Purification and Some Enzymatic Features of Cytochrome-c Oxidase from Facultative Alkalophilic *Bacillus* YN-2000 Grown at pH 8

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(Received 28th May, 1990, revised 28th April, 1991)

Summary: A cytochrome-c oxidase was highly purified from the Facultative Alkalophilic bacterium, Bacillus YN-2000, grown at pH 8, by ion exchange chromatography, gel-filteration and hydrophobic chromatography. The enzyme contained 8.5 n mol heme a/mg protein. It contained 1.2 mol of protoheme, 1 mol of heme c, 2.0 g atoms of copper, 2.3 g atoms of iron and 2.1 g atoms of magnesium per mol of heme a. The enzyme molecule found to be composed of two subunits with Mrs of 52,000 and 41,600 and the molecular weight of the enzyme was estimated to be 93,600 per minimal structural unit. Only protoheme reacted with CO and CN^-. Heme a did not react with CO; cytochrome-a_3 did not seem to be present in the enzyme. The enzyme oxidized 314 mol of horse ferrocytochrome-c per heme a per sec at pH 6.5. A change in an alkaline pH of growth medium did not make any effect upon the cytochrome contents of the oxidase.

Introduction

During recent years, bacteria belonging to Thermophilic and Alkalophilic families gained much attentions due to the specialities of their respiratory chain components that make them enable to survive in an extra ordinary severe growth conditions. Particularly much efforts have been given to elucidate the structural-functional mechanism of cytochrome-c oxidase, an enzyme known to catalyze oxidation of ferrocytochrome-c by molecular oxygen along with concomitant transformation of energy to an electrochemical proton gradient [1-3].

The possibility about the occurrence of an aco-type cytochrome-c oxidase had already been reported by Sone et al [4] with a Thermophilic bacterium PS3. However, it was revealed that the amount of air supplied to the culture medium controlled the relative distribution of cytohrome contents; namely cytochrome-o production was enhanced under oxygen limited conditions, while cytochrome-aa_3-type cytochrome-c oxidase was dominated under sufficiently aerated conditions.

In our recent findings that Facultative Alkalophilic bacterium, *Bacillus* YN-2000, grown at pH 10, produces aco-type cytochrome-c oxidase [5] but not aa_3-type oxidase despite the cultivation of this bacterium with vigorous aeration, under which bacterium such as *N. winogradski* and *N. europaea* produced aa_3-type cytochrome-c oxidase suggested that there may be some obstacles in the transport of oxygen through cytoplasmic membrane of the bacterium. In present investigation, we cultivated facultative alkalophilic *Bacillus* YN-2000 at pH 8, to evaluate a possible significant effect by the growth medium pH in the transport of oxygen through the cytoplasmic membrane of the bacterium, that may consequently result in an alteration in the cytochrome contents of the oxidase. We obtained the same aco-type oxidase with almost similar enzymatic characteristics as had been observed in the case of Facultative Alkalophilic Bacillus YN- 2000, grown at pH 10.

Experimental

Organism

The facultative alkalophilic bacterium, *Bacillus* YN-2000, used in the present study was kindly provided by Drs. Y. Nosoh and N. Koyama (Tokyo Institute of Technology, Tokyo). The bacterium was aerobically grown in the medium as described in [6]. The large scale cultivation of the organism was performed in 500 litres of the above medium with a stainless steel fermenter of 600 liters volume.

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Reagents

Horse cytochrome-c (Type VI) and cardiolipin (from bovine heart) were purchased from Sigma Chemical (U.S.A). DEAE-Toyopearl was product of Tosoh Corporation (Tokyo), and Tween 20 was a product of Wako Pure Chemical Industries (Osaka). Hydroxylapatite was prepared according to the method describe in [7]. All other chemicals were of the highest grade commercially available.

Physical and Chemical Measurements

Absorption spectra were recorded with Hitachi model 220A and Schimadzu model MPS-2000 spectrophotometer, using a 1 cm light path cuvette at room temperature. The contents of heme a. protoheme, and heme c in the oxidase were determined on the basis of millimolar extinction coefficients at the α-peak of their pyridine ferrohemochromes; 26[8], 34.4 [9], and 29.1 [10] for the hemochromes of heme a, protoheme, and heme c, respectively. The contents of heme a and protoheme were determined with HCl-acetone extract of the enzyme, while that of heme c was determined with the residue obtained after the extraction with the HCl-acetone of the enzyme. The metal content of the enzyme was determined by atomic absorption spectrophotometer.

The CO complex of the oxidase was prepared by incubating the reduced enzyme under 100% CO atmosphere in a Thunberg-type cuvette for 10 min or more. The CN-complex of the reduced enzyme was prepared by adding anaerobically to 10 mM KCN to 2.8 μM oxidase in a Thunberg-type cuvette and then the oxidase was reduced by the addition of small amount of $Na_2S_2O_4$, as the CN-complex was very autoxidizable.

The purity of the enzyme was analyzed by native polyacrylamide gel electrophoresis in the presence of Triton X-100 by the method of Davis [11]. Estimation of apparent molecular weight was performed by polyacrylamide gel electrophoresis in the presence of SDS by the method of Laemmli [12]. The sample for SDS-polyacrylamide gel electrophoresis was prepared by incubating the enzyme with 2% SDS in the presence or absence of 5% β-mercaptoethanol at 25°C for 24 h before being subjected to electrophoresis. The presence of heme a and heme c and the oxidase activity in the gel were detected by using heme-staining reagents [13] and "Nadi" reagents [14], respectively.

In estimation of the apparent molecular weight by SDS-gel electrophoresis, horse cytochrome-c (Mr 12,500), carbonic anhydrase (Mr 29,000), ovalbumin (Mr 43,000), bovine serum albumin (Mr 68,000), and Phosphorylase a (Mr 94,000) were used as the marker proteins. Protein content was determined by the method Lowery *et al* [15] with slight modifications [16].

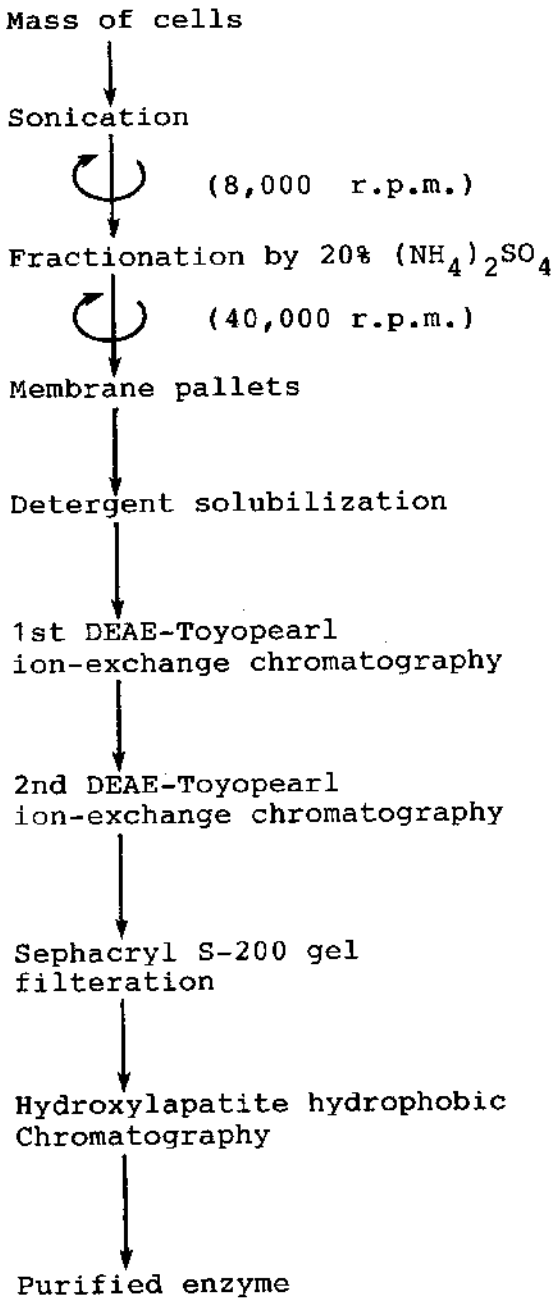
Assay of Oxidase Activity

The reaction mixture for determination of the cytochrome-c oxidase activity of the enzyme contained 7.2 μM horse ferrocytochrome c, 2-10 nM heme a of the enzyme, and 25 mM sodium phosphate buffer, pH 6.5 in total volume of 1.0 ml. The reaction was measured spectrophotometrically; the decrease in the absorbance at 550 nm was followed with time. In the determination of the oxygen consumption activity, the reaction mixture contained 145.5 μM horse ferrocytochrome c, 6.8 μM heme a of the enzyme and 25 mM sodium phosphate buffer, pH 6.5 in a total volume of 2.0 ml. The oxygen consumption by the oxidase was measured with Clark-type oxygen electrode (Oxygen analyzer, MP-1000, Iijima Products, Tokyo), equipped with a temperature controlled cell.

Results and Discussion

Purification of Oxidase

(Flow scheme 1) Frozen Cell (about 100 g in a centrifugally packed state) were suspended in 120 ml) of 10 mM Tris-HCl buffer, pH 8.0 containing 10 mM disodium-EDTA and 10 μM PMSF. The suspension was treated three times with a sonic oscillator for a total period of 30 min and centrifuged at 10,000 x g for 15 min. Ammonium sulphate was then added to the supernatant up to 20% saturation followed by centrifugation at 105,000 x g for 60 min. The reddish pellets obtained (about 12.5 g) were suspended in 100 ml of Tris-HCl buffer, pH 8.0, containing 0.3 M KCl and centrifuged at 187,000 x g for 60 min to remove the soluble proteins. The pallets thus obtained (6.4 g) were again suspended in 80 ml of 10 mM Tris-HCl buffer, pH 8.0 and dialyzed against 1 litre of 10mM Tris-HCl buffer,



Flow Scheme I: Purification of cytochrome-c oxidase from Bacillus YN-2000 grown at pH 8.

pH 8.0 for 12 h at 4°C. To the dialyzed suspension. Triton X- 100 was added up to final concentration of 1.5% and the suspension thus treated was incubated for 30 min at 2°C followed by centrifugation at 105,000 x g for 60 min. The reddish suspension thus obtained was subjected to Ist chromatography on a DEAE-Toyopearl column (2.8 x 8.5 cm) which had been equilibrated with 10 mM Tris-HCl buffer, pH 8.0, containing 1% Triton X-100. The enzyme adsorbed at the column was eluted with the linear gradiant solution produced from 600 ml each of 10 mM Tris-HCl buffer, pH 8.0, containing 1% Triton X-100 and the buffer containing 1% Tirton X-100 and 0.3 M NaCl. A large amount of various cytochrome-b and some cytochrome c were removed by the chromatography (Fig. 1A). The eluates which contained both cytochrome-a and cytochrome-c were dialyzed against 1 liter of 10 mM Tris-HCl buffer, pH 8.0, containing 1% Triton X-100, and then subjected to 2nd chromatography on a DEAE Toyopearl column (2.8 x 14.5 cm) that had been equilibrated with the same buffer as used for the above dialysis. The adsorbed enzyme was eluted with a linear gradiant (0.2 -0.4 M NaCl) produced in 600 ml of buffer used for the equilibration. The enzyme solution was then subjected to the gel filtration with a Sephacryl S-200 column which had been equilibrated with 50mM Tris-HCl buffer, pH 8.0, containing 1% Triton X-100 and 0.25 M NaCl. The elutes containing both cytochrome-a and cytochrome-c from the column (Fig. 1, B) were dialyzed against 2 litres of 10 mM Tris-HCl buffer, pH 8.0 for 5 h and then applied to a hydroxylapatite column (2.2 x 7 cm) that had been equilibrated with 5 mM Na-phosphate buffer, pH 8.0, containing 0.3% Triton X-100. The column was washed firstly with equilibrating buffer (100 ml), next with 10 mM Na-phosphate buffer, pH 8.0 (100 ml) containing 0.4% Triton X-100, and then with 50 mM Na-phosphate buffer, pH 8.0 (100 ml) containing 0.5% Triton X-100. Finally the oxidase was eluted with 100 mM Na- phosphate buffer, pH 8.0 containing 0.5% Triton X-100. The purification steps are summarized in flow scheme 1 and in Table 1. The purified enzyme contained 8.5 nmol heme a/mg protein, and the total recovery of activity was 7.8%.

Spectral Properties

The absorption spectra of the purified cytochrome-c oxidase is shown in (Fig. 2 A). The oxidized form of the enzyme showed absorption peaks at 410, 530, and 595 nm, and its reduced form prepared by the addition of a small amount of $Na_2S_2O_4$ showed peaks at 417,521,550 and 603 nm

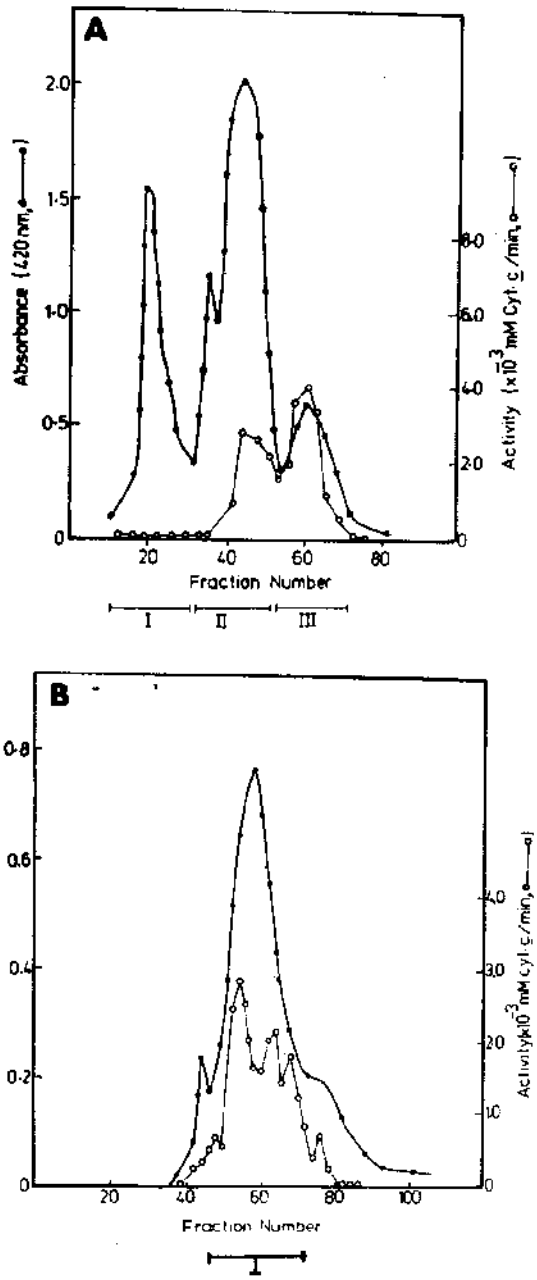


Fig.1: (A) Elution profile of Ist DEAE-Toyopearl chromatography. Cytochrome-c oxidase activity was measured with each of fractions as described in Materials and Methods and expressed as μM ferrocytochrome c oxidized per min. I, Cyt. b; II, Cyts. b and c III, Cyt. a. (B) Elution profile in Sephacryl S-200 gel-filteration chromatograph. Cytochrome-c oxidase activity was measured as described in (A).

Table 1: Purification of cytochrome-c oxidase from Bacillus YN- 200 cultivated at pH 8.

Experimental Steps	Protein Conc. mg/ml	Total Vol. ml	Total Protein mg	Heme a/ Protein nmol/mg	Specific Activity[a]	Recovery[b] (%)
Fraction solubilized with Triton X-100	5.8	95	551	0.298	245	100.00
Ist DEAE-Toyopearl	1.0	75	75	1.23	1030	57.2
2nd DEAE-Toyopearl	0.39	55	21.4	2.89	2175	34.5
Sephacryl S-200	0.19	49	9.3	4.5	3062	21.1
Hydroxyl-apatite	0.14	22	3.0	6.8	4112	9.3
Finally purified	0.9	2.8	3.7	8.5	4222	7.8

[a] μmol of ferrocytochrome c (horse) oxidized per μmol of the oxidase per min.

[b] Calculated from the total activity (μmol of ferrocytochrome c (horse) oxidized per min).

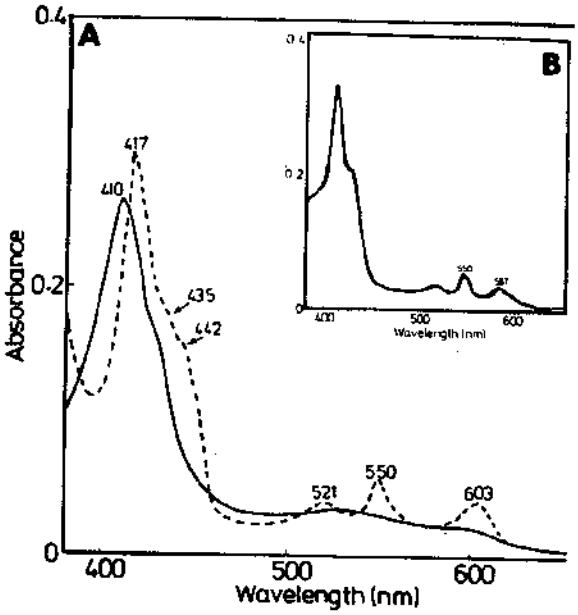


Fig.2: (A) Absorption spectrum of Bacillus YN-2000 cytochrome-c oxidase. The enzyme was dissolved in 10μM Tris-HCl buffer, pH 8.0, containing 0.5% Triton X-100, at the concentration of 2.1 μM heme a. ----- oxidized from ——— reduced with $Na_2S_2O_4$. (B) Absorption spectrum of pyridine ferrohemochrome. To 8.2 mM heme a were added 0.1 N NaOH, 5% pyridine, and a small amount of $Na_2S_2O_4$.

and shoulders at around 435 and 442 nm. The spectral properties suggested that the oxidase contains heme-c as well as heme-a. As the spectra did not show any distinct peak around 440 nm as had observed in case of Thermophilic bacteria [17-18] but showed a shoulder around 560 nm, the presence of protoheme in the oxidase was suspected.

The pyridine ferrohemochrome of the enzyme showed absorption peaks at 550 and 587 nm (Fig. 2, B) and the HCl-acetone extracts of the enzyme showed peaks at 553 and 586 nm (Fig. 3,A), whereas the hemochrome of the precipitate obtained when the enzyme was treated with HCl-acetone showed a peak at 549 nm (Fig. 3, B). It is evident from the absorption peaks of pyridine ferrohemocromes, that the enzyme contains protoheme, heme-c and heme-a.

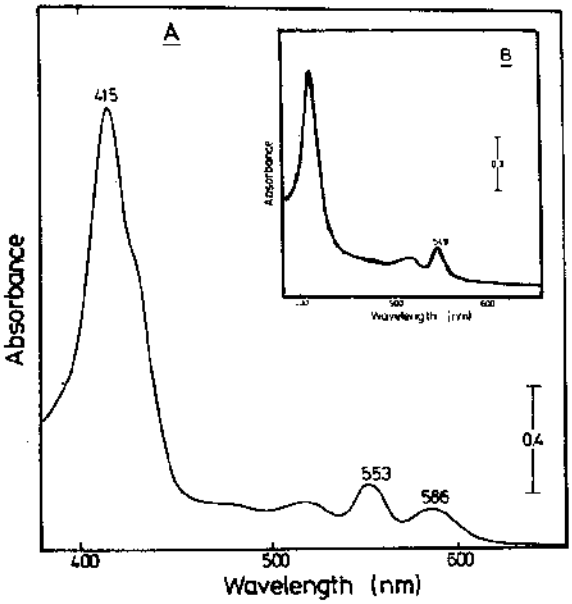


Fig.3: (A) Absorption spectrum of pyridine ferrohemochrome of HCl-acetone extracts from Bacillus YN-200 cytochrome-c oxidase and (B) the hemochrome of the precipitate obtained on treating the enzyme with HCl-acetone. The precipitate was dissolved in 0.1 N NaOH, and the final volume was the same as that of (A).

Effect of ligands

Fig. 4 shows the effect of CO on the absorption spectrum of the reduced enzyme. When CO introduced in the reduced form of oxidase, the absorbance of the γ-peak at 417 nm increased markedly, a distinct peak appeared at around 443

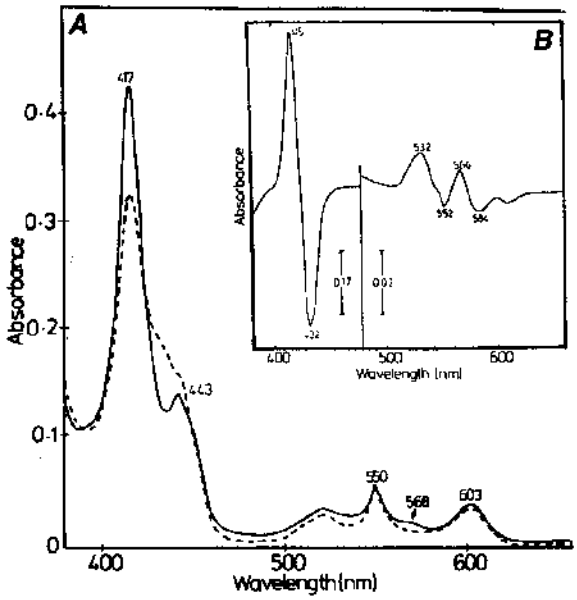


Fig.4: (A) Absorption spectrum of CO-complex of Bacillus YN-2000 cytochrome-c oxidase. The solution of the reduced enzyme used for the experiment in Fig. 2 was incubated under a 100 % CO atmosphere in a Thunberg-type cuvette. (B) Difference spectrum, reduced + CO minus reduced.

nm and a small broad peak appeared at around 568 nm, while the position of the peaks at 550 and 603 nm were not affected significantly (Fig. 4,A). The CO difference spectrum, reduced + CO minus reduced, of the enzyme showed peaks at 415, 532 and 566 nm and troughs at 432, 552 and 584 nm (Fig. 4 B). When CO-reduced and CO difference spectrum were taken separately at pH 7 and pH 10, no change could be observed in the absorption peaks. These results seems to indicate that heme a of the enzyme did not react with CO, while protoheme combined with the compound. Figure 5 shows the absorption spectrum of CN-enzyme complex. When 10 mM KCN was added to the resting form of the enzyme, the absorption peaks at 409 nm shifted to 413 nm. Subsequent reduction with $Na_2S_2O_4$ caused the peak at 413 nm to shift to 416 nm. The peaks at 550 and 603 nm were not affected by CN^- (Fig. 5). These results suggest that heme a and heme c in the oxidase do not react with cyanide, either.

Catalytic Properties

When 163.3 μM horse ferrocytochrome-c was oxidized by the enzyme, 37.6 μM oxygen was consumed; the ratio of ferrocytochrome-c oxidized to

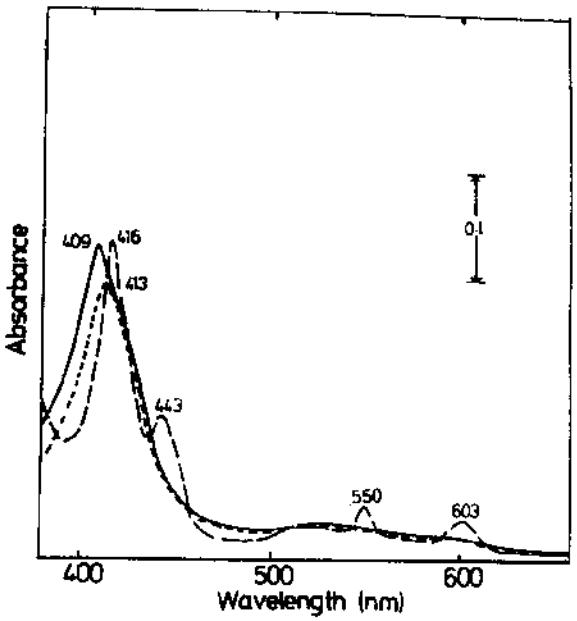


Fig.5: Effect of KCN on the absorption spectrum of Bacillus YN- 2000 cytochrome-c oxidase. The enzyme was dissolved in a buffer as described in the ligend to Fig. 2. In order to prepare the CN- complex of the reduced enzyme, 10 μM KCN was added to the oxidized enzyme and then the enzyme was reduced with a small amount of $Na_2S_2O_4$. ----,oxidized; ----, oxidized + KCN: ----, CN- complex was reduced with $Na_2S_2O_4$.

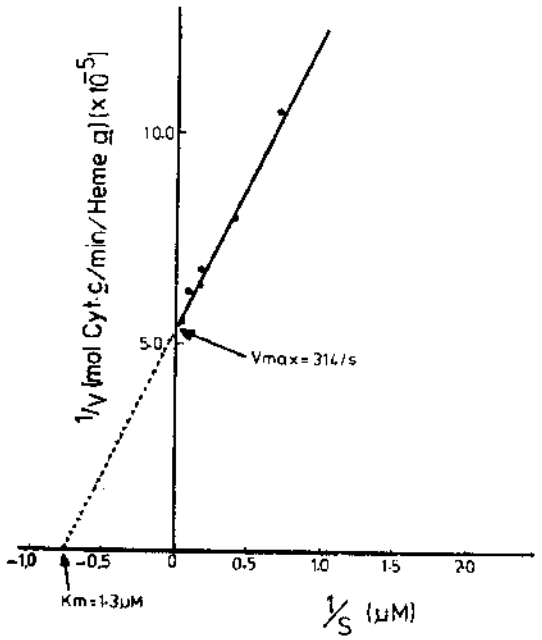


Fig.6: Lineweaver-Burk plot for the oxidiation of horse ferrocytochrome-c by Bacillus YN-2000 cytochrome-c oxdase. The reaction mixture contained 25 μM phosphate buffer, pH 6.0, 4.1 μM oxidase and various amounts of cytochrome c (2.5-30 μM).

oxygen consumed was 4.3:1. One mole of the enzyme oxidized 314 mol of horse ferrocytochrome-c per sec at pH 6.5 and Km for hourse ferrocytochrome-c of the enzyme was 1.3 μM (Fig. 6).

Effect of added lipids

The effect of added lipids on oxygen consumption by the enzyme was determined by use of the Clark-type oxygen electrode. The reaction mixture contained 6.45 μM horse cytochrome-c, 0.24 μM oxidase. 0.62 mM Na-ascorbate and 0.12 mM TMPD, and 25 mM Na-phosphate buffer, pH 6.5 in a total volume of 2.0 ml. The reaction was started by the addition of oxidase. Thirty seconds after the reaction had been started, cardiolipin (49 g/ml) was added. The oxygen consumption was increased about 1.9 fold. When the cardiolipin was replaced by the Tween 20 (2 mg/ml), no stimulating effect could be observed.

Subunit Structure and Heme contents

When the enzyme treated with 2% SDS in the absence of β-mercaptoethanol was subjected to SDS-gel electrophoresis, bands corresponding to protein species with Mrs of 52,000 and 41,600, respectively were observed on staining with Coomassie Brilliant Blue. The band for the protein species of lower molecular weight was a little diffused (Fig. 7). When the enzyme treated with 2% SDS and 5% β- mercaptoethanole for 24 h was subjected to SDS-gel electrophoresis, the band corresponding to Mr of 41,600 was converted into a distinct band. When the same gel was treated with the heme staining reagent, one band corresponding to Mr of 52,000 was stained (results not shown). On the basis of the above results, the molecular weight estimated to be 93,600 per minimal structural unit. The purified enzyme contained 8.5 nmol heme a/mg protein. It contained 1.23 mole of protoheme, 2.0 g atoms of copper, 2.5 g atoms iron, and 1.8 g atoms magnesium per mol of heme a.

Our previous findings have shown that, bacterium Facultative Alkalophilic *Bacillus*, YN-2000, grown at pH 10, possess a novel aco-type cytochrome-c oxidase, in which the ligand binding site is cytochrome-o instead of cytochrome-a_3 [5] as has been commonly found among Alkalophiles and Thermolphile [2-3]. In the present investigation,

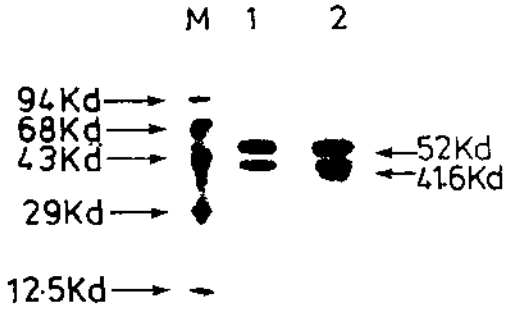


Fig. 7: SDS-gel electrophoresis of Bacillus YN-2000 cytochrome-c oxidase. The gel was stained with Coomassie Brilliant Blue. Lane 1, in the presence of β-mercaptoethanol. M, marker proteins; from top to bottom, phosphorylase a (Mr 94,000), bovine serum albumin (mr 68,000), ovalbumin (Mr 43,000), carbonic anhydrase (Mr 29,000), and horse cytochrome c (Mr 12,500).

strain of Alkalophilc *Bacillus* YN-200 was grown at pH 8 and the cytochrome-c oxidase obtained is exhibiting the same structural and functional characteristics as had been found in the case of Facultative Alkalophilic *Bacillus* YN-2000, grown at pH 10. The oxidase purified in the current study contain 1 mole of each heme-a, protoheme and heme c in the minimal structural unite (Mr 93,600), but it does not show any trace of heme-a3 component, whereas heme-a constituent of the oxidase does not react with CO or CN^-. It is clearly evident by the CO-absorption spectra that the absorption peak at 550 nm attributable to heme-c is not effected by CO, while a broad peak appears at 568 nm suggesting that protoheme has reacted with CO. Furthermore, when the absorption spectrum of CO reduced and CO difference spectrum were taken at pH 7 and pH 10, no significant change could be observed. The enzyme shows high cytochrome-c oxidizing activity; Lineweaver-Burk plot showed that 1 mole of the enzyme oxidizes 314 mol of horse ferrocytochrome-c per sec at pH 6.5.

Although the enzyme does not show presence of heme a3, it reduces oxygen to water with ferrocytochrome-c as the ratio of (in mole) ferrocytochrome-c oxidized to O_2 consumed is 4:1. As it is clearly evident from the absorption spectra that heme a and heme c of the enzyme do not react with CO and CN^-, thus the enzyme will react with O_2 at protoheme. Furthermore, the enzyme contains 2.0 g atoms of copper per mole of heme a, it is suggested that metal will also participate in the reduction of O_2 as has been observed in the case of aa3-type cytochrome-c oxidase.

Previously it was suggested that under limited aeration conditions during the growth of bacterium, cytochrome-o probably substituted by cytochrome a3 [3], resulting in the appearance of cytochrome-o as a constituents of oxidase enzyme. By considering these possibilities, bacterium in the present investigation was cultivated with sufficient aeration conditions, under which bacteria such as *N. winogradski* and *N.europaes* grows spontaneously and produces aa3-type cytochrome-c oxidase. To explore a cause of aco-type cytochrome-c oxidase production instead of aa3-type oxidase in Facultative Alkalophilic *Bacillus* YN-200, it was assumed that the high pH of the growth media may exert some hindrence in the transportation of O_2 through the cytoplasmic membrane of the bacterium, subsequently resulting in substitution of cytochrome-a3 by cytochrome-o. For this reason we cultivated the same Alkalophilic strain of bacteria at lower alkaline pH, i.e. pH 8, and obtained the same oxidase as had been purified from the Facultative Alkalophilic *Bacillus* grown at pH 10. These data further confirm the existence of a novel aco-type cytochrome-c oxidase among the Facultative Alkalophilic *Bacillus* YN-2000 strain and revealed that the high alkaline pH of the growth medium does not play any significant role in the hindrence of O_2 transportation through the cytoplasmic memberane of the bacterium.

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