Generation of protonic potential by the *bd*-type quinol oxidase of *Azotobacter vinelandii*

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Received 31 July 1997

Abstract Inside-out subcellular vesicles of *Azotobacter vinelandii* are found to produce ΔpH and ΔΨ (interior acidic and positive) when oxidising malate or menadiol. These effects are inherent in both Cyd^+Cyo^- (lacking the *o*-type oxidase) and Cyd^-Cyo^+ (lacking the *bd*-type oxidase) strains. They appear to be myxothiazol-sensitive in the Cyd^-Cyo^+ strain but not in the Cyd^+Cyo^- strain. The H^+/e^- ratio for the terminal part of respiratory chain of a *bd*-type oxidase overproducing strain is established as being close to 1. It is also shown that NADH oxidation by the vesicles from the Cyd^-Cyo^+ strain is sensitive to low concentrations of myxothiazol and antimycin A whereas that of the Cyd^+Cyo^- strain is resistant to these Q-cycle inhibitors. It is concluded that (i) the *bd*-type oxidase of *A. vinelandii* is competent in generating a protonic potential but its efficiency is lower than that of the *o*-type oxidase and (ii) Q-cycle does operate in the *o*-type cytochrome oxidase terminated branch of the *A. vinelandii* respiratory chain and does not in the *bd*-type quinol oxidase terminated branch. These relationships are discussed in the context of the respiratory protection function of the *bd*-type oxidase in *A. vinelandii*.

Key words: Respiratory protection; *bd* oxidase; Cytochrome *d*; *o* Oxidase; Protonic potential; Energy coupling; Q-cycle; *Azotobacter vinelandii*

1. Introduction

In this group, it was recently suggested [1–3] that one of the basic functions of cellular respiration consists in antioxygen defence of the cell by means of lowering concentrations of O_2 and its one-electron reductants. To avoid limitation by energy consumers, respiratory systems responsible for the defence function were assumed to be non-coupled (or coupled but only partially) to energy conservation. As a precedent for such an antioxygen defence mechanism, phenomenon of the respiratory protection of *Azotobacter* nitrogenase complex has been considered.

It is known that the nitrogenase mechanism of N_2 reduction is strongly inhibited by O_2. This is why many prokaryotes containing this enzyme system cannot reduce N_2 under aerobic conditions. *Azotobacter* is an exception to this rule. It can assimilate N_2 within a wide range of the ambient O_2 concentrations [4,5]. To explain this feature of *Azotobacter*, Dalton and Postgate [6,7] introduced the concept of respiratory protection. They hypothesised that very high activity of the Azotobacter respiratory enzymes allows intracellular $[O_2]$ to be maintained at a safely low level.

A. vinelandii possesses at least two terminal oxidases belonging to *o*- and *bd*-types [8]. Moreover, genes encoding for an oxidase of cbb_3-type have been recently described in the *A. vinelandii* genome [9]. It has been shown that the *bd*-type oxidase dominates in cells growing diazotrophically or under high oxygen tension [10,11]. It has also been shown that deletion in the *bd*-type oxidase genes abolishes N_2 fixation whereas that in the *o*-type oxidase genes is without such an effect [12,13]. Thus it is the *bd*-type oxidase that is responsible for the respiratory protection of the nitrogenase complex.

The primary structures as well as the sets of redox groups and subunit compositions of the *A. vinelandii bd*-type oxidase are similar to those of the *Escherichia coli bd*-type oxidase. Nevertheless, these oxidases clearly differ in induction conditions. The *A. vinelandii bd*-type oxidase is induced by an $[O_2]$ increase whereas the *E. coli* enzyme is induced under conditions when protonic potential decreases [14], in particular by lowering the O_2 concentration [15]. This is why Poole has suggested that the *bd*-type oxidases of *A. vinelandii* and *E. coli* perform different functions [8].

On the basis of some indirect evidence, Acrell and Jones assumed that the *A. vinelandii bd*-type oxidase is not coupled to energy conservation [16]. In this paper, we have tried to directly verify the above assumption. It was found that the *A. vinelandii bd*-type oxidase is, in fact, coupled to the generation of protonic potential but the coupling efficiency is two-fold lower than that of the *o*- or aa_3-type oxidases. An indication was obtained that the Q-cycle operates in the respiratory chain branch terminated by the *o*-type cytochrome oxidase but not the *bd*-type quinoloxidase.

2. Materials and methods

The following *A. vinelandii* strains were a generous gift of Professor R.K. Poole: (I) Cyd^+Cyo^- strain DL10 (UW136 *cyo*::Tn*903* Rif^R Km^R) [13], (ii) Cyd^-Cyo^+ strain MK5 (UW136 *cydB*::Tn*5* Rif^R Km^R) [12], and (iii) the *bd*-type oxidase overproducing strain MK8 (UW136 *cydR*::Tn*5* Rif^R Km^R) [12].

The cells were grown in a modified Burk's medium BS or BSN [17] in a shaker (200 rpm) at 37°C.

To obtain subcellular vesicles, the cells were harvested by centrifugation (10 000 × *g*, 10 min) and washed twice with 50 mM NaCl, 10 m KH_2PO_4 and 1 mM $MgSO_4$, pH 7.5 (medium 1). The sediment was suspended in 20 mM HEPES, 5 mM $MgSO_4$, 25 mM K_2SO_4, 0.5 mM DTT and 0.5 mM EDTA, pH 7.5 (medium 2). The mixture was passed through a French press at 16 000 psi. Unbroken cells and cell debris were removed by centrifugation at 22 500 × *g* (10 min). Subcellular membrane vesicles were sedimented at 50 000 × *g* (90 min). The final pellet was suspended in medium 2 to obtain protein concentration of about 35–40 mg ml^{-1} and stored in liquid nitrogen.

To measure the H^+/e^- ratio, the cells were grown on plates (agar

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Abbreviations: ΔΨ, transmembrane difference in electric potentials; CCCP, *m*-chlorocarbonylcyanide phenylhydrazone; ΔpH, transmembrane difference in pH; DTT, dithiothreitol

PII S0014-5793(97)01047-8

medium BS or BSN) for 16–18 h. Then the cells were washed off the plate using liquid medium BS or BSN and were grown in these media for 4–6 h at 37°C with shaking (200 rpm). The grown cells were sedimented at 27000×*g* (5 min) and washed twice with 60 mM KCl, 2 mM $MgSO_4$ (medium 3). The pellet was suspended in the same medium (final concentration, 8–10 mg protein ml^{-1}).

The measurements were carried out in an anaerobic 1.3 ml chamber by means of a pH electrode, using medium 3 at cell concentration about 0.45 mg protein ml^{-1}. The pH value of the mixture was adjusted to 6.8 by adding KOH. Then 20 mM KSCN was added. The mixture became anaerobic in 10–15 min due to the O_2 consumption by the cells. After this, 0.2 mM capsaicin (8-methyl-*N*-vanillyl-6-nonenamide), 50 mm vitamin K_3 and 2 mM DTT were added. An O_2 pulse was made by adding 2.58 nmol O_2 (10 ml of air saturated at 25°C water solution). The pH electrode was calibrated with 10 nmol argon-saturated HCl. To estimate the H^+/e^- ratio, the linear extrapolation method was employed according to [18].

Generation of ΔpH by subcellular vesicles was detected using acridine orange. Fluorescence of this probe was measured with a MPF-4 fluorimeter at 530 nm (excitation, 492 nm). The mixture contained 20 mM HEPES, 60 mM KCl, 2 mM $MgSO_4$, 4 mM acridine orange and vesicles (20–30 mg protein ml^{-1}), pH 7.5. A 2 ml sample was preincubated for 5 min. ΔpH generation was initiated by adding the respiratory substrate.

Generation of ΔΨ by subcellular vesicles was monitored by Oxanol VI. The sample contained 20 mM HEPES, 60 mM KCl, 2 mM $MgSO_4$, 1.25 mM Oxanol VI and vesicles (25–50 mg protein ml^{-1}), pH 7.5. The mixture was preincubated for 5 min prior to addition of the respiratory substrate. The ΔΨ level was estimated spectrophotometrically (dual-wave measurements of the optical density difference at 625–587 nm) using an Aminco DW2000 spectrophotometer.

NADH oxidase activity of the vesicles was measured with a Hitachi 557 spectrophotometer (340–400 nm). The sample contained 20 mM HEPES, 60 mM KCl, 2 mM $MgSO_4$, 0.9 mM gramicidin D and vesicles (1–5 mg protein ml^{-1}), pH 7.5. The reaction was initiated by adding 0.12 mM NADH. The NADH molar extinction coefficient was assumed to be 6.22×10^3 M^{-1} cm^{-1}.

Protein concentration was measured by the Lowry method using bovine serum albumin (Serva, type V) as a standard.

3. Results and discussion

In the first series of experiments, generation of ΔpH and ΔΨ by *A. vinelandii* subcellular vesicles was measured. To this end, three strains were used, namely DL10 (Cyd^+Cyo^-) [13], MK8 ($CydR^-$, deletion in a regulatory gene required for *bd*-type oxidase repression) [12] and MK5 (Cyd^-Cyo^+) [12]. In Fig. 1A,B acridine orange responses reporting the ΔpH formation are shown. It is seen (Fig. 1A) that malate or menadiol oxidations by *A. vinelandii* DL10 (Cyd^+Cyo^-) strain resulted in strong quenching of the acridine orange fluorescence, which suggests ΔpH formation on the vesicles membrane (interior acidic). This process was inhibited by the Na^+/H^+ exchanger monensin as well as protonophorous uncoupler CCCP (Fig. 1A, traces a and b) and stimulated by valinomycin (not shown in the figure). The K^+/H^+ exchanger nigericin and channel-former alamathicin (not shown) were also inhibitory whereas myxothiazol was without effect (Fig. 1A, trace c). Similar results were obtained with the *bd*-type oxidase overproducing strain MK8 (data not shown). On the other hand, the MK5 (Cyd^-Cyo^+) strain was shown to form ΔpH in a myxothiazol-sensitive fashion (Fig. 1B).

Then ΔΨ generation by the *A. vinelandii* vesicles was studied with Oxanol VI. One can see (Fig. 1C) that malate oxidation by the DL10 strain (Cyd^+Cyo^-) resulted in an Oxanol VI response indicating formation of ΔΨ by the vesicles (interior positive). The response was stimulated by monensin (converting ΔpH to ΔΨ) and completely inhibited by CCCP or valinomycin.

The above data indicate that the *A. vinelandii bd*-type terminal oxidase is competent in generating both constituents of the protonic potential, i.e. ΔpH and ΔΨ.

In the next series of experiments, the H^+/e^- ratio of the *bd*-type oxidase was measured. To this end, we used the MK8 (Δ*cydR*) strain where *bd*-type oxidase was overproduced whereas the contribution of other terminal oxidases was negligible [12]. It was found that addition of a small amount of O_2 to the anaerobic suspension of MK8 cells resulted in a transient acidification of the medium, the H^+/e^- ratio being 0.93 ± 0.05 (five measurements were done). The result of a typical O_2 pulse is shown in Fig. 2. The H^+/e^- ratio for the *bd*-type oxidase was the same for cells grown in the presence of NH_3 (in BSN medium) as well as those grown diazotroph-

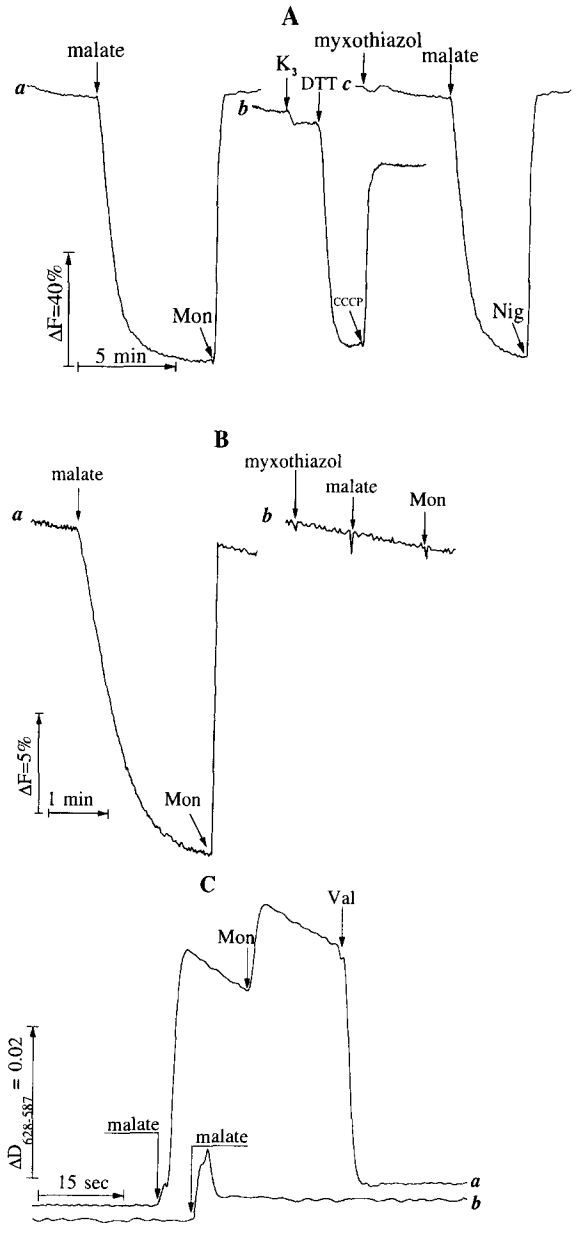

Fig. 1. Generation of protonic potential by subcellular vesicles from *A. vinelandii* DL10 (Cyd^+Cyo^-) (A,C) and MK5 (Cyd^-Cyo^+) (B). Additions: 5 mM malate, 50 mM vitamin K_3, 2 mM DTT, 2.5 mM monensin, 50 nM nigericin, 5 mM CCCP, 2.5 mM valinomicin and 0.5 mM myxothiazol. In C, trace b, the incubation medium was supplemented with 5 mM CCCP.

ically (in BS medium) (data not shown). Moreover, the measured stoichiometry for the *bd*-type oxidase was not changed in the presence of 50 mM cyanide (Fig. 2) whereas such concentration of this inhibitor completely arrested transient acidification of the medium after O_2 pulses on the cells of the strain MK5 lacking the *bd*-type oxidase (data not shown). Thus, one can conclude that the H^+/e^- stoichiometry of the *A. vinelandii* *bd*-type oxidase is equal to 1 just as for *bd*-type oxidases from *E. coli* [19] and *Bacillus subtilis* [20]. This is two times lower than for the oxidases belonging to the haem-copper superfamily [21].

A further indication of the lower efficiency of the *bd*-terminated branch of the *A. vinelandii* respiratory chain was obtained when sensitivity of respiration to Q-cycle inhibitors was tested. It was found that NADH oxidation by the subcellular vesicles from the *bd*-type oxidase deleted MK5 strain proved to be sensitive to both myxothiazol and antimycin A, $I_{0.5}$ being 7.4 ± 0.5 nM and 50 ± 1.5 nM, respectively (Fig. 3). On the other hand, the respiration of vesicles from the *bd*-type oxidase overproducing MK8 strain was resistant to low concentrations of myxothiazol and antimycin A (Fig. 3). These data indicate that the Q-cycle does operate in the *o*-type cytochrome oxidase terminated chain and does not in the *bd*-type quinol oxidase terminated chain.

In other experiments performed in this group, it was shown that in *A. vinelandii* high $[O_2]$ and low $[NH_3]$ (conditions favourable to induce the *bd*-type oxidase [10,11]) lower the level of the energy-coupled NADH dehydrogenase I and increase that of the non-coupled NADH dehydrogenase II [22].

Assuming that the $H^+\backslash e^-$ ratio for the coupled NADH dehydrogenase I, the Q-cycle, and the *o*-type oxidase are equal to 2 [23], 1 [24] and 2 [21], respectively, we may conclude that transport of one electron from NADH to oxygen along the *o*-type oxidase terminated chain is coupled to translocation of 5 H^+. Such high efficiency is inherent in cells growing at low $[O_2]$ and in the presence of NH_3 when oxygen danger is minimised or nitrogenase is not necessary for the cell to survive.

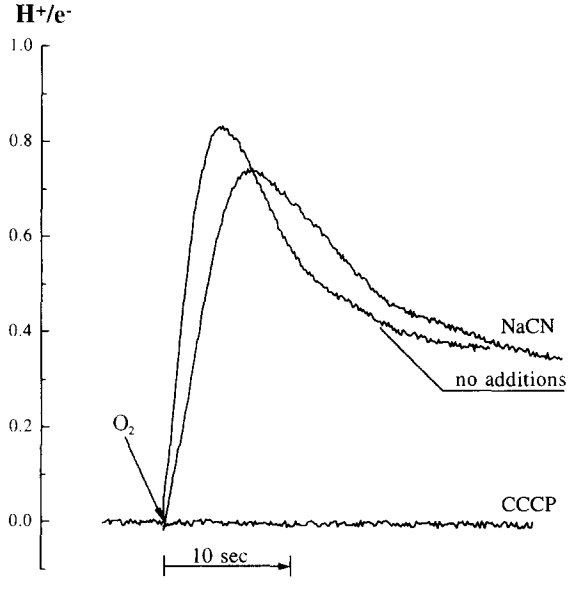

Fig. 2. The H^+/e^- stoichiometry of the *bd*-type oxidase in *A. vinelandii* MK8 (the *bd* oxidase superproducent) cells grown in the BSN medium. Additions: 50 mM NaCN and 5 mM CCCP.

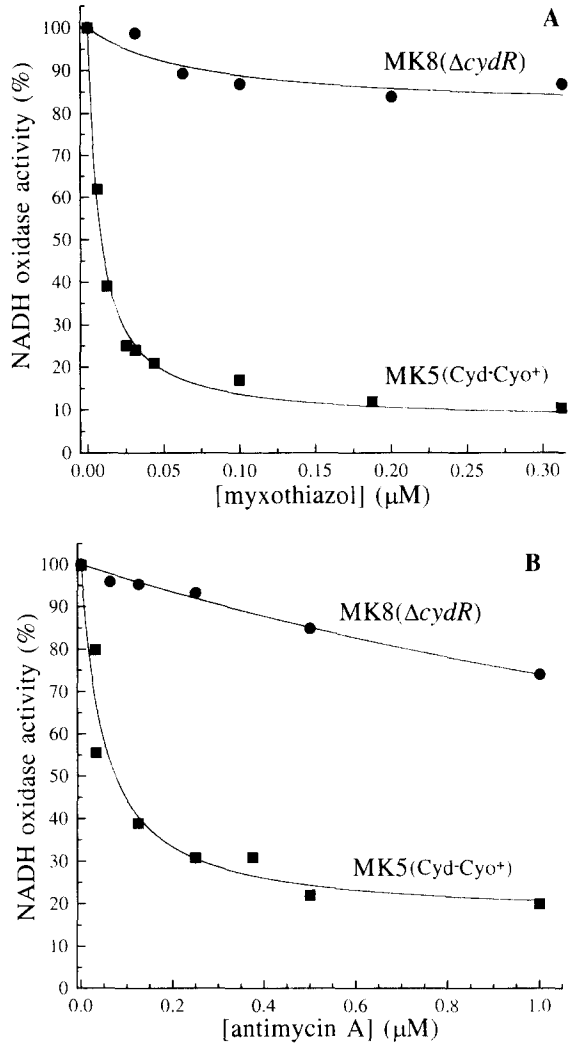

Fig. 3. Effects of myxothiazol and antimycin A on the NADH oxidase activity of subcellular vesicles from *A. vinelandii* MK8 (the *bd* oxidase superproducent) and MK5 (Cyd^-Cyo^+). Activity without the inhibitors was 2.16 mmol min^{-1} mg $protein^{-1}$ for vesicles from strain MK8 and 0.48 mmol min^{-1} mg $protein^{-1}$ for vesicles from strain MK5.

On the other hand, at high $[O_2]$ and in the absence of NH_3, electron transfer is switched to the simplified low-efficiency respiratory chain. In this chain, quinone reduced by non-coupled NADH dehydrogenase II is directly oxidised by the 'partially coupled' *bd*-type oxidase (H^+/e^- stoichiometry is equal to 1 instead of 2) with no Q-cycle involved. This means that the low-efficiency chain must consume 5 times more oxygen than the high-efficiency chain to produce the same amount of ATP. It is not surprising, therefore that respiratory protection is performed by the low-efficiency chain.

Interestingly, in plant mitochondria a completely non-coupled respiratory chain seems to be involved in antioxygen defence. It is composed of non-coupled NAD(P)H dehydrogenases and non-coupled cyanide-resistant quinoloxidase [25]. For *A. vinelandii*, possessing no photosynthetic apparatus, it would be too risky to employ completely non-coupled respiratory chain to lower intracellular $[O_2]$. To perform this function, it uses very active, partially coupled respiration of 5-fold lower efficiency than the completely coupled respiration.

Acknowledgements: The authors express their gratitude to Dr. R.A. Murtasina for help in the microbiological part of this work, Professor R.K. Poole for providing the *A. vinelandii* strains and INTAS and the Russian Foundation for Basic Research for financial support (Grant INTAS-RFBR 95-1259).

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