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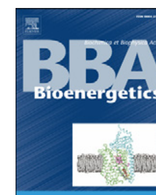

Substrate binding and the catalytic reactions in cbb_3-type oxidases: The lipid membrane modulates ligand binding

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ABSTRACT

Heme–copper oxidases (HCuOs) are the terminal components of the respiratory chain in the mitochondrial membrane or the cell membrane in many bacteria. These enzymes reduce oxygen to water and use the free energy from this reaction to maintain a proton-motive force across the membrane in which they are embedded. The heme–copper oxidases of the cbb_3-type are only found in bacteria, often pathogenic ones since they have a low K_m for O_2, enabling the bacteria to colonize semi-anoxic environments. Cbb_3-type (C) oxidases are highly divergent from the mitochondrial-like aa_3-type (A) oxidases, and within the heme–copper oxidase family, cbb_3 is the closest relative to the most divergent member, the bacterial nitric oxide reductase (NOR). Nitric oxide reductases reduce NO to N_2O without coupling the reaction to the generation of any electrochemical proton gradient. The significant structural differences between A- and C-type heme–copper oxidases are manifested in the lack in cbb_3 of most of the amino acids found to be important for proton pumping in the A-type, as well as in the different binding characteristics of ligands such as CO, O_2 and NO. Investigations of the reasons for these differences at a molecular level have provided insights into the mechanism of O_2 and NO reduction as well as the proton-pumping mechanism in all heme–copper oxidases. In this paper, we discuss results from these studies with the focus on the relationship between proton transfer and ligand binding and reduction. In addition, we present new data, which show that CO binding to one of the c-type hemes of CcoP is modulated by protein–lipid interactions in the membrane. These results show that the heme c-CO binding can be used as a probe of protein–membrane interactions in cbb_3 oxidases, and possible physiological consequences for this behavior are discussed.
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1. Introduction

Aerobic respiration plays a fundamental role for life on Earth and is the most exergonic metabolism known. In aerobic organisms, electron transfer to oxygen through a series of membrane-bound protein complexes is coupled to the maintenance of a transmembrane electrochemical proton gradient that is used e.g. for the synthesis of ATP.

Terminal oxidases catalyze the last step in the respiratory chain; reduction of oxygen to water (see Eq. (1a)). The respiratory chain enzymes are located in the mitochondrial inner membrane in eukaryotes or the inner cell membrane in bacteria. Most terminal oxidases belong to the family of heme–copper oxidases (HCuOs), where the name describes their catalytic O_2-binding center which is composed of a heme and a copper ion. The superfamily of HCuOs is defined by the primary sequence of their catalytic subunit, which is composed of twelve transmembrane helices with six invariant histidines ligating three cofactors; a high-spin heme (one His ligand) and a copper (three His ligands) in the catalytic site and an additional low-spin heme (two His ligands). HCuOs couple the exergonic O_2-reduction to the generation of a proton electrochemical gradient across the membrane in two ways; first, electrons and protons used to reduce O_2 to H_2O (Eq. (1a)) are derived from opposite sides of the membrane; electrons from donors (often a cyt. c) in the 'outside' solution, and protons from the 'inside'. Second, HCuOs are proton pumps, i.e. they translocate protons through the protein across the membrane (Eq. (1b)). In many HCuOs, four protons are translocated across the membrane for every oxygen reduced to water (i.e. in Eq. (1b), $n=4$), but this number appears variable (see below).

$$O_2 + 4e^-_{out} + 4H^+_{in} \rightarrow 2H_2O \quad (1a)$$

$$nH^+_{in} \rightarrow nH^+_{out} \quad (1b)$$

The best known HCuOs are of the type found in mitochondria, the *aa_3-type* (or A, see below) *oxidases*. These oxidases contain, in their catalytic subunit I, a low-spin heme a and a high-spin heme a_3. Together with a nearby Cu-ion (Cu_B), heme a_3 forms the catalytic site of oxygen reduction. There is an additional redox cofactor, Cu_A, bound

Abbreviations: NOR, bacterial nitric oxide reductase; HCuO, heme–copper oxidase

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to subunit II, a membrane-anchored protein (see Fig. 1). Cu_A is the initial acceptor of electrons from soluble cyt. *c*, and electrons are then transferred via heme *a* to the active site. Protons are transferred through two defined pathways from the negative (N-, or in-) side of the membrane up to the catalytic site, the D- and the K-pathways (see below), where the D-pathway transfers the pumped protons.

Heme–copper oxidases of the type called cbb_3 (the names aa_3 or cbb_3 refers to the types of hemes found in the complexes) were first found in nitrogen-fixing and pathogenic bacteria, where they are expressed under low oxygen tension and have a lower K_m for O_2 than A-type HCuOs [1]. The cbb_3 oxidase is the only O_2-reducing HCuO in several pathogens, including *Helicobacter pylori* [2], which is the major cause of gastric ulcers, making the cbb_3 oxidase a potential drug target. The homology between cbb_3-and aa_3-type oxidases is low (about 14% sequence identity [3]) and the residues constituting the D-pathway (see above) are missing in cbb_3s.

Sequence alignments have also identified another, divergent member of the family of HCuOs; the bacterial NO-reductase (NOR) [4,5]. The NORs perform one step in the denitrification process, reducing NO to N_2O (see Eq. (2)).

$$2NO + 2e^- + 2H^+ \rightarrow N_2O + H_2O \quad (2)$$

Instead of the copper in the catalytic site (Cu_B), NORs contain a non-heme iron (Fe_B), which is thought to be important for efficient NO reduction. NORs are also present in some pathogens (that are not denitrifiers), where its role is to detoxify the NO produced by the host's immune defense. A major difference between the NORs and O_2-reducing HCuOs is that NORs do not couple reduction of NO, which is as exergonic as O_2 reduction, to the generation of a proton gradient. NO reduction by NOR is thus completely non-electrogenic [6–8] and protons (c.f. Eq. (2)) are taken up from the outside (periplasmic) solution [8].

Among the O_2-reducing HCuOs, the cbb_3-type is the closest relative to NOR, and interestingly, cbb_3-type oxidases have substantial NO reduction activities ($\geq 2\ e^- s^{-1}$) [9,10] in contrast to the aa_3-type which show no significant such activity [11,12]. It is not known if this activity plays any physiological role for the pathogenicity of the cbb_3-containing bacteria, but it could help counteracting the immune defense of the host, just like for some 'true' NORs.

There are many significant differences between aa_3-type and cbb_3-type oxidases, e.g. the lack in cbb_3 of most of the amino acids found to be important for proton pumping in the aa_3-type, the capability of the cbb_3s to reduce NO, and the complex characteristics of CO binding in cbb_3s. These issues will be discussed in this paper, and we will further show evidence that CO binding properties in cbb_3 can be used as a probe reporting on protein–lipid interactions in the membrane.

Investigating the molecular details of the reasons for the differences between these subfamilies of heme–copper oxidases should help elucidate the mechanism of O_2 and NO reduction in HCuOs, the general pumping mechanism in all HCuOs, as well as the degree to which NO reduction is coupled to proton pumping in cbb_3, and give us a better understanding of the evolution of the HCuO family.

1.1. Cbb_3 or C-type heme–copper oxidases

The oxygen-reducing members of the heme–copper oxidase superfamily have previously been classified into three main subfamilies denoted A-, B-, and C-types [3,13]. The A family comprises the enzyme found in mitochondria and other well-studied and structurally defined aa_3-type HCuOs from e.g. *Rhodobacter* (*R.*) *sphaeroides* and *Paracoccus* (*P.*) *denitrificans* (see e.g. [14–19]) as well as the bo_3 oxidase from *Escherichia* (*E.*) *coli* [20]. The best studied member of the B family is the ba_3 oxidase from *Thermus thermophilus* (see e.g. [21,22]). The C family containing the cbb_3 oxidases are found strictly in bacteria, and the cbb_3 complex has been purified and characterised from e.g. *Pseudomonas* (*P.*) *stutzeri* [23], *P. denitrificans* [24], *Bradyrhizobium japonicum* [1], *R. sphaeroides* [25,26], and *Vibrio* (*V.*) *cholerae* [27]. NORs form two distinct families, the q- and cNORs [28], where the letters q and c indicate that they use quinol or soluble proteins such as cyt. *c* as the electron donor.

Fig. 1 shows a schematic comparison between cNOR, cbb_3-, and aa_3-type oxidases. In cbb_3, the catalytic subunit CcoN is related to subunit I of the aa_3-type and the NorB of NOR and is the core subunit defining the members of the HCuO family. CcoN contains the high-spin heme b_3-Cu_B catalytic site and a low-spin heme *b*. CcoO is a membrane-anchored protein containing one *c*-type heme and has no counterpart in other O_2-reducing HCuOs, but is related to NorC in cNORs [4]. CcoP, also anchored to the membrane via one trans-membrane helix, contains two *c*-type hemes [23] and has no counterpart in

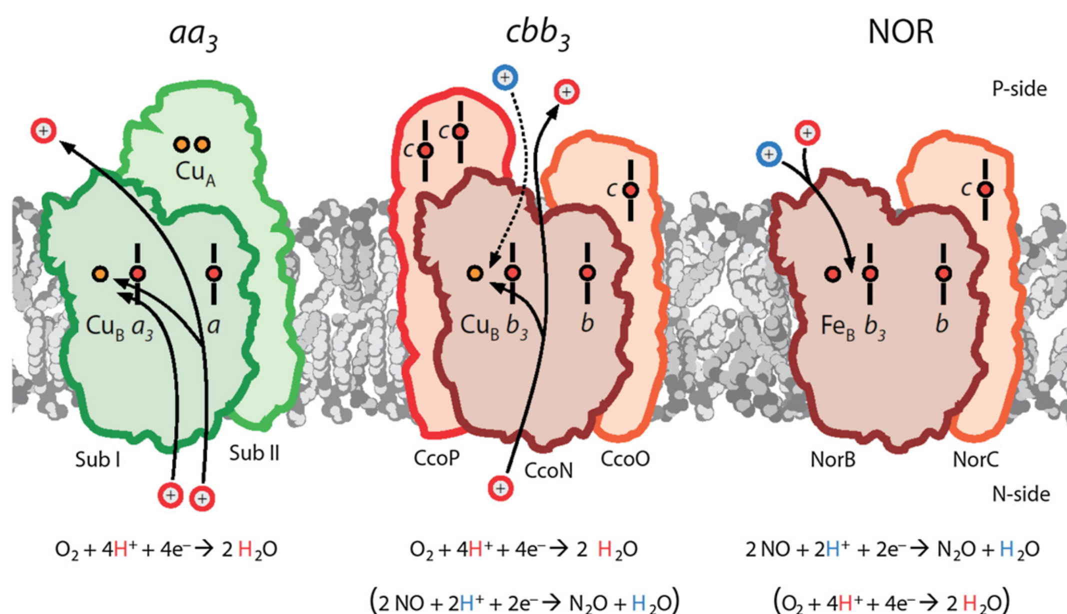


Fig. 1. Schematic comparison of cbb_3 oxidases (middle) to cNORs and aa_3-type oxidases. The N and P refers to the net negative (N) and positive (P) side of the membrane. The (main) physiological and secondary activities of the enzymes are indicated. The figure illustrates the suggested existence of only one proton input pathway in cbb_3, compared to two (D- and K-) in aa_3. Also shown is from which side of the membrane protons are taken up, including the suggestion [10] that protons for NO reduction in cbb_3 come from the P-side.

neither the O_2- nor NO-reducing HCuOs. *Cbb*$_3$ oxidases also contain a fourth small subunit, CcoQ, which has a stabilising role on the complex [29,30].

In contrast to the two (D- and K-) pathways for protons in *aa*$_3$-type oxidases, *cbb*$_3$ oxidases presumably have only one pathway [31], analogous to the K-pathway, for delivering protons from the cytosol to the catalytic site. NORs must have a proton pathway leading from the periplasm to the active site (see above).

In the *aa*$_3$ oxidases, electrons are transferred from cytochrome *c* via the Cu_A center in subunit II to heme *a* in subunit I and on to the catalytic site. The sequence of electron transfers in *cbb*$_3$ is not known, the CcoO presumably acts as the initial electron acceptor from cyt. *c* in species where the CcoP gene is missing [32], whereas the CcoP subunit could take over this role where present.

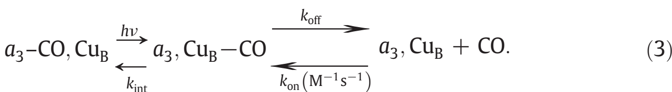
There are no published crystal structures available for the *cbb*$_3$ oxidases or the NORs (but see below), crystallisation trials for *cbb*$_3$ [33,34] have so far yielded poorly diffracting crystals. Models of the catalytic subunit have been generated based on the homology to the known oxidase structures (for CcoN models see [27,35,36] and for NorB models [8,37]).

Although clearly related, it is not known which of the different HCuO subfamilies came first during evolution. Considering that O_2 was not present in considerable amounts in the early atmosphere on Earth, the hypothesis that the O_2-reducing HCuOs, starting with the *cbb*$_3$s, evolved from the NORs [4], although attractive, has been challenged [3] as more sequences have been reported.

2. CO binding to the cbb3-type oxidases; comparison to other HCuOs

Carbon monoxide is often used as a substrate analogue of oxygen, and typically binds to pentacoordinated (or hexa-coordinated if one ligand can be displaced) ferrous (Fe^{2+}) heme groups. The heme–CO bond is photolabile such that a short laser flash can be used to break it, and studies of the recombination of CO to the heme have been used extensively for HCuOs as a probe of the detailed environment of the O_2-binding site.

In *aa*$_3$-type oxidases, such as those from mitochondria and *R. sphaeroides*, CO binds to the reduced heme a_3. Upon flash photolysis of the a_3–CO bond, CO migrates to Cu_B from which it leaves with a time constant of ~1 µs (k_{off} ~10^6 s^{-1} in Eq. (3)) [38]. CO rebinding occurs in a single phase with a time constant of 10–20 ms at 1 mM CO (see e.g. [39,40]). At very high CO concentrations (>20 mM), the time constant saturates at ~1 ms (k_{int}~10^3 s^{-1} in Eq. (3)) due to rate limitation by the transfer of CO back to heme a_3 from Cu_B [38].



$$a_3\text{–CO}, \text{Cu}_B \underset{k_{int}}{\overset{h\nu}{\rightleftarrows}} a_3, \text{Cu}_B\text{—CO} \underset{k_{on}(\text{M}^{-1}\text{s}^{-1})}{\overset{k_{off}}{\rightleftarrows}} a_3, \text{Cu}_B + \text{CO}. \qquad (3)$$

In the cNOR from *P. denitrificans*, after flash photolysis of the heme b_3–CO bond, CO rebinding is very rapid with a time constant of ~5 µs (at 1 mM CO) and there is no evidence for involvement of the Fe_B in the process [41].

In the *cbb*$_3$ oxidase from *P. stutzeri*, the CO binding characteristics are markedly different from both A-type and cNOR HCuOs [42]. First, in addition to the active site heme b_3, CO binds also to one of the *c*-type hemes in CcoP, as evidenced by two different components with differing kinetic difference spectra in the rebinding process [42], something we observe also for the *cbb*$_3$ oxidase from *R. sphaeroides* (see Fig. 2). The process with an observed rate constant of 2×10^4 s^{-1} (at 1 mM CO) peaks at ~415 nm and it is ascribed to CO rebinding to a *c*-type heme (cf. [42]), whereas the process with a rate constant of ~200 s^{-1} (at 1 mM CO) peaks at ~425 nm and is ascribed to CO rebinding to the b_3 heme. Fig. 2A shows a typical trace at 430 nm,

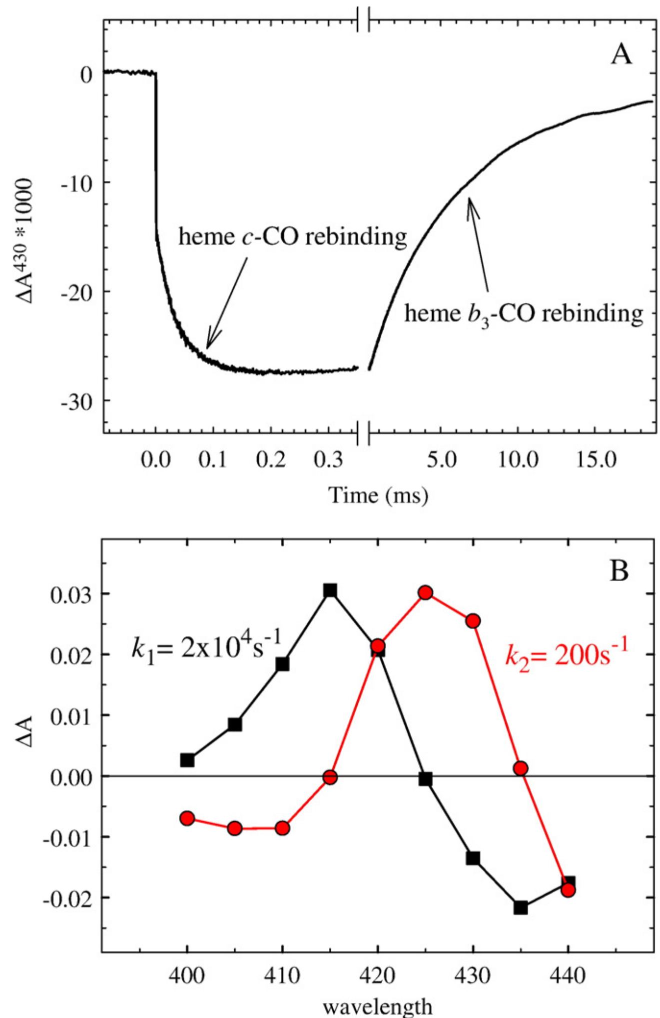


Fig. 2. A) CO recombination to the fully reduced *cbb*$_3$ oxidase from *R. sphaeroides*, studied at 430 nm. Two major components with rate constants of 2×10^4 s^{-1} and 200 s^{-1} are observed. B) Kinetic difference spectrum of the $k_1=2\times10^4$ s^{-1} and $k_2=200$ s^{-1} phases, ascribed to *c*-heme-(k_1) and *b*-heme (k_2) CO rebinding. Experiments performed as in [10]. Experimental conditions: 25 mM Hepes, pH 7.5, 100 mM NaCl, 0.03% DDM, 3 µM PMS, 3 mM ascorbate, ~50 µM dithionite, 1 mM CO and $T=298$ K.

where the two rebinding processes are clearly resolved as the absorbance changes associated with the two reactions (see Fig. 2B) have different signs. The two components are discussed separately below.

2.1. CO binding to the b_3 heme

CO rebinding to the b_3 heme occurs with a rate constant of ~200 s^{-1} in *R. sphaeroides cbb*$_3$ (see Fig. 2), similar to the $k=800$ s^{-1} observed with the *P. stutzeri cbb*$_3$ [42]. This rate constant does not vary with CO concentrations down to a few µM ([42] and our own data), showing that it is limited by a first-order process, presumably the transfer of CO from Cu_B [43] (see Eq. (3)). Stavrakis et al. [43] further showed that CO never leaves Cu_B in between the transfer from and back to heme b_3. This off-rate from Cu_B in the ms-range is in sharp contrast to the time constant ~1 µs observed with the *aa*$_3$ [38]. Several reports have identified a link between the reduction state/ligand binding to Cu_B and the properties of the D-pathway in *aa*$_3$-type oxidases (see e.g. [44,45]), and it has been discussed that the overlap of the pathway for pumped protons with the channel for gases puts restrictions on the flexibility of gas binding in the *aa*$_3$ oxidases [46]. The *cbb*$_3$ oxidases do not have a D-pathway for protons (discussed further below), they have different CO binding

properties as well as a lower K_m for O_2 than the aa_3 oxidases, properties that might be structurally linked.

The CO binding properties of the catalytic site are also important parameters for the application of the so-called flow-flash technique (see below).

2.2. CO binding to a c-heme of CcoP

In the *P. stutzeri* cbb_3, CO binds to one of the *c* hemes in CcoP only after reduction with dithionite [42], indicating a very low midpoint potential for the CO binding heme. Since one of the hemes in CcoP was shown to have an unusual bis-His ligation [23] (instead of the common His/Met ligation), this *c*-heme was the proposed site of CO interaction, losing one His ligand upon CO binding. In contrast to the situation in *P. stutzeri*, the *R. sphaeroides* cbb_3 binds CO to a *c*-type heme to the same extent upon reduction with ascorbate as in the presence of dithionite (data not shown), which means that the CO binding *c*-type heme is not necessarily of very low reduction potential.

The CO binding to CcoP in cbb_3 from *P. stutzeri* was suggested to perhaps indicate a physiological role as a ‘heme-based sensor’, reporting on environmental conditions, such as O_2 levels. This suggestion was based on earlier evidence for the involvement of the cbb_3 oxidase in such processes [26,47,48], leading to the repression of photosynthetic genes at high O_2 in *R. sphaeroides*, although the mechanism for how the O_2 tension is sensed and the signal transduced is largely unknown. On the other hand, even soluble horse heart cyt. *c* can bind CO in mutant forms or when denatured (see e.g. [49,50]), or when interacting with cardiolipin [51], suggesting that the CO binding observed for CcoP might arise from structural changes associated with solubilisation/purification. In this scenario, both *c* hemes in CcoP have the common His/Met ligation in the membrane, and one Met is exchanged for His during purification of the complex.

In our hands, the cbb_3 from *R. sphaeroides* shows a variable degree (between different preparations) of CO binding to a *c*-type heme. Furthermore, when reconstituted into soy bean lipid vesicles, there is no (or very little, varying between experiments) CO binding to CcoP, as evidenced by CO recombination studies showing the rapid (with rate constant $(k) = 2\times10^4\ s^{-1}$) phase disappearing upon reconstitution whereas CO rebinding to heme b_3 is unaffected (Fig. 3A). This effect seems specific in terms of lipids used in the reconstitution process, reconstituting cbb_3 into soy bean lipids as well as into a mix resembling that of the native *R. sphaeroides* membrane [52] leading to a decrease in the relative amount of CO binding to CcoP, whereas reconstituting into vesicles made from cardiolipin (CL) leads to a dramatic increase (see Fig. 3B).

The variable degree of CO binding to CcoP that we observe in the purified, detergent-solubilised cbb_3 appears at first to indicate that the heme *c*-CO binding is an artefact from solubilisation and/or purification. However, the observation that it is a *reversible* effect (and not simply irreversible damage) changing as the lipid environment changes might indicate a more complex scenario. In this context, we also observe no clear correlation between CO binding to CcoP and catalytic O_2-reducing activity.

CcoP is anchored in the membrane via one predicted transmembrane (TM) helix, and we assume the changes occurring in the vesicles are due to interactions between the vesicle lipids and this TM helix (illustrated schematically in Fig. 4) since there is no effect of adding the same solubilised lipid mixtures solubilised (as done in Ref. [51] for the effects on cyt. *c*) to the detergent-purified cbb_3 (data not shown). The effects on the TM helix presumably propagates to the CO binding heme, possibly affecting the affinity for the original His/Met or His/His ligands (see above), which in turn could lead to a change in the propensity to exchange a protein ligand for CO.

In terms of a physiological role for the effects we observe, we can speculate on a scenario where ligand binding to CcoP induces

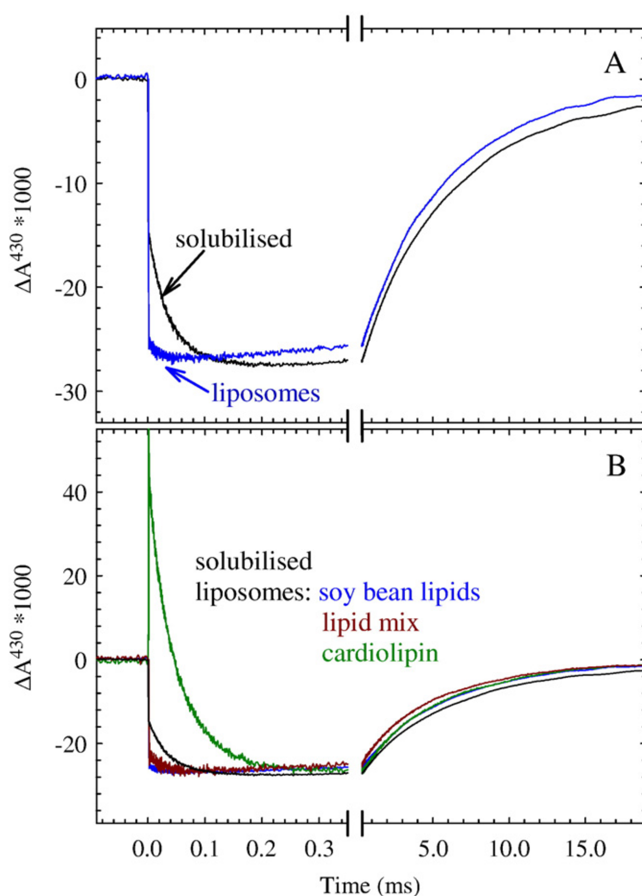


Fig. 3. Comparison of CO recombination characteristics in detergent-solubilised and liposome-reconstituted cbb_3. A) Liposomes made the ‘standard’ way with soy bean lipids. B) CO recombination in cbb_3 reconstituted with different lipid mixes. Liposomes made as in [10]. The lipid mix was 40% DOPE, 30% DOPC, 20% DOPG and 10% CL (cardiolipin) to correspond roughly to the native *R. sphaeroides* membrane [52]. Other experimental conditions are as in Fig. 2. The traces were normalised to the same amplitude for the slower phase to aid comparison.

conformational changes propagating to the transmembrane region, affecting the interaction between subunits or between CcoP and some other sensor on the cytosolic side. In this context, it is interesting to note that the proposed interaction between the *R. sphaeroides* cbb_3 oxidase and the PrrB kinase, involved in controlling expression of photosynthetic genes, appears to require the membrane-spanning domain of PrrB [26]. Alternatively, our results are consistent with changes in environment leading to changes in the composition of the lipid membrane [52], sensed by CcoP, resulting in altered ligand-binding properties as well as an altered interaction with the PrrB.

These results are also part of the increasing body of evidence for the importance of protein–lipid interactions in membrane proteins. For example, it has been recognized that many membrane proteins such as the aa_3-type HCuO [53] have specific, highly conserved lipid binding sites which are believed to play important roles in membrane protein structure, assembly and/or activity. Furthermore, the choice of detergents/lipids for solubilisation is critical for the stability of these proteins (for reviews, see e.g. [54,55]).

3. O_2 reduction in cbb$_3$ oxidases

In aa_3-type oxidases, reduction of O_2 proceeds via a series of reasonably well-defined intermediates; starting with the fully reduced (four available electrons) oxidase, O_2 binds to heme a_3 with a time constant of ~10 µs (at 1 mM O_2) forming the so-called A intermediate. The O–O bond is then broken and reduced by four

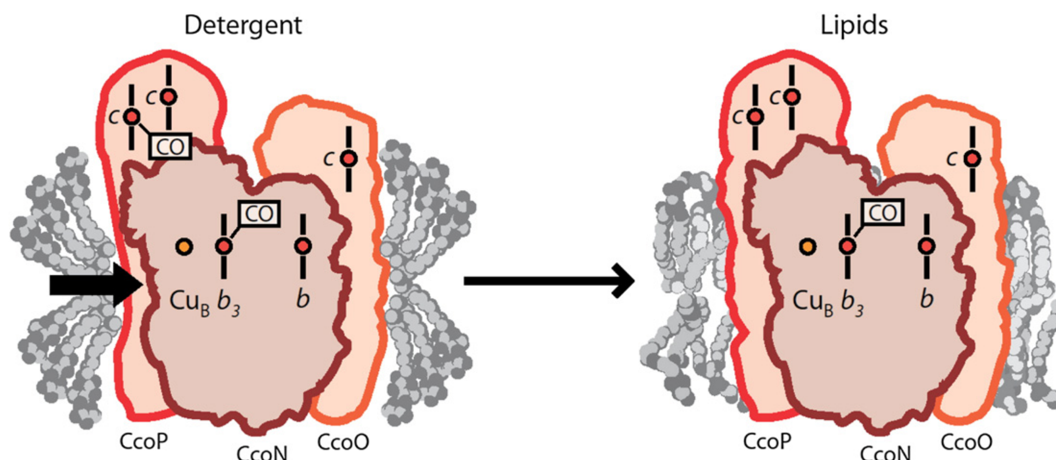


Fig. 4. A schematic illustration of the observed effect on CO binding to CcoP in *cbb*$_3$-liposomes. In detergent micelles, the TM helix of CcoP is in a different conformation (indicated by the black arrow to the left). This conformational change propagates to one of the *c* hemes, leading to a higher affinity for CO. When reconstituted into a lipid membrane, the CcoP conformation 'relaxes', and the affinity for CO is decreased.

electrons in a single step forming the so-called peroxy or 'P' intermediate with a time constant of ~30 μs in the fully reduced enzyme (see e.g. [56,57]). A proton is then taken up with a time constant of 100 μs, resulting in formation of the ferryl (F) intermediate, which is further reduced to form the oxidised (O) intermediate by transfer of the last electron from Cu_A, together with a second proton with a time constant of ~1 ms.

The name 'peroxy' is used for historical reasons even though a ferryl ($Fe^{4+}=O^{2-}$) state is already found at the catalytic site [58]. With the fully reduced enzyme, three of the four electrons required come from the catalytic site (heme $a_3^{2+} \rightarrow a_3^{4+}$, $Cu_B^{+} \rightarrow Cu_B^{2+}$) and the fourth from heme *a*. When the two-electron reduced enzyme is the starting point, the 'P' intermediate presumably contains a radical at a nearby Tyr (Y-288 in *R. sphaeroides aa*$_3$), as the donor of the 4th electron [59]. This tyrosine is crosslinked to a histidine ligand to Cu_B (H-284) [19,60] and considered part of the catalytic core, where it was suggested to donate a hydrogen atom (a proton together with an electron) during turnover [61]. Such a His-Tyr crosslink is also observed in the *ba*$_3$ oxidase from *T. thermophilus* [22,62].

In *cbb*$_3$ oxidases, the sequence of oxygen reduction intermediates is unknown. The equivalent of the Tyr-288 in helix VI is missing in the primary sequence of CcoN, but another Tyr in helix VII was suggested by modelling to fulfil the same role [27], and the presence of the His-Tyr crosslink later verified [63,64]. A Tyr-His crosslink is thus observed in all major (A, B and C) classes of O_2-reducing HCuOs, but not in the NORs, indicating that it is vital for the O_2-reducing or proton-pumping mechanism (see below).

For the *aa*$_3$-type HCuOs, the reaction mechanism has been extensively studied using the so-called 'flow-flash' technique. In this technique, fully reduced enzyme with carbon monoxide (CO) bound to the high-spin heme is mixed in a stopped-flow apparatus with an oxygenated solution. Since CO and O_2 bind at the same site, the reaction with O_2 is limited by the dissociation rate of CO, which is slow (in *aa*$_3$ oxidases). However, if a short laser flash is applied after complete mixing, the photolabile Fe–CO bond is broken and the binding of dioxygen and its subsequent step-wise reduction can be followed using time-resolved spectroscopy. The flow-flash technique has been used in combination with various detection techniques and has yielded a large body of information about the catalytic cycle of these HCuOs, e.g. the formation and decay rates as well as the chemical structure of the different intermediates, the sequence of electron transfers and the timing and pathways for proton transfer to the catalytic site and across the membrane (see e.g. [58,65–68]). For the flow-flash technique to work, a number of conditions have to be fulfilled: first, the spontaneous dissociation of CO (the CO-off rate) from the reduced high-spin heme must be slow enough to allow for mixing in the stopped-flow apparatus before the laser flash is applied. Secondly, after dissociation of CO by the flash, the rate of oxygen binding to the heme has to be faster than that of CO recombination. How long CO stays bound to Cu_B is also an important parameter, since even though O_2 seems able to bind to the high-spin heme while CO is still bound to Cu_B (or the rate of dissociation from Cu_B increases in the presence of O_2, see below), cleavage of the O–O bond presumably requires the participation of an unliganded Cu_B. The complex behaviour of CO binding in *cbb*$_3$, as well as the overlapping spectra of the three *c*-type hemes have hampered detailed kinetic studies using the flow-flash technique. The study performed in our laboratory (focussed on comparing proton transfer characteristics between the reaction with O_2 and NO (see below)), identified a major phase of heme oxidation with a time constant of ~1 ms, concomitant with proton uptake from solution [10], but no chemical intermediates were identified. This 1 ms phase could be limited by the escape of CO from Cu_B (see above) and we presume that CO is lost to solution instead of rebinding to heme b_3 (as occurs in CO atmosphere [43]) when O_2 is bound to the heme.

4. NO reduction in cbb$_3$ oxidases

In addition to O_2 reduction, the *cbb*$_3$ oxidases from *R. sphaeroides* and *P. stutzeri* have been found to catalyze NO reduction [9,10]. This is presumably a general property of the *cbb*$_3$-type oxidases, and we have found NOR activity also in the *cbb*$_3$ from *V. cholerae*.

The mechanism of NO reduction is not known for *cbb*$_3$ oxidases, and also poorly understood in the 'true' bacterial NORs (for a review see [28]). NO is also reduced by the *ba*$_3$ from *T. thermophilus* (but slower than in the *cbb*$_3$), where the product was shown to be nitrous oxide, N_2O [11], just like for NORs (see Eq. (2)). For NOR, models for the catalysis have been suggested that involve either a 'trans' mechanism [7,69,70] where one NO binds to each of the metals in the binuclear site, or 'cis' mechanisms where two NOs bind either to the non-heme Fe_B [71], or consecutively to heme b_3 such that the second NO binds to the intermediate formed upon binding of the first [72]. In the latter suggested scenario, an intermediate hyponitrite dianion ($N_2O_2^{2-}$) coordinating in between the two irons would form [72].

Binding of NO also to the oxidised active site, as observed for both NOR and *cbb*$_3$ [73–75] leads to additional reaction paths and complicates the interpretation of experimental results.

The reason why some O_2-reducing HCuOs can also reduce NO while others can't is not known, but has been suggested to be related

to the higher affinity of Cu_B for ligands such as CO found in the NO-reducing HCuOs [11]. If the higher CO affinity implies a higher NO affinity, the Cu_B-bound NO might facilitate reductive coupling to the NO bound at heme b_3. A similarity between cbb_3 and NOR, possibly also related to the ability to reduce NO (by avoiding the ferrous-NO complex during turnover, see [76]) is that the active site heme b_3 has been found to have a low midpoint potential in both enzymes, ~60 mV for *P. denitrificans* NOR [76] and ~−60 mV for the cbb_3 from *R. sphaeroides* [77], although such a low midpoint potential was not observed in the cbb_3s from *P. stutzeri* [2] or *B. japonicum* [78].

One plausible difference between O_2− and NO reduction is that the pK_as of intermediates formed at the active site during reduction of O_2 are much higher (>12, see e.g. [79]) than those suggested to form during NO reduction [80]. We suggested that with the lower pK_as formed during NO reduction, protons cannot be pulled from the 'normal' (used for O_2 reduction) proton donor [10]. In the cbb_3s, the identity of the immediate proton donor to the active site during O_2 reduction is not known, but in the aa_3-type HCuOs, this proton donor, Glu-286 [81], (*R. sphaeroides* aa_3 numbering) has a pK_a>9 [79]. So, presuming that the 'normal' proton donor, has a pK_a which is too high to be deprotonated by the NO-intermediates, protons would instead be pulled from a donor with a lower pK_a and we speculated that the presence of such alternative proton donors could be part of the reason why some HCuOs can reduce NO [10].

Two conserved glutamate residues towards the periplasmic surface in CcoN were further suggested to be possible low pK_a proton donors, and these glutamates are present in cbb_3s but not in other O_2-reducing HcuOs. This suggestion was based on the conservation of these glutamate residues in the NORs where they were suggested to form the entry point for the proton transfer pathway from the periplasm into the active site. In NOR, these glutamates are important for catalytic activity [82] and there was a specific, large shift in the pK_a for the rate of proton-coupled electron transfer when one of the glutamates (Glu-122 in NorB from *P. denitrificans*) was replaced with an aspartate [83]. In wild-type NOR, the pK_a of the same reaction is 6.6 [84], much lower than that for the proton donor during O_2 reduction in aa_3 oxidases (see above).

It should be noted however, that in the crystal structure of a qNOR, the equivalent of the Glu-122 is a ligand to a Ca^{2+} ion, additionally ligated by the heme propionates of hemes b and b_3 (Y. Shiro, personal communication, unpublished). Such an intricate lattice of interactions presumably affecting the midpoint potentials of both hemes b and b_3 might in itself lead to the observed effects of the mutations.

5. Proton transfer in cbb$_3$

In aa_3-type HCuOs, the D-pathway for protons leads from an aspartate (D-132, *R. sphaeroides* aa_3 numbering) at the cytoplasmic surface up to a glutamate (Glu-286) close to Cu_B, and the K-pathway leads through a lysine (K-362) up to the active site. The D-pathway is presumably used for six or more of the eight protons transferred per turnover, the protons used during oxidation of the active site and the pumped protons. The K-pathway is used for the remaining one or two protons that are taken up during reduction of the active site [85,86]. The output pathway for pumped protons, leading from the active site to the outside solution, is largely unknown.

Sequence alignments have shown that in the cbb_3-type oxidases there is no equivalent to the D-pathway, and the pattern of conserved residues supports the presence of only one proton input pathway up to the catalytic site, spatially analogous to the K-pathway [3,31]. Even within subgroups of cbb_3-type oxidases, no alternative pattern of conserved residues that could form a second pathway is found [31]. Although proton pathways can be composed entirely of water molecules, the known examples of membrane protein proton pathways all have at least a few participating polar/ionizable side chains, presumably in order to provide specificity for protons over other cations [87].

Despite lacking the pathway responsible for conducting the pumped protons in the aa_3-type oxidases, the cbb_3-type oxidases have been shown to pump protons [88,89], but with varying stoichiometries [24]. Purified cbb_3 from *B. japonicum* reconstituted into lipid vesicles showed pumping of about half as many protons as observed with an aa_3-type oxidase [88]. The same scenario with only one conserved proton pathway analogous to the K-pathway and pumping with a lower stoichiometry is observed also for the ba_3 oxidase in *T. thermophilus* [90], suggesting that the two properties are linked. If the basic mechanism of the pump is the same throughout the O_2-reducing HCuOs, and made up from conserved residues, there is very little to choose from; only (strictly, five out of) the six cofactor-ligating histidines are completely conserved, and these are conserved also to the non-pumping NORs [31]. As discussed above, although not conserved in the primary sequence, the existence of a Tyr-His crosslink in the active site seems conserved in the O_2-reducing HCuOs, and not in the NORs, making it a good candidate for being involved in the pumping mechanism. Furthermore, the suggested 'evolutionary migration' [63] from one position to the other could be linked to the low K_m for O_2 in cbb_3 oxidases if the position of the Tyr in the aa_3 oxidases interferes with gas delivery. Alternatively, the two different positions of the Tyr-His crosslink could be seen as evidence that this crosslink arose twice, independently, during evolution from a common ancestor that did not reduce O_2 (but possibly NO).

In cNOR, even though NO reduction is similarly exergonic to O_2 reduction, NORs do not pump protons, and substrate protons are taken from the periplasmic solution [6–8]. Since electrons are donated from a cyt. *c* in the periplasm, the reaction is completely non-electrogenic. Several explanations for NORs not conserving energy have been suggested; e.g. considering the toxicity of NO, NOR might have evolved to favor rapid reduction and tight binding over energetic efficiency [28]. Alternatively, the reason is related to NO reduction not resulting in formation of intermediates with high enough pK_as (as discussed above), if this is required for the pumping mechanism [8,80]. Simplified, NO reduction by NOR is non-electrogenic due to the protein or due to the substrate, which means that investigation of the coupling between NO reduction and proton translocation in the cbb_3 oxidases should enable resolving of the question. The reason for this is that the cbb_3s, in contrast to the NORs, pump protons during O_2 reduction, so all required structural elements are present. We recently investigated the coupling of NO reduction to proton translocation in the cbb_3s by using the flow-flash technique in combination with both optical and electrometric detection in liposome-reconstituted cbb_3 [10]. When comparing the reaction of the fully reduced cbb_3 with either O_2 or NO, we found that oxidation of heme groups in cbb_3 proceeded to the same extent with the two substrates, but that the build-up of electrical potential was much smaller with NO. This presumably means that no protons are pumped with NO, as is the case for NO reduction by NOR. Furthermore, the lack of electrochemical potential generation during NO reduction in cbb_3 is consistent with the chemical protons (see Eq. (2)) coming from the 'outside' solution. We suggested that this is due to the location of the proton donors (see above), i.e. with O_2 the donor sits in the pathway leading from the 'inside', whereas with NO, the alternative lower pK_a donor sits towards the 'outside' [10]. Experiments aimed at verifying this suggestion by direct measurements of proton uptake/extrusion during a single turnover are under way in our laboratory.

If in cbb_3s, NO reduction is uncoupled from proton pumping as suggested, it means that the properties of the intermediates formed are important in the general pumping mechanism of all HCuOs, placing restraints on the models for such a mechanism.

6. Summary

In summary, we have discussed some key differences in the mechanisms of ligand interactions between the aa_3-(A) and cbb_3-(C)

type oxidases. First, the binding of CO to the cbb_3 oxidases is very complex, e.g. CO binds to both heme b_3 and one of the *c* hemes in CcoP. We have shown that CO binding to this *c*-type heme is modulated by the lipid membrane, which may be of physiological relevance for the involvement of the cbb_3 oxidases in reporting on O_2 levels. Our study is also an additional example of the importance of specific lipid–protein interactions for the structure and function of membrane proteins. Second, we have discussed the ability of the cbb_3 oxidases to reduce NO and possible features specific to cbb_3 oxidases enabling this catalytic function, as well as the presumed uncoupling of the proton pump from NO reduction. We also propose that the characteristic features of ligand interactions and the existence of only one proton pathway in cbb_3 oxidases are functionally linked.

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