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CYTOCHROME OXIDASE FROM *PSEUDOMONAS AERUGINOSA* III. REDUCTION OF HYDROXYLAMINE

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SUMMARY

Pseudomonas aeruginosa cytochrome oxidase, which reduces nitrite and oxygen, is also capable of reducing hydroxylamine to ammonia.

The K_m for hydroxylamine reduction is $6 \cdot 10^{-4}$ M compared to $5 \cdot 10^{-5}$ M for nitrite reduction. NADH, NADPH, reduced *P. aeruginosa* cytochrome c_{551}, and reduced *P. aeruginosa* copper protein were ineffective as electron donors for hydroxylamine reduction whereas reduced pyocyanine and methylene blue acted as electron mediators.

Hydroxylamine reduction did not require the presence of Mn^{2+} or FAD and was not inhibited by prolonged dialysis versus sodium diethyldithiocarbamate. Cyanide, nitrite, and CO were very effective inhibitors.

Removal of heme *d* and its reconstitution, as well as inhibition by CO, suggest that the reduction of hydroxylamine, like the reduction of nitrite or oxygen, proceeds via the heme *d*.

INTRODUCTION

Pseudomonas aeruginosa cytochrome oxidase (ferrocytochrome c_2 : oxygen oxidoreductase, EC 1.9.3.2) functions in the terminal respiration of cells in *P. aeruginosa* [1–11]. The enzyme can utilize either molecular oxygen or nitrite as electron acceptors. Evidence for the ability of the purified enzyme to utilize hydroxylamine as an electron acceptor is presented in this paper.

Crude extracts of *P. aeruginosa* have previously been shown to reduce hydroxylamine to ammonia, and a soluble fraction has been isolated that required the presence of FAD and Mn^{2+} for enzymatic activity [12]. The enzyme responsible for that activity, however, has not been isolated.

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EXPERIMENTAL

Materials

Pyocyanine was prepared from phenazine methosulfate as described by Jagendorf and Margulies [13]. Methylene blue and benzylviologen were obtained from Schwarz-Mann. Sulfanilimide, *N*-1-napthylethylenediamine dihydrochloride, and diethyldithiocarbamic acid were products of Eastman. Palladized asbestos was obtained from K and K Laboratories, Inc., Plainview, N.Y. All other chemicals were analytical reagent grade.

Methods

Enzyme. P. aeruginosa cytochrome oxidase was isolated and purified from *P. aeruginosa* according to the procedure of Gudat et al. [10]. A concentrated solution of the enzyme (about 300 μM heme *c*) in 0.01 M sodium phosphate, pH 6, was taken to 50 % saturation with $(NH_4)_2SO_4$ and allowed to stand at 22 °C for a few days. The crystals which formed were spun down in a clinical centrifuge, washed with 50 % $(NH_4)_2SO_4$, redissolved in 0.01 M phosphate buffer, pH 6, and then dialyzed overnight against the same buffer at 4 °C. The crystallized enzyme had an $A_{410\,nm}/A_{280\,nm}$ ratio of 1.19, and oxidized 1.4 μmoles of cytochrome c_{551}/minute per mg protein when assayed as described by Gudat et al. [10].

Assay of hydroxylamine reductase activity. Hydroxylamine reductase activity was assayed either by measuring the oxidation of electron donors such as reduced pyocyanine and reduced methylene blue, or by estimating chemically using the method of Novak and Wilson [14], the rate of disappearance of hydroxylamine in the assay medium. A typical assay was conducted as follows: 30 ml of a stock solution containing 0.25 mM pyocyanine and 0.05 M sodium phosphate, pH 7, were evacuated and gassed with pure N_2 in a 50-ml test tube having a side-arm and fitted at the top with a rubber serum cap. The resulting blue anaerobic solution was then titrated until colorless with a dilute anaerobic solution of sodium dithionite by means of a microsyringe fitted with a long needle. Alternatively, the solution could also be reduced by the addition of a few mg of palladized asbestos and gassing with pure H_2 following evacuation. After the solution became colorless, it was evacuated again and then gassed with pure N_2. For the spectrophotometric assay, 2.5 ml of the reduced solution was transferred to a 1-cm cuvette fitted with a rubber serum cap which had been evacuated and filled with pure N_2. The transfer was made anaerobically using a hypodermic syringe. Hydroxylamine, from a freshly prepared anaerobic solution of hydroxylamine · hydrochloride, was injected to a final concentration of 0.9 mM. The reaction was initiated by injecting 20 μl of an anaerobic solution of the pure enzyme and the reaction was followed at 690 nm with a Gilford spectrophotometer attached to a Heathkit recorder. The ε_{mM} for oxidized pyocyanine was taken as 4.5 at 690 nm [13]. When the reaction was followed chemically, serum-capped test tubes with side arms instead of cuvettes were used. The reaction was carried out at 30 °C and the disappearance of hydroxylamine was measured from 0.5-ml aliquots of the assay mixture by the method of Novak and Wilson [14]. Boiled enzyme blanks were included as controls, but it was found that, at the substrate concentrations described, there was no nonenzymatic reduction of hydroxylamine.

Estimation of ammonia. Ammonia was estimated using the method of Chaykin

[15]. Identical results were obtained when the ammonia in the assay medium was determined directly or when the ammonia was allowed to diffuse overnight into HCl using Conway dishes. The enzymatic reactions were allowed to proceed until all the hydroxylamine had been reduced before aliquots were removed for the estimation of ammonia.

Removal of heme d and subsequent reconstitution. 100 µl of a 300 µM solution of the pure enzyme were added to 2 ml of acetone at −20 °C containing 0.012 M HCl. The resulting mixture was shaken thoroughly and the precipitate spun down at maximum speed in a clinical centrifuge. The pellet was washed twice with the acidified acetone and finally resuspended in 0.5 ml of 0.2 M potassium phosphate, pH 6, at 0 °C. The acetone containing the heme was extracted with 0.05 ml of 2 M NaOH, centrifuged to separate the phases, and the top layer of acetone removed. Residual acetone was removed by blowing gently with a stream of pure N_2. The pH of the extract was then lowered to between 9 and 10 by adding sufficient 0.5 M potassium phosphate, pH 6, at 0 °C.

For the reconstitution the apoprotein and heme *d* were mixed and solid, freshly crystallized, urea was added until the solution clarified (between 4–6 M urea). The clarified solution was dialyzed versus 0.01 M sodium phosphate, pH 6, for 6 h and centrifuged to remove any precipitate.

Protein was determined by the method of Lowry et al. [16] and the concentration of the enzyme was determined from its absorption spectrum recorded versus buffer in a Beckman DK-2A spectrophotometer. A ε_{mM} of 30.2 at 549 nm for the reduced cytochrome was used.

RESULTS

Effects of electron donor, substrates and pH on hydroxylamine reductase activity

Michaelis–Menten constants for pyocyanine and methylene blue as electron donors were determined to be in the order of $1 \cdot 10^{-5}$ M and $4 \cdot 10^{-6}$ M, respectively. Maximum velocity was achieved using 10^{-5} M methylene blue and $3 \cdot 10^{-5}$ M pyocyanine. Reduced pyridine nucleotides were ineffective as electron donors.

In the reduction of hydroxylamine, maximum velocity was achieved when concentrations of $9 \cdot 10^{-4}$ M or greater hydroxylamine were used. The K_m for hydroxylamine, using pyocyanine as an electron donor, was $6 \cdot 10^{-4}$ M. This is 6-fold higher than the K_m of 10^{-4} M for nitrite reduction using pyocyanine as electron donor and one order of magnitude higher than the K_m of $5.4 \cdot 10^{-5}$ M for nitrite reduction using *P. aeruginosa* cytochrome c_{551} as electron donor (Fig. 1A, B).

The specific activity of the purified enzyme was 22 µmoles of hydroxylamine reduced/min per mg protein at 30 °C at saturating concentrations of hydroxylamine and pyocyanine. The velocity of the reaction was proportional to the concentration of enzyme added.

A pH optimum of 7.2 was found for the reduction of hydroxylamine using pyocyanine as electron donor. Using the same electron donor, a pH optimum of 6.5 was found for nitrite reduction (Fig. 2).

Stoichiometry and product of reaction

When the reduction of hydroxylamine was followed both spectrophotometri-

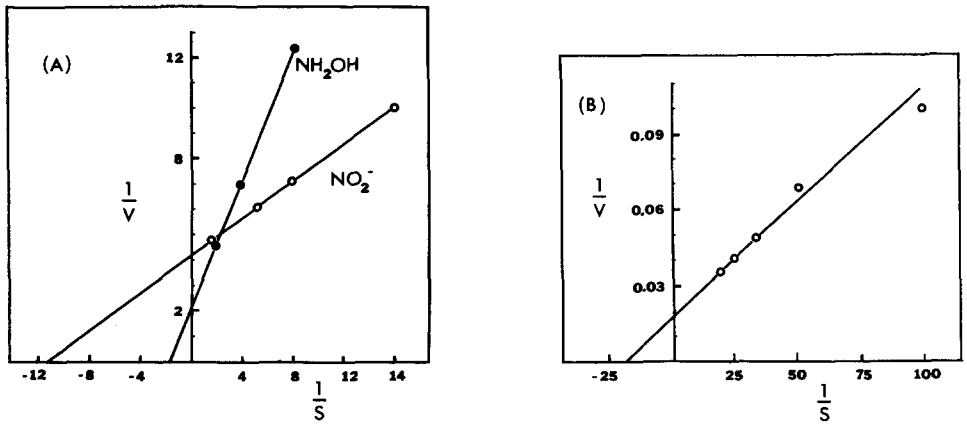

Fig. 1. Lineweaver–Burk plots of hydroxylamine reductase and nitrite reductase activities of *P. aeruginosa* cytochrome oxidase. (A) Lineweaver–Burk plot for hydroxylamine and nitrite reductases using reduced pyocyanine as electron donor. Velocity (v) is measured as ΔA 690 nm/min, and substrate (S) is the mM concentration of hydroxylamine. (B) Lineweaver–Burk plot for nitrite reductase using reduced *P. aeruginosa* cytochrome c_{551} as electron donor; v is measured as the ΔA 551 nm/min and S is the mM concentration of nitrite. Assays were performed spectrophotometrically as described in Experimental. Amount of the pure enzyme used per assay was 0.013 mg (20 μl).

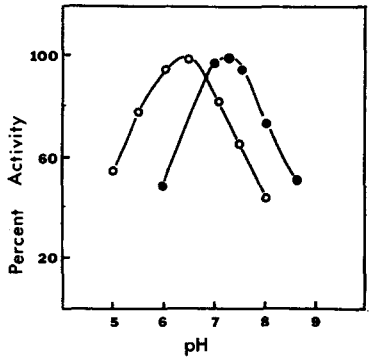

Fig. 2. Effect of pH on the reduction of hydroxylamine and nitrite by *P. aeruginosa* cytochrome oxidase using reduced Pyocyanine as electron donor. The enzymatic reactions were assayed spectrophotometrically as described in Experimental. The amount of pure enzyme used was 0.013 mg (20 μl). The concentrations of pyocyanine and hydroxylamine used were $2.5 \cdot 10^{-4}$ M and $9 \cdot 10^{-4}$ M, respectively. ○–○, nitrite reduction; ●–●, hydroxylamine reduction.

cally and chemically, it was found that for every mole of pyocyanine oxidized, an equivalent amount of hydroxylamine was reduced. It was also observed that hydroxylamine was completely converted to ammonia. The overall reaction can therefore be written as

reduced pyocyanine + NH_2OH →	pyocyanine + NH_4OH
or	or
reduced methylene blue	methylene blue

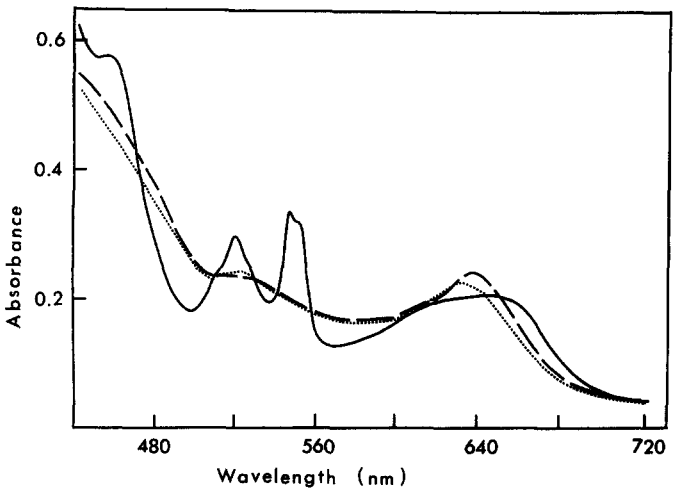

Fig. 3. Effect of hydroxylamine on the spectrum of *P. aeruginosa* cytochrome oxidase. Spectra were recorded using a Beckman DK-2A ratio recording spectrophotometer. The oxidase was in 0.1 M sodium phosphate, pH 7, and the same buffer was contained in the reference cuvette. ——, anaerobic solution of the cytochrome reduced by titration with dithionite or ascorbate; – – –, anaerobic solution of the oxidized cytochrome; · · · ·, addition of 0.1 mM hydroxylamine to an anaerobic solution of the reduced cytochrome.

Spectral properties of the enzyme in the presence of hydroxylamine

Hydroxylamine did not alter the absorption spectrum of the oxidized *P. aeruginosa* cytochrome oxidase. When added to an anaerobic, reduced (by dithionite or ascorbate) sample, however, the enzyme became oxidized and the absorption maximum of heme *d* shifted about 5 nm lower than when the enzyme had been oxidized in the absence of hydroxylamine (Fig. 3).

TABLE I

EFFECT OF INHIBITORS ON HYDROXYLAMINE REDUCTION BY *P. AERUGINOSA* CYTOCHROME OXIDASE

Reduced pyocyanine was used as electron donor and the reaction assayed spectrophotometrically as described in Experimental. 20 μl (0.013 mg) of the pure enzyme were used. Hydroxylamine concentration was 0.9 mM.

Inhibitor	Concentration (M)	% inhibition
Cyanide	10^{-6}	80
Nitrite	10^{-6}	100
CO	one atmosphere	100
Sodium azide	10^{-2}	0
Sodium sulfide	10^{-2}	0
Hydrazine	10^{-2}	0
p-Mercuribenzoic acid	10^{-3}	0

Effect of inhibitors on hydroxylamine reductase activity

When the enzyme was dialyzed for 24 h against 1 mM sodium diethyldithiocarbamate in 0.01 M sodium phosphate, pH 7, and then redialyzed against 0.01 M sodium phosphate buffer, pH 7, no significant loss in hydroxylamine reductase or nitrite reductase activities was observed.

Table I shows the effect of a number of inhibitors on hydroxylamine reductase activity. Cyanide was very effective as a competitive inhibitor with a K_i of $1.5 \cdot 10^{-7}$ M. The K_i was determined using $2 \cdot 10^{-7}$ M, $5 \cdot 10^{-7}$ M and 10^{-6} M concentrations of inhibitor each against three separate concentrations of hydroxylamine. CO, at atmospheric pressure, completely inactivated the enzyme. However, sodium azide, sodium sulfide, hydrazine, and *p*-mercuribenzoic acid were ineffective as inhibitors at relatively high concentrations.

Even at saturating concentrations of hydroxylamine, the presence of 10^{-6} M nitrite completely inhibited hydroxylamine reductase activity. Conversely, 1 mM hydroxylamine had no effect on the rate of reduction of nitrite at 10^{-6} M nitrite as determined both spectrophotometrically and chemically. It was not possible to determine the K_i and type of inhibition by nitrite since the small amount of nitrite was reduced rapidly even at high concentrations of hydroxylamine. Once all the nitrite was reduced, the inhibition of hydroxylamine reduction was immediately relieved (see Fig. 4).

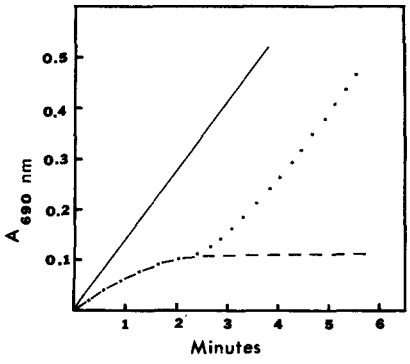

Fig. 4. Inhibition of hydroxylamine reduction by nitrite. Reactions were assayed spectrophotometrically as described in Experimental. 20 µl (0.013 mg) of enzyme were used in each assay. ——, 10^{-3} M hydroxylamine, no nitrite; – – – –, $5 \cdot 10^{-5}$ M nitrite, no hydroxylamine; · · · ·, $5 \cdot 10^{-5}$ M nitrite plus 10^{-3} M hydroxylamine.

Removal and reconstitution of heme d

Neither heme *d* nor the apoprotein (still containing heme *c*) alone was effective in the reduction of hydroxylamine. Table II shows that most of the hydroxylamine reductase could be recovered by reconstitution just as was observed for oxidase activity. The use of urea in the reconstitution procedure was essential for the high recovery of both activities.

Effect of reduced P. aeruginosa cytochrome c_{551} and copper protein as electron donors

Although *P. aeruginosa* cytochrome c_{551} and copper protein were more effective than pyocyanine as electron donors for nitrite reduction (K_m for cytochrome c_{551},

TABLE II

RECONSTITUTION OF HEME *d* AND APOPROTEIN (CONTAINING HEME *c*)

Removal of heme *d*, reconstitution, and assay of cytochrome c_{551} oxidation and hydroxylamine reduction activities were described in Experimental. Concentration of *P. aeruginosa* cytochrome oxidase (native or reconstituted) was determined using a ε_{mM} of 30.2 for the absorption at 549 nm for the reduced cytochrome.

Enzyme	nmoles cytochrome c_{551} oxidized/min per nmole *P. aeruginosa* cytochrome oxidase	Recovery (%)	nmoles NH_2OH reduced/min per nmole *P. aeruginosa* cytochrome oxidase	Recovery (%)
Native enzyme	91.7	100	760.5	100
Apoprotein	0	0	0	0
Heme *d*	0	0	0	0
Reconstituted enzyme	86.3	94	564.7	74

$0.1 \cdot 10^{-4}$ M; for copper protein, $0.17 \cdot 10^{-4}$ M and for pyocyanine $0.9 \cdot 10^{-4}$ M), they were totally ineffective when hydroxylamine was used as the electron acceptor. Table III shows the relative rates of hydroxylamine reduction using cytochrome c_{551}, copper protein and pyocyanine as electron donors. Hydroxylamine did not change the spectral properties of the oxidized or reduced cytochrome c_{551} or copper protein. It was also noted that although cytochrome c_{551} or copper protein were ineffective as electron donors in hydroxylamine reduction, they were very effective as electron donors for nitrite reduction even in the presence of high concentrations of hydroxylamine.

TABLE III

COMPARISON OF *P. AERUGINOSA* CYTOCHROME c_{551}, COPPER PROTEIN AND PYOCYANINE AS ELECTRON DONORS IN HYDROXYLAMINE REDUCTION

Hydroxylamine reductase activity was assayed spectrophotometrically as described in Experimental. Saturating concentrations (100 μM) of cytochrome c_{551} and copper protein were reduced by dithionite and excess dithionite removed by shaking in air before evacuation. A pH of 6.5 instead of 7.0 was used for all three assays. 20 μl (0.013 mg) of the enzyme were used for each assay.

Electron donor	μmoles of electron donor reduced/min per mg *P. aeruginosa* cytochrome oxidase
Pyocyanine	17
Cytochrome c_{551}	0.7
Copper protein	0.4

DISCUSSION

It has been observed that actively denitrifying bacteria possess an active hydroxylamine reductase. Walker and Nicholas [12] showed that crude extracts of *P. aeruginosa* were capable of reducing hydroxylamine to ammonia. They partially purified the enzyme and found that it required both FAD and Mn^{2+} for activity. Similarly,

Kono and Taniguchi [17] also characterized a *c*-heme containing hydroxylamine reductase from *Micrococcus denitrificans*. However, no FAD was required for enzymatic activity. Hydroxylamine reductase activity has also been identified and associated with sulfite reductases from *Escherichia coli* [18, 19] and *Salmonella typhimurium* [20], but it is not known whether the activities are only coincidental or are physiologically significant properties in these bacteria. In *P. aeruginosa*, however, Fewson and Nicholas [21] showed that the actively denitrifying bacteria could simultaneously assimilate nitrate. It is therefore possible that their hydroxylamine reductase serves a physiological function.

The hydroxylamine reductase reported in this paper differs from that reported by Walker and Nicholas [12] in that no FAD or Mn^{2+} were required for its activity. Other properties such as the K_m and the product of the reaction were similar to their enzyme. It is possible that there is more than one hydroxylamine reductase in *P. aeruginosa*. Multiplicity of hydroxylamine reductase has been reported in *Neurospora crassa* [22].

The reduction of hydroxylamine by *P. aeruginosa* cytochrome oxidase required the presence of heme *d* on the protein as was shown by the reconstitution experiment. Complete inhibition by CO also indicated that the enzymatic reduction probably proceeds via heme *d*. Hydroxylamine reduction is very sensitive to cyanide, but azide, sulfide and *p*-mercuribenzoic acid had no effect even at relatively high concentrations. Unlike the Walker and Nicholas enzyme [12] it was not inhibited by hydrazine.

There is no doubt that the primary physiological function of the *P. aeruginosa* cytochrome oxidase is nitrite reduction. This is supported by the fact that the K_m of the enzyme for nitrite is 10-fold less than that for hydroxylamine, and that hydroxylamine reduction is inhibited by the presence of very low concentrations of nitrite.

It was interesting to note that although pyocyanine was effective as an electron donor for both nitrite and hydroxylamine reduction, the natural electron donors of nitrite reduction, cytochrome c_{551} and copper protein, were ineffective as electron donors for hydroxylamine reduction. Wharton et al. [11] showed that in the reaction between copper protein and the oxidase, a complex is formed rapidly between copper protein and the oxidase followed by the donation of an electron to the heme *c* moiety of the oxidase. It is not known whether the inability to utilize copper protein in hydroxylamine reduction results in either an inhibition of electron transfer from the copper protein to the heme *c* moiety of the oxidase or an inhibition of the intramolecular electron transfer between the *c* and *d* hemes in the presence of hydroxylamine. Experiments directed to resolve this have so far been inconclusive.

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