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IDENTIFICATION OF NITRIC OXIDE AND NITROUS OXIDE AS PRODUCTS OF NITRITE REDUCTION BY PSEUDOMONAS CYTOCHROME OXIDASE (NITRITE REDUCTASE)

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SUMMARY

Both NO and N_2O were identified by GC/MS as gaseous products of nitrite reduction catalyzed by the cytochrome oxidase (nitrite reductase) purified from Pseudomonas aeruginosa. Gas production was inhibited by cyanide. The enzyme also catalyzed the reduction of NO to N_2O. No N_2 was identified as a consequence of either nitrite or NO reduction.

INTRODUCTION

Dissimilatory denitrification by many microorganisms has been proposed by a number of investigators[1] to involve the following reactions:

$$NO_3^-(4+) \longrightarrow NO_2^-(3+) \longrightarrow NO(2+) \longrightarrow N_2O(1+) \longrightarrow N_2(0)$$

although other schemes have been proposed. The final product, dinitrogen, is evolved by the organism into the environment. Among the many organisms that have the capacity to participate in dissimilatory denitrification is Pseudomonas aeruginosa whose nitrite reductase has been studied intensively. This enzyme was first isolated by Okunuki and his colleagues as a cytochrome oxidase (ferrocytochrome c-551:oxidoreductase, EC 1.9.3.2) (1) but was demonstrated later by them to be a nitrite reductase as well (2). This enzyme, which has since been isolated and studied in a number of laboratories (3-5), contains two heme c and two heme d_1 moieties bound to a dimer of about 120,000 daltons (3-4). It has been proposed that the product of nitrite reduction is NO since optical spectra identical to its NO-complex were observed when the enzyme, reduced either with ascorbic acid or hydroquinone, was reacted anaerobically with $NaNO_2$ (6). Additional support for

[1] For discussions of denitrification see reviews by W. J. Payne (1973) Bacteriol. Rev. 37, 409-452, and by C. C. Delwiche and B. A. Bryan (1976) Ann. Rev. Microbiol. 30, 941-962.

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this view has been obtained by EPR spectroscopy in our laboratory (7). However, because no direct measurements of the gaseous products of nitrite reduction by the enzyme isolated from P. aeruginosa have been described, we would like to report our findings obtained by using a combination of gas chromatography and mass spectrometry (GC/MS).

MATERIALS AND METHODS

Materials. All inorganic reagents were of analytical grade. NADH, menadione (sodium bisulfite form), FMN, and crystalline bovine albumin were purchased from Sigma Chemical Co. Ascorbic acid (U.S.P. grade) was obtained from Merck. NO (min. purity 98.5%) and helium (zero grade) were products of Matheson while [^{15}N]$NaNO_2$ (99 atom %) was from ICN. Pseudomonas cytochrome oxidase (nitrite reductase) and cytochrome c-551 were purified from extracts of P. aeruginosa as described by Gudat et al. (4).

Reaction Mixture. The reaction mixture used to reduce nitrite employed NADH as an electron source. The NADH was coupled to the reduction of cytochrome c-551 and the nitrite reductase by means of a NADH-quinone reductase purified in our laboratory from extracts of P. aeruginosa (unpublished results). This reaction mixture of 2.7 ml in an 8-ml, side-armed test tube fitted with a rubber serum cap, contained 30 μmol $NaNO_2$, 6 nmol cytochrome c-551, 5 nmol menadione (sodium bisulfite form), 300 nmol FMN, 0.5 mg of NADH-quinone reductase, and 2.0 mg of Pseudomonas cytochrome oxidase (nitrite reductase) in 0.05 M potassium phosphate, pH 6.0. Anaerobic conditions were achieved by at least ten cycles of evacuation and flushing with helium over a period of about 30-45 min. The reaction was initiated by adding 0.3 ml (60 μmol) of NADH which had been made anaerobic by exhaustive flushing with helium. The reaction mixture was incubated with shaking at 25°C under 1 atm. of helium. Samples of the gas phase were withdrawn by means of a 100-μl gas-tight syringe inserted through the serum cap.

Analytical Methods. Protein was determined by the method of Lowry et al. (13) using bovine albumin as a standard. The concentration of cytochrome c-551 was calculated from ϵ (551 nm) of 28.3 nM^{-1} cm^{-1} (14) for the dithionite-reduced protein. For gas chromatography/mass spectrometry (GC/MS) a Hewlett-Packard model 5982 mass spectrometer in combination with a Hewlett-Packard model 5933 data system was used. For gas chromatographic separation a 5 ft. x 2 mm glass column packed with Chromosorb 102 (80/100 mesh) was used with both the injection port and column oven at room temperature (26°C). The initial flow of helium was 3 ml/min. The effluent from this column was allowed to flow directly into the source of the mass spectrometer which was operating in the electron impact mode with electron energy equal to 70 eV and a source temperature of 180°C. GC retention times and mass settings were first evaluated for standard gas samples from full mass scans prior to analysis of experimental samples in a "selected ion" mode. Quantitation was obtained from computer integration of the mass ion peak for each substance observed.

RESULTS

Reduction of Nitrite. GC/MS analyses of the gaseous products of nitrite reduction by Pseudomonas cytochrome oxidase (nitrite reductase) indicated, as seen in Fig. 1, that both [^{14}N]NO and [^{14}N]N_2O were generated. The concentration of NO reached a maximum about 30 min into the reaction and

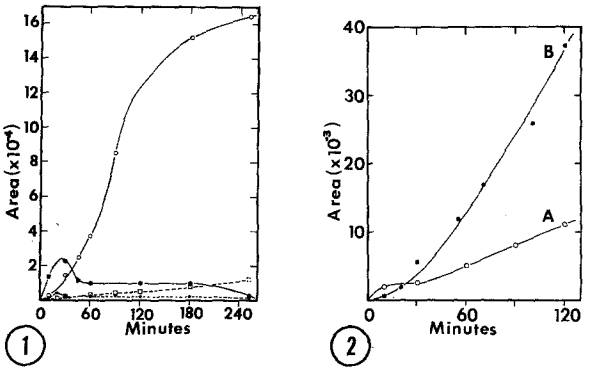

Figure 1. Formation of nitric oxide and nitrous oxide from nitrite by Pseudomonas cytochrome oxidase (nitrite reductase)

The reaction mixture contained in a final volume of 3 ml, 30 μmol $NaNO_2$, 5 nmol menadione (sodium bisulfite), 300 nmol FMN, 60 μmol NADH, 6 nmol cytochrome c-551, 0.5 mg NADH-quinone reductase, 2.0 mg Pseudomonas enzyme, and 0.05 M phosphate buffer, pH 6.0. The reaction vessel contained 1 atm. of helium and was incubated at 25°C. The gas phase was analyzed by GC/MS as described in Methods. Symbols, (•) NO; (o) N_2C; (★) NO control (no enzyme); (□) N_2O control (no enzyme).

Figure 2. Formation of nitrous oxide from nitric oxide by Pseudomonas cytochrome oxidase (nitrite reductase)

The reaction mixture was the same as that described in Figure 1 except that NO was used in place of nitrite. A, the reaction vessel contained 1 atm. of NO and was incubated at 25°C; B, the reaction medium without NADH was first incubated for 30 min under 1 atm. of NO; then 2.7 ml were transferred anaerobically to a sealed reaction vessel that contained 1 atm. of helium; after incubation for 10 min at 25°C the reaction was initiated by adding anaerobically 0.3 ml (60 μmol) of NADH. The gas phase was analyzed by GC/MS as described in Methods.

then declined slightly to a steady-state level which was maintained until it decreased after about 180 min. The concentration of N_2O increased rapidly after an initial lag and exceeded that of NO within 45 min following the start of the reaction. As seen in Fig. 1, a control system, which included all ingredients except the oxidase (reductase), produced some NO and N_2O but these products were present at significantly lower levels than those detected in the presence of the enzyme. A second control system, which contained only $NaNO_2$ and NADH in buffer, failed to yield any detectable amounts of NO or N_2O. If the reaction was carried out in the presence of 10^{-3} M KCN the generation of both NO and N_2O was reduced to the levels seen for the controls. When [^{15}N] $NaNO_2$ was used as the terminal electron acceptor the products of reduction in the presence of the enzyme were identified as [^{15}N] NO and [^{15}N] N_2O. However, no [^{15}N] N_2 was observed indicating that the system did not produce dinitrogen.

Another assay system, which utilized ascorbic acid rather than NADH as an electron source, also yielded both NO and N_2O as products of nitrite reduction. In this system, however, it was found that ascorbic acid alone reduced $NaNO_2$ to yield significantly large quantities of NO and N_2O. For that reason we relied primarily on the assay system that used NADH as an electron source.

Reduction of NO. Since N_2O was identified as a product of nitrite reduction, experiments were conducted to determine whether Pseudomonas cytochrome oxidase (nitrite reductase) could catalyze the reduction of NO when it was added to the reaction vessel in the gaseous state. Fig. 2A shows the results of such an experiment and indicates further that N_2O can be produced as a result of NO reduction. The rate of N_2O production from 1 atm. of NO using NADH as the electron source was less than that determined when nitrite was used as the initial terminal acceptor of electrons. In another experiment the reaction mixture, exclusive of NADH, was incubated with 1 atm. of NO for 30 min at 25°C with gentle agitation. Three ml of this solution were then transferred anaerobically by means of a gas-tight syringe to a sealed, 8-ml reaction tube containing 1 atm. of helium. After gently agitating the tube for 10 min at 25°C the reaction was initiated by adding NADH as for the other analyses. Fig. 2B shows that N_2O was a product of this reaction in the presence of the Pseudomonas enzyme. Interestingly, the rate of N_2O production in this experiment was higher than when the reaction was carried out in the presence of 1 atm. of NO. In addition and for as yet unexplained reasons, the amount of NO in the gas phase increased significantly after adding NADH to the reaction mixture and, after about 30 min, was approximately equal to the level of NO present in the control vessel at zero time. This liberation of NO was dependent on the presence of the enzyme since a control did not show any increase in the amount of gaseous NO when NADH was added.

DISCUSSION

GC/MS analyses of the gaseous products of nitrite reduction catalyzed by Pseudomonas cytochrome oxidase (nitrite reductase) clearly show that both NO and N_2O are produced. The pattern of gas production, as seen in Fig. 1, suggests a precursor-product relationship involving NO and N_2O, respectively. The presence of NO reductase activity in our preparations is also indicated by the reduction of NO which was added to the reaction vessels in the gaseous state. These results are similar to the findings of Sawhney and Nicholas (10) who showed by mass spectrometry that both NO and N_2O are gaseous products of nitrite reduction

catalyzed by a cytochrome cd which they purified from Thiobacillus denitrificans, and to those of Matsubara and Iwasaki (11) who found both nitrite and NO reductase activities in a cytochrome cd purified from Alcaligenes faecalis. Our results differ from those of Sawhney and Nicholas (10), however, in that they found N_2O appearing in the gas phase at a faster initial rate than NO.

Whether all nitrite reductases of the cytochrome cd type can reduce NO remains a question since, in contrast to the results cited above, LeGall et al. (12) and Zumft et al. (13) could detect only NO as a product of nitrite reduction catalyzed by enzymes purified from T. denitrificans and P. perfectomarinus, respectively. However, Zumft and Vega (14), working with a membrane fraction from P. perfectomarinus, identified N_2O as a product of the reduction of both nitrite and NO. Since these membranes contained cytochrome cd nitrite reductase, they tentatively attributed the NO reductase activity to that enzyme. It is possible that the NO reductase activity present in these preparations may be a function of species or strain. Alternatively, the presence in our preparation of a contaminating enzyme, which catalyzes the reduction of NO to N_2O, may be an explanation. However, such a contaminant would have to possess an extremely high activity since our preparations of the enzyme are essentially homogenous electrophoretically (4). Even if the purified nitrite reductase also catalyzes the reduction of NO it is not yet possible to claim that it functions in vivo as the major (or even a minor) NO reductase of P. aeruginosa. That function would depend on several factors including the presence of another NO reductase and the relative affinity and turnover for NO. To our knowledge there are no reports in the literature describing a distinct NO reductase from P. aeruginosa. However, the presence of such an enzyme is made plausible by the work of Payne and his associates (15) who obtained three fractions from extracts of P. perfectomarinus which reduced 1) nitrite to NO, 2) NO to N_2O, and 3) N_2O to N_2, respectively.

There has been some discussion by Hollocher and his co-workers (16-17) and by Zumft and Vega (14) as to whether NO is a free or a bound intermediate during the dissimilatory denitrification of nitrite. St. John and Hollocher (16) concluded that N_2O was released as a product from cells of P. aeruginosa in the presence of nitrite but that NO must remain bound since they could detect it only in trace amounts in the gas phase of their reaction medium. Our data with the isolated Pseudomonas cytochrome oxidase (nitrite reductase) certainly indicate that NO can be a free gaseous product of nitrite reduction and are consistent with a mechanism whereby NO is rebound and reduced to N_2O. We cannot, of course, exclude the

possibility that some NO remains complexed to the enzyme where it undergoes reduction to N_2O.

The lack of $[^{15}N]N_2$ in the gas phase of the reaction vessels demonstrates that our purified preparation of Pseudomonas cytochrome oxidase (nitrite reductase) lacks the capacity to reduce N_2O to N_2. This indicates that another enzyme in the organism is responsible for catalyzing that reaction.

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