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Over-expression of *cbaAB* genes of *Bacillus stearothermophilus* produces a two-subunit SoxB-type cytochrome *c* oxidase with proton pumping activity

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Abstract

We constructed expression plasmids containing *cbaAB*, the structural genes for the two-subunit cytochrome bo_3-type cytochrome *c* oxidase (SoxB type) recently isolated from a Gram-positive thermophile *Bacillus stearothermophilus*. *B. stearothermophilus* cells transformed with the plasmids over-expressed an enzymatically active bo_3-type cytochrome *c* oxidase protein composed of the two subunits, while the transformed *Escherichia coli* cells produced an inactive protein composed of subunit I without subunit II. The oxidase over-expressed in *B. stearothermophilus* was solubilized and purified. The oxidase contained protoheme IX and heme O, as the main low-spin heme and the high-spin heme, respectively. Analysis of the substrate specificity indicated that the high-affinity site is very specific for cytochrome *c*-551, a cytochrome *c* that is a membrane-bound lipoprotein of thermophilic *Bacillus*. The purified enzyme reconstituted into liposomal vesicles with cytochrome *c*-551 showed H^+ pumping activity, although the efficiency was lower than those of cytochrome aa_3-type oxidases belonging to the SoxM-type.

Keywords: SoxB-type heme-copper oxidase; Cytochrome bo_3; Respiratory chain; Cytochrome *c*-551; Proton pump; (*Bacillus stearothermophilus*)

1. Introduction

Gram-positive spore-forming thermophilic bacilli, such as *Bacillus* PS3 and *Bacillus stearothemophilus* (*Bst*) contain cytochrome caa_3-type cytochrome *c* oxidase as the main respiratory terminal oxidase [1–3]. Cytochrome caa_3 belongs to the SoxM-type heme-copper oxidase family [4,5] based on its primary sequence and enzymatic function [1,6]. *Bacillus* caa_3-type oxidase contains *c*-type cytochrome as an extra domain covalently fused to the C-terminus of subunit II [6], and addition of another cytochrome *c* hardly accelerates electron transfer to the caa_3-type oxidase from the b_6c_1-type quinol-cytochrome *c* oxidoreductase [7,8], or from an artificial electron donor, TMPD [9]. Cytochromes caa_3 and b_6c_1 form a quinol-oxidizing supercomplex, and they constitute the main part of the respiratory chain in these bac-

Abbreviations: *Bst*, *Bacillus stearothermophilus*; CCCP, carbonylcyanide *m*-chlorophenyl-hydrazone; MEGA 9+10, 1:1 mixture of *n*-nonanoyl *N*-methylglucamide and *n*-decanoyl *N*-methylglucamide; ORF, open reading frame; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine

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teria [10]. We also found two alternative oxidases in the *Bst* K17 mutant strain, which was derived from a wild-type *Bst* K1041 and lacks a caa_3-type oxidase [11]. One of these oxidases is quinol-oxidizing cytochrome *bd* [11,12] and the other is a novel terminal oxidase that catalyzes the oxidation of cytochrome *c*-551 [13,14]. Cytochrome *c*-551 is a membrane-bound lipoprotein with a molecular mass of 10.5 kDa [15]. The $b(o/a)_3$-type cytochrome oxidase, detected as an *o*-type oxidase in *Bacillus* PS3 for the first time [16], has been purified from *Bst* K17 [13]. This oxidase is composed of two subunits with apparent molecular masses of 56 and 19 kDa, as determined by a Ferguson plot, and it contains protoheme IX, heme O, heme A and Cu in a ratio of 1:0.7:0.2:3 [13]. CO difference spectra indicated that the high-spin heme is mainly heme O and partly heme A. This heterogeneity is the reason why the enzyme was designated as cytochrome $b(o/a)_3$. The enzyme oxidized cytochrome *c*-551 with a maximum velocity (V_{max}) of 190 s^{-1} and an affinity (K_m) of 0.14 μM for cytochrome *c*-551 under high ionic strength conditions [13].

DNA sequencing and Northern blotting analyses indicated that the two genes, *cbaA* and *cbaB*, compose an operon and encode subunits I (61 kDa) and II (17 kDa), respectively [14]. The deduced amino acid sequence shows the highest similarity to the ba_3-type oxidase of *Thermus thermophilus* [17,18], indicating that it belongs to the SoxB group [19,20]. SoxB-type oxidases, which have a single transmembrane helix in their subunit II, have been identified and cloned from archaea, such as *Sulfolobus acidocaldarius* [21], *Acidianus ambivalens* [22] and *Natronobacterium pharaonis* [23] in addition to *T. thermophilus* [18], while the majority of the heme-copper oxidases including the mitochondrial ones are the SoxM-type, whose subunit II contains two transmembrane helices. All cytochrome *c* oxidases, including *Bst* cytochrome bo_3, conserve the $-HX_nCXEXCX_3HX_2M-$ motif as the Cu_A-ligating site, except that the glutamate residue is substituted with glutamine in *T. thermophilus* ba_3-type oxidase [18]. Site-directed mutagenesis experiments with *Paracoccus denitrificans* aa_3-type oxidase demonstrated that this glutamate is indispensable for the binding of Mg^{2+}/Mn^{2+}, which stands at the interface between subunits II and I, and the site-directed mutant (E247Q in subunit II) showed a reduced turnover number of cytochrome *c* oxidation [24].

The SoxB-type oxidases are also different from the usual SoxM-type cytochrome *c* oxidases in lacking the so-called D-channel structure for H^+ translocation in subunit I, while amino acid residues ligating to heme irons are common with the SoxM-type [20]. In this respect, cytochrome ba_3-type oxidase from *T. thermophilus* was recently reported to oxidize cytochrome *c*-552 of the bacterium [25] and to pump H^+, although with a less efficiency than SoxM-type oxidases [26]. We have now succeeded in over-expressing *cbaAB* genes in *Bst*, which possibly opens a way to elucidate the structure–function relationship of the H^+-pumping heme-copper oxidase family from a new angle. Here we report the over-expression system of *Bst* bo_3-type oxidase and the characterization of the over-produced enzyme, including its H^+ pumping activity.

2. Materials and methods

2.1. Materials

The transformable strain K1041, kindly given by Dr. I. Narumi [27], was used as *Bst* unless otherwise described. An expression plasmid for this bacterium, pSTE12 [28], was used to construct the plasmids for over-production of *Bst* cytochrome bo_3. Cytochrome *c*-551 of *Bacillus* PS3 was over-expressed in *Bst* K1041 and purified as previously described [29]. MEGA 9 and MEGA 10 were purchased from Dojin (Kumamoto). DEAE-Toyopearl anion exchange gel, hydroxyapatite and TMPD were obtained from Tosoh (Tokyo), Bio-Rad (Hercules), and Wako (Kyoto), respectively. Proteins used as molecular mass standards and cytochromes *c* of yeast and bovine heart were purchased from Sigma (St. Louis). Ferrocytochrome *c* was prepared as described previously [1]. Other reagents were of analytical grade.

2.2. Construction of expression plasmids for E. coli and Bst

First we prepared pUCcba1 for the expression in *E. coli* by introducing the *Nde*I fragment of the pCBA31 plasmid to *Nde*I *plus Sma*I-cut pCBA21,

which covers 3 kbp DNA including the *cbaAB* operon and the ORF 5′-upstream of the operon (cf. Fig. 1 of [14]). Gene manipulations were performed as described in [30]. The whole *Bst* DNA dissected from pUCcbaI was introduced into pSTE12 [27] for the expression in *Bst* by using *Eco*RI and *Pst*I at the multi-cloning sites. The resulting plasmid was named pSTEcba1. We also prepared pUCcba2 and pSTEcba2 from pUCcba1 by deleting the upstream ORF as follows: a pair of primers, 5′-GTBCARCC-GYTSGARAARGT-3′ (ba-1) and 5′-ARMACVAC-RCGYTCYTGCGG-3′ (ba-2), were designed for replacement at the *Nde*I site (at 1128th in the cloned genomic DNA (in Fig. 1 of [14]). The polymerase chain reaction was carried out with pUCcba1 as the template, and the product was used for deletion of the ORF portion of pUCcba1. pSTEcba2 was prepared from pUCcba2 just as pSTEcba1 was prepared from pUCcba1. The structures of pSTEcba2 for the expression in *Bst* are shown in Fig. 1.

2.3. Transformation and cell culture

Bst was transformed by electroporation using a Bio-Rad Gene Pulser apparatus with a pulse controller by the method of Narumi et al. [28] as described previously [29]. Preculture in a test tube containing L-broth with 2 μg/ml tetracycline was carried out overnight at 48°C. A 3-ml aliquot was inoculated to 500 ml culture medium consisting of 2×L-broth containing 0.2% KH_2PO_4 and 2 μg/ml tetracycline in a baffled flask (1 liter), and the culture was carried out for 12 h with moderate shaking (120 rpm). Cells were then harvested and membranes were prepared as described previously [1].

2.4. Purification of cytochrome bo_3 and reconstitution into vesicles

Cytochrome bo_3 was prepared from the wild-type cells transformed with pSTEcba2 essentially as described previously for the purification of the enzyme from the caa_3-deficient *Bst* strain K17 [13], except that the following step was added: the over-expressed membrane fraction (1 g protein), having been washed twice with 2% (w/v) Na-cholate, was solubilized at 10 mg protein/ml with a 1:1 mixture of 0.5% (w/v) *n*-nonanoyl *N*-methylglucamide (MEGA 9) and *n*-decanoyl *N*-methylglucamide (MEGA 10) (MEGA 9+10) in 100 mM NaCl, 1 mM EDTA, and 20 mM Na-Pi buffer, pH 6.0 as described previously [13]. The solubilized membranes were centrifuged at 140 000×*g* for 40 min, and the supernatant obtained was applied to a DEAE-Toyopearl column (1.6×4 cm) without dialysis to absorb the cytochrome caa_3. The pass-through fraction containing cytochrome bo_3 was dialyzed against 20 mM Na-Pi buffer, pH 6.0, and the succeeding steps were carried out as previously described [13].

Reconstitution into liposomal vesicles (0.5 ml) was carried out using cytochrome bo_3 (0.30 nmol), cytochrome *c*-551 (0.33 nmol) and soybean P-lipids (20 mg) by the freeze–thaw–sonication method [31].

2.5. Measurement of oxidase activity and optical spectrum

Oxidase activity was polarographically measured with an oxygen electrode (YSI 4500) in a semi-closed glass cell (2.5 ml) at 40°C. The standard reaction medium contained 15 nM b_3-type oxidase, 0.4 μM cytochrome *c*-551, 125 μM TMPD, 5 mM Na-ascorbate, 1 mM $MgSO_4$, 150 mM KCl and 1 mM Na-Pi buffer, pH 6.7. The reaction was initiated by adding cytochrome *c*-551 of *Bacillus* PS3 to 2.5 ml of the reaction mixture containing the other constituents with stirring. Absorption spectra were measured by a Beckman DU-70 spectrophotometer. Air-oxidized enzyme was taken and then a few grains of solid sodium dithionite were added to it to obtain its reduced form. Contents of cytochromes aa_3 and *c*-551 were determined from reduced *minus* oxidized difference spectra using millimolar extinction coefficient differences of 21.3 at 604 *minus* 630 nm [1], and 24.3 at 551 nm [15]. The content of *b*-type cytochromes was tentatively calculated using a millimolar extinction coefficient difference of 22 at 560 *minus* 575 nm. For the kinetic analyses, cytochrome *c* oxidase activity of the purified enzyme was continuously monitored with a pH electrode (Beckman 39030) in terms of the pH increment due to the difference between H^+ absorption caused by reduction of the final electron acceptor O_2 and H^+ production by oxidation of the initial electron donor ascorbate, as described elsewhere [9]. H^+ absorption was titrated by adding adequate volumes of 10 mM HCl to

each assay. TMPD oxidase activity eluted from columns was spectrophotometrically measured at 22°C by monitoring the absorbance increment at 562 nm by using a millimolar extinction coefficient, $\Delta E = 10.5$ mM^{-1} cm^{-1} [32].

2.6. Other analyses

Detection of H^+ pumping upon yeast ferrocytochrome *c* addition was carried out as described previously [31]. The protein concentration was determined as described by Lowry et al. [33] after precipitation with 5% trichloroacetic acid in the presence of 0.05% sodium deoxycholate. Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli [34] except that boiling of the protein samples was omitted.

Heme A content was calculated from the redox difference spectra of its pyridine hemochrome by the method of Berry and Trumpower [35]. The content of protoheme IX *plus* heme O was calculated as their sum using the extinction coefficient for protoheme IX [35]. Hemes were also analyzed by reverse-phase chromatography as described previously [13].

3. Results

3.1. Transformation with cbaAB operon

At first we tried to over-express cytochrome bo_3 using pSTEcba1 for *Bst* and pUCcba1 for *E. coli*, but in vain. We then constructed pSTEcba2 and pUCcba2 (Fig. 1), in which the unidentified ORF upstream of the *cbaAB* operon was eliminated. Both pSTEcba2 and pUCcba2, containing the *cbaAB* operon and its own putative promoter or about 100 bp span just upstream of the initiation codon of *cbaB*, were able to transform *Bst* and *E. coli*, respectively. Preliminary experiments showed that both the *Bst*/pSTEcba2 and *E. coli*/pUCcba2 cells harvested at the late stage of growth looked darker than the cells at the early stage or the untransformed cells. In fact, the redox difference spectra of the membrane fraction from *Bst*/pSTEcba2 at the early stationary phase showed a prominent α-peak at 560 nm, which was 3–5 times higher than the peak of the wild-type cells

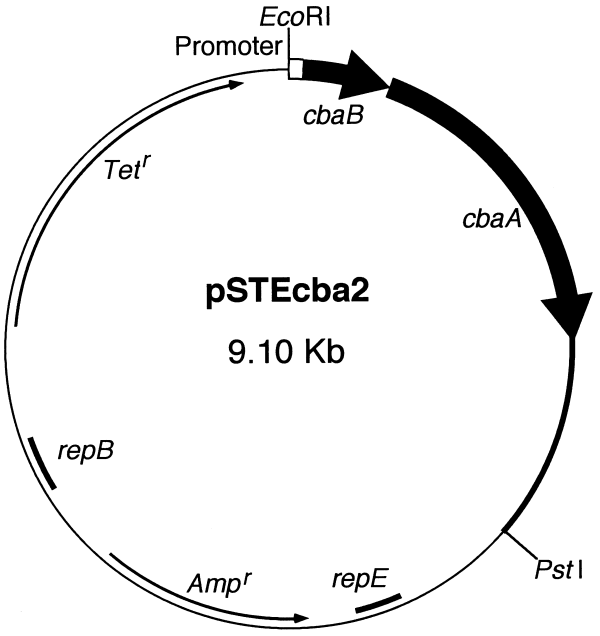

Fig. 1. Structure of pSTEcba2. Tet^r gene and ori-pAM-alpha 1 are active in *Bst* while Amp^r and ori-177 are active in *E. coli*. The *cbaAB* operon containing the natural promoter and structure genes for subunits I and II for *Bst* cytochrome bo_3 is inserted in the pSTE plasmid [27] at *Eco*RI and *Bam*HI.

per mg basis. Similar intensification of the 560 nm peak was also observed with the membrane from *E. coli*/pUCcba2.

3.2. Over-expression of two-subunit cytochrome bo_3

Since the extra formation of *b*-type cytochrome is probably due to over-expression of CbaAB, the extent of the expression of *cbaAB*, was monitored by measuring the spectra in order to optimize the growth condition. Prominent expression of cytochrome bo_3 occurred in cells at the stationary phase cultured with shaking at 120 rpm. Under this air-limited condition, the growth curve was proportional to time from 3 to 8 h after inoculation, indicating that the growth at that region was limited by the air supply, and then it reached the stationary phase. The content of *b*-type cytochromes in *Bst*/pSTEcba2 under this optimal condition was 1.5–2.5 nmol, while that of untransformed *Bst* cell was 0.4–0.7 nmol/mg membrane protein. The transformed cells cultured with vigorous shaking at 200 rpm contained about 1.0 nmol of *b*-type cytochromes at the early station-

ary phase, in which the content was higher than those in the log phases. Expression of the genes was also tried in *E. coli*. The *b*-type cytochrome content of the *E. coli*/pUCcba2 cells harvested at a late stage of growth was 0.7–1.2 nmol/mg membrane protein, while that of the untransformed cells was 0.1–0.2 nmol/mg membrane protein.

Fig. 2 shows an electrophoregram of the membrane fragments and purified cytochrome bo_3. Compared with the control membranes from *E. coli* (lane 2) and *Bst* cells (lane 4), a new band appeared at the position corresponding to an apparent mass of 43 kDa in the transformant membranes from *E. coli*/pUCcba2 (lane 3) and *Bst*/pSTEcba2 cells (lanes 5 and 6). This 43-kDa band seems to be due to the CbaA product, the subunit I of the bo_3-type oxidase, as already reported for *Bst* K17 mutant cells [13]. The 19-kDa bands due to the subunit II (CbaB) product were also observable in *Bst*/pSTEcba2 membranes (lanes 5 and 6), but was not seen in *E. coli*/pUCcba2 membranes (lane 3). The 43- and 19-kDa bands were also observed in the purified enzyme from *Bst*/pSTEcba2 (lane 7).

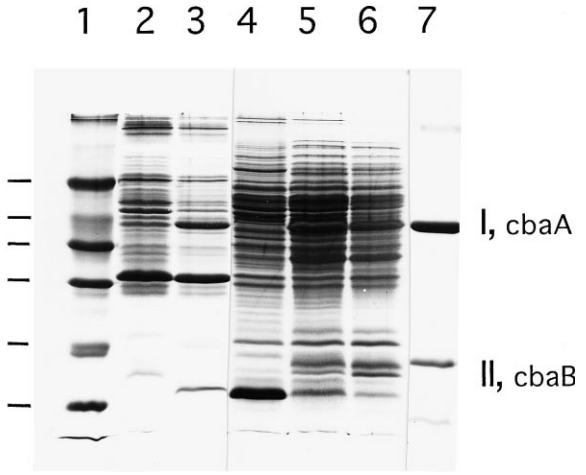

Fig. 2. SDS-PAGE patterns of CbaAB product over-expressed in *E. coli* and *Bst* cells and cytochrome bo_3 purified from the *Bst*/pSTEcba2. Coomassie brilliant blue was used for protein staining. About 50 μg of each membrane fraction was used for the electrophoresis except for lanes 1 and 6. Lane 1, marker proteins (bars at the lefthand side show 66, 45, 36, 29, 20 and 14 kDa); lane 2, *E. coli*. control membranes; lane 3, membranes from *E. coli*/pUCcba2; lane 4, *Bst* control membrane; lane 5, membranes from *Bst*/pSTEcba2; lane 6, membranes from another *Bst*/pSTEcba2; lane 7, the purified bo_3-type oxidase (5 μg).

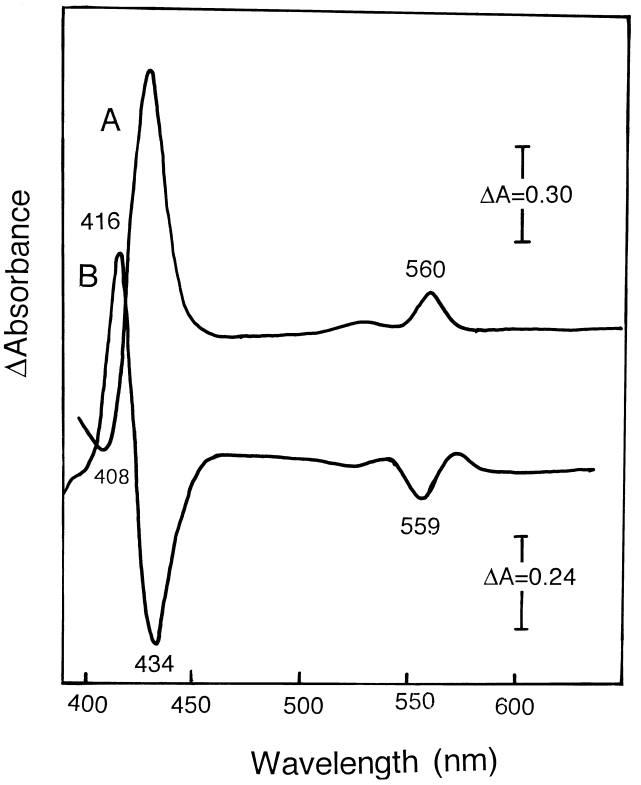

Fig. 3. Difference spectra of the bo_3-type oxidase from the transformed *Bst* cells. A, $Na_2S_2O_4$-reduced *minus* oxidized (as prepared); B, CO-reduced *minus* reduced. The enzyme used was 5.0 μM in 100 mM Tris-HCl buffer, pH 8.0.

It was rather difficult to confirm the presence of cytochrome *c*-551-dependent TMPD oxidase activity of cytochrome bo_3 [13] in wild-type *Bst* membranes, since the thermophilic bacilli contain caa_3-type cytochrome *c* oxidase that actively oxidizes TMPD, and therefore the increment of the activity due to cytochrome bo_3 appears to be relatively small. In order to confirm the expression of active cytochrome bo_3, we observed effects of cyanide concentration on the TMPD oxidase activity of the *Bst*/pSTEcba2 membranes, because the three terminal oxidases in thermophilic *Bacillus* show different susceptibility to cyanide [11,13,36]. The titration curve for cyanide inhibition of the TMPD oxidase of *Bst*/pSTEcba2 membranes was biphasic; 25% was inhibited by the addition of cyanide at concentrations lower than 10 μM, while the rest was inhibited at 50–100 μM. A low concentration of cyanide below 10 μM scarcely inhibited the respiration of the wild *Bst* cells [36]. Our previous data showed that I_{50} of the purified cytochrome bo_3 was much lower [13] than that of

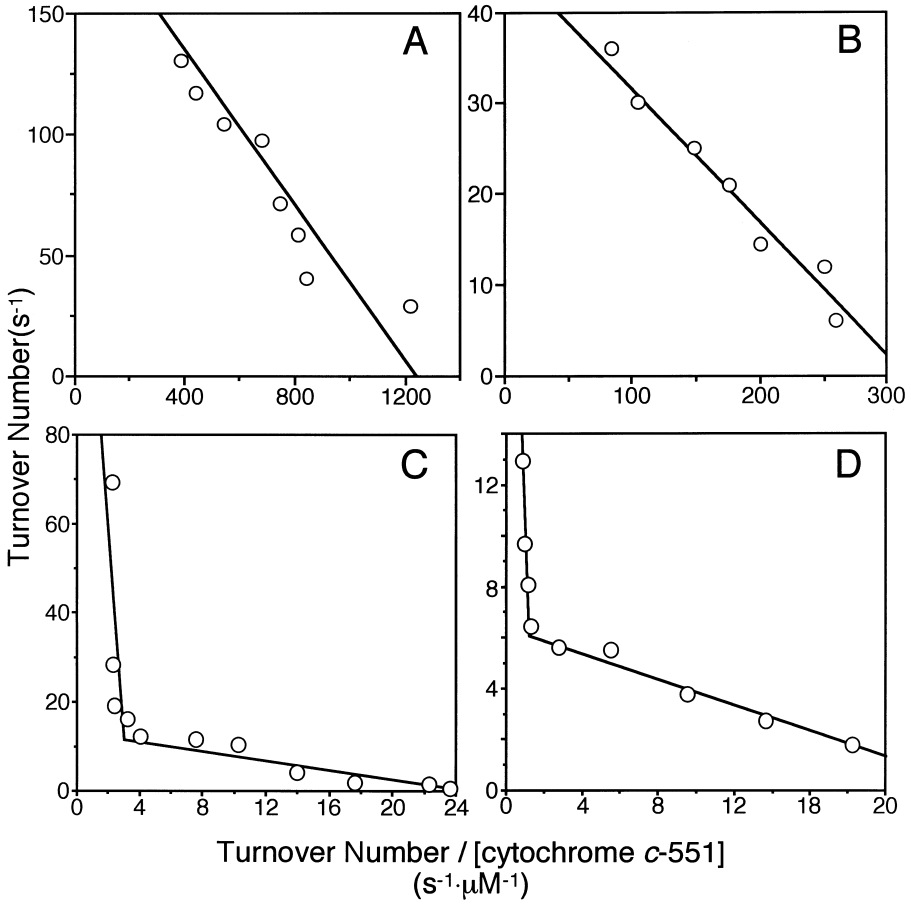

Fig. 4. Eadie–Hofstee plots for cytochrome *c*-551 oxidation by cytochrome bo_3 under different conditions. The activity was potentiometrically measured with a glass electrode using 0.05–0.08 nmol enzyme at 40°C in a 2.5-ml glass cell. (A) The reaction medium contains 200 mM KCl, 1 mM $MgSO_4$ and 0.5 mM Pi buffer, pH 6.7, 10 mM sodium ascorbate and 0.3 mM TMPD. (B) The same conditions as A except that the KCl concentration was 10 mM and $MgSO_4$ was omitted.(C) the same as A, but TMPD was omitted.(D) the same as B, but TMPD was omitted.

cytochrome caa_3, which was about 50 μM [37]. On the other hand, no additional TMPD oxidase activity was observed in the *E. coli*/pUCcba2 membranes compared with the control membranes that characteristically contain a very low TMPD oxidase activity. Furthermore, this TMPD oxidase activity was not stimulated by the addition of cytochrome *c*-551. It is thus likely that the CbaA product expressed in *E. coli* cells is not active.

3.3. Purification of cytochrome bo3 and its molecular properties

The cytochrome bo_3 was purified from the membrane fraction of *Bst*/pSTEcba2 grown under moderate aeration of the early stationary growth phase with essentially the same procedure as that reported previously for the purification from K17 [13], with an important additional step. The membrane proteins solubilized in the presence of 100 mM NaCl was applied to a DEAE-Toyopearl column, which cytochrome bo_3 passed through, while most of the cytochrome caa_3 was absorbed too. This pass-through step seems important to eliminate the caa_3-type oxidase which is abundant in the wild-type of thermophilic *Bacillus*; and after this step, we could follow cytochrome bo_3 as the major cytochrome on the columns. The yield was 10–20% overall, and the final preparation was almost pure as judged by SDS-PAGE (lane 7 in Fig. 2).

The redox difference spectrum (Fig. 3A) showed the α-peak at 560 nm like the enzyme from K17

mutant cells. It is noteworthy that the minor peaks found at 595 and 614 nm in the K17 enzyme [13] were invisible, suggesting that the heme composition is more homogeneous in the over-expressed enzyme. The low-spin heme mainly contributing to the absorption may be protoheme IX. The difference absorption spectrum of CO-reduced *minus* reduced form of the oxidase (Fig. 3B) shows peaks at 572, 537 and 416 nm and troughs at 559 and 434 nm, indicating that the heme species bound with CO is heme O or protoheme. There was no indication that another heme, such as heme A, is present at the high-spin heme site of the O_2/CO-binding binuclear center. We previously found heterogeneity of the high-spin heme in the preparation from K17 [13], and therefore the enzyme was designated as cytochrome $b(o/a)_3$. Pyridine hemochrome and reverse-phase chromatography analyses also showed that heme A content was less than 7% of the total amount of hemes in the present preparation.

3.4. Catalytic properties and H^+ pumping

We previously reported that cytochrome *c*-551, at a very low concentration, markedly activates the TMPD oxidase activity of cytochrome bo_3 purified from K17, especially under high ionic strength conditions [13]. In Fig. 4, the rate of cytochrome *c*-551 oxidation and TMPD oxidation are plotted against $V/[c\text{-}551]$ under two ionic strength conditions. In the presence of TMPD, the oxidase activity followed typical Michaelis–Menten kinetics showing a K_m value for cytochrome *c*-551 of 0.14 μM under both low and high ionic strength conditions and a V_{max} of 190 s^{-1} under the high ionic condition (Fig. 4A), while the V_{max} was 45 s^{-1} under the low ionic strength (Fig. 4B). In the absence of TMPD, the oxidase activity showed a biphasic dependency on cytochrome *c*-551 under both low and high ionic strength conditions. Under the high ionic condition, the K_m and V_{max} values for the high affinity phase were 0.5 μM and 12 s^{-1} and those for the low affinity phase, 45 μM and 158 s^{-1} (Fig. 4C). Under the low ionic strength condition, the former values were 0.3 μM and 6.5 s^{-1} and the latter values 30 μM and 67 s^{-1} (Fig. 4D). This kind of biphasic kinetics has been well known for mitochondrial cytochrome aa_3 [38]. In the previous paper, we reported that only cytochrome *c*-551 ($K_m = 0.15$ μM), but neither horse heart cytochrome *c* nor yeast cytochrome *c*, was very effective in activating the TMPD oxidase activity [13]. It is thus likely that the high affinity site of the oxidase is very specific for cytochrome *c*-551, whereas the low-affinity site is so less specific that it can even be occupied by TMPD. The K_m values of the low affinity site for cytochrome *c*-551, yeast cytochrome *c* and TMPD were 30, 33 and 29 μM, respectively, which were measured in the presence of 1 μM cytochrome *c*-551 under the high ionic strength condition (not shown).

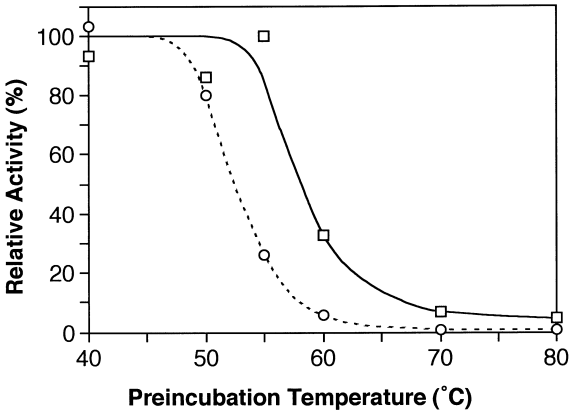

Fig. 5. Protective effects of phospholipids against heat denaturation. The cytochrome bo_3 preparation (1 nmol) were reconstituted into liposomes (1 ml) with 10 mg soybean P-lipids by the freeze–thaw–sonication method [31] or used as prepared without adding P-lipids. The heat treatment was carried out in the reaction medium containing 50 mM phosphate buffer at pH 6.7, 200 mM KCl, 1 mM $MgSO_4$ and 1 mM EDTA. After the treatment, cytochrome *c*-551 was added to a final concentration of 0.5 μM. The TMPD oxidase activity, started by adding sodium ascorbate (5 mM) and TMPD (0.3 mM), was measured polarographically. □, (with P-lipids) vesicles containing 0.05 nmol cytochrome bo_3 and 0.5 mg P-lipids were used: ○, (without P-lipids) 0.05 nmol cytochrome bo_3 was used. The turnover numbers without preincubation were 76 s^{-1} with P-lipids, and 160 s^{-1} without P-lipids.

Stability of bo_3-type oxidase was studied by changing the preincubation temperature as shown in Fig. 5. The enzyme reconstituted into vesicles with soybean P-lipids was stable up to 55°C, while the enzyme without P-lipids was greatly injured at that temperature. The difference of the turnover numbers between the enzyme with and without P-lipids (the latter was about two-fold of the former, see legend for Fig. 5) may be due to scrambling of the enzyme with P-lipids; half of the enzyme facing the inside of

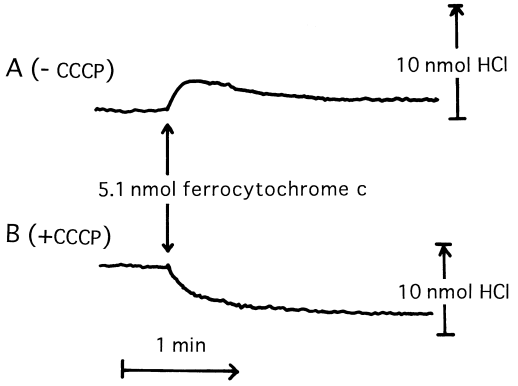

Fig. 6. H^+ pumping by cytochrome bo_3 vesicles containing cytochrome*c*-551 upon the yeast ferrocytochrome *c* pulse. A 75-μl aliquot of vesicles reconstituted as described in Section 2 were suspended in 2 ml of the reaction medium containing 20 mM KCl, 2.5 mM $MgSO_4$, 0.4 μg/ml valinomycin and 0.2 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethane sulfonate buffer, pH 6.6. the reaction was carried out at 38°C and followed with a pH meter. A, control: B, CCCP (2 μg) added.

the vesicles could not bind exogenously added cytochrome *c*-551. Lipophilic and small TMPD molecules seem to be able to penetrate the liposomal membrane almost freely and donate electrons to the enzyme via cytochrome *c*-551 at the high affinity site.

We determined if cytochrome bo_3 reconstituted into liposomal vesicles with cytochrome *c*-551 pumps H^+. Acidification, indicating H^+ ejection, occurred upon a yeast ferrocytochrome *c* pulse, and on the contrary alkalinization showing consumption of H^+ forming H_2O, was observed in the presence of an uncoupler CCCP (Fig. 6). The H^+/e^- ratio, the maximal amount of H^+ ejected/ferrocytochrome *c* added, was 0.4. We prepared the liposomal vesicles several times using different cytochrome bo_3 preparations with various lipid/protein ratios, but no higher value than 0.5 was obtained, suggesting that cytochrome bo_3 was a H^+-pump, but either its efficiency is not as high as the SoxM-type enzymes such as *Bst* cytochrome caa_3 [31] or its H^+ pump activity is very labile. A similar result with a low H^+/e^- stoichiometry was observed in *T. thermophilus* cytochrome ba_3 [26].

4. Discussion

Previously, cytochrome $b(o/a)_3$ was prepared from the K17 strain, a mutant of *Bst* K1041 lacking cytochrome caa_3 oxidase [13]. This mutant seems to contain cytochrome *bd*-type quinol oxidase as the main terminal oxidase [11,12,36], and the contents of cytochrome $b(o/a)_3$ were low and varied from batch to batch of the cell culture. We have succeeded to overexpress this enzyme with the cytochrome composition of bo_3 by transforming the wild-type *Bst* cell with pSTEcba2. The K17 mutant strain could not be transformed successfully, although it would be advantageous to use it since this would exclude the contamination of very active cytochrome caa_3.

The reason why pSTEcba1 did not induce expression of cytochrome bo_3 is not known at present, but the following facts are noteworthy: efficiency of transformation was much lower with pSTEcba1 than that with pSTEcba2, and surviving *Bst*/pSTEcba1 cells contained only a small amount of the plasmid. It seems likely that the unidentified ORF just 5′-upstream of the *cbaAB* operon is harmful to the bacterium in a high copy number, since we succeeded to over-express cytochrome bo_3 by using pSTEcba2 devoid of the ORF. We obtained membranes containing 1.0–1.5 nmol cytochrome bo_3 per mg protein from the *Bst* transformant with pSTEcba2. Since the promoter itself was very weak, leading to the low content of mRNA of *cbaAB* in the original *Bst* cells [14], the high content of cytochrome bo_3 is probably due to the high copy number of the plasmid. The fact that cytochrome bo_3 contents increased until the early stationary phase with moderate shaking indicates that the promoter responds to such microaerobic conditions.

We call the present enzyme cytochrome bo_3 since the high spin heme was mostly heme O. The previous preparation of this enzyme from the K17 strain contained appreciable amounts of heme A in addition to heme O, and therefore we called cytochrome $b(o/a)_3$ [13]. This difference seems due to the lower oxygen tension of the present culture medium. CtaA, heme O oxygenase to produce heme A [39], probably needs relatively high oxygen concentration for its catalytic activity.

Concentration dependencies on TMPD and cytochrome *c*-551 (Fig. 4) suggest that cytochrome bo_3 contains two substrate binding sites; the high affinity site is very specific to cytochrome *c*-551, while the low affinity site is relaxed in terms of substrate spe-

cificity. Although the structure of the high affinity site has not been elucidated, an analysis with phylogenetic trees by the neighbor joining method showed that cytochrome *c*-551 belongs to the group of *Bacillus* small *c*, which is rather close to cyanobacterial cytochrome c_6, but apart from mitochondrial cytochrome *c* or the related proteobacterial cytochrome c_2 [40]. Another specific feature of the bo_3-type cytochrome *c*-551 oxidase is its activity increment under high ionic strength conditions. The activity was enhanced by NaCl and KCl with a similar concentration dependency and also enhanced by $MgSO_4$, but at lower concentrations [13]. The measurement of kinetic constants indicated that high ionic strength mainly enhanced the velocity of internal electron transfer of the enzyme rather than lowering the affinity to bind the substrate (Fig. 4). This kinetic behavior is somewhat different from that of cytochrome *(c)*aa_3-type (SoxM-type) oxidases, and thus it is important to elucidate the specific interaction between cytochrome *c*-551 and cytochrome bo_3. In this respect, it would be interesting to the test cytochrome ba_3 from *T. thermophilus*, which is at present only the other eubacterial cytochrome *c* oxidase belonging to the SoxB-type. This enzyme catalyzes oxidation of cytochrome *c*-552 from the same bacterium [41], which was recently claimed to be a specific substrate with a high turnover number [25], and pumps H^+ with a H^+/e^- ratio of 0.5 [26].

In order to test the proton pumping activity, cytochrome *c*-551 was added to cytochrome bo_3 prior to liposomal vesicle formation to fill the high affinity site. The addition of reduced yeast cytochrome *c* resulted in acidification of the external medium (Fig. 6). The obtained H^+/e^- ratio, however, was about one half of the value usually observed with SoxM-type oxidases [42], in accordance with the result reported for *T. thermus* ba_3-type oxidase [26]. Such a H^+/e^- ratios lower than 1.0 were previously observed even for the SoxM-type oxidases, but only when the enzymes were heat-treated or suffered a prolonged aging [43,44]. It is also worth mentioning that our H^+/e^- ratio measurement with resting cells of a *Bst* mutant having cytochrome bo_3 as the main terminal oxidase without having caa_3-type and *bd*-type oxidases showed that the H^+/e^- ratio at the terminal oxidase-level was about 0.5 (Koyanagi and Sone, to be published). We are thus tempted to speculate that absence of several chargeable amino acid residues in the hydrophobic region in subunit I of the SoxB-type oxidases, which might constitute the H^+ pathway (especially the D channel, [14]), may be the cause of this lower H^+/e^- ratio of the SoxB-type cytochrome *c* oxidases.

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