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Studies on the Physiological Electron Donor for Cytochrome *C* Oxidase in *Nitrobacter winogradskyi*

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Abstract: Terminal respiratory system of an autotrophic nitrite- oxidizing bacterium, *Nitrobacter winogradskyi* was reconstructed with the purified components *in vitro*. Cytochrome aa_3-type cytochrome *c* oxidase, soluble cytochrome *c* oxidase, soluble cytochrome *c*-550 and membrane-bound *c*-551 were purified from the bacterium to electrophoretically homogeneous states, respectively. Oxidation's of the soluble ferro-cytochrome *c*-550 and membrane–bound ferrocytochrome *c*-551 by the cytochrome *c* oxidase was kinetically analyzed. Value of affinity constant (Km) and maximum velocity (Vmax) for the soluble cytochrome *c*-550 were 5.78 μ M and 251.5 sec^{-1} respectively, while values of Km and Vmax for the membrane–bound cytochrome *c*-551 were 3.2 μM and 88.7 sec^{-1}respectively. The oxidation of membrane–bound ferro-cytochrome *c*-551 with the cytochrome *c* oxidase was accelerated by the addition of soluble cytochrome *c*-550, while oxidation of soluble cytochrome *c*-550 with enzyme was inhibited by the addition of membrane bound cytochrome *c*-551. Although membrane-bound cytochrome *c*-551 could be electron donor for cytochrome *c* oxidase, the soluble cytochrome *c*–550 seems to function as electron mediator between membrane bound cytochrome *c*-551 and cytochrome *c* oxidase *in vivo*.

Key words: Cytochromes, respiratory chain, electron donor, *Nitrobacter winogradskyi*

Introduction

Nitrobacter winogradskyi is a chemoautotroph which acquires the energy for life process by oxidizing nitrite to nitrate (Buchanan and Gibbons,1974). Aleem and Nason,1959 prop osed an electron transport system for the oxidation of nitrite in this bacterium. The system was composed of cytochrome a_1c_1 (nitrite cytochrome *c* oxidoreductase) (Paul and Clark (1989) and Buchanan and Gibbons (1974), soluble cytochrome *c* (Tanaka *et al.*, 1983 and Fukuoka *et al.*, 1987) membrane bound cytochrome *c* (Nomoto *et al.*,1993) and cytochrome aa_3 (cytochrome *c* oxidase) (Tanaka *et al.*,1982). The electron transfer system of bacterium involving cytochrome a_1c_1, cytochrome *c*-550 and cytochrome aa_3 have been proposed by Yamanaka and Fukumori (1988).

Aleem and Sewell (1981) proposed the energy dependent electron transfer between nitrite and cytochrome *c* mediated by cytochrome a_1c_1 purified from *Nitrobacter winogradskyi*. Although Sundermeyer *et al.*(1984). have obtained nitrite–oxidoreductase from *Nitrobacter hamburgenesis*, the enzyme possess no heme *a* and further, shows no nitrite-cytochrome *c* reducing activity. These results suggest that heme *a* in cytochrome a_1c_1 may function as a mediator o f electr on transport between nitrite and cytochrome *c*. Cyto chrome aa_3 oxidase which functions as the terminal oxidase in the aerobic respiratory chain (Yamanaka and Fukumori, 1988).

Nomoto *et al.*(1993) have proposed cytochrome *c*-550 and cytochrome aa_3 (Fig. 1). And also suggested that the cytochrome *c* can also mediate the electron transport between cytochrome a_1c_1 and cytochrome aa_3.

In this experim ent study, examine the physiological donor for cytochrome aa_3. We have purified soluble cytochrome *c*-550, membrane bound cytochrome *c*–551 and cytochrome *c* oxidase from the bacterium and determined kinetics parameter for the oxidation of cytochrome *c* oxidase and further the following electron transport system of the bacterium.

Cytochrome a_1c → membrane bound cytochrome *c*-551
soluble cytochrome *c* –550 → cytochrome aa_3.

Materials and Methods

Organism and culture: *Nitrobacter winogradskyi* was grown in the inorganic mineral salts liquid medium (Aleem and Alexander, 1958) with slight modification 1000 ml subcultivation medium contained these components $NaNO_2$ 1.0g; K_2HPO_4, 0.5g ; NaCl,0.3g; mixture of metal salts 10.0ml mixture of metal salts per 1000 ml of deionized water $MgSO_4$. $7H_2O$, 10.0g; $MnSO_4$.4-$6H_2O$,2.5g; $CuSO_4$. $5H_2O$, 10.0g;$(NH_4)_6MO_2$. $4H_2O$, 5.0g; $CaCl_2$,2.0g;$FeCl_3$. $6H_2O$,0.5g; EDDHA., 1.0g and distilled water 1.0L. The mixture of metal salts sterilized separately to prevent the formation of sediments the pH of the medium was adjusted to 7.8 ±. 0.5 with concentrate H_2SO_4. Large-scale cultivation of the organism was done in 500 L of the medium in a stainless steel fermented of 600 L capacity (Yamanaka *et al.*, 1982)

The initial cultivation was carried out in 200 L of the medium inoculated with 5L seed culture obtained at 28°C for 5-6 days (the period was dependent the consumption of nitrate) with vigorous aeration. During the cultivation, the nitrate monitored in every day (Nicholas and Nason, 1957). When the concentration of nitrate has decreased to approximately 10mM, it was raised to 50-60 mM by addition of sterilized KNO_2. When the total amounts of KNO_2 consumption was reach ed 80-100 m mole L^{-1}, then the cells were harvested. About 80g of centrifugal packed cells were obtained from 500 L culture.

Physical and chemical measurements: Spectrophotometeric determinations were carried out Shimadzu MPS –2000 multipurpose spectrophotometer, using 1cm light path cuvettes. Concentration of cytochrome *c*, soluble cytochrome *c* and membrane bound cytochrome *c* were determined spectrophotometerically by ϵ mM (at the α peak) of pyridine ferrohomochrome *a* of 26mM^{-1} cm^{-1}(Lamberg *et al.*, 1962) and pyridineferrohemochrome *c* of 29.1$mM^{-1}cm^{-1}$ (Drabkin, 1942). Purities were checked by polyacrylamide gel electrophoresis in the presence of SDS by the method of Laemmli (1970). The sample was heated at 100°C in sample buffer containing 1% SDS for 1 min. The gel concentration was 15%.

Assay for enzyme activity: The enzymatic activity of cytochrome *c* oxidase was spectrophotometrically by following the decreased in the absorbance at 550 nm of ferrocytochrome *c*. The standard reaction mixture contained 1-7μM cytochrome *c* and 0.3 nM cytochrome *c* oxidase and 25 mM phosphate buffer pH 6.5 in a total volume of 1.0 ml.

Results

Purification of soluble cytochrome *c*-550 *N.winogradskyi*: Frozen cells of *N.winogradskyi* about (15g) centrifugally packed state were suspended in 100ml of 50 mM Tris-HCl pH–8.0, containing 0.1 mM EDTA and 10 μM PMSF then treated twice with sonic- oscillator (20KH_2, 150W. Heat system U. S.A) for 20 min. The suspension was centrifuged at 6,000 x g for 20 min. to remove the unbroken cells. Cell free extract (the supernatant) was centrifuged at 166,000 x g for 1 h .The precipitate (the membrane fraction) were used for the purification's of membrane-bound cytochrome *c*-551 and cytochrome aa_3. The soluble fraction was fractionated by addition of solid ammonium sulfate, the saturation degree was elevated step to final 80% saturation through 60 and 70% saturation's. Then the resulting solution was centrifuged at 6,000 x g for 20 min .The supernatant was obtained and applied to a DEAE-cellulose column (2.8 x 3 cm) which had been previously equilibrated with 20mM Tris-HCl buffer, pH-8.0 contains 0.1 M EDTA and 80% saturated ammonium sulfate. The red colour material that was eluted adsorbed on the top of the column was eluted with 20mM Tris-HCl buffer, pH-8.0, 1mM EDTA and 0.2 M NaCl. The fraction which contain soluble cytochrome *c*-550 were combined and dialyzed against 10mM sodium phosphate buffer, pH-6.0. After dialysis for 2 h, the resulting solution was charged on a column (2.8 x 30 cm) which had been equilibrated with the same buffer as used for dialysis. The cytochrome ***c*** adsorbed on the column was eluted with a linear gradient of 200 ml each of 10 mM phosphate buffer pH-6.0 and the buffer contained 100 mM NaCl. Cytochrome *c* –550 was eluted at 60 mM NaCl (Fig. 2). Finally cytochrome *c* was concentrated using small CM-cellulose column, and then used as purified sample. All the purification steps were conducted at 4° C.

Molecular properties of the bacterial soluble cytochrome *c*-550: The absorption spectra of the purified soluble cytochrome *c*-550 in the visible region showed an absorption peak at 410 nm in the oxidized form and peaks at 416, 521 and 550 nm in the reduced form (Fig. 3). Soluble cytochrome *c*-550 was purified to an electrophoretically homogenous state and its molecular weight was estimated to be about 12,600 from the result of SDS-polyacrylamide gel electrophoresis (Fig. 4).

Purification of membrane bound cytochrome *c*-551 from *N.winogradskyi*: Membrane which was prepared by the method as mention above was suspended in 50 mM Tris-HCl buffer pH-8.0, containing 1mM PMSF, 1mM EDTA. Then, non-ionic detergent Triton X-100 was added to the suspension up to the final concentration of 1% (w/v). The resulting suspension was gently stirred in an ice bath for 8 h and centrifuged at 166,000 x g for 60 min. The suspension thus obtained was applied to a DEAE-Toyoperal 650M (3 x 20 cm) which had been equilibrated with 20 mM Tris-HCl, pH-8.0, 0.2% Triton X-100. 0.1 mM EDTA and 0.1mM PMSF. Cytochrome *c* oxidase was adsorbed on the column, while the membrane bound cytochrome *c* was passed through the column. Passed fraction were collected and dialyzed against 20mM sodium phosphate buffer, pH-7.0, containing 0.2%Triton X-100, 0.1mM PMSF, 0.1mM EDTA. After dialysis, it was applied to a CM-cellulose (3 x 20 cm) which had been equilibrated with the same buffer as used for dialysis. Membrane –bound cytochrome *c* was adsorbed on the column, then it was eluted by a linear gradient of 200 ml each of the same buffer containing 0.3 M NaCl. Separation of the membrane-bound cytochrome *c* –551 by the column chromatography was profile in Fig.5. Eluted cytochrome *c* fraction was collected, dialyzed and the concentrated cytochrome with using a small size CM –cellulose was used as the final purified preparation of the membrane-bound cytochrome *c*-551.

Molecular properties of the bacterial membrane bound cytochrome *c*-551: Membrane bound cytochrome *c*-551 showed an absorption peak at 410 nm in the oxidized form, while it showed peaks at 416,522 and 551 nm in reduced form (Fig. 6), Molecular weight of the bacterial membrane –bound cytochrome *c* –551 was estimated about 13,200 from SDS –polyacrylamide gel electrophoresis (Fig. 4). Heme staining showed that the membrane bound cytochrome *c*-551 preparation in the present study, had no contamination of soluble cytochrome *c* –550.

Purification of cytochrome *c* oxidase: Cytochrome *c* oxidase was extracted with Triton X-100 from membrane fraction .The cytochrome aa_3 was absorbed on the DEAE-Toyoperal column as describe above. The column was extensively washed with the same buffer as used for the equilibration to remove the membrane bound *c*-type cytochromes, the enzyme was eluted with 10mM Tris HCl buffer pH-8.0 contains 0.2 M NaCl and 1% Triton X-100. The eluted fraction which showed cytochrome *c* oxidase activity were combined and dialyzed against 10mM Tris-HCl pH-8.0, containing 1% Triton X-100. Then, the resulting solution was applied to a second DEAE-Toyoperal 650 M column (3 x 20 cm) which had been equilibrated with 10mM Tris-HCl buffer pH-8.0, containing 1% Tween 20 and 10 μM PMSF. After an extensive washing with the same buffer as used for equilibration, the enzyme absorbed on the column was eluted with a linear gradient produced from 400 ml each of the same buffer for washing and the buffer containing 0.2 M NaCl (Fig. 7). Cytochrome *c* oxidase fraction was pooled and dialyzed against 10mM Tris-HCl buffer, pH-8.0, contains 1% Tween 20, 10 μM PMSF. Finally, the enzyme preparatbn was concentrated with a small size DEAE-Toyoperal column and used as for purification preparation.

Molecular properties of the bacterial cytochrome *c* oxidase: The absorption spectra of the purified cytochrome *c* oxidase peaks appeared at 422 and 600 nm in the oxidized form of the enzyme (Fig. 8). Complete reduction of the required more than 10 min when dithionite was used as the artificial reductant. The α and β peaks were shifted 605 to 443 nm respectively (Fig.8).

The enzyme was composed of subunits of the molecular weights of 40,000 and 27,000, which were calculated on SDS-polyacrylamide gel electrophoresis (Fig. 9). The protein with molecular weight of 80,000,seems to be aggregates of subunit I.

Kinetic analysis of oxidation of the bacterial cytochrome *c* catalyzed by cytochrome *c* oxidase: Oxidation of soluble ferro-cytochrome *c*–550 and the membrane bound ferrocytochrome *c*-551 catalyzed by *N.winogradskyi* cytochrome *c* oxidase were measured by the method as described in