# OXIDATION OF HYDROXYLAMINE BY PARTICLES FROM NITROSOMONAS

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Cell-free preparations from Nitrosomonas europaea have been shown to oxidize hydroxylamine to nitrite (3, 6). This reaction is greatly enhanced by addition of suitable electron acceptors such as mammalian cytochrome c, methylene blue, or phenazine methosulfate (6). In this communication we present data on the participation of various components of the respiratory chain in the oxidation of hydroxylamine by particles prepared from Nitrosomonas europaea.

The bacteria, grown in batch culture (6), were well washed with Tris buffer (0.05 M, pH 7.5) to remove nitrite. The cells were then suspended in the buffer and disrupted with a Mullard ultrasonic probe (20 kilocycles/second) at 0° for 20 minutes. The resulting homogenate was centrifuged at 15,000 × g for 20 minutes to remove cell debris and the supernatant fraction (A) recentrifuged at 144,000 × g for 60 minutes in a Spinco Model L ultracentrifuge. The pellet (B) formed was resuspended in Tris buffer and the

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supernatant fraction which was centrifuged further at 144,000 × g for 16 hours yielded a second pellet (C) which was also taken up in the buffer. In air, fractions A, B, and C oxidized hydroxylamine to nitrite in the presence of catalytic amounts of mammalian cytochrome c. Under anaerobic conditions substrate amounts of cytochrome were reduced by hydroxylamine but nitrite was not formed. The supernatant fraction left after centrifuging for 16 hours was devoid of these enzymic activities.

The hydroxylamine-mammalian cytochrome c reductase activity of the particles was assayed in the presence of $10^{-3}$ M KCN which inhibits cytochrome oxidase and nitrite production. When the reaction mixture contained substantially more mammalian cytochrome c than hydroxylamine, the data given in Table I support the following stoichiometry:

(1) 2e + 2 cytochrome c $Fe^{+3} \rightarrow$ 2 cytochrome c $Fe^{+2}$

TABLE I

STOICHIOMETRY OF HYDROXYLAMINE-MAMMALIAN CYTOCHROME C REDUCTASE ACTIVITY OF PARTICLES

The final reaction mixture contained the following: KCN, 3 μmoles; Tris-HCl buffer, 122.5 μmoles; oxidized cytochrome c (Sigma, Type III, from horse heart), 63 mμmoles; enzyme (pellet C), 21.3 μg; and $NH_2OH$ in the amounts shown. Final volume = 3 ml; temperature = 23°. The enzyme was preincubated for 5 minutes in the mixture in the absence of $NH_2OH$. At the end of this time $NH_2OH$ was added and the maximum reduction of cytochrome c was determined by following absorbancy at 551 mμ by a Beckman DU spectrophotometer. Under these conditions reduced cytochrome c was not reoxidized and nitrite was not formed.

| mμmoles $NH_2OH$ added | mμmoles cytochrome c reduced | % Theoretical yield* |
|---|---|---|
| 5 | 9.7 | 97 |
| 10 | 19.2 | 96 |
| 15 | 27.6 | 92 |

*Based on the following reaction: 2e + 2 cytochrome c $Fe^{+3} \rightarrow$ 2 cytochrome c $Fe^{+2}$

Similar results were obtained with this reaction mixture under anaerobic conditions, or when it was treated with CO. Quinacrine hydrochloride at 1.7 mM markedly inhibited the oxidation of hydroxylamine and this effect was reversed by adding 2.9 mM FAD as shown in Table II.

TABLE II

SOME PROPERTIES OF PARTICLES THAT HAVE HYDROXYLAMINE-MAMMALIAN CYTOCHROME C REDUCTASE ACTIVITY

The final reaction mixture contained KCN, 3 μmoles; Tris-HCl buffer, 122.5 μmoles; oxidized cytochrome c (Sigma, Type III, from horse heart), 63 mμmoles; enzyme (A), 0.5 mg; and the various additions indicated in the following amounts: $NH_2OH$, 0.2 μmole; quinacrine hydrochloride, 5 μmoles; FAD 8.6 μmoles; and DPNH, 0.9 μmoles. Final volume = 3 ml; temperature = 23°. The enzyme was preincubated for 5 minutes in the mixture in the absence of $NH_2OH$, FAD, or DPNH. At the end of this time these were added as indicated in the table and the rate of reduction of cytochrome c was determined by following the rate of change in absorbancy at 551 mμ by means of a Beckman DU spectrophotometer.

| Experiment | Additions | mμmoles cytochrome c reduced per minute |
|---|---|---|
| 1 | $NH_2OH$ | 13.8 |
| 2 | $NH_2OH$ + quinacrine hydrochloride | 3.9 |
| 3 | $NH_2OH$ + quinacrine hydrochloride + FAD | 10.0 |
| 4 | DPNH | 3.1 |

The particulate preparation oxidized DPNH at only one-fifth the rate obtained with hydroxylamine. Amytal at 4 mM or antimycin A at 3.33 μg/ml did not inhibit the reduction of cytochrome c by either hydroxylamine or DPNH. Under conditions described in Table II cytochrome c was not reduced by either DL-β-hydroxybutyrate or succinate.

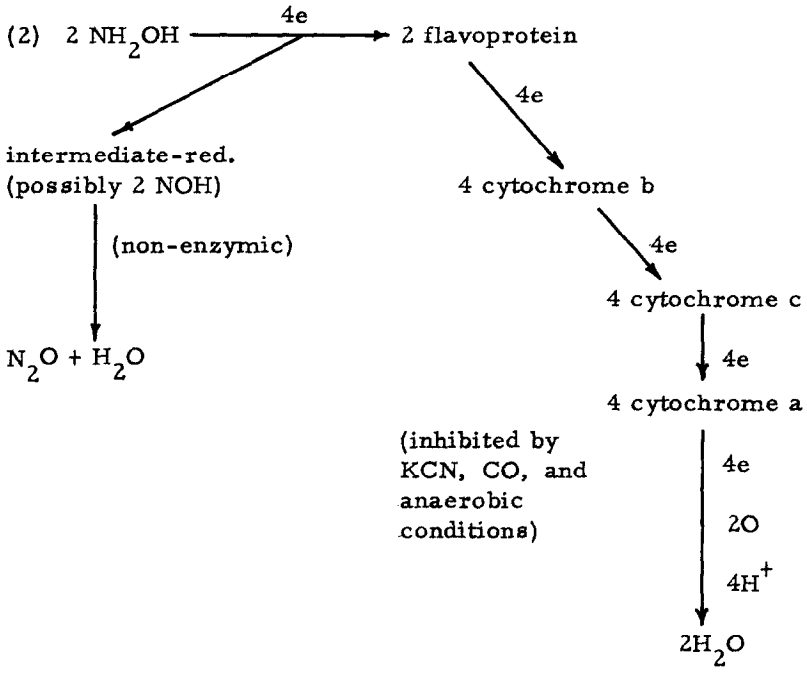
Cytochromes b, c, and a and flavin were detected in the particles by spectrophotometric methods. Addition of $Na_2S_2O_4$, DPNH, or hydroxyl-

amine to the particles resulted in absorption maxima (mμ) at 600, 560, 551, 521, and 415; and a decreased absorbancy at 450 mμ. Analysis of the particles (B) for heme components by the pyridine hemochromogen method (2) and for total flavin by difference spectra (4) was as follows (mμmoles/mg protein): cytochrome c, 3.82; cytochrome b, 0.34; cytochrome a, 0.50; and flavin, 0.30.

A gas was formed when the pellets were incubated with hydroxylamine and methylene blue or mammalian cytochrome c under an atmosphere of nitrogen in a Warburg apparatus. The main component of the gas (>90%) was nitrous oxide which was identified in a recording mass spectrometer. This finding would be in accord with the formation of a nitrogenous intermediate at the oxidation-reduction level (+1) of the nitroxyl radical (NOH) (5). Nitrous oxide, however was not oxidized to nitrite aerobically by the particulate preparations neither did it reduce cytochrome c anaerobically. Thus it appears that nitrous oxide is not utilized in this reaction and is likely to be a degradation product of an unstable intermediate(s) formed during the oxidation of hydroxylamine to nitrite. When the reaction mixture, incubated anaerobically with cytochrome c for 60 minutes, was subjected to intermittent evacuation, the subsequent introduction of air resulted in the oxidation of the reduced mammalian cytochrome c but nitrite was not formed. Thus the unstable intermediate was removed from the reaction mixture. The subsequent addition of hydroxylamine to this system however resulted in nitrite production.

The addition of DPNH under aerobic conditions inhibited the oxidation of hydroxylamine to nitrite.

These results suggest that the following electron transfer components function during the oxidation of hydroxylamine in Nitrosomonas:




(3) intermediate-red. + carrier-ox. → intermediate-ox. + carrier-red.

(4) intermediate-ox. + 1/2 $O_2$ + 2 cytochrome a-red. $\xrightarrow{\text{(inhibited by KCN, CO and anaerobic conditions)}}$ $HNO_2$ + 2 cytochrome a-ox.

(5) over-all reaction:

$$NH_2OH + O_2 \rightarrow H_2O + HNO_2$$

It is likely that cytochrome a has a dual function, forming water in the electron transfer sequence depicted by equation 2 or nitrite from an oxidized nitrogenous intermediate as shown in equation 4. It is of interest that a purified nitrite reductase from Pseudomonas aeruginosa has also been shown to have cytochrome oxidase activity (7, 8).

Aleem et al. (1) have recently suggested that nitrohydroxylamine is the intermediate formed by an initial oxidative condensation of hydroxylamine and nitrite as follows:

(6) $NH_2OH + HNO_2 + 1/2\ O_2 \rightarrow NO_2 \cdot NHOH + H_2O$

Our experiments however show conclusively that substrate amounts of the intermediate are formed (Table I) under anaerobic conditions and in the absence of nitrite as determined by the sensitive sulfanilamide method (6).

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