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The release of nitric oxide from denitrifying cells of *Paracoccus denitrificans* by an uncoupler is the basis for a new oscillator

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Micromolar concentrations of the uncoupler 3-chlorophenylhydrazonepropanedinitrile caused the release of nitric oxide from the cells of *Paracoccus denitrificans* respiring nitrite. At the same time, a new component with an absorption maximum at 573 nm could be observed in the difference spectra. Damped oscillations of the concentration of the cytochrome *c*-NO complex were observed during the trapping of released NO by added mammalian cytochrome *c*; reduced cytochrome *c* accumulated simultaneously in the reaction mixture.

Denitrification; Uncoupler; Nitric oxide; Cytochrome *c*; Damped oscillation; (*Paracoccus denitrificans*)

1. INTRODUCTION

Denitrification is anaerobic respiration in which ionic oxoanions of nitrogen are reduced to gaseous products. As a rule, the intermediates in the conversion of NO_3^- to N_2 are present in very small concentrations. However, their identification is relatively easy after specific modulation of the activities of denitrifying enzymes. In *Paracoccus denitrificans*, the antibiotics antimycin and mucidin inhibited nitrite reductase and consequently blocked the denitrification on the level of NO_2^-, whereas acetylene or a mild acidic pH caused the accumulation of N_2O due to the inhibition of N_2O reductase [1,2]. In the present work, a similar approach has been applied to prove the involvement of free nitric oxide by using an uncoupler of a phenylhydrazonepropanedinitrile type as a perturbing agent.

2. MATERIALS AND METHODS

P. denitrificans (NCIB 8944 obtained as CCM 982) was grown anaerobically at 30°C in 1-l flasks filled with a medium [3] containing 50 mM sodium succinate as the source of carbon and 10 mM sodium nitrate as the terminal acceptor. Cells were harvested in the late exponential phase of growth, and then washed and suspended in 0.1 M sodium phosphate buffer, pH 7.3. All optical measurements were performed at 30°C on the spectrophotometer Shimadzu UV-3000. The detection of NO was based upon the formation of a complex with mammalian cytochrome *c*, measured at the wavelength pair 563–580 nm, the method representing a variant of the procedure suggested previously [4]; for monitoring the reduction of cytochrome the wavelength pair 550–535 nm was used. Cytochrome *c* (horse heart) was obtained from Koch-Light Laboratories (Colnbrook, England) and the uncoupler 3-chlorophenylhydrazonepropanedinitrile (carbonyl cyanide *m*-chlorophenylhydrazone, CCCP) from Sigma (St. Louis, MO, USA).

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3. RESULTS

Using mammalian cytochrome *c* as a scavenger it was established that micromolar concentrations of the uncoupler bring about an intensive release of NO from the nitrite-respiring cells of *P. denitrificans*. The obtained traces (fig.1) were marked by lags with the length inversely proportional to the final rates of the production of the NO complex. As was shown by a series of titrations similar to that in fig.1, the intercepts common to the extended linear parts of the curves shifted down proportionally to the amounts of the

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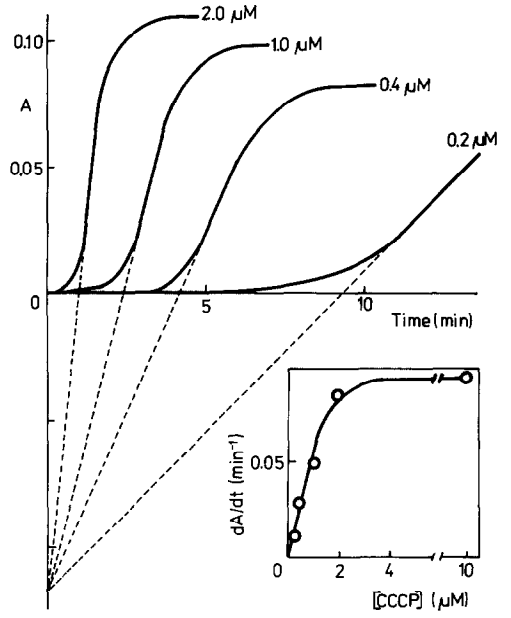

Fig.1. Formation of cytochrome *c*-NO complex in the presence of uncoupler. The closed cuvette previously gased with nitrogen contained in 2 ml, 0.1 M Na-phosphate pH 7.3, 38 μM cytochrome *c*, the given concentration of CCCP and 1.2 mg dry wt of *P. denitrificans* cells. The reaction was started with the addition of 10 μl of 1 M $NaNO_2$. The values dA/dt in the inset correspond to the slopes of dashed straight lines.

cells used. This clearly excludes the trivial possibility that the lags were caused by the reaction of NO with residual traces of oxygen. At sufficiently low initial concentrations of nitrite the time-course of the NO complex produced exhibited a characteristic shape with several damped oscillations (fig.2). The decomposition of the transiently accumulated NO complex was always accompanied by an increase in the concentration of reduced cytochrome *c*.

Under conditions of previous experiments it was possible to neglect the contribution of intracellular compounds to the final time-course of absorbance. The changes in the absorption of denitrifying cells after the addition of uncoupler were studied in an independent experiment by means of the technique of difference spectra. From the results in fig.3 it follows that the first stage of nitrite reduction was accompanied by the appearance of a marked maximum at 573 nm. Later this effect was overlapped by the formation of the minima at 552 and 521 nm, suggesting the reoxidation of cytochromes in cells due to the presence of uncoupler.

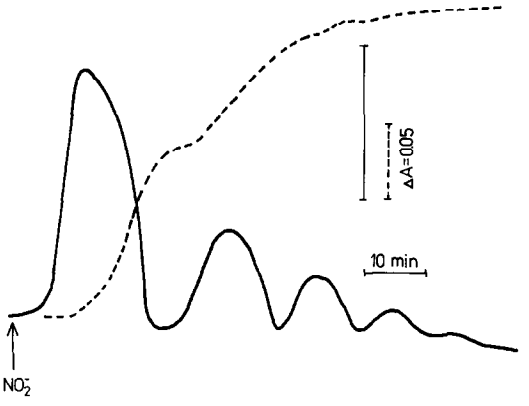

Fig.2. Time-course of the absorbance of NO complex (unbroken trace) or reduced cytochrome *c* (dashed trace). The reaction was started with the addition of 10 μl of 1 M $NaNO_2$ to 2 ml of the anaerobic mixture containing 0.1 M Na phosphate pH 7.3, 36 μM cytochrome *c*, and 1.5 mg dry wt of *P. denitrificans* cells.

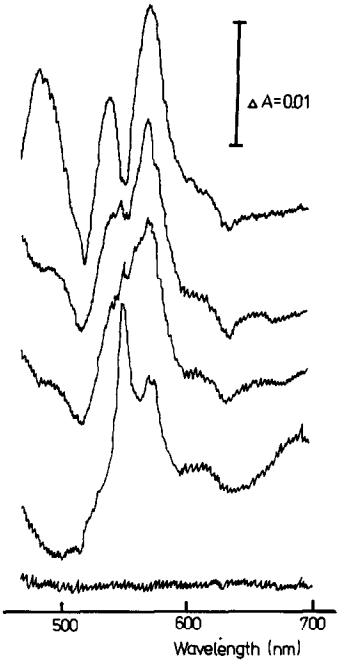

Fig.3. Spectral changes during the utilization of nitrite in the presence of uncoupler. Both cuvettes contained 3 ml of 0.1 M Na phosphate pH 7.3 with 8 mg dry wt of *P. denitrificans* cells. After gasing with nitrogen and adding 6.7 μM CCCP into the sample cuvette, the baseline was registered (bottom). This was followed by the addition of 30 μl of 1 M $NaNO_2$ into both cuvettes and by the scanning of difference spectra at the speed of 50 nm · min^{-1} without delay between successive scans.

4. DISCUSSION

The results obtained indicate that nitric oxide can be looked upon as an obligatory intermediate of denitrification in *P. denitrificans*, its formation being less sensitive to the effect of uncoupler than its further transformation. The accumulated NO may form a '573 nm complex' with a hitherto unidentified cellular component similar to the one described for other denitrifying bacteria [5] and can also be trapped by extracellular mammalian cytochrome *c* (cf. [4]). The conclusions concerning the involvement of NO and the presence of a separate NO reductase in *P. denitrificans* are supported by further independent observations: the energization of cells in the presence of NO [6], the different sensitivities of NO_2^- and NO reductases to detergents [7] and the formation of nitrosylhaemoglobin with extracellular haemoglobin during denitrification [8]. Obviously, the type of uncoupler used may become a useful tool for the study of denitrification on the level of intact cells. In further work it will be necessary to decide whether its influence depends on the decrease of the proton-motive force or on its binding to denitrifying enzymes. The first possibility, according to which one of the redox reactions of the denitrification pathway would require the presence of the proton-motive force, would suggest an interesting parallel to the bioenergetics of nitrification [9] and methanogenesis [10] in other bacteria.

In the observed oscillations (fig.2) the following processes probably participate: (i) the diffusion of nitrite into the periplasmic space of cells and its reduction to NO by the dissimilatory nitrite reductase, (ii) the binding of NO to extracellular cytochrome *c*, and (iii) the decomposition of the NO complex providing reduced cytochrome *c* along with the regeneration of nitrite. The sum of processes (i) to (iii) represents the transfer of one reducing equivalent from the respiratory chain to external cytochrome *c* by means of the recycling redox pair NO_2^-/NO. The observed reduction of cytochrome *c* (fig.2) fits well into this picture. However, in the appearance of oscillations the competition of intrinsic NO-binding proteins and external cytochrome *c* for NO may also take place. Another possibility, namely the involvement of kinetic signals destabilizing the steady-state conditions of the denitrification pathway, was demonstrated recently by the finding that NO inhibits the N_2O reductase in *Pseudomonas perfectomarina* [11].

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