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Nitric oxide is a potent inhibitor of the *cbb*$_3$-type heme-copper oxidases

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ABSTRACT

C-type heme-copper oxidases terminate the respiratory chain in many pathogenic bacteria, and will encounter elevated concentrations of NO produced by the immune defense of the host. Thus, a decreased sensitivity to NO in C-type oxidases would increase the survival of these pathogens. Here we have compared the inhibitory effect of NO in C-type oxidases to that in the mitochondrial A-type. We show that O_2-reduction in both the *Rhodobacter sphaeroides* and *Vibrio cholerae* C-type oxidases is strongly and reversibly inhibited by submicromolar NO, with an inhibition pattern similar to the A-type. Thus, NO tolerance in pathogens with a C-type terminal oxidase has to rely mainly on other mechanisms.

1. Introduction

Heme-copper oxidases (HCuOs) are integral membrane proteins that catalyze reduction of oxygen to water as the final reaction of the respiratory chain, and conserve the energy thereby released by contributing to the proton electrochemical gradient. All HCuOs have a homologous catalytic subunit with twelve transmembrane helices containing 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. The HCuO superfamily is classified into three major subfamilies denoted A-, B-, and C-type [1,2]; in addition, the bacterial NO-reductases (NOR) belong to the same family where they form their own subfamily.

In the mitochondrial, or A-type HCuOs, electrons flow from the donor cytochrome (cyt.). *c* to a dinuclear Cu_A-site in subunit (SU) II, and from there into the low-spin heme *a* in SU I before reaching the binuclear heme a_3-Cu_B active site where O_2 is bound and reduced. 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. Both B- and C-type HCuOs differ from the A-type in that they presumably use only a K-pathway analogue for proton transfer [2,4–6].

The C (*cbb*$_3$) family of oxidases are found mainly in bacteria, and are often expressed under semi-anaerobic conditions as they show a low K_m for O_2 [7]. They are the O_2-reducing HCuOs evolutionarily closest to the NORs and most distant from the A-type [1,8]. The C-type enzymes also have the highest NO-reduction activity, excepting the true NORs [9,10]. In C-type HCuOs, the catalytic subunit CcoN contains the high-spin heme b_3-Cu_B catalytic site and a low-spin heme *b*. C-type HCuOs further typically consist of two membrane-anchored *c*-cytochromes; CcoO with one *c*-type heme, and CcoP with two *c*-type hemes, where electrons are presumed to enter from the donor cyt. *c*. The C-type HCuO from *Pseudomonas* (*P.*) *stutzeri* was recently structurally defined at atomic resolution [11].

C-type oxidases are often found in human pathogens since the lower K_m for O_2 enables colonization of microaerophilic habitats. In some pathogens, such as *Helicobacter* (*H.*) *pylori* and *Neisseria* (*N.*) *meningitidis*, the C-type HCuO is the only terminal oxidase, and therefore a potential drug target [12]. Pathogenic bacteria are likely to encounter elevated NO concentrations as NO is produced by macrophages in the immune defense of the host, and an increased NO tolerance of C-type oxidases would benefit the pathogens that harbor it.

For the mitochondrial A-type oxidase there is a large body of literature concerning inhibition by NO, which is a potent reversible inhibitor of respiration (for a recent review, see [13]). In the

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mitochondrial HCuO, NO binds rapidly, with high affinity (with IC_{50}s in the nM range [14]), and to several different intermediates in a range of different modes (see e.g. [15,16]).

In this study, we have compared the sensitivity to NO inhibition of the C-type HCuOs from both *Vibrio* (*V.*) *cholerae* and *Rhodobacter* (*R.*) *sphaeroides* to that of the A-type HCuO from *R. sphaeroides*. Our aim was to investigate whether the C-type HCuOs exhibit general insensitivity to NO inhibition. Furthermore, we wanted to learn if the NO-reduction activity in C-type HCuOs influenced the inhibition behavior. Our results show that O_2-reduction in C-type oxidases is strongly and reversibly inhibited by NO with the IC_{50} in the nanomolar range, similar to A-type HCuOs. In the two HCuOs from *R. sphaeroides*, the IC_{50} is higher for the C-type than for the A-type HCuO, but the overall behavior is very similar. In the *V. cholerae* cbb_3, however, the IC_{50} is very similar to that found in *R. sphaeroides* aa_3. These differences in the details of the inhibition will be discussed, but it is clear that mechanisms other than general NO insensitivity for C-type oxidases must be operating in pathogens with an increased NO tolerance.

2. Materials and methods

2.1. Protein purification

The *R. sphaeroides* Δcbb_3 strain carrying the plasmid pUI2803NHIS [17] was grown, and the cbb_3 wildtype purified as in [5].

The *V. cholerae* Δcbb_3 strain with plasmids for the polyhistidine-tagged cbb_3 protein were grown and the cbb_3 wildtype was purified essentially as in [18] but with the following modifications: the elution from the Ni^{2+}-agarose resin was performed using a gradient of 5–200 mM imidazole, and the major *V. cholerae* cbb_3 peak that elutes around 100 mM imidazole was collected. Imidazole was removed by repeated dilutions and concentrations in 20 mM Tris, pH 8.0, 100 mM NaCl, 0.05% β-D-dodecyl maltoside (DDM), and the protein flash frozen in liquid nitrogen and stored at −80 °C.

2.2. NO stock solutions and other reagents

The NO solution was prepared by bubbling NO (100% or 5% NO (in N_2)) gas into water made anaerobic by bubbling with N_2 as in [19]. Sodium nitrite (Sigma) stock solution was prepared in a concentration of 1–10 mM. Equine cytochrome (cyt.) *c*, and bovine hemoglobin (Hb) were from Sigma.

2.3. Steady-state activity measurements and data handling

The steady-state O_2 reduction activity of the different HCuOs was measured using a Clark-type electrode (Hansatech). The reaction chamber was filled (total of 1 mL) with 100 mM HEPES-KOH, pH 7.3, 50 mM KCl, 0.07% DDM, 10 mM ascorbate, 0.2 mM N,N,N′,N′-tetramethyl-*p*-phenylenediamine (TMPD), and 18 μM cyt. *c*. For measurements with the cbb_3 from *V. cholerae*, a 100 mM HEPES-NaOH at pH 7.3, 100 mM NaCl, 0.07% DDM buffer was used. Oxygen consumption was initiated by the addition of the enzymes, and different amounts of NO were added when the oxygen level had decreased to either 200 or 60 μM. The starting activity varied somewhat between preparations of the HCuOs, but was typically ~700 e^-/s for *Rs* aa_3, 350 e^-/s for *Rs* cbb_3 and 150 e^-/s for *Vc* cbb_3.

Instead of reporting IC_{50} as the NO concentration that gives 50% inhibition, we fitted the inhibitory effect of increasing amounts of added NO on the turnover activity of aa_3/cbb_3 as in [20], to Eq. (1) using Sigmaplot (Systat Software). Note that this is not a fit of dissociation constants for the enzyme-NO complex, as it acts as a competitive inhibitor (at least partly), but rather a way of reporting IC_{50}s using all available data. Note that because of very tight NO-binding (especially at lower O_2), the [NO] in Eq. (1) refers to the unbound [NO] in solution taking the enzyme concentration into account.

$$V_{obs} = \frac{V_0}{1 + [NO]/IC_{50}} \quad (1)$$

3. Results

3.1. NO inhibition of catalytic activity in C-type oxidases

NO inhibits catalytic O_2 reduction in C-type oxidases strongly and reversibly, as shown in Fig. 1. When ~1 μM NO is added to both A (Fig. 1A) and C-type oxidases (Fig. 1B and C), catalytic turnover is completely inhibited, but activity is regained after a certain amount of time, presumably because NO is slowly degraded in the presence of O_2, indicating that recovery occurs as NO is released from the active site. If hemoglobin (Hb) is added after NO, the recovery speeds up, as observed previously in A-type oxidases [13], since Hb can compete with the HCuOs for NO binding. We note that there is no significant difference in the time it takes to regain 50% activity between the *R. sphaeroides* A- and C-type oxidase (Table 1), which means that the NO-reduction activity exhibited by the C-, but not the A-type oxidase [9,10], does not significantly influence this time (see Section 4). The final recovered activity after Hb addition is slightly larger for the cbb_3s (90% of the original value, see Table 1) than for the *Rs* aa_3 (80%), which might be related to the sensitivity to NO_2^- (see below).

When the NO solutions are prepared, especially from only 5% NO, there is a risk that some NO is converted to nitrite by reacting with small amounts of residual O_2. Therefore we also tested the inhibitory effect of nitrite on turnover of the A- and C-type HCuOs. The results show that there is inhibition by nitrite, but at higher concentrations (at least several μM for *R. sphaeroides* aa_3 and cbb_3 and even more for *V. cholerae* cbb_3) than with NO and with a different inhibition pattern (data not shown). Interestingly, in the *R. sphaeroides* aa_3, when 10 μM NO_2^- is added at 60 μM O_2, inhibition recovers after a certain time, much like when adding NO, possibly indicating that some NO_2^- is converted to NO, as previously suggested for the mitochondrial aa_3 [21]. Basically, however, NO_2^- inhibition occurs at high enough concentrations that we assume it does not significantly influence our NO inhibition data.

3.2. Determination of the IC_{50} for NO inhibition in C-type oxidases

We titrated the effect on activity as a function of added NO in the three HCuOs used in Fig. 1, as exemplified by the data shown for *R. sphaeroides* cbb_3 in Fig. 2. Plots of the O_2-reduction rate immediately after NO addition as a function of added NO in the two HCuOs (aa_3 and cbb_3) from *R. sphaeroides* at both higher (~200 μM) and lower (~60 μM) O_2 are shown in Fig. 3, and fits of the data to Eq. (1) are shown as solid lines. The obtained IC_{50} values are 60 nM and 280 nM NO at 200 μM O_2 (A), and ~6 nM NO and 60 nM NO at 60 μM O_2 (B) for the *R. sphaeroides* aa_3 and cbb_3, respectively. Fig. 4 shows the comparison between *R. sphaeroides* and *V. cholerae* cbb_3 giving IC_{50}s for the *Vc* cbb_3 of 30 nM NO (A; 200 μM O_2) (A) and ~4 nM NO (B; 60 μM O_2).

It should be noted that the conditions of the *V. cholerae* cbb_3 assay are different (as horse heart cyt. *c* is not a good substrate, see [22]) from the conditions in which the two *R. sphaeroides* HCuOs are assayed; hence, the IC_{50} values might not be as easily compared. If the rate of electron input into the *Rs* HCuOs is increased by increasing the amount of cyt. *c* in the assay, the initial turnover rate increases somewhat and the sensitivity to NO