Suppression by Nitrate of Enzymatic Reduction of Nitric Oxide[1] (34192)

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Barbaree and Payne (1) found gas chromatographic methods, known to be reliable for determining the components of specifically prepared mixtures of gases (2), useful in the analysis of products of denitrification carried out by *Pseudomonas perfectomarinus*—cells and extracts. Employing these procedures, we can now demonstrate routinely that nitric and nitrous oxides (as well as nitrogen) are products of nitrate dissimilation by enzymes in cell-free extracts of this bacterium. It has been generally known for some time, however, that in growing cultures of denitrifiers the flow of reduction is delayed. Before the oxides and nitrogen are released, nitrite accumulates nearly stoichiometrically with the quantity of nitrate reduced (3). Only after nitrate is depleted does dissimilative nitrate reduction go rapidly to completion (*e.g.*, release of gaseous nitrogen). This blockage occurs in *P. perfectomarinus* as in other denitrifiers and might be explained either (i) by a delay in induction of enzymes for reduction of nitrate and the intermediate nitrogenous oxides until nitrate is removed, or (ii) by suppression by nitrate of the functioning of the reductive enzymes further along the pathway. The purpose of this paper is to present results supporting the latter explanation.

Materials and Methods. We cultured *P. perfectomarinus* as described by Rhodes *et al.* (4) in tryptone–yeast extract–sea salt medium containing nitrate (TYSN). Cells were harvested by centrifugation after 20 hr and washed twice in 0.052 *M* $MgCl_2$, pH 7.0. From these we prepared cell-free extracts as before (1) and fractionated with ammonium sulfate. The precipitate from 40 to 70% saturation of the extract contained all the dentrifying enzymes. This crude fraction was dialyzed against 0.02 *M* phosphate buffer, pH 7.0, for 12 hr with three evenly spaced changes of buffer and then assayed. For certain experiments, the dialyzed fraction was loaded onto a diethylaminoethyl cellulose (DEAE, Whatman DE-52) column, 2.5 × 3.5 cm, and discontinuous elution gradients were employed further to fractionate the protein. We concentrated the column eluates with an Amicon model 50 ultrafiltration cell (UM-10 membrane) before assaying for activity. Protein contents of the extracts were measured by the biuret method (5) and by absorption at 280 nm. Gas chromatographic analyses were performed as described by Barbaree and Payne (1).

Release of nitrogen and the nitrogenous gases detectable by gas chromatography was accomplished by supplying the following components: dialyzed extract or eluted fraction as source of enzymes, reduced nicotinamide adenine dinucleotide (NADH) as the electron donor, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) as cofactors, and the appropriate oxide of nitrogen as the terminal electron acceptor (6). Before initiation of reactions by the addition of NADH and the appropriate terminal electron acceptor, we sparged the reaction mixtures with helium to provide anaerobiosis. The mixtures were then incubated in rubber-capped Warburg flasks on a shaking platform in a water bath at 30°. At intervals we obtained samples of the atmosphere above each reaction mixture with a 500-μl gas tight syringe (Hamilton Co., Whittier, Calif.) and injected them into the analyzer.

Results and Discussion. We observed (as have other workers previously with a number of denitrifiers) the accumulation of nitrite in TYSN cultures of *P. perfectomarinus*. Using for inoculum aerobically grown cells that had none of the denitrifying enzymes at the beginning, we found that the nitrite concentration increased in the cultures for 5–6 hr

[1] Supported by ONR Grant No. 3677(01) and NIGMS Training Grant 1 T01 GM01968-01.

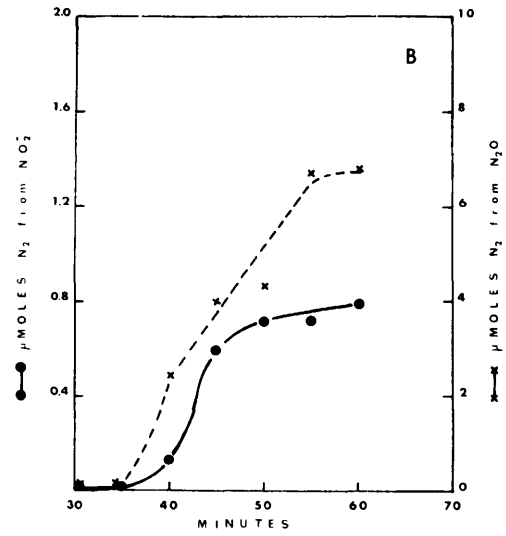

FIG. 1. Induction of nitrite and nitrous oxide reducing enzymes. Aerobically grown cells were incubated in the presence of nitrate in TYSN, and at 5-min intervals, samples were treated with 100 μg/ml of chloramphenicol. The treated cells were harvested and washed twice with 0.052 *M* $MgCl_2$ containing the same quantity of chloramphenicol. Conditions of assay: washed cells, 0.5 ml (from sample registering 500 Klett 420 units); phosphate buffer, pH 7.0, 750 μmoles; potassium or sodium nitrite, 15 μmoles, or nitrous oxide, approx. 15 μmoles; chloramphenicol, 500 μg; final liquid volume, 5 ml. Incubated at 30° for 20 min.

after inoculation and was rapidly depleted thereafter. Yet, all the enzymes needed for reduction of nitrite and nitrous oxide (the first and probably last intermediate) to nitrogen were present during the last 4–5 hr of this period. But, they were not functioning effectively. Assays of cells taken at 5-min intervals, treated and kept in contact with choloramphenicol while they were washed and assayed, revealed that these enzymes were in fact demonstrable after 40, but not 35, min of incubation (Fig. 1). Quantity of enzyme reached maximal at 55–60 min. Chloramphenicol was employed to stop protein synthesis. Inclusion of the inhibitor during both sampling and testing was further insurance against possibly continuing effects of induction. For further assurance, we demonstrated in control experiments with fully adapted cells that this antibiotic does not affect the functioning of the denitrifying enzymes. Thus it seems certain that the activity at each point on the curves is a true reflection of the quantity of enzyme produced up to that time.

We concluded from these observations that the presence of nitrate suppressed one or more subsequent reductive steps in the pathway and accordingly assayed nitrite, nitric oxide, and nitrous oxide reductase activities in the presence of varying quantities of nitrate (Fig. 2). Reduction of nitrous oxide to nitrogen was not affected (curve 1), but nitric oxide accumulated in the presence of nitrate (curve 2). This might be credited solely and simply to enrichment by nitrate reduction of the nitrite supply. But the diminished quantity of nitrous oxide produced from nitric oxide reduction in the presence of nitrate (curve 3) indicates that this latter is the reductive step sensitive to nitrate.

We were gratified then to find the suppressive effect exerted by nitrate on nitric oxide reduction extended to enzymes in the fraction that had been freed of reductive activi-

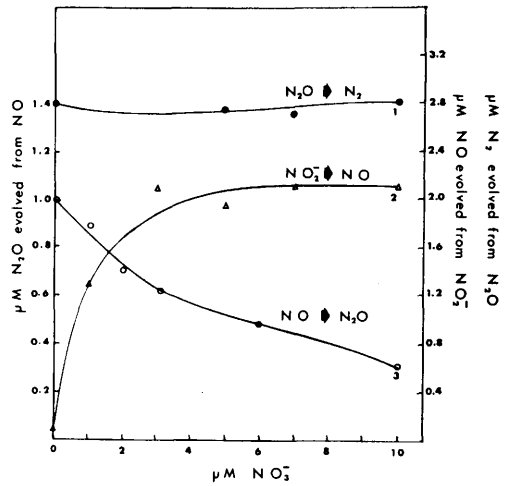

FIG. 2. Effect of nitrate on nitrite, nitric oxide, and nitrous oxide reductase activities in crude cell-free extracts. Conditions of assay: NADH, 1 μmole; FAD, 0.5 μmole; FMN, 0.5 μmole; Tris [tris(hydroxymethyl)aminomethane] buffer, pH 8.0, 250 μmoles; protein, 30 mg; final liquid volume, 5ml. The appropriate terminal electron acceptor was then added: nitric oxide, 5% of atmosphere; nitrous oxide, approx. 15 μmoles; or potassium or sodium nitrite, 2 μmoles. Final liquid volume, 5 ml; incubated at 30° for 30 min. Nitrogen was not detectable in experiments described by curves 2 and 3.

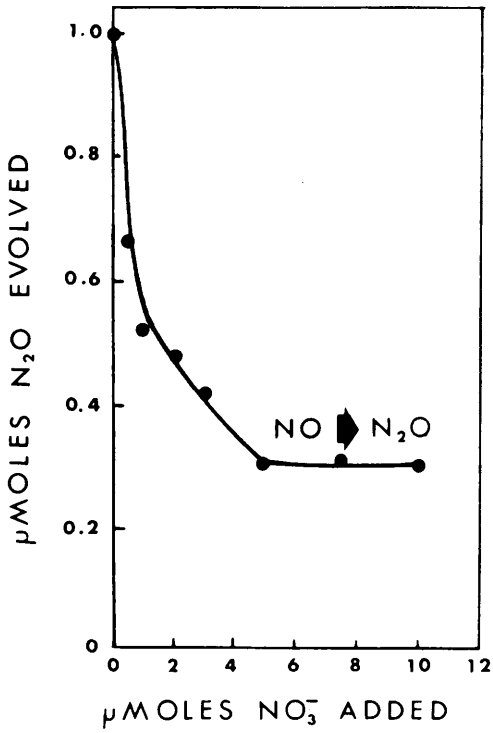

FIG. 3. Effect of nitrate on reduction of nitric oxide by somewhat purified enzyme fraction which did not reduce nitrate, nitrite, or nitrous oxide. The active enzymes were obtained by elution from a DEAE column with 0.23 *M* phosphate buffer. Conditions of assay: NADH, 1 μmole; FMN, 0.5 μmole; FAD, 0.5 μmole; Tris buffer, pH 8.0, 100 μmole; protein, 0.5 mg; nitric oxide, 5% of atmosphere. Final liquid volume, 5 ml; incubated at 30° for 30 min. Nitrogen was not detected.

ties for nitrate, nitrite, and nitrous oxide (Fig. 3). This confirmed the point of sensitivity in the pathway. In this somewhat purified system the nitrate was not converted to nitrite and thus could only be exerting its effect as *nitrate*. We have no indication yet of the nature of this suppression. Monovalent cations appear not to be influential in these reactions, for either potassium or sodium nitrate served equally well in equimolar quantity. The pH did not change during the course of the reactions. The only indication of difference we noted was derived from preliminary results which indicate that reduction of nitric oxide is less sensitive to metal chelators than nitrite and nitrous oxide reductase activities in crude extracts.

Cove and Pateman (7) recently observed that synthesis of enzymes for *assimilative* nitrate and nitrite reduction in *Aspergillus nidulans* is induced by either compound but controlled by a regulator and two structural genes. In our studies of *dissimilative* nitrate reduction, we found that nitrate, nitrite, nitric oxide, and nitrous oxide reductases are all induced by growth at the expense of the first, second, and fourth named compounds (nitric oxide does not support growth); but we have not yet investigated genetic control of synthesis. The data in Fig. 1 suggest, however, that synthesis of the entire system occurs simultaneously, for the capacity for reduction of both the first and probably the last intermediate became obvious at the same time. There may thus be one regulator gene for denitrification, and it may be affected by several inducers. It is very likely repressed by oxygen (8).

Summary. We found that the entire array of enzymes in *Pseudomonas perfectomarinus* that account for reduction of nitrate to nitrogen, including that which reduces nitrous oxide to nitrogen, was synthesized in 40, but not 35, min of incubation in a complete medium containing nitrate. Nitrite accumulated during the first 5–6 hr of culture despite the fact that we could demonstrate enzymes in crude cell-free extracts that reduced nitrite, nitric oxide, and nitrous oxide. To explain this, we found that nitrate suppressed the activity of nitric oxide reducing enzyme in crude extracts, as well as that in a fraction containing none of the other relevant reducing systems.

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Received May 22, 1969. P.S.E.B.M., 1969, Vol. 132.