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A Novel Cytochrome $b(o/a)_3$-Type Oxidase from *Bacillus stearothermophilus* Catalyzes Cytochrome *c*-551 Oxidation[1]

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Gram-positive thermophilic *Bacillus* species contain cytochrome caa_3-type cytochrome *c* oxidase as their main terminal oxidase in the respiratory chain. To identify alternative oxidases, we isolated several mutants from *B. stearothermophilus* defective in the caa_3-type oxidase activity [Sakamoto, J. *et al.* (1996) *FEMS Microbiol. Lett.* 143, 151-158]. A novel oxidase was isolated from membrane preparations of one of the mutants, K17. The oxidase was composed of two subunits with molecular masses of 56 and 19 kDa, and contained protoheme IX, heme O, heme A, and Cu in a ratio of 1:0.7:0.2:3. CO difference spectra indicate that the high-spin heme is mainly heme O. These results suggest that the enzyme belongs to the heme-copper oxidase family and is a cytochrome $b(o/a)_3$-type oxidase, whose high-spin heme is mainly heme O and partly heme A. The enzyme oxidized cytochrome *c*-551, which is a membrane-bound lipoprotein of thermophilic *Bacillus*. The turnover rate of the activity ($V_{max}=190\ s^{-1}$) and its affinity for cytochrome *c*-551 ($K_m=0.15\ \mu M$) were much higher than those for yeast and equine heart cytochromes *c*. The oxidase activity was enhanced by the presence of salts and inhibited by sodium cyanide with a K_i value of 19 μM. The enzyme kinetics suggests that cytochrome *c*-551 is the natural substrate to this oxidase. Furthermore, the oxidase had similarity to cytochrome ba_3-type oxidase from *Thermus thermophilus* in the subunit composition, partial amino acid sequence, and prosthetic groups, and therefore is suggested to belong to a unique subgroup of the heme-copper oxidase family together with the *Thermus* enzyme and archaeal oxidases such as *Sulfolobus* SoxABCD.

Key words: *Bacillus stearothermophilus*, ba_3-type cytochrome *c* oxidase, bo_3-type oxidase, cytochrome *c*-551, thermophilic bacteria.

Gram-positive spore-forming thermophilic *Bacilli* such as *Bacillus* PS3 and *B. stearothermophilus* contain cytochrome caa_3-type cytochrome *c* oxidase as the respiratory terminal oxidase (complex IV) (*1-3*). The cytochrome caa_3 belongs to the heme-copper respiratory oxidase family (*4, 5*) based on its prosthetic groups, primary sequence and enzymatic function (*1, 6*). Compared with aa_3-type cytochrome *c* oxidases from mitochondria and *Paracoccus denitrificans*, the *Bacillus* caa_3-type oxidase contains *c*-type cytochrome as an extra domain covalently fused to C-terminus of subunit II. 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 (complex III) (*7*) or from an artificial electron carrier, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD) (*8*). These observations suggest that the *c*-type cytochrome domain of subunit II is a functional equivalent to cytochrome *c* mediating electron transfer from complex III to complex IV. Equivalence of these two *c*-type cytochromes was also suggested by comparing the transient kinetic properties of a *Bacillus* caa_3-type oxidase and those of a complex between mitochondrial aa_3-type oxidase and cytochrome *c* (*9*). On the other hand, thermophilic *Bacillus* possesses cytochrome *c*-551, which is a membrane-bound lipoprotein with a molecular mass of 10.5 kDa and is regarded as an electron donor to a terminal oxidase (*10, 11*). Cytochrome *c*-551 can in fact donate electrons to caa_3-type oxidase, but it scarcely accelerates electron transfer from cytochrome b_6c_1 or TMPD to the oxidase. Moreover, the extent of its expression is not parallel with that of caa_3-type oxidase. Therefore, it is reasonable to hypothesize that the bacteria possess an alternative cytochrome *c* oxidase which physiologically utilizes the cytochrome *c*-551 as its substrate (*12, 13*). Since the presence of high oxidase activity due to the caa_3-type complex might affect the expression and/or detection of alternative oxidases, we isolated *B. stearothermophilus* mutants defective in caa_3-type oxidase activity to various extents (*14*). A second oxidase was identified in one of the mutants and purified to homogeneity, but it was a cytochrome *bd*-type

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Abbreviations: MEGA 9+10, 1 : 1 mixture of *n*-nonanoyl *N*-methylglucamide and *n*-decanoyl *N*-methylglucamide; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; SHAM, salicylhydroxamic acid; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine.

enzyme and oxidized quinol instead of cytochrome *c* (*14*), like its homologues in *Escherichia coli* and *Azotobacter vinelandii* (*15-17*). Therefore, the bacterium should possess another unidentified terminal oxidase, which oxidizes cytochrome *c*-551.

Here we report purification and characterization of *Bacillus* cytochrome $b(o/a)_3$-type oxidase, whose high-spin heme is mainly heme O and partly heme A. This novel oxidase shows a high catalytic activity in oxidizing cytochrome *c*-551 with high affinity, suggesting that the oxidase is a physiological acceptor of electrons from cytochrome *c*-551 in an alternative electron-transfer pathway. Furthermore, the oxidase has similarity to ba_3-type oxidase of *Thermus thermophilus* (*18, 19*). Accumulated sequence data indicate that heme-copper oxidases can be classified into three subfamilies based on the sequence similarity of their subunit I; SoxM-, SoxB-, and FixN-types, as named by Saraste and Castresana (*20*). Most heme-copper oxidases so far sequenced are SoxM-type molecules and their subunit II contains two transmembrane α-helices. *Thermus* ba_3-type oxidase, together with SoxABCD oxidase from an archaeon, *S. acidocaldarius* (*21*), composes the small subgroup of SoxB-type and they have only one putative transmembrane helix in their subunit II, although the subunit has sequence similarity to those of SoxM-type oxidases (*20, 22, 23*). Cytochrome cbb_3-type oxidases are referred to as FixN-type. This paper is the first report on an SoxB-type oxidase purified from Gram-positive bacteria.

MATERIALS AND METHODS

Enzyme Preparation—The K17 mutant was isolated from *B. stearothermophilus* K1041 as described previously (*14*). Growth and membrane preparation were performed as described elsewhere (*24*). After having been washed twice with 2% (w/v) Na-cholate, the membranes (1 g protein) were solubilized at 10 mg protein/ml with 1% (w/v) of a 1 : 1 mixture of *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 sodium phosphate buffer, pH 6.0. The 100-ml mixture was centrifuged at 140,000×*g* for 40 min to obtain the supernatant, which was then dialyzed against 20 mM sodium phosphate buffer, pH 6.0, supplemented with 1% (w/v) MEGA 9+10, and applied to a DEAE-Toyopearl column (1.0×6.4 cm). The column was washed with 30 ml of a buffer containing 0.5% (w/v) MEGA 9+10, 1 mM EDTA, and 20 mM sodium phosphate, pH 6.0, and then proteins were eluted using a 100-ml linear gradient from 0 to 70 mM NaCl in the same buffer. The oxidase activity was eluted with 20 mM NaCl. Peak fractions were combined and applied to a hydroxyapatite column (0.5×1.5 cm) and proteins were eluted stepwise with solutions containing 0.5% (w/v) MEGA 9+10 and increasing concentrations of sodium phosphate. The activity was eluted in fractions of 0.3 and 0.4 M sodium phosphate buffer.

Enzymatic Assay and Optical Spectroscopy—Cytochrome *c* oxidase activity of the purified enzyme was continuously monitored with a pH electrode (Iwaki Glass) in termes 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 acceptor ascorbate, as described elsewhere (*8*). The standard reaction medium contained 15 nM $b(o/a)_3$-type oxidase, 0.4 μM cytochrome *c*-551, 125 μM *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD), 5 mM Na-ascorbate, 1 mM $MgSO_4$, 150 mM KCl, 1 mM NaP_i, 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 at 30°C with stirring. H^+ absorption was titrated by adding adequate volumes of 10 mM HCl to each assay. Turnover number was expressed as mol of electrons transferred per mol of hemes of the enzyme per second. TMPD oxidase activity eluted from columns was spectrophotometrically measured at 22°C by monitoring the increase in A_{562}, and was calculated by using a millimolar extinction coefficient, $\Delta\varepsilon_{562} = 10.5\ mM^{-1}\cdot cm^{-1}$ (*14*).

Redox difference spectra were recorded using a Beckman DU-70 spectrophotometer at room temperature. Air-oxidized enzyme was taken and then a few grains of solid sodium dithionite were added to it to obtain its reduced form. Heme A content was calculated from redox difference spectra of its pyridine hemochrome by the method of Berry and Trumpower (*25*). The content of protoheme IX *plus* heme O was calculated as their sum using the extinction coefficient for protoheme IX (*25*).

Other Analyses—Protein concentration was determined as described by Lowry *et al.* (*26*) 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 (*27*) except that boiling of protein samples was omitted. Gels were stained with Coomassie Brilliant Blue for detection of total proteins and with *o*-tolidine to detect peroxidase activity due to heme (*28*). Molecular weights of polypeptides were estimated by Ferguson plot analysis (*29*) using 10, 12, 14, and 16% (w/v) acrylamide gels. For sequence analysis, proteins were separated by SDS-PAGE and electro-transferred to a polyvinylidene difluoride membrane, as described by Towbin *et al.* (*30*). The membrane was washed extensively with water to remove glycine, treated with 0.6 N HCl at room temperature for 24 h to release a possible N-terminal formyl group (*31*), and applied to a pulse-liquid peptide sequencer (Applied Biosystems, model 477A). Proteolytic fragments for sequencing were obtained using *Staphylococcus aureus* V8 protease as described previously (*32*). For heme analysis, hemes were extracted from enzyme preparations with acetone containing 10% concentrated HCl as described previously (*13*). After centrifugation, hemes in the supernatant were extracted into ether. The heme-containing upper phase was separated and the ether was evaporated under a stream of nitrogen. The hemes were dissolved in 80 μl of 30% acetonitrile and a 10-μl aliquot was applied to a reverse-phase HPLC column Hikarisil-C18 (Asahi Kasei, Kawasaki). The hemes were eluted with a gradient from 30 to 100% (v/v) acetonitrile in water containing 0.05% trifluoroacetic acid at a flow rate of 0.5 ml/min. Copper contents of enzymes were measured with a polarized Zeeman atomic absorption spectrophotometer (Hitachi, model Z-6100).

Materials—Cytochrome *c*-551 of *Bacillus* PS3 was overexpressed in *B. stearothermophilus* K1041 and isolated as previously described (*11*). Among the oxidase inhibitors, quercetin and salicylhydroxamic acid (SHAM) were gener-

ous gifts from Dr. Nobuko Minagawa of Niigata Institute of Pharmacology, and *p*-benzoquinone and 2,6-dimethyl-*p*-benzoquinone were from Drs. Mariko Watanabe, Tatsushi Mogi, and Yasuhiro Anraku of the University of Tokyo. *N*-2-Hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, MEGA 9 and MEGA 10 were purchased from Dojin (Kumamoto). DEAE-Toyopearl anion exchange gel, hydroxyapatite, polyvinylidene difluoride membranes, and TMPD were obtained from Tosoh (Tokyo), Bio-Rad (Hercules), Millipore (Bedford), and Wako (Kyoto), respectively. Proteins used as molecular mass standards and cytochromes *c* of yeast and bovine heart were purchased from Sigma. Other reagents were of analytical grade.

RESULTS

Purification of the Cytochrome c Oxidase—In order to identify an alternative cytochrome *c* oxidase operating in *B. stearothermophilus*, we have isolated a mutant named K17, which is defective in caa_3-type oxidase (*14*). A novel oxidase was purified from membrane preparations of the mutant, by following the enzymatic activity oxidizing TMPD, an artificial substrate for cytochrome *c* oxidase (Table I). After having been solubilized with MEGA 9+10, the oxidase was subjected to anion-exchange chromatography and then to hydroxyapatite chromatography. Figure 1 shows SDS-PAGE analysis of fractions eluted from the hydroxyapatite column. The peak oxidase activity was eluted in fraction 13, the first fraction obtained with 0.4 M sodium phosphate. The activities of fractions 9 through 14 were 14, 35, 32, 32, 100, and 20% of that of fraction 13, respectively. The only main proteins seen in the peak fraction were two polypeptides with apparent molecular masses of 42 and 19 kDa, which were also eluted in other fractions in parallel with the activity. Ferguson plot analysis indicated that the molecular masses of the two peptides were 56 and 19 kDa, which is consistent with the observation that the largest hydrophobic subunits of oxidases migrate faster than expected for soluble proteins of the same sizes under some conditions, including those of Laemmli's system (*2*). The size of the second polypeptide is much smaller than subunit II of many heme-copper oxidases, but similar to that of ba_3-type oxidase from *T. thermophilus* (*19*). Densitometric scanning of gels stained with Coomassie Brilliant Blue indicated that the ratio of the larger polypeptide to the smaller polypeptide was 1:0.19. Based on the molecular weights estimated from the Ferguson plot, their molar ratio was calculated to be 1:0.53. The reason why the ratio is less than unity might be attributable to instability of the 19-kDa polypeptide and its low stainability with Coomassie Brilliant Blue, as in the case of the *Thermus* enzyme (*19*). This polypeptide tended to be lost faster than the 56-kDa polypeptide during storage after the enzyme was isolated. The N-terminal sequences of the 19-kDa peptide and one of its proteolytic fragments were MHIHKYEKIWLTF- and LNIVVAAFSFTPNAIEI-PKGAKV-, respectively. The former sequence is similar to that of amino acid residues 9-21 in subunit II of *Thermus* oxidase (-KAILAYEKGWLAF-), and the latter to that of residues 81-103 (-VYVLAFAFGYQPNPIEVPQGAEI-) (*19*). Furthermore, the -EXXW- motif underlined above is conserved among the only transmembrane segment of subunit II of SoxB-type oxidases, which is located close to the N-terminus, and the second transmembrane segment of subunit II of SoxM-type oxidases, which is located several tens of residues away from the N-terminus (*19, 22*). These comparisons suggest that the novel *Bacillus* oxidase is similar to SoxB-type enzymes. The alignment described above was supported by the nucleotide sequence of genes cloned by using the peptide sequence to design probes (unpublished data). The cloned genes also demonstrated global sequence similarity between the *Bacillus* and *Thermus* enzymes. Hence, the 56- and 19-kDa polypeptides are referred to as subunits I and II, hereafter.

Prosthetic Groups of the Oxidase—Figure 2 shows the absorption spectra of reduced and oxidized forms of the purified oxidase. The redox difference spectrum showed a main α-peak at 560 nm and minor peaks at 595 and 614 nm. The spectrum of pyridine hemochromes of the enzyme showed a peak at 555 nm and a smaller peak at 589 nm. The wavelength of the former peak is between that of protoheme IX (557 nm) and heme O (553 nm) (*33*), suggesting that the enzyme contains both protoheme and heme O. The peak at 589 nm indicates heme A. Assuming that the molar extinction coefficient for heme O is the same as that for protoheme, the amount of heme O *plus* protoheme was calculated to be 16.9 nmol/mg protein and that of heme A, 1.64 nmol/mg protein. Based on the fact that the oxidase is a heterodimer composed of the two subunits with molecular masses of 56 and 19 kDa, the content of heme O *plus*

TABLE I. **Purification of the cytochrome *c* oxidase.**

Sample	Protein (mg)	TMPD oxidase activity			
		Total activity (μmol/min)	Recovery (%)	Specific activity (μmol/mg/min)	Purification (-fold)
Membranes	2,900	408	100	0.141	1
Cholate washed	310	297	72.8	0.961	6.83
MEGA extract	167	60.1	14.7	0.359	2.55
DEAE peak	23.4	22.2	5.4	0.951	6.75
Hydroxyapatite peak	16.7	18.2	4.5	1.10	7.71

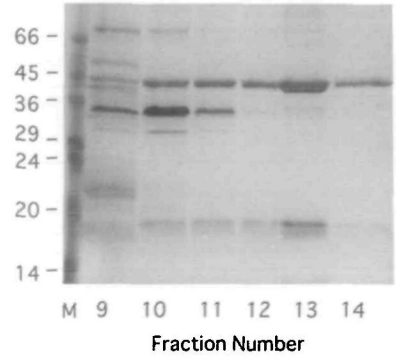

Fig. 1. **SDS-PAGE analysis of the eluates from the hydroxyapatite column.** Membrane preparation of *B. stearothermophilus* was solubilized and applied to a DEAE-Toyopearl column. Pooled peak fractions of TMPD oxidase activity from the column were then applied to a hydroxyapatite column and stepwise eluted with Na-P_i buffer as described under "MATERIALS AND METHODS." Aliquots (20 μl/lane) of eluate fractions were applied to 13.5% (w/v) acrylamide gel for SDS-PAGE. M indicates molecular mass standards. Fraction number 9, the last fraction of 0.2 M NaP_i eluate; 10 through 12, fractions obtained with 0.3 M NaP_i; 13 and 14, first two fractions with 0.4 M NaP_i.

protoheme and that of heme A were 1.29 and 0.125 mol/mol enzyme, respectively. There was no indication of heme C or D in the absorption spectra. The absence of heme C was confirmed by heme staining of the purified oxidase with *o*-tolidine in a gel after SDS-PAGE (data not shown).

In order to estimate the ratio of protoheme and heme O, total hemes were extracted from the oxidase and analyzed by reverse-phase chromatography, as shown in Fig. 3. Another run, shown in the figure, was done as a standard for retention times of hemes, using a preparation of supercomplex composed of b_6c_1-type quinol-cytochrome *c* oxidoreductase and cao_3-type cytochrome *c* oxidase obtained from *Bacillus* PS3 (*7, 34, 35*). Three hemes were detected in the novel oxidase and the ratio of protoheme:heme O was 1:0.68. The content of heme A was lower than those of the other two hemes, confirming the result of spectroscopic

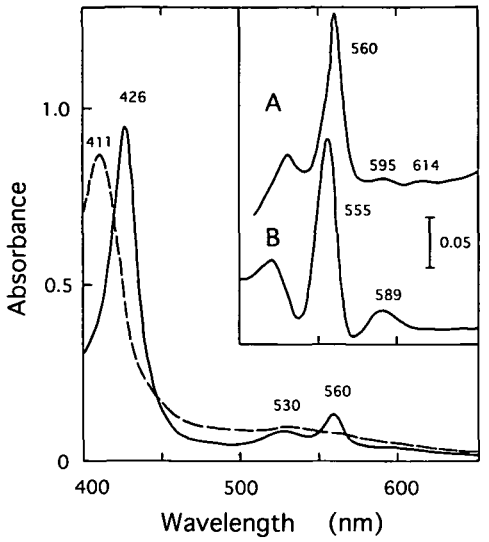

Fig. 2. **Absorption spectra of the cytochrome *c* oxidase.** The purified oxidase at 0.35 mg protein/ml was air-oxidized (broken line) or reduced with hydrosulfite (solid line). Inset: redox difference spectra of intact enzyme (A) and pyridine hemochromes of the enzyme (B).

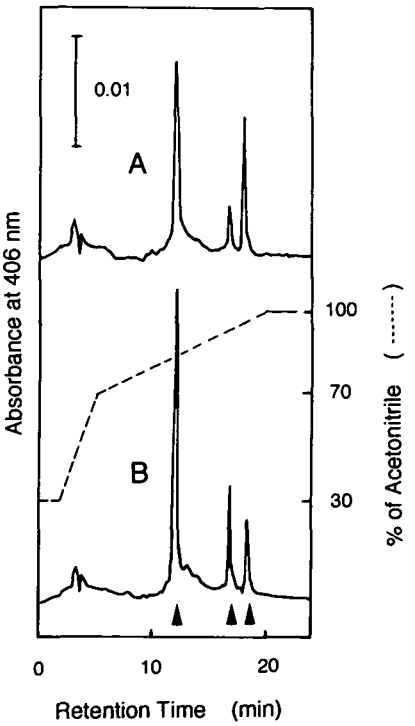

Fig. 3. **Reverse-phase HPLC elution profiles of hemes.** Hemes were extracted from 0.6 nmol of $b(o/a)_3$-type oxidase (A) or $b_6c_1 + cao_3$-type super-complex (B), and analyzed by reverse-phase HPLC. Absorbance of eluates was monitored at 406 nm. The broken line indicates the profile of the acetonitrile gradient. The arrowheads indicate retention times for protoheme (11.8 min), heme A (16.5), and heme O (17.7) from the left.

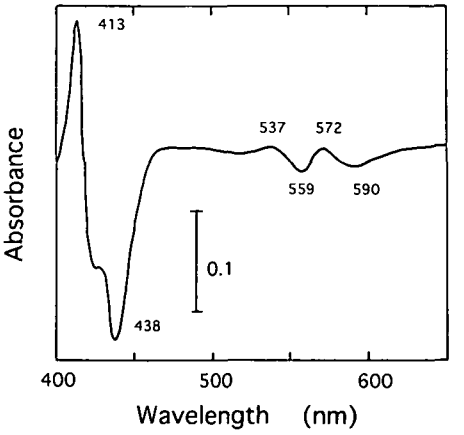

Fig. 4. **Difference spectrum of CO-reduced minus reduced form of the cytochrome *c* oxidase.** The purified oxidase at 0.7 mg protein/ml was reduced with hydrosulfite (baseline) and then bubbled with CO gas for 1 min.

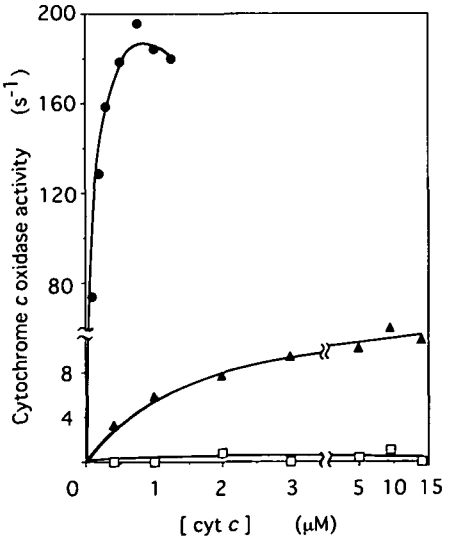

Fig. 5. **Substrate specificity of the cytochrome *c* oxidase.** The oxidase was incubated at 15 nM with the indicated concentrations of cytochrome *c*-551 of *Bacillus* PS3 (●), and cytochromes *c* from yeast (▲) and horse heart (□) in the presence of 125 μM TMPD, 5 mM Na-ascorbate, 1 mM $MgSO_4$, 150 mM KCl, and 1 mM NaP_i at pH 6.7 and 30°C. The oxidase activity was measured as described under "MATERIALS AND METHODS." Turnover number was expressed as mol of electrons transferred per mol of hemes of the enzyme per second.

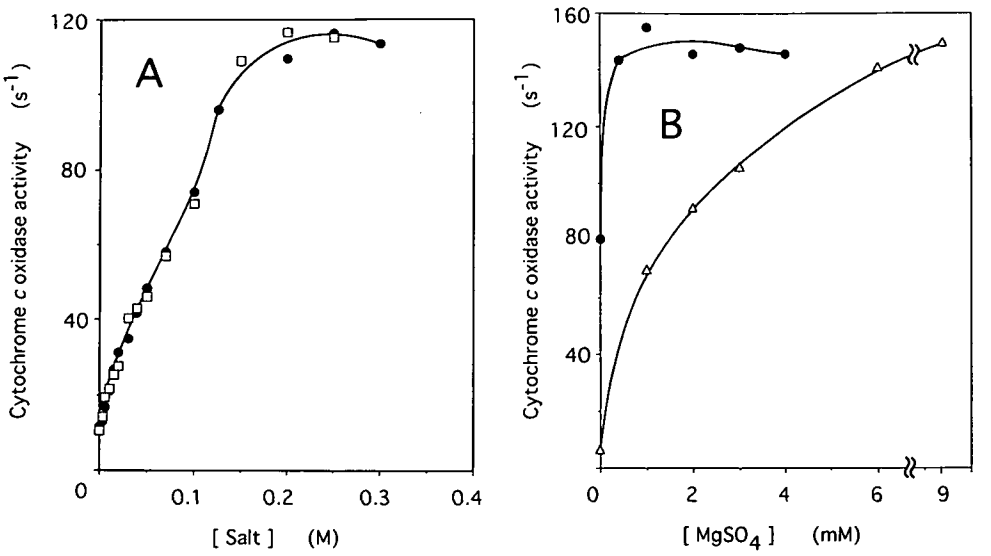

Fig. 6. **Salt concentration dependency of the cytochrome *c* oxidase activity.** The oxidase activity was measured in the presence of 0.4 μM cytochrome *c* as described for Fig. 5, except for the following: A, $MgSO_4$ was omitted and the concentrations of NaCl (□) and KCl (●) were varied as indicated; B, the concentration of $MgSO_4$ was varied in the presence (●) or absence of 150 mM KCl (△).

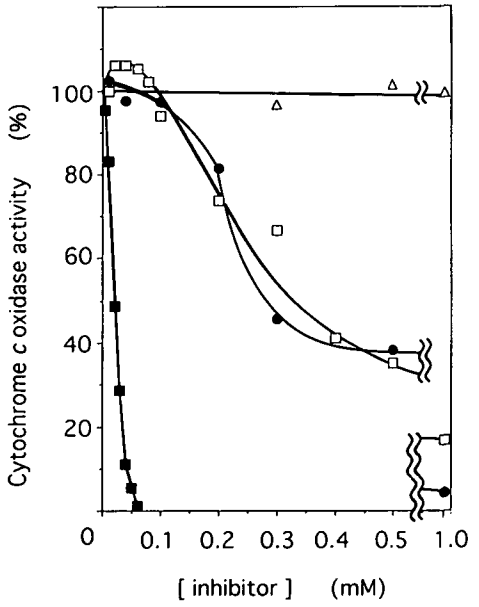

Fig. 7. **Effects of inhibitors on the cytochrome *c* oxidase activity.** The enzyme activity was measured in the presence of 0.4 μM cytochrome *c* as described for Fig. 5, except that the reaction mixtures included the indicated concentrations of quercetin (△), 2,6-dimethyl-*p*-benzoquinone (●), $ZnCl_2$ (□), and NaCN (■).

measurement. There was no indication of heme D or A_s, of which the latter is a heme A analogue with a geranylgeranyl group replacing the farnesyl group and was recently identified in the *Sulfolobus* oxidase (*22*). By combining the data from absorption spectroscopy and HPLC analysis, the amounts of protoheme, heme O, and heme A were estimated to be 0.77, 0.52, and 0.125 mol/mol enzyme. The difference absorption spectrum of CO-reduced minus reduced form of the oxidase (Fig. 4) shows peaks at 572, 537, and 413 nm and troughs at 590, 559, and 438 nm, indicating that the main heme species bound with CO is heme O or protoheme. Troughs in γ-band areas are generally at about 429–431 nm in the case of heme O or protoheme, whereas they are at about 441–444 nm in the case of heme A (*2*), so the intermediate wavelength obtained here (438 nm) suggests that the heme bound with CO is heterogeneous, including both heme O (or protoheme) and heme A. The shoulder at 428 nm may also suggest heterogeneity of high-spin heme, but the entity responsible for it is not known. The Cu content of the oxidase was measured with an atomic absorption spectrophotometer and found to be 29.8 nmol/mg protein. Based on the same assumption as described above, this corresponds to 2.26 mol/mol enzyme.

Enzymatic Activity—Figure 5 shows the substrate specificity of the purified cytochrome *c* oxidase. Three cytochromes *c* were used as substrates, including cytochrome *c*-551 of *Bacillus* PS3 (*11*). The V_{max} value for cytochrome *c*-551 was 190 s^{-1}, much higher than those for yeast cytochrome *c* (14 s^{-1}) and equine heart cytochrome *c* (less than 1 s^{-1}), while the K_m value was 0.15 μM, being lower than that for yeast cytochrome *c* (14 μM). The oxidase activity was highly dependent on salt concentrations as shown in Fig. 6. The activity was increased as the NaCl concentration was raised, followed by saturation at about 0.2 M NaCl. A similar result was obtained with KCl. The enzymatic activity was also enhanced by $MgSO_4$ both in the absence and presence of 150 mM KCl and reached about the same maximum. The pH optimum was 6.7 (data not shown). The standard reaction conditions were chosen based on these observations. The effects of several inhibitors on the enzyme activity were measured under the standard conditions and some of the results are presented in Fig. 7. The enzyme was sensitive to NaCN ($K_i = 19$ μM) and NaN_3 ($K_i = 0.5$ mM), which are inhibitors of heme-copper oxidases, but insensitive to quercetin and SHAM, which inhibit cyanide-resistant oxidases in some microorganisms (*36*). *p*-Benzoquinone and 2,6-dimethyl *p*-benzoquinone, inhibitors of quinol oxidases, inhibited the *Bacillus* oxidase with K_i values of 0.82 and 0.27 mM, respectively, which are more than or about 100-fold larger than the values of the inhibitors for *E. coli* quinol oxidases (*37, 38*). $ZnCl_2$ slightly activated the enzyme at concentrations lower than 60 μM and inhibited it at higher concentrations with a K_i value of 0.34 mM.

DISCUSSION

The novel cytochrome *c* oxidase was purified from the mutant K17 of *B. stearothermophilus* K1041 deficient in cytochrome caa_3-type oxidase. The electrophoretic data demonstrate that the new oxidase is composed of two subunits with molecular masses of 56 and 19 kDa. The size of the larger subunit is comparable to that of subunit I of heme-copper terminal oxidases from *Bacillus* or proteobacteria, whereas that of the second subunit is almost half of that of their subunit II (*2*), but comparable to that of subunit II of ba_3-type oxidase from *T. thermophilus* (*19*). The *Bacillus* oxidase contains protoheme IX, heme O, and a small amount of heme A as chromophores. No heme C, D, or A_s was detected. The total amount of hemes O *plus* A was roughly equal to that of protoheme. In general, high-spin hemes at the O_2-reducing site of heme-copper terminal oxidases are hemes A, O, or A_s, which contain long hydrophobic side chains such as farnesyl or geranylgeranyl groups, except for cbb_3-type (FixN-type) oxidases in proteobacteria (*4, 22*). It has also been reported that either heme A or O can be bound to the O_2-reducing site of some oxidases, such as $b(a/o)_3$-type oxidase from *Acetobacter aceti* (*39*), $ca(a/o)_3$-type oxidase from *Bacillus* PS3 (*35*), and $a(a/o)_3$-type oxidases from cyanobacteria (*40, 41*), depending on conditions of cell growth. In these cases, heme O is preferentially bound to high-spin heme sites, while low-spin heme sites tend to be occupied by original protoheme or heme A. Based on these findings, we concluded that the majority of the oxidase presented here was bo_3-type and about one-fifth of the total oxidase was ba_3-type. The amount of Cu atom was about three times that of protoheme or, in other words, one and a half times the total amount of all hemes. This is consistent with the fact that cytochrome *c* oxidases contain two Cu atoms at the Cu_A site and one at the Cu_B site on the O_2-reducing binuclear center (*4, 5*). The relative amounts of hemes and Cu to the polypeptides were a little lower than stoichiometric values. This may have been caused by loss during preparation.

Cytochrome *c*-551 of thermophilic *Bacillus* does not accelerate electron transfer to caa_3-type oxidase (complex IV) from TMPD (*8*) or from cytochrome b_6c_1-type reductase (complex III) within b_6c_1-caa_3 super-complex (*7, 34*). Furthermore, cytochrome *c*-551 is increased (*10*) while caa_3-type oxidase is decreased under air-limited conditions (*12, 13, 35*). It was also shown that the *c*-type cytochrome domain in subunit II of the caa_3-type oxidase functions quite similarly to cytochrome *c* (*8, 9*). These findings indicate that cytochrome *c*-551 is not necessary for electron transfer to the terminal oxidase. As demonstrated in this paper, the cytochrome $b(o/a)_3$-type enzyme oxidized cytochrome *c*-551 at high turnover rate ($V_{max}=190$ s^{-1}) with high affinity ($K_m=0.15$ μM). These results suggest that cytochrome *c*-551 is the physiological substrate of this alternative oxidase. However, in order to reach a conclusion as to their roles, it will be necessary to measure the affinities of these oxidases to oxygen.

It was reported that an "*o*"-type oxidase is evolved with a concomitant decrease in the amount of $ca(a/o)_3$-type oxidase under highly air-limited conditions in wild cells of *Bacillus* PS3 and *B. stearothermophilus* (*12, 13, 35*). Cytochrome b_6c_1-type reductase is present under both conditions. Therefore, the cytochrome bo_3 identified in this study may be the suggested "*o*"-type oxidase. Another possibility is that cytochrome ba_3 is the original form of the oxidase in wild-type cells although the affinity of the apoenzyme to heme A or the extent of heme A synthesis is reduced in the mutant K17, resulting in occupation by heme O of the original heme A site. Whichever is the case, identification of the oxidase might have been hampered by the presence of very high activity due to the main caa_3-type oxidase and the coexistence of various *b*-type cytochromes, including b_6c_1-type reductase and succinate dehydrogenase, in wild cells. In mutant K17, the expression of $b(o/a)_3$-type oxidase is enhanced in some way and complements the deficiency in caa_3-type cytochrome *c* oxidase. This mutant also contains cytochrome *bd*-type quinol oxidase, as previously reported (*14*). The ratio of these two alternative oxidases varied from batch to batch of cell culture, however, and it has not yet been found which factor determines the ratio.

The subunit composition, the size and the low stainability of subunit II, the content of prosthetic groups, and the partial peptide sequence suggest similarity of the novel *Bacillus* enzyme to cytochrome ba_3-type oxidase in *T. thermophilus* (*18, 19*). The *Thermus* oxidase was also reported to oxidize TMPD and equine cytochrome *c* (*18*), though its natural substrate is not known. Moreover, the *Thermus* enzyme did not exhibit saturation behavior with these two substrates even at high concentrations (*18*). Thus, *Bacillus* $b(o/a)_3$-type oxidase may be preferable in studying physiological enzymatic activity.

The cytochrome *c* oxidase activity was enhanced by NaCl and KCl with very similar concentration dependency (Fig. 6). The enzyme was also activated by $MgSO_4$, but at lower concentration, and the maximal extent was about the same in the presence and absence of KCl. These findings suggest that the activation is mainly due to ionic strength. A similar salt effect was observed for oxidase activity of membrane preparations (data not shown). In general, cytochrome *c* is thought to interact with SoxM- or SoxB-type cytochrome *c* oxidase on the Cu_A-binding domain of subunit II extruding to the P-phase (*2*). Crystallography of the two SoxM-type cytochrome *c* oxidases from *Paracoccus* and bovine cardiac mitochondria revealed precise stereo structure of the oxidases at atomic resolutions (*42, 43*). Subunit II of the *Paracoccus* oxidase comprises an N-terminal segment in the P-phase, two transmembrane α-helices, a globular Cu_A domain which contains a ten-stranded β-barrel, and a C-terminal α-helix. In contrast, sequence alignment shows that subunit II of SoxB-type oxidase does not contain the N-terminal segment in periplasm, the first transmembrane helix and the C-terminal helix. Furthermore, SoxB-type cytochrome *c* oxidases lack some amide group-containing or acidic amino acid residues that are conserved among SoxM-type oxidases and that may be involved in electrostatic interactions with a cluster of conserved lysines on cytochrome *c* (*19, 44*). Some of these differences may explain the characteristic salt dependency and the tight substrate specificity of the *Bacillus* oxidase. More kinetic measurements are needed to determine the properties, for example, the parameters affected by the salts and the sidedness of the salt effect.

Recently, two other archaeal oxidases, cytochrome aa_3 from *Acidianus ambivalens* (*45*) and cytochrome ba_3 from

Natronobacterium pharaonis (*46*), were also shown or suggested to be of SoxB-type. To our knowledge, the novel *Bacillus* enzyme is the first oxidase belonging to this subfamily to have been identified or purified from an organism outside of archaea and *Thermus*.

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