

Evidence for an NO-rebound mechanism for production of N₂O from nitrite by the copper-containing nitrite reductase from *Achromobacter cycloclastes*

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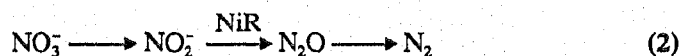
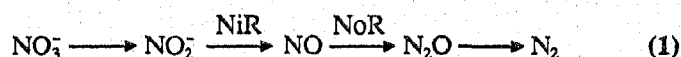
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Reduction of NO₂⁻ by the Cu-containing nitrite reductase from *Achromobacter cycloclastes* produces NO as the primary product initially, but as NO accumulates, NO production levels-off and N₂O production becomes significant. Reaction of the enzyme with NO₂⁻ in the presence of NO increases the amount of N₂O product significantly, while trapping the NO product as nitrosylhemoglobin or rapid removal of NO by sparging results in no detectable N₂O production. Reaction of the enzyme with ¹⁵NO₂⁻ in the presence of ¹⁴NO results in rapid formation of the mixed isotope product (¹⁴N, ¹⁵N)O in ca. 45% yield. In contrast, the presence or absence of NO has no effect on N₂O production by a prototypical heme cd₁-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 N₂O requires that the released NO must rebound to the enzyme to combine with a second NO₂⁻ or a species derived therefrom.

Denitrification; Nitrite reductase; Nitrosyl; Isotope labelling; Reaction mechanism; N₂O 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 N₂O on a single enzyme, nitrite reductase (NiR), (Eqn. 2), remains unclear.



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 obli-

gatory 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 cd₁ [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 NO₂⁻. On the other hand, recent ¹⁸O labelling studies have shown that the extent of equilibration with H₂O of N₂O derived from NO₂⁻ is significantly less than that of N₂O 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 ¹⁵N 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 N₂O 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 NO₂⁻ by both the Cu- and heme cd₁-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 NO₂⁻ reduction by both types of enzyme [22,23]. In this communication, we present evidence that NO binding to the Cu NiR of

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Achromobacter cycloclastes is essential for N₂O 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 μ M 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 Poropak 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 ¹⁵N-containing N₂O 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 mM 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 NO₂⁻ has been consumed (Table I). In contrast, the rate of N₂O production is approximately constant but relatively low, such that after 30 min the amount of NO₂⁻ converted to N₂O is ca. 3% of that converted to NO. Clearly, the presence of NO has a different effect on the production of NO vs N₂O. The simplest explanation for this observation is that NO is a substrate for the Cu NiR, being reduced to N₂O. The data in Table I, line 2, show that this is incorrect, however.

In order to examine further the relationship between NO and N₂O production, experiments were designed to examine the yield of N₂O 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

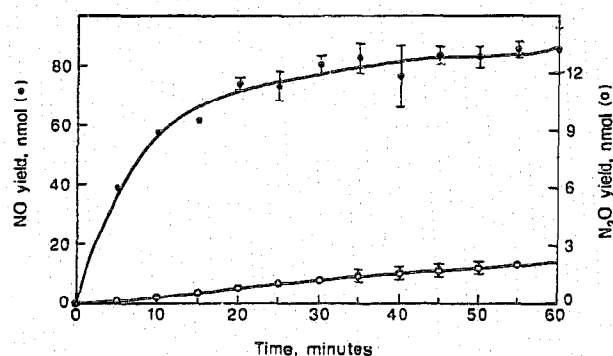
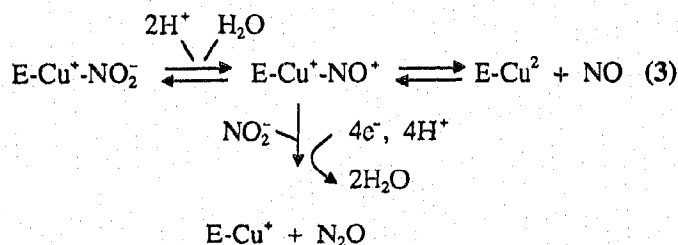


Fig. 1. Progress curve for NO and N₂O production from NO₂⁻ by *A. cycloclastes* NiR. Conditions as in Table I, line 1.

the reaction mixture at the start resulted in a tripling of the N₂O 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 N₂O (Table I, lines 4 and 5), but removed the inhibition of NO₂⁻ 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 NO₂⁻ to a final concentration of 5 mM to saturate the deoxyHb; this resulted in the production of NO and N₂O 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 ¹⁵NO₂⁻ in the presence of ¹⁴NO under the same conditions used in the experiment of Table I, line 3, resulted in rapid formation of the mixed isotope (¹⁴N, ¹⁵N)O, which constituted 40–50% of the total amount of N₂O produced (Table II). Thus, incorporation of NO into product N₂O is rapid.

The above results are most consistent with a minimal scheme for N₂O 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 NO₂⁻ (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

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