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An electrogenic nitric oxide reductase

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ABSTRACT

Nitric oxide reductases (Nors) are members of the heme-copper oxidase superfamily that reduce nitric oxide (NO) to nitrous oxide (N_2O). In contrast to the proton-pumping cytochrome oxidases, Nors studied so far have neither been implicated in proton pumping nor have they been experimentally established as electrogenic. The copper-A-dependent Nor from *Bacillus azotoformans* uses cytochrome c_{551} as electron donor but lacks menaquinol activity, in contrast to our earlier report (Suharti et al., 2001). Employing reduced phenazine ethosulfate (PESH) as electron donor, the main NO reduction pathway catalyzed by Cu_ANor reconstituted in liposomes involves transmembrane cycling of the PES radical. We show that Cu_ANor reconstituted in liposomes generates a proton electrochemical gradient across the membrane similar in magnitude to cytochrome aa_3, highlighting that bacilli using Cu_ANor can exploit NO reduction for increased cellular ATP production compared to organisms using cNor.

1. Introduction

Biological and chemical N_2 fixation are pivotal for the supply of terrestrial life with the indispensible element nitrogen. In denitrification, the nitrogen oxides are successively reduced back to molecular nitrogen (Eq. (1)) employing four specific reductases for the reduction of nitrate (Nar), nitrite (Nir), NO (Nor) and N_2O (N_2Or), respectively [1,2]:

$$\begin{aligned}\text{Nitrate}\,(NO_3^-) \rightarrow \text{Nitrite}\,(NO_2^-) \rightarrow \text{Nitric Oxide}\,(NO)\\ \rightarrow \text{Nitrous Oxide}\,(N_2O) \rightarrow \text{dinitrogen}\,(N_2)\end{aligned} \quad (1)$$

Prokaryotic Nors are integral membrane enzymes (distinct from the soluble P450 eukaryotic Nors [3,4]), which catalyze the N–N bond formation between two NO molecules consuming two protons and two electrons and producing one N_2O and one water (Eq. (2))[1,2,5–7]:

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

Sequence analysis classifies Nors as members of the heme-copper oxidase (HCO) superfamily [3,8–11]. The proton pumping quinol and cytochrome *c* oxidases (Coxs) are the other members of the HCO superfamily. Both enzyme families share a similar catalytic core, which consists of a low-spin heme (*b* in Nors and *a* or *b* in Coxs) and a binuclear metal center (BNC) where the NO or O_2 reduction chemistries take place [2,6,7,12–15]. The BNC in Nors contains a high-spin heme b_3 [6,13–15] and a non-heme iron designated Fe_B. In Coxs the BNC consists of Cu_B and a high-spin heme of type a_3, o_3 or b_3 [12,16]. The members of the HCO superfamily show great overall structural similarity in their membrane-buried catalytic subunit that contains 12–14 transmembrane α-helices (TMHs) and six conserved histidine residues that coordinate the redox centers [10].

Coxs and Nors show cross-reactivity with their respective electron acceptors (O_2 and NO, resp.) [17–20] A notable kinetic property of all Nors is that the activity is quadratic with respect to NO concentration yielding non-Michaelis–Menten behavior [6,12,21]. In addition, Nors are substrate-inhibited at low micromolar NO concentrations resulting in sigmoidal reaction progress curves [6,12,21].

Three prokaryotic classes of Nor have been identified: cytochrome *c* oxidizing cNor [13,22,23]; quinol-dependent qNor [24–26] and the Nor from *Bacillus azotoformans* (qCu_ANor) reported to receive electrons from menaquinol or cytochrome c_{551} [3,27]. However, here we report that the *Bacillus* enzyme in fact lacks menaquinol activity (see Section 3) and have changed its name from qCu_ANor to Cu_ANor.

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The structure of the heterodimeric cNor from the Gram-negative *Pseudomonas aeruginosa* has recently been solved [28]. Computational, mutational and structural analyses of *Pseudomonas* and *Paracoccus* cNors (52% sequence identity [29]), suggested that the enzyme takes both electrons and protons from the periplasm rendering it non-electrogenic [28–31], which was borne out experimentally [22,29,31]. In contrast, the crystal structure of the quinol-dependent qNor from the Gram-positive *Geobacillus stearothermophilus* [26] revealed the presence of a putative proton transfer pathway between the cytoplasmic aqueous phase and the BNC suggesting that NO reduction by qNor could be electrogenic. Cu_ANor has so far only been found in bacilli [11,21,27]. Biochemical and protein N-terminal and genomic sequence analyses show that the enzyme from *B. azotoformans* is hetero-trimeric [8,21,27]. Cu_ANor accepts electrons from the endogenous cytochrome c_{551} [27], which is negatively charged (p$I \sim 3$, $E_m = 140$ mV). Cu_ANor contains a di-copper site (Cu_A) [21] similar to cytochrome *c* dependent Coxs [32]. Like cNor and qNor the enzyme has one heme *b* and a BNC consisting of heme b_3 and Fe_B [21].

Among the HCO superfamily, Coxs are established as proton pumps conserving energy in the form of a proton electrochemical gradient by employing two processes [32–34]. In the first process, the four protons needed for water formation (chemical protons) are taken up from the negative side of the membrane (e.g. cytoplasm) while electrons enter the enzyme from the positive side of the membrane (periplasm). The second process involves protons being pumped across the membrane from the negative side to the positive side (Eq. (3)) [32,35]:

$$O_2 + (4+n)H^+_{cyto} \rightarrow 2H_2O + nH^+_{peri} \quad (3)$$

Here *n* is the number of protons pumped ($n = 4$ for Type A and $n = 2$–4 for Types B and C Cox) [36,37]. The subscripts *cyto* and *peri* indicate the cytoplasmic (negative) and periplasmic (positive) sides of the membrane, respectively.

In this study the ability of Cu_ANor from *B. azotoformans* to couple NO reduction to the formation of a membrane potential was investigated. To this end we used protein-reconstituted liposomes. Our results provide the first experimental evidence that NO reduction by Cu_ANor in closed liposomes creates a proton electrochemical gradient indicating the energy conserving capability of the enzyme.

2. Materials and methods

2.1. Chemicals

Soybean asolectin was purchased from Fluka BioChemika, N-Dodecyl β-D-maltoside (LM) from Affymetrix and equine heart cytochrome *c*, Carbonyl cyanide 3-chlorophenylhydrazone (CCCP), valinomycin, phenazine ethosulfate (PES), safranine O and phenol red from Sigma–Aldrich. Saturated NO solutions (2 mM) were prepared by flushing anaerobic MilliQ water with 100% NO gas at room temperature.

2.2. Preparation of proteoliposomes (PLs)

Expression and purification of Cu_ANor, cytochrome aa_3 and cNor from *Paracoccus denitrificans* were performed as described in refs. [6,21,38], respectively. Soybean asolectin was dissolved in chloroform and dried with nitrogen gas to form a thin phospholipid film. The film was hydrated with 1 mL of 0.1 M K^+-HEPES pH 7.4 per 10 mg phospholipids. The resulting liposomes were fractured using 4 cycles of freezing in liquid nitrogen and thawing (75 °C). To obtain a uniform suspension of small unilamellar vesicles, the liposomes were extruded 15 times over a membrane with 0.2 μm pore size (Nuclepore Track-Etch Membrane, Whatman). The vesicle suspension was diluted to 5 mg/mL and incubated for 30 min with 6 mM LM to destabilize the vesicles before the addition of 0.1 μM or 0.5 μM of cytochrome aa_3 or Cu_ANor or cNor. In the case of the mixed aa_3/Cu_ANor-PLs or aa_3/cNor-PLs, 0.5 μM of each enzyme was added. The enzyme-containing liposome suspension was incubated for 1 h. Methanol-washed Bio-Beads (Bio-Rad SM-2) were used to adsorb the detergent in two successive steps (300 and 150 mg Bio-Beads/mL liposomes for 1, 5 h each step, respectively). The phospholipid and enzyme contents were determined in the final PL preparation from the phosphate content [39,40] and the Soret absorbance [6,27], respectively. Using cytochrome *c* the orientation of cNor was determined at 40 ± 5% right-side out a value similar to Cu_ANor (see Section 4).

2.3. Activity measurements

The activity of Cu_ANor-PLs and cNor-PLs was monitored optically from the formation of oxidized PES ($\varepsilon_{387\,nm} = 26\ mM^{-1}\,cm^{-1}$). At pH 7.6, reduced PES (PESH, estimated p*K* ~3.7 [41]) is a two-electron plus one proton donor, which we confirmed experimentally. Thus the NO consumption rate was calculated according to Eq. (4):

$$PESH + H^+ + 2NO \rightarrow PES^+ + N_2O + H_2O \quad (4)$$

2.4. Determination of the respiratory control ratio (RCR)

The V_{max} values were determined by simulation of the time course of the reaction according to the model (Eq. (5)) proposed earlier [6]:

$$\frac{v}{[E]} = \frac{V_{max}}{1 + K_2 * \left(\frac{1}{[NO]} + \frac{K_1}{[NO]^2}\right) + \frac{[NO]}{K_i}} \quad (5)$$

where K_1 and K_2 are the apparent binding constants for first en second NO molecules. The K_i term is the inhibition constant proposed to represent the binding of NO to the oxidized form of the enzyme [6]. The respiratory control ratio (RCR) was calculated by dividing the V_{max} values of the uncoupled PLs by that of the coupled PLs. The PLs were assayed in 20 mM HEPES + 45 mM KCl + 45 mM sucrose, pH 7.6. For complete uncoupling, 40 μM CCCP was added with or without 50 μM valinomycin. The RCR for cytochrome aa_3 PLs (aa_3-PLs) was determined based on the decrease in absorbance of reduced cytochrome *c* at 550 nm.

2.5. Measurement of the membrane potential ($\Delta\psi$)

Fluorescence quenching of safranine O [42,43] was used to monitor the formation of $\Delta\psi$ by the PLs. The assay with Cu_ANor-PLs and cNor-PLs contained 5 μM safranine O, 40 mM sucrose, 40 mM KCl, 60 μM PES^+ and 20 mM ascorbate, pH 7.6. Cu_ANor-PLs were also assayed without ascorbate in the presence of 60 μM PESH (with or without addition of 60 μM or 1 mM PES^+). The reaction was started with 10, 20 or 40 μM NO. The $\Delta\psi$ formation by aa_3-PLs was monitored in the same buffer without PES using 10 μM equine heart cytochrome *c* as electron donor and ~260 μM O_2 as electron acceptor in the presence of 20 mM ascorbate to prevent inner-filter quenching effects on the fluorescence by cytochrome *c*. The measurements were carried out in a Cary Eclipse (Varian) fluorescence spectrophotometer at room temperature using excitation and emission wavelengths of 495 and 585 nm, respectively. Additions were made after removing the cuvette from the fluorescence spectrophotometer. The reaction buffer was mixed thoroughly using a magnetic stirrer prior to