Control of respiration rate in non-growing cells of *Paracoccus denitrificans*

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By means of fluorimetric measurement and by direct determination of intracellular NAD^+ and NADH contents, it was proved that the respiration rate of *Paracoccus denitrificans* cells utilizing glucose is limited by processes preceding NADH oxidation in the respiratory chain, so that the membrane NADH dehydrogenase is not saturated by its substrate. In the separated membrane fraction on saturation with exogenous NADH the main limiting factor is represented by NADH:ubiquinone oxidoreductase.

INTRODUCTION

The non-fermenting bacterium *Paracoccus denitrificans* can utilize glucose as a source of carbon and energy during heterotrophic growth. Glucose is metabolized by means of the pentose phosphate cycle and the Entner–Doudoroff pathway; the pyruvate formed in the reaction is oxidized to CO_2 in the tricarboxylic acid cycle (Forget & Pichinoty, 1965; Dunstan *et al.*, 1984). The reoxidation of the cytoplasmic pool of NADH is carried out by the membrane-bound NADH:ubiquinone oxidoreductase, with a known subunit composition (George & Ferguson, 1984), which passes reduction equivalents into the branched respiratory chain of the bacterium (Stouthamer, 1980; Ferguson, 1982).

In a previous paper (Kučera *et al.*, 1983a), the distribution of the electron flow among the individual terminal acceptors (NO_3^- , NO_2^- and N_2O) was measured in cells grown anaerobically on glucose. From the observed rates of terminal-acceptor reduction, the intensities of actual electron flows in the terminal branches of the respiratory chain were calculated. Thus it appeared that their sum remained constant throughout the experiment, even though the individual fluxes changed significantly, depending on acceptor concentrations. Interpreting the result it was suggested that the total rate of respiration was limited as early as at the level of the entrance of the reduction equivalents into the respiratory chain (Kučera *et al.*, 1983a).

In the present paper attention has been directed towards a more detailed description of factors limiting the respiration rate of cells of *P. denitrificans*. Two experimental systems were studied in parallel: membrane fragments oxidizing exogenous NADH, and intact cells in which only the NADH formed endogenously in the cytoplasm via the metabolism of glucose was oxidized.

MATERIALS AND METHODS

Micro-organism

The bacterium *Paracoccus denitrificans* (N.C.I.B. 8944) obtained from the Czechoslovak Collection of Micro-organisms (CCM 982) was grown anaerobically for 24 h at 30 °C in 3 ml flasks filled with a medium containing 50 mM-glucose, 10 mM- KNO_3 , 30 μM -ferric citrate and additional inorganic salts (Kučera *et al.*, 1983a). Membranes were derived from cells, which had

been disintegrated with a French press, by differential centrifugation (Burnell *et al.*, 1975). The modified biuret method (Szarkowska & Klingenberg, 1963), with bovine serum albumin as a standard, was used for the determination of the concentration of membrane proteins.

Analytical methods

O_2 consumption was measured with a Clark oxygen electrode in a 3 ml electromagnetically stirred closed vessel. NADH oxidation was monitored as the A_{340} . The time course of the degree of reduction of cytochromes *c* was obtained by scanning the difference spectrum of the cell suspension at 1 min intervals against the suspension oxidized with excess of solid $\text{K}_3\text{Fe}(\text{CN})_6$. A complete 100% reduction in the measured cuvette was achieved by adding solid $\text{Na}_2\text{S}_2\text{O}_4$. In the fluorimetric registration of redox state of the pool of intracellular nicotinamide nucleotides the excitation wavelength was 340 nm, the emission wavelength as 460 nm (Harrison, 1976), and the width of the two slits was 1 mm. Zero fluorescence corresponded to a medium not containing cells and the value of 100 was taken from an anaerobic suspension of cells in the presence of glucose. In all cases 0.1 M-Tris/HCl, pH 7.5, at 25 °C was the medium. N_2O was added in aqueous solution (Kučera *et al.*, 1983a), and O_2 was introduced in the form of H_2O_2 , which is quickly decomposed by the catalase activity of the cells (Kučera *et al.*, 1986b).

The oxidized and the reduced forms of NAD were extracted from the sample of cell suspension as described by Wimpenny & Firth (1972). On the basis of model experiments with pure coenzymes, a slight modification of the method, involving replacing 0.1 M-HCl with 0.2 M and 3 M-KOH with 2 M solutions, proved suitable. To determine the content of the coenzymes in the extracts, a sensitive recyclicalization reaction catalysed by horse liver alcohol dehydrogenase in the presence of *p*-nitroso-*NN*-dimethylaniline and butan-1-ol was used (Rasmussen *et al.*, 1972; Kovář & Kučera, 1982). When this procedure was used, none of the interferences mentioned by Wimpenny & Firth (1972) was observed.

RESULTS

Membrane fragments derived from cells of *Paracoccus denitrificans* grown anaerobically on glucose exhibited

high NADH oxidase activity (of the order of hundreds of nmol of O_2 /min per mg of protein). The pH optimum of the reaction in 0.1 M-Tris/HCl was found to be in the range 7.5–7.8. The initial NADH oxidation rate was dependent in a hyperbolic manner on substrate concentration. Kinetic parameters determined were: K_m (NADH) = 14 μ M and V = 610 nmol of NADH/min per mg of protein. The NADH oxidase activity of the particles was enhanced 2–3-fold by the uncoupler (1 μ M) carbonyl cyanide *m*-chlorophenylhydrazone.

Because of their nitrate reductase content, the mem-

Fig. 1. Effect of nitrate on the rates of NADH and O_2 consumption in respiration by membrane fragments of *P. denitrificans*

The experiment was carried out in an oxygraphic vessel or in a spectrophotometric cuvette which contained 3 ml of 0.1 M-Tris/HCl buffer, pH 7.5; 0.2 mg of membrane protein was used, and the final concentrations of NADH and NO_3^- were 0.33 mM and 6.4 mM respectively.

branes possessed the ability of reducing nitrate together with O_2 (cf. John, 1977). The addition of nitrate resulted in a fall in O_2 consumption rate to about one-half; however, the NADH oxidation rate remained unchanged (Fig. 1). Thus it can be assumed that the resulting respiration rate of membrane fragments is limited by electron flux through the NADH:ubiquinone oxidoreductase segment of the respiratory chain.

Unlike membrane fragments, the respiratory chain of intact *P. denitrificans* cells does not oxidize exogenous NADH, but only NADH generated in the catabolism of the growth substrate in the cytoplasm. The reoxidation of the intracellular pool of nicotinamide nucleotides after the respiration has commenced can be monitored fluorimetrically (for overview see, e.g., Harrison, 1976). The result of a typical experiment in which the action of four terminal acceptors (O_2 , NO_3^- , NO_2^- , N_2O) was studied is shown in Fig. 2; for comparison the simultaneous time courses of reduction of cytochromes *c* measured spectrophotometrically are given. The results in Fig. 2 show that the degrees of reduction of cytochromes *c* differed in connection with different distributions of the electron flow among the terminal branches of the respiratory chain. The monophasic profile of the curves reflects the fact that, under given conditions, the denitrification intermediates NO_2^- and N_2O are not accumulated (cf. Kučera *et al.*, 1983a, 1986a). On the other hand, fluorescence values (and thus also the degrees of reduction of intracellular nicotinamide nucleotides) coincided in all four cases, which demonstrates the identical flow of the redox equivalents through the nicotinamide nucleotide pool. The same conclusion can also be reached by comparing the electron flows to terminal acceptors calculated from the time interval needed for acceptor consumption, the number of mol of the applied acceptor and the number of electrons accepted in the reduction ($H_2O_2 = 2$, $NO_3^- = 5$, $NO_2^- = 3$ and $N_2O = 2$). For the experiment in Fig. 2, mutually close values were thus obtained: 100.0 (O_2), 96.0 (NO_3^-), 107.1

Fig. 2. Time course of redox alterations of intracellular pool of nicotinamide nucleotides and cytochromes *c* in cells of *P. denitrificans*

For description of conditions see the Materials and methods section; 8 mg dry wt. of cells was used; 3 ml of Tris/HCl medium contained 20 mM-glucose, and additions of terminal acceptors were 2 μ mol (H_2O_2 , NO_3^- , NO_2^-) or 2.5 μ mol (N_2O).

Table 1. Redox state of intracellular pool of NAD⁺ in anaerobiosis and during utilization of terminal acceptors

A 2 ml suspension of cells of *P. denitrificans* (5 mg dry wt.) in Tris medium containing 20 mM-glucose and the corresponding terminal acceptor (5 mM) was incubated for about 3 min at 25 °C. The sample lacking terminal acceptor was made anaerobic by gassing with N₂. After that, the reaction was stopped by adding HCl or KOH and the cell-free extract was separated for determination of NAD⁺ of NADH content (see the Materials and methods section). The internal volume of cells, used for calculation of intracellular concentrations of coenzymes, was 2.7 ml/g dry wt. (Karlovský *et al.*, 1982). The variation coefficient established for ten repetitions of the analysis of NAD⁺ and NADH in samples was 11 % and 13 % respectively.

Terminal acceptor	Intracellular concn. (mM)		Reduction (%)
	NAD ⁺	NADH	
O ₂	1.1	0.02	1.8
Nitrate	1.1	0.02	1.8
Nitrite	1.2	0.03	2.4
None	1.0	0.47	32.0

(NO₂⁻) and 125.0 (N₂O), in nmol of electrons/min per mg dry wt.

Although the results of the above experiment (Fig. 2) prove a limitation of *P. denitrificans* cell respiration at a site before terminal branching, they do not make it possible to decide whether the limitation occurs in a similar way to that in the membrane fragments, i.e. at the level of NADH dehydrogenase, or already in preceding metabolic processes. The knowledge of actual intracellular concentrations of NADH, necessary for making this decision, is lacking, i.e. the calibration of the fluorescence signal from Fig. 2. The essential values were experimentally obtained by extraction of nicotinamide nucleotides from cells and determination of their content by a one-enzyme recyclization reaction (see the Materials and methods section). From the result of a typical experiment given in Table 1, it follows that after the respiration has started a significant reoxidation of the intracellular nicotinamide nucleotide pool occurs (from 32 % reduction during anaerobiosis to about 2 % during respiration). Also, in respiring cells the total sum of NAD⁺ plus NADH is slightly lower, which might be attributed to their phosphorylation and other metabolic conversions. The resulting cytoplasmic NADH concentration is comparable with the value of the Michaelis constant of NADH dehydrogenase. This probably means that in the respiration of *P. denitrificans* cells the NADH dehydrogenase is not saturated by its substrate and that the resulting respiration rate is greatly limited by processes which are responsible for the formation of NADH in the cell.

DISCUSSION

The present results, linking up with our previous paper (Kučera *et al.*, 1983a), show that the rates of respiration of membrane fragments and of intact cells of *Paracoccus denitrificans* are controlled by different steps.

In membranes with saturation guaranteed by means of exogenous NADH addition, an obvious limiting factor is represented by the activity of the NADH:ubiquinone oxidoreductase segment. This follows from the results in Fig. 1, according to which in the competition of O₂ and NO₃⁻ for reducing equivalents, which takes place at the level of the ubiquinone pool (Kučera *et al.*, 1984a,b), the NADH oxidation rate is not affected. The limiting activity of the segment can be due to a low value of the enzyme turnover, its low content in the membrane, or due to the respiration control connected with the translocation of protons in that domain of the respiratory chain (Boogerd *et al.*, 1981); this follows from the existence of respiratory control in membrane vesicles. A proof of an effect of protonmotive force on respiration by means of an uncoupler cannot be performed with intact cells, since uncoupling in this case concomitantly slows down the access of the substrate to the cell.

On the other hand, in the regulation of the respiration of intact cells the decisive control function is obviously that of processes preceding the reactions in the respiratory chain. This conclusion may be drawn from the high reoxidation of the NAD pool after respiration has started (Fig. 2 and Table 1). The limitation can operate as early as at the level of glucose transport into the cell similarly as was observed with another growth substrate, succinate (Kučera *et al.*, 1983b). However, the regulatory role of cytoplasmic dehydrogenase cannot be excluded, either. For a stationary NADH concentration comparable with the *K_m* value for NADH dehydrogenase and at a large excess of NAD⁺ (NAD⁺/NADH ≈ 50), the course of respiration could be effectively adjusted by the rate of NADH production in the cytoplasm according to the metabolic needs of the cell. In connection with this, it is noteworthy that the reduction of NAD⁺ by the key enzyme of glucose metabolism in *P. denitrificans*, glucose-6-phosphate dehydrogenase, is effectively inhibited by physiological concentrations of ATP (Slabas & Whatley, 1977). It can therefore be assumed that effects of this type represent some feedback-regulation signal linking respiration to the utilization of glucose (NADH generation), thus providing the homeostasis of the phosphorylation potential of the cell.

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