Terminal oxidases of *Azoarcus* sp. BH72, a strictly respiratory diazotroph

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Abstract The nitrogen-fixing, grass-associated bacterium *Azoarcus* sp. BH72 was characterized with respect to its terminal oxidases. Inhibitory respiratory analysis revealed the presence of at least one cytochrome *c* oxidase and one quinol oxidase. The cytochrome *c* oxidase was preferably used by the cells under aerobic, whereas the quinol oxidase seemed to be dominant under microaerobic, nitrogen-fixing conditions. Differential spectroscopy and heme analysis of the membrane preparations indicated that the cytochrome *c* oxidase is probably of the *cb* type.

Key words: Nitrogen fixation; Cytochrome oxidase; Quinol oxidase; Heme; *Azoarcus*

1. Introduction

Oxygen plays an important role in biological nitrogen fixation. Different diazotrophs possess a variety of adaptive mechanisms which allow energy generation with the most efficient electron acceptor (oxygen) while protecting oxygen-sensitive nitrogenase, a key enzyme for nitrogen fixation. Rhizobia take advantage of low oxygen concentrations inside legume nodules, where symbiotic nitrogen fixation takes place [3]. Free-living microaerophilic azospirilla have a strong aerotactic response which navigates them into niches where oxygen concentration is optimal for both energy generation and nitrogen fixation [4]. Free-living aerobes of the genus *Azotobacter* have a very high overall concentration of electron transport complexes per cell, resulting in high respiration rates, which maintain low intracellular concentrations of oxygen [5]. Adaptation to changes in oxygen concentration in the environment at the level of electron transport is a common mechanism for bacteria. All nitrogen-fixing bacteria studied so far have a branched electron transport chain, in which different terminal oxidases catalyse the oxidation of either quinol or cytochrome *c*. Some branches of electron transport were shown to be specific for nitrogen fixation or low oxygen concentrations. *Azotobacter vinelandii* employs two quinol oxidases for respiration, however only one of them, cytochrome *bd*, is essential for oxygen-tolerant nitrogen fixation [6]. For aerobic growth, *Bradyrhizobium japonicum* uses the cytochrome *c* oxidase of the aa_3 type [7]. When fixing nitrogen in the root nodules of soybeans, the bacteria face extremely low O_2 concentrations (3–30 nM) [8,9]. Under these conditions and during anaerobic growth on nitrate, a cytochrome *c* oxidase of the *cbb′* type [10] with high affinity to oxygen [11] is expressed, which is necessary for symbiotic nitrogen fixation [12].

Similar low oxygen concentrations may be encountered by other root-associated nitrogen-fixing bacteria when they occur in aggregates inside roots of plants. Plant-associated members of the genus *Azoarcus* spp. were detected in high numbers inside roots of Kallar grass (*Leptochloa fusca* (L.) Kunth) grown in Pakistan [13,14]. They can also invade roots of rice in gnotobiotic culture [15]. They are Gram-negative, strictly respiratory Proteobacteria of the beta subgroup [16,17], which fix nitrogen only under microaerobic conditions [18]. *Azoarcus* sp. BH72 can develop into a state of augmented rates of respiration and efficiency of nitrogen fixation at nanomolar O_2 concentrations [19], which is accompanied by formation of intracytoplasmic membrane stacks, so-called 'diazosomes' [20]. Since the apparent affinity for oxygen of the in vivo O_2 uptake increases under these conditions to a $K' = 15$ nM, *Azoarcus* sp. BH72 is likely to possess a branched respiratory chain terminated by different oxidases.

In order to characterize terminal oxidases used by *Azoarcus* sp. BH72, we compared aerobically and microaerobically (nitrogen-fixing) grown cells with respect to the spectral characteristics and heme composition of their membranes and by inhibitory respiratory analysis. Cells appeared to use a cytochrome oxidase (most likely of the *cbb′* type) preferably under aerobic conditions, whereas cells fixing nitrogen microaerobically mainly used a quinol oxidase of the *bb′* type.

2. Materials and methods

2.1. Bacterial strains, growth conditions and membrane isolation

Azoarcus sp. strain BH72 [13] was grown aerobically in SM medium [21] supplemented with 0.3% of Bacto Tryptone, 0.1% of yeast extract, 0.1% of NaCl, and 0.05% of NH_4Cl at 37°C and 250 rpm to an OD_{578} of 0.4–0.7. For nitrogen-fixing, microaerobic growth, nitrogen-free SM medium was used. Cells were grown in a 2 l fermentor (Braun Biotech, Melsungen, Germany) at a dissolved oxygen concentration of 1–5 μM.

Bacillus subtilis OI1085 (wild type) [22], *Escherichia coli* GO103 (Cyd^-) and GO104 (Cyo^-) [23] were grown to the late exponential growth phase in LB broth supplemented with appropriate antibiotics.

A previously described method [24] was used to prepare membrane fractions. Cells were harvested and resuspended in 50 mM potassium phosphate buffer, containing 2 mM $MgCl_2$ and 1 mM PMSF, pH 7.0. The suspension was then passed three times through the French press at 1400 kg/cm^2. Unbroken cells were removed by centrifugation at $10\,000 \times g$ for 30 min and the supernatant was centrifuged at

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Abbreviations: PMSF, phenylmethylsulfonyl fluoride; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine; HPLC, high-performance liquid chromatography; HQNO, 2-heptyl-4-hydroxyquinoline *N*-oxide; Heme nomenclature: We have followed the lead of Puustinen and Wikström [1] in the nomenclature of heme structures. Isolated hemes are indicated by upper-case letters (A, B, O, D), whereas when the hemes are within their natural protein structures, italic lower-case letters are used (*a*, *b*, *o*, *d*). The cytochrome nomenclature is used as suggested by Poole and Chance [2], according to the IUB Nomenclature Committee

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110000×*g* for 2 h. The membrane pellet was frozen at −70°C and then stored at −20°C. Total protein in membrane fractions was measured by the BCA assay (Pierce Chemical Co., Rockford, IL) using bovine serum albumin as a standard.

2.2. Visible light difference spectrophotometry

Membrane samples in 50 mM potassium phosphate buffer (pH 7.0) containing 2 mM $MgCl_2$ were either oxidized with air or by addition of a crystal of potassium ferricyanide or reduced by addition of a few grains of sodium dithionite. The reduced-minus-oxidized difference absorption spectra were recorded at room temperature on a Shimadzu 3000 spectrophotometer with 1 nm band width, a light path of 10 mm, 0.3 s response time and a scan speed of 0.5 nm s^{-1}.

CO-bound reduced-minus-reduced difference spectra were recorded after reducing the membrane samples with a few grains of sodium dithionite and then flushing the sample cuvette with 100% CO for at least 5 min. Spectra were recorded 20 min after flushing with CO.

2.3. Heme extraction and HPLC analysis

Non-covalently bound hemes were extracted from membrane samples using the method of Sone and Fujiwara [25] with modifications [26,27]. Aliquots of membrane preparations (5 to 15 mg of protein) were dissolved in 0.5 ml of acetone/HCl (19:1, v/v) and incubated for 20 min on a rotary mixer. After centrifuging for 2 min at 14000×*g*, 1 ml of ice-cold water and 0.3 ml of ethyl acetate were added to the supernatant, and the sample was vortexed for 30 s and centrifuged again. The ethyl acetate phase was recovered and the solvent removed by a vacuum concentrator. The residues were dissolved in ethyl acetate/acetonitrile (1:1 v/v).

The heme composition was analyzed by HPLC using a Microsorb C18 reversed phase column. Hemes were eluted by acetonitrile (0.5% trifluoroacetic acid)/water (0.5% trifluoroacetic acid) gradients and detected spectrophotometrically at 406 nm (Hewlett-Packard 1050 detector). The hemes were identified by comparing with the retention times of heme standards (A, B, O, D) prepared from *B. subtilis* membranes (hemes A, B), *E. coli* GO103 membranes (hemes B, O) and *E. coli* GO104 membranes (hemes B, D), similarly to a previously described procedure [27].

2.4. Measurement of oxygen uptake

Oxygen consumption in bacterial suspensions was measured using a Clark-type electrode and an oxygen monitor (Yellow Springs Instrument Co, Yellow Springs, OH) connected to a channel of the MacLab data recording system (Model MK-III, Analog Digital Instruments, Boston, MA). Cells were washed and suspended in 50 mM potassium phosphate buffer pH 7.0, and 0.25% of ethanol was added as a substrate if not otherwise indicated. The data collected was stored and analyzed using a Macintosh IISi computer [4].

3. Results

3.1. Inhibitory respiratory analysis

The presence of ubiquinol:cytochrome *c* oxidoreductase (the bc_1 complex) was revealed by inhibitory analysis using antimycin A, HQNO, and myxothiazol. The bc_1 complex is a highly conserved region of redox chains: its structure and function are very similar in different bacteria and in mitochondria. The action of the above-mentioned inhibitors on the bc_1 complex of *Azoarcus* is expected to be as in other bacteria [28]. Both antimycin A and HQNO inhibit quinone reduction by cytochrome *b*, whereas myxothiazol inhibits quinol oxidation by the Rieske iron-sulfur protein which is specific for the bc_1 complex. Endogenous respiration of the cells without an added carbon source was only 37% of that in the presence of ascorbate plus TMPD (500 μM and 250 μM, respectively), which donates electrons directly to cytochrome *c*. This indicated the presence of cytochrome *c* oxidase(s) in aerobically grown cells. When used alone, any of the three inhibitors caused a significant decrease in the respiration rate, indicating the presence of the bc_1 complex. A mixture

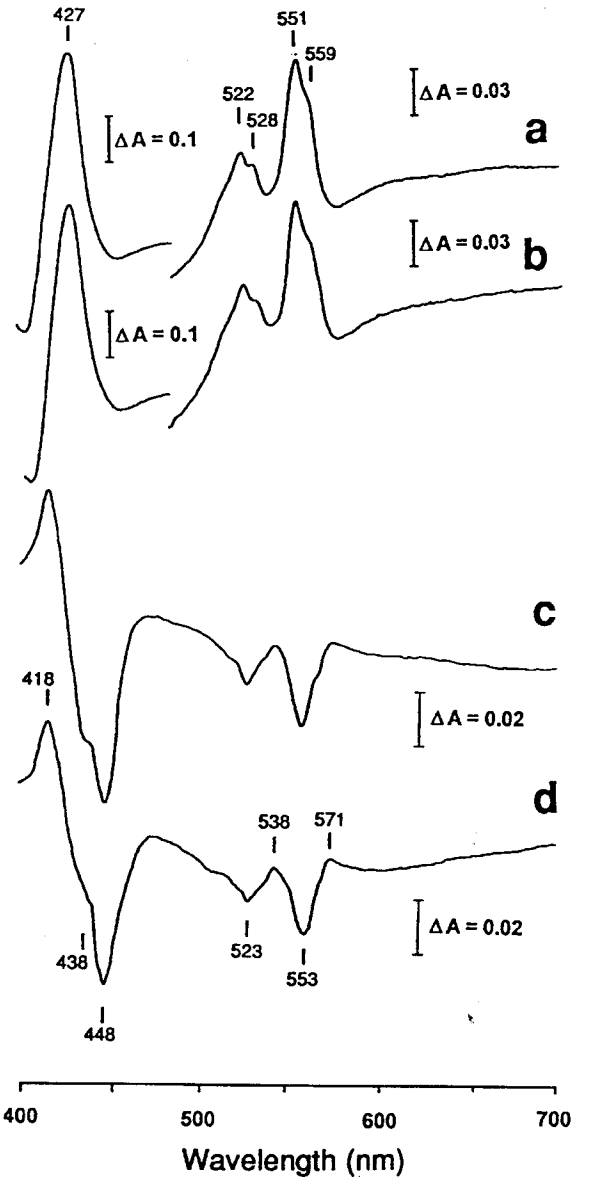

Fig. 1. Optical spectra of membrane preparations of *Azoarcus* sp. BH72 grown under aerobic (a,c) and microaerobic, nitrogen-fixing (b,d) conditions. (a,b) Dithionite-reduced *minus* air-oxidized difference spectra. (c,d) (CO+dithionite-reduced)-*minus*-reduced difference spectra.

of HQNO, antimycin A and myxothiazol (75, 100 and 100 μM, respectively) inhibited ethanol-dependent respiration by 69% in aerobic cells, which could be fully restored by addition of ascorbate plus TMPD. In contrast, cells fixing nitrogen microaerobically were significantly less sensitive to inhibitors of the bc_1 complex. The same mixture of inhibitors decreased respiration in microaerobically grown cells only by 18%.

Aerobically and microaerobically grown cells also showed a different pattern of inhibition by cyanide. The threshold for cyanide in aerobically grown cells was approx. 0.025 μM (inhibition of respiration by 4%), whereas respiration in microaerobically grown cells was unaffected by up to 0.1 μM KCN. Inhibition of respiration by 15% in aerobically and microaerobically grown cells was achieved by addition of 0.05 and 0.5 μM KCN, respectively. When ascorbate was used as an electron donor instead of ethanol, aerobic cells were even more

sensitive to cyanide: 0.01 μM KCN inhibited respiration by 11%.

3.2. Difference spectroscopy

Dithionite-reduced minus air- or ferricyanide-oxidized difference spectra of membrane preparations from aerobically and microaerobically grown cells looked identical (Fig. 1a,b), revealing an intense Soret band at 427 nm, and highly asymmetric α and β bands typical of the cytochrome *c* oxidase of the *cbb'* type, a newly described class of oxidase in the heme-copper superfamily (for recent reviews, see [29,30]). An alpha peak at 551 nm and β peak at 522 nm are indicative of *c*-type heme, and shoulders at 559 nm (α peak) and 528 nm (β peak) are indicative of *b*-type heme (Fig. 1a,b). Essentially the same peaks were identified for the purified *cbb'* oxidase complexes from *B. japonicum* [10], *Paracoccus denitrificans* [31,32], *Rhodobacter sphaeroides* [33], and *R. capsulatus* [34,35]. No evidence for either heme *a* (band at 600–610 nm) or heme *d* (band at 620–640 nm) was seen on the differential spectra (Fig. 1a,b).

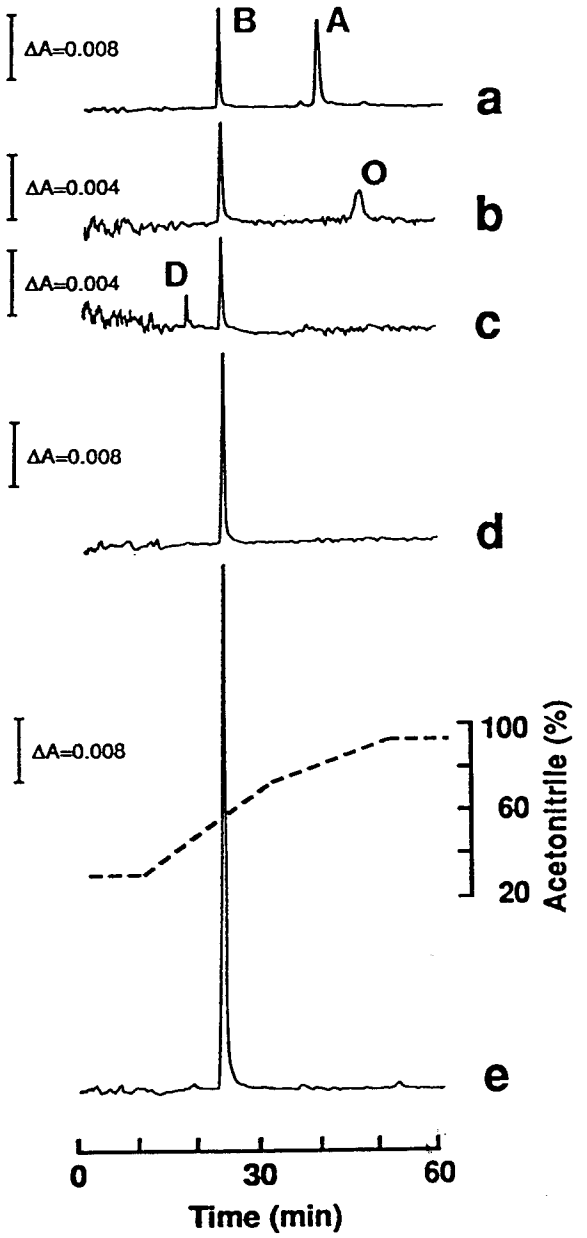

Fig. 2. Elution profiles for the reversed-phase HPLC of non-covalently bound heme compounds extracted from control membranes (a–c) and aerobically (d) or nitrogen-fixing microaerobically (e) grown *Azoarcus* sp. BH72. The dashed line (in e) shows an elution gradient of acetonitrile (30–100%) in water containing 0.5% trifluoroacetic acid. The elution peaks of standard hemes A, B, D, and O are indicated. Control membranes were isolated from *Bacillus subtilis* OI1085 (a), *Escherichia coli* GO103 (b), and *E. coli* GO104 (c).

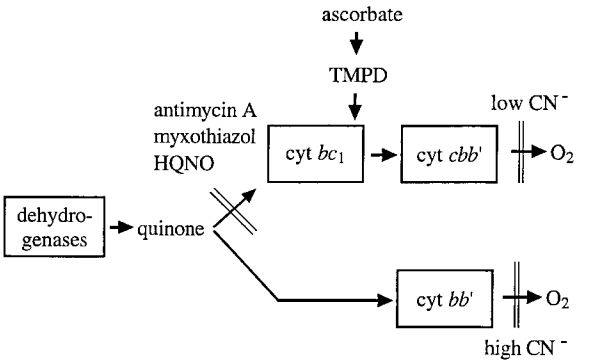

Fig. 3. Putative respiratory pathways in *Azoarcus* sp. BH72. See text for explanations.

Additional evidence for the cytochrome containing hemes *c* and *b* to be a dominant terminal oxidase in *Azoarcus* cells came from the CO-binding spectra (Fig. 2c,d), which look similar to those of the purified *cbb'* complex from *B. japonicum* [10] and *P. denitrificans* [31]. Troughs at 523 and 553 nm implicate CO complexing mainly with *c*-type hemes.

3.3. Heme analysis

Comparative heme analysis of membrane fractions revealed that both aerobically and microaerobically grown *Azoarcus* sp. BH72 contained only heme B (Fig. 2d,e). Hemes A, O and D obtained from the control strains (Fig. 2a–c) were not detectable, whereas heme B was present abundantly in *Azoarcus* sp. BH72.

4. Discussion

The data demonstrate that *Azoarcus* sp. BH72 utilizes at least two different terminal oxidases. The respiratory inhibitory analysis indicated that one of the oxidases is a cytochrome *c* oxidase and another is a quinol oxidase. Both oxidases are functional under aerobic and microaerobic conditions. However, under aerobic conditions, the cytochrome *c* oxidase appears to be dominant, whereas in microaerobically grown nitrogen-fixing cells the quinol oxidase seemed to be preferably used. The following lines of evidence support this statement. (i) Inhibitors of the bc_1 complex, which affect cytochrome *c* oxidases, dramatically (but not completely) inhibited respiration in aerobically grown cells. In microaerobically grown cells, the effect of the inhibitors was much less pronounced. In both cases, ascorbate plus TMPD, which donates electrons directly to cytochrome *c*, completely restored respiration. (ii) Respiration of aerobically grown cells was more sensitive to cyanide than that of microaerobically grown cells. Generally, cytochrome *c* oxidases (caa_3, *cbb'*) are more sensitive to cyanide than quinol oxidases (*bo*, *bd*).

We suggest that the cytochrome *c* oxidase of *Azoarcus* sp. BH72 is of the *cbb'* type. The reduced-minus-oxidized difference spectra and the CO difference spectra of membranes from aerobically and microaerobically grown cells revealed major peaks typical of the purified *cbb'* oxidases from different Proteobacteria [10,30–34]. No evidence for a cytochrome *c* oxidase of the aa_3 type or a quinol oxidase of the *bd* type was obtained from the spectra. Most importantly, we have recently obtained genetic evidence from PCR amplification and sequencing for the presence of genes highly homologous to *cbb'* oxidase genes of *B. japonicum* and *R. capsulatus* (F. Macht and B. Reinhold-Hurek, unpublished data).

The quinol oxidase is probably of the *bb'* type. No evidence for a quinol oxidase of the *bd* type was obtained from the spectra. Heme analysis revealed heme B (protoheme) as the only non-covalently bound heme in membranes of *Azoarcus* sp. BH72. Hemes A, O and D were not detected.

A scheme of the putative respiratory pathways in *Azoarcus* sp. BH72 is shown in Fig. 3. Cytochrome *c* oxidases of the *cbb'* type were recently identified in several bacterial species [10,30–34]. Quinol oxidases of the *bb'* type are less common, but they were also identified in *P. denitrificans* [31] and in *Bacillus* sp. FTU [27]. The organization of the respiratory system of *Azoarcus* sp BH72 closely resembles that of *Paracoccus* [36]. A difference seems to be the absence of cytochrome aa_3 in the former. Remarkably, *Azoarcus* sp. BH72 appears to be the first bacterium reported so far, which does not modify protoheme in order to achieve a flexibility of the respiratory system. Usually, eubacteria modify heme B to heme O and further to heme A, and archaea appear to have even more complex modifications of protoheme [26]. Whether the protoheme modification resulting in novel cytochromes occurs in *Azoarcus* sp. BH72 during a physiological state of 'hyperinduction' at nanomolar oxygen concentrations [19] awaits elucidation.

Bacteria that oxidize quinol through a cytochrome bc_1 complex also possess at least one alternative quinol oxidase, which transfers electrons directly from quinol to oxygen (for a recent review, see [28]). It was suggested that the existence of additional quinol oxidases allows bacteria to escape toxins that act on the bc_1 complex. In addition, quinol oxidases may also balance the energy requirements of the cell against the requirements for oxidation of NADH and similar metabolites [28]. Terminal oxidases with different oxygen affinities assist in adaptation of bacteria to a broad range of oxygen concentrations in the environment. Such an adaptation is crucial for nitrogen-fixing bacteria that have an aerobic type of metabolism. Different species of rhizobia use cytochrome *c* oxidase of the aa_3 type as the dominant oxidase during aerobic growth [7,37,38]. Under microaerobic conditions, the alternative cytochrome *c* oxidase of the *cbb'* type is induced in rhizobia [10,11] as well as in *R. sphaeroides* [33] and in *P. denitrificans* [34]. In *B. japonicum*, this oxidase is essential for symbiotic nitrogen fixation [12] and it has an extremely high affinity to oxygen with a K_m value of 7 nM [11].

Azoarcus sp. BH72 appears to possess a unique pattern of utilization of its terminal oxidases. It is unusual in using the cytochrome *c* oxidase of the *cbb'* type as the dominant oxidase for aerobic growth and the quinol oxidase of the *bb'* type as the major oxidase for microaerobic growth. Whether either of the oxidases is essential for nitrogen fixation and hyperinduction [19] of *Azoarcus* sp. BH72 will be revealed by mutational analysis.

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