Nitrite Oxidase, A Particulate Cytochrome Electron Transport System from Nitrobacter*

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The somewhat obscure pathway and mechanism of nitrite oxidation in the obligately chemautotrophic bacterial genus Nitrobacter have been receiving increasing attention. Lees and Simpson (1957) using contaminated cell suspensions of Nitrobacter reported cytochrome absorption peaks at 589, 551 and 520-525 mμ upon addition of nitrite or dithionite. More recently Aleem and Alexander (1958) demonstrated a nitrite oxidising system in cell-free Nitrobacter extracts which was stimulated by the addition of iron and inhibited by low concentrations of cyanide.

The present preliminary paper demonstrates that the nitrite-oxidizing activity of cell-free preparations of uncontaminated Nitrobacter cultures resides solely in a cytochrome-containing particle designated as nitrite oxidase; and implicates a sequence of electron transport from nitrite to molecular oxygen via cytochrome c-like and cytochrome oxidase-like components. The specific requirements of this system for inorganic iron, and the stabilization by glutathione of the cell-free enzyme system during storage are also reported.

Nitrite oxidase activity of cell-free extracts prepared from Nitrobacter agilis cells (ATCC No. 9482), grown and disrupted by sonic oscillation as described by Aleem and Alexander (1958), was determined by measuring the

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decrease in nitrite concentration (Bratton et al., 1939) in aerated 0.5 ml. reaction mixtures incubated for 10 to 25 minutes at room temperature.

Nitrite oxidase activity of cell-free extracts requires molecular oxygen, as evidenced by the lack of nitrite disappearance under anaerobic conditions, and is confined solely to a particulate fraction. Centrifugation of the crude cell-free extract at 10,000 x g for 30 minutes gives an opalescent supernatant solution (fraction S-10,000) containing more than 80% of the nitrite oxidase activity. Recentrifugation of fraction S-10,000 at 144,000 x g for one hour yields an inactive clear supernatant solution (fraction S-144,000) and a red pellet (fraction P-144,000) possessing approximately 50% of the activity. The presence of added glutathione (10^{-3} M final concentration) during sonication of Nitrobacter cells results in cell-free extracts whose nitrite oxidase is considerably more stable during storage, undergoing only a 10 to 20% loss in activity after two weeks in the frozen condition compared to a 60 to 80% loss in the controls.

Examination in the Cary[1] or split-beam[2] recording spectrophotometers of intact cells or cell-free preparations possessing nitrite oxidase activity e.g., fractions S-10,000 (not shown) and P-144,000, with added nitrite showed absorption maxima in the 550 and 520 mμ regions (representative of the alpha and beta peaks of a cytochrome component of the c type) and in the 585-590 mμ and 438 mμ regions indicative of the alpha and gamma peaks of a cytochrome a-type, probably a_1 (Fig. 1). However, the addition of nitrite to fraction S-144,000 which lacked nitrite oxidase activity, resulted in a small absorption maximum at 550 mμ, and in almost all cases a negligible peak at 585-590 mμ. Added dithionite or cyanide gave essentially similar peaks (not shown), but of several-fold greater magnitude, and also produced an absorption maximum at 415 mμ corresponding to the gamma peak of cytochrome c. The effect of cyanide

1 We are grateful to Dr. Bernard L. Strehler of the U.S. Public Health Service for performing most of the spectrophotometric measurements on a model 14 Cary spectrophotometer equipped with a light scattering device and the sensitive slide.

2 This was kindly performed by Drs. Britton Chance and Ronald W. Estabrook at the Johnson Foundation, University of Pennsylvania.

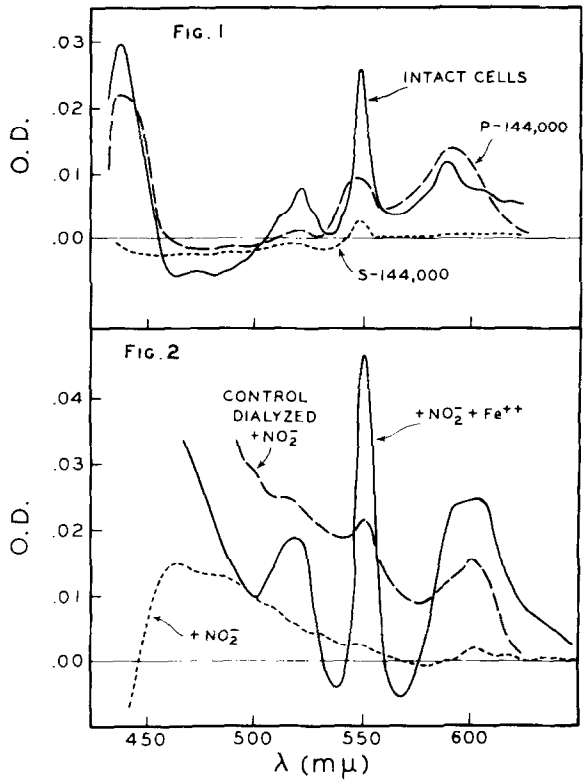

Fig. 1. Steady State-Oxidized difference spectra of intact Nitrobacter cells and cell-free fractions P-144,000 and S-144,000 upon addition of KNO_2.

Fig. 2. Steady State-Oxidized difference spectra of cyanide-dialyzed and control dialyzed fractions P-144,000 upon addition of KNO_2 and $FeSO_4$. The "$+NO_2^-$" and "$+NO_2^- + Fe^{++}$" curves are for cyanide-dialyzed fraction P-144,000.

is tentatively ascribed to an inhibition of the cytochrome a_1-like portion of the electron transport scheme and to the presence of endogenous substrate. The above results suggest that both cytochrome c- and cytochrome a_1-like components, presumably oriented as an integrated system in the particle, are necessary for nitrite oxidase activity. The suggestion, by Lees and Simpson (1957), that cytochrome 551 alone is concerned with nitrite oxidation therefore seems unlikely. The specificity of nitrite as a substrate for nitrite oxidase is indicated by the failure of added succinate, DPNH or lactate to produce any of the above reduced cytochrome absorption maxima.

The absence of hemoglobin, catalase and peroxidase in fractions P-144,000 and S-144,000 was demonstrated by direct spectrophotometric examination[2] with added potassium ferricyanide, hydrogen peroxide and methyl hydrogen peroxide, respectively (Chance and Maehly, 1955).

The stimulation of nitrite oxidase activity by added iron, originally reported by Aleem and Alexander (1958), is somewhat variable, some preparations failing to respond to the added metal ion. It is now possible to demonstrate consistently a specific requirement for iron by nitrite oxidase of fraction P-144,000 by means of the cyanide-dialysis procedure of Nicholas and Nason (1954). A 50 to 100% restoration of activity in fraction P-144,000, which had been completely inactivated by the cyanide-dialysis procedure, was accomplished specifically by Fe^{+++} or Fe^{++} (4×10^{-3} M). Copper, zinc, manganese, cobalt, nickel, tungstate, magnesium, vanadate, molybdate and borate ions were without effect. Tungstate and copper enhanced the non-enzymatic disappearance of nitrite.

The above cyanide-dialyzed fraction P-144,000 when examined spectrophotometrically, showed appreciably smaller cytochrome peaks upon addition of nitrite as compared to the dialyzed control (Fig. 2). The addition of ferrous ions as $FeSO_4$ (5×10^{-3} M final concentration) alone or together with nitrite resulted in the appearance of the reduced cytochrome *c*- and cytochrome a_1-like absorption maxima (Fig. 2). This can probably be attributed to the non-enzymatic reduction of the cytochrome *c*-like component by Fe^{++} (Weber *et al.*, 1956). The role of inorganic iron in nitrite oxidase is not clear, however, since the addition of ferric ions plus nitrite failed to restore the steady state difference spectrum of the cyanide-dialyzed system. It has also not been possible thus far to demonstrate the enzymatic reduction of Fe^{+++} by nitrite in the presence of fraction P-144,000 aerobically or anaerobically.

Thus far it has not been possible to observe a flavin component spectrophotometrically or to show a flavin effect in the nitrite oxidase system. Atabrine dihydrochloride at final concentrations of 10^{-3} M inhibited the nitrite oxidizing activity of intact cells and fraction P-144,000 approxima-

tely 60 and 25%, respectively. Quinine sulfate at the same concentration caused a 30% inhibition in both cases. These inhibition results are indefinite with regard to suggesting a flavin involvement.

One interpretation of the above data would picture the nitrite oxidase system as a series of enzymatic steps involving the transfer of electrons from nitrite to molecular oxygen via cytochrome c- and cytochrome a_1-like components as indicated in the following electron transport sequence:

$$NO_2^- \rightarrow \text{cytochrome } c \rightarrow \text{cytochrome } a_1 \rightarrow O_2$$

It is quite possible that iron may be functioning between nitrite and cytochrome c. The exact site and role of iron in this scheme as well as the possible concomitant occurrence of phosphate esterification are now under investigation.

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