$ | Minor blue shift of heme *a* | 4 mM (average for two $Na^{+}$ binding sites, 2 $Na^{+}$ compete with 1 $Ca^{2+}$) |
| $Li^{+}$ | No effect below 0.1 M | 20 mM (average for two sites, 2 $Li^{+}$ compete with 1 $Ca^{2+}$) |
| $K^{+}$ | No effect below 0.2 M | > 100 mM |
| $H^{+}$ ($H_3O^{+}$) | Blue shift | pK = 5.4 |

The table summarizes the data obtained in this group.

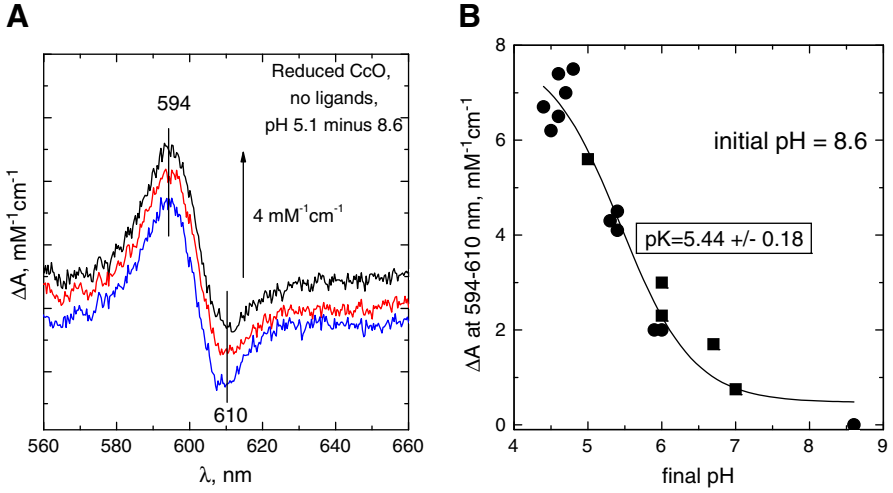


**Fig. 3.** The $H^{+}$-induced blue shift of heme *a* absorption in the reduced unliganded CcO. CcO (1.3–1.7 μM) in 25 mM HEPES/Tris buffer, pH 8.6, with 0.1% dodecylmaltoside, 30 mM $K_2SO_4$, 1 mM EGTA and 1 mM BAPTA were reduced by 10 mM dithionite/40 μM ruthenium hexamine and the spectrum at pH 8.6 was taken as a baseline. Calibrated amounts of acetic acid were added to reach the desired values of pH. Final pH was checked with a pH-meter. (A) The 3 spectra have been obtained in 15, 20 and 30 min after pH shift from 8.6 to 5.1. (B) pH-dependence of the blue shift. The panel gives amplitude of the difference spectrum induced by acidic pH-jump from 8.6 to the value indicated on the abscissa scale.

were able to influence the $Ca^{2+}$-induced shift or produce a shift by their own" [9].

In variance with this conclusion, it was found later on that $Na^+$ ions compete with calcium for binding with the site [7,11,12,16]. Subsequent studies in this group entailed further refinement and/or revision of the initial conclusions. Some of the findings are illustrated in Fig. 2 and are summarized in Table 1. As shown in Fig. 2A, divalent metal cations, $Ca^{2+}$ and $Mg^{2+}$, bind with CcO bringing about virtually identical red shift of heme *a* absorption spectrum, although ca. 5000-fold higher concentrations of $Mg^{2+}$ are required to induce the shift. Special care was taken to make certain that the effect of $Mg^{2+}$ was not due to admixture of $Ca^{2+}$.

In contrast, monovalent metal cations ($Na^+$, $Li^+$, $K^+$) do not perturb the spectrum of CcO significantly (Fig. 2A); in any case, there is no red shift. Sodium ions are likely to bring about a very small blue shift of heme *a* spectrum in agreement with earlier observations on *Paracoccus denitrificans* D477A mutant CcO [16] corroborated by the recent results of Marechal et al. [17] on the bovine enzyme. It is not excluded that $Li^+$ or $K^+$ may induce a similar blue shift at very high concentrations.

Despite the absence of pronounced absorption changes, binding of the monovalent metal cations with CBS is revealed by their antagonistic effect on calcium binding. As shown in Fig. 2B, 50 mM $Na^+$ and $Li^+$ strongly counteract the induction of the red shift of heme *a* by $Ca^{2+}$, increasing the apparent $K_d$ for $Ca^{2+}$ binding with the enzyme. A small effect of 100 mM $K^+$ (2–3-fold increase in $K_d$ for calcium) is also observed, but it cannot be fully guaranteed at this time that the effect is not due to admixture of $Na^+$.

The most significant disagreement of our work with the original paper by Saari et al. [9] is concerned with the effect of protons. In contrast to [9], we found that $H^+$ does not induce the same red shift of heme *a* as $Ca^{2+}$, but can bring about a marked blue shift of cytochrome oxidase spectrum (spectrum 6 in Fig. 2A, Fig. 3A).

It is to be emphasized that repeating the experiment exactly as described in Ref. [9], i.e., measuring the changes of the absorption spectrum of unliganded fully-reduced CcO induced by a shift of pH from 7.8 to 6.3 in the presence of 200 μM EGTA, we were able to reproduce a partial red shift of heme *a* very similar to that shown in Fig. 3 of Ref. [9] (data not shown). However, this effect is likely to be an artifact. At pH 8 EGTA is a strong $Ca^{2+}$ chelator and sequesters efficiently adventitious calcium in the reaction buffer. When pH is shifted to 6.3, affinity of EGTA for $Ca^{2+}$ decreases greatly and part of the $Ca^{2+}$ sequestered by EGTA at alkaline pH is released to the buffer bringing about the observed partial red-shift of heme $a^{2+}$. For example, at 20 μM of adventitious calcium in the reaction medium which is a realistic number if no special precautions are taken, concentration of free calcium in the presence of 200 μM EGTA will be ~1 nM at pH 8, but around 2 μM at pH 6.3 (or ~1 μM at 10 μM of adventitious $Ca^{2+}$).

Indeed, if the same experiment was performed with 1 mM BAPTA in addition to 0.2 mM EGTA, no red shift was induced by acidification of CcO from pH 7.8 to pH 6.3, but rather a small blue shift which increased in size with lowering pH (cf. Fig. 3). The difference spectrum developed with a half-time of 3–5 min and was stable for at least half an hour (Fig. 3A). A similar $H^+$-induced difference spectrum characteristic of a blue shift was observed also with the fully reduced CO-complexed enzyme (data not included). Therefore, the response is likely to be associated with heme $a^{2+}$. Also in the KCN-inhibited mixed-valence CcO, acidification brought about spectral changes indicating the blue shift of heme $a^{2+}$, although of a somewhat different line shape (Fig. 2A, trace 6). The asymmetric difference spectrum with an intensive trough at ca. 607 nm and a weaker broad maximum at ca. 590 nm may indicate that under aerobic conditions, the acidification-induced blue shift of the reduced heme $a^{2+}$ is accompanied by partial oxidation of the heme.

Amplitude of the $H^+$-induced difference spectrum of the reduced CcO grows with lowering the final pH. Results of a titration in which the $H^+$-jump was performed from pH 8.6 to different acidic pH levels are shown in Fig. 3B. The points can be approximated formally by a titration curve for a single protonatable group with pK 5.4 ± 0.2. However, this approximation is to be considered as provisional. First, it is not quite clear whether the dependence levels off indeed at acidic pH. Points far below pH 5 are required to verify plateau on the acidic side, but that will be detrimental to the enzyme. Second, it is not possible at the moment to say whether the $H^+$-induced spectral shift is reversible because after acidification to pH 5.5 or below, return to pH 8–9 always resulted in partial (15–20%) oxidation of the hemes despite the presence of dithionite.

The nature of the $H^+$-induced blue shift remains to be established. The amplitude and the width of the difference spectrum indicate somewhat stronger displacement of the α-peak position of heme *a* than that induced by $Ca^{2+}$. It is currently assumed that the α-band of heme $a^{2+}$ is shifted in CcO from ~590 to 605 nm relative to low-spin complexes of isolated heme *a* because of strong hydrogen bonding of the carbonyl substituent of heme A with Arg38 of subunit I [3,23–25]. It is possible that the observed $H^+$-induced blue shift is caused by protonation of some group(s) resulting in weakening or breaking the hydrogen bond of Arg38 with the carbonyl of heme *a*. In case of the experiments in the presence of KCN, replacement of one of the axial histidines of heme *a* by cyanide can be an additional factor (this possibility was pointed out by Marten Wikstrom during the discussion of the data). Other types of spectroscopy, like MCD and Resonance Raman, would be useful in order to interpret the $H^+$-induced blue shift of the absorption spectrum of the reduced CcO.

### 3.2. Dependence of the calcium-induced inhibition of cytochrome c oxidase activity on the turnover rate

As shown recently [18,19], $Ca^{2+}$ binding at the CBS inhibits cytochrome *c* oxidase at low turnover rates of the enzyme (<10 $s^{-1}$), whereas no inhibition is observed at high turnover numbers (~100 $s^{-1}$ at pH 8). We have studied the dependence of the calcium-induced inhibition on the turnover rate of CcO in more detail. The results are summarized in Fig. 4A. The oxidase turnover was assayed and its rate was varied in 3 ways. In the first set of experiments, CcO activity was measured as oxygen consumption rate in the presence of ascorbate and cytochrome *c* at moderate ionic strength (100 mM HEPES–Tris), and turnover rate was varied by increasing the concentration of cytochrome *c* (up to 1.7 μM, filled squares). In the second set, the same ascorbate/cytochrome *c* oxidase activity was measured at constant concentration of cytochrome *c* of 2.5 μM and turnover rate was attenuated by increasing the ionic strength of the medium with 0.1–0.4 M choline chloride (open squares). In the third type of experiments, CcO activity was measured at high ionic strength (0.4 M choline chloride added) at high redox potential of cytochrome *c*. To this end the experiments were performed in the absence of ascorbate with 1:1 mixture of the ferrous and ferric cytochrome *c*, following oxidation of added ferrocytochrome *c* spectrophotometrically [19]. The turnover rate was varied by increasing the concentration of the total added cytochrome *c* (2–25 μM of each form) and the initial reaction rates were determined. In each type of the above described experiments, the rates in the presence of 100 μM EGTA were compared with the rates in the presence of 100 μM EGTA and 200 μM added $Ca^{2+}$.

Under all the conditions, calcium ions bring about 2–3-fold inhibition of cytochrome oxidase activity at the lowest turnover rates of the enzyme confirming the data in [19]. In the case of oxygen consumption measurements, the inhibition is gradually released as the TN increases, so that there is almost no inhibition at TN > 20 $s^{-1}$. At not too high ionic strength, the dependence of the extent of inhibition vs turnover rate can be linearized reasonably well as the $(\text{inhibition})^{-1}$ vs TN plot (Fig. 4B). The plot extrapolates to 55–70% maximal inhibition at zero TN. If the reaction rate was varied by increasing the ionic strength at constant concentration of cytochrome *c*, the dependence of inhibition on turnover rate appeared to be not hyperbolic but rather sigmoidal with a lag phase up to ~5 $s^{-1}$ but also in this case the inhibition was essentially released above TN of 10 $s^{-1}$. At the same time, if

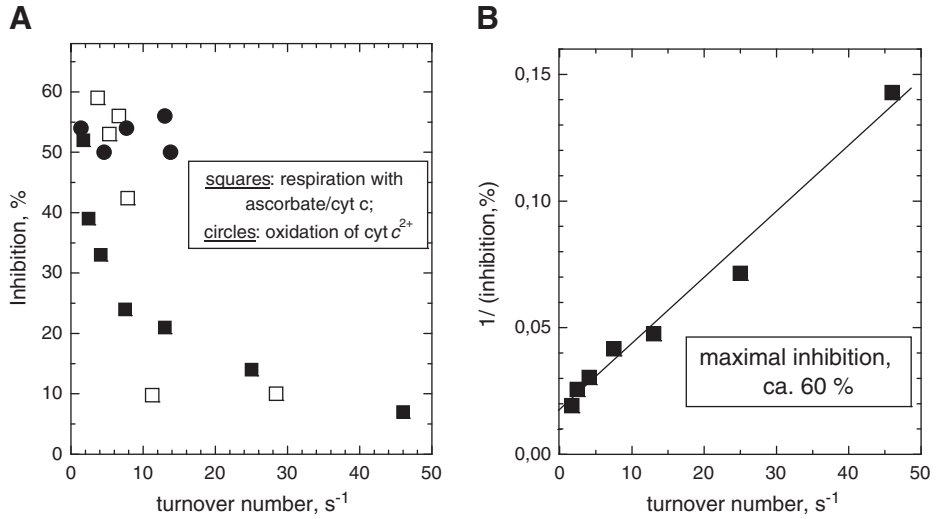


**Fig. 4.** Effect of turnover rate on the inhibition of CcO by calcium. (A). *Filled squares*: 125 nM CcO in 100 mM HEPES/Tris buffer pH 7.5 with 0.1% dodecyl-maltoside and 0.1 mM EGTA. CcO activity was measured as oxygen consumption with an oxygen electrode in the presence of 5 mM ascorbate and increasing concentrations of cytochrome *c* (0.2–5 μM). *Open squares*. The same basic conditions, but respiration was measured at a constant concentration of cytochrome *c* of 2.5 μM. The turnover rate was varied by increasing the ionic strength of the buffer with 0.1–0.4 M choline chloride. *Filled circles*. Activity was measured by following the oxidation of ferrous cytochrome *c* spectrophotometrically at 550 minus 535 nm. To decrease the reaction rate, the ionic strength was increased by 0.4 M choline chloride, and ferric cytochrome *c* was included in the medium to provide for 1:1 mixture of the ferrous and ferric form. The turnover rate was varied by changing the concentration of the added ferrocytochrome *c* in the range 1–25 μM but always at 1:1 ratio with the ferric cytochrome *c*, i.e., $E_h$ of the donor at the reaction onset was kept constant and high. CcO concentration, 25 nM. In each type of the experiments, the turnover rate was compared for experiments in the presence of 100 μM EGTA and 100 μM EGTA + 200 μM added $Ca^{2+}$. Each point is an average for 3–5 measurements. (B) Linearization of the concentration dependence of the inhibition for the experiments that are shown by filled squares in panel (A).

ferrocytochrome *c* oxidation measurements at high ionic strength were carried out in the presence of equimolar amount of the oxidized cytochrome *c*, the ~60% inhibition by $Ca^{2+}$ persisted throughout the available range of turnover rate (up to 14 $s^{-1}$ at 25 μM of cytochrome *c*).

The main inference from this part of the results for further research may be as follows:

(1) The 2–3-fold inhibition of CcO by $Ca^{2+}$ at low turnover rate of the enzyme can be reproduced with different activity assays.
(2) In case of the most common assay of CcO activity (oxygen consumption in the presence of ascorbate and cytochrome *c*) when cytochrome *c* is kept almost fully reduced by excess ascorbate, the maximal inhibition extrapolates to ~60% at zero TN and vanishes as turnover rate increases above ~10 $s^{-1}$. This dependence of the inhibition on turnover rate explains why the inhibitory effect of calcium was not noticed earlier since most of the studies were carried out at TN well above 10 $s^{-1}$.
(3) The calcium-induced inhibition is favored by high redox potential of cytochrome *c*.

The release of the calcium-induced inhibition upon increase of concentration of the ferrous cytochrome *c* might imply not only dependence of the inhibition on the turnover rate, but also antagonistic relationships between the binding of $Ca^{2+}$ at the CBS and binding of cytochrome $c^{2+}$ at its docking site, although the two binding sites are well separated spatially. In such a case, ferrous cytochrome *c* would be expected to decrease CcO affinity for $Ca^{2+}$. Preliminary experiments have not revealed any effect of 10 μM cytochrome $c^{2+}$ on CcO affinity for $Ca^{2+}$ (see below, Fig. 6).

As pointed out by one of the reviewers of this paper, there are certain similarities between the inhibitory effects of $Ca^{2+}$ and $Zn^{2+}$ on CcO. Both metal cations inhibit cytochrome oxidase activity binding at the *P*-side of the membrane [19,26–28], although there are additional $Zn^{2}$-binding sites at the *N*-side [27,29,30]. As with $Ca^{2+}$, the inhibitory action of low (few μM) concentrations of $Zn^{2+}$ from the *P*-side of the membrane has been observed only at low turnover rates of CcO attained in the state of respiratory control [26], and the maximal inhibition was not complete reaching, only 60–75% [26]. The effects of both $Zn^{2+}$ and $Ca^{2+}$ have been proposed to arise from inhibition of the exit proton pathway in CcO [19,26,27]. In view of these similarities it has to be emphasized that there is no evidence for $Zn^{2+}$ binding at the Cation Binding Site of CcO [9,30], although interaction of $Zn^{2+}$ with some of the *P*-surface exposed residues in the exit of the putative H-channel in bovine CcO cannot be fully excluded.

Notably, modulation of CcO steady-state kinetics by phosphorylation and nucleotides is also most clearly observed at the enzyme turnover rates below 10–20 $s^{-1}$ (e.g., [31,32] and refs. therein). It can be suggested that while searching for modulation of CcO and other respiratory enzymes by potential physiological effectors, it may be reasonable to extend the studies to enzyme turnover rates in the physiologically relevant range (low rates), rather than always keeping the activity close to $V_{max}$.

### 3.3. High affinity binding of calcium with "slow" form of CcO

Initially, affinity of CcO for calcium was characterized by $K_d$ of ~30 μM [8]. At that time, it was not known yet that $Ca^{2+}$ binding is counteracted by $Na^{+}$, and the high value of $K_d$ may be explained by the presence of $Na^{+}$ in the buffers. Subsequent studies performed in the absence of $Na^{+}$ and in the presence of calcium buffers gave consistently a value of $K_d$ around 1 μM for bovine CcO at slightly alkaline pH 7.5–8 [12,16]. A somewhat higher value of $K_d$ = 11 μM has been reported recently by P. Rich and coworkers with the aid of ATR-FTIR technique [17]. These data were obtained at slightly acidic pH = 6.5 and, accordingly, $K_d$ of 17 μM at pH 6 was determined in this group following the absorption shift of heme $a^{2+}$ (unpublished data of T.V. Vygodina).

We were then excited to observe a much higher affinity of CcO for calcium in one of our recent preparations of bovine CcO. Accidentally, the preparation was obtained with characteristics of the so-called "slow" form of CcO [33,34] as evidenced by a position of the Soret band of the oxidized enzyme at ~418 nm instead of 424–425 nm and by slow binding of cyanide with major fraction of the oxidized enzyme. With this preparation, a conventional activation procedure (reduction by dithionite for 15–30 min followed by aerobic gel-filtration) did not result in restoration of the 424 nm Soret maximum, typical of the fast enzyme,

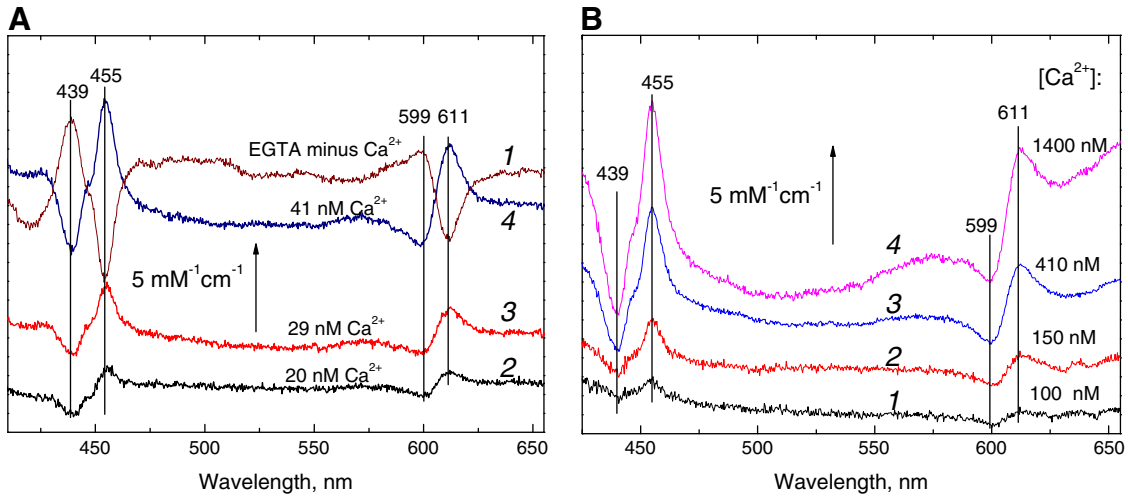


**Fig. 5.** Binding of calcium with "slow" CcO at nanomolar concentrations of the cation. 1.8 μM "slow" (A) or "fast" (B) CcO in 100 mM basic buffer pH 8 was converted to mixed-valence cyanide complex in the presence of 5 mM ascorbate, 0.1 mM TMPD and 4 mM KCN. Addition of 3 mM EGTA (A) or 5 mM HEDTA (B) brought about reversal of the red shift induced by adventitious calcium (cf. difference spectrum 1 in panel A), and the resulting absolute spectrum in the presence of excess chelator was taken as a baseline. Difference spectra vs the baseline induced by addition of increasing concentrations of $CaCl_2$ are shown. Concentrations of free $Ca^{2+}$ are indicated in figure panels.

and cyanide binding was stimulated but slightly. Experiments on the $Ca^{2+}$ binding were performed with this preparation either as isolated or after the nominal activation procedure without marked differences in the results.

The results are illustrated in Figs. 5 and 6. As shown in Fig. 5A, in the "slow" preparation the $Ca^{2+}$-induced red shift of heme $a^{2+}$ fully develops at free calcium concentration of ~40 nM (spectra 2–4). Under the same conditions, control experiments with the enzyme from one of the previous "fast" preparations show almost no response at 100 nM $Ca^{2+}$ and saturation is achieved only at several μM of the cation (Fig. 5B, Fig. 6). $K_d$ of 1.2 μM for calcium binding was also reproduced in a control titration with a preparation of CcO ("fast" enzyme) kindly provided by the laboratory of prof. A.D. Vinogradov.

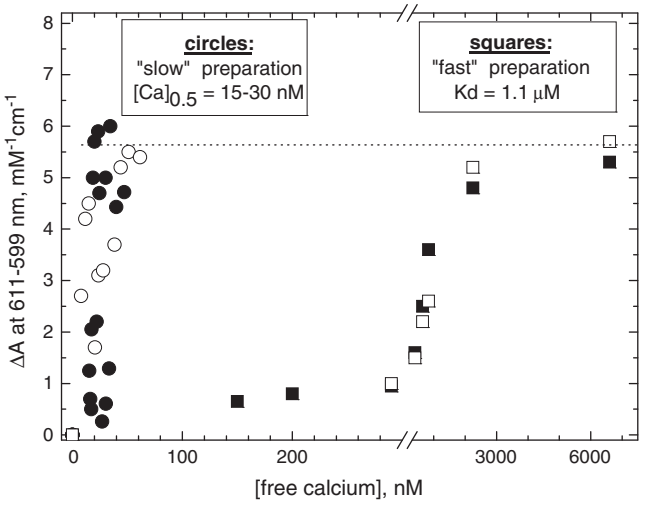


**Fig. 6.** Titrations of the amplitude of the $Ca^{2+}$-induced spectral shift of heme *a* in the "fast" and "slow" preparations of CcO. Basic conditions, as in Fig. 5. CcO concentration was 1.5–2 μM. In the case of the "slow" preparation (*circles*), 1 or 3 mM EGTA has been used as the $Ca^{2+}$ buffer. Data for three titrations in the forward direction with $Ca^{2+}$ (*filled circles*) and two titrations in the reversed direction with EGTA (*open circles*) are included. The dashed horizontal line indicates average maximal amplitude of the red shift induced by excess $Ca^{2+}$ in different titrations of the "slow" oxidase. In the control experiment with the "fast" preparation (*squares*), 5 mM HEDTA has been used to buffer free calcium concentration. *Open squares*, the reaction mixture contained additionally 10 μM cytochrome $c^{2+}$ in order to test possible anticooperative interactions between the binding of $Ca^{2+}$ and cytochrome $c^{2+}$ with CcO (cf. Section 3.2 under Results and discussion).

Concentration dependence of the calcium-induced spectral shift in the "slow" preparation of CcO was studied using EGTA, BAPTA or HEDTA as calcium buffers. Results of several independent titrations with 1–3 mM EGTA as the buffer and performed in both directions, i.e. increasing calcium or increasing EGTA, are showed in Fig. 6. It is to be noted that the titration curves deviated significantly from the normal binding isotherm and showed strong sigmoidicity, particularly in the direct titrations (increasing $Ca^{2+}$ concentration). Hysteretic behavior and significant scatter between the individual titrations were observed, so we consider these results as preliminary until the reasons for this odd behavior are figured out. Nevertheless, all the titrations showed full response attained below 0.1 μM of the cation (Fig. 6). Possibly, there may be problems of equilibration at high affinities for the cation and relatively low rate constants for binding and dissociation of $Ca^{2+}$ ($k_{on}$ = 5–10 × $10^3$ $M^{-1}$ $s^{-1}$ [15,35]). In view of the complications, correct values of $K_d$ remain to be determined and we use empirical [free $Ca^{2+}$]$_{0.5}$ values to characterize the binding. These values varied for separate repetitive titrations in Fig. 6 in the range 15–30 nM. Importantly, $Na^+$ ions decreased the affinity of the "slow" oxidase for calcium ions, like what was observed with the "fast" enzyme. Thus in the presence of 10 mM $Na^+$ corresponding to physiological concentration of sodium ions in cytoplasm, the $Ca^{2+}$-induced spectral shift of heme $a^{2+}$ titrated in the "slow" CcO with [free $Ca^{2+}$]$_{0.5}$ of 110–120 nM (data not shown).

The high affinity of the "slow" preparation for calcium as revealed by the spectral shift of heme *a* was accompanied by other known effects of calcium on the enzyme in the same concentration range. Thus 50 nM free calcium brought about increased reduction of heme *a* equilibrated with the ferrocyanide/ferricyanide redox buffer (data not included) consistent with the ~20 mV positive shift in $E_m$ of heme *a* induced by $Ca^{2+}$ [16,36].

In this connection, it is interesting that high-affinity binding of $Ca^{2+}$ ($K_d$ = 6 nM) was observed earlier for CcO from *Rhodobacter sphaeroides* with D485A substitution [15], whereas homologous CcO mutant D477A from *P. denitrificans* revealed $K_d$ of ~1 μM [13,16]. Time-resolved studies of $Ca^{2+}$ binding with the D485A mutant oxidase from *R. sphaeroides* showed that the binding was not a single-step process. As one possible interpretation of those data, initial rapid binding of $Ca^{2+}$ with relatively low affinity of 0.2 μM is followed by a slow spectrally silent transition to the high-affinity complex with $K_d$ of 6 nM [15]. It is tempting to suggest that also the bovine enzyme can exist in the states with low ($K_d$ ~$10^{-6}$ M) and high affinities for the cation ($K_d$ ~ $10^{-8}$–$10^{-7}$ M).

Whether the "slow" form of CcO is present in the cells and is a physiologically relevant form of the enzyme is an interesting question that remains to be explored.

As a word of caution, we would emphasize that at the moment we are not yet in the position to conclude unambiguously which features of our accidental "slow" preparation exactly are responsible for the high-affinity binding of $Ca^{2+}$. It is conceivable that bovine hearts from a slaughter-house is not as standard biological material as the tissues from laboratory animals. Notably, the second isolation of CcO from the same batch of bovine heart mitochondria that were kept deep-frozen yielded a preparation with the Soret peak of the oxidized form at 424 nm, and this preparation showed the usual affinity for $Ca^{2+}$ with $K_d$ close to 1 μM. Therefore, the high affinity for calcium may be indeed related to the "slow" form of CcO rather than to some unusual characteristics of the heart tissue used for the preparation. Experiments to establish whether conventional procedures converting "fast" CcO to the "slow" form, e.g. by low pH treatment [33,34,37], will result in increased affinity of the enzyme for calcium are in progress.

There are several speculations that may be inferred provisionally from this part of the results.

(1) CcO can exist in a form with very high affinity for $Ca^{2+}$ that is able to "sense" the cation at submicromolar concentrations close to those in the resting cells.
(2) Tightly-bound $Ca^{2+}$ could participate in fastening CcO in the "slow" form.
(3) Transition of CcO from the "fast" to the "slow" form is known to be associated with the structural changes around the oxygen-reducing center, $a_3/Cu_B$, whereas the CBS locates near the electron entry domain of CcO (CuA, heme *a*). If transition of CcO from the "fast" to the "slow" form is associated with increased affinity of the CBS for $Ca^{2+}$ ions, this effect may point out the communication between the two spatially and functionally separate sites. Such communication could play an important role in tuning the electron and proton transfer through the enzyme.

Other indications to long range structural communication between the electron entry site and the binuclear center [38,39] were pointed out by a reviewer of this paper. In particular, the effects induced by R481K replacement in *R. sphaeroides* CcO [38,40] allow us to suggest that ligation of $Ca^{2+}$ by D442 and S441 in bovine oxidase could transmit structural perturbation to the hemes via the arginines R439 and R438 (homologous to R482 and R481 in *R. sphaeroides*) that make bonds to propionates of the hemes *a* and $a_3$ and which are located in the same cytoplasmic loop between the transmembrane helices XI and XII as the calcium ligating residues S441 and D442. Conceivably, transmission of a structural perturbation from the CBS to the binuclear site could play a role in the mechanism of CcO inhibition by calcium.

### 3.4. General discussion: possible physiological significance of $Ca^{2+}$ and $Na^+$ interaction with the Cation Binding Site of cytochrome oxidase

It is a question of obvious interest and significance — what physiological role of CcO regulation by metal cations may be. As pointed out by the reviewers of this and previous [19] papers, the inhibition of CcO by $Ca^{2+}$ may be thought to contradict a conventional concept of oxidative phosphorylation being stimulated by $Ca^{2+}$ (cf. [41,42] and refs. therein). In our opinion, this contradiction may be apparent because the stimulating effect of $Ca^{2+}$ on oxidative phosphorylation and its inhibitory effect on CcO are expected to occur in the cell at different concentrations of extramitochondrial calcium. As discussed in [19], $K_d$ for $Ca^{2+}$ binding with "fast" CcO at typical intracellular concentration of $Na^+$ (~10 mM) is around 10 μM. Therefore, any significant inhibitory effect of the cation on CcO in the cell will occur only at concentration of free $Ca^{2+}$ above some 5 μM. At the same time, activation of the mitochondrial Krebs cycle dehydrogenases as well as of the citrin- or aralar-type substrate transporters responsible for the stimulation of the mitochondrial respiration is observed at concentrations of extramitochondrial free $Ca^{2+}$, ca. 0.3–5 μM [43–45]. So an increase in cytoplasmic concentration of free $Ca^{2+}$ up to a few μM can stimulate oxidative phosphorylation, whereas CcO will remain fully protected from the calcium inhibition by sodium ions.

At the same time, during the $Ca^{2+}$ outbursts from ER or extracellular stores, local cytoplasmic concentrations of $Ca^{2+}$ can rise transiently well above 10 μM (the values as high as 20–100 μM are cited in [46,47]) in the "microdomains" near mitochondria forming an interface with the sarco–endoplasmic reticulum [48,49], particularly in the contractile and excitable tissues. At the peaks of the $Ca^{2+}$-spikes, $Na^+$ can be displaced from the Cation Binding Site by calcium and transient inhibition of CcO by extramitochondrial $Ca^{2+}$ is expected to take place. The inhibition will be released shortly as the extruded $Ca^{2+}$ is taken back by ER, sucked in by mitochondria and diffuses outside the "microdomains" so that its local concentration goes below ~5 μM. Biphasic effect of calcium on the mitochondrial respiration – i.e. stimulation up to ~2 μM of extramitochondrial $Ca^{2+}$ followed by inhibition at higher concentrations – can be found, for instance, in Refs. [45,50].

The short term inhibition of CcO at the peaks of the calcium spikes may have several functional implications as briefly considered below.

#### 3.4.1. Prevention of excessive $Ca^{2+}$ uptake by mitochondria

Transient inhibition of CcO during the $Ca^{2+}$ spikes can reduce excessive uptake of $Ca^{2+}$ by mitochondria driven by the respiratory chain. This will lower the risk of mitochondria overloading with calcium with all the negative consequences. Besides, it will reduce energy expenses for superfluous calcium transportation to the matrix via the $Ca^{2+}$-uniporter followed by its subsequent release to cytoplasm via the $Na^+/Ca^{2+}$ exchanger, both processes consuming energy of membrane potential [47]. Damping the effects of the oscillations of extramitochondrial calcium would be particularly reasonable in the case of mitochondria in the beating heart.

#### 3.4.2. Generation of ROS by mitochondria

Stimulation of electron input to the respiratory chain by activation of the dehydrogenases and substrate transporters [43–45] and simultaneous inhibition of CcO at the peaks of $Ca^{2+}$ efflux from ER or extracellular stores in response to various stimuli could have a cumulative effect resulting in increased reduction of the respiratory chain components. Accordingly, stimulation of the mitochondrial ROS production by $Ca^{2+}$ spikes is anticipated. This transient ROS production may be involved in the calcium-dependent intracellular signaling [49,51].

#### 3.4.3. Modulation of intracellular calcium signal transmission through inhibition of $Ca^{2+}$ uptake by mitochondria

When calcium is released from ER or extracellular stores, it is taken up actively by mitochondria and this uptake modulates the intracellular calcium waves. Interplay between ER and mitochondria that operate as dynamic calcium stores shapes the intracellular calcium signal in time and space [49,51,52]. The uptake of $Ca^{2+}$ by mitochondria is driven primarily by membrane potential across the inner mitochondrial membrane generated by the respiratory chain. Therefore, inhibition of CcO at the peak concentrations of cytoplasmic $Ca^{2+}$ may be expected to finely tune the shaping of the calcium signals in the cell.

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## References


[1] S. Ferguson-Miller, G.T. Babcock, Heme/copper terminal oxidases, Chem. Rev. 7 (1996) 2889–2907.
[2] R.B. Gennis, Coupled proton and electron transfer reactions in cytochrome oxidase, Front. Biosci. 9 (2004) 581–591.
[3] I. Belevich, M.I. Verkhovsky, Molecular mechanism of proton translocation by cytochrome *c* oxidase, Antioxid. Redox Signal. 10 (2008) 1–29.
[4] S. Yoshikawa, K. Muramoto, K. Shinzawa-Itoh, Proton-pumping mechanism of cytochrome *c* oxidase, Annu. Rev. Biophys. 40 (2011) 205–223.
[5] A.A. Konstantinov, Cytochrome *c* oxidase: intermediates of the catalytic cycle and their energy-coupled interconversion, FEBS Lett. 586 (2012) 630–639.
[6] M.M. Pereira, M. Santana, M. Teixeira, A novel scenario for the evolution of haem-copper oxygen reductases, Biochim. Biophys. Acta 1505 (2001) 185–208.
[7] S. Yoshikawa, K. Shinzawa-Itoh, R. Nakashima, R. Yaono, N. Inoue, M. Yao, M.J. Fei, C.P. Libeu, T. Mizushima, H. Yamaguchi, T. Tomizaki, T. Tsukihara, Redox-coupled crystal structural changes in bovine heart cytochrome *c* oxidase, Science 280 (1998) 1723–1729.
[8] M. Wikstrom, H. Saari, A spectral shift of heme *a* induced by calcium ions, Biochim. Biophys. Acta 408 (1975) 170–179.
[9] H. Saari, T. Pentilla, M. Wikstrom, Interaction of $Ca^{2+}$ and $H^+$ with heme A in cytochrome oxidase, J. Bioenerg. Biomembr. 12 (1980) 325–338.
[10] C. Ostermeier, A. Harrenga, U. Ermler, H. Michel, Structure at 2.7 A resolution of the *Paracoccus denitrificans* two-subunit cytochrome *c* oxidase complexed with an antibody Fv fragment, Proc. Natl. Acad. Sci. U. S. A. 94 (1997) 10547–10553.
[11] H. Mkrtchyan, T. Vygodina, A.A. Konstantinov, $Na^+$-induced reversal of the $Ca^{2+}$-dependent red shift of cytochrome *a*. Is there a hydronium output well in cytochrome *c* oxidase? Biochem. Int. 20 (1990) 183–190.
[12] A. Kirichenko, T.V. Vygodina, H.M. Mkrtchyan, A.A. Konstantinov, Specific cation-binding site in mammalian cytochrome *c* oxidase, FEBS Lett. 423 (1998) 329–333.
[13] U. Pfitzner, A. Kirichenko, A.A. Konstantinov, M. Mertens, A. Wittershagen, B.O. Kolbesen, G.C.M. Steffens, A. Harrenga, H. Michel, B. Ludwig, Mutations in the $Ca^{2+}$-binding site of the *Paracoccus denitrificans* cytochrome *c* oxidase, FEBS Lett. 456 (1999) 365–369.
[14] S. Riistama, L. Laakkonen, M. Wikstrom, M.I. Verkhovsky, A. Puustinen, The calcium binding site in cytochrome $aa_3$ from *Paracoccus denitrificans*, Biochemistry 38 (1999) 10670–10677.
[15] A. Lee, A. Kirichenko, T. Vygodina, S.A. Siletsky, T.K. Das, D.L. Rousseau, R.A. Gennis, A.A. Konstantinov, $Ca^{2+}$-binding site in *Rhodobacter sphaeroides* cytochrome *c* oxidase, Biochemistry 41 (2002) 8886–8898.
[16] A.V. Kirichenko, U. Pfitzner, B. Ludwig, C.M. Soares, T.V. Vygodina, A.A. Konstantinov, Cytochrome *c* oxidase as a calcium binding protein. Studies on the role of a conserved aspartate in helices XI–XII cytoplasmic loop in cation binding, Biochemistry 44 (2005) 12391–12401.
[17] A. Marechal, M. Iwaki, P.R. Rich, Structural changes in cytochrome *c* oxidase induced by binding of sodium and calcium ions: an ATR-FTIR Study, J. Am. Chem. Soc. 135 (2013) 5802.
[18] T.V. Vygodina, A.A. Konstantinov, $Ca^{2+}$-induced inhibition of the mammalian cytochrome *c* oxidase, Biochim. Biophys. Acta 1797 (2010) 102.
[19] T. Vygodina, A. Kirichenko, A.A. Konstantinov, Direct regulation of cytochrome *c* oxidase by calcium ions, PLoS ONE 8 (2013) e74436.
[20] S. Yoshikawa, A cytochrome *c* oxidase proton pumping mechanism that excludes the $O_2$ reduction site, FEBS Lett. 555 (2003) 8–12.
[21] L.R. Fowler, S.H. Richardson, Y. Hatefi, A rapid method for the preparation of highly purified cytochrome oxidase, Biochim. Biophys. Acta 64 (1962) 170–173.
[22] C. Patton, S. Thompson, D. Epel, Some precautions in using chelators to buffer metals in biological solutions, Cell Calcium 35 (2004) 427–431.
[23] K. Carter, G. Palmer, Models of the two heme centers in cytochrome oxidase, J. Biol. Chem. 257 (1982) 13507–13514.
[24] G.T. Babcock, P.M. Callahan, Origin of the cytochrome *a* absorption red shift: a pH-dependent interaction between its heme *a* formyl and protein in cytochrome oxidase, Biochemistry 22 (1983) 452–461.
[25] S. Riistama, M.I. Verkhovsky, L. Laakkonen, M. Wikstrom, A. Puustinen, Interaction between the formyl group of heme *a* and arginine 54 in cytochrome $aa_3$ from *Paracoccus denitrificans*, Biochim. Biophys. Acta 1456 (2000) 1–4.
[26] D.A. Mills, B. Schmidt, C. Hiser, E. Westley, S. Ferguson-Miller, Membrane potential-controlled inhibition of cytochrome *c* oxidase by zinc, J. Biol. Chem. 277 (2002) 14894–14901.
[27] S.S. Kuznetsova, N.V. Azarkina, T.V. Vygodina, S.A. Siletsky, A.A. Konstantinov, Zinc ions as cytochrome *c* oxidase inhibitors: two sites of action, Biochem. Mosc. 70 (2005) 128–136.
[28] T.V. Vygodina, W. Zakirzyanova, A.A. Konstantinov, Inhibition of membrane-bound cytochrome *c* oxidase by zinc ions: high-affinity $Zn^{2+}$-binding site at the *P*-side of the membrane, FEBS Lett. 582 (2008) 4158–4162.
[29] A. Aagaard, A. Namslauer, P. Brzezinski, Inhibition of proton transfer in cytochrome *c* oxidase by zinc ions: delayed proton uptake during oxygen reduction, Biochim. Biophys. Acta 1555 (2002) 133–139.
[30] K. Muramoto, K. Hirata, K. Shinzawa-Itoh, S. Yoko-o, E. Yamashita, H. Aoyama, T. Tsukihara, S. Yoshikawa, A histidine residue acting as a controlling site for dioxygen reduction and proton pumping by cytochrome *c* oxidase, Proc. Natl. Acad. Sci. U. S. A. 104 (2007) 7881–7886.
[31] M. Huttemann, I. Lee, A. Pecinova, P. Pecina, K. Przyklenk, J.W. Doan, Regulation of oxidative phosphorylation, the mitochondrial membrane potential, and their role in human disease, J. Bioenerg. Biomembr. 40 (2008) 445–456.
[32] S. Helling, M. Huttemann, R. Ramzan, S.H. Kim, I. Lee, T. Muller, E. Langenfeld, H.E. Meyer, B. Kadenbach, S. Vogt, K. Marcus, Multiple phosphorylations of cytochrome *c* oxidase and their functions, Proteomics 12 (2012) 950–959.
[33] A.J. Moody, C.E. Cooper, P.R. Rich, Characterisation of "fast" and "slow" forms of bovine heart cytochrome-*c* oxidase, Biochim. Biophys. Acta 1059 (1991) 189–207.
[34] A.J. Moody, "As prepared" forms of fully oxidised haem/Cu terminal oxidases, Biochim. Biophys. Acta 1276 (1996) 6–20.
[35] A. Kirichenko, Interaction of Cytochrome *c* Oxidase With Cations, (Master Thesis) M.V. Lomonosov Moscow State University, Moscow, 1995.
[36] T.V. Vygodina, V.V. Ptushenko, A.A. Konstantinov, $Ca^{2+}$ binding to cytochrome *c* oxidase affects redox properties of heme *a*, Biochim. Biophys. Acta 1777 (2008) S110–S111.
[37] A.J. Moody, C.E. Cooper, R.B. Gennis, J.N. Rumbley, P.N. Rich, Interconversion of fast and slow forms of cytochrome *bo* from *Escherichia coli*, Biochemistry 34 (1995) 6838-6684.
[38] D.A. Mills, L. Geren, C. Hiser, B. Schmidt, B. Durham, F. Millett, S. Ferguson-Miller, An arginine to lysine mutation in the vicinity of the heme propionates affects the redox potentials of the hemes and associated electron and proton transfer in cytochrome *c* oxidase, Biochemistry 44 (2005) 10457–10465.
[39] Y. Zhen, B. Schmidt, U.G. Kang, W. Antholine, S. Ferguson-Miller, Mutants of the $Cu_A$ site in cytochrome *c* oxidase of *Rhodobacter sphaeroides*: I. Spectral and functional properties, Biochemistry 41 (2002) 2288–2297.
[40] G. Branden, M. Branden, B. Schmidt, D.A. Mills, S. Ferguson-Miller, P. Brzezinski, The protonation state of a heme propionate controls electron transfer in cytochrome *c* oxidase, Biochemistry 44 (2005) 10466–10474.
[41] A.I. Tarasov, E.J. Griffiths, G.A. Rutter, Regulation of ATP production by mitochondrial $Ca^{2+}$, Cell Calcium 52 (2012) 28–35.
[42] B. Glancy, W.T. Willis, D.J. Chess, R.S. Balaban, Effect of calcium on the oxidative phosphorylation cascade in skeletal muscle mitochondria, Biochemistry 52 (2013) 2793–2809.
[43] R.M. Denton, Regulation of mitochondrial dehydrogenases by calcium ions, Biochim. Biophys. Acta 1787 (2009) 1309–1316.
[44] J. Satrustegui, B. Pardo, A. Del Arco, Mitochondrial transporters as novel targets for intracellular calcium signaling, Physiol. Rev. 87 (2007) 29–67.
[45] F.N. Gellerich, Z. Gizatullina, S. Trumbeckaite, N.P. Nguyen, T. Pallas, O. Arandarcikaite, S. Vielhaber, E. Seppet, F. Striggow, The regulation of OXPHOS by *extra*mitochondrial calcium, Biochim. Biophys. Acta 1797 (2010) 1018–1027.
[46] A. Spat, G. Szanda, G. Csordas, G. Hajnoczky, High and low calcium-dependent mechanisms of mitochondrial calcium signalling, Cell Calcium 44 (2008) 51–63.
[47] E.J. Griffiths, D. Balaska, W.H.Y. Cheng, The ups and downs of mitochondrial calcium signalling in the heart, Biochim. Biophys. Acta 1797 (2010) 856–864.
[48] M.T. Alonso, C. Villalobos, P. Chamero, J. Alvarez, J. García-Sancho, Calcium microdomains in mitochondria and nucleus, Cell Calcium 40 (2006) 513–525.
[49] V. Eisner, G. Csordas, G. Hajnoczky, Interactions between sarco–endoplasmic reticulum and mitochondria in cardiac and skeletal muscle — pivotal roles in $Ca^{2+}$ and reactive oxygen species signaling, J. Cell Sci. 126 (2013) 2965–2978.
[50] F.N. Gellerich, Z. Gizatullina, H.P. Nguyen, S. Trumbeckaite, S. Vielhaber, E. Seppet, S. Zierz, B. Landwehrmeyer, O. Riess, S. von Horsten, F. Striggow, Impaired regulation of brain mitochondria by extramitochondrial $Ca^{2+}$ in transgenic Huntington disease rats, J. Biol. Chem. 283 (2008) 30715–30724.
[51] D.E. Clapham, Calcium signalling, Cell 131 (2007) 1047–1058.
[52] S.Y. Cheranov, J.H. Jaggar, Mitochondrial modulation of $Ca^{2+}$ sparks and transient $K_{Ca}$ currents in smooth muscle cells of rat cerebral arteries, J. Physiol. 556 (2004) 755–771.
[53] T. Tsukihara, K. Shimokata, Y. Katayama, H. Shimada, K. Muramoto, H. Aoyama, M. Mochizuki, K. Shinzawa-Itoh, E. Yamashita, M. Yao, Y. Ishimura, S. Yoshikawa, The low-spin heme of cytochrome c oxidase as the driving element of the proton-pumping process, Proc. Natl. Acad. Sci. U. S. A. 100 (2003) 15304–15309.
[54] K. Kamiya, M. Boero, M. Tateno, K. Shiraishi, A. Oshiyama, First-principles molecular dynamics study of proton transfer mechanism in bovine cytochrome *c* oxidase, J. Phys. Condens. Matter 19 (2007) 365220.
[55] K. Kamiya, M. Boero, M. Tateno, K. Shiraishi, A. Oshiyama, Possible mechanism of proton transfer through peptide groups in the H-pathway of the bovine cytochrome *c* oxidase, J. Am. Chem. Soc. 129 (2007) 9663–9673.