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THE PARTICIPATION OF CYTOCHROMES IN THE PROCESS OF NITRATE RESPIRATION IN *KLEBSIELLA (AEROBACTER) AEROGENES*

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SUMMARY

1. The participation of cytochromes in the membrane-bound, nitrate and oxygen respiratory systems of *Klebsiella (Aerobacter) aerogenes* has been investigated. The membrane preparations contained the NADH, succinate, lactate and formate oxidase systems, and in addition a high respiratory nitrate reductase activity.

2. Difference spectra indicated the presence of cytochromes *b*, a_1, *d* and *o*. Cytochromes of the *c*-type could not be detected in these membranes. Both cytochrome *b* content and respiratory nitrate reductase activity were the highest in bacteria grown anaerobically in the presence of nitrate.

3. Cytochrome *b* was the only cytochrome which, after being reduced by NADH, could be partially reoxidized anaerobically in the presence of nitrate. Furthermore, nitrate caused a lower aerobic steady state reduction only of cytochrome *b*.

4. NADH oxidase and NADH-linked respiratory nitrate reductase activities were both inhibited by antimycin A, 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide and KCN. NADH oxidase activity was selectively inhibited by CO, while azide was found to inhibit only the respiratory nitrate reductase. In the presence of azide, nitrate did not affect the level of reduction of cytochrome *b*.

5. The evidence presented suggests that cytochrome *b* is a carrier in the electron transport systems to both nitrate and oxygen; from cytochrome *b* branching occurs, with one branch linked to the respiratory nitrate reductase and one branch linked to oxidase systems, containing the cytochromes a_1, *d* and *o*.

INTRODUCTION

Klebsiella (Aerobacter) aerogenes can utilize nitrate as the sole nitrogen source for growth (nitrate assimilation) and, under anaerobic conditions, also as terminal electron acceptor instead of oxygen (nitrate respiration). In both processes nitrate is reduced to nitrite through a nitrate reductase. In the assimilatory process, the nitrite is further reduced to NH_4^+ by the action of a nitrite reductase. Previous work from this laboratory suggested that the electron transport system to nitrate is different for the two physiological processes, though the same nitrate reductase may act as terminal electron acceptor[1,2]. However, the actual pathways of electron transport to nitrate in both cases are still largely unknown. So far we have established that ubiquinone-8,

Abbreviation: HQNO, 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide.

the sole quinone present in this bacterium[3], acts as an electron carrier in both the NADH-linked respiratory nitrate reductase and the NADH oxidase system[3-4]. Furthermore, some evidence was obtained suggesting that a *b*-type cytochrome is involved in the electron transport to nitrate in the process of nitrate respiration[2,3,5]. On the other hand, it was found recently that the nitrate assimilation process in aerobic cultures of *K. aerogenes* does not require *b*-type cytochromes as electron carriers[6].

In this paper we describe more detailed investigations of the cytochrome system of *K. aerogenes* membranes, which contain, in addition to various oxidase activities, a high respiratory nitrate reductase activity. The participation of cytochromes in the nitrate respiratory system was investigated by analyzing the effects of nitrate on the oxidation–reduction kinetics of the cytochromes in these membranes, and by utilizing inhibition studies.

MATERIALS AND METHODS

Growth of organism and preparation of membranes

K. aerogenes (strain S 45) was grown anaerobically at 30 °C on minimal nitrate *plus* NH_4^+ medium to a cell density corresponding to 120 mg (dry weight) per l as described previously[1]. The cells were harvested, washed and disrupted by sonication as described by Van 't Riet *et al.*[1]. The membrane fraction was obtained by centrifugation of the cell-free extract at $120000 \times g$ for 60 min. The pellets were resuspended in 0.065 M sodium–potassium phosphate buffer (pH 7.4) and sonicated[1] once more for 2 min to remove endogenous substrates. After centrifugation at $120000 \times g$ for 60 min, the membrane pellets were washed with the same buffer. The membrane fraction was prepared freshly for each experiment, and had a high level of respiratory nitrate reductase activity[3]. In some experiments (*cf.* Table I) membrane preparations obtained from bacteria, grown anaerobically on minimal NH_4^+ medium[1] or aerobically on minimal nitrate medium[1], were also used.

Measurement of oxidase and nitrate reductase activities

O_2 uptake was measured at 25 °C using a Clark oxygen electrode. The reaction mixture contained 38 μmoles sodium–potassium phosphate buffer (pH 7.4), 1.2 μmoles substrate, 8 μmoles $MgCl_2$, 1 μmole EDTA and an appropriate amount of membranes in a final volume of 1.5 ml. NADH-linked respiratory nitrate reductase activity was determined at 25 °C under anaerobic conditions by recording the decrease in NADH content in the presence of nitrate as described previously[3].

Spectra and cytochrome levels

Difference spectra of membrane suspensions (7.75 mg protein/ml in 0.025 M sodium–potassium phosphate buffer, pH 7.4) were recorded at room temperature (21 °C) in an Aminco–Chance split-beam spectrophotometer, and at the temperature of liquid N_2 (77 °K) in a Perkin–Elmer 356 double beam spectrophotometer. The contents of cytochromes *b*, a_1 and *d* were determined by measuring the absorbance difference at the wavelengths 559–576 nm, 592–578 nm, and 630–615 nm, respectively, in the dithionite-reduced *minus* oxidized difference spectra at 21 °C. Cytochrome *o*

was determined from the CO–dithionite-reduced *minus* dithionite-reduced difference spectra using the wavelength pair 416–426 nm.

Dual-wavelength spectrophotometry

The oxidation–reduction kinetics of the individual cytochrome components of *K. aerogenes* membranes were determined using an Aminco–Chance dual-wavelength spectrophotometer. All measurements were carried out at 21 °C in a reaction mixture, containing 75 μmoles sodium–potassium phosphate buffer (pH 7.4), 15 μmoles $MgCl_2$, 3 μmoles EDTA, 4.0–4.5 mg membrane protein and glass double-distilled water to a final volume of 3.0 ml. The mixture was aerated, and subsequently the reaction was initiated by the addition of 10 μmoles NADH. The effects of nitrate or of nitrite on the reduction levels of cytochromes were studied under strictly anaero bic conditions in Thunberg-type cuvettes. The following wavelength pairs were employed in these experiments: cytochrome *b*, 559–576 nm; cytochrome a_1, 592–578 nm; and cytochrome *d*, 630–615 nm.

Cytochrome *o* may contribute to the recorded absorbance changes at 559–576 nm, the wavelength pair used for cytochrome *b*. However, from the reduced *minus* oxidized difference spectra and the CO-reduced *minus* reduced difference spectra it was estimated according to the method of Sinclair and White[7] that cytochrome *o* in this case contributes less than 10% to the absorbance changes at 559–576 nm (*cf.* also ref. 8).

RESULTS

The low temperature (77 °K) dithionite-reduced *minus* oxidized difference spectrum of *K. aerogenes* membranes is shown in Fig. 1. This spectrum indicates the presence of a *b*-type cytochrome with maxima at 426, 526 and 554 nm, cytochrome a_1 with shoulders at 435 and 590 nm, and cytochrome *d* with a characteristic band at 625 nm and a trough at 645 nm. The bands in the room temperature reduced *minus* oxidized difference spectrum (not shown) were somewhat less sharp, and shifted a few nm towards the red, but they also suggested the presence of the same cytochrome set: cytochrome *b* (428, 529 and 559 nm), a_1 (shoulder at 440 and a broad band at about 592 nm) and *d* (630 nm). The NADH-reduced *minus* oxidized difference spectra were very similar to the dithionite-reduced ones, apart from the somewhat lower anaerobic state reduction levels of the various cytochromes (*cf.* also Table II). None of these difference spectra gave evidence for the presence of *c*-type cytochromes in membranes of *K. aerogenes*, irrespective of growth conditions.

The low temperature CO-reduced *minus* reduced difference spectrum (lower trace in Fig. 1) indicates the presence of cytochromes *o* (maxima at 416, 538 and 564 nm), a_1 (band at 430 nm and a little absorption in the 600-nm region) and *d* (633 nm). The troughs at 426 and 552 nm are due to the reduced form of cytochrome *o* in the reference cuvette.

The relative cytochrome contents and oxidase system activities of membranes obtained from bacteria grown under various conditions are given in Table I. The data show that under growth conditions in which a high respiratory nitrate reductase activity is obtained (anaerobic growth in nitrate *plus* NH_4^+ medium), cytochrome *b* (in contrast to the other cytochromes) is formed in large amounts as well. This

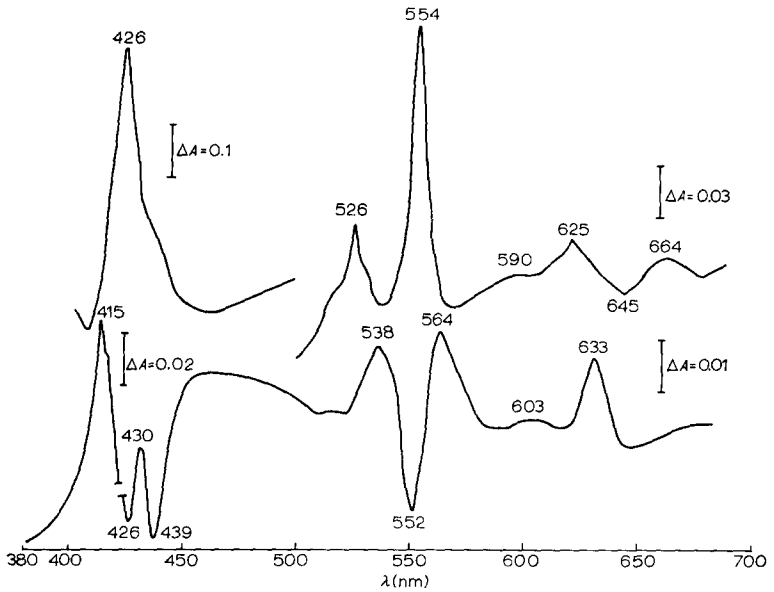

Fig. 1. Low-temperature (77 °K) difference spectra of membranes from *K. aerogenes*, grown anaerobically on minimal nitrate *plus* NH_4^+ medium[1]. Upper trace, dithionite-reduced *minus* oxidized difference spectrum; Lower trace, dithionite-reduced + CO *minus* dithionite-reduced difference spectrum, recorded after a treatment with CO for 2 min.

TABLE I

OXIDASE ACTIVITIES AND CYTOCHROMES CONTENTS OF MEMBRANES FROM *K. AEROGENES* GROWN UNDER DIFFERENT CONDITIONS

The growth conditions of *K. aerogenes*, the preparation of the membranes, the determination of cytochrome levels and the measurements of oxidase activities are all described in Materials and Methods. Oxidase activities are expressed as natoms O_2 per min per mg protein, and the respiratory nitrate reductase activity is expressed as nmoles NADH/min per mg protein. Cytochrome contents are expressed as absorbance differences per 10 mg membrane protein/ml as described in Materials and Methods.

Growth conditions	*Nitrogen source*	*Oxidase activities*				*Nitrate reductase activity*	*Cytochromes*		
		NADH	*Succinate*	*Lactate*	*Formate*		*b*	a_1	*d*
Aerobic	NO_3^-	700	35	22	15	0	0.048	< 0.001	0.004
Anaerobic	NH_4^+	720	60	20	40	80	0.080	0.015	0.015
Anaerobic	$NO_3^- + NH_4^+$	721	60	20	42	464	0.195	0.010	0.013

finding, which is in agreement with the results obtained by other authors[9-11], suggests that cytochrome *b* may be a component of the electron transport system to nitrate. Furthermore, it can be seen that in cells cultured aerobically the cytochrome levels are rather low, in particular cytochrome a_1 is nearly absent in these cells.

Oxidase systems are also given in Table I. The presence of nitrate under anaerobic conditions of growth led to a 6-fold increase of the NADH–nitrate reductase

activity, but had no influence on the oxidase activities. The increase of nitrate reductase activity was also found with intact cells, when formate was used as electron donor instead of NADH. It should be noted, however, that the formate-nitrate reductase activity, postulated to be the most important pathway of electron transport to nitrate in *Escherichia coli*[10], was found to be very low in membranes, since the formate dehydrogenase of *K. aerogenes* showed a high lability and was easily inactivated by cell disruption procedures[2]. The low formate oxidase activity in isolated membranes of *K. aerogenes* (Table I) can be explained in the same manner. The addition of FAD, FMN, or NAD^+ did not effect any of the measured oxidase activities.

In order to investigate whether the cytochromes participate in the nitrate respiratory system the effects of nitrate on the level of reduction of the cytochromes in the anaerobic state were determined by dual-wavelength spectrophotometry. The changes in the reduction levels of individual cytochrome components of *K. aerogenes* membranes under various conditions are recorded in Fig. 2, and the quantitative data are summarized in Table II. On the addition of NADH an aerobic steady state reduction (A in Fig. 2) is attained which persists until all the O_2 dissolved in the reaction mixture has been consumed; at this point the cytochromes become further reduced and pass into the anaerobic reduced state (B in Fig. 2). The anaerobic addition of nitrate to the membranes in this anaerobic reduced state results in a rapid partial reoxidation of cytochrome *b* to the nitrate-reducing steady state (C in Fig. 2; *cf.* ref. 12). The latter state persists until the added nitrate is reduced to nitrite through the respiratory nitrate reductase; then cytochrome *b* returns slowly to a new anaerobic reduced state (D in Fig. 2), which represents a somewhat lower reduction level than the original anaerobic reduced state as a result of a slight oxidation by the nitrite formed (see below). In the case of cytochromes a_1 and *d*, the addition of nitrate in the anaerobic state produces hardly any change in the reduction levels. Only a very small rapid reoxidation is observed, followed by a slow, continuously

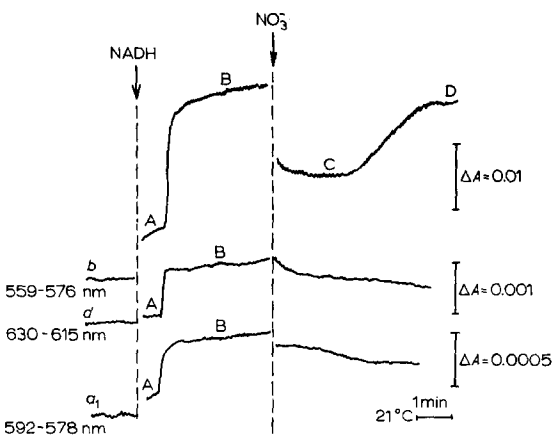

Fig. 2. Effect of nitrate on the reduction levels of the NADH-reduced cytochromes *b*, a_1 and *d* of *K. aerogenes* membranes, as determined by dual-wavelength spectrophotometry (for experimental details see Materials and Methods). An upward deflection of the traces indicates reduction. A, aerobic steady state reduction; B, anaerobic state reduction; C, steady state reduction in the presence of nitrate (in a starting concentration of 1 mM); D, anaerobic state reduction in the presence of formed nitrite (in a final concentration of approximately 1 mM).

proceeding reoxidation process (Fig. 2). Apparently, this secondary slow reoxidation is not caused by nitrate itself, but rather by an increasing amount of nitrite, formed enzymatically from nitrate in the course of the experiments. This idea is substantiated by the results which are summarized in Table II. Whereas the effect of nitrate on the reduction levels of cytochromes *b*, a_1 and *d* is concentration-independent (within the limits investigated), the relative reoxidation of the reduced cytochromes by nitrite depends on the nitrite concentration added. The reoxidation of cytochromes by nitrite has no physiological significance, since separate experiments demonstrated

TABLE II

EFFECT OF NITRATE AND NITRITE ON THE REDUCTION LEVELS OF CYTOCHROMES IN *K. AEROGENES* MEMBRANES

Reduction levels of cytochromes, expressed as percentage of total reduction by dithionite, were determined by dual-wavelength spectrophotometry as described in Materials and Methods. NADH was used as substrate. All values represent at least 6 different membrane preparations. The values marked with * refer to the situation immediately after the addition of the indicated compound (*cf.* Fig. 2).

Oxidation–reduction state	*Additions*	*Reduction of cytochromes (%)*		
		b	a_1	*d*
Aerobic steady state	None	30	33	10
	NO_3^- (1 mM)	13	33	10
Anaerobic reduced state	None	95	96	95
	NO_3^- (0.3–1.7 mM)	49	88*	90*
	NO_2^- (1 mM)	76	85*	85*
	NO_2^- (2 mM)	69	70*	70*

TABLE III

EFFECT OF INHIBITORS ON THE NADH OXIDASE AND NADH-LINKED NITRATE REDUCTASE SYSTEMS OF *K. AEROGENES* MEMBRANES

Antimycin A and HQNO were added to the reaction mixture (1.5 ml) as an methanolic solution (0.1 ml) 15 min prior to the addition of NADH. The presence of methanol in this low final concentration was found to have no influence on the measured enzymatic activities. All values represent at least five preparations.

Inhibitor	*Concn and time of exposure*	*% inhibition*	
		NADH oxidase	*Respiratory nitrate reductase*
Antimycin A	400 nmoles/mg protein	56	54
HQNO	500 nmoles/mg protein	76	77
KCN	0.8 mM	78	84
NaN_3	0.2 mM	0	96
CO	2–5 min	90	4

that a NADH-linked nitrite reductase activity is completely absent in these *K. aerogenes* membranes.

The effects of various respiratory inhibitors on the NADH oxidase and the NADH-linked respiratory nitrate reductase activities are shown in Table III. Both activities are strongly inhibited by antimycin A and 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO) (two inhibitors presumably acting at the cytochrome *b* region), and also by cyanide, the classical inhibitor of cytochrome oxidase. Two other inhibitors of terminal oxidases, azide, and CO, appeared to have a more differential effect. CO blocks the NADH oxidase activity almost completely, but has no influence on the measured nitrate reductase activity. On the other hand, azide acts as a selective inhibitor of the respiratory nitrate reductase activity.

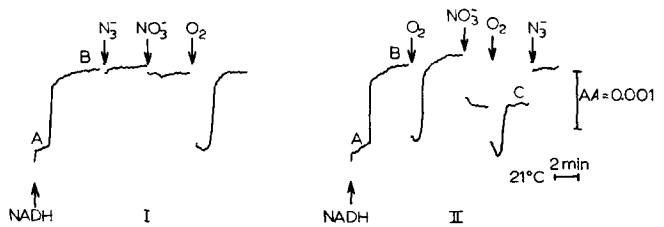

Fig. 3. The effect of 1 mM azide on the redox changes of cytochrome *b*, as measured by dual-wavelength spectrophotometry. Experimental conditions were as described in Materials and Methods, except that 0.30 mg of membrane protein was added to each cuvette. Nitrate (1 mM) was added as indicated. The reduction states A, B and C are defined under Fig. 2. (I) Addition of azide in the anaerobic state. (II) Addition of azide in the nitrate-reducing steady state.

In view of its property to inhibit selectively the nitrate respiratory system, the influence of azide on the redox changes of cytochrome *b* was studied (Fig. 3). In the presence of azide nitrate does not cause a significant reoxidation of cytochrome *b* (Fig. 3-I). The small change in the reduction of cytochrome *b* by adding nitrate in the presence of azide may reflect an incomplete inhibition of the nitrate reductase activity under these experimental conditions. Azide does not prevent the reoxidation of cytochrome *b* by oxygen. Similarly, by the addition of azide the reduction of cytochrome *b* passes almost immediately from the nitrate-reducing steady state into the anaerobic reduced state (Fig. 3-II). By the same procedure as described in Fig. 3, it was found that azide had no influence on the unphysiological reoxidation of cytochromes a_1 and *d* by nitrite shown in Fig. 2.

The results obtained so far indicate that cytochrome *b* is a component of both the oxygen and the nitrate respiratory system, whereas cytochromes a_1 and *d* do not participate in the electron transport to nitrate. This conclusion is also supported by the observation that the addition of nitrate to membranes in the aerobic steady-state results in a lower reduction level of cytochrome *b* only, whilst giving no change in the reduction levels of cytochromes a_1 and *d* (see Table II).

DISCUSSION

Difference spectra of *K. aerogenes* membranes, obtained from bacteria grown under conditions in which a high respiratory nitrate reductase activity is induced, indicate the presence of cytochromes *b*, a_1, *d* and *o*.

The CO-reduced *minus* reduced difference spectra show that CO complexes were formed with the cytochromes a_1, *d* and *o* (Fig. 1). Since CO does not inhibit the NADH-linked respiratory nitrate reductase system (Table III), it seems rather unlikely that these cytochromes are involved in the electron transport to nitrate as was suggested for other bacteria[13,14]. Kinetic experiments confirmed the idea that cytochromes a_1 and *d* do not participate in the nitrate respiratory system; the addition of nitrate in the anaerobic state hardly changed the reduction levels of these cytochromes. The slow reoxidation of the reduced cytochromes in these cases appeared not to be caused by nitrate itself, but rather by nitrite formed enzymatically from nitrate in the course of the experiments. The reoxidation of reduced cytochromes by nitrite was found to be non-enzymatic. Nitrite cannot act as a terminal electron acceptor in *K. aerogenes* under the growth conditions used. The observed oxidation of cytochromes by nitrite was a rather unexpected result, since it has been reported by other authors[15,16] that nitrite does not cause a significant oxidation of cytochromes. Therefore, the reported reoxidation patterns of reduced cytochromes by the addition of an excess amount of nitrate in bacterial systems with an active nitrate reductase[15,17,18] may be due to the nitrite formed.

The low aeroble steady state reduction of cytochrome *d* suggests that this cytochrome may act as terminal oxidase. The place and function of cytochrome a_1, which has an aerobic steady state reduction nearly equal to that of cytochrome *b* remains obscure.

In the present experiments the amount of cytochrome *b* appeared to be the highest under growth conditions, that also favour the synthesis of respiratory nitrate reductase. In *Escherichia coli* addition of nitrate to the medium had a similar effect on the cellular amount of cytochrome *b*[15] and this cytochrome was considered to be a component of the formate–nitrate reductase pathway[10,15,19]. Ruiz-Herrera and DeMoss[10] found two types of cytochrome *b* in *E. coli*: cytochrome b_{555} (measured at 77 °K) functions in the formate–nitrate reductase pathway in exponential-phase cells, whereas cytochrome b_{558} may participate in the NADH oxidase system. In our difference spectra no evidence for the presence of more than one *b*-type cytochrome was found.

In the present dual-wavelength experiments NADH, and not formate, was used as electron donor, since in our membrane preparations the formate-linked nitrate reductase activity was very low, probably as a result of the inactivation of the formate dehydrogenizing enzymes during cell disruption. Furthermore, it has been shown that NADH acts as a physiological donor for the respiratory nitrate reductase in *K. aerogenes*[20]. Recently, it was also found for *E. coli* that formate is not the obligatory electron donor for nitrate respiration, and that formate can be replaced, at least partially, by NADH[21].

Cytochrome *b* was the only cytochrome which, after being reduced by NADH, could become reoxidized to a nitrate-reducing steady state. Furthermore, the addition of nitrate to membranes in the aerobic steady state resulted in a lower reduction level of cytochrome *b*, while giving no change in the reduction levels of cytochromes a_1 and *d* (*cf.* Table II). The presence of the selective inhibitor of the nitrate reductase system, azide, eliminated the effects of nitrate on the reduction levels of cytochrome *b*. These data permit the conclusion that cytochrome *b* is a component of both the oxygen and the nitrate respiratory system. The results of studies with respiratory in-

hibitors are consistent with this conclusion. Antimycin A and HQNO inhibited the electron transport to oxygen and to nitrate, both to the same extent.

The evidence available indicates that the electron transport systems to oxygen and nitrate in *K. aerogenes*, induced for high respiratory nitrate reductase activity, have a common part, starting with ubiquinone-8[3] and ending with cytochrome *b*. From this cytochrome branching occurs, with one branch linked to the respiratory nitrate reductase and one branch linked to oxidase systems, containing the cytochromes a_1, *d* and *o*. The data presented could also be interpreted to mean that the electron transport pathways to nitrate and oxygen are completely parallel, and that a part of the electron carriers are identical for the two parallel chains. However, in view of the observed competition between the electron transfer to nitrate and to oxygen, both in intact bacteria[2] and in membrane preparations[3] the existence of a branched electron transport chain seems more likely.

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