A UNIQUE NITRIC OXIDE-BINDING COMPLEX FORMED BY DENITRIFYING PSEUDOMONAS AERUGINOSA

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SUMMARY

Extracts of denitrifying Pseudomonas aeruginosa cells showed difference absorption maxima at 542 nm and 573 nm after exposure to nitric oxide. The 573 nm maximum decreased in intensity with time and nitrous oxide was concomitantly released in a linear manner. The rate of decrease in light absorbance at 573 nm was enhanced by the addition of L-malate. These results suggest that this complex represents a nitric oxide-cytochrome$_{573}$ intermediate in the enzymatic conversion of nitric oxide to nitrous oxide.

INTRODUCTION

Pseudomonas aeruginosa belongs to a unique group of non-fermenting bacteria which can respire either with oxygen as the terminal electron acceptor or, anaerobically, with nitrogen oxides as terminal electron acceptors. Physiologically, the latter phenomenon is "anaerobic respiration" and is specifically termed denitrification. It is viewed as the concluding step of the nitrogen cycle with the conversion of nitrate and nitrogen oxides to elemental nitrogen. Denitrification is thought to occur in a stepwise manner as follows:

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$$

Recent investigators, however have questioned whether nitric oxide is an obligatory intermediate of the denitrification pathway (1). In this regard we report the presence of an apparent nitric oxide-cytochrome complex in extracts of denitrifying P. aeruginosa which appears to function in the bimolecular formation of nitrous oxide. This complex was not observed in aerobically-grown cells.

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METHODS

P. aeruginosa PAO (formerly Holloway strain 1) was used throughout this study. The organism was grown aerobically in a basal salts medium (2) supplemented with 0.1% yeast extract and 0.5% glucose. The medium was further supplemented with 1% KNO_3 for anaerobic growth.

Cultures were harvested during late exponential phase or early stationary phase of growth. The cells were washed twice with 0.05 M potassium phosphate, pH 7, and stored at -20° C until used. The frozen cells were then thawed, suspended in 0.05 M potassium phosphate, pH 7, and passed three times through a French pressure cell (18,000 psi) in the cold. Whole bacteria and fragments were removed by centrifuging at 5,000 x g for 20 min at 4° C. The cell extract was dialyzed against a 500-fold volume of cold 0.05 M potassium phosphate, pH 7, and then kept in ice. Samples were transferred to reaction vessels as needed. Protein was determined by the semi-microbiuret procedure (3).

Spectrophotometric analysis was performed with a Beckman Acta V recording spectrophotometer set on double beam. Difference spectra were obtained for cell extracts contained in 3-ml quartz cuvettes (light path, 1 cm) fitted with serum bottle stopers. The appropriate volume of gas or reagent solution was injected with a hypodermic needle into the extract-containing cuvette using a 0.5-ml syringe and the reaction mixtures were scanned through the desired range of wavelengths.

Reduction of nitrite or of nitric oxide to nitrous oxide was followed by gas chromatography as described previously (4). A Carle AGC 111 gas chromatograph unit, equipped with an Infotronics digital integrator and a 1 mv Omni stripchart recorder, was used for these studies.

RESULTS

The effect of nitric oxide on the difference spectrum between 500 nm and 600 nm is shown in Fig. 1A. Absorption maxima at 542 nm and 573 nm appeared immediately upon the addition of nitric oxide; and, as little as 160 nmoles of nitric oxide per mg of protein elicited this response. On the other hand, addition of nitrite at twice the molar concentration of nitric oxide did not cause an immediate appearance of these absorption maxima other than the expected oxidation in the cytochrome c region as indicated by the large trough between 550 nm and 560 nm (Fig. 1B); but, incubation of the cell extract with nitrite for 10 min resulted in the formation of a small peak at 573 nm (Fig. 1C). If, instead, high concentrations of nitrite were injected (e.g., 10 μmoles per mg of protein), the 573 nm absorption maximum developed as rapidly as when nitric oxide was used. In the latter case, the difference spectrum resembled that which was observed when nitric oxide was

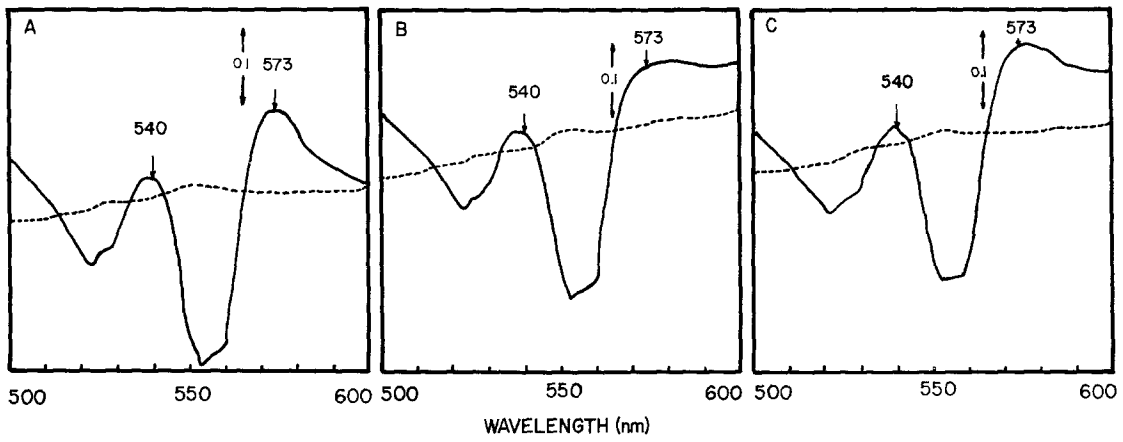

FIGURE 1. The effects of nitric oxide (NO) and nitrite (NO_2^-) on the difference spectrum between 500 nm and 600 nm of cell extracts from denitrifying P. aeruginosa. Each cuvette contained 25 mg of cell extract protein and the final volumes were 2.5 ml and 2.6 ml respectively for the cuvettes containing NO and NO_2^-. The base line, as represented by the dashed line, is shown for each reaction. A, 4 µmoles of NO were injected into the sample cuvette and the difference spectrum (NO-treated minus untreated) was recorded. B, 10 µmoles of NO_2^- was injected into the sample cuvette and the difference spectrum (NO_2^--treated minus untreated) was recorded. C, same as "B" above except that after injection of NO_2^- the reaction mixture was incubated 10 min before the difference spectrum was recorded.

supplied and the 573 nm absorption peak persisted over a long period of time. Thus, the effect of nitrite was interpreted to be indirect and attributable to its reduction to nitric oxide by nitrite reductase. The rapid and prolonged appearance of the 573 nm absorption maximum when high concentrations of nitrite were used is thought to be due to the inhibitory effect of nitrite on nitric oxide reductase (5) resulting in the accumulation of nitric oxide. Nitrous oxide, nitrate or nitrogen did not cause the formation of the 573 nm absorption peak. The peak was considered to be specific for nitric oxide. Interestingly, however, all of these compounds except nitrogen were capable of oxidizing the c-type cytochromes. Of considerable importance is the additional fact that the 573 nm nitric oxide complex was not detected in aerobically-grown P. aeruginosa.

We reasoned that if the 573 nm complex did indeed represent an intermediate between nitric oxide and nitrous oxide, then we should be able to

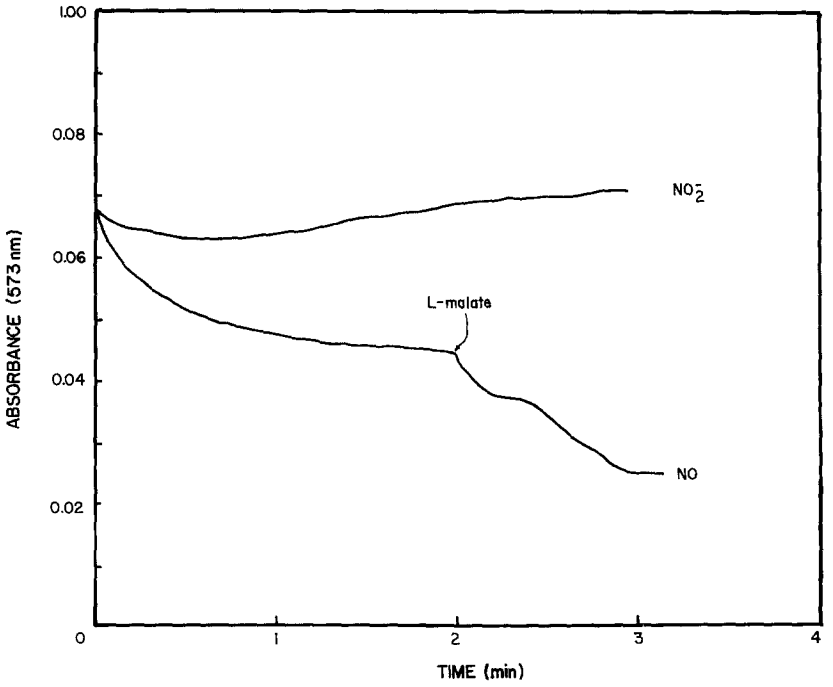

FIGURE 2. Absorbance at 573 nm monitored with time after treatment of a cell extract of denitrifying P. aeruginosa with nitric oxide (NO) and nitrite (NO_2^-). Curve NO; the extract was treated with NO as described in Fig. 1A and the reference system was untreated. L-Malate (2 μmoles) was added to the sample cuvette at the point indicated by the arrow. Curve NO_2^-; the sample was treated as described in Fig. 1B and the change in absorbance monitored with time.

see a decrease in absorption with time as the nitric oxide is converted to nitrous oxide. Furthermore, the rate of the reaction should increase upon addition of an electron donor. Thus, according to prediction, after the generation of the 573 nm absorption peak by the introduction of nitric oxide, a decrease in absorbancy at 573 nm occurred. The rate of decrease was clearly enhanced by the addition of L-malate (Fig. 2). (The latter compound was showned previously to serve as an electron donor supporting active transport in membrane vesicles of P. aeruginosa [2,6].) Moreover, when gas chromatographic analysis of the atmosphere over the reaction mixture in the cuvette was carried out, nitrous oxide was detected. A separate experiment with the same extract demonstrated the formation of nitrous oxide from nitric oxide

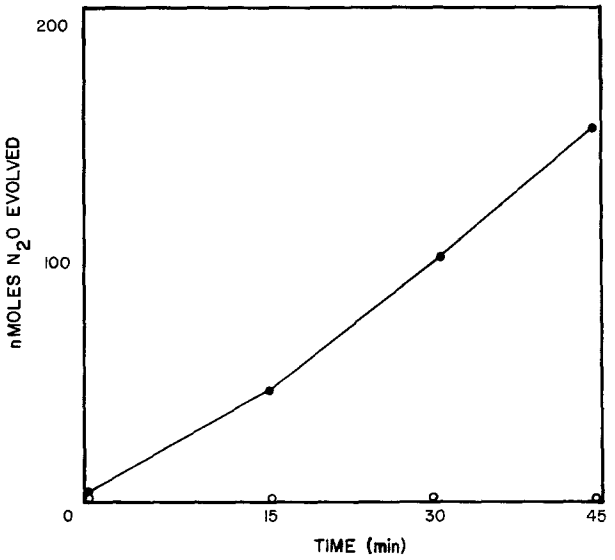

FIGURE 3. The headspace of a 5-ml stoppered, serum bottle containing 1 ml of cell extract (24 mg protein/ml) of denitrifying P. aeruginosa was injected with 250 µl of nitric oxide (NO). Samples (200 µl) were removed at time intervals for gas chromatographic analysis. Closed circles, fresh cell extract. Open circles, cell extract heated for 5 min in a boiling water bath.

to be linear with time (Fig. 3). Heat-treated cell extract exhibited no nitric oxide reduction. Thus, these data clearly indicate that nitric oxide was being enzymatically converted to nitrous oxide.

DISCUSSION

Doubt has been expressed that nitric oxide is a true intermediate of denitrification. This question was partially resolved when nitric oxide reductase activity was detected in cell extracts prepared from denitrifying microorganisms (4,7). Unlike nitrite or nitrous oxide, however, nitric oxide is not released from cells, even transiently, under ordinary conditions (4,5). Moreover, a nitric oxide-nitrite reductase complex was reportedly observed in a purified preparation of cytochrome cd from P. aeruginosa (8). This complex exhibited an absorption maximum at 551 nm with a shoulder at 570 nm. It was postulated that the complex represented the second step in

the enzymatic reduction of nitrite to nitric oxide although little directly supportive evidence was presented.

We have presented evidence herein for a clearly separate factor, with characteristics of a cytochrome, in cell extracts from denitrifying P. aeruginosa. This factor specifically binds nitric oxide and absorbs at 542 nm and 573 nm. The absorbance suggests that the factor might be a high wavelength cytochrome b species or a low wavelength d species. (There is no known cytochrome c species with an absorbance as high a 573 nm.) Spectrophotometric and gas chromatographic evidence strongly suggests that this factor has a physiological role in the enzymatic conversion of nitric oxide to nitrous oxide. The factor was detected only in cell extracts of denitrifying P. aeruginosa and not in cell extracts of aerobically grown bacteria.

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