CHARACTERIZATION OF A MEMBRANE-BOUND NITRATE REDUCTASE FROM Azotobacter chroococcum

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SUMMARY: Nitrate reductase from the aerobic bacterium Azotobacter chroococcum is a soluble enzyme with the characteristic features of Pichinoty's type B nitrate reductase. When cell suspensions of A. chroococcum are repeatedly subcultured in liquid medium with nitrate as the nitrogen source, most of the nitrate-reducing activity is incorporated into the cytoplasmic membrane. The properties of the particulate nitrate reductase closely resemble Pichinoty's type A enzyme

According to Pichinoty's proposal (1,2), there exist two types of bacterial nitrate reductase which differ from one another in several of their properties. They have been designated enzyme A and B. Briefly, nitrate reductase A associated with cell membrane is synthesized at a high level when capable bacteria are grown in anaerobic cultures at the expense of nitrate, thus playing an essential role in the anaerobic respiration of nitrate. Nitrate reductase B is a soluble protein which has an assimilatory function in bacterial species that form it when grown aerobically with nitrate as the sole nitrogen source. Nevertheless, several strict aerobic species that assimilate nitrate produce enzyme A (1).

In previous work from this laboratory the preparation and characterization of a soluble nitrate reductase from the aerobic, nitrogen-fixer Azotobacter chroococcum was described (5,6). The properties of this enzyme lead us to classify it as a Pichinoty enzyme B.

We wish to report now that when Azotobacter chroococcum cells are repeatedly subcultured in liquid cultures containing nitrate,

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they gradually develop, besides the already described soluble enzyme, a new nitrate reductase activity which is incorporated into the constitutive portion of the respiratory chain. Our results indicate that a cytochrome of the b-type is involved in the catalysis of the particulate nitrate reductase activity, and that menadione stimulates the enzymatic electron transport chain from several reductants to nitrate.

MATERIALS AND METHODS. Azotobacter chroococcum (strain ATCC-4412 from the Salamanca University collection) was used throughout this study. Stock cultures of bacteria were kept as 3 ml suspensions in 60% (v/v) glycerol at -20°C. A preculture of A. chroococcum was made by inoculating 250 ml of the nitrate-containing growth medium (5) with 3 ml of stock culture in a conical flask and shaking it at 27°C under air in a giratory shaker at 200 rpm. After 36 h, 30 ml of this culture was used to inoculate 1 litre of the liquid medium in a 2 litre conical flask and then growth was allowed to proceed for 12-14 h under the above experimental conditions. The cells were transferred into a liquid medium at a 2% proportion and grown at 27°C for 12 h with vigorous shaking. After an additional period of 12 h without shaking, this culture was again used to inoculate a fresh liquid medium. The same kind of subculture was daily repeated.

12-14 h old cells from a culture grown with vigorous shaking under air were always used for the preparation of subcellular fractions. After harvesting the cells at 0-4°C by centrifugation at 10.000 g for 10 min and washing them once in 0.1 M potassium phosphate buffer, pH 7.0, they were resuspended in the same buffer to a final concentration of 1 g wet wt. in 10 ml of buffer. The suspension of whole cells was disrupted by passage through a French pressure cell at 5000 lb/in^2. The resultant mixture was centrifuged at 3000 g for 20 min at 4°C. The supernatant will be referred to as the cell-free extract. This was centrifuged at 39.000 g for 20 min in order to obtain the large particles fraction. The turbid supernatant was further centrifuged at 110.000 g for 150 min to yield a reddish brown small particle sediment and a yellowish supernatant. The upper two-thirds of this supernatant was carefully removed with a teflon tube joined to a syringe and will be referred to as the high speed supernatant. The resuspension of the above sediments was made with 0.1 M potassium phosphate buffer, pH 7.0.

MVH:NO_3Rase* activity was carried out as previously described (11) Procedures for the measurement of the electron transport to nitrate from NAD(P)H, malate and succinate are described in the legends to the corresponding Tables. One unit of enzyme activity is defined as that which forms 1 µmole of nitrite/min. Specific activity is expressed as units/mg protein.

*Abbreviations: MVH:NO_3Rase, reduced methyl viologen: nitrate reductase; NADH:NO_3Rase, NADH-supported nitrate reductase activity.

Table I. Intracellular distribution of nitrate reductase activity of A. chroococcum

Fraction	MVH:NO_3Rase		NADH:NO_3Rase	
	Units	Specific Activity (munits/mg)	Units	Specific Activity (munits/mg)
Cell-free extract	46	300	11	60
Large particles	13	300	0.6	20
Small particles	54	1100	10	200
High-speed supernatant	5	30	0.4	10

The subcellular fractions were prepared from cells subcultured in liquid medium for twenty days. NADH:NO_3Rase activity was determined in Warburg manometer flasks under an atmosphere of argon. The reaction mixture contained, in a final volume of 2 ml, the following in μmoles: potassium phosphate buffer, pH 7.0, 150; KNO_3, 10; NADH, 0.6; and an appropriate amount of the respective fractions. The reaction was carried out at 25°C for 10 min. The measurement of nitrite formed was as previously described (11).

RESULTS AND DISCUSSION. Table I shows the distribution of nitrate reductase activity among the different subcellular fractions of A.chroococcum cells that had been subcultured in the growth medium for 20 days. As can be seen, most of the MVH:NO_3Rase and the NADH:NO_3Rase activity was confined to the particulate materials; the activity of the high speed supernatant was very low. A similar experiment carried out with A. chroococcum cells that were subcultured in liquid medium for less than ten days, did not show any nitrate reductase activity associated with the particulate material.

It can be shown that the nitrate reductase activity present in the small particles reduced nitrate along linear rates from the outset, when either NADH, dithionite-reduced methyl viologen, or just dithionite were used as the electron donors. On the contrary, the

Table II. Effect of menadione on the particle-bound nitrate reductase activity from A. chroococcum

Electron donor	Addition	Nitrate reductase Specific activity (munits/mg)
None	K_3	0
NADH	–	70
NADH	K_3	234
NADPH	–	19
NADPH	K_3	58
Malate	–	28
Malate	K_3	87
Succinate	–	26
Succinate	K_3	32

Experimental conditions were as described in Table I for NADH:NO_3Rase activity. NAD(P)H, 0.3 mM; malate, 10 mM; succinate, 10 mM; K_3, dissolved in 0.05 ml of methanol, was added at a final concentration of 0.25 mM.

nitrate reductase activity remaining in the high speed supernatant, which could not use NADH nor dithionite alone as an electron donor, behaved like the previously described soluble enzyme, i.e., the rate of nitrate reduction diminished as a function of time during its assay with dithionite-reduced methyl viologen as the reductant (5). In this respect, the particulate nitrate reductase resembles the bacterial enzyme A (2).

Since the initial work of Wainwright (7) on the effect of menadione derivatives on nitrate reductase activity, the role of quinones on the nitrate-reducing electron transport chains has more recently been reinvestigated (2,8).

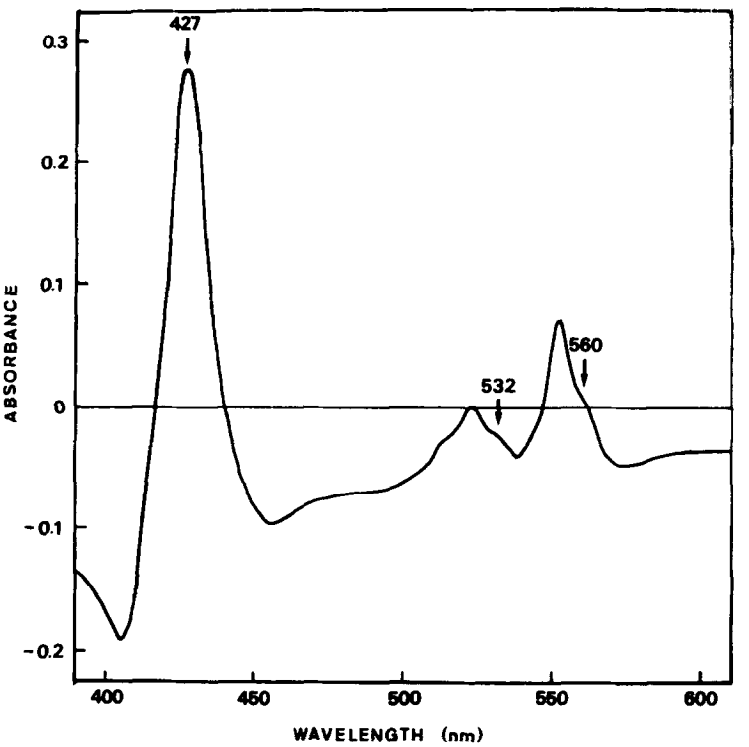

Fig. 1. Reduced minus oxidized difference spectra of nitrate reductase-containing small particles from A. chroococcum. The base line was recorded with 2.5 ml of the small particles (4 mg protein/ml) in each cuvette. A few crystals of $Na_2S_2O_4$ were then added to the sample cuvette and the difference spectrum was recorded after 5 min.

We were able to show that menadione greatly stimulated the particulate nitrate reductase activity as assayed with a variety of elec tron donors. It can be seen in Table II that K_3 increased the elec - tron transport to nitrate by the Azotobacter particles with NAD(P)H or malate. K_3 was without effect on the succinate-supported nitrate reductase activity. No other quinones were tested.

Nitrate reductase activity of small particle preparations from A. chroococcum decreased to a very low level when the cells were transferred to an ammonia - containing culture medium, thus indica - ting the apparent adaptive nature of the particulate enzyme.

Dithionite-reduced minus oxidized difference spectra of A. chroo-

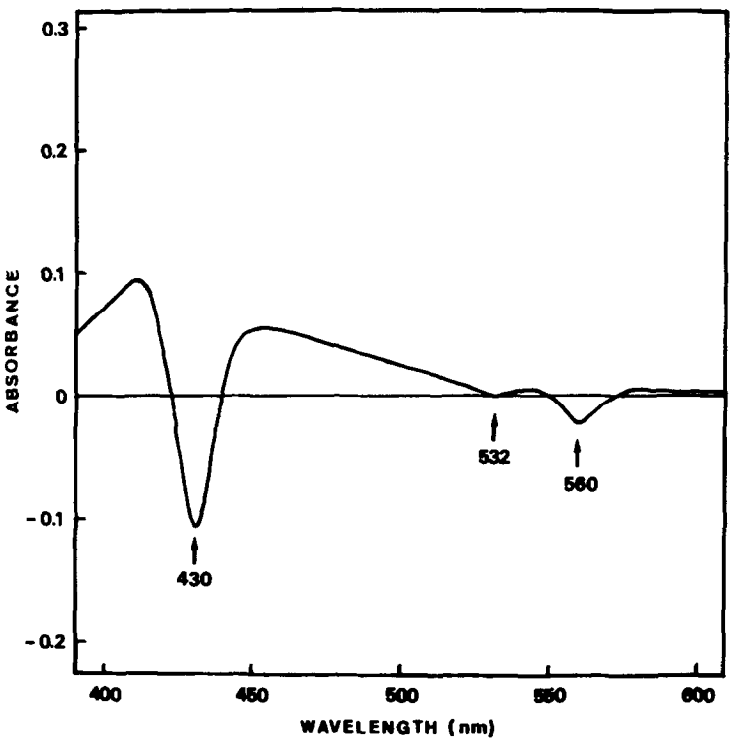

Fig. 2. Difference spectra of the small particles reduced by $Na_2S_2O_4$ versus its reoxidation by nitrate. The base line was obtained as in Fig. 1 in the presence of $Na_2S_2O_4$ in both cuvettes. Solid potassium nitrate was then added to the sample cuvette and the difference spectrum was recorded after 10 min.

coccum small particles exhibiting nitrate reductase activity are shown in Fig. 1 . The cytochrome components are rather similar to those reported for several Azotobacter species (9,10). It could be shown that the peak at 427 nm and the shoulder at 560 nm, as well as one of the fused β-bands observed at 520-534 nm, were oxidized by nitrate, as can be seen in Fig. 2 . This demonstrates that cytochrome b_1 was acting as a cofactor for the enzymatic reduction of nitrate. As is known, cytochrome b_1 plays a functional role in assimilatory nitrate reductase from different eukaryotes (3,4),and bacterial respiratory nitrate reductase (2).

Interestingly enough, the cytochrome spectra of small particles

proceeding from cells grown on ammonia were similar to the one shown in Fig. 1. Furthermore, the difference spectra of a small particle preparation from A. chroococcum cells of a nitrate-containing liquid culture just coming from a stock culture in glycerol were also identical with that shown in Fig. 1. As already stated, these particles lacked nitrate reductase activity and hence their cytochrome b component should not be oxidized by nitrate. As expected, it was not.

It is tempting to assume that, under the specified liquid subcultures, A. chroococcum cells undergo a spontaneous mutation to a strain in which the nitrate reductase activity is mainly associated with the cytoplasmic membranes. The physiological significance of this mutation remains to be clarified. Work is now in progress to determine what role, if any, the oxygen tension of the medium may play on the appearance of the membrane-bound nitrate reductase.

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