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Electrochemistry suggests proton access from the exit site to the binuclear center in *Paracoccus denitrificans* cytochrome *c* oxidase pathway variants

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ABSTRACT

Two different pathways through which protons access cytochrome *c* oxidase operate during oxygen reduction from the mitochondrial matrix, or the bacterial cytoplasm. Here, we use electrocatalytic current measurements to follow oxygen reduction coupled to proton uptake in cytochrome *c* oxidase isolated from *Paracoccus denitrificans*. Wild type enzyme and site-specific variants with defects in both proton uptake pathways (K354M, D124N and K354M/D124N) were immobilized on gold nanoparticles, and oxygen reduction was probed electrochemically in the presence of varying concentrations of Zn^{2+} ions, which are known to inhibit both the entry and the exit proton pathways in the enzyme. Our data suggest that under these conditions substrate protons gain access to the oxygen reduction site via the exit pathway.

1. Introduction

The concerted movement of protons and electrons is a common feature of many energy-transducing complexes including the photosynthetic reaction center (RC), the cytochrome (cyt) bc_1 complex (cyt bc_1), the NADH ubiquinone oxidoreductase (complex I) or cyt *c* oxidase (CcO). These enzymes generate an electrochemical proton gradient across the membrane by the vectorial uptake and release of protons at the lipid bilayer in a manner coupled to electron transfer and which is then used to drive the synthesis of ATP. Earlier observations have indicated that transition metal binding in energy transducing components can be used to identify residues involved in proton transfer pathways in proton translocating enzymes. It was reported that transition metal ions such as Zn^{2+} and Cd^{2+} can inhibit the proton transfer activity of bacterial RCs [1,2]. Zn^{2+} binding to the RC from *Rhodobacter sphaeroides*, decreases the rate of proton transfer to the acidic residue named E212, becoming a rate-limiting step [2]. The X-ray crystal structure of the RC with bound Zn^{2+} revealed that the Zn^{2+} binding cluster D124, H126 and H128 of the RC subunit H is involved in the pathway of the first proton delivery to Q_B- at the entry point [3]. Also the inhibition of proton translocation by Zn^{2+} in the bc_1 complex (complex III) [4,5] and inhibition of proton pumping in complex I [6] have been described. The IC_{50} for the inhibition of the respiratory complexes were estimated to be in the mid-micromolar range for complexes I and IV and in the mid nanomolar range for complex III. Under physiological conditions, the levels of free Zn^{2+} in mitochondria are close to zero. However, pathologic conditions may increase Zn^{2+} concentrations, thus inhibiting oxidative phosphorylation basically at the level of complex III [7]. It is not clear whether the inhibition of complexes I and IV are of physiological relevance.

As for complex IV, it was shown that zinc inhibits the proton translocation and causes selective uncoupling from electron transfer [8–10] and it can thus be used as a tool to investigate proton pumping. Two separate proton pathways have been suggested for the bacterial CcO, the so-called D- and K-pathways [11–14], leading from the N-(negative) side (cytoplasmic side) of the membrane towards the heme-copper binuclear site. As identified in the

Abbreviations: cyt bc_1, bc_1 complex; cyt, cytochrome; CcO, cytochrome *c* oxidase; complex I, NADH ubiquinone oxidoreductase; RC, reaction center; Tris, tris(hydroxymethyl) aminoethane

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structure of the CcO from *Paracoccus denitrificans*, studied here, the D-pathway starts at D124 (*P. denitrificans* CcO amino acid numbering), close to the cytoplasmic surface of CcO [15,16]. It appears to be the only pathway required when the fully reduced CcO reacts with molecular oxygen [14]. The K-pathway, named after the conserved amino acid K354, is located close to the protein/lipid interface and leads straight to the active site. This pathway may be involved in the delivery of the first one or two protons during the reduction of the oxidized enzyme. These proton uptake pathways were studied using Zn^{2+} and Cd^{2+} binding experiments describing the action of the cations from inside the liposomes on the entrance proton channels [17]. The metal binding sites have been studied by anomalous difference Fourier analyses of *R. sphaeroides* CcO crystals in the presence of cadmium revealing a binding site in the proposed initial proton donor/acceptor of the K pathway, E101 (E78 in *P. denitrificans*) of subunit II [18].

The number of Zn^{2+} binding sites and their affinities has been studied in detail. It was reported that the presumed inhibition of the exit pathway by Zn^{2+} with apparent Ki of 5–10 μM was observed only in the highly energized membrane, while ~1 mM Zn^{2+} was required to observe inhibition from the P-side in the deenergized state [19]. As shown later on by Kuznetsova et al. [20] at least two Zn inhibitory sites are located at the opposite sides of the membrane. Binding of Zn^{2+} with the exit proton pathway (from the P-side of the membrane) in the oxidized CcO required either very long preincubation with μM concentrations of Zn^{2+} and the enzyme turning over, or ~mM concentrations of Zn^{2+} [19,20]. Vygodina et al. [21] reported that the inhibition of the exit pathway may occur rapidly with Ki of about 1 μM, provided some high potential non-heme center (e.g. CuB) is reduced prior to onset of the reaction with oxygen.

Here we apply electrochemical experiments on Zn^{2+} inhibited CcO for the study on these mutants. The technique is based on porous three-dimensional gold nanoparticle electrodes obtained by drop-casting a concentrated gold colloid on the surface of a polycrystalline gold substrate. These electrodes allow both an exceptionally high surface coverage and a reversible direct electron transfer with the immobilized enzyme [22–24]. After immobilization of CcO, the catalytic current and the oxygen reduction potential can be investigated in presence of oxygen [23].

2. Materials and methods

2.1. Gold nanoparticles synthesis

15 nm gold nanoparticles were synthesized following a procedure defined by Turkevich et al. [25] and refined by Frens [26]. Briefly, 125 ml of a 1 mM solution of $HAuCl_4$ was allowed to boil before addition of 13 ml of a 39 mM sodium citrate aqueous solution. After appearance of the red dark color, the solution was maintained under boiling for 10 min before cooling at room temperature. Finally, gold nanoparticles were centrifuged at 10000 rpm for 30 min in order to remove 95% of the supernatant to obtain a concentrated solution of gold nanoparticles.

2.2. Protein preparation

Wild type, K354M, D124N and D124N/K354M mutant enzyme CcO from *P. denitrificans* have been purified as previously described [27,28]. To perform protein film voltammetry, depletion of the amount of detergent of the samples was necessary. To decrease the amount of detergent of the samples, the stock protein was diluted with 50 mM Tris buffer for the zinc inhibition measurements or with 50 mM phosphate buffer. The sample was then reconcentrated to 100 μM for gold surface deposition.

2.3. Electrochemistry

A 2 mm diameter polycrystalline gold electrode was polished with diamond paste and activated in a 0.1 M H_2SO_4 solution by maintaining the potential at +2.21 V for 5 s, −0.14 V for 10 s and cycling 100 times between +0.01 V and +1.71 V at 4 V/s. To verify the cleanliness of the surface, a last scan between +0.01 V and +1.71 V was performed. The resulting electrode was subsequently modified by deposition of 3 drops of 3 μl concentrated gold nanoparticles solution. Each drop was allowed to dry under air. Then, the electrode was immersed in a 1:1 ethanolic solution of mercaptohexanol and hexanethiol (1 mM) and kept at 4 °C overnight. The resulting electrode was rinsed with ethanol and dried under argon. Then 3 μl of a 100 μM protein sample were deposited on the surface and left overnight under argon at 4 °C. Finally, the electrode was rinsed with fresh buffer to remove the excess of non-adsorbed protein. The measurements were performed in a standard three electrode cell connected to a Princeton Applied Research VERSASTAT 4 potentiostat. An AgCl/Ag 3 M NaCl reference electrode was used together with a platinum wire as counter electrode. All the potentials mentioned in this article are referred to a standard hydrogen electrode (SHE). All the voltamograms were recorded with a scan rate of 0.02 V s^{-1}. The samples were first studied anaerobically and then cycled in the presence of oxygen.

2.4. Zinc inhibition

Zinc inhibition was carried out by adding (0–2 ml) of a 2 mM buffered $ZnCl_2$ solution to the 20 ml initial Tris buffer solution. 10 min equilibration time was required before the measurement. In control experiments 2 mM cyanide was added (see Suppl. Materials) leading to a loss of the catalytic current. IC_{50} were determined from the plots of catalytic current vs Zn^{2+} concentration.

3. Results

3.1. Electrocatalytic reaction of variants in the proton pathways

Fig. 1 shows the catalytic current obtained for wild type (black line), K354M (dotted line), D124N (dark grey) variants and the D124N/K354 double variant (light gray) of CcO from *P. denitrificans*.

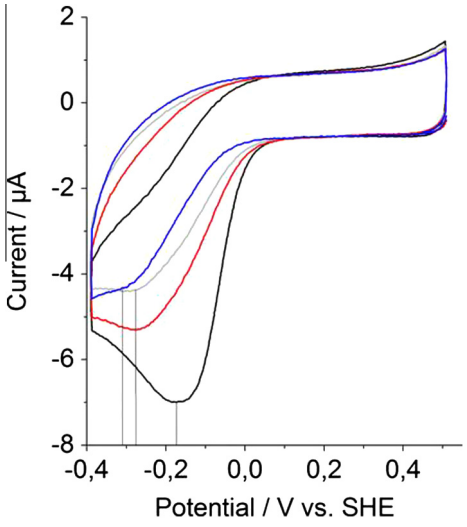

Fig. 1. Voltamograms of WT (black), K354M (light gray), D124N (red), D124N/K354M (blue) CcO from *P. denitrificans* at pH 7 (v = 0.02 V/s, 20 °C). The reduction potentials are −0.18 V for wild type, −0.28 V for the K354M and D124N variants and −0.31 V for the double variant (potential vs SHE).

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