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Cytochrome *c* oxidase, ligands and electrons

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Abstract

We present hereby an overview of the reactions of cytochrome *c* oxidase, the terminal enzyme of the mitochondrial respiratory chain, with ligands (primarily oxygen) and electrons, pointing out where necessary unresolved facts or questionable interpretations.

Keywords: Cellular respiration; Oxidase; Intermediates; Electron transfer; Mechanisms

1. Introduction

The reaction of cytochrome *c* oxidase (CcOX) with O_2 has naturally attracted a lot of attention over the last decades because of its vital role in cellular respiration, given that the basic chemistry catalyzed by this enzyme is the reduction of O_2 to water with free energy gain to sustain bioenergetics. Therefore in this review, we illustrate the main aspects of the reaction of the reduced enzyme with O_2, as well as the relevant electron transfer processes involving the metal centers of this complex and intriguing integral membrane protein. Since the discovering of the "Respiratory enzyme" by Otto Warburg, CO proved to be a valuable ligand competing with O_2 at the active site of CcOX; thus, although it is a very poor poison of respiration (its concentration should exceed O_2 by more than 100-fold to achieve inhibition), we present important information available on the CO adduct of the reduced enzyme. Finally, we have reviewed the main features of the reactions of NO with CcOX, because over the last 10 years this gas was shown to be a potent, yet reversible inhibitor of respiration; its efficiency in blocking oxidase activity is such that in vivo basal levels of NO are sufficient to control respiration, the biochemical mechanisms involved being more complex than anticipated.

We have decided to limit our attention to work carried out with the purified enzyme in detergent solution, and have focused on mechanisms and general features of the role of the metals in the reactions of the reduced enzyme with these gaseous ligands, and of the pertinent electron transfer processes. Thus, we have not tackled the most challenging and yet unresolved function of CcOX, i.e. the mechanism of transmembrane proton pumping crucial to bioenergetics [1–8].

2. Structural information

The 3D structure of the enzyme is now available not only for beef heart CcOX [9–12], but also for three other cytochrome *c* oxidases, namely the aa_3-type oxidases from *Paracoccus denitrificans* [13–15] and from *Rhodobacter sphaeroides* [16], and the ba_3-type oxidase from the thermophilic bacterium *Thermus thermphilus* [17]; moreover also the structure of a ubiquinol oxidase (bo_3-type from *Escherichia coli*) [18] is available. Most of the structural information obtained by X-ray diffraction is for the completely oxidized enzyme, but in some cases

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the fully reduced state either unliganded (beef heart and *P. denitrificans* enzymes) or complexed with CO (beef heart enzyme) have been solved as well.

A schematic view of the crucial metals of beef heart CcOX is depicted in Fig. 1 (top panel). The bimetallic Cu_A site, which accepts electrons from cyt *c* [19–22] is located in a globular domain of subunit II protruding into the mitochondrial intermembrane space (the periplasmic space in bacteria). The tween Cu_A metals accept one electron from reduced cyt *c*, and in turn reduce rapidly (by intramolecular eT) heme *a*, located 19 Å away (Cu_A-to-Fe_a distance). The latter redox center is a bis-histidine low-spin heme, bound to subunit I and buried within the membrane-embedded part of the enzyme; the reduction of heme *a* by Cu_A is thus expected to be electrogenic and this was experimentally verified (see below). Although eT to heme *a* occurs without reorganization of the Fe coordination, structural changes around this site (which are believed to be crucial for the proton pumping mechanism) were observed upon reduction of the beef heart enzyme ([11,12], see below). From heme *a*, electrons are transferred intramolecularly to the active site, heme a_3 and Cu_B, where the gaseous ligands (O_2, CO and NO) bind. A pre-requisite for O_2 and CO binding, but not for NO, is the complete reduction of this binuclear site, as discussed below.

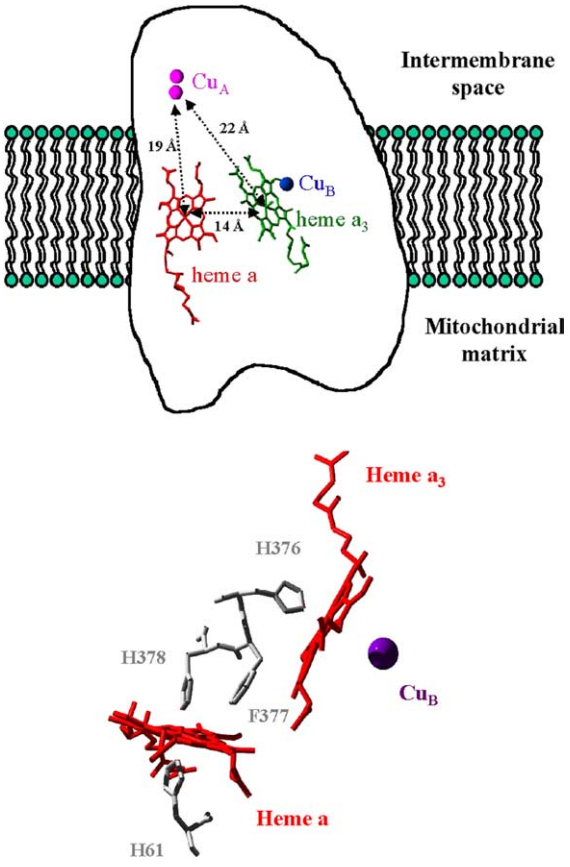


Fig. 1. Overall structure of CcOX and its redox active metals. From the Protein Data Bank coordinates of the oxidized beef heart CcOX, deposited by Yoshikawa et al. [11]. *Top*: Schematic representation of the redox metals of beef heart CcOX with their relative distances. *Bottom*: Structure of heme *a* and heme a_3. Heme *a* and heme a_3 are at a short distance (14 Å between the two metals) and connected by three residues on helix X of subunit I (His376, Phe377 and His378).

Heme *a* and heme a_3 (Fe_a-to-Fe_{a3} distance of 14 Å) are at approximately the same depth within the membrane-spanning moiety of subunit I (Fig. 1); this notwithstanding, eT from heme *a* is electrogenic, being coupled to an uptake of protons from the mitochondrial matrix (the cytoplasmic phase in bacteria). The iron of heme a_3 and Cu_B form a bimetallic site; the distance between the two metals depends on the redox-state in the beef heart enzyme (4.9 Å in the fully oxidized and 5.2 Å in the fully reduced state [11]), whereas it is independent of redox state (5.2 Å) in the *P. denitrificans* enzyme [15]. Heme a_3 is coordinated by one histidine (H376) [1] on the "proximal" side, whereas Cu_B is coordinated by three histidines (H290, H291 and H240). A totally unexpected finding, revealed by the structural work on beef heart CcOX, is that one of the Cu_B histidine ligands (H240) makes a covalent bond with an adjacent tyrosine (Y244). Such a covalent bond has been observed in most of the oxidase structures available, although in the *R. sphaeroides* enzyme the pertinent electron density suggested a mixed population with and without the Tyr–His bond [16]. The effect of the cross-link with H240 would be to lower the pK_a of Y244, making this residue a possible proton donor for O_2 intermediates bound at the active site [11]. Interestingly, Y244 has been postulated to be in a radical state in one of the O_2 intermediates of the catalytic cycle ([23], see below). However this Tyr residue is not conserved in the most divergent members of the superfamily of heme-copper oxidases, the so-called cbb_3-type oxidases [24].

An additional feature that became apparent from examination of the model of the oxidized beef heart enzyme is the presence of a magnesium ion at the interface between subunits I and II [9]. Such a metal, that can be replaced by manganese in bacterial cytochrome oxidases, is bound to H368, D369, E198 of subunit II and three water molecules in the bovine enzyme. Interestingly, E198 of subunit II is also coordinating Cu_A at the peptide carbonyl, suggesting a structural role of Mg in the stabilization of the Cu_A site; consistently, the Mg/Mn site is missing in the quinol oxidases which lack the Cu_A site. The Mg/Mn site is located on the top of the heme a_3-Cu_B site and it has been more recently suggested to be involved also in the exit pathway for protons/water molecules (see below).

In the fully oxidized state, an electron density between the iron of heme a_3 and Cu_B was identified in

[1] The aminoacid numbers refer to the beef heart enzyme, unless specified.

most of the available structures, although interpretation of this density is quite controversial. In the beef heart enzyme a peroxide was proposed to bridge the two metals [11], whereas in *P. denitrificans* a H_2O *plus* a OH^- were consistent with this electron density [14]. In contrast, in the *R. sphaeroides* [16] and in the *T. thermophilus* [17] enzymes this density was fitted to just one bridging oxygen (OH^- or a H_2O). The presence of a OH^- (or a H_2O) as a ligand of oxidized Cu_B had been inferred from analysis of EXAFS and ENDOR spectroscopic results [25]. Although undetected by X-ray crystallography, experimental evidence strongly suggests that a Cl^- ion is bound to oxidized Cu_B (replacing the OH^-) when the enzyme is purified in the presence of chloride, conferring characteristic spectroscopic and ligand binding properties [25–30]. Whatever the identity of the bridging ligand(s), in the fully reduced state the electron density between the two metals vanishes, indicating dissociation [11,15]. In the beef heart enzyme, together with the small but significant increase (0.3 Å) in the distance between heme a_3 and Cu_B, a redox-coupled structural change was also reported at the heme *a* moiety. Such a change, linked to the reduction of heme *a* as assessed by FTIR [31], was initially observed in the beef heart enzyme at a resolution of 2.35 Å [11] and more recently confirmed at 1.8/1.9 Å resolution [12]; however it has not been detected in the *Paracoccus* enzyme at 3.3 Å resolution [14]. This structural transition involves D51, which is connected through a hydrogen bond network to the formyl group of heme *a*. Upon reduction, D51 becomes exposed to the intermembrane space and a water channel, linking heme *a* to the matrix space, increases its volume; hence the suggestion that this structural transition may be at the heart of proton pumping in the beef heart enzyme. This hypothesis, though controversial, appears supported by very recent site-directed mutagenesis data [12].

Ligand binding to the fully reduced enzyme does not appear to induce major conformational changes, as inferred from inspection of the 3D structure of the CO adduct of reduced beef heart CcOX at 2.8 Å [11]. CO binds to ferrous heme a_3 in a bent configuration at 2.5 Å from Cu_B, suggesting a very weak interaction with this metal; upon binding, no significant structural changes of the protein moiety are detected by reference to the structure of the fully reduced enzyme.

In summary, at the present resolution CcOX appears as a fairly rigid protein, showing some conformational changes upon reduction but none upon ligand (CO) binding. However, extensive studies by FTIR spectroscopy provide evidence for structural changes upon reduction of and/or ligand binding (see [32] for a review on this issue); hence it would not be surprising if subtle but possibly significant conformational changes will become evident when the structure will be available at higher resolution.

Table 1
Overall rate constants for the reactions of reduced beef heart cytochrome *c* oxidase with O_2, CO and NO

	O_2	CO	NO
k_{on} (M^{-1} s^{-1})	$\sim 2 \times 10^8$	8×10^4	$0.4–1 \times 10^8$
k_{off} (s^{-1})	?	0.023	4×10^{-3}

Buffer: 0.1 M phosphate pH = 7.0–7.5. Detergent: 1% Tween 80 or 0.1% dodecyl-β-D-maltoside. Temperature = 20 °C. Relevant Refs.: [33–38,48].

3. Rates of reaction in the formation of the adducts

The reaction of O_2, CO and NO with the fully reduced binuclear center is a very fast process, which has been investigated over the last 40 years by rapid-mixing, temperature-jump, flash (or laser) photolysis, or a combination of the latter two techniques (flow-flash [33]). Photolysis proved particularly useful, taking advantage of the photolabile bond between CO and ferrous heme a_3. In the concentration range up to 1 atm of the gaseous ligands, the combination reaction follows simple bimolecular kinetics. The relevant bimolecular rate constants, reported in Table 1, were calculated from the ligand concentration dependence of the observed reactions in the binding to reduced heme a_3 of the beef heart enzyme.

Table 1 reports also the dissociation rate constants for CO and NO, as obtained by replacement experiments [33,34]; it is clear that both ligands have a very high affinity for reduced heme a_3. In the case of O_2, the reaction pattern is more complex, comprising a series of intermediates that will be discussed below. Here it may suffice to point out that the affinity of O_2 for reduced heme a_3-Cu_B in the very first O_2 adduct (called compound **A**), is quite low (K_d in the mM range) [35,36], but immediate eT to the bound O_2 leads to "kinetic trapping" [37], which makes it impossible to define an overall dissociation rate constant comparable to that determined for CO and NO.

4. Mechanism of ligand binding

A "gating" role of Cu_B^+ in the mechanism of CO binding was initially presumed on the basis of a classical experiment reported by Alben et al. [38], who made use of FTIR spectroscopy. These authors observed that upon illumination of CO-bound fully reduced CcOX at very low temperature (below 140 K), photolysis of the heme a_3^{2+}-CO bond was associated to loss of the typical C–O stretching frequency at 1963 cm^{-1} and to the appearance of a new band at 2062 cm^{-1}. The latter corresponds to a Cu^+–CO adduct, similar or identical to that of CO-hemocyanin, indicating that photolyzed CO diffuses a short distance in the site and binds to

Cu_B^+. This result provided a clue to the interpretation of the temperature dependence of the photolytic experiments reported in 1976 by Scharrock and Yonetani [39].

Many years later, time-resolved IR and UV–Vis spectroscopy was employed to investigate the kinetics of CcOX following CO photolysis, from picosecond onwards (see [40] and [41] for reviews). These studies showed that in less than 1 ps, CO is transferred from the Fe_{a3} to Cu_B [42], and from there it migrates into the bulk; at room temperature, the half time of the transient Cu_B–CO adduct was estimated to be about 1.5 μs in the beef heart enzyme [43]. A role for Cu_B^+ in the kinetic mechanism of CO binding was shown at room temperature by Woodruff and coworkers [43]. By performing laser-photolysis experiments at high CO pressures (up to 20 atm), these authors demonstrated that the observed rate constant for CO rebinding to reduced heme a_3 does not follow a linear, but rather a hyperbolic profile as a function of pCO. These data were interpreted as the evidence that Cu_B^+ acts as a *gate* for exogenous ligands. Data can indeed be accounted for by a kinetic model whereby CO, on the way to heme a_3, pre-equilibrates with Cu_B^+ with low affinity ($K = 87\ M^{-1}$), being thereafter rapidly transferred to heme a_3 (at $k = 1030\ s^{-1}$). The involvement of Cu_B was demonstrated in very elegant experiments on wild-type *E.coli* bo_3 quinol oxidase and on two mutants of this enzyme lacking Cu_B [44]; while the hyperbolic behavior was observed with the wild-type enzyme, loss of Cu_B was associated to disappearance of saturation kinetics, the observed rate constant following second order behavior, up to 20 atm ([CO] = 20 mM at 20 °C).

The next intriguing question is whether Cu_B^+ is the *gate* also for O_2 and NO on their pathway to the heme in the active site of CcOX. To address this issue, Bailey et al. [36] investigated the kinetics of O_2 binding to beef heart CcOX, by performing flow-flash experiments up to elevated O_2 pressures at room temperature. In this case as well, the observed rate constant for O_2 combination followed a hyperbolic behavior, yielding an asymptotic value of $\sim 1 \times 10^6\ s^{-1}$ and a half-saturation O_2 concentration of ~7.7 mM. Interpretation of these data, however, is perhaps more problematic than in the case of CO, because of the following problem. The experimental design demands that the enzyme is initially in the fully reduced CO-bound state; after mixing with an O_2-containing solution, a laser pulse leads to (instantaneous) CO dissociation, which allows the reaction with O_2 to begin. It cannot be excluded that in the case of O_2, the saturation kinetics may simply depend on the fact that O_2 binding may be rate limited by CO dissociation from Cu_B^+; it is in fact suspicious that the maximal rate constant at which O_2 binds ($\sim 1 \times 10^6\ s^{-1}$) is not very different from the rate of CO dissociation from Cu_B^+ ($7 \times 10^5\ s^{-1}$) [43]. Finally, even if O_2 could directly bind to heme a_3 bypassing CO still bound to Cu_B, some hindrance may affect the rate.

To circumvent this technical problem, Einarsdóttir and co-workers [45] developed a clever method for the rapid in situ production of O_2 by photodissociation of a synthetic caged dioxygen carrier. Unfortunately, due to the relatively low quantum yield of O_2 photoproduction, maximum concentration achieved was ~90 μM, which did not allow the authors to exclude a possible interference of CO in the flow-flash O_2 experiments illustrated above.

If the detailed mechanism of O_2 binding has still some unsettled features, this is even more so for NO. Like CO, photolysis experiments have shown that also NO transfers from heme a_3 to Cu_B, as detected by EPR experiments at ~5 K [46,47]. As reported by Blackmore et al. [48], there is no indication of deviation from a second-order behavior up to 1 mM NO, but as far as we know the kinetics of binding has not been measured at elevated [NO] as yet. In summary, it may be argued that, *by analogy with CO*, also the other gaseous ligands (O_2 and NO) are expected to use Cu_B^+ as a *gate* for combination with the reduced heme a_3 in the active site, but conclusive evidence is still lacking.

The relatively low affinity of Cu_B^+ for CO (and perhaps O_2 and NO) observed with the beef heart enzyme deserves some additional comment. This is not a universal property of all heme-copper oxidases since a number of bacterial oxidases have affinities of Cu_B^+ for CO much higher (more than 100-fold) than the beef heart enzyme. Interestingly, most of the oxidases displaying an unusually high affinity are preferentially expressed by hyperthermophilic microorganisms (like the ba_3-type cytochrome oxidase from *T. thermophilus* or the aa_3-type quinol oxidase from *Acidianus ambivalens*) or by mesophilic microorganisms growing under microaerobic conditions (as in the case of those expressing cbb_3-type oxidases). Since under these conditions environmental O_2 is limiting, we entertained the hypothesis [49] that if the enhanced affinity observed for CO reflects a higher affinity for O_2 as well, this may reveal an adaptative advantage for these microorganisms, by facilitating the reaction of their oxidases with O_2. To date, the structural basis for modulation of the variable Cu_B affinity is essentially unknown. In spite of these established differences, the structure of the heme a_3-Cu_B center in the cytochrome ba_3 from *T. thermophilus* [17] seems virtually identical to that of the beef heart enzyme at the present resolution; the only significant difference is a considerably larger length of the bond between the proximal histidine and the Fe of heme a_3 in the oxidized bacterial enzyme (3.3 Å) as compared to the mammalian enzyme (1.9 Å) [11].

In conclusion, the kinetic pathway for CO (and possibly also O_2 and NO) binding to and dissociation from reduced heme a_3 involves a low-affinity transient interac-

tion with reduced Cu_B, which is therefore presumed to be located on the entry/exit channel leading to the active site of the reduced enzyme. In the beef heart enzyme at room temperature, the affinity of the gaseous ligands for Cu_B^+ is very low indeed, while it was recently shown that in oxidases from other species the affinity is much higher (with possible physiological implications).

5. Oxidation state of the metals and the NO/O_2 competition

As outlined above, NO is a quick, potent yet reversible inhibitor of cellular respiration. Clear-cut evidence for this phenomenon, already anticipated by Carr and Ferguson [50], was independently provided by several groups in 1994 [51–53]. Such an inhibitory effect was subsequently shown to be due to binding of NO to CcOX, based (among other facts) on the competition between O_2 and NO in achieving inhibition (see [54,55] for reviews). To provide a ball-park figure, CcOX activity is halved at [NO] = 60 nM when [O_2] = 30 μM [52]; thus NO is a potent inhibitor of CcOX activity, $\approx 10^4$-fold more effective than CO. Such an observation immediately raised enormous interest because it was obvious that even a modest increase in intracellular NO fluxes would increase inhibition of respiration, with unforeseen consequences to bioenergetics. This scenario became more intriguing after the discovery of a *bona-fide* mitochondrial NO-synthase [56–59], which suggested that controlling NO fluxes in the mitochondrion would be a sensitive and reversible mechanism of modulating cellular respiration.

However, a glance at the relative rate constants for the binding of O_2 and NO to reduced CcOX reveals a conundrum, because the fairly similar values of the combination rates (see Table 1) cannot account for the fast efficient inhibition by NO. This stimulated extensive investigations on the mechanism of inhibition, and two groups [60,61] published an acceptable hypothesis to explain this apparent inconsistency, based on the following considerations.

It is unequivocally established that binding of CO to CcOX demands that both metals in the binuclear center are fully reduced; the first compelling evidence was provided by an analysis of redox titrations of CcOX under CO, as reported by Lindsay et al. [62]. Subsequently, this was confirmed by kinetic experiments using CO as a trap for reduced metals [63] and extended to O_2; thus it is generally accepted that both O_2 and CO can bind with physiologically meaningful affinities only to a fully reduced heme a_3-Cu_B site. However, this seems not to be the case for NO, which binds to reduced heme a_3 even when the binuclear center is only half reduced (an intermediate in the reduction pathway indicated as $\mathbf{E_1} \leftrightarrow \mathbf{E_2}$, see below). Experimental evidence in support of this hypothesis was recently provided by Giuffrè et al. [64] exploring the reaction of NO with a mutant of *Paracoccus* CcOX, the K354M mutant in the so-called K proton-conducting channel [13].

In conclusion, NO can efficiently bind to the enzyme when the binuclear site is both fully or half reduced, while O_2 cannot. Simulations carried out using a simplified mechanism [61] indicated that this unique possibility can account for the observed inhibition and for the O_2/NO competition. Definitive proof may demand the availability of a protocol to isolate state $\mathbf{E_1}/\mathbf{E_2}$ with a partially reduced binuclear site to allow direct experiments with NO (which should bind) and with O_2 (which should not).

6. A role for Cu_B in NO-reductase activity

Giuffrè et al. [65] were the first to show that *bona-fide* CcOXs (the ba_3 oxidase and the caa_3 oxidase, both from *T. thermophilus*) are endowed with NO reductase activity, catalyzing the reaction $2NO + 2H^+ + 2e^- \rightarrow N_2O + H_2O$, while beef heart CcOX is not. This finding was subsequently extended to the cytochrome cbb_3 from *Pseudomonas stutzeri* [66]. Interestingly, the latter enzyme displayed the highest NO reductase activity, an intriguing finding given that the cbb_3-type oxidases are the most divergent members of the heme-copper oxidases superfamily, sharing notable similarities with bacterial NO-reductase [67]. Here, we wish to present some consideration on the role of Cu_B in accounting for the efficiency (or lack of) NO-reductase activity by oxidases. The hypothesis that we put forward [65] was based on the idea that this accessory activity critically depends on the affinity of reduced Cu_B for the gaseous ligand (a feature outlined above); according to this hypothesis, the higher affinity of Cu_B for CO should correlate with a higher NO-reductase activity.

Recent experimental work carried out by time-resolved FTIR spectroscopy by Varotsis and co-workers [68–70] seems to support our hypothesis. These authors, by extending pioneering studies carried out more than 10 years ago [40], provided evidence that the three above-mentioned bacterial oxidases (from *T. thermophilus* and *P. stutzeri*) that display measurable NO-reductase activity are indeed characterized by very peculiar ligand binding dynamics at the Cu_B site. As outlined above, in the beef heart enzyme the photodissociated CO escapes the active site via a transient adduct on Cu_B^+ ($t_{1/2} \sim 1.5$ μs) [43]. In contrast, in the *T. thermophilus* ba_3 oxidase [69] and in the *P.stutzeri* cbb_3 oxidase [68], photolyzed CO instantly binds to the nearby Cu_B, but instead of diffusing out in the solvent, it re-combines with heme a_3 at longer times ($t_{1/2} \sim 24$ ms and $t_{1/2} \sim 140$ μs for the ba_3 and the cbb_3 enzymes, respectively). These new data, carried out at 1 mM CO, are further indication that the affinity constant of Cu_B^+ for CO is

unusually high in these enzymes ($K > 10^4$ M^{-1}), as compared to the beef heart oxidase ($K = 87$ M^{-1}). The behavior of *T. thermophilus caa*$_3$ displaying some NO-reductase activity [65], is somewhat intermediate, with partial (~35%) escape of Cu_B-bound CO from the site, followed by back-transfer to heme a_3 ($t_{1/2} \sim 20$ ms) [70]. More recently, a "docking site" for gaseous ligands has been hypothesized near the ring A propionate of the *Thermus* ba_3 enzyme, with possible physiological relevance [71].

In conclusion, modulation of the coordination stereochemistry of reduced Cu_B may control the affinity of this metal for the gaseous ligands, a pre-requisite to carry out the complex chemistry involved in the reduction of two NO molecules to N_2O. The major catalytic subunit of bacterial NO-reductase displays some suggestive structural similarities with sub I of CcOX, having a binuclear site constituted by a protoheme and a non-heme Fe [72]. On this basis it was proposed by Saraste and Castresana [73] and by van der Oost et al. [74] that oxidases and bacterial NO-reductases share a common phylogeny. It will be interesting to see how far site directed mutagenesis of the Cu_B site will be able to engineer a classical CcOX to acquire high NO-reductase activity.

7. Formation and decay of the O_2 intermediates

A major effort has been invested in the characterization of the intermediates in the reaction of O_2 with reduced CcOX (see [41,75,76] for reviews). The vast majority of the results made use of the "flow-flash" approach introduced by Gibson and Greenwood [33] over 40 years ago. As outlined above, the CO derivative of beef heart CcOX is mixed with an oxygenated buffer, and immediately (<0.1 s) hit with a short pulse of light. The binuclear site *stripped* of CO is free to bind O_2, and the formation and decay of several intermediates can be followed from μs upwards by time-resolved spectroscopy (mostly UV/Vis absorption and Resonance Raman). It may be recalled that the availability of a super-rapid mixing device (dead-time ~20 μs) made it possible to confirm some of the results obtained starting from the fully reduced enzyme, thus in the absence of CO [77]. Obviously the considerations that follow are also based on analysis of the O_2 intermediates when trapped at low or ultra-low temperatures, using essentially a triple-trapping technique introduced by Chance [78].

A scheme showing the intermediates in the reaction with O_2 is reported in Fig. 2. Upon CO-photolysis, O_2 rapidly ($t_{1/2} \sim 10$ μs) combines with reduced heme a_3 yielding the first intermediate, called compound **A**; the rate of formation of this species, a real O_2 adduct analogous of the oxy-ferrous complex in hemoglobin or myoglobin [79], is [O_2] dependent. Compound **A** is short-lived due to very fast delivery of electrons to bound O_2. Depending on the possibility of eT from heme *a*, **A** yields two (apparently) distinct intermediates, collectively called **P** (from peroxy). If an electron is available on heme *a*, compound **A** decays to the so-called $\mathbf{P_R}$ intermediate concomitantly with the oxidation of heme *a* ($t_{1/2} \sim 40$–50 μs). Alternatively, if further electrons are not available on the protein metals beyond

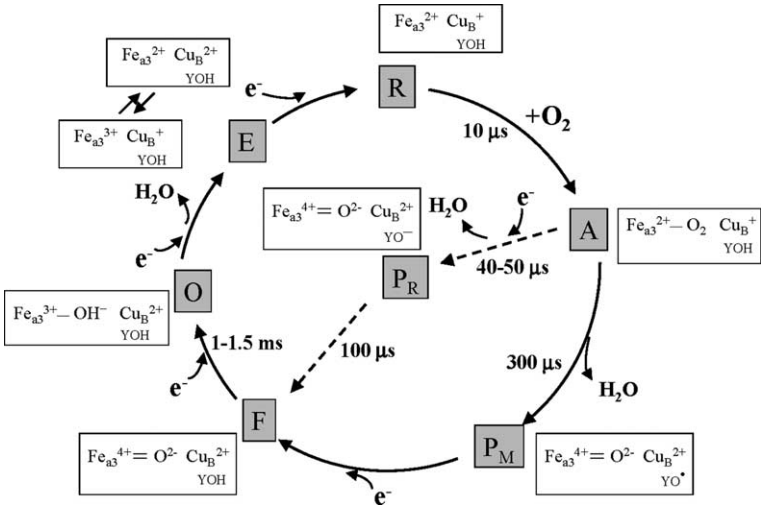


Fig. 2. The catalytic cycle. Scheme of the catalytic cycle of CcOX with the indication of the redox and the ligation state of the metals in the active site. The scheme also reports the putative state of Tyr244 in each intermediate and the half-time of the different transitions, as measured in the reaction of the fully reduced enzyme with O_2 by the flow-flash approach of Gibson and Greenwood [33]. Whenever indicated, electrons are transferred to the active site via heme *a*. The oxidized species **O** is fully reduced to **R**, by two single-electron donations via formation of a putative half-reduced intermediate **E**, in which the donated electron is either on Fe_{a3} or on Cu_B (the former being indicated as $\mathbf{E_1}$ and the latter as $\mathbf{E_2}$). Upon reaction with O_2, **R** converts into **A**, which decays to **F** via formation of either $\mathbf{P_R}$ or $\mathbf{P_M}$, depending on the redox state of heme *a*. Afterwards, the oxidized state **O** is restored from **F** through transfer of a further electron. Tyr244 seems to have radical character in the $\mathbf{P_M}$ intermediate only.

those residing on the heme a_3-Cu_B site, compound **A** decays to the species called $\mathbf{P_M}$ ($t_{1/2} \sim 300$ μs): the latter intermediate can be obtained in a relatively stable form (few minutes) by oxygenation of the so-called mixed valence CO-enzyme (i.e. CcOX with CO bound to reduced heme a_3 and Cu_B and oxidized heme *a* and Cu_A) [80].

The assessment of the chemistry of the **P** intermediates has been very complex and indeed it is still a matter of debate (see for instance the recent report by van Eps et al. [81]). The initial hypothesis [82] that **P** could be a peroxy-complex with oxidized heme a_3 and Cu_B had to be revised, and now-a-days there is a consensus on the idea that $\mathbf{P_M}$ and $\mathbf{P_R}$ are both oxo-ferryl adducts. This conclusion, already suggested by Weng and Baker [83], was strongly supported by extensive Resonance Raman spectroscopic investigations (see references in [84]), and eventually confirmed (at least for $\mathbf{P_M}$) by mass-spectrometry [85]. The finding that $\mathbf{P_M}$ is an oxo-ferryl adduct has important implications: first of all, the O–O bond is already cleaved (with production of a H_2O molecule) and, therefore, both O atoms are already at oxidation level = −2. This demands overall delivery of 4 electrons, though only 3 are formally available in the heme a_3-Cu_B center (2 from Fe_{a3} and 1 from Cu_B), leading to the suggestion that a protein residue could provide the missing electron. From examination of the 3D structure of beef heart CcOX, an involvement of Tyr244 was suggested and a radical character of Tyr244 in intermediate $\mathbf{P_M}$ was later confirmed by radioactive iodide labeling [23]; however, it remains to be established whether Tyr244 actually acts as the primary electron donor in the O_2 reduction or if its radical character is just the result of an intramolecular radical migration. Since in the case of $\mathbf{P_R}$ the fourth electron for O_2 reduction is provided by heme *a*, in this intermediate the occurrence of a protein residue seems unlikely, though not yet excluded.

By reference to Fig. 2, it may be seen that $\mathbf{P_M}$ and $\mathbf{P_R}$ both convert into the same intermediate (called **F**), but following distinct pathways and kinetics. The decay of $\mathbf{P_R}$ is fast ($t_{1/2} \sim 100$ μs) without demanding further eT from heme *a*. This is not the case for $\mathbf{P_M}$ that can proceed in the catalytic cycle only at the rate at which it is reduced by heme *a*. The resulting intermediate **F** is also an oxo-ferryl intermediate, as assessed by time resolved Resonance Raman spectroscopy [86]; thus, it is likely that the radical residue in $\mathbf{P_M}$ is the electron acceptor in the $\mathbf{P_M} \rightarrow \mathbf{F}$ transition. Finally, **F** coverts to the fully oxidized state **O** upon arrival of a further electron from Cu_A/heme *a* ($t_{1/2} \sim 1$–1.5 ms).

Recent experimental data published by Wikstrom's group [8,87] indicate that the proton pumping yield of liposome reconstituted CcOX depends on the *age* of the oxidized enzyme. In particular they observed that the number of H^+ translocated across the membrane upon reduction of **O** is greater if the proton pump is tested immediately after oxidation of the reduced enzyme **R**. They correlated this important observation with the discovery of *pulsed* CcOX reported by Antonini et al. [88]. These authors demonstrated that the functional and spectroscopic properties of oxidized CcOX produced by reaction of **R** with O_2 are different from those of the *resting* enzyme, an unexpected finding which at the time helped to rationalize a number of previously obscure observations; they also measured the rate of the *pulsed*-to-*resting* decay which was shown to be quite variable depending on conditions. This original observation was confirmed and extended by others [89] that called the same state *fast* on the basis of the reaction rate with cyanide. Wilson et al. [90] proposed a general two-state model involving a transition between *pulsed* and *resting*, which may now have acquired significance for the proton pumping function of CcOX thanks to the discovery by Bloch et al. (see [8] for a discussion of the phenomenon).

A point of general interest may be made to contrast the very short half-life of the intermediates with their populations at steady-state, during turnover. This is an aspect which is often overlooked, since it is generally felt that the population of these intermediates (say **P** and **F**) at steady-state should be minuscule given that their decay rates are so fast. This is the case when and if the full complement of 4 electrons is already stored in the enzyme, as for the fully reduced species. However this is often not applicable during turnover, given that the rate of electron entry from cyt *c* (via Cu_A and heme *a*) can become rate limiting. Under these conditions, the population of the intermediates can grow, as shown for example in the simulation reported in Fig. 3. This consideration was particularly important in assessing the likelyhood of the reaction of NO with $\mathbf{P_M}$ and **F** during turnover [91].

8. The reductive pathway

It is universally accepted that Cu_A is the electron entry site in CcOX [19–22,92]. The eT pathway can be schematically depicted as:

$$c \rightarrow Cu_A \rightarrow \text{heme } a \rightarrow [\text{heme } a_3\ Cu_B] \rightarrow O_2 \qquad (1)$$

A great deal of work has been published regarding the kinetic and structural determinants of the reaction of cyt *c* and CcOX. The problem will be discussed by reference to Fig. 4, which highlights a number of amino acid side chains on the surface of sub I and sub II of *Paracoccus* CcOX which were shown to be involved in the formation of the productive complex between cyt *c* and its oxidase. The more significant points are summarized by reference to the work by Witt et al. [93–95] and Ferguson-Miller and co-workers [92,96], where site directed mutagenesis of (respectively) *Paracoccus* and *Rhodobacter* oxidases was employed.

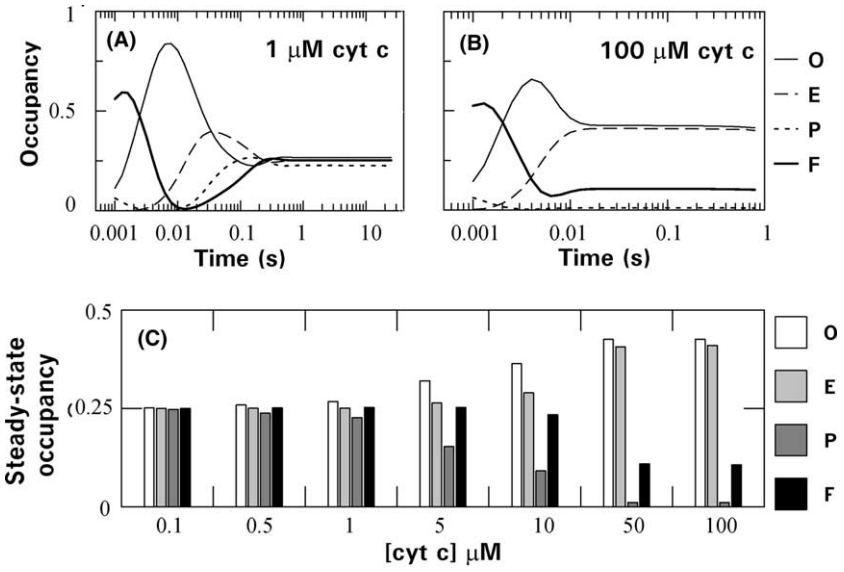


Fig. 3. Steady-state simulations. Output of the simulations (Panels A and B) carried out by Giuffrè et al. [91], according to a simplified version of the scheme in Fig. 2, not including the $\mathbf{P_R}$ intermediate. The resulting time dependence (from 1 ms to 100 s) of the relative population intermediates **O**, **E**, $\mathbf{P_M}$ and **F** is depicted at 1 μM (A) and 100 μM (B) cytochrome *c*. Panel C: Steady-state occupancy of the intermediates at different concentrations of cytochrome *c*. At low cytochrome *c* concentration, electron supply from heme *a* becomes limiting and the steady-state occupancy of intermediates $\mathbf{P_M}$ and **F** raises, approaching the occupancy of the intermediates **O** and **E**. Simulations have been carried out at $[O_2] = 280$ μM; the steady-state occupancy of the **R** state (not shown) is therefore extremely small under all conditions.

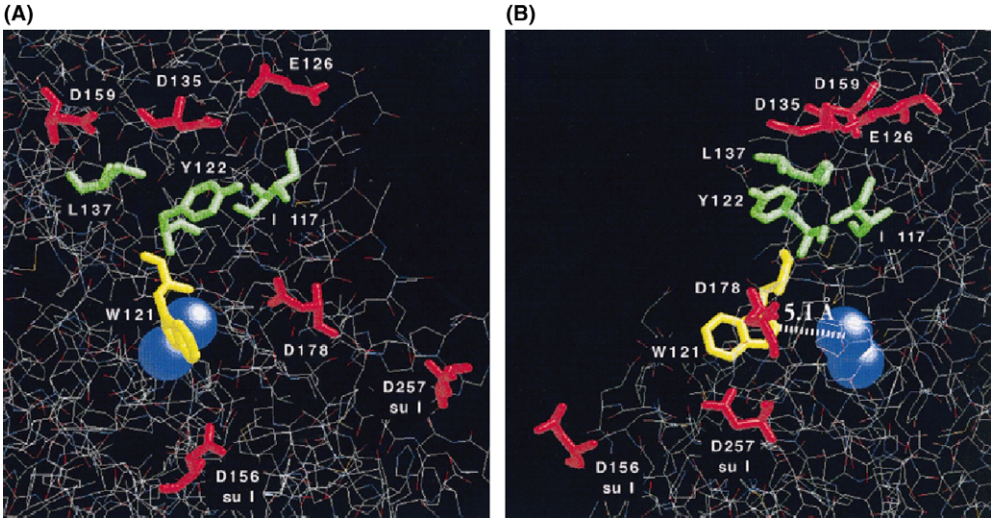


Fig. 4. Cytochrome *c* docking site on the *P. denitrificans* cytochrome oxidase. A frontal (A) and a side view (B) of the site. The highlighted residues (from subunit I and II) were proven by site-directed mutagenesis [95] to have a role in the interaction with cytochrome *c*. Residues are numbered according to the *P. denitrificans* oxidase sequence; those belonging to subunit I are indicated (su I). Trp121, the electron entry site in the enzyme, is depicted in yellow, while acidic and hydrophobic residues in red and green, respectively. The Cu_A metals are in blue. Reproduced from Witt et al. [94] with permission.

As shown in Fig. 4, the contact surface presents a number of negatively charged residues forming a crown which contains in the middle 4 uncharged side chains that provide a hydrophobic patch for the fine turning of the partners interface yielding optimal eT. A crucial role was assigned by Witt et al. [94] to Trp121 (numbering according to *Paracoccus*) based on the properties of the mutant W121Q. With the exception of ba_3-oxidase where Trp121 is replaced by Phe88 [17], this conserved aromatic residue sits on top of Cu_A (at 5.1 Å) and is considered as the electron entry site into the enzyme (as shown by burst and steady-state kinetics). Interestingly the affinity for cyt *c* of W121Q is essentially unchanged compared to *wt*, while k_{cat} drops to ~1% [94]. The negatively charged residues form a cluster that helps the docking of cyt *c* by interaction with its Lys residues (already known to be involved in the contact surface) [97,98].

Mutagenesis studies [94,95,99] as well as the effect of ionic strength on the burst and steady-state kinetics yielded a fairly convincing picture of the role of electrostatic and hydrophobic interactions in the correct docking for efficient eT. At very low non physiological ionic strength (below ~30 mM), a very stable (electrostatically stabilized) 1:1 complex can be isolated [100,101]. Consistently, under these conditions dissociation of the reaction product (oxidised cyt *c*) is slow and rate limiting in steady-state experiments. As the ionic strength is increased, however, the turnover number increases first and then decreases, following a bell-shaped curve [94,101]. The balance achieved at intermediate values (say μ = 50–100 mM depending on enzyme source, conditions, etc.) is optimal for the formation of a transient complex guided by favourable but partially screened electrostatic interactions, which being mobile can adjust by short range hydrophobic interactions for eT via the crucial Trp121 to achieve efficient rate-distance stereochemistry (Fig. 4).

The role of the other residues in the promiscuous patch has been quantitatively assessed by preparing and characterizing several mutants of the charged and uncharged residues. It is of interest that when the soluble cytochrome c_{552} reacts with ba_3 CcOX from *Thermus thermophilus*, it appears that the complex formation is driven by hydrophobic interactions [102]. Particularly interesting is the mutation Y122K in *Paracoccus* CcOX which induces a significant change in K_M (increased by 3- to 5-fold), perfectly understood based on repulsive effects [94]. This same mutation is observed in quinol oxidase which may be underlying the vastly different nature of the two substrates, cyt *c* and quinol. More data and all relevant older literature may be found in Witt et al. [94] and Ferguson-Miller et al. [4,92,96].

In summary, the information available from the 3D structure and from the kinetics of mutants of different CcOX strongly supports the hypothesis that there is a single productive cyt *c* binding site on the enzyme, mostly involving the solvent exposed surface of sub I and sub II protruding towards the intermembrane space. As far as subunit III, it has been speculated that the additional negative patch (1Glu and 2Asp residues) exposed on its surface may be viewed as a ''parking position'' [95] for a second incoming cyt *c* molecule, enhancing turnover even at ionic strength ~50–100 mM. Functional assays have been recently carried out using soluble or vesicles reconstituted CcOX treated with antibodies raised against conserved amino acid sequences in extramembranous portions of subunit III; depending on the targeted sequence, some activation (30–50%) or inactivation (15%) of the electron transfer has been reported [103], suggesting a regulatory role of subunit III.

A problem that remains open to interpretation is the significance of the biphasic Eadie–Hofstee plots observed in steady-state kinetic data, initially reported by Ferguson-Miller et al. [104]. This observation was originally interpreted to imply two cyt *c* binding sites on CcOX, but in view of the information now available, this interpretation is not wholly satisfactory. Alternative explanations have been offered [105–108], but this point remains as yet obscure; given that the biphacisity tends to vanish at ionic strengths 50 mM, this phenomenon may have limited physiological significance. Nevertheless, it must be explained for a more complete understanding of the in vitro properties of the enzyme.

9. Internal electron transfer processes

Internal eT proceeds by rapid (μs) redox re-equilibration between the reduced Cu_A site ($1e^-$ per 2 metals) and heme *a*. This reaction has been extensively characterized and shown to be a simple relaxation process, with $\tau^{-1} \sim 10^4\ s^{-1}$ and an equilibrium favouring the reduction of heme *a* ($K \sim 3.5$) [19,109–111]. As shown in Fig. 1, this eT step is electrogenic as the electron moves from the surface of the protein to the middle of the mitochondrial membrane, with creation of a membrane potential [112–116]. Direct eT from reduced Cu_A to the binuclear heme a_3-Cu_B site has not been convincingly disproven but is considered unlikely [117]. A specific role of the Mg^{2+} bound to the beef heart CcOX, Mn^{2+} in some bacterial species [118,119], has been debated, based on experiments of deuterium exchange at the Mg^{2+}/Mn^{2+} site [120,121]. According to the structure [9], this site is located in a water pool above the active site, and a rapid exchange of protons or water occurs between the coordination sphere of the metal and the pool (which in turn is connected with the bulk). If confirmed, a pathway for the exit of protons or water produced during turnover may have been identified.

It is generally agreed that eT to the binuclear active site proceeds via heme *a* in two single steps involving (in principle) a partially reduced binuclear centre with the electron residing either on heme a_3 (**E_1**) or on Cu_B (**E_2**). Heme *a* and heme a_3 lie across helix X of sub I (Fig. 1, bottom panel), which provides two His residues (376 and 378) as coordination sites for the iron of the two hemes. The rather short distance between them (14 Å Fe-to-Fe [10]) supports the view that eT should be very rapid indeed [122]. Assuming no effect on eT from a reorganization energy term, Dutton and coworkers [123] calculated from their rate-vs-distance standard correlation plot that eT should proceed at $\sim 10^8\ s^{-1}$. However, the report of ultra-fast heme–heme eT compatible with this figure [124,125] has been challenged [126].

A clever experiment by Boelens and Wever [127], later extended by other authors, showed that the reverse eT from reduced heme a_3 to oxidized heme *a*-Cu_A is very rapid ($\tau \sim 3$ μs) [35,126,128–131]. These experiments

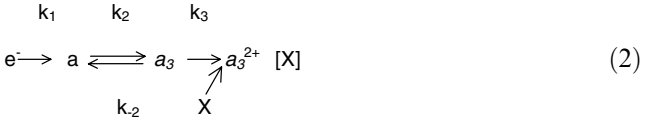
were carried out starting from the so-called mixed-valence CO complex, initially characterized by Greenwood et al. [80], in which only the binuclear site is reduced and bound to CO, i.e. $a^{3+}Cu_A^{2+}$ [a_3^{2+} CO Cu_B^{+}]. Upon photolysis of the heme a_3-bound CO, an electron moves back to heme *a* and Cu_A, the extent of the back transfer being variable from ~10% in the beef enzyme up to ~100% in the *Rhodobacter* enzyme [132]. These back eT rates pertain to the CO-stripped photoproduct state which is likely to have the configuration proper of the CO-bound species, and thus different from that of the fully oxidized enzyme (see above). Comparison of the 3D structure of the oxidized, fully reduced and CO-bound states shows relatively small differences in the protein but some at the heme a_3-Cu_B site (see above); at the limited resolution available today it would be risky to correlate structural information to the barrier for eT, but it may be noticed that the Fe_{a3}-to-Cu_B distance increases upon reduction, at least in the beef heart enzyme [11]; on the other hand no structural change was observed upon CO binding to the reduced enzyme.

The rate of heme a_3 reduction followed in the stopped-flow by mixing the fully oxidized enzyme with a large excess of reductant (cyt c^{2+} or Ru(II)-hexamine) is, on the other hand, much slower being in the millisecond time range; generally rate constants ranging from even less than 1 s^{-1} to as much as ~160 s^{-1} have been reported [63,133–135].

Understanding why the apparent rate of reduction of the binuclear oxidized heme a_3-Cu_B site by reduced heme *a* is so much slower (ms) than expected from the rates (μs) of reverse eT experiments starting from the mixed-valence CO complex, has been (and still is) a puzzle; indeed alternative interpretations have been proposed. Since this internal kinetic event is the slowest step in the whole series of eT processes, and thus is the rate-limiting stage in turnover, it may be important to outline the problem and discuss the alternative interpretations by reference to some papers dealing specifically with this point [136,137].

10. Is the rate-limiting step proton or electron transfer?

To account for the slow accumulation of reduced heme a_3 which is observed when the fully oxidized enzyme is challenged with reductants, two different mechanisms have been proposed. The following simplified scheme may help discussion:



(2)

where e^- is an electron donor (Ru(II)-hexamine or cyt c^{2+}) and X is a *trapping* ligand, i.e. a ligand that binds with sufficiently high affinity to the reduced heme a_3-Cu_B center, and thereby drains the reaction to the right. If the proposition is to single out k_2 (the rate constant of internal eT to heme a_3), experimental conditions must be adjusted to drive the reaction to the right and to fulfil the condition $k_1[e^-]$ and $k_3[X] > k_2$ and k_{-2} (all processes conforming to a first order or pseudo-first order rate equation). Both conditions can be tested changing the concentration of the reductant and of the *trapping* ligand.

The alternative mechanisms proposed are as follows.

Thermodynamic control – It is based on the finding that k_2 and k_{-2} are extremely fast (10^4 to 10^5 s^{-1}) as assessed by reverse eT experiments, presumably pertaining to the configuration characteristic of the reduced enzyme; under these conditions, since the equilibrium is strongly in favour of reduced heme *a* (i.e. $k_{-2} > k_2$), the slow rate of accumulation of reduced heme a_3 may be accounted for. Verkhovsky et al. [136] have therefore suggested that although eT is very fast (μs) even in the oxidized enzyme, the apparent rate constant for the reduction of heme a_3 is slow because thermodynamics favors reduced heme *a*. Although only a small fraction (say <10%) of reduced heme a_3 is populated on a very short timescale (i.e. at rates of ~10^5 s^{-1}), this quota can nevertheless combine with a *trapping* ligand X draining reaction (2) to the right. Because redox titrations have shown that low pH tends to stabilize heme a_3^{2+}, Verkhovsky et al. [136] postulated that the *trapping* ligand X, driving reaction (2) to right, is the H^+ which diffuses to the (membrane) buried active site slowly enough to account for the relatively slow (ms) rate of reduction of heme a_3 *vis-à-vis* a very rapid (μs) intrinsic eT from heme *a*. The pH dependence of this reduction process, already discussed by Malatesta et al. [63], is consistent with the thermodynamic hypothesis, although at acidic pH the apparent rate constant increases only by a factor of 3/pH unit. Verkhovsky et al. [136] also reported that the time courses of accumulation of reduced heme a_3 and of H^+ uptake (followed with phenol red as a pH indicator) are synchronous; this interesting result, however, is of no value to disprove one of the two mechanisms, since synchrony is expected also if eT per se was rate-limiting (see below), with reduction of heme a_3 being coupled to rapid H^+ uptake by a redox-linked ionisable group. Finally, site-directed mutagenesis allowed to show that the internal eT is associated to a proton uptake through the so-called K pathway. Mutation of residues in this pathway indeed causes an impaired reduction of heme a_3 via heme *a* [64,135,138–141], but once again this result does not prove that eT is never rate-limiting in the wild-type enzyme.

Kinetic control – This hypothesis assumes that in the configuration proper of the oxidized enzyme, the rate of eT from heme *a* to heme a_3 is in the ms time range because of an increased configurational energy barrier.

In this case increasing progressively the rates of reduction of heme *a* and of the binding of the *trapping* ligand X, the rate of formation of reduced heme a_3 should become the limiting kinetic step in the binding of X (making reduction and ligation synchronous). Under these conditions, the rate of heme a_3 reduction should become independent of the concentration of both the reductant and the *trapping* ligand alike, and an estimate of k_2 in the fully oxidized configuration of the binuclear centre should be obtained. As a test, an experimental protocol to probe eT to heme a_3 by mixing the oxidized (*fast* or *pulsed*) enzyme with a reductant solution containing X = NO or CO, which bind quickly to reduced heme a_3, has been developed [63,137]. Brunori et al. [137], working with a *fast* preparation [142] of beef heart CcOX in the presence of chloride, showed that the rate constant for the formation of heme a_3^{+2}-NO adduct is not only independent of the concentration of Ru(II)-hexamine but also of NO over a very large concentration range (~10–500 μM) (Fig. 5). This result is still difficult to explain unless one assumes that eT from reduced heme *a* to oxidized heme a_3 (i.e. k_2 in scheme (2)) is rate-limiting the binding of X. Given that the internal eT is very fast (μs) in the configuration of the CO-photolyzed state (see above), Brunori et al. [137] postulated that reduction of a fully oxidized binuclear heme a_3-Cu_B site involves a significant contribution from a reorganizational energy term, which increases the barrier for eT. The crystallographic results (see above) show some structural changes upon reduction of the enzyme, but it is premature to conclude whether these are consistent or inconsistent with the latter hypothesis.

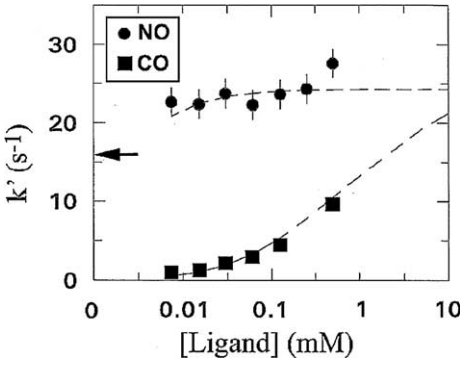


Fig. 5. Reduction of the heme a_3-Cu_B site in the presence of CO or NO. Rate constants for the formation of reduced heme a_3-Cu_B, as measured by stopped-flow spectrophotometry in the presence of CO and NO, acting as *trapping* ligands. Due to its higher affinity for heme a_3^{2+}, NO drives the internal electron transfer to heme a_3 more efficiently than CO; the observed rate constant for the formation of heme a_3^{2+}-NO is in the ms time range and essentially invariant over a wide NO concentration range; the bold arrow pointing the *Y* axis indicates the rate constant observed in the absence of NO. This kinetic behaviour is indication of a rate-limit at the level of heme a_3 reduction. Modified from Brunori et al. [137].

In summary, there is substantial agreement about the bare experimental observation, that starting from fully oxidized CcOX the rate of formation of reduced heme a_3 is in the ms time range, even with a large excess of reductant; nevertheless, the two alternative mechanisms proposed are still under scrutiny, and additional crystallographic data at higher resolution may hopefully be revealing.

11. Abbreviations

CcOX	cytochrome *c* oxidase
cyt *c*	cytochrome *c*
eT	electron transfer

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References

[1] G.T. Babcock, M. Wikström, Nature 356 (1992) 301–309.
[2] A.A. Konstantinov, J. Bioenerg. Biomembr. 30 (1998) 121–130.
[3] H. Michel, Biochemistry 38 (1999) 15129–15140.
[4] D.A. Mills, L. Florens, C. Hiser, J. Qian, S. Ferguson-Miller, Biochim. Biophys. Acta 1458 (2000) 180–187.
[5] D. Zaslavsky, R.B. Gennis, Biochim. Biophys. Acta 1458 (2000) 164–179.
[6] S. Yoshikawa, FEBS Lett. 555 (2003) 8–12.
[7] P. Brzezinski, G. Larsson, Biochim. Biophys. Acta 1605 (2003) 1–13.
[8] D. Bloch, I. Belevich, A. Jasaitis, C. Ribacka, A. Puustinen, M.I. Verkhovsky, M. Wikström, Proc. Natl. Acad. Sci. USA 101 (2004) 529–533.
[9] T. Tsukihara, H. Aoyama, E. Yamashita, T. Tomizaki, H. Yamaguchi, K. Shinzawa-Itoh, R. Nakashima, R. Yaono, S. Yoshikawa, Science 269 (1995) 1069–1074.
[10] T. Tsukihara, H. Aoyama, E. Yamashita, T. Tomizaki, H. Yamaguchi, K. Shinzawa-Itoh, R. Nakashima, R. Yaono, S. Yoshikawa, Science 272 (1996) 1136–1144.
[11] S. Yoshikawa, K. Shinzawa-Itoh, R. Nakashima, R. Yaono, E. Yamashita, N. Inoue, M. Yao, M.J. Fei, C.P. Libeu, T. Mizushima, H. Yamaguchi, T. Tomizaki, T. Tsukihara, Science 280 (1998) 1723–1729.
[12] 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, Proc. Natl. Acad. Sci. USA 100 (2003) 15304–15309.
[13] S. Iwata, C. Ostermeier, B. Ludwig, H. Michel, Nature 376 (1995) 660–669.

[14] C. Ostermeier, A. Harrenga, U. Ermler, H. Michel, Proc. Natl. Acad. Sci. USA 94 (1997) 10547–10553.
[15] A. Harrenga, H. Michel, J. Biol. Chem. 274 (1999) 33296–33299.
[16] M. Svensson-Ek, J. Abramson, G. Larsson, S. Tornroth, P. Brzezinski, S. Iwata, J. Mol. Biol. 321 (2002) 329–339.
[17] T. Soulimane, G. Buse, G.P. Bourenkov, H.D. Bartunik, R. Huber, M.E. Than, EMBO J. 19 (2000) 1766–1776.
[18] J. Abramson, S. Riistama, G. Larsson, A. Jasaitis, M. Svensson-Ek, L. Laakkonen, A. Puustinen, S. Iwata, M. Wikström, Nat. Struct. Biol. 7 (2000) 910–917.
[19] K. Kobayashi, H. Une, K. Hayashi, J. Biol. Chem. 264 (1989) 7976–7980.
[20] B.C. Hill, J. Biol. Chem. 266 (1991) 2219–2226.
[21] B.C. Hill, J. Biol. Chem. 269 (1994) 2419–2425.
[22] F. Malatesta, F. Nicoletti, V. Zickermann, B. Ludwig, M. Brunori, FEBS Lett. 434 (1998) 322–324.
[23] D.A. Proshlyakov, M.A. Pressler, C. DeMaso, J.F. Leykam, D.L. DeWitt, G.T. Babcock, Science 290 (2000) 1588–1591.
[24] G. Buse, T. Soulimane, M. Dewor, H.E. Meyer, M. Bluggel, Protein Sci. 8 (1999) 985–990.
[25] Y.C. Fann, I. Ahmed, N.J. Blackburn, J.S. Boswell, M.L. Verkhovskaya, B.M. Hoffman, M. Wikström, Biochemistry 34 (1995) 10245–10255.
[26] R.A. Scott, P.M. Li, S.I. Chan, Ann. N.Y. Acad. Sci. 550 (1988) 53–58.
[27] A.J. Moody, C.E. Cooper, P.R. Rich, Biochim. Biophys. Acta 1059 (1991) 189–207.
[28] C.S. Butler, H.E. Seward, C. Greenwood, A.J. Thomson, Biochemistry 36 (1997) 16259–16266.
[29] A. Giuffrè, G. Stubauer, M. Brunori, P. Sarti, J. Torres, M.T. Wilson, J. Biol. Chem. 273 (1998) 32475–32478.
[30] M. Fabian, L. Skultety, C. Brunel, G. Palmer, Biochemistry 40 (2001) 6061–6069.
[31] D. Okuno, T. Iwase, K. Shinzawa-Itoh, S. Yoshikawa, T. Kitagawa, J. Am. Chem. Soc. 125 (2003) 7209–7218.
[32] R.B. Gennis, FEBS Lett. 555 (2003) 2–7.
[33] Q.H. Gibson, C. Greenwood, Biochem. J. 86 (1963) 541–554.
[34] P. Sarti, A. Giuffrè, E. Forte, D. Mastronicola, M.C. Barone, M. Brunori, Biochem. Biophys. Res. Commun. 274 (2000) 183–187.
[35] M.I. Verkhovsky, J.E. Morgan, M. Wikström, Biochemistry 33 (1994) 3079–3086.
[36] J.A. Bailey, C.A. James, W.H. Woodruff, Biochem. Biophys. Res. Commun. 220 (1996) 1055–1060.
[37] M.I. Verkhovsky, J.E. Morgan, A. Puustinen, M. Wikström, Nature 380 (1996) 268–270.
[38] J.O. Alben, P.P. Moh, F.G. Fiamingo, R.A. Altschuld, Proc. Natl. Acad. Sci. USA 78 (1981) 234–237.
[39] M. Sharrock, T. Yonetani, Biochim. Biophys. Acta 434 (1976) 333–344.
[40] W.H. Woodruff, J. Bioenerg. Biomembr. 25 (1993) 177–188.
[41] O. Einarsdóttir, Biochim. Biophys. Acta 1229 (1995) 129–147.
[42] R.B. Dyer, K.A. Peterson, P.O. Stoutland, W.H. Woodruff, J. Am. Chem. Soc. 113 (1991) 6276–6277.
[43] O. Einarsdóttir, R.B. Dyer, D.D. Lemon, P.M. Killough, S.M. Hubig, S.J. Atherton, J.J. Lopez-Garriga, G. Palmer, W.H. Woodruff, Biochemistry 32 (1993) 12013–12024.
[44] D.D. Lemon, M.W. Calhoun, R.B. Gennis, W.H. Woodruff, Biochemistry 32 (1993) 11953–11956.
[45] N. Van Eps, I. Szundi, O. Einarsdóttir, Biochemistry 39 (2000) 14576–14582.
[46] S. Yoshida, H. Hori, Y. Orii, J. Biochem. (Tokyo) 88 (1980) 1623–1627.
[47] R. Boelens, H. Rademaker, R. Pel, R. Wever, Biochim. Biophys. Acta 679 (1982) 84–94.
[48] R.S. Blackmore, C. Greenwood, Q.H. Gibson, J. Biol. Chem. 266 (1991) 19245–19249.
[49] A. Giuffrè, E. Forte, G. Antonini, E. D'Itri, M. Brunori, T. Soulimane, G. Buse, Biochemistry 38 (1999) 1057–1065.
[50] G.J. Carr, S.J. Ferguson, Biochim. Biophys. Acta 1017 (1990) 57–62.
[51] M.W. Cleeter, J.M. Cooper, V.M. Darley-Usmar, S. Moncada, A.H. Schapira, FEBS Lett. 345 (1994) 50–54.
[52] G.C. Brown, C.E. Cooper, FEBS Lett. 356 (1994) 295–298.
[53] M. Schweizer, C. Richter, Biochem. Biophys. Res. Commun. 204 (1994) 169–175.
[54] C.E. Cooper, Trends Biochem. Sci. 27 (2002) 33–39.
[55] P. Sarti, A. Giuffrè, M.C. Barone, E. Forte, D. Mastronicola, M. Brunori, Free Radic. Biol. Med. 34 (2003) 509–520.
[56] L. Kobzik, B. Stringer, J.L. Balligand, M.B. Reid, J.S. Stamler, Biochem. Biophys. Res. Commun. 211 (1995) 375–381.
[57] T.E. Bates, A. Loesch, G. Burnstock, J.B. Clark, Biochem. Biophys. Res. Commun. 213 (1995) 896–900.
[58] P. Ghafourifar, C. Richter, FEBS Lett. 418 (1997) 291–296.
[59] A. Tatoyan, C. Giulivi, J. Biol. Chem. 273 (1998) 11044–11048.
[60] J. Torres, V. Darley-Usmar, M.T. Wilson, Biochem. J. 312 (1995) 169–173.
[61] A. Giuffrè, P. Sarti, E. D'Itri, G. Buse, T. Soulimane, M. Brunori, J. Biol. Chem. 271 (1996) 33404–33408.
[62] J.G. Lindsay, C.S. Owen, D.F. Wilson, Arch. Biochem. Biophys. 169 (1975) 492–505.
[63] F. Malatesta, P. Sarti, G. Antonini, B. Vallone, M. Brunori, Proc. Natl. Acad. Sci. USA 87 (1990) 7410–7413.
[64] A. Giuffrè, M.C. Barone, M. Brunori, E. D'Itri, B. Ludwig, F. Malatesta, H.W. Mueller, P. Sarti, J. Biol. Chem. 277 (2002) 22402–22406.
[65] A. Giuffrè, G. Stubauer, P. Sarti, M. Brunori, W.G. Zumft, G. Buse, T. Soulimane, Proc. Natl. Acad. Sci. USA 96 (1999) 14718–14723.
[66] E. Forte, A. Urbani, M. Saraste, P. Sarti, M. Brunori, A. Giuffrè, Eur. J. Biochem. 268 (2001) 6486–6491.
[67] W.G.Zumft, J. Inorg. Biochem. (2004), this volume.
[68] S. Stavrakis, K. Koutsoupakis, E. Pinakoulaki, A. Urbani, M. Saraste, C. Varotsis, J. Am. Chem. Soc. 124 (2002) 3814–3815.
[69] K. Koutsoupakis, S. Stavrakis, E. Pinakoulaki, T. Soulimane, C. Varotsis, J. Biol. Chem. 277 (2002) 32860–32866.
[70] E. Pinakoulaki, T. Soulimane, C. Varotsis, J. Biol. Chem. 277 (2002) 32867–32874.
[71] C. Koutsoupakis, T. Soulimane, C. Varotsis, J. Am. Chem. Soc. 125 (2003) 14728–14732.
[72] W.G. Zumft, Microbiol. Mol. Biol. Rev. 61 (1997) 533–616.
[73] M. Saraste, J. Castresana, FEBS Lett. 341 (1994) 1–4.
[74] J. van der Oost, A.P. de Boer, J.W. de Gier, W.G. Zumft, A.H. Stouthamer, R.J. van Spanning, FEMS Microbiol. Lett. 121 (1994) 1–9.
[75] S. Ferguson-Miller, G.T. Babcock, Chem. Rev. 96 (1996) 2889–2908.
[76] T. Kitagawa, J. Inorg. Biochem. 82 (2000) 9–18.
[77] S. Takahashi, Y.C. Ching, J. Wang, D.L. Rousseau, J. Biol. Chem. 270 (1995) 8405–8407.
[78] B. Chance, N. Graham, V. Legallais, Anal. Biochem. 67 (1975) 552–579.
[79] E. Antonini, M. Brunori, Hemoglobin and Myoglobin in their Reactions with Ligands: Frontiers in Biology, North-Holland Publishing Co, Amsterdam, 1971.
[80] C. Greenwood, M.T. Wilson, M. Brunori, Biochem. J. 137 (1974) 205–215.
[81] N. Van Eps, I. Szundi, O. Einarsdóttir, Biochemistry 42 (2003) 5065–5073.
[82] B. Chance, C. Saronio, J.S. Leigh Jr., Proc. Natl. Acad. Sci. USA 72 (1975) 1635–1640.
[83] L.C. Weng, G.M. Baker, Biochemistry 30 (1991) 5727–5733.
[84] G.T. Babcock, Proc. Natl. Acad. Sci. USA 96 (1999) 12971–12973.

[85] M. Fabian, W.W. Wong, R.B. Gennis, G. Palmer, Proc. Natl. Acad. Sci. USA 96 (1999) 13114–13117.
[86] S. Han, Y.C. Ching, D.L. Rousseau, Nature 348 (1990) 89–90.
[87] M.I. Verkhovsky, A. Jasaitis, M.L. Verkhovskaya, J.E. Morgan, M. Wikström, Nature 400 (1999) 480–483.
[88] E. Antonini, M. Brunori, A. Colosimo, C. Greenwood, M.T. Wilson, Proc. Natl. Acad. Sci. USA 74 (1977) 3128–3132.
[89] G.M. Baker, M. Noguchi, G. Palmer, J. Biol. Chem. 262 (1987) 595–604.
[90] M.T. Wilson, J. Peterson, E. Antonini, M. Brunori, A. Colosimo, J. Wyman, Proc. Natl. Acad. Sci. USA 78 (1981) 7115–7118.
[91] A. Giuffrè, M.C. Barone, D. Mastronicola, E. D'Itri, P. Sarti, M. Brunori, Biochemistry 39 (2000) 15446–15453.
[92] Y. Zhen, B. Schmidt, U.G. Kang, W. Antholine, S. Ferguson-Miller, Biochemistry 41 (2002) 2288–2297.
[93] H. Witt, V. Zickermann, B. Ludwig, Biochim. Biophys. Acta 1230 (1995) 74–76.
[94] H. Witt, F. Malatesta, F. Nicoletti, M. Brunori, B. Ludwig, J. Biol. Chem. 273 (1998) 5132–5136.
[95] H. Witt, F. Malatesta, F. Nicoletti, M. Brunori, B. Ludwig, Eur. J. Biochem. 251 (1998) 367–373.
[96] K. Wang, L. Geren, Y. Zhen, L. Ma, S. Ferguson-Miller, B. Durham, F. Millett, Biochemistry 41 (2002) 2298–2304.
[97] S. Dopner, P. Hildebrandt, F.I. Rosell, A.G. Mauk, M. von Walter, G. Buse, T. Soulimane, Eur. J. Biochem. 261 (1999) 379–391.
[98] J.A. Kornblatt, J. Theodorakis, G.H. Hoa, E. Margoliash, Biochem. Cell. Biol. 70 (1992) 539–547.
[99] V. Drosou, F. Malatesta, B. Ludwig, Eur. J. Biochem. 269 (2002) 2980–2988.
[100] J.K. Dethmers, S. Ferguson-Miller, E. Margoliash, J. Biol. Chem. 254 (1979) 11973–11981.
[101] J. Wilms, E.C. Veerman, B.W. Konig, H.L. Dekker, B.F. van Gelder, Biochim. Biophys. Acta 635 (1981) 13–24.
[102] O. Maneg, B. Ludwig, F. Malatesta, J. Biol. Chem. 278 (2003) 46734–46740.
[103] A. Jeannine Lincoln, N. Donat, G. Palmer, L.J. Prochaska, Arch. Biochem. Biophys. 416 (2003) 81–91.
[104] S. Ferguson-Miller, D.L. Brautigan, E. Margoliash, J. Biol. Chem. 251 (1976) 1104–1115.
[105] B. Michel, H.R. Bosshard, Biochemistry 28 (1989) 244–252.
[106] S.H. Speck, D. Dye, E. Margoliash, Proc. Natl. Acad. Sci. USA 81 (1984) 347–351.
[107] K.M. Sinjorgo, J.H. Meijling, A.O. Muijsers, Biochim. Biophys. Acta 767 (1984) 48–56.
[108] P. Brzezinski, B.G. Malmström, Proc. Natl. Acad. Sci. USA 83 (1986) 4282–4285.
[109] J.E. Morgan, P.M. Li, D.J. Jang, M.A. el-Sayed, S.I. Chan, Biochemistry 28 (1989) 6975–6983.
[110] L.P. Pan, S. Hibdon, R.Q. Liu, B. Durham, F. Millett, Biochemistry 32 (1993) 8492–8498.
[111] O. Farver, O. Einarsdóttir, I. Pecht, Eur. J. Biochem. 267 (2000) 950–954.
[112] D. Zaslavsky, A.D. Kaulen, I.A. Smirnova, T. Vygodina, A.A. Konstantinov, FEBS Lett. 336 (1993) 389–393.
[113] D.L. Zaslavsky, I.A. Smirnova, S.A. Siletsky, A.D. Kaulen, F. Millett, A.A. Konstantinov, FEBS Lett. 359 (1995) 27–30.
[114] A.A. Konstantinov, S. Siletsky, D. Mitchell, A. Kaulen, R.B. Gennis, Proc. Natl. Acad. Sci. USA 94 (1997) 9085–9090.
[115] M. Ruitenberg, A. Kannt, E. Bamberg, B. Ludwig, H. Michel, K. Fendler, Proc. Natl. Acad. Sci. USA 97 (2000) 4632–4636.
[116] M.I. Verkhovsky, A. Tuukkanen, C. Backgren, A. Puustinen, M. Wikström, Biochemistry 40 (2001) 7077–7083.
[117] J.J. Regan, B.E. Ramirez, J.R. Winkler, H.B. Gray, B.G. Malmström, J. Bioenerg. Biomembr. 30 (1998) 35–39.
[118] J.P. Hosler, M.P. Espe, Y. Zhen, G.T. Babcock, S. Ferguson-Miller, Biochemistry 34 (1995) 7586–7592.
[119] H. Witt, A. Wittershagen, E. Bill, B.O. Kolbesen, B. Ludwig, FEBS Lett. 409 (1997) 128–130.
[120] L. Florens, B. Schmidt, J. McCracken, S. Ferguson-Miller, Biochemistry 40 (2001) 7491–7497.
[121] B. Schmidt, J. McCracken, S. Ferguson-Miller, Proc. Natl. Acad. Sci. USA 100 (2003) 15539–15542.
[122] C.C. Moser, J.M. Keske, K. Warncke, R.S. Farid, P.L. Dutton, Nature 355 (1992) 796–802.
[123] C.C. Page, C.C. Moser, X. Chen, P.L. Dutton, Nature 402 (1999) 47–52.
[124] M.I. Verkhovsky, A. Jasaitis, M. Wikström, Biochim. Biophys. Acta 1506 (2001) 143–146.
[125] J.E. Morgan, M.I. Verkhovsky, A. Puustinen, M. Wikström, Biochemistry 32 (1993) 11413–11418.
[126] A. Namslauer, M. Branden, P. Brzezinski, Biochemistry 41 (2002) 10369–10374.
[127] R. Boelens, R. Wever, Biochim. Biophys. Acta 547 (1979) 296–310.
[128] H.J. Harmon, J. Bioenerg. Biomembr. 20 (1988) 735–748.
[129] M. Oliveberg, B.G. Malmstrom, Biochemistry 30 (1991) 7053–7057.
[130] M.I. Verkhovsky, J.E. Morgan, M. Wikström, Biochemistry 31 (1992) 11860–11863.
[131] O. Einarsdottir, K.E. Georgiadis, A. Sucheta, Biochemistry 34 (1995) 496–508.
[132] M. Branden, A. Namslauer, O. Hansson, R. Aasa, P. Brzezinski, Biochemistry 42 (2003) 13178–13184.
[133] F. Malatesta, G. Antonini, P. Sarti, M. Brunori, Biophys. Chem. 54 (1995) 1–33.
[134] M. Tarasev, B.C. Hill, Arch. Biochem. Biophys. 400 (2002) 162–170.
[135] F.L. Tomson, J.E. Morgan, G. Gu, B. Barquera, T.V. Vygodina, R.B. Gennis, Biochemistry 42 (2003) 1711–1717.
[136] M.I. Verkhovsky, J.E. Morgan, M. Wikström, Biochemistry 34 (1995) 7483–7491.
[137] M. Brunori, A. Giuffrè, E. D'Itri, P. Sarti, J. Biol. Chem. 272 (1997) 19870–19874.
[138] J.P. Hosler, J.P. Shapleigh, D.M. Mitchell, Y. Kim, M.A. Pressler, C. Georgiou, G.T. Babcock, J.O. Alben, S. Ferguson-Miller, R.B. Gennis, Biochemistry 35 (1996) 10776–10783.
[139] S. Junemann, B. Meunier, R.B. Gennis, P.R. Rich, Biochemistry 36 (1997) 14456–14464.
[140] T.V. Vygodina, C. Pecoraro, D. Mitchell, R. Gennis, A.A. Konstantinov, Biochemistry 37 (1998) 3053–3061.
[141] M. Wikström, A. Jasaitis, C. Backgren, A. Puustinen, M.I. Verkhovsky, Biochim. Biophys. Acta 1459 (2000) 514–520.
[142] T. Soulimane, G. Buse, Eur. J. Biochem. 227 (1995) 588–595.