Biochimica et Biophysica Acta, 459 (1977) 88–95

BBA 47223

NITRITE REDUCTASE SYSTEM INVOLVED IN THE TERMINAL OXIDATION OF THE *STREPTOMYCES GRISEUS* RESPIRATORY PARTICLE

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(Received June 18th, 1976)
(Revised manuscript received September 10th, 1976)

SUMMARY

A nitrite reductase system which was associated with the electron transfer system of the respiratory particle in *Streptomyces griseus* was studied.

The electron transfer pathway consisted of the cytochrome oxidase and the nitrite reductase systems under aerobic and anaerobic conditions respectively, and these systems showed the exact opposite response to 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide and azide. Azide inhibited specifically the nitrite reductase system. It seems that cytochrome *d* works as the nitrite reductase and the reduced cytochrome *b* works as an intermediate electron donor for cytochrome *d* respectively.

The respiratory particle also had a hydroxylamine reductase activity and ammonia was identified as the product of hydroxylamine reduction by the respiratory particle.

A terminal electron transfer pathway in *Streptomyces griseus* was proposed.

INTRODUCTION

There have been many reports [1-15] on a *d*-type cytochrome which acts as cytochrome oxidase in bacteria. Yamanaka and his coworkers [6–9] have reported that *Pseudomonas aeruginosa* cytochrome oxidase (*d*-type cytochrome) is also a nitrite reductase. A similar system has also been reported in *Micrococcus denitrificans* by Newton [13]. In previous papers, Inoue [16, 17] demonstrated that the respiratory particles of *Streptomyces griseus* contain cytochromes *a*, *b*, *c* and *d*, cytochrome oxidase, cytochrome *c* peroxidase and nitrite reductase activities; and that cytochrome *d* acts as a member of the aerobic respiratory system. In addition, it was also reported that under anaerobic conditions, cytochromes *b* and *d* in the substrate-reduced respiratory particle were immediately oxidized by the addition of nitrite at pH values around 6.0–6.6; furthermore the cytochrome *d* was found to be functioning as an important component in the nitrite reducing system. It was suggested that under aerobic and anaerobic conditions these two terminal oxidase activities were working in the electron transfer system of the respiratory particle. However, it was not clear

whether cytochrome *d* of *S. griseus* acted as a terminal oxidase under aerobic conditions as shown for *P. aeruginosa* or *M. denitrificans*.

This paper describes some properties of the nitrite reducing system in the electron transfer system of the *S. griseus* respiratory particle.

MATERIALS AND METHODS

Culture conditions and the preparation of the respiratory particle (the precipitate between 10 000 and 67 000 $\times g$ of a sonic disrupted mycelium suspension) were the same as described previously [16]. The respiratory particle was designated 67P.

The spectrophotometric experiment. For aerobic studies: standard cells (optical length, 1 cm) were used. The total reaction mixture was 1.0 ml. The difference spectrum (difference between the substrate-reduced and oxidized state) was measured at 4 or 5 min intervals. Thunberg type cells (optical length 1 cm) were used for anaerobic studies and the substrate was added from the side arm. A total of 1.5 ml of the reaction mixture was placed in the cell which was evacuated by a vacuum pump until the outer surface of the cell became wet with condensed water. During the evacuation, each cell was shaken to help expell dissolved air. The substrate was then added to the main chamber and the assay initiated. For the reference cell, the substrate was omitted from the reaction mixture and replaced by water.

Reaction mixtures are described in the table or figure legends. All the experiments were replicated at least three times.

A Beckman DB-G spectrophotometer equipped with a Log converter was used for all the spectrophotometric studies.

Measurement of oxidative and reductive activities. NADH oxidase (under aerobic conditions), nitrate, nitrite and hydroxylamine reductase activities (under anaerobic conditions) were measured spectrophotometrically by the change in the optical density at 340 nm due to NADH oxidation. Nitrite reductase activity was also observed by the change of the difference spectrum of cytochrome components which resulted in the oxidation of cytochromes *b* and *d* in the substrate-reduced respiratory particles by nitrite addition under anaerobic conditions.

Ammonia was determined using Conway's microdiffusion analysis [18] followed by Nesslerization.

Protein was determined either by the method of biuret formation [19] or according to Lowry et al. [20].

2-*n*-Heptyl-4-hydroxy-quinoline-*N*-oxide(HOQNO) was obtained from Sigma Chemical Corp., St. Louis, Mo. and NADH was from Boehringer Mannheim, Germany. Carbon monoxide and nitric oxide were obtained from commercial sources and were passed through an alkaline pyrogallol solution to remove contaminating oxygen or oxides of nitrogen.

RESULTS

The influence of nitrate and nitrite on succinate-reduced 67P under aerobic conditions

The influence of nitrate and nitrite on the reduction of cytochrome components by succinate addition were examined. In the previous work [17], nitrite was added after cytochrome components of the respiratory particle were reduced by lactate, but

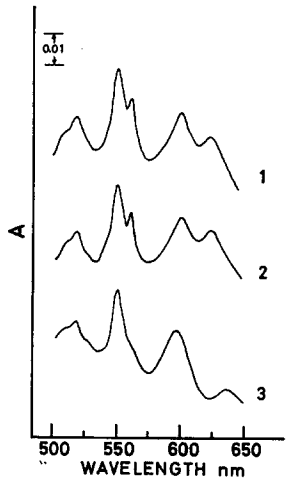


Fig. 1. The influence of nitrate and nitrite on the cytochrome components in succinate oxidation. The reaction mixtures contained: 0.2 M phosphate buffer (pH 6.0), 0.2 ml; 67P, 0.1 ml; 0.2 M $NaNO_3$, $NaNO_2$ or water, 0.1 ml; 0.2 M succinate, 0.1 ml; and water to a final volume of 1.0 ml. The temperature was 30 °C and the protein content 3.5 mg/ml (aerobic conditions). Difference spectra (reduced state minus oxidized state) were assayed at 28 min after the reaction was initiated. The oxidized state of the cytochromes was obtained by aeration. Curve 1: control (with succinate); 2: with $NaNO_3$; and 3: with $NaNO_2$.

in this experiment, nitrite was added prior to the reaction. The difference spectra (Fig. 1) appeared after the dissolved oxygen was expelled from the reaction mixture. All cytochrome components (cytochromes *a* at 600 nm, *b* at 558 and 562 nm, *c* at 550 nm and *d* at 625 nm, respectively) were reduced by succinate addition (curve 1); nitrate (curve 2) had no effect. The presence of nitrite (curve 3), however, caused the oxidation of the reduced cytochromes *b* and *d*, and the difference maximum of cytochrome *d* shifted to the red (from 625 to 633 nm). Although the difference spectra were assayed at 4-min intervals, the spectra at 28 min of reaction remains as shown in Fig. 1. The difference spectra in the presence of nitrite obtained were similar to the one shown in a previous paper [17].

The reduction of nitrate, nitrite and hydroxylamine in the presence of NADH by 67P under anaerobic conditions

It was examined whether the *Streptomyces* respiratory particles have the reducing activities of nitrate, nitrite and hydroxylamine linked to NADH oxidation. NADH was not oxidized by 67P (Fig. 2A, curve 1) under anaerobic conditions. However, NADH was slightly oxidized by 67P in the presence of nitrite, but not affected by nitrate (curve 3 and 2). Since it was found that the optical density of NADH at 340 nm was decreased by the non-enzymatic reaction in the presence of nitrite at the acidic pH, curve 3 with nitrite in Fig. 2A was corrected for the non-enzymatic reaction (curve 8). NADH was considerably oxidized by 67P in the presence of hydroxylamine (curve 4). These results indicate that 67P has both the nitrite reductase and the hydroxylamine reductase activities. Ammonia was detected as the

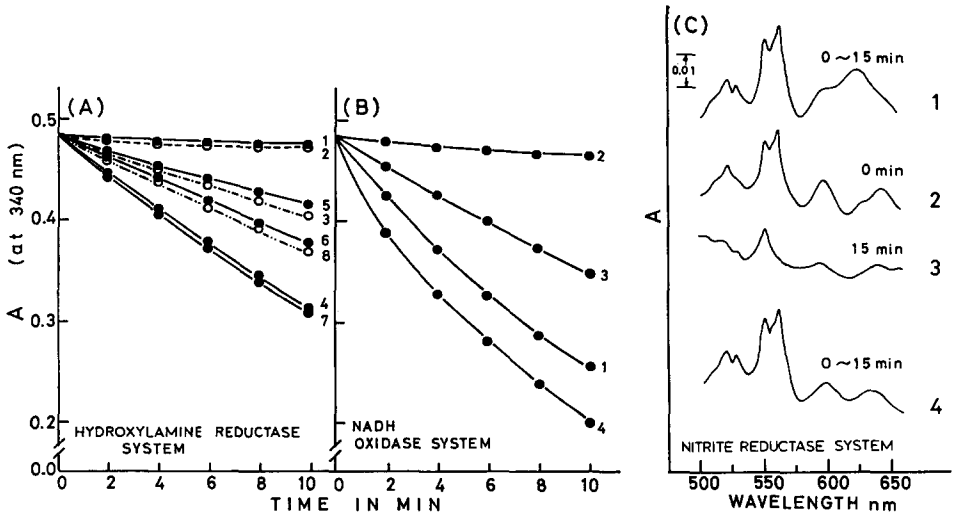


Fig. 2. The influence of inhibitors on the anaerobic and the aerobic respiratory systems linked to NADH oxidation. The reaction mixtures for Figs. 2A and 2B contained: 0.2 M phsophate buffer (pH 6.0), 0.3 ml; 67P, 0.05 ml; 0.4 M $NaNO_3$, $NaNO_2$, NH_2OH or water, 0.3 ml; inhibitors or water, 0.03 to 0.15 ml; 1 mM NADH, 0.15 ml; and water to a final of volume 1.5 ml. The temperature was 30 °C and the protein content 120 μg/1.5 ml. (A) Hydroxylamine reduction including nitrate and nitrite reduction under anaerobic conditions. Curve 1: without $NaNO_3$, $NaNO_2$ or NH_2OH; 2: with $NaNO_3$ (nitrate reduction); 3: with $NaNO_2$ (nitrite reduction); 4: with NH_2OH; 5: with NH_2OH and KCN; 6: with NH_2OH and HOQNO; 7: with NH_2OH and NaN_3; and 8: without 67P, with NADH and $NaNO_2$ (non-enzymatic reaction). Curves 4 to 7: hydroxylamine reduction. (B) NADH oxidase system under aerobic conditions. Curve 1: control (with NADH); 2: with HOQNO; 3: with KCN; and 4: with NaN_3. Oxidant was air. The reaction mixtures for Fig. 2C contained: 0.2 M phosphate buffer (pH 6.0), 0.3 ml; 67P, 0.15 ml; 0.4 M $NaNO_2$ or water, 0.3 ml; inhibitors or water, 0.03 to 0.15 ml; 1 mM NADH, 0.03 ml and water to a final volume of 1.5 ml. The temperature was 30 °C and the protein content 3.2 mg/1.5 ml. (C) Nitrite reduction under anaerobic conditions. Curve 1: control (without $NaNO_2$ or inhibitors); 2 and 3: with $NaNO_2$; and 4: with $NaNO_2$ and NaN_3. Difference spectra were assayed every 5 min, and 0 min assayed immediately after NADH addition. In Figs. 2A, 2B and 2C, NaN_3: 30 mM (final concentration); HOQNO: 60 μM, and KCN: 40 mM.

TABLE I

PRODUCTS IN THE HYDROXYLAMINE REDUCING SYSTEM UNDER ANAEROBIC CONDITIONS

The reaction mixtures contained: 0.2 M phosphate buffer (pH 6.0), 0.3 ml; 67P, 0.3 ml; 0.4 M $NaNO_3$, $NaNO_2$, NH_2OH or water, 0.6 ml; 10 mM NADH or water, 1.2 ml and water to a final volume of 3 ml. The reaction mixture was incubated at 30 °C for 30 min and the protein content was 720 μg/ml. A Thunberg tube was used and evacuated by a vacuum pump for 6 min.

Salts (80 mM)	Ammonia (mM)
None (control)	0.018
$NaNO_3$	0.000*
$NaNO_2$	0.000*
NH_2OH	0.615*

* The control value was subtracted.

product in the hydroxylamine reductase system, but was not found in either the nitrite reductase or the nitrate reductase systems (Table I).

The influence of inhibitors on nitrite, hydroxylamine reductase and NADH oxidase activities of the 67P

The influence of inhibitors on both the anaerobic and the aerobic respiratory systems of 67P were examined. The activity of nitrite reduction linked to NADH oxidation under anaerobic conditions was very minor (Fig. 2A, curve 3). Therefore, the influence of inhibitors on the nitrite reductase activity was observed by the change of the difference spectrum which was produced by the oxidation of cytochromes *b* and *d* in the NADH-reduced 67P by nitrite addition under anaerobic conditions. All cytochrome components were kept in the reduced state during each assay period (Fig. 2C, Curve 1). Although 67P, even in the presence of nitrite, was immediately reduced by the addition of NADH, the reduced cytochromes *b* and *d* were immedately oxidized (Fig. 2C, curves 2 and 3). This oxidation was completely inhibited by azide (Fig. 2C, curve 4) and this inhibition was also observed at a concentration of 1 mM of azide which was relatively low. The oxidations of the reduced cytochromes *b* and *d* by nitrite were not inhibited by the addition of cyanide or HOQNO, and those spectra obtained were similar to Fig. 2C (curves 2 and 3). The influence of inhibitors on the oxidation of cytochromes *b* and *d* in the succinate-reduced 67P was examined, because no attention needs to be paid to the non-enzymatic reaction of the substrate by nitrite. The results obtained were quite similar to those shown in Fig. 2C.

HOQNO inhibited hydroxylamine reductase activity by about 40 %, but azide had no effect (Fig. 2A, curves 6 and 7). While HOQNO almost completely inhibited NADH oxidase activity without nitrate, nitrite or hydroxylamine under aerobic conditions, azide showed a stimulating effect on it (Fig. 2B, curves 2 and 4). Curve 1 in Fig. 2B is the control. These three enzyme activities were relatively insensitive to 40 mM concentration of cyanide under the given conditions (Fig. 2A, curve 5, 2B, curve 3). These results indicate that azide is a specific inhibitor for the nitrite reductase activity of 67P in the presence of respiratory substrate under anaerobic conditions.

DISCUSSION

Egami et al. [21] in *Escherichia coli*, Downey [22] in *Bacillus stearothermophilus*, and Garrett and Nason [23] in *Neurospora crassa* demonstrated that the cytochrome *b* participates in the nitrate reductase system. On the other hand, Horio et al. [24], Yamanaka et al. [7], Yamanaka and Okunuki [25], Walker and Nicholas [10], Lam and Nicholas [11], Newton [13], Layne and Nason [5], and Iwasaki and Matsubara [14] reported that the nitrite reductase of *Pseudomonas aeruginosa*, *Micrococcus denitrificans*, *Azotobacter vinelandii* and *Alcaligenes faecalis* have two heme cytochromes (cytochrome *cd*) in its molecule, and that the reduced cytochrome *c* of these bacteria acts as a hydrogen donor for cytochrome *d*. The previous [17] and the present study have demonstrated that the reduced cytochromes *b* components (*b*-558 and 562) and *d* are instantaneously oxidized by the addition of nitrite at the acidic pH, but not by nitrate when the 67P was reduced either by NADH, succinate, or lactate under anaerobic conditions. The addition of nitrite resulted in the oxidation of the reduced cytochromes *b* and *d* under anaerobic conditions. These results suggest

that the cytochrome *b* components participate in the nitrite reducing system, and that the reduced cytochrome *b* may act as a hydrogen donor for cytochrome *d* in the nitrite reductase system.

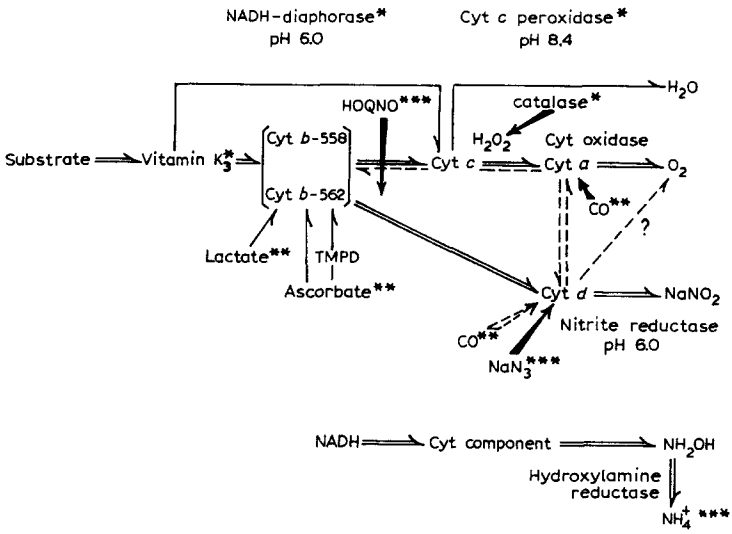
Ammonia was produced in the hydroxylamine reductase system when NADH was oxidized by 67P under anaerobic conditions. Yamanaka et al. [7] have demonstrated that nitric oxide was formed as a product of the nitrite reduction of *Pseudomonas cytochrome* oxidase (= *P.* nitrite reductase), and nitrite and nitric oxide had the same effect on the dithionite-reduced cytochrome *d*. They demonstrated that a shoulder at 570 nm appeared in the absorption spectrum of the reduced *Pseudomonas* cytochrome oxidase by the addition of nitrite or nitric oxide. The previous [17] and the present results (Fig. 1 curve 3) suggested that nitric oxide might be the product in the nitrite reducing system of the 67P. This is suggested by the difference maximum of *Streptomyces* cytochrome *d* which was produced by either the substrate- or dithionite-reduced 67P in the presence of nitrite or the dithionite-reduced 67P added with nitric oxide, was shifted to the red with quite similar pattern (from 625 to 633 nm) which was also observed in the reaction at pH 6.0. This finding indicated that nitrite and nitric oxide affected to *Streptomyces* cytochrome *d* in a similar way as *Pseudomonas* nitrite reductase.

Nitrite reductase activity was found to be very sensitive to azide but insensitive to HOQNO. However, these inhibitors show the opposite effect on NADH oxidase activity (Figs. 2B and 2C). The findings that the reduced cytochrome *d* was instantaneously oxidized by nitrite and its oxidation was completely inhibited by azide under anaerobic conditions, strongly suggested that the cytochrome *d* of *S. griseus* might be functioning as a nitrite reductase under anaerobic conditions like bacterial cytochrome *d*. The stimulating effect of azide on NADH oxidase activity may be due to the fact that the metal(s) interfering NADH oxidase activity were removed by azide as a metal-chelating agent. Since nitrite and hydroxylamine reductase activities responded to HOQNO and azide differently, it was suggested that nitrite and hydroxylamine were reduced by different enzymes. As previously demonstrated [16], the electron transfer system of *S. griseus* under aerobic conditions was inhibited by the low concentrations of cyanide at 500 μM and this inhibition diminished with time. In the present work, NADH oxidase, nitrite and hydroxylamine reductase activities were relatively insensitive to cyanide at 40 mM. All the cytochrome components in the substrate-reduced 67P were oxidized by the addition of hydroxylamine under anaerobic conditions (data not shown). This latter result suggests that some cytochrome components were participating in the hydroxylamine reducing system.

The hydrogen was donated to nitrite via cytochromes *b* and *d* through nitrite reductase activity under anaerobic conditions and to oxygen via cytochrome *a* through cytochrome oxidase activity in the absence of nitrite under aerobic conditions. Therefore, cytochrome *d* works as a member of cytochrome chain under aerobic conditions [17]. As shown in Fig. 2C, (curves 2 and 3), however, cytochromes *a* and *c* were oxidized gradually after cytochromes *b* and *d* were oxidized instantaneously in the presence of nitrite under anaerobic conditions. In this case, the hydrogen seems to be donated to nitrite via cytochromes *c*, *a* and *d*, or via cytochromes *a*, *c*, *b* and *d* due to a back-flowing of the hydrogen among cytochromes *b*, *c* and *a* through nitrite reductase activity.

From the above considerations, the terminal electron transfer pathway of the

S. griseus respiratory particle may be proposed as follows:



Scheme I. The terminal electron transfer pathway of *Streptomyces griseus.* Cyt, cytochrome; – – –, hypothetical reaction; *, ref. 16; **, ref. 17; ***, present paper; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine.

It was previously shown [17] that carbon monoxide inhibited the oxidation of NADH and succinate, and was bound to the reduced cytochrome *d* in the absence of nitrite under aerobic conditions. The difference spectrum of CO-binding cytochrome *d* was shifted to the red (from 625 to 630 nm). A similar difference spectrum was also observed in the dithionite-reduced cytochrome *d* by the addition of CO. However, there is no direct evidence that CO inhibits nitrite reductase activity in the presence of nitrite under anaerobic conditions.

ACKNOWLEDGEMENTS

I wish to thank K. Sekine for his assistance in performing these experiments.

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