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THE ROLE OF UBIQUINONE IN LINKING NITRATE REDUCTASE AND CYTOCHROME *o* TO THE RESPIRATORY CHAIN OF *PARACOCCUS DENITRIFICANS*

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Three alternatives of the mode of branching in the ubiquinone-cytochrome *b* region of the anaerobic respiratory chain of *Paracoccus denitrificans* were experimentally tested. It was found that the view that the constitutive cytochrome *b*-560 or *b*-566 serves as an electron donor for the nitrate reductase is incompatible with the proposed scheme of the cyclic electron flow in the bc_1 segment. By means of the extraction procedure, the extent of reduction of ubiquinone was determined in cells utilizing oxygen and nitrate in the presence of antimycin. It was found that the redox response of ubiquinone was consistent with what had been predicted by the pool model of Kröger and Klingenberg, extended for more than one terminal acceptor. Our results are in support of the assumption that in cells of *P. denitrificans* ubiquinol (QH_2) has a function of an electron donor both for nitrate reductase and cytochrome *o*.

Introduction

The denitrifying bacterium *Paracoccus denitrificans* grown anaerobically in the presence of nitrate synthesizes terminal reductases catalyzing successive dissimilatory reduction of nitrate down to molecular nitrogen and an alternative pathway to oxygen which is constituted by cytochrome *o* [1,2]. These enzymes are attached to the constitutive respiratory chain closely resembling that of mitochondria [3]. Three alternatives of the mode of linking nitrate reductase and cytochrome *o* are considered in the literature (see Fig. 1). Constitutive cytochrome *b*-560 or *b*-566 was assumed to be a donor for nitrate reductase [1,3,4] (see line 1a in Fig. 1) because of the insensitivity of the nitrate respiration to antimycin and due to the fact that the reoxidation of *b* cytochromes was observed in the presence of nitrate [5]. The scheme 1b proposed by Whatley [6] takes into account the finding [7] that cytochrome *b*-560 and *b*-566 of *P. denitrificans* are functionally arranged in accor-

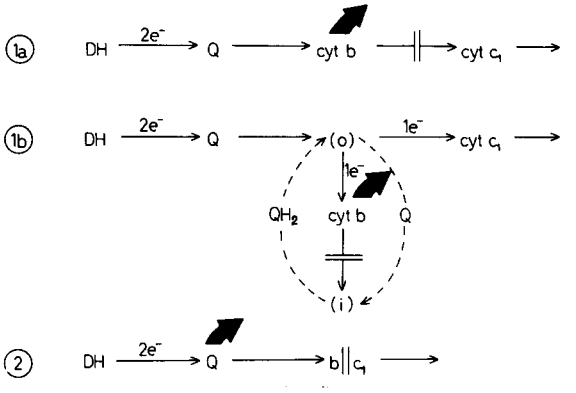


Fig. 1. Possible alternatives of the branching point to nitrate reductase and cytochrome *o* in the anaerobic respiratory chain of *Paracoccus denitrificans*. The linkage of inducible components is presented by arrow. The interruptions, ⫞⫟, of pathways designate the antimycin inhibition, Cytochrome *b* means *b*-560 and *b*-566. Dashed lines in 1b mean the diffusion of Q and QH_2 between the centres (o) and (i), DH, dehydrogenase.

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

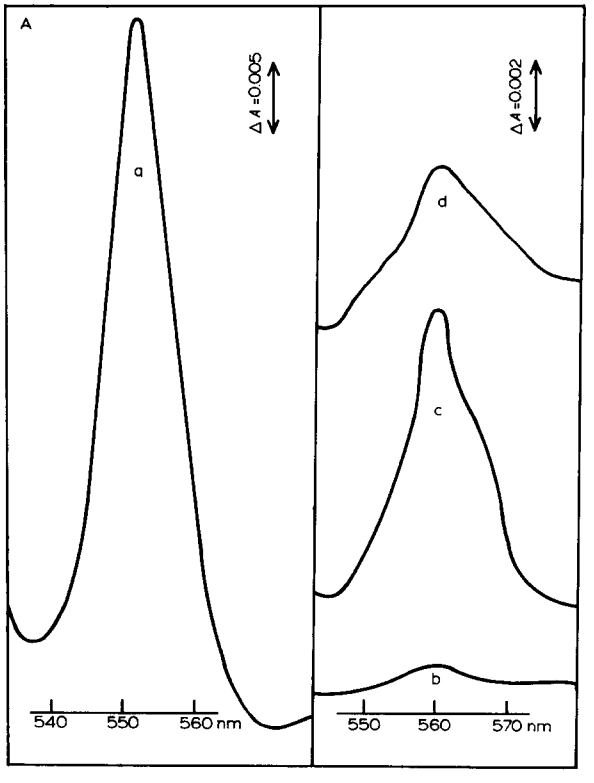
dance with the cyclic scheme of Mitchell's ubiquinone cycle. Recently, ubiquinone was suggested to serve as the branching point [2] (see item 2 in Fig. 1). The present study brings some quantitative data on the redox behaviour of ubiquinone and cytochrome *b* in the respiratory chain of *P. denitrificans* which can help to decide among the alternatives.

Materials and Methods

P. denitrificans (NCIB 8944) was grown anaerobically in the medium according to Tait [8] with succinate as the carbon source and nitrate as the terminal acceptor. Cells were harvested in the early stationary growth phase. Spectrophotometrical measurements were performed with Cary 118 C. The redox state of ubiquinone in intact cells was determined by means of the extraction technique described previously [9] using pyrogallol as antioxidant.

Results and Discussion

As for mechanism 1b (Fig. 1), it is characteristic that in the presence of antimycin the reduction of nitrate is possible only in the presence of an electron acceptor abstracting electrons from a site located behind cytochrome c_1. With respect to the fact that nitrite and nitrous oxide as intermediates of nitrate reduction represent such a kind of acceptor [1,2], the sensitivity of the nitrate re-



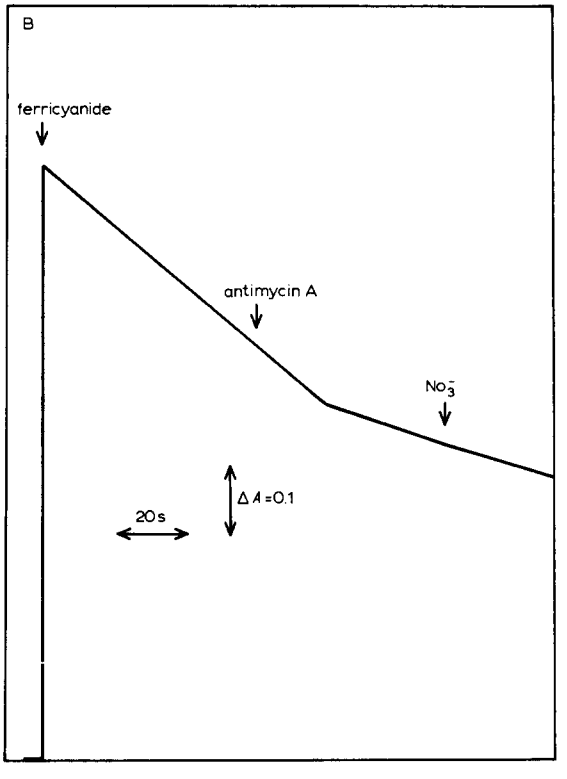


Fig. 2. (A) Spectral behaviour of cytochromes of *P. denitrificans* cells in the presence of ferricyanide. The sample cuvette contained 3 ml of medium consisting of 0.25 M sucrose, 0.1 M Tris-HCl buffer (pH 7.3) and 0.1 M succinate together with 7.58 mg dry wt. of cells; the reference cuvette contained the same amount of cells in 3 ml 0.1 M phosphate buffer (pH 7.3) with a few crystals of solid ferricyanide. (a) Anaerobiosis after oxygen consumption; (b) 50 μl 0.5 M ferricyanide added to the sample cuvette; (c) 10 μg antimycin added to the sample cuvette; (d) 50 μl 1 M $NaNO_3$ added to the sample cuvette. (B) Reduction of ferricyanide by *P. denitrificans* cells. The sample and reference cuvette contained 3 ml of the medium described in A and cells of 1.86 mg dry wt. The reaction was started by the addition of 5 μl of 0.5 M ferricyanide to the sample cuvette. Additions: 5 μg antimycin A; 50 μl 1 M $NaNO_3$.

ductase reaction to antimycin should be markedly enhanced when working with membrane fragments (depleted of nitrite- and nitrous oxide-reductases) rather than with intact cells. This effect has not been observed so far. The postulated enhancement of the electron flow through the cytochrome bc_1 complex on reoxidation of *b* cytochromes with nitrate was experimentally tested using ferricyanide as the artificial electron acceptor (see Fig. 2). It can be seen that ferricyanide added to an anaerobic suspension of *P. denitrificans* cells was progressively reduced. At the same time, cytochromes of the respiratory chain were fully oxidized. The action of antimycin in saturating inhibitory concentration led to the decrease of the rate of ferricyanide reduction to about 30% of the original value and simultaneously, the reduction of cytochromes *b* occurred. The antimycin-insensitive ferricyanide reductase activity was probably caused by ferricyanide taking electrons from the dehydrogenase region in cells of increased cytoplasmic membrane permeability or from a redox centre different from the *c*-type cytochromes localized on the outer aspect of plasmatic membrane, as was observed in the case of other bacteria [10]. It was found in an independent experiment that under these conditions (i.e., at redox equilibration of cytochromes *c* by ferricyanide in the presence of antimycin), the cells used exhibited high nitrate reductase and oxidase activities (not shown). In keeping with this, the nitrate addition brought about the reoxidation of cytochromes *b*. However, with the reduction of nitrate taking place at the same time, the ferricyanide reduction was not significantly enhanced. In view of the fact that no stimulation of ferricyanide reductase activity was observed, we may conclude that the participation of main cytochromes *b*-560 and *b*-566 in the ubiquinone cycle [7] is incompatible with their functioning as electron donors for nitrate reduction as suggested by Whatley [6].

The reoxidation of cytochromes *b* by nitrate (see Ref. 5 and Fig. 2) need not necessarily be the consequence of their function as donors for nitrate reductase, but may reflect changes in the extent of ubiquinone reduction. As discussed in the review [2], the respiratory chain of *P. denitrificans* may be arranged in multiprotein complexes analogous to mitochondria, with the ubiquinone pool as the donor of reducing equivalents for the bc_1 complex and complexes of nitrate reductase and cytochrome *o*. To date, only qualitative data obtained with fluorescence probe on the redox behaviour of ubiquinone in *P. denitrificans* are available [11,12].

If ubiquinone constitutes a common pool of reducing equivalents for nitrate reductase, cytochrome *o* and bc_1 complex (cf. item 2 in Fig. 1), we may expect that its redox response can be described by means of the ubiquinone pool model as derived by Kröger and Klingenberg [13] for mitochondrial oxidation. The applicability of this model for bacterial respiration requires its extension for the actual utilization of more than one terminal acceptor. Such a form as can be experimentally tested is deduced in the Appendix. It is evident from the final equation (A-4) that in the case of blocking the electron flow through the bc_1 complex with antimycin and using nitrate and oxygen as terminal acceptors the following relation holds:

$$\frac{1}{\phi_{O_2}} + \frac{1}{\phi_{NO_3}} = \frac{1}{\phi_{O_2+NO_3}} + 1 \qquad (1)$$

TABLE I

REDOX STATE OF UBIQUINONE IN *P. DENITRIFICANS* CELLS IN THE PRESENCE OF ANTIMYCIN

The extraction of 10–20 mg dry wt. of intact cells in 0.5 ml of the medium consisting of 0.25 M sucrose, 0.1 M Tris-HCl buffer (pH 7.3) 0.1 M succinate and 1 μg antimycin/mg dry wt. was performed with methanol/light petroleum mixture [9] containing 1 mg of pyrogallol as antioxidant per 1 ml of solvent mixture. The content of oxidized ubiquinone amount was determined spectrophotometrically after reduction with potassium borohydride. The total amount of ubiquinone in cells was determined always in parallel samples after alkaline extraction [9] and its average value for given type of cells was 1.12 μmol/g dry wt. Oxygen was brought into bacterial suspension by aeration and agitation, anaerobic conditions were achieved by nitrogen presence, nitrate concentration was 20 mM. In the absence of antimycin the extent of ubiquinone reduction was found to be 15% in utilizing O_2 (batch II); upon addition of 4 mM KCN, ubiquinone was completely reduced.

Acceptor	Batch I		Batch II	
	100ϕ (%)	$1/\phi$	100ϕ (%)	$1/\phi$
Oxygen	81	1.23	73	1.37
Nitrate	58	1.72	53	1.89
Oxygen + nitrate	56	1.79	40	2.50

where ϕ means the extent of reduction of ubiquinone pool ($0 < \phi < 1$) in the presence of saturating amounts of terminal acceptors. From the results of experiments examining the fractional extent of ubiquinone reduction (see Table I), the values for the left- and right-hand side of Eqn. 1 were established. They equal for batch I of bacteria 2.95 (left) and 2.79 (right) and for batch II 3.26 (left) and 3.50 (right). Taking into account the mean deviation of the estimates of the reductoin extent of ubiquinone which was about $\pm 3\%$ the calculated values for the left- and right-hand side of Eqn. 1 are in good accordance.

From the validity of the relation above, it follows that the intensity of the electron flow to terminal acceptors is proportional to the reduction degree of ubiquinone. This statement makes the special case of scheme 1a – i.e., the limitation of the electron flow by the redox state of cytochrome(s) *b* ('*b* pool model') – less probable. Taking into account the rapid achievement of the equilibrium of the reaction:

$$QH_2 + 2\ \text{cyt}\ b^{3+} \rightleftharpoons Q + 2\ \text{cyt}\ b^{2+}$$

with the equilibrium constant K it follows that in this case the electron flow should be proportional to the term $(1 + \sqrt{(1-\phi)/K\phi})^{-1}$. In contrast to this, the deduced relation presumes the proportionality to ϕ. Another special case of scheme 1a (Fig. 1) may be provided by the Q pool model. However, in scheme 1a the condition implied in the Appendix, i.e., the independence of pathways, distributing electrons to terminal acceptors, does not hold. Unlike 1a, according to scheme 2, oxygen may affect the nitrate reduction only by oxidizing the common pool of ubiquinone. The indirect support of the view that ubiquinone functions as an electron donor for nitrate reductase is offered by the observation that duroquinol also serves as a donor for nitrate reductase in an antimycin insensitive reaction [14]. The sensitivity of nitrate reduction to HQNO may indicate the participation of a low-potential *b* type cytochrome as a part of the nitrate reductase complex [14] although such a component was not revealed by the potentiometric analysis of anaerobically grown cells [15]. Finally, it should be emphasized that our conclusions are based on the measurements performed under steady-state conditions of the respiratory chain. Unambiguous conclusions on the role of ubiquinone and cytochrome *b* could be attained by more distinctive approaches such as by transient-state kinetics. Further parameters are under exploration.

Appendix

Let us consider a homogeneous pool of the redox carrier (ubiquinone) communicating with m substrates by means of pathways of capacities $V_{\text{red},i}$; $i = 1,\ldots,m$ and with n terminal acceptors by means of independent pathways of capacities $V_{\text{ox},j}$; $j = 1,\ldots,n$ (for the definition of V_{red}, V_{ox}, see Ref. 13). In the stationary state when all substrates and n terminal acceptors are in saturating concentrations, the statement holds that the reduction rate of the pool equals the rate of its oxidation, i.e.:

$$(1-\phi)\sum_{i=1}^{m} V_{\text{red},i} = \phi \sum_{j=1}^{n} V_{\text{ox},j} \qquad \text{(A-1)}$$

where ϕ is the extent of pool reduction ($0 < \phi < 1$). Adapting (A-1) results in:

$$\frac{1}{\phi} = 1 + \sum_{j=1}^{n} \left(V_{\text{ox},j} \Big/ \sum_{i=1}^{m} V_{\text{red},i} \right) \qquad \text{(A-2)}$$

For the reduction level of pool ϕ_j in the presence of only the jth terminal acceptor, the following equation holds:

$$\frac{1}{\phi_j} = 1 + \frac{V_{\text{ox},j}}{\sum_{i=1}^{m} V_{\text{red},i}}, \quad j = 1,\ldots,n \qquad \text{(A-3)}$$

From Eqns. (A-2) and (A-3), the final relation bteween ϕ and ϕ_j, $j = 1,\ldots,n$ can easily be obtained in the form:

$$\frac{1}{\phi} = 1 - n + \sum_{j=1}^{n} \frac{1}{\phi_j} \qquad \text{(A-4)}$$

In the text above a special case of Eqn. (A-4) for $n = 2$ was considered.

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