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OXIDATION-REDUCTION TITRATION OF TWO CYTOCHROMES b IN AZOTOBACTER VINELANDII

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Summary: Two b-type cytochrome components, differing in their midpoint redox potentials as well as their reactivities toward CO, are demonstrated in the membrane particle of Azotobacter vinelandii by potentiometric titration. The low redox component is revealed to be CO-reactive while the higher redox component is not. Accordingly, the later cytochrome is believed not to be directly involved in the reduction of oxygen while the former component is the cytochrome o of Azotobacter.

Azotobacter vinelandii is a free-living obligate aerobe which uses molecular oxygen as a terminal acceptor for its respiration. A branched electron transport chain was first proposed in this organism by Jones and Redfearn, based on their inhibition kinetics and action spectral studies (4). This scheme has been subjected to some minor revision, and some critical questions have recently been raised (6,11). This is due partly to the complex spectral change exhibited by the cytochrome components during oxidation-reduction reactions of the electron transferring process and, to some extent, due to the lack of understanding of these redox carriers such as cytochrome b and cytochrome o in this organsim. The heterogenous kinetics observed for the reduction of b-type cytocrhome(s) was an example of such a case (4). So far there has been little reported on the redox potentials of the cytochrome components in A. vinelandii cells. Tissieres (8) was the first to report the redox potentials of both purified cytochromes c_4 and c_5. We have previously established that cytochrome o, when co-purified with cytochrome c_4, had a low midpoint potential(11). We have assumed that solubilization and purification had no major effect on the midpoint of cytochrome o, and that in its membrane-bound form cytochrome o perhaps also had a low redox midpoint. However, there was no evidence for supporting such a presumption (11). In this study I present results obtained by measuring redox potentials of two b-type cytochrome components in the membrane particles of

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A. vinelandii, and show that cytochrome o is indeed different from a classical b-type cytochrome.

MATERIALS AND METHODS

A. vinelandii strain o was grown in N_2-fixing conditions with 1% sodium acetate as the sole carbon source. Cells were harvested at late log phase and the membrane particle was prepared as described previously (9). The membrane preparations were washed once and either used fresh or stored at -27°C until used. Protein concentrations in the membrane preparations were determined by the biuret method of Gornall et al. (3), with bovine serum albumin as standard.

Oxidation-reduction titration was performed in a vessel described by Dutton (2) and details of the titration are described by Yang et al. (11). When the titration was performed in the presence of CO, the preparations were first reduced completely and then kept under anaerobic conditions before and during bubbling with CO.

RESULTS AND DISCUSSION

The membrane particle of A. vinelandii is rich in cytochrome b, its molar concentraiton (average from 1.12 to 1.45 μM) is slightly higher than cytochrome c(c_4+ c_5, 0.95 to 1.28 μM) in the preparations studied here. The midpoint potential of one b-type cytochrome (cytochrome b_{561}) is determined to be +122 mV at pH 7.4 as shown in Figure 1A. This component shows no CO-binding activity. In the presence of saturated CO, cytochrome b_{561} is titrated completely as in the absence of CO, suggesting that this component not to be CO-reactive. However, in the presence of CO, cytochrome b_{561} has a slightly lower midpoint, with an $E_{m,7.4}$= 100 mV compared with its midpoint of +122 mV in the absence of CO, as the results show in Figure 1 (A and B). The 22 mV difference observed may be considered within the experimental error. The midpoint of another cytochrome b (b_{559}) is determined to be -30 mV, about 150 mV lower than cytochrome b_{561}, as the results shown in Figure 2. When titrated in the presence of CO, the reduced form of cytochrome b_{559} binds CO and hence resists oxidative titration. Thus, cytochrome b_{559} identified operationally as cytochrome o due to its binding to CO in the membrane particle, is titrated and separated from cytochrome b_{561} potentiometrically. Although the solubilized cytochrome o and membrane-bound form (b_{559}) varied slightly in their maximal absorption peaks in the visible region (557-558 nm in the previous reports vs. 559 nm in this communication), the variation appears too small to assess the extent of spectral alteration which might have been caused by detergent solubilization and purification processes (9,11). Judged

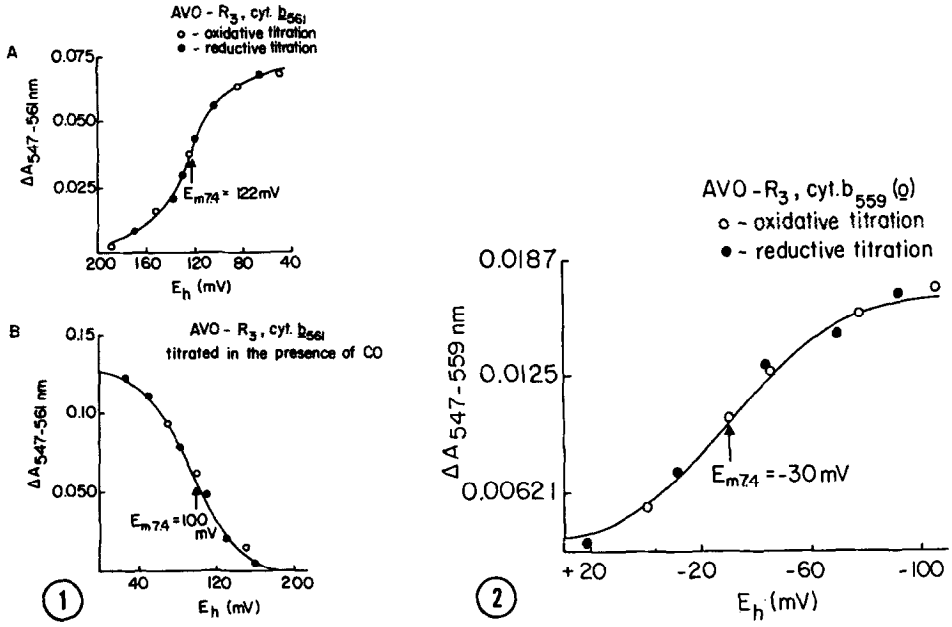

Figure 1. The course of oxidation-reduction of cytochrome b_{561} in intact electron transport particle of A. vinelandii in the absence (A) and in the presence of CO (B). Membrane particle was suspended in 50 mM of potassium phosphate buffer, pH 7.4 and the protein concentrations were (A) 5.6 mg/ml, (B) 8.8 mg/ml. The redox mediators used were 30 μM of phanazine methosulfate, phenazine ethosulfate and diaminodurol. Oxidative-titration(o) was initiated by small aliquots of potassium ferricyanide and reductive-titration (•) by sodium dithionite. A theoretical n=1 line is drawn through the points in each case.

Figure 2. The oxidation-reduction potential of cytochrome b_{559} (cytochrome o) in the membrane particle of A. vinelandii. The experimental conditions are the same as in Figure 1 except the mediator dyes were 10 μM of 2-hydroxy-1,4-naphthanquinone and pyocyanine and 25 μM of phenazine ethosulphate. The protein concentrations were 8.8 mg/ml. (o) represents oxidative-titration and (•) reductive-titration. A theoretical n=1 line is drawn though the points.

from the midpoint potential in its membrane-bound form and from the little change observed in its absorption maximum, it is assumed that the heme component of this oxidase is not affected by the detergent solubilization or by other purification procedures.

It is unlikely that cytochrome b_{561} is composed of two different components as several attempts to resolve the membrane-bound b_{561} into its possible constituents were unsucessful. We have also failed to seperate spectrally cytochrome b_{561} from b_{559} undoubtedly due to their largely overlapping spectral regions. Nonetheless, the present results appear to confirm that the CO-reactive cytochrome b_{559} component in the membrane particle is the cytochrome o component

which has a low redox potential as previously predicted (10, 11), while cytochrome b_{561} with a relatively high potential is the classical b-type cytochrome that has often been reported in A. vinelandii yet none of its properties has ever been described (4, 5).

Few reports deal with bacterial b-type cytochromes primarily because of the difficulties in seperating these components by spectral means and in isolating them from their membrane-bound state. Shipp reported early that in late log phase growing A. vinelandii, cells produced two to three b-type cytochrome components, as revealed in a cold temperature profile using the method of fourth-order finite difference analysis (7). More recent evidence (6) indicated that the respiratory chain scheme of Downs and Jones (1) cannot explain the inhibition pattern of malate oxidation. In the scheme Downs and Jones proposed that in vitro substrates such as NADH , NADPH and succinate were channeled into cytochrome b (presumably b_{561}). Although NADH, NADPH and succinate oxidations were all completely inhibited by about 10 uM divalent metal ion (such as Zn^{++}), however,malate oxidation was about 100 times less sensitive to such inhibition (6). The implication was that malate and NADH (also NADPH and succinate) might not share the same electron transfer pathway as proposed by Jones and his co-workers. The results presented here are not incompatible with the proposed scheme in presuming that cytochrome b_{561} with a midpoint of +122 mV could serve as electron donors for both cytochromes c_4(and c_5) and d (1). Because both cytochromes $c_4$and d all have higher potentials than b_{561} (11, and T. Yang unpublished work). Consequently, it is thermodynamically plausible that cytochrome b_{561} may play a key role in branching the electron flux into the termial oxidases such as cytochromes d and o. The low redox potential of cytochrome o , as report here, may imply that this oxidase terminates respiration with low efficiency of energy conservation if the electron is coupled to oxidative phosphorylation (site III) as it was proposed by Downs and Jones (1). However, it is also possible that the intermediate(s) formation by cytochrome o and oxygen (9) could be a high redox product which then would certainly generate less free energy than anticipated.

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