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Regulation of the hexaheme nitrite/nitric oxide reductase of *Desulfovibrio desulfuricans*, *Wolinella succinogenes* and *Escherichia coli*

A mass spectrometric study

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Dissimilatory nitrite reduction, carried out by hexaheme proteins, gives ammonia as the final product. Representatives of this enzyme group from 3 bacterial species can also reduce NO to either ammonia or N_2O. The redox regulation of the nitrite/nitric oxide activities is discussed in the context of the denitrifying pathway.

Hexaheme cytochrome; Nitrite reductase; Nitric oxide reductase; Mass spectrometry; *Desulfovibrio desulfuricans*; *Wolinella succinogenes*; *Escherichia coli*

1. INTRODUCTION

Dissimilatory nitrite reductases occur in several forms. In denitrifying bacteria, either non-heme copper [1,2] or cd_1 hemes serve as redox centers [3,4] in nitrite reductases and primarily release nitric oxide, which is subsequently reduced to nitrous oxide apparently by a *bc* cytochrome [5]. The N_2O is then reduced to dinitrogen by a multi-copper enzyme [6–9]. As the enzyme forming the N-N bond, and thus reversing N-fixation, nitric oxide reductase attracts much interest [10–14].

Despite controversy about the intermediacy of NO in denitrification, recent evidence ranging from kinetic [15] and chemical analysis [16] to mutagenesis [17], the presence of the enzyme within vesicles [18] and enzyme purification [5] combines to substantiate the validity of accepting NO as an intermediate.

Ammonia-generating nitrite reductases have typically been purified from spinach and display a complex active site comprised of a siroheme coupled to a single [4Fe,4S] center [19]. Nitrite reduction by hexaheme enzymes from strictly and facultatively anaerobic bacteria, such as *Desulfovibrio desulfuricans* (ATTC 27774) [20], *Wolinella succinogenes* [21], *Escherichia coli* [22], *Vibrio alginolyticus* [23] and *V. fischeri* [24], also give rise directly to ammonia. In concert, EPR and Mössbauer measurements indicate that one high-spin and 5 low-spin hemes function in each molecule of this type nitrite reductase [25]. Two pairs of magnetically interacting hemes (low-spin/low-spin; low-spin/high-spin) operate in this complex. The high-spin heme binds NO and appears to be the enzyme-active site. Reaction of nitrite with fully reduced enzyme reoxidizes the low-spin hemes, but the EPR spectrum reveals persistence of the high-spin heme in the NO-bound form. Since the six-electron reduction of nitrite yields no NO as a free intermediate [25,26], NO appears to exist as an enzyme-bound and not as a gaseous product during the nitrite-to-ammonia transit.

In view of such involvement of NO with the hexaheme nitrite reductases, we have studied the capacity of representatives of this group to reduce free NO. Their influence in the pathway, NO_2^- to N_2 is discussed as is the redox regulation of the nitrite/nitric oxide reduction activities in these multi-heme systems.

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Abbreviations: EPR, electron paramagnetic resonance

2. MATERIALS AND METHODS

Desulfovibrio desulfuricans (ATCC 27774), *Wolinella succinogenes* (VPI 10659) and *Escherichia coli* K12 strain CB900 were grown at 37°C under nitrate/nitrite respiring conditions as described by Liu and Peck [20], Yoshinari [27] and Aboud-Jaoude et al. [28], respectively. Purification of hexaheme nitrite reductases from all three proceeded as described by Liu et al. [20–22]. Analytical grade reagents and chemicals, and gases freed from oxygen by passage through a BASF catalyst, were used. Determining ammonia production as previously described [20], units of specific nitrite reductase activities of purified hexaheme were observed as follows: *D. desulfuricans*, 778; *W. succinogenes*, 770; and *E. coli*, 520. A unit equals the amount of protein required to convert 1 μmol nitrite to 1

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μmol ammonia · min^{-1}. Identical assay procedures were employed [20] when nitric oxide reduction to ammonia was determined.

2.1. *Mass spectrometry measurements*

We used a VG model 1380 mass spectrometer equipped for direct gas introduction to the ion source. The air-tight vessel used was connected by a vacuum line and a cold trap to the instrument's ion source [29]. In a typical experiment, 10 ml of 25 mM Tris-HCl buffer (pH = 7.6) was introduced into the reaction vessel and thoroughly purged of oxygen (mass peak 32) with purified argon gas. The following additions were made by gas tight syringe: sodium ascorbate, 20 μmol; phenazine methosulfate (PMS), 0.2 μmol; sodium nitrite (^{15}N), 100 μmol or nitric oxide (^{15}N), 40 μmol. After assuring that the levels of the components were stable, 10 nmol of hexaheme nitrite reductase were added to initiate reaction. Assay of a control system lacking the enzyme accompanied each experiment.

Nitric oxide gas (^{15}N), free of detectable $^{15}N_2O$, was prepared from acidified sodium nitrite (^{15}N) or was generated in the reaction vessels by the use of the copper nitrite reductase of *Achromobacter cycloclastes* acting on sodium nitrite (^{15}N). When NO (gas) served as electron acceptor, ethylene diamine tetraacetic acid (EDTA) was added to prevent non-enzymatic production of N_2O [30].

Enzyme activities were monitored by peak height variations related to appearance or consumption of component of interest. Use of ^{15}N-labeled components ensured unambiguous assignments. Mass peaks 31 (^{15}NO), 46 ($^{15}N_2O$) and 32 (O_2) were successively scanned by a peak jumping system monitored by an Apple II data acquisition system. A complete acquisition cycle lasted about 20 sec. The rates of change in concentration of ^{15}NO and $^{15}N_2O$ were recorded each min for 25 up to 50 min. Ammonia was trapped out and not detected in this system. Sensitivity towards the different components was measured and calibrations against pure components made as in [29].

3. RESULTS AND DISCUSSION

The hexaheme nitrite reductases reduce nitrite to ammonia in a six-electron step [13]. We now find that the use of dithionite as electron donor for the *D. desulfuricans* hexaheme enzyme yielded comparable rates of ammonia production when either nitrite or nitric oxide served as electron acceptor (Table I). Supplying ascorbate-PMS as electron donor gave only low yields of ammonia from either acceptor. Mass spectrometry then revealed that the hexaheme cytochrome nitrite reductases from *D. desulfuricans, W. succinogenes* and *E. coli* (Fig. 1A,B,C) reduced to N_2O (mass peak 46) the NO (mass peak 31) that had been generated at the expense of ascorbate-PMS by the *A. cycloclastes* copper enzyme before addition of the hexaheme enzyme. Concomitantly, as in many previous experiments, a low background of N_2O (mass peak 46)

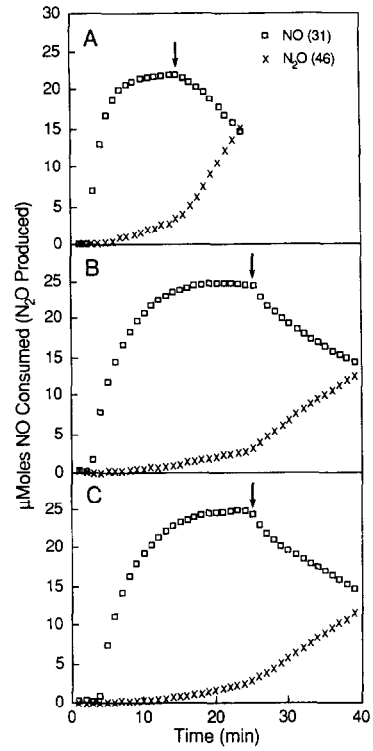

Fig. 1. NO consumption and N_2O production by hexaheme nitrite reductases (added at the arrows) at the expense of NO produced by the nitrite reductase from *A. cycloclastes*. (A) - *D. desulfuricans;* (B) - *W. succinogenes;* (C) - *E. coli.*

slowly accumulated during reduction of nitrite to NO. We found calculation of specific activity rates for NO reduction more conveniently based on the rates of N_2O release than on rates of decrease in NO concentration (Table II), for replenishing production of NO by the *A. cycloclastes* enzyme apparently continued even after the addition of the hexaheme cytochrome. A correction was made for this low background rate of N_2O resulting from the persistant action of the copper nitrite reductase. The specific activities of the hexahemes reducing NO to N_2O were unexpectedly much greater than that reported for the purified nitric oxide reductase from *P. stutzeri* [5].

The *A. cycloclastes* nitrite reductase contributed only NO to the overall activity of the system; exogenously

Table I

Specific Activity* of nitrite reductase from *D. desulfuricans* (ATCC 27774) utilizing different electron donors and nitrite or nitric oxide as electron acceptor

Source of electrons	Nitrite	Nitric Oxide
Dithionite	778	860
Ascorbate + PMS	28	35
NADPH + Crude Extr.	17	-

*The enzymatic unit equals the amount of reductase required to convert 1 μmol nitrite or nitric oxide to 1 μmol ammonia · min^{-1}.

Table II

Mass spectrometric measurement of Specific Activity* of nitric oxide reductase at the expense of electrons provided by ascorbate-PMS

Source of hexaheme cytochrome	NO (gas)	NO (**)
D. desulfuricans	535	360
W. succinogenes	-	133
E. coli	-	135

*The enzymatic unit equals the amount of reductase required to convert 1 μmol NO to 1/2 μmol of N_2O · min^{-1}.

**NO generated from the reaction of *A. cycloclastes* nitrite reductase with nitrite.

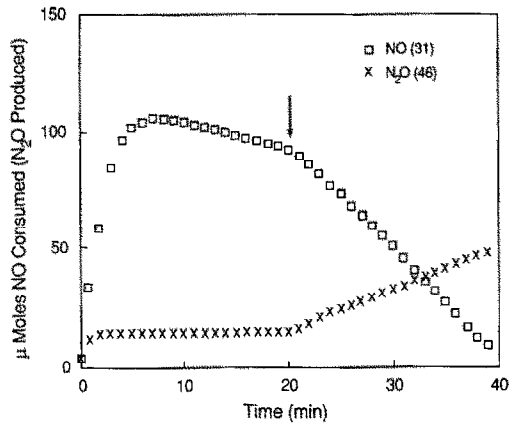

Fig. 2. NO consumption and N_2O production by *D. desulfuricans* (ATCC 27774) hexaheme nitrite reductase (added at the arrow) using exogenously supplied chemically generated NO gas. The *A. cycloclastes* copper protein was not present.

supplied NO gas was also immediately reduced to N_2O at the expense of ascorbate-PMS by the hexaheme nitrite reductase of *D. desulfuricans* with no *A. cycloclastes* enzyme present (Fig. 2) and with EDTA present to preclude non-enzymatic, ferrous ion-catalyzed reduction of NO [30].

Demonstration of a new catalytic capability for this hexaheme enzyme and the indication given that free as well as bound NO can be involved in nitrite reductase activity represent two major interests in this work. In studies of the role of NO in denitrification, three hypotheses have been considered [15,31]: (i) NO was not a plausible intermediate; (ii) NO was a free intermediate but kinetically controlled at low levels; or (iii) NO was an enzyme-bound intermediate. Recent studies indicate that NO is kinetically competent as an intermediate [15]. Moreover, trapping of free NO [32] and continued utilization of NO by nitrite reductase-less mutants [14] or by copper nitrite reductase-inhibited extracts [33] are consistent with NO being an intermediate. Purification of a nitric oxide reductase, a *bc* cytochrome from *B. stutzeri* [5], provides the strongest inference yet in favor of the involvement of NO as a free intermediate. Now, surprisingly, our studies demonstrate that several hexaheme cytochromes from non-denitrifiers can reduce free NO to ammonia when low-potential electron donors are available or, like denitrifiers, to N_2O when high-potential electron donors serve as sources, following these pathways:

$$\begin{array}{l} \nearrow \\ NO_2^- \\ \searrow \end{array} \begin{array}{l} [NO] \rightarrow NH_4^+ \text{ (with low potential electron donors)} \\ \\ [NO] \rightarrow N_2O \text{ (with high-potential electron donors)} \end{array} \quad \begin{array}{l} \nwarrow \\ NO\ (gas) \\ \swarrow \end{array}$$

This observation may explain how *W. succinogenes*, which usually yields ammonia and does not ordinarily produce NO or N_2O during growth dependent upon respiratory reduction of nitrate and nitrite, can nevertheless reduce NO to N_2O [34]. Unlike *D. desulfuricans* or *E. coli*, *W. succinogenes* produces a N_2O reductase as well [27]. The capacity to reduce free NO to either ammonia or N_2O has physiological significance in the environment, in particular as far as enterobacteria such as *E. coli* are concerned. In addition, our study shows that a mechanism that could prevent the inhibition of iron-sulfur proteins, which are considered to be the target for NO produced from arginine by macrophages [35] is present in these organisms. Removal of nitrite toxicity by reduction to N_2O has been strongly inferred as a function for the copper nitrite reductase of a rhizobium strain [36], in which such electron transfer does not support growth or appear linked to energy conservation [37].

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