FEBS Letters 587 (2013) 770–774

FEBS Letters

journal homepage: www.FEBSLetters.org

The heme-copper oxidase superfamily shares a Zn^{2+}-binding motif at the entrance to a proton pathway

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ARTICLE INFO

Article history:
Received 6 December 2012
Revised 30 January 2013
Accepted 31 January 2013
Available online 8 February 2013

Edited by Stuart Ferguson

Keywords:
cbb_3
Proton transfer
K-pathway
Nickel
Liposome
Nitric oxide reductase

ABSTRACT

Heme-copper oxidases (HCuOs) catalyse the reduction of oxygen, using the liberated free energy to maintain a proton-motive force across the membrane. In the mitochondrial-like A-type HCuOs, binding of heavy metal ions at the surface of the protein inhibits proton transfer. In bacterial C-type oxidases, the entry point to the proton pathway is on an accessory subunit unrelated to any subunit in A-type HCuOs. Despite this, we show here that heavy metal ions such as Zn^{2+} inhibit O_2-reduction very similarly in C-type as in A-type HCuOs, and furthermore that the binding site shares the same Glu-His motif.

1. Introduction

Heme-copper oxidases (HCuOs) are integral membrane proteins that form the last component of the respiratory chain, catalysing the reduction of oxygen to water (see Eq. (1)). All HCuOs have a homologous catalytic subunit with twelve transmembrane helices harbouring six invariant histidines which ligate three cofactors; a high-spin heme and a copper ion (Cu_B) in the catalytic site and an additional low-spin heme. HCuOs conserve energy from O_2-reduction by generating a proton electrochemical gradient across the membrane. This is achieved by using electrons from donors in the 'outside' solution and protons from the 'inside' of the membrane, and also by translocating protons across the membrane. In the mitochondrial-like A-type HCuOs, four protons are translocated across the membrane for every oxygen reduced to water (i.e. in Eq. (1), $n = 4$).

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

The HCuO superfamily is classified into three major subfamilies denoted A-, B-, and C-type [1,2], and also bacterial NO-reductases (NOR) belong to the HCuO family (see below), where they form their own subfamily.

In the mitochondrial-like, or A-type HCuOs, the catalytic subunit I contains the Cu_B, the low-spin heme *a* and the high-spin heme a_3. There is an additional redox cofactor, Cu_A, bound to subunit II, a membrane-anchored protein. Cu_A is the acceptor of electrons from the donor, soluble cyt. *c*. Protons are transferred through two defined pathways (see [3] for a recent review on proton pathways in the HCuO family) up to the catalytic site, the D- and the K-pathway. The D-pathway is the main pathway for both chemical and pumped protons (in total 6–7 H^+ per O_2). The K-pathway is used for protons (1–2) taken up during the reduction of the active site and starts with a glutamate in subunit II ($E101^{II}$ in *Rhodobacter sphaeroides* aa_3) [4,5], which is the only residue in either the D- or K-pathways not found in subunit I.

The B- and C-type HCuOs differ from the A-type in that the D-pathway is missing in the sequence, and they presumably use only one proton transfer pathway, analogous in its spatial location to the K-pathway [2,6].

The C (cbb_3) family of oxidases are found exclusively in bacteria, and is the O_2-reducing HCuO most distantly related to the A-type, and the closest to NOR. The C-type HCuO from *Pseudomonas stutzeri* was recently structurally defined at atomic resolution [7]. In C-type HCuOs, the catalytic subunit CcoN, related to subunit I of the A- and B-type, contains the high-spin heme b_3-Cu_B catalytic

Abbreviations: HCuO, heme-copper oxidase; NOR, bacterial nitric oxide reductase; *c*NOR, cytochrome *c*-dependent NOR; DDM, β-D-dodecyl maltoside; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine; FCCP, carbonyl cyanide-*p*-trifluoromethoxyphenyl-hydrazon; RCR, respiratory control ratio

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0014-5793/$36.00
http://dx.doi.org/10.1016/j.febslet.2013.01.069

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 NOR. CcoP, also anchored to the membrane via one trans-membrane helix, contains two *c*-type hemes and has no counterpart in any other HCuO.

The K-pathway analogue in C-type HCuOs was suggested [7] to start at the Glu-49 (*P. stutzeri* cbb_3 numbering) in CcoP. We recently showed that this Glu (Glu-25^P in *R. sphaeroides* cbb_3) is needed for efficient proton transfer [8], thus identifying it as a crucial part of the proton transfer pathway. The Glu-49 in CcoP is also here the only residue in the proton pathway not located in CcoN. Thus, all O_2-reducing HCuO subfamilies have the entry point to the K-pathway (analogue) in one of the accessory subunits.

Bacterial NO-reductases (NOR) also belong to the HCuO superfamily, although their physiological role is to reduce NO to N_2O. In the best characterised NORs, the *c*NORs, one major difference to the O_2-reducing HCuOs is that NO-reduction, although as exergonic as O_2-reduction, is non-electrogenic and protons and electrons are derived from the same side of the membrane (see [3] and references therein). Hence, *c*NOR has neither a D- nor a K-pathway analogue, as confirmed by the crystal structure of *c*NOR from *Pseudomonas aeruginosa* [9].

Many proton-transferring membrane proteins, e.g. the bacterial photosynthetic reaction center (RC) are inhibited by heavy metal cations such as Zn^{2+}, Ni^{2+} and Cd^{2+} [10]. This presumably occurs because the metals tend to bind to acidic and histidine residues found at the entry point to the proton-transferring pathways (where they form a 'proton-collecting antenna' [11]). In A-type HCuOs, Zn^{2+} has been shown to inhibit catalytic turnover [12,13], proton transfer during oxidation [14], release of pumped protons [15] and to decrease the pumping stoichiometry [15,16]. Also, other heavy metal ions such as Cd^{2+} and Ni^{2+} inhibit O_2-reduction in A-type HCuOs [12].

In a crystal structure of the A-type HCuO from *R. sphaeroides*, binding of Cd^{2+} was observed at the entrance to the K-pathway, where it was ligated by Glu-101^{II} and a nearby histidine on the same subunit, H-96^{II} [17]. In a crystal of the bovine oxidase soaked with Cd^{2+} or Zn^{2+}, the metals were found bound to a His at the entrance to the D-pathway [18].

In C-type HCuOs, there is no evolutionary relationship between the CcoO or CcoP with the subunits II or III in A-type HCuOs. Despite this, the arrangement of histidines surrounding the glutamic acid at the entry point to the K-pathway analogue is similar to that found in the A-type. In this work, we have investigated the effect of heavy metal ions on catalytic turnover in the C-type (cbb_3) from *R. sphaeroides*. Our results show that the inhibition pattern of Zn^{2+} and Ni^{2+} is very similar between the A- and C-type HCuOs, and that furthermore, a histidine (His-18^P) in the CcoP subunit close to the Glu (Glu-25^P) that defines the proton entry point is part of the metal binding site.

2. Materials and methods

2.1. Construction of site-directed mutant forms and protein purification

Construction of E25^{P}A and E25^{P}Q was described earlier [8]. Both H18^{P}A and H124^{N}A were constructed in the same manner as in [8]. Briefly, the ccoNOPQ fragment (BamHI-EcoRI) from pUI2803NHIS subcloned to pBluescript SK+ was used for site directed mutagenesis with the Quikchange kit (Stratagene). Mutagenic primers were from Eurofins, restriction enzymes from Fermentas, and sequencing performed by Eurofins. The ccoNOPQ containing the mutation was ligated back to pRK-415. The plasmid containing the mutation was transformed into S-17-1, and finally transferred into the *R. sphaeroides* Δcbb_3 strain by conjugation. The *R. sphaeroides* Δcbb_3 strain carrying the plasmid pUI2803NHIS [19] with mutations were grown, and the cbb_3 wildtype and variants were purified as in [8]. The growth of *Escherichia coli* expressing *c*NOR and its purification was done as in [20].

2.2. Steady-state activity measurements and data handling

The steady-state O_2-reduction activity of cbb_3 was measured using a Clark-type electrode (Hansatech). The reaction chamber was filled (total of 1 ml) with 50 mM Hepes-KOH, pH 7.4, 50 mM KCl, 0.03% β-D-dodecyl maltoside (DDM), 5 mM ascorbate, 0.5 mM *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD), and 20–40 μM horse heart cytochrome *c*. For measurements with cbb_3–liposomes 100 mM Hepes-KOH at pH 7.4 was used.

$ZnSO_4$–$7H_2O$ (Sigma), $NiSO_4$–$6H_2O$ (Sigma), and $CaCl_2$–$2H_2O$ (Scharlan) were used for metal stock solutions and prepared by serial dilutions. Na–EDTA (Scharlan) at pH 7.4 was used as a chelator. The metal solutions were added directly to the measuring chamber after the addition of cbb_3. A few subsequent additions of metal solution were made, followed by addition of excess EDTA before the measurement was repeated. Because of the limited solubility of $Zn(OH)_2$ at pH > 7 [21], we did not use Zn^{2+}-concentrations above 0.5 mM when fitting the data.

The effect of metals added on the turnover activity of cbb_3 was fitted, as in [12], to Eq. (2) using Sigmaplot (Systat Software):

$$V_{obs} = \frac{V_0}{1 + [M^{2+}]/K_i} \quad (2)$$

If the fit was not satisfactory, a background rate V_b was added to the equation to account for a non-zero turnover rate at high metal ion concentration. Alternatively, the data obtained was fitted to two inhibitory sites [12] according to Eq. (3):

$$V_{obs} = \frac{V_0^1}{1 + [M^{2+}]/K_i^1} + \frac{V_0^2}{1 + [M^{2+}]/K_i^2} \quad (3)$$

2.3. Reconstitution of the cbb_3 into phospholipid vesicles

Purified soybean lipids (L-α-phosphatidylcholine, Sigma–Aldrich, 40 mg/ml) in 2% cholate, 100 mM Hepes-KOH, pH 7.4 were mixed and sonicated. The *R. sphaeroides* cbb_3 was then added and the mixture incubated in room temperature for one hour with occasional shaking. The mixture was then passed through a PD-10 column (GE Healthcare) resulting in incorporation of the cbb_3 into small unilamellar vesicles (see [22]). The respiratory control ratio (RCR, see also [8]) was measured as the ratio of the rates of O_2-reduction after and before the addition of valinomycin/carbonyl cyanide-*p*-trifluoromethoxyphenyl-hydrazon (FCCP). The RCR was typically above 6, indicating that the vesicles prepared by this method are well sealed. The absolute turnover activity in uncontrolled vesicles was similar to that in the solubilised enzyme.

3. Results

3.1. Turnover oxygen reduction activity in *R. sphaeroides* cbb_3 variants

Both the H214^{N}A and H18^{P}A variants show high O_2-reduction turnover, with 120% and 90% of the wildtype activity, respectively.

3.2. Metal ion inhibition of O_2-turnover in solubilised cbb_3 and cNOR

Zn^{2+} affects the rate of O_2-reduction in cbb_3, a titration of observed turnover rate versus added Zn^{2+} is shown in Fig. 1. Ni^{2+} also inhibits turnover, whereas Ca^{2+} shows very little inhibition. The K_is