Oxygen Regulation of Nitrate Uptake in Denitrifying *Pseudomonas aeruginosa*

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Oxygen had an immediate and reversible inhibitory effect on nitrate respiration by denitrifying cultures of *Pseudomonas aeruginosa*. Inhibition of nitrate utilization by oxygen appeared to be at the level of nitrate uptake, since nitrate reduction to nitrite in cell extracts was not affected by oxygen. The degree of oxygen inhibition was dependent on the concentration of oxygen, and increasing nitrate concentrations could not overcome the inhibition. The inhibitory effect of oxygen was maximal at approximately 0.2% oxygen saturation. The inhibition appeared to be specific for nitrate uptake. Nitrite uptake was not affected by these low levels of aeration, and nitrite reduction was only partially inhibited in the presence of oxygen. The regulation of nitrate respiration at the level of transport by oxygen may represent a major mechanism by which the entire denitrification pathway is regulated in *P. aeruginosa*.

Biological denitrification is defined as the production of gaseous nitrogen by the microbial reduction of nitrogen oxides and is an example of anaerobic respiration (24). In this process nitrogen oxides, in the absence of molecular oxygen, serve as respiratory electron acceptors and are reduced by a unique series of complex enzymes which conserve energy in several of the reductive steps by electron transport phosphorylation. The pathway for denitrification is as follows (12): NO$_3^-$→NO$_2^-$→NO→N$_2$O→N$_2$.

The enzymes of nitrate respiration are only formed when specific conditions for denitrification are present (8, 13). Anaerobic conditions alone are sufficient to partially de-repress the enzymes involved in nitrate respiration, but the presence of nitrate or nitrite intensifies the response (23).

The synthesis of denitrifying enzymes is repressed in the presence of oxygen (2, 7, 21). Oxygen blocks the synthesis of respiratory nitrate reductase in *Escherichia coli* at the level of transcription and at some later step of enzyme formation (7). Although the derepression of denitrifying enzymes is thought to require strict anaerobiosis, the activity of some of the preformed enzymes in the pathway may persist under aerobic conditions (8, 23).

Ever since the classic experiments of Gayon and Dupetit in 1886, it has been known that oxygen inhibits the reduction of nitrate and the formation of dinitrogen by denitrifying bacteria (4, 16). Addition of oxygen to cultured cell suspensions or reaction mixtures containing extracts of denitrifiers results in inhibition of the pathway at some level and thus a rapid loss of the capacity for reduction of nitrate and other nitrogen oxides (1, 6, 7, 12).

Until recently there has been very little work of a quantitative nature showing the magnitude of oxygen inhibition in relation to the partial pressure of oxygen during nitrate respiration. Furthermore, the significance of this inhibitory effect remains unclear because the site at which oxygen inhibits nitrate respiration has not been well defined. The effects of oxygen on nitrate respiration have been studied mainly by growing bacteria under anaerobic conditions, measuring nitrate reduction rates (i.e., nitrate disappearance or nitrous oxide formation), introducing oxygen, and observing any changes in the rates of nitrate respiration. As a result of this methodology, the effect of oxygen on nitrate entry into the cell has been overlooked as a major potential regulatory mechanism in nitrate respiration. The present work dealt with the identification of the level at which oxygen inhibition of nitrate respiration occurs in a denitrifying organism such as *Pseudomonas aeruginosa*.

**MATERIALS AND METHODS**

**Organism and growth conditions.** *P. aeruginosa* PAO1 was used. The organism was maintained on tryptic soy agar slants. To induce the nitrate respiration system, the organism was grown as follows: a large loopful of a 24- to 36-h tryptic soy agar slant culture was used to inoculate a 1-liter Erlenmeyer flask which contained 1 liter of tryptic soy broth supplemented with 50 mM filter-sterilized potassium nitrate. This culture was flushed with argon to establish anaerobic conditions, sealed with a stopper that allowed for gas release, and incubated at 37°C. The culture was vigorously stirred with a magnetic stir bar to prevent clumping and was incubated until the late log phase (20 to 24 h).

The cells were harvested by centrifugation at 10,410 g for 10 min at 23°C, and the pellet was washed twice with room temperature 10 mM Tris hydrochloride buffer (pH 7.4) and suspended at a concentration of 1.5 g (wet weight) per 50 ml of Tris buffer.

**Determination of oxygen, nitrate, and nitrite.** Changes in the concentration of oxygen and nitrate were measured in either a 50- or a 225-ml glass reaction vessel equipped with a Clark-type oxygen electrode (Yellow Springs Instrument Co. model 53 Oxygen Monitor) and an Orion nitrate electrode (model 93-07), in conjunction with a double junction reference electrode (model 90-02) as described by John (6). The nitrate electrode was connected to an Orion digital ion analyzer (model 601A). The signals from both the oxygen and nitrate electrodes were fed into two potentiometric recorders to obtain continuous traces. The nitrate electrode was also used to monitor nitrite concentrations if no nitrate was present. However, the response of the electrode to nitrite was of a smaller magnitude than that to nitrate. Total nitrite levels (internal and external) were determined by a colorimetric reaction after extraction with 5 N acetic acid (17). For the colorimetric reaction, a solution of 0.8% sulfanilic acid (reagent A) and 0.85% N-(1-naphthyl)
ethylendiamine (reagent B) was prepared in 5 N acetic acid. For the time course assays, 0.3 ml of sample was added to 2.1 ml of ice-cold 5 N acetic acid. After the addition of reagent A (0.3 ml), the sample was vortexed and 0.3 ml of reagent B was added to obtain a color reaction. The samples were read in a Gilford spectrophotometer at 545 nm after they were centrifuged for 5 min in a Beckman Microfuge (15,600 × g) to remove the cells. The absorbance of the sample was read against a standard curve to obtain the nitrite concentration. Calibration of the oxygen electrode was performed by measuring the stoichiometric conversion of hydrogen peroxide to water and oxygen by catalase (14). In our system 1% air dissolved in Tris buffer was equivalent to 22 nmol of O₂ per ml of buffer.

Uptake studies. For most studies, cell suspensions at a density of 1.5 to 2.0 g (wet weight) per 50 ml of 10 mM Tris hydrochloride buffer (pH 7.4) were placed in a glass vessel which was closed to the atmosphere and which possessed ports for the oxygen and nitrate electrodes, substrate addition, sample withdrawal, and gas flushing. A 10⁻¹ dilution (0.1 ml) of an antifoam emulsion (Antifoam B Emulsion, Dow Corning Corp.) was added to the cell suspension, and the suspension was vigorously stirred to prevent clumping and to maintain a homogeneous mixture. Glucose was added as an energy source to a final concentration of 10 mM. The cell suspension was flushed with argon several minutes before each experiment to ensure anaerobic conditions. This was ascertained by the use of the oxygen electrode to measure dissolved oxygen tension. To initiate the reaction, potassium nitrate or potassium nitrite was added to the cell suspensions to the desired concentrations (final concentrations, 0.2 to 1 mM). When the effects of aeration on the uptake of nitrate or nitrite were to be determined, air was vigorously bubbled through the cell suspension at a rate of 120 to 150 ml per min.

Preparation of cell extracts. Cells grown and harvested as described above were suspended in 10 mM Tris hydrochloride buffer (pH 7.4) at a density of 1 g (wet weight) per 3 ml of buffer. This suspension was placed in a glass sonicating vessel which was maintained in an ice bath throughout the procedure. Sonication was performed with a Branson Sonicator by the application of five 30-s bursts at 1-min intervals. The crude extract was centrifuged twice at 11,950 × g for 10 min at 4°C, and the supernatant fraction was collected, brought to 50 ml with 10 mM Tris buffer, and maintained under an argon atmosphere until needed.

Protein determinations. The amount of protein in the various samples was determined by the method of Lowry et al. (18) after the samples were boiled in a water bath for 2 min in the presence of 2 M NaOH. Bovine serum albumin was used as a standard. Spectrophotometric measurements were performed with a Gilford spectrophotometer.

RESULTS

Oxygen inhibition of nitrate utilization by whole cells. The disappearance of nitrate from the medium of a cell suspension of denitrifying P. aeruginosa was easily monitored with a nitrate electrode (Fig. 1). Nitrate utilization began immediately after its addition, and all external nitrate was consumed in less than 5 min. The rate of nitrate utilization was determined to be 140 nmol of NO₃⁻ per min per mg of protein (Fig. 1, inset). The effect of oxygen on nitrate utilization by a denitrifying culture of P. aeruginosa is shown in Fig. 2. Aeration of the cell suspension immediately and completely inhibited nitrate utilization. However, this inhibition was readily reversed by shifting the cell suspension back to anaerobic conditions, and in fact the cell suspension could be exposed to several shifts with the same results (data not shown). It is interesting that whereas oxygen inhibition appeared to be instantaneous, recovery from inhibition took approximately 30 to 60 s. This delay probably occurred because it took that long for the cell suspension to once again become completely anaerobic. The rates of nitrate utilization, calculated from the data in Fig. 2, before (91 nmol of NO₃⁻ per min per mg of protein) and after (89 nmol of NO₃⁻ per min per mg of protein) the suspension was shifted back to anaerobic conditions demonstrate that the reversion of inhibition was complete.

Effect of aeration on nitrate reduction by cell extracts. Nitrate utilization by denitrifying cells is a function of two processes, nitrate transport into the cell and subsequent reduction of nitrate inside the cell. The nitrate electrode cannot differentiate between nitrate uptake and intracellular nitrate reduction in whole-cell suspensions. To discern at which level the inhibition of nitrate utilization by oxygen occurs, cell extracts of denitrifying cells were prepared from cells grown under conditions identical to those described for the experiments for which results are shown in Fig. 1 and Fig 2. These extracts were used to determine what effect, if any, oxygenation had on the rate of nitrate reduction. The rationale was that if nitrate uptake is inhibited by aeration but reduction is not, we should be able to observe nitrite formation in cell extracts in the presence of oxygen.
nitrate reduction by cell extracts was linear as a function of time even in the presence of oxygen (Fig. 3). It should also be noted that upon aeration of the extract (A), the rate of nitrite formation increased, and upon shifting back to anaerobic conditions (B), the rate of nitrite formation decreased to what it had been before aeration. This apparent increase in the rate of nitrite formation during aeration of the extract was probably due to a partial inhibition of nitrite reductase by oxygen, which resulted in a higher rate of nitrite accumulation. Purified nitrite reductase from various denitrifiers has been shown to be a cd-type cytochrome in which the d component possesses oxidase activity and can reduce oxygen to water. In the presence of oxygen, nitrite reduction is inhibited and nitrite reductase behaves as an oxidase (8).

The rates of nitrite formation during the anaerobic and aerobic experimental periods were 24.7 and 30.2 nmol of NO$_2^-$ per min per mg of protein, respectively. On the basis of these rates, we calculated an 18% inhibition of the rate of nitrite reduction upon oxygenation of cell extracts. We repeated this experiment with several different extract preparations, and inhibition of nitrite reduction varied from 12 to 21%.

Effect of aeration on nitrite accumulation. When nitrate was added to a cell suspension of denitrifying *P. aeruginosa*, nitrate accumulation could be detected after several minutes (Fig. 4). As the cell suspension was aerated (Fig. 4A), total levels of nitrite (internal plus external) decreased until anaerobic conditions were reestablished (Fig. 4B). We interpret these results along with the data shown in Fig. 2 and 3 as follows. Under anaerobic conditions, nitrate is transported into the cell where it is converted to nitrite, which is subsequently reduced by the sequence of reactions of the denitrification pathway. Upon aeration of the culture, nitrate transport into the cell stops and any nitrate present in the cell is quickly reduced to nitrite, which is then further reduced. The ability of *P. aeruginosa* to reduce nitrite during transient aerobic conditions may be of some importance to the organism since it results in the quick depletion of nitrite, which even at low concentrations inhibits aerobic respiration or respiration-driven processes or both (15, 24). When anaerobic conditions are reestablished, nitrate transport into the cell resumes.

Effect of oxygen concentration on nitrate uptake. The effect of increasing oxygen concentration on the rate of nitrate utilization is shown in Fig. 5. Aeration of the culture resulted in a decrease in the rate of nitrate utilization, with complete inhibition occurring when aeration of the medium reached 0.21% oxygen saturation (dissolved oxygen tension, 4.6 nmol of O$_2$ per ml of Tris buffer). Recently, Trevors (20) reported that in soil slurries in vitro denitrification did not occur when oxygen levels in the gas phase were 0.28 μmol per ml or above. By using the Bunsen coefficient for oxygen in water ($\beta = 0.031$ at 20°C), it can be estimated that the DOT above which denitrification was inhibited in soil slurries was approximately 8.68 nmol of O$_2$ per ml. When the differences in the nature of the samples and the experimental conditions used in the two investigations are considered, these values appear to be in close agreement.

These results indicate that *P. aeruginosa* can detect very
In facultative anaerobic bacteria the synthesis of enzymes involved in anaerobic electron transport is repressed by aerobiosis. This general regulatory phenomenon has been termed the oxygen effect (7). Although not entirely clear in all instances, it has been thought by most workers that oxygen may affect dissimilatory nitrate reduction (i) by suppressing the formation of enzyme systems that catalyze these reactions, (ii) by directly interfering with the action of the enzyme systems when they are already present in bacteria, (iii) by diverting the flow of electrons from one respiratory system (nitrate) to another (oxygen), or (iv) by a combination of these processes (7, 10).

Until recently there has been very little work of a quantitative nature showing the magnitude of oxygen inhibition of nitrate respiration in relation to the partial pressure of oxygen during denitrification. The first quantitative approach to the problem of nitrate reduction was made by Stickland (19), who determined the influence of oxygen at various partial pressures on the reduction of nitrate to nitrite by cell suspensions of _E. coli_. Under conditions of aeration that should have maintained an equilibrium of oxygen distribution, he found that as little as 1% oxygen inhibited nitrate reduction to nitrite by 50%, whereas approximately 3% oxygen resulted in 93% inhibition of nitrate reduction.

In contrast, Marshall et al. (11), Sacks and Barker (16),

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**DISCUSSION**

The obvious economic importance of losses of nitrogen from the soil due to the action of denitrifying bacteria has stimulated investigations into factors affecting this process. The mechanism by which nitrate respiration is affected by oxygen is particularly interesting.
and other workers have reported the occurrence of denitrification under aerated conditions and in the presence of oxygen concentrations as high as 5%. It has been suggested that since oxygen levels were not monitored in these experiments, reports of aerobic denitrification may be attributed to the fact that the cultures were not adequately oxygenated (3, 5). This conclusion is further supported by the observation that in certain environments anaerobic processes such as denitrification may occur even though the surrounding environment is well oxygenated, due to the presence of anaerobic microenvironments (22).

In a recent study, Kawakami et al. (8) investigated the effect of oxygen levels on the reduction of nitrite to nitrous oxide by nitrite-grown Paracoccus denitrificans. They found that aeration of the cell suspension reversibly inhibited this process but that some activity was present at air concentrations as high as 2 to 10%. This is in agreement with our findings on nitrite uptake by whole cells; we found that aeration (1.8% air) did not inhibit transport of nitrite and only partially inhibited nitrite reductase.

Hochstein et al. (5) working with continuous cultures of Paracoccus halodenitrificans observed that, upon increasing the oxygen concentration, nitrogen production shifted first to nitrous oxide, then to nitrite, and finally to nitrate. They concluded that nitrous oxide reductase (which reduces N₂O to N₂) is most sensitive to oxygen inhibition, followed by nitrite reductase, and that the least sensitive step is nitrate reduction.

It can be surmised from the literature that the denitrification pathway is affected by oxygen at several different levels. At the level of reduction of nitrate to nitrite, there is evidence that the synthesis of enzymes of the pathway is repressed in the presence of oxygen (7). It has also been established that nitrous oxide reductase activity is irreversibly inhibited by very low oxygen concentrations (1, 5, 9). Although it has been observed that reduction of nitrate to nitrite is affected by oxygen in whole-cell preparations, very little is known about the nature of the inhibition or the possible role this phenomenon may play in the regulation of the denitrification pathway. Our results indicate that at least for P. aeruginosa the inhibitory effect is immediate and reversible. Furthermore, the inhibition appeared to be at the level of nitrate uptake, since it was observed in whole-cell preparations but not in cell extracts. Nitrite uptake by whole cells was not affected by oxygen at the levels tested; however, nitrite reduction itself, whether by whole-cell suspensions or by cell extracts, was partially inhibited in the presence of oxygen. The regulation of the denitrification pathway at the level of nitrate transport represents a primary mechanism by which the entire pathway may be regulated.

Most denitrifying bacteria of significance in soil are basically aerobes and prefer to utilize oxygen as the terminal electron acceptor. The oxygen-respiring enzymes are thought to remain functional during denitrification, albeit at low levels. Since transfer of an electron pair from substrate to oxygen provides more energy than does the equivalent transfer to nitrate (12), it would be advantageous for a denitrifying organism to be able to respond to transient changes in oxygen concentration and divert electron flow from one respiratory system (nitrate) to another (oxygen) without inactivating the denitrifying enzymes. From this point of view the regulation of nitrate transport by oxygen provides the organism with a tremendously flexible means of immediately reacting to changes in oxygen tension.

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**LITERATURE CITED**


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