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Separate binding sites for antimycin and mucidin in the respiratory chain of the bacterium *Paracoccus denitrificans* and their occurrence in other denitrifying bacteria

Igor KUČERA, Raimund HEDBÁVNÝ and Vladimír DADÁK
Department of Biochemistry, Faculty of Science, J. E. Purkyně University, Kotlářská 2, 611 37 Brno, Czechoslovakia

By means of the method of fluorimetric titration it has been shown that mucidin does not affect the attachment of antimycin to membranes from anaerobically grown *Paracoccus denitrificans*. The fluorimetric titration with antimycin can be used in the determination of the amount of the cytochrome bc_1 complex in the membrane. In cells inhibited with antimycin, the oxidation of cytochromes *c* was accompanied by the reduction of cytochrome *b*; in the presence of mucidin this effect did not take place. The results, which indicated a difference in binding sites, were interpreted in terms of the Q-cycle [Mitchell (1976) J. Theor. Biol. **62**, 327–367; Trumpower (1981) Biochim. Biophys. Acta **639**, 129–155]. Comparable sensitivity towards antimycin and mucidin was shown by other typical denitrifying bacteria: *Pseudomonas stutzeri* and *Alcaligenes xylosoidans*, subspecies *denitrificans*.

INTRODUCTION

In a previous paper (Kučera *at al.*, 1984*a*) we showed that the antibiotic mucidin was an effective inhibitor of the respiratory chain of the denitrifying bacterium *Paracoccus denitrificans*, acting in the region of cytochromes *b* and c_1. Antimycin as well as mucidin blocked the electron flow to the terminal acceptors NO_2^- and N_2O, and partly to O_2, whereas the reduction of nitrate was not affected. The observed different degrees of reduction of cytochromes *b* and *c* in the presence of antimycin and/or mucidin have resulted in the presentation of a scheme suggesting the location of binding sites for the two inhibitors (cf. Fig. 5 in Kučera *et al.*, 1984*a*).

The present paper deals with a detailed study of the interaction of the inhibitors with the respiratory chain of *P. denitrificans*. The experimentally proved difference between the two binding sites will be discussed in relation to the mechanism of the electron transport in the cytochrome bc_1 segment of the respiratory chain. It is also shown here that the sensitivity towards cytochrome bc_1 inhibitors of the mitochrondrial respiration is not limited to *P. denitrificans* [the assumed evolutionary ancestor of mitochondria (John & Whatley, 1975)], but that it is also exhibited by other characteristic denitrifying bacteria.

MATERIALS AND METHODS

Micro-organisms

Paracoccus denitrificans [CCM (Czechoslovak Collection of Microorganisms) 982], *Pseudomonas stutzeri* (CCM 2660) and *Alcaligenes xylosoxidans* subsp. *denitrificans* formerly deposited as *A. denitrificans* (CCM 2945), obtained from CCM were cultured under anaerobic conditions statically in 1 litre of medium containing 50 mM-succinate as a source of carbon and 10 mM-nitrate as the terminal acceptor of electrons (Kučera *et al.*, 1983). In the case of *A. xylosoxidans* an extra amount (5 g/litre) of beef extract (Difco, Detroit, MI, U.S.A.) was present as well as peptone (3 g/litre) (Imuna, Šarišské Michal'any, Czechoslovakia). The cells were harvested by centifugation (30 min, 6000 *g*), washed with 50 mM-sodium phosphate buffer, pH 7.3, and the thick suspension cooled to 0 °C and used the same day. The membranes were prepared by means of lysozyme and osmotic lysis of *P. denitrificans* cells as described by Burnell *et al.* (1975). *Escherichia coli* K12 (Ymel) (Bachmann, 1972) was obtained from Dr. Koukalová, Biophysical Institute ČSAV, Brno, Czechoslovakia. It was cultivated aerobically for 8 h at 37 °C with M9CA medium (Maniatis *et al.*, 1982) supplemented with 10 μM-ferric citrate. The cells were disrupted by sonicating for 8 min in 0.1 M-sodium phosphate, pH 7.3, using an MSE 500 W ultrasonic disintegrator, the unbroken cells being removed by centrifugation (10000 *g* for 10 min). The membrane fraction was collected by centrifugation at 100000 *g* for 60 min, washed with sodium phosphate, and suspended in the same medium. For protein determination the modified biuret method was used (Szarkowska & Klingenberg, 1963).

Optical measurements

Dual-wavelength measurements were performed on a Shimadzu UV-3000 spectrophotometer; the difference spectra were measured with a Cary 118C instrument. Fluorimetric titration with antimycin was carried out in an Aminco–Bowman spectrofluorimeter. The wavelengths of excitation and emission were 350 nm and 430 nm respectively, a 1 mm slit being used. At the concentration range applied, mucidin did not affect the excitation or the emission spectrum of antimycin.

Measurement of activities

O_2 consumption was measured by means of the Clark oxygen electrode in a closed electromagnetically stirred vessel of 2.5 ml volume at 30 °C. Nitrate reductase activity of cells was determined in closed chambers filled with N_2 at 30 °C containing 2 ml of the medium. The

reaction was started by adding 1 mM-$NaNO_2$. After 20 min incubation the reaction was stopped with 0.5 ml of a saturated solution of zinc acetate, the mixture centrifuged (5 min, 5500 *g*), and the nitrite remaining in the supernatant was determined (Snell & Snell, 1949). The anaerobic conversion of nitrate to nitrite was monitored in a similar way, the initial $NaNO_3$ concentration being 10 mM.

Chemicals

Antimycin and lysozyme were from Sigma (Taufkirchen, Germany); mucidin (strobilurin A) produced by the basidiomycete *Oudemansiella mucida* was obtained as a gift from Dr. Vladimir Musílek, Mikrobiologický ústav ČSAV, Vídenská 1083, 14220 Praha 4-Krč., Czechoslovakia. The concentrations of inhibitors in the ethanolic stock solutions were determined spectrophotometrically (Becker *et al.*, 1981). The other substances were of Czechoslovak origin, all of analytical purity.

RESULTS AND DISCUSSION

As described previously for submitochondrial particles (Berden & Slater, 1972), on binding of antimycin to membranes of *P. denitrificans* there is quenching of its fluorescence. During the titration of membranes with added antiycin, therefore, the fluorescence starts increasing only after the occupation of binding sites for the inhibitor. An example of an experimentally obtained fluorescence titration curve is shown in Fig. 1. The fact that the horizontal part of the curve reflects the attachment of the inhibitor to the bc_1 segment of the respiratory chain is apparent from the parallel course of the inhibition of the activity of NADH oxidase. The observed residual NADH oxidase activity corresponds to the activity of the alternative terminal oxidase, which abstracts reduction equivalents before the bc_1 segment, probably in the region of ubiquinone (Kučera *et al.*, 1984*a*,*b*; Parsonage *et al.*, 1986). The position of the bend in titration curves is given by the titre of antimycin (mean value 0.5 nmol/mg of protein). If the M_r of the bc_1 complex is 121000 (Yang & Trumpower, 1986), it would imply (in the case of a firm bond and a stoichiometry of 1:1) that, in the membrane preparation used, the bc_1 complex takes up 6% of the proteins present.

Additional observations support the view that the first part of the curves given in Fig. 1 reflect the binding of antimycin to the bc_1 complex. These are as follows.

(i) It was shown, by using a molar difference absorbance coefficient ($\epsilon_{560-575}$) of 21.8 $mM^{-1} \cdot cm^{-1}$ for the dithionite-reduced minus ascorbate-reduced complex (Berry & Trumpower, 1985), that the membrane fraction used in the experiments contained 0.9 nmol of cytochrome *b* per mg of protein. The estimated amount (0.5 nmol of antimycin bound to 1 mg) is approximately one half, in accordance with the known stoichiometry of antimycin binding to the mitochondrial bc_1 complex (Berden & Slater, 1972).

(ii) A fluorimetric titration of the type presented in Fig. 1 was carried out with an complex of ubiquinol oxidase purified from membranes as described by Berry & Trumpower (1985); in this case also the stoichiometry of antimycin binding with respect to the content of cytochrome *b* was maintained (results not shown).

(iii) The NADH oxidase activity of membrane fraction derived from *E. coli* was not affected by antimycin, even

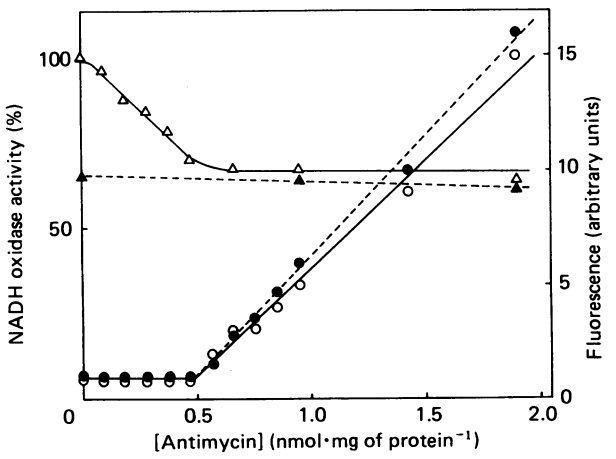

Fig. 1. Titration of *P. denitrificans* membranes with antimycin

The medium (2 ml of 50 mM-Tris/HCl, pH 7.3) for fluorimetric titration contained 2 mg of membrane proteins (○, ●) and, for the titration of NADH oxidase, 0.2 mg of protein (△, ▲). In some cases mucidin was also present (25 nmol/mg of protein) (●, ▲). An NADH oxidase activity of 100% corresponded to a value of 1.9 μmol of $O_2 \cdot min^{-1} \cdot mg$ of $protein^{-1}$.

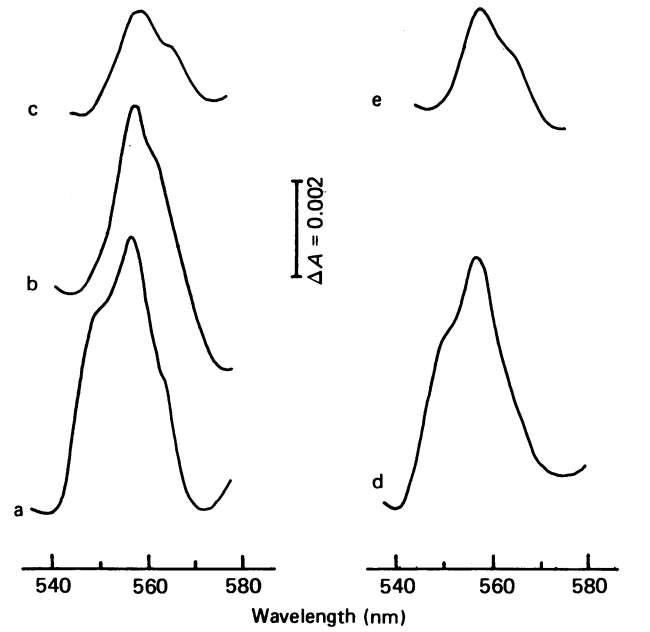

Fig. 2. Redox behaviour of cytochromes of cells of *P. denitrificans*

The cuvette contained 3 ml of 0.1 M-sodium phosphate buffer, pH 7.3, with 8 mM-sodium succinate and 4.4 mg dry wt. of cells. The content of the reference cuvette was oxidized with solid ferricyanide. On exhausting O_2 in the sample cuvette and the addition of 10 μmol of $NaNO_2$ and 9.1 nmol of antimycin, a difference spectrum was measured at the rate of 0.5 $nm \cdot s^{-1}$ (a). The recording of the spectrum was repeated on adding 10 μmol of $K_3Fe(CN)_6$ (b) and 19.4 nmol of mucidin (c). In another experiment the curvette contained 10 μmol of $NaNO_2$ and 19.4 nmol of mucidin (d), after which 10 μmol of $K_3Fe(CN)_6$ was added (e).

at the titre of 25 nmol/mg of protein. The insensitivity of respiration of *E. coli* to antimycin corresponded to the course of fluorimetric titration of the membranes with antimycin, where the initial quenching, as was found with membranes of *P. denitrificans*, was not observed.

Mucidin, unlike antimycin, does not fluoresce, and the course of membrane titrations could be monitored only from the decrease in NADH oxidase activity (not shown). In this way the titre which evokes the maximum inhibitory effects was determined to be about 2 nmol/mg of protein. Under conditions where the medium contained ten times as much mucidin as the above-cited titre, fluorescence titration with antimycin had a course coincident with that in the absence of mucidin (Fig. 1). Hence it follows that the attached mucidin does not affect the attachment of antimycin. Owing to the small difference in affinity of membranes for antimycin and mucidin (see above), it is probable that the two inhibitors do not compete with each other and attach to separate sites in the bc_1 segment of the respiratory chain.

John & Papa (1978) showed that the pulse of O_2 introduced to the membranes from anaerobically grown *P. denitrificans* in the presence of NADH and antimycin brings about the reduction of cytochrome b_{566}. We observed a similar effect ('extra-reduction') when examining the time course of the absorbance differences at 566 nm and 575 nm on addition of electron acceptors such as nitrite or ferricyanide to an anaerobic suspension of cells containing antimycin. The 'extra-reduction', however, did not occur in the presence of either mucidin or both mucidin and antimycin in the suspension (results not shown). Similarly to nitrite, ferricyanide takes electrons from *c*-type cytochromes (Kučera *et al.*, 1984*a*, *b*), but the extent of reduction of cytochromes *c* is lower in the presence of ferricyanide. Measuring the difference of absorbances at 550 and 540 nm in anaerobically grown cells of *P. denitrificans* at pH 7.3 (0.1 M-sodium phosphate) with 8 mM-succinate and 3.3 mM-nitrite, 36% of cytochromes *c* was found in the reduced state. The extent of reduction decreased to 19% on inhibiting the cells with antimycin and further to 7% on addition of mucidin (100% means the anaerobic state in a mixture lacking terminal acceptor and 0% the oxidation state caused by ferricyanide).

The combined action of nitrite and ferricyanide on the redox state of cytochromes in *P. denitrificans* is shown in Fig. 2. Here nitrite (spectrum a) was added first to cells inhibited by antimycin, followed by the addition of ferricyanide (spectrum b). The oxidation of cytochrome *c* (decrease in absorption maximum at 550 nm) was accompanied by a marked growth of the degree of reduction of cytochrome b_{566}; the change in the redox state of cytochrome b_{560} was less evident. In an analogous experiment with mucidin (d) there was no reduction of cytochromes *b* due to the action of ferricyanide (e). Moreover, the addition of mucidin resulted in the reoxidation of cytochromes *b* reduced earlier in the presence of antimycin (cf. b and c). The arrangement of the experiment shown in Fig. 2 makes the redox alterations of cytochromes *b* more striking by working at a high oxidation state of cytochromes *c* synthesized in high amounts in anaerobically grown cells.

The results can be interpreted on the basis of a mechanism of electron transfer analogous to the Q-cycle in mitochondria (Mitchell, 1976; Trumpower, 1981). It can be assumed that the flow of reduction equivalents through the bc_1 segment is non-linear and that cytochromes *b* interact with ubiquinone at two centres, one of them being blocked by mucidin and the other by antimycin. During the oxidation of cytochromes *c* by an electron acceptor, a reducing agent for cytochrome *b* is generated in the centre sensitive to mucidin. The agent probably consists of ubiquinone ($Q^{-\cdot}$) as a product of the one-electron oxidation of ubiquinol (QH_2). The con-

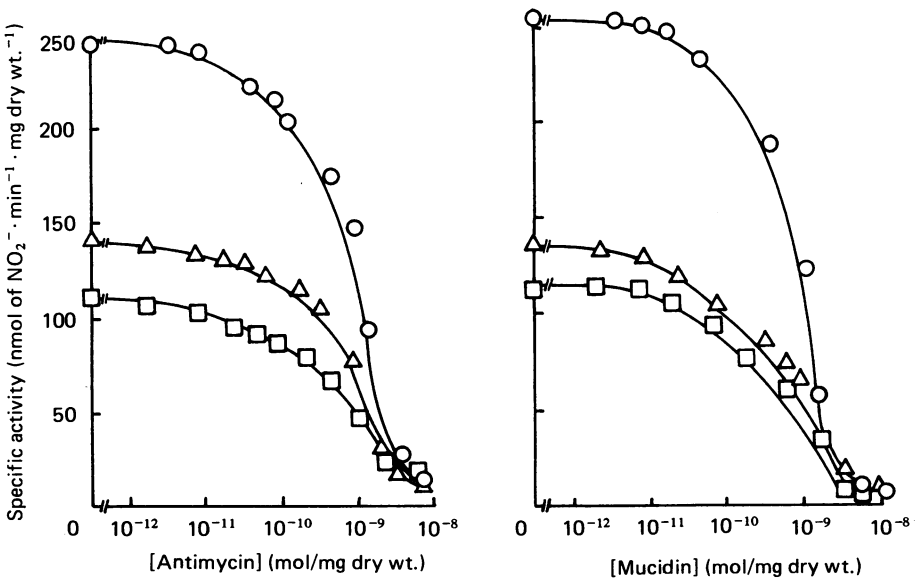

Fig. 3. Inhibition of nitrite reductase activity of denitrifying bacteria with antimycin and/or mucidin

A 2 ml portion of 0.1 M-sodium phosphate buffer, pH 7.3, contained the following amounts of cells (mg dry wt.): *Paracoccus denitrificans* (△) 1.5; *Pseudomonas stutzeri* (○) 1.4; *Alcaligenes xylosoxidans* (□) 1.2; 20 mM-succinate and 1 mM-$NaNO_2$ were also present. For a detailed description of the determination of inhibition, see the Materials and methods section.

comitant presence of antimycin prevents the redox equilibration of reduced cytochromes *b* with the ubiquinone pool. On adding mucidin the intermediate reducing agent does not arise, and cytochromes *b* are therefore substantially less reduced, independently of the presence of the terminal acceptor for electrons (cf. Fig. 2). The abolition of the 'extra-reduction' brought about on mucidin addition is a special feature indicating redox equilibration of cytochrome *b* with other redox components under these conditions. A clear elucidation of the effect remains to be achieved, but it might be connected with somewhat lower specificity of ferricyanide as an artificial electron acceptor (Kučera *et al.*, 1984*a*).

The information that the sensitivity towards bc_1 inhibitors is inherent even in the prokaryotic bc_1 segment, which has an essentially simple structure in comparison with the mitochondrial complex (Yang & Trumpower, 1986), has been utilized for testing its presence in other typical representatives of the denitrifying genera *Pseudomonas* and *Alcaligenes*. Fig. 3 demonstrates that both antimycin and mucidin effectively inhibit the nitrite reductase activity of cells of *P. stutzeri* and *A. xylosoxidans* in comparable titres. The concentrations of inhibitors (1.0 nmol/mg dry wt.) did not prevent the anaerobic reduction of nitrate. In their presence the stoichiometric accumulation of nitrite was observed at the rate of 110 nmol/mg of dry wt. in *P. stutzeri* and of 102 nmol per mg of dry wt. in *A. xylosoxidans*. Without the addition of inhibitor, nitrite did not accumulate at pH 7.3. These properties of the tested bacteria are similar to that revealed in anaerobically grown cells of *P. denitrificans* (Kučera *et al.*, 1984*a*). Another similarity can be seen in an incomplete inhibition of the oxidase activity of cells at the saturating concentration of inhibitors. In cells of *P. stutzeri* there was at most 30% inhibition of the oxidase activity; in *A. xylosoxidans*, 10% inhibition was found (medium: 0.1 M-sodium phosphate plus 20 mM-succinate). The results of inhibition studies confirm the fact that in these strains also the bc_1 segment is a part of only one terminal pathway of the respiratory chain, as is the case in the bacterium *P. denitrificans* (Kučera *et al.*, 1984*a*).

The main contribution of the present paper can be seen in supporting the hypothesis about the functioning of the Q-cycle in the respiratory chain of denitrifying bacteria. Further research in this area, e.g. the employment of new purification methods for bacterial bc_1 complexes (Ljungdahl *et al.*, 1987), raises interesting possibilities for studying the comparative and evolutionary biochemistry of anaerobic respiration.

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