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THE EFFECT OF UNCOUPLER ON THE DISTRIBUTION OF THE ELECTRON FLOW BETWEEN THE TERMINAL ACCEPTORS OXYGEN AND NITRITE IN THE CELLS OF *PARACOCCUS DENITRIFICANS*

Igor Kučera and Vladimír Dadák

Department of Biochemistry, J. E. Purkyně University, Kotlářská 2, CS-61137 Brno (Czechoslovakia)

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The preferential utilization of oxygen, the terminal acceptor, in anaerobically grown cells of *Paracoccus denitrificans* was abolished in the presence of uncoupler (3 μM carbonyl cyanide m-chlorophenylhydrazone) which brought about a switch to the reduction of nitrite. It has been proved by measuring the redox state of cytochromes that this effect is due to the inhibition of the electron flow to oxygen caused by nitrite, which attains the site of its inhibitory action when the membrane potential is lowered.

The denitrifying bacterium *Paracoccus denitrificans* grown anaerobically in the presence of nitrate can utilize O_2, NO_3^-, NO_2^- and/or N_2O as terminal acceptors (for review cf. [1-3]). The mechanism of the distribution of the electron flow to the individual terminal acceptors has been studied for several years [4-11]. This paper shows that the reduction of the transmembrane potential of the cells due to the uncoupler action markedly affects the distribution of the electron flow between O_2 and NO_2^- and a plausible mechanism of this effect is discussed.

MATERIAL AND METHODS

Paracoccus denitrificans (N.C.I.B. 8944) was grown anaerobically at 30°C in a medium described before [9], only glucose being replaced by 50 mM sodium succinate. The cells were harvested at an early stationary phase of growth, washed with 0.1 M Na phsophate buffer pH 7.3 and suspended in the same medium.

In measuring the consumption of nitrite under aerobic conditions the cells were shaken vigorously at 300 oscillations min^{-1} at 25°C in 100 ml conical flasks containing 20 ml of

Abbreviation: CCCP, carbonyl cyanide m-chlorophenylhydrazone

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the basic medium of the following composition: 0.25 M sucrose, 50 mM sodium succinate, 50 mM Tris/HCl pH 7.3, supplemented by 0.2 mM sodium nitrite. At the time indicated 2 ml aliquots of the reaction mixture were transferred to 1 ml of saturated solution of uranyl acetate, centrifuged, and in the supernatant nitrite concentration was determined colorimetrically 12.

Oxygen consumption was measured with the aid of the Clark-type electrode in a closed electromagnetically stirred vessel of 3.5 ml volume filled with the basic medium.

The difference spectra were followed in closed cuvettes containing 3.5 ml of the basic medium using spectrophotometer Cary 118 C. On oxygen exhaustion the proper terminal acceptor was added and after a 3 min. interval the difference spectrum was registrated. The content of the reference cuvette was kept oxidized by adding solid potassium ferricyanide; the maximum reduction of cytochromes was reached by adding solid $Na_2S_2O_4$.

RESULTS

Fig. 1 presents the time courses of nitrite concentrations in incubating different amounts of cells of *Paracoccus denitrificans* in the aerated medium. From the initial rates of decrease in nitrite concentration and from the employed amounts of dry weight specific nitrite reductase activities can be calculated. With the higher density of the suspension these values increased slightly evidently due to decreasing aeration; in the experiment

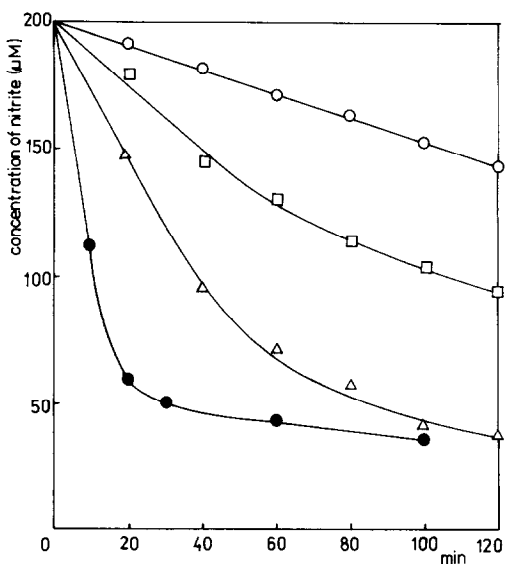

Figure 1

Nitrite reduction by the cells of *Paracoccus denitrificans* under aerobic conditions and CCCP effect. Conditions of measuring see Material and Methods. The employed dry weight of cells were: 4.3 mg (○,●), 8.5 mg (□), and/or 17.0 mg (△). In one case (●) also CCCP was present in 3 μM concentration.

in Fig. 1 they varied between 2.2 and 3 nmol NO_2^- min^{-1} mg dry weight $cells^{-1}$. The presence of the uncoupler CCCP at the concentration of 3 μM resulted in a sharp increase in the specific nitrite reductase activity to the value of 40.5 nmol NO_2^- min^{-1}mg dry weight $cells^{-1}$, i.e. to a value 18 times higher than that of the control sample. From Fig. 1 it can also be seen that irrespective of the presence of the uncoupler the rate of nitrite reduction was strongly slowed down after the concentration of NO_2^- dropped below the value of about 40 μM.

Oxygen consumption by the cells under similar conditions as those in the experiment in Fig. 1 can be seen from oxygraphic records in Fig. 2. The specific oxidase activity of cells expressed as the initial rate of oxygen consumption (record a) equalled 38.3 nmol O_2 min^{-1}mg dry weight $cells^{-1}$. Immediately on addition, CCCP stimulated respiration twice, followed by its gradual deceleration (record b). Nitrite at a similar concentration as in the experiment in Fig. 1 strongly inhibited the respiration, but only in the presence of CCCP (records c,d), this inhibition being of temporary limited character.

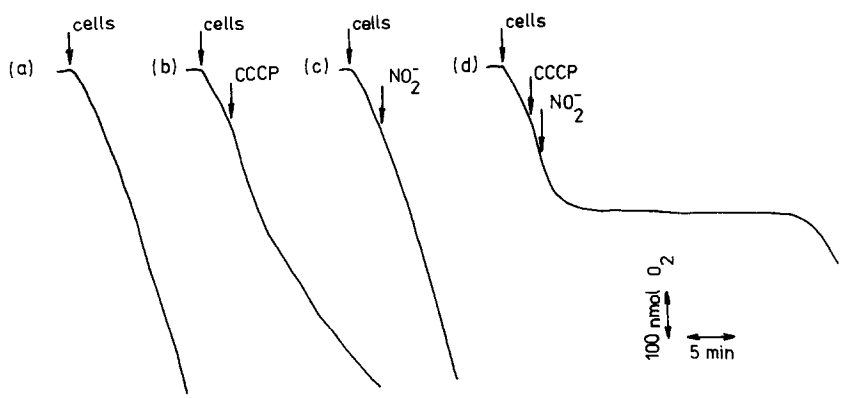

Figure 2

CCCP and nitrite effect on the rate of oxygen reduction by the cells of *Paracoccus denitrificans*. Conditions of measuring see Material and Methods. Indicated additions: cells (0.9 mg dry weight) CCCP (10 nmol, resulting concentration 2.9 μM), NO_2^- (1 μmol, resulting concentration 0.29 mM).

From comparing the results in Figs. 1 and 2 it clearly follows that in *P. denitrificans* the uncoupler causes the switching of the electron flow from oxygen to nitrite. This switch is almost quantitative, since the original electron flow to oxygen, 38.3 x 4 = 153.2 nmol $e^{-}min^{-1}$mg dry weight $cells^{-1}$ corresponds roughly to the resulting flow to nitrite, 40.5 x 3 = 121.5 nmol $e^{-}min^{-1}$mg dry weight $cells^{-1}$. The difference of about 20 % can be due to the changes in redox equivalent influx to the respiratory chain during the experiment (cf. the shape of records a and b in Fig. 2).

For the elucidation of the mechanism of the described effect of the uncoupler difference spectra of cytochromes in intact cells were registered (see Fig. 3). Owing to a lower sensitivity of the spectrophotometric measurement it was necessary to use a higher concentration of dry weight of cells and to dose oxygen in the form of hydrogen peroxide. In these

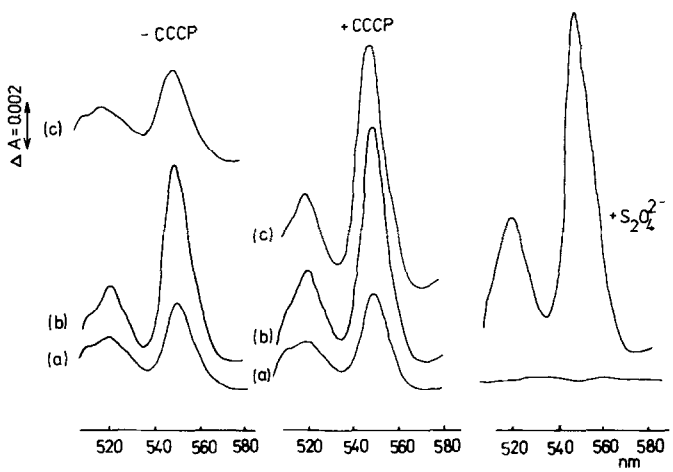

Figure 3

CCCP effect on the redox state of cytochromes in utilizing terminal acceptors O_2 and NO_2^- by the cells of *Paracoccus denitrificans*. Conditions of measuring see Material and Methods. 4.3 mg dry weight cells were used. CCCP concentration was 2.9 µM, additions of terminal acceptors were: (a) 20 µmol H_2O_2, (b) 5 µmol NO_2^-, and (c) 20 µmol H_2O_2 + 5 µmol NO_2^-. In a separate experiment it was verified that at the time of spectrum registration (after 3 minutes) the terminal acceptors were not consumed.

experiments it appeared that while in the absence of the uncoupling agent the difference spectrum of cells utilizing the mixture of NO_2^- + O_2 (c) corresponded to the spectrum of cells utilizing O_2 (a), the presence of uncoupler, in the case of the mixture of NO_2^- + O_2 (c), resulted in the increase in the degree of cytochrome reduction to the value corresponding to the utilization of NO_2^- (b). This finding witnesses the fact that in the presence of the uncoupler and a sufficient nitrite concentration oxygen cannot function as a terminal acceptor.

DISCUSSION

It is possible to consider two main mechanisms by means of which the drop in the transmembrane potential due to the uncoupler can change the distribution of the electron flow between the terminal acceptors O_2 and NO_2^- in the cells of *Paracoccus denitrificans* : (1) Since a higher membrane potential was found with O_2 utilization than with NO_2^- utilization [13,14], the reduction of O_2 might inhibit the reduction of NO_2^- via the membrane potential in a similar way as the photosynthetic transfer of electrons inhibits respiration in *Rhodopseudomonas capsulata* [15]. This inhibition might be removed by means of an uncoupler. (2) The uncoupler can affect the distribution of the negatively charged ion NO_2^- between the cytoplasm and the medium, thus changing its local concentration in the vicinity of the active centres of respiratory enzymes on the inner aspect of the cytoplasmic membrane. Since in *P. denitrificans* nitrite reductase is located in the periplasmic space [5,16], whereas the active centres of terminal oxidases (cytochromes *o* and aa_3) are situated on the inner aspect of cytoplasmic membrane (cf. [3]), this could be the true reason for the inhibition of terminal oxidases by nitrite. Its dependence on the membrane potential may result in the loss

of oxygen ability to compete effectively with nitrite for the influx of redox equivalents in the respiratory chain.

The result of experiments presented in Fig. 3 confirms the latter mechanism, since it follows from it, that in the presence of the uncoupler nitrite inhibits the electron flow to oxygen by interferring in the terminal part of the respiratory chain. This resulted in establishing the same degree of cytochromes reduction as found in the absence of oxygen. A further support for mechanism (2) is the finding [11], that in permeabilized cells the concentration of nitrite causing a 50 % inhibition of the oxidase activity (I_{50}) is three orders lower than in intact cells. Since I_{50} of nitrite in permeabilized cells or in those in the presence of the uncoupler is comparable with the Michaelis constant of nitrite reductase for nitrite (I_{50} = 15 μM [11], K_m = 46 μM [17] and/or 6 μM [18]), the effect of the inhibition of terminal oxidases appears at all utilizable concentrations of nitrite (cf. Fig. 1).

The described effect is a valuable complement of earlier information on the distribution of the electron flow in the branched respiratory chain of *P. denitrificans* [4-11], since it shows that the decrease in the membrane potential can cancel the physiological consequences of the respiratory chain topology of this bacterium, consisting in the preferential utilization of oxygen as the terminal acceptor.

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