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Evidence for an NO-rebound mechanism for production of N_2O from nitrite by the copper-containing nitrite reductase from *Achromobacter cycloclastes*

M.A. Jackson[1], J.M. Tiedje[2] and B.A. Averill[1]

[1]*Department of Chemistry, University of Virginia, Charlottesville, VA 22901, USA* and [2]*Department of Crop and Soil Science, Michigan State University, East Lansing, MI 42283, USA*

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Reduction of NO_2^- by the Cu-containing nitrite reductase from *Achromobacter cycloclastes* produces NO as the primary product initially, but as NO accumulates, NO production levels-off and N_2O production becomes significant. Reaction of the enzyme with NO_2^- in the presence of NO increases the amount of N_2O product significantly, while trapping the NO product as nitrosylhemoglobin or rapid removal of NO by sparging results in no detectable N_2O production. Reaction of the enzyme with $^{15}NO_2^-$ in the presence of ^{14}NO results in rapid formation of the mixed isotope product (^{14}N, ^{15}N)O in ca. 45% yield. In contrast, the presence or absence of NO has no effect on N_2O production by a prototypical heme cd_1-containing nitrite reductase. These results are consistent with formation of a labile Cu^+–NO^+ species in the copper enzyme, which normally decomposes to NO. Production of N_2O requires that the released NO must rebind to the enzyme to combine with a second NO_2^- or a species derived therefrom.

Denitrification; Nitrite reductase; Nitrosyl; Isotope labelling; Reaction mechanism; N_2O production

1. INTRODUCTION

Despite many detailed studies on intact bacteria, crude extracts, and purified enzymes [1–4], the mechanism of denitrification continues to be the object of controversy. Although it is now generally accepted that denitrifying bacteria possess a distinct nitric oxide reductase (NoR) activity and that at least a significant portion of the total nitrogen flux occurs via a stepwise pathway with NO as an intermediate (Eqn. **1**), the importance of an alternative direct pathway in which 2 nitrite ions are reduced to N_2O on a single enzyme, nitrite reductase (NiR), (Eqn. **2**), remains unclear.

$$NO_3^- \longrightarrow NO_2^- \xrightarrow{NiR} NO \xrightarrow{NoR} N_2O \longrightarrow N_2 \qquad (1)$$

$$NO_3^- \longrightarrow NO_2^- \xrightarrow{NiR} N_2O \longrightarrow N_2 \qquad (2)$$

On the one hand, quantitative studies of NO levels during denitrification by several organisms have been interpreted as indicating that only the stepwise pathway of Eqn. (1) is operative, such that NO is a free obligatory intermediate in denitrification [5–8]. This conclusion is consistent with the recent purification and characterization of NoR's as heme proteins [9,10], with the fact that mutants lacking the heme cd_1 [11] or Cu NiR's (R. Ye and J.M. Tiedje, unpublished results) are still capable of reducing NO, and with the fact that most isolated NiR's produce NO as the major product upon reduction of NO_2^-. On the other hand, recent ^{18}O labelling studies have shown that the extent of equilibration with H_2O of N_2O derived from NO_2^- is significantly less than that of N_2O derived from NO [12], indicating that, for some organisms at least, NO is not an obligatory intermediate. This conclusion is supported by a number of isotope labelling and trapping studies [13–16], by the fact that the ^{15}N isotope effect increases with increasing nitrite concentration, suggesting that 2 nitrite ions combine prior to the first irreversible step [17,18], and by the fact that purified NiR's do in at least some cases produce significant amounts of N_2O that cannot be attributed to chemical side reactions [19–21].

In order to determine the origin of some of these apparently contradictory findings, we have examined the reduction of NO_2^- by both the Cu- and heme cd_1-containing NiR's in some detail. The problem is complicated by the fact that one of the products, NO, is known to be a potent inhibitor of NO_2^- reduction by both types of enzyme [22,23]. In this communication, we present evidence that NO binding to the Cu NiR of

Correspondence address: B.A. Averill, Department of Chemistry, University of Virginia, Charlottesville, VA 22901, USA. Fax: (1) (804) 924 3710.

Achromobacter cycloclastes is essential for N_2O production by the enzyme.

2. EXPERIMENTAL

Nitrite reductases were isolated from *A. cycloclastes* [24] and *Pseudomonas stutzeri* [25] as previously described and stored frozen until used. Bovine hemoglobin was obtained from Sigma. Deoxyhemoglobin was prepared by the method of Doyle [26]. The β-93 thiols were blocked according to Winterbourn and Carrell [27]. The resulting hemoglobin was converted to the deoxy form with repeated vacuum/argon cycles until the visible spectrum of deoxyhemoglobin was obtained. All samples were maintained under anaerobic conditions. To measure the concentration of nitrosylhemoglobin (HbNO), a sample of the reaction mixture containing Hb was placed in a cuvette, and a visible spectrum acquired from 450 nm to 700 nm. The single band at 557 nm representing deoxyhemoglobin is split into two bands at 542 and 572 nm in HbNO.

Nitric oxide was from Scott Specialty Gases or Matheson and was bubbled through 1 M NaOH prior to use. NO has an Ostwald coefficient [28] of 0.04708, which gives an aqueous solution under 1% NO that is 21 μM in NO. For assays containing added nitric oxide, an aqueous solution of NO was transferred to the assay bottle via anaerobic syringe, and the mixture was stirred until equilibrium was attained. All GC analyses were performed on a Shimadzu Mini-2 GC with an ECD operating at 300°C and a 2m Poropack Q column at 70°C. The instrument utilized a closed system similar to that described by Kaspar and Tiedje [29]. Assays were run in 13 ml serum bottles stoppered with butyl rubber stoppers; the reaction mixtures were usually 3 ml. For assays utilizing the purge technique, the system was modified so that oxygen-free argon was passed through a diaphragm pump, a 150 ml serum bottle, and then through the gas sampling valve on the GC. Mass spectrometric analyses of ^{15}N-containing N_2O were performed as described previously [13,15].

Assays that contained hemoglobin were initiated by the addition of nitrite to a final concentration of 0.5 mM. The assay solutions contained the following: 2 mM NADH, 0.1 mM phenazine methosulfate (PMS) or thionine, 2 mM EDTA, and 2.6 nM Cu NiR, in 50 mM MES buffer, pH 6.3. The heme concentration was approximately equal to the nitrite concentration.

3. RESULTS AND DISCUSSION

3.1. *Effect of NO on Cu NiR activity*

A typical progress curve for *A. cycloclastes* NiR (Fig. 1) shows that the rate of NO production slows rapidly after an initial 'burst', and then levels-off rapidly. After 30 min, only about 3% of the available NO_2^- has been consumed (Table I). In contrast, the rate of N_2O production is approximately constant but relatively low, such that after 30 min the amount of NO_2^- converted to N_2O is ca. 3% of that converted to NO. Clearly, the presence of NO has a different effect on the production of NO vs N_2O. The simplest explanation for this observation is that NO is a substrate for the Cu NiR, being reduced to N_2O. The data in Table I, line 2, show that this is incorrect, however.

In order to examine further the relationship between NO and N_2O production, experiments were designed to examine the yield of N_2O in the presence of nitrite and varied amounts of NO. Thus, addition of amounts of NO comparable to those produced in a typical assay to

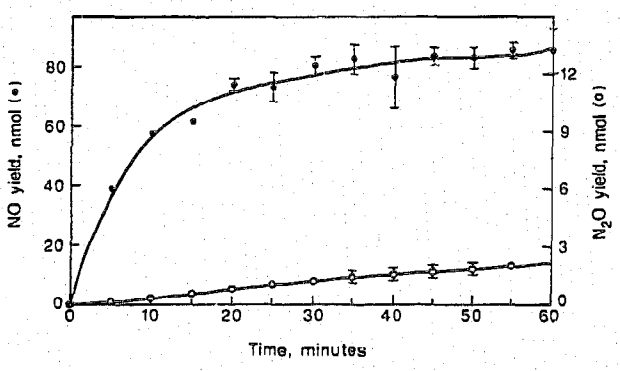
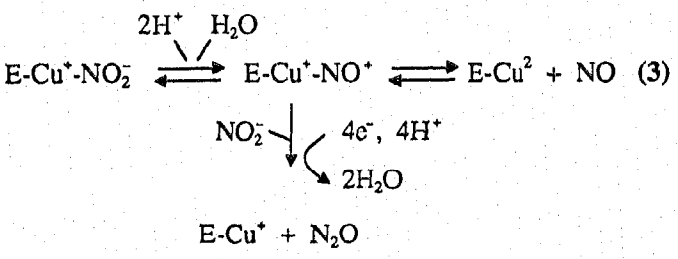


Fig. 1. Progress curve for NO and N_2O production from NO_2^- by *A. cycloclastes* NiR. Conditions as in Table I, line 1.

the reaction mixture at the start resulted in a tripling of the N_2O produced (Table I, line 3). In contrast, rapid removal of NO either by trapping as HbNO by reaction with deoxyHb or by sparging, resulted in no detectable amounts of N_2O (Table I, lines 4 and 5), but removed the inhibition of NO_2^- reduction as expected, resulting in much larger yields of NO. To show that the Cu NiR was not adversely affected by the presence of HbNO or deoxyHb, a reaction mixture comparable to that in Table I, line 4, was allowed to incubate for 30 min, followed by addition of NO_2^- to a final concentration of 5 mM to saturate the deoxyHb; this resulted in the production of NO and N_2O in the usual fashion, but in somewhat lower yields (data not shown). Production of NO in the sparging experiments appeared to be limited by enzyme denaturation caused by the vigorous bubbling necessary for efficient removal of NO.

Finally, reduction of $^{15}NO_2^-$ in the presence of ^{14}NO under the same conditions used in the experiment of Table I, line 3, resulted in rapid formation of the mixed isotope (^{14}N, ^{15}N)O, which constituted 40–50% of the total amount of N_2O produced (Table II). Thus, incorporation of NO into product N_2O is rapid.

The above results are most consistent with a minimal scheme for N_2O formation such as that shown in Eqn. 3:



In this scheme, the key species is a labile copper-nitrosyl species [30], from which loss of NO is rapid compared to attack by a second NO_2^- (in a fashion similar to that originally proposed for the heme NiR's [31]). The key species, E-Cu^+-NO^+, can be formed either from either

Table I

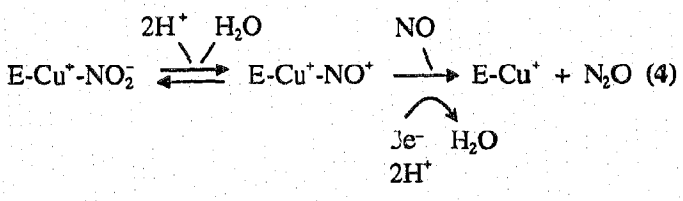
NO and N_2O yields from *A. cycloclastes* nitrite reductase[a]

	nmol NO	nmol N_2O
1) Control[b]	80	1.2 ± 0.3
2) NiR + 45 nmol NO[c]	–	0.0
3) 1 + 45 nmol NO	–	3.7 ± 0.8
4) 1 + Hb[d]	840[e]	0.0
5) 1 with sparging[f]	4100	0.0

All reaction times were 30 min. [b]Control contained a 3.0 ml solution of 1.0 mM NO_2^-, 2 mM EDTA, 1 mM NADH, 0.1 mM PMS, and 2.6 nM NiR in a 13 ml serum bottle. [c]Solution (1) but lacking NO_2^-. [d]See experimental section for details. [e]Based on [HbNO]. [NO_2^-] for this reaction was 0.5 mM. [f]Sparging experiments were performed using a 50 ml solution of (1) in a 150 ml serum bottle. Sparging rates were 3.7 l·min^{-1}.

from NO_2^- *or from NO via a 'rebound' mechanism*, which increases the concentration of the E-Cu^+-NO^+ intermediate, making attack by NO_2^- more probable. In contrast, rapid removal of NO by trapping or sparging drives the reaction to the right, suppressing N_2O formation.

An alternative that also agrees with the data, if the rate of NO release is rapid, and is equally plausible is shown in Eqn. 4:



This differs in that formation of the N–N bond occurs after $1e^-$ reduction of one NO_2^- to NO as shown in Eqn. 3, and cannot be distinguished from the scheme of Eqn. 3 based on available data.

The observation that NO inhibits its own formation at concentrations that favor N_2O formation suggests that NO can bind to the enzyme in two different modes. One results in N_2O formation via Eqn. 3 of 4 or some variant thereof, while the second suppresses NO formation altogether. A plausible site for the second NO binding is the Type 1 or 'blue' copper center, inasmuch as reversible bleaching of the CuNiR [32] and other blue copper proteins [33] by NO has been observed. This would presumably suppress electron transfer to the active site.

Table II

Incorporation of NO into N_2O

	Time (min) from injection of $^{15}NO_2^-$		
	5	10	15
$^{45}N_2O/^{45}N_2O+^{46}N_2O$	43±9	42±7	46

Reaction conditions as in Control for Table I but using $^{15}NO_2^-$. Values are for duplicate runs except for the measurement after 15 min.

Table III

NO and N_2O production by *P. stutzeri* JM300 nitrite reductase

	NO (nmol)	N_2O (nmol)
(1) Control[a]	100±10	0.55±0.03
(2) 1 + 40 nmol NO	70	0.28±0.05
(3) 1 with sparging[b]	770	2.52

[a]The control contained a 3 ml solution of 1 mM NO_2^-, 1 mM NADH, 0.1 mM thionine, 2 mM EDTA, and 0.8 nM NiR in a 13 ml bottle. [b]60 ml of solution (1) containing 3.3 nM NiR in a 150 ml bottle.

3.2. *Effect of NO on heme cd_1 NiR activity*

As shown by the data in Table III, the heme cd_1 NiR from *P. stutzeri* produces only small amounts of N_2O (ca. 1% of the total reduced N oxides), and the proportion of N_2O product is not particularly sensitive to addition of excess NO or to its removal by sparging. Instead, addition of NO results only in significant inhibition of NO production, consistent with the formation of stable ferroheme protein–NO complexes [34,35]. There is no evidence that NO interacts with the heme cd_1 enzyme in a productive fashion to enhance N_2O production.

3.3. *Conclusions*

The data presented above demonstrate a novel role for NO in the production of N_2O from nitrite by the Cu-containing NiR from *A. cycloclastes*. These results indicate that *A. cycloclastes* and, by implication, other organisms containing Cu NiR's possess a pathway for incorporation of nitrogen from NO into N_2O that is absent in organisms containing heme cd_1 NiR's. This may have substantial implications for interpretation of isotope exchange studies, which are generally carried out using relatively high (and non-physiological) NO concentrations.

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