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Biochimica et Biophysica Acta 1411 (1999) 147–158

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Gene structure and quinol oxidase activity of a cytochrome *bd*-type oxidase from *Bacillus stearothermophilus*

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Received 10 November 1998; received in revised form 7 January 1999; accepted 27 January 1999

Abstract

Gram-positive thermophilic *Bacillus* species contain cytochrome caa_3-type cytochrome *c* oxidase as their main terminal oxidase in the respiratory chain. We previously identified and purified an alternative oxidase, cytochrome *bd*-type quinol oxidase, from a mutant of *Bacillus stearothermophilus* defective in the caa_3-type oxidase activity (J. Sakamoto et al., FEMS Microbiol. Lett. 143 (1996) 151–158). Compared with proteobacterial counterparts, *B. stearothermophilus* cytochrome *bd* showed lower molecular weights of the two subunits, shorter wavelength of α-band absorption maximum due to heme D, and lower quinol oxidase activity. Preincubation with menaquinone-2 enhanced the enzyme activity up to 40 times, suggesting that, besides the catalytic site, there is another quinone-binding site which largely affects the enzyme activity. In order to clarify the molecular basis of the differences of cytochromes *bd* between *B. stearothermophilus* and proteobacteria, the genes encoding for the *B. stearothermophilus bd* was cloned based on its partial peptide sequences. The gene for subunit I (*cbdA*) encodes 448 amino acid residues with a molecular weight of 50 195 Da, which is 14 and 17% shorter than those of *Escherichia coli* and *Azotobacter vinelandii*, respectively, and CbdA lacks the C-terminal half of the long hydrophilic loop between the putative transmembrane segments V and VI (Q loop), which has been suggested to include the substrate quinone-binding site for the *E. coli* enzyme. The gene for subunit II (*cbdB*) encodes 342 residues with a molecular weight of 38 992 Da. Homology search indicated that the *B. stearothermophilus cbdAB* has the highest sequence similarity to *ythAB* in *B. subtilis* genome rather than to *cydAB*, the first set of cytochrome *bd* genes identified in the genome. Sequence comparison of cytochromes *bd* and their homologs from various organisms demonstrates that the proteins can be classified into two subfamilies, a proteobacterial type including *E. coli bd* and a more widely distributed type including the *B. stearothermophilus* enzyme, suggesting that the latter type is evolutionarily older. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Cytochrome *d*; Gram-positive bacterium; Menaquinol; Thermophilic bacterium

1. Introduction

Most aerobic organisms contain heme-copper oxidases as their main terminal enzyme complexes in the respiratory chains [1,2]. These oxidases, including mitochondrial cytochrome aa_3-type cytochrome *c* oxidases, are regarded to compose a large superfamily, based on their functional and structural similarities.

Abbreviations: MEGA 9+10, 1:1 mixture of *n*-nonanoyl *N*-methylglucamide and *n*-decanoyl *N*-methylglucamide; MK, menaquinone; orf, open reading frame; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecylsulfate; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine; UQ, ubiquinone

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PII: S0005-2728(99)00012-2

Cytochrome *bd*-type quinol oxidases are the only known bacterial terminal oxidases out of this superfamily, and operate in γ-proteobacteria as alternative electron-transfer pathways (see [3,4] for review). Cytochrome *bd* from *Escherichia coli* is a heterodimer protein encoded by *cydAB* operon, and the deduced amino acid sequences show that the sizes of subunits I and II are 58 and 43 kDa, respectively [5,6]. Out of three metal centers for oxidoreduction, the low-spin protoheme (*b*-558) is demonstrated to have their axial ligands in subunit I and the high-spin protoheme (heme *b*-595) is also suggested to have one in the same subunit [7,8], while the heme *d* ligand has not yet been located. Heme *b*-595 and heme *d* are suggested to compose a heme-heme binuclear center where dioxygen molecules are reduced [9]. The enzyme does not pump H^+ unlike heme-copper oxidases, but catalyzes transmembrane electron transfer. A similar cytochrome *bd* has been identified from *Azotobacter vinelandii*, another proteobacterium in the γ subdivision [10,11].

Gram-positive spore-forming thermophilic bacilli such as *Bacillus* PS3 and *Bacillus stearothermophilus* contain a heme-copper oxidase, cytochrome caa_3-type cytochrome *c* oxidase, as the main respiratory terminal oxidase [12–14]. On the other hand, mesophilic bacilli such as *Bacillus subtilis* and *Bacillus cereus* have aa_3-type quinol oxidase as the dominant terminal complex in vegetative cells, although the presence of the caa_3-type cytochrome *c* oxidases were also confirmed [15–17]. The presence of cytochrome *d* in Gram-positive bacteria was reported for several *Bacillus* species [18–20], though they had not been purified until recently because of their low contents [21]. We previously identified a cytochrome *bd*-type quinol oxidase operating in a caa_3-deficient mutant strain of *B. stearothermophilus* K1041, named K17, and purified it from membrane preparations of the strain [22]. Redox difference spectra of this cytochrome *bd* showed α-band absorption peaks at 560 nm due to low-spin protoheme *b* and at 618 nm due to heme *d*. The latter is about 10 nm blue-shifted compared with those of proteobacterial cytochromes *bd*. The enzyme was composed of two subunits. Ferguson plot analysis of sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) showed that the sizes of subunits I and II are 52 and 40 kDa, respectively, which seem appreciably lower than those of the *E. coli* subunits. In addition, the enzymatic activity of the oxidase was much lower than that of proteobacterial *bd*. Similar results were also demonstrated for the alkalophile *Bacillus firmus* [23].

Here we report several properties of the enzyme purified from *B. stearothermophilus* K17, including the fact that its duroquinol oxidase activity was highly accelerated by preincubation with menaquinone-2 (MK-2) or ubiquinone-1 (UQ-1). We also have cloned and sequenced their structural genes. These are the first cytochrome *bd* genes which were isolated from an organism outside the γ subdivision of proteobacteria and whose protein products are characterized. Comparison of the sequences of these identified cytochromes *bd* and homologous sequences found in bacterial and archaeal genomes indicates that those can be divided into two subfamilies; a proteobacterial type including *E. coli bd* and a more widely distributed type including the *B. stearothermophilus* enzyme. A few amino acid residues are completely conserved across the both subfamilies.

2. Materials and methods

2.1. Materials

Cytochrome *bd* was solubilized with 1% (w/v) of a 1:1 mixture of *n*-nonanoyl *N*-methylglucamide (MEGA 9) and *n*-decanoyl *N*-methylglucamide (MEGA 10) from membranes of the caa_3-deficient mutant strain K17 isolated from *B. stearothermophilus* K1041, and purified with DEAE-Toyopearl and hydroxyapatite chromatography [22]. *E. coli* cytochrome *bd*, *p*-benzoquinone and 2,6-dimethyl-*p*-benzoquinone were generous gift from Dr. Tatsushi Mogi and Dr. Yasuhiro Anraku of the University of Tokyo. MKs and UQs were a kind gift from Eisai. *N*-2-Hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, MEGA 9 and MEGA 10 were purchased from Dojin (Kumamoto). TMPD and *Staphylococcus aureus* V8 protease were purchased from Wako (Kyoto). DEAE-Toyopearl anion exchange gel, hydroxyapatite and polyvinylidene difluoride membranes were obtained from Tosoh (Tokyo), Bio-Rad (Hercules) and Millipore (Bedford), 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.

2.2. Enzymatic activity

Quinol oxidase was spectrophotometrically measured using tetramethyl-*p*-benzoquinol (duroquinol) as substrate, unless otherwise stated. Standard conditions for the measurement were as follows. Cytochrome *bd*, purified in the presence of 0.5% (w/v) MEGA 9+10 and 200 mM sodium phosphate (pH 6.0), was preincubated with 200 μM MK-2 for 2 h at 45°C. Duroquinone at 5 mM in the presence of 50% (v/v) ethanol was reduced to quinol with a few grains of sodium hydrosulfite and diluted 100-fold with buffer containing 1 mM ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid and 20 mM sodium phosphate (pH 6.5) to scavenge residual hydrosulfite with dissolved dioxygen. The reaction was started by adding 470 μl of the duroquinol-containing reaction mixture to 30 μl of the MK-2-preincubated cytochrome *bd* and continuously monitored at 45°C as increase in $A_{264.5}$ minus $A_{284.5}$ with a Hitachi two-wavelength spectrophotometer, model 556. Absorbance increment in the absence of enzyme was subtracted from total absorbance increment as baseline. The oxidase activity was calculated by using the millimolar extinction coefficient, $\Delta\varepsilon_{264.5-284.5} = 19.5$ mM^{-1} cm^{-1}.

2.3. Cloning and sequencing of the gene

For cloning of *B. stearothermophilus* cytochrome *bd*, a set of primers were designed for polymerase chain reaction (PCR) targeting the partial amino acid sequences of subunit II; 5′-ATGACG(C/T)TGGA(A/G)GTNAT(A/C/T)GG for MTLEVIG at the N-terminus [22] as a sense primer, and 5′-CT(A/G)AT(A/G)CCNTT(C/T)(A/G)ACAAGCG for DYGKLF found in an peptide cleaved by V8 protease as an antisense primer. PCR (94°C, 2 min/45°C, 1 min/72°C, 2 min for 30 cycles) was performed with *B. stearothermophilus* K1041 genomic DNA (1 μg) as the template and the resultant 500-bp product was ligated into pUC118. The amplified 500-bp fragment, labeled with digoxigenin by the method of the manufacturer's protocol (Boehringer Mannheim), was used for cloning cytochrome *bd* genes; i.e., K1041 genomic DNA cleaved by *Fba*I, *Pvu*II, *Nde*I or *Eco*T22I *plus Sna*BI was size-separated by gel electrophoresis and ligated into pUC118 digested by corresponding restriction enzyme(s). The resultant plasmids were selected by colony hybridization using the probe at 50°C in 5× SSC (0.45 M NaCl, 0.045 M sodium citrate buffer, pH 7.0) containing 0.5% blocking reagent (Boehringer Mannheim), 0.1% sodium lauroyl sarcosinate, and 0.02% SDS. DNA fragments were sequenced by the dye-primer method with pUC118 in a Shimazu DNA sequencer, model PSQ1000. General gene manipulations were carried out according to Sambrook et al. [24]. Multiple alignment and phylogenetic trees were constructed by CLUSTAL W [25] and hydropathy profiles were made by GENETYX ver 6.2.

2.4. Other analyses

Redox difference spectra were recorded using a Beckman DU-70 spectrophotometer at room temperature. A spectrum of air-oxidized enzyme was taken and then a few grains of solid sodium hydrosulfite were added to it to obtain its reduced form. For CO-difference spectra, the purified oxidase was reduced as above (baseline) and then bubbled with CO gas for 1 min. Protein concentration was determined as described by Lowry et al. [26] after precipitated with 5% trichloroacetic acid in the presence of 0.05% sodium deoxycholate. SDS-PAGE was performed with minigels (6 cm×8 cm) as described by Laemmli [27], except that boiling of protein samples was omitted. For sequence analysis, proteins were separated by SDS-PAGE and electro-transferred to a polyvinylidene difluoride membrane, as described by Towbin et al. [28]. 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 [29], and applied to a pulse-liquid peptide sequencer (Applied Biosystems, model 477A). Proteolytic fragments for sequencing were obtained using *S. aureus* V8 protease as described previously [30].

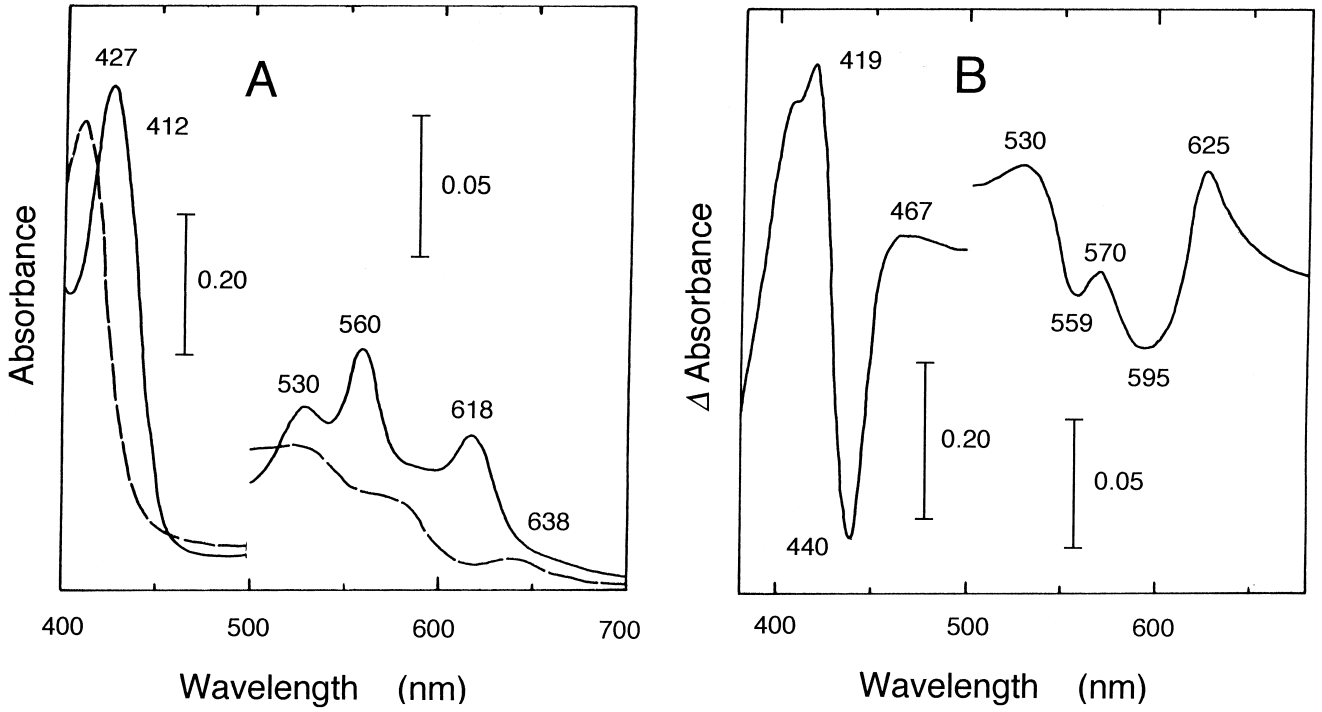

Fig. 1. Absorption spectra of *B. stearothermophilus* cytochrome *bd*-type oxidase. (A) Absolute spectra of the purified oxidase (0.3 mg protein/ml) air-oxidized (broken line) or reduced with sodium hydrosulfite (solid line). (B) Difference spectrum of CO-reduced minus reduced form of the oxidase at the same concentration.

3. Results

3.1. Absorption spectra

Fig. 1A shows the absolute absorption spectra of reduced and oxidized forms of the purified cytochrome *bd*-type oxidase of *B. stearothermophilus*. The spectrum of the reduced enzyme showed α peaks at 618 and 560 nm, a β peak at 530 nm and a γ peak at 427 nm, while that of the air-oxidized form showed a γ peak at 412 nm with a small α peak at 638 nm. Reduced pyridine hemochromes of the enzyme showed α peaks at 613 and 557 nm, confirming that the 618-nm peak of the intact enzyme is due to heme D and the 560-nm peak to protoheme IX. The wavelength due to heme D (618 nm) was shorter by about 10 nm than the *E. coli* and *A. vinelandii* counterparts [5,11,31,32]. A similarly short wavelength was reported for cytochrome *bd* of the alkalophile *B. firmus* [23].

The CO-difference spectrum (Fig. 1B) shows peaks at 625, 570, 530 and 419 nm, and troughs at 595, 559 and 440 nm. Although the shape of this spectrum was similar to that of the *E. coli* enzyme, it is noteworthy that the wavelength pair of 625 and 595 nm

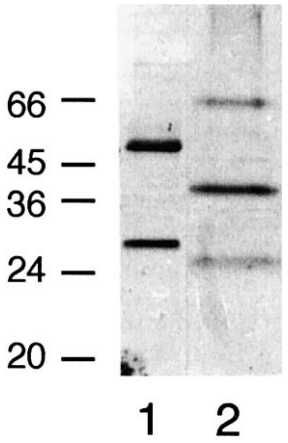

Fig. 2. Comparison of *B. stearothermophilus* cytochrome *bd* with that of *E. coli* on SDS-PAGE. Cytochromes *bd* (3 μg each) of *E. coli* (lane 1) and *B. stearothermophilus* (lane 2) were applied to 13.5% (w/v) acrylamide gel. Molecular mass standards are bovine serum albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde 3-phosphate dehydrogenase (36 kDa), trypsinogen (24 kDa) and trypsin inhibitor (20 kDa).

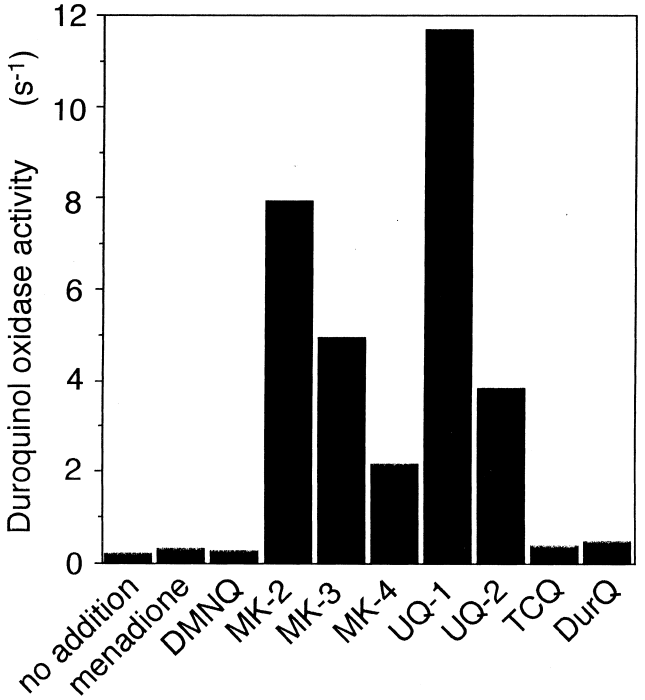

Fig. 3. Effects of preincubation with various quinones on cytochrome *bd*-type quinol oxidase. Duroquinol oxidase activity was measured as described in Section 2, except that the oxidase was preincubated with or without 200 μM of 2-methyl-1,4-naphthoquinone (menadione), 2,3-dimethyl-1,4-naphthoquinone (DMNQ), MK-2, MK-3, MK-4, UQ-1, UQ-2, tetrachloro-*p*-benzoquinone (TCQ), or tetramethyl-*p*-benzoquinone (duroquinone, DurQ).

was again shorter than the equivalent of the *E. coli* enzyme. Fig. 2 directly compares cytochromes *bd* purified from *B. stearothermophilus* and *E. coli* on SDS-PAGE. The band at 65 kDa in the lane for the *B. stearothermophilus* cytochrome *bd* is a complex of subunits I and II which resisted dissociating in the presence of SDS, as previously identified [22]. Both of the two subunits of *B. stearothermophilus bd* migrated faster than the corresponding *E. coli* subunits, confirming the differences of their molecular sizes estimated separately for the enzymes of these two organisms [5,6,22].

3.2. Quinol oxidase activity

The quinol oxidase activity of *B. stearothermophilus* cytochrome *bd* was as low as 0.3–1.3 s^{-1} in turnover number. Low activity of *bd*-type oxidase was also reported for *B. firmus* [23]. MKs are generally more labile to ultraviolet light than UQs. The cytochrome b_6c_1-type quinol-cytochrome *c* oxidoreductase (complex III) of thermophilic *Bacillus* PS3 was previously reported to be activated by preincubation with MKs and suggested to have a quinone-binding site important for enzyme activity [33]. Fig. 3 shows the effects of preincubation with several quinones on the duroquinol oxidase activity of cytochrome *bd*. Although the intrinsic quinone of *B. stearothermophilus* cells is MK-7 as in *Bacillus* PS3 and *B. subtilis*, MK-2 showed a better activation, probably because quinones with longer isoprenoid side-chains are less soluble in reaction mixture and have lower restoring ability under experimental conditions. UQ-1 showed even higher activation than MKs, suggesting that the structure of side chains at 5th and 6th positions of the quinone ring is not an absolute determinative factor of the preference of quinones. The activity increased with increase in the preincubation time until 2 h, and then gradually decreased. The highest values of turnover number achieved were 12–30 s^{-1} in several experiments, which are still much lower than those for *E. coli bd*-type ubiquinol oxidase (500–1000 s^{-1}) [5,31].

The oxidase activity was not much dependent on NaCl concentrations between 0 to 450 mM. The pH and temperature optima were at 6.2 and 45°C, respectively. The *B. stearothermophilus* cells optimally grow at 65°C, however, the isolated oxidase was inactivated with such a high temperature. Table 1 shows the effects of several inhibitors on the quinol oxidase activity. The enzyme was sensitive to $ZnCl_2$, *p*-benzoquinone and 2,6-dimethyl-*p*-benzoquinone, which are known to inhibit *E. coli bd*-type oxidase

Table 1
Effects of inhibitors on the quinol oxidase activity

Inhibitor	Concentration for 50% inhibition (mM)
p-Benzoquinone	0.12
2,6-Dimethyl-*p*-benzoquinone	0.065
$ZnCl_2$	0.20
NaCN	0.50
NaN_3	8.2

The purified oxidase was preincubated for 10 min with various concentrations of inhibitors and then the enzyme activity was measured as described in Section 2.

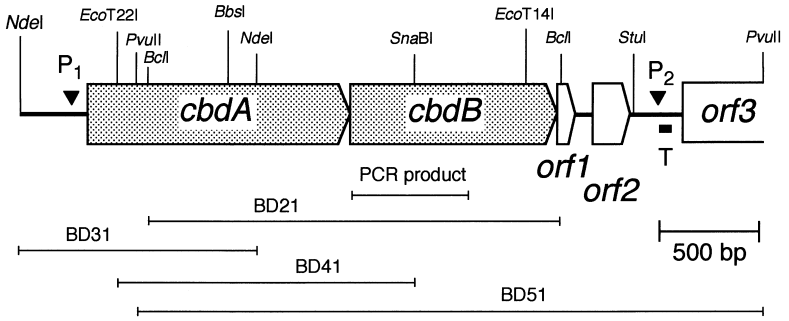

Fig. 4. Physical map of the *B. stearothermophilus cbdAB* gene cluster. Four clones (from BD21 to BD51) cover the whole *cbd* operon and a part of *orf3*. Closed triangles (P_1 and P_2), their putative promoters; a closed rectangle (T), a terminator sequence. The nucleotide sequence was deposited in DDBJ/EMBL/GenBank under accession number AB016894.

as well [34]. The *B. stearothermophilus* oxidase was also inhibited by NaCN and NaN_3 with 50%-inhibitory concentrations (IC_{50}) of 0.50 and 8.2 mM, respectively. These values indicate that the *B. stearothermophilus* enzyme is more susceptible to those anionic inhibitors than *E. coli bd*-type oxidase [5,31].

3.3. CbdAB operon encoding subunits of bd-type oxidase

In addition to the N-terminal sequences of the two subunits [22], sequences of their proteolytic fragments digested with *S. aureus* V8 protease were also determined. The results were TSSHAPLILFGTLEEDNEVK for subunit I and NAAGVALDYGKLFASPL for subunit II. A pair of PCR primers targeting the gene for subunit II were designed, and the obtained PCR product of about 500 bp was used as a probe to clone the whole operon in chromosomal DNA from wild type cells. Fig. 4 shows the map of the genes encoding the both subunits and adjacent open reading frames (orfs). The amino acid sequence deduced from the nucleotide sequence from the wild type cells matched fairly well the four partial sequences of the protein purified from K17 cells. This result not only proves that the cloned genes correspond to the protein, but also confirms that K17 strain is not a contaminant but a mutant derived from the wild cells of *B. stearothermophilus* K1041. The genes encoding subunits I and II are referred to *cbdA* and *cbdB*, respectively, hereafter. *B. stearothermophilus* CbdA comprises 448 amino acid residues of 50 195 Da, and CydB 342 residues of 38 992 Da. These sequences do not show the highest similarity to *cydAB* of *B. subtilis*, which is identified in the whole genome sequence [35] as a homolog of *E. coli cydAB* encoding cytochrome *bd*, but do so to *ythAB* of *B. subtilis*, which might be the second set of cytochrome *bd* genes. There is a putative promoter sequence upstream of *cbdA*, whereas there are not consensus sequences for binding of regulatory proteins; TTGAT-N_4-ATCAA for Fnr and TATTT for ArcA. These two factors are known in *E. coli* to regulate its terminal oxidases [36]. *CbdAB* was followed by two open reading frames, *orf1* and *orf2*, and then by a terminator sequence. *Orf1* and *orf2* may encode 33- and 96-residue peptides, respectively. *E. coli cydAB* genes are followed by *orfC* and *orfD*, encoding 37- and 97-residue peptides, respectively, and these four genes were demonstrated to composed an operon [37]. *B. stearothermophilus orf1* and *2* are not similar in sequence to *E. coli orfC* and *D*. The putative operon of these four orfs were followed by *orf3* in the same strand. The amino acid sequence deduced from *orf3* shows 55.3% identity to *E. coli* SugE protein, which suppresses a *groEL* mutation and mimics the effect of GroE overexpression, acting as a chaperone. The orf also shows 42.0% identity to a *B. subtilis* hypothetical gene *ykkC*, which is however located far from *ythAB* in the chromosomal DNA.

3.4. Characteristics of CbdA

CydAs from *E. coli* and *A. vinelandii* have been suggested to have 7 putative membrane-spanning helices [6,10]. The hydropathy plot of *B. stearothermophilus* CbdA (Fig. 5) and sequence comparison indicate that this polypeptide also has the 7 hydrophobic segments, in addition to another highly hy-

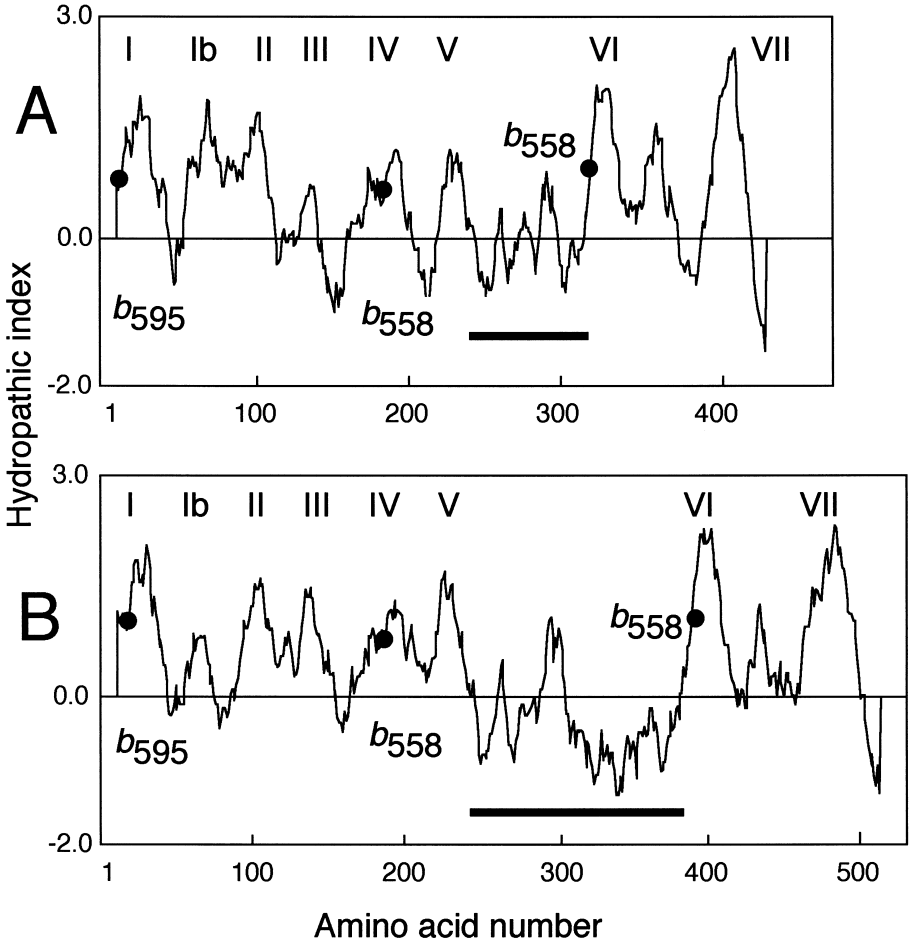

Fig. 5. Hydropathy profiles of *B. stearothermophilus* CbdA and *E. coli* CydA. Hydropathic index for *B. stearothermophilus* CbdA (A) and *E. coli* CydA [6] (B) was calculated according to Kyte and Doolittle [54] with window width of 21 residues. Roman numbers, putative transmembrane segments described for Fig. 7; closed circles, axial ligands for hemes *b*-595 and *b*-558; bars, positions of the Q loop.

drophobic region, designated Ib, between segments I and II. Fig. 6 compares sequences of subunits of various cytochromes *bd*, including putative ones only found in genomic sequences without their protein products identified. *B. stearothermophilus* CbdA lacks the C-terminal half of the long hydrophilic loop between putative transmembrane segments V and VI. This loop in *E. coli* is as long as 140 amino acid residues and referred to as 'Q loop', since it has been suggested to contain quinone-binding site [38–40]. All of subunit I and its homologs from Gram-positive bacteria reported so far lack half of the loop, whereas most of the subunit from proteobacteria, except *Pseudomonas aeruginosa* CioA [41], maintain the region. It is noteworthy that CydAs of a cyanobacterium *Synechocystis* sp. PCC6803 [42] and an archaeon *Halobacterium* sp. NRC-1 (GenBank accession number AF029621) also lacks the region in their genes. The phylogenetic tree of subunit I (Fig. 7A) shows that the 12 members can clearly be divided into two subfamilies; the proteobacterial type with the C-terminal half of loop V/VI and the more widely distributed type without the region.

Putative amino acid ligands for protohemes, His-21 for high-spin heme *b*-595, His-186 and Met-325 for low-spin heme *b*-558 are all conserved. Several residues around these ligands are also conserved, as well as the N-terminal half of the Q loop. Several residues in membrane-spanning helices, especially in segments II, III and Ib are conserved, in addition to a completely conserved motif GRQPW in loop VI/VII.

A. Region around helices I and II of subunit I

```
Bst.CbdA  20 VHIIYATIGVGVPLMIAIAQWVGIRKNDMHYILLARRWTRGFVITVAVGVVTGTAIGLQLSLLWPNFMQLAGQVISLPLFMET-FAFFFE 108
Bsu.CbdA  12 FHIIFATLGVGLPLMILVAELIYQKTKDDHYAIMAKRWTKAQAVLLGVAIPTGTIAGTQLALLWPGFMEVIGRVMSLPFQIEI-YAFFVE 100
Bsu.CydA  17 FHFLFVPMSIGLVFMVALMETLYLVKKNELYLKMAKFWGHLFLINFAVGVVTGILQEFQFGLNWSDYSRFVGDVFGAPLAIEALLAFFME 106
S68.CydA  22 FHMLWPVLTTGMGIYLVIIEGLWLKTRNLDYYHHARFWARLYVLNFGIGVATGLPMAFQFGLNWAPFSESVGDFFGTVLGFEGTMAFMLE 111
Pae.CioA  20 FHIIFPAITIGLASYLAVLEGLWLKTNEEVYRDLYHFWSKIFAVNFGMGVVSGLVMAYQFGTNWSHFSDFAGSITGPLLTYEVLTAFFLE 109
HNR.CydA  34 VHIIFASLSIGLAPFIIYFTWKDVRTNEQRYARLRSFWVKVFAVGFVMGTVTGIPMSFQFGTNFPQFATIAGELIGGPLAFEAKMAFFLE 123
Eco.CydA  18 YHFLFVPLTLGMAFLLAIMETVYVLSGKQIYKDMTKFWGKLFGINFALGVATGLTMEFQFGTNWSYYSHYVGDIFGAPLAIEGLMAFFLE 107
Kpn.CydA  18 YHFLFVPLTLGMAFLLAIMETVYVLSGKQIYKDMTKFWGKLFGINFALGVATGLTMEFQFGTNWSYYSHYVGDIFGAPLAIEGLMAFFLE 107
Avi.CydA  20 YHFLFVPLTLGMTFLLAIMESVYVMTGKQVYKDMVKFWGKLFGINFALGVTTGITMEFQFGTNWAYYSHYVGDIFGAPLAIEGLTAFFLE 109
Eco.CbdA  18 YHFLFVPLTLGLIFLLAIMETIYVVTGKTIYRDMTRFWGKLFGINFALGVATGLTMEFQFGTNWSFYSNYVGDIFGAPLAMEALMAFFLE 107
              *        *                   *      *              *     *            *         *   **  *
             b595          I                                  Ib                           II
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B. Loop region between helices V and VI of subunit I

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Bst.CbdA 232 VASALVGDLSGKFLAEYQPEKLAAAEWHFETSSH------APLILFGTLEEDNE-VKYALEIPYALSILAHNH--PAAVVTGLNDIPEDE 312
Bsu.CbdA 227 LLTALNGHESAQMLYEYQPEKLAGAEGLFETRSH------APLAIGGFTDPNEEKVKWAIEIPWALSFLAANR--FDTVVKGLNAFPRDE 308
Bsu.CydA 229 LGVGLSGHMQAEHLMESQPMKMAASEGLWEDSGD-----PAAWTAFATIDTKNEKSSNEIKVPYALSYLAYQK--FSGSVKGMKTLQAEY 311
S68.CydA 234 PLQIFIGHLSAEQVSVHQPAKLAAMEALWETVPAKT---PAAWSVVALPNDKAEKNDWELSIPGALSYILELKPQLDKPIQGLKDWAPVD 320
Pae.CioA 232 PVQALIGDAHGLNTLEHQPAKIAAMEGHWDNSSGE----PTPLILFGWPDMQREETRFKVEIPVLGSLILKHS--LTEPIPALKDFPPED 315
HNR.CydA 250 PFQAVHGDAYGRHVEDTQPQKFAAMEAHYETGSADLHLLAFPKSLDALTDPRTE-NLFTVSLPGIGSFLASGG-DFDAEVIGLNEY-EEN 336
Eco.CydA 232 LSVIVLGDESGYEMGDVQKTKLAAIEAEWETQPA-----PAAFTLFGIPDQEEETNKFAIQIPYALGIIATRS--VDTPVIGLKELMVQH 314
Kpn.CydA 232 LSVIVLGDESGYEMGDVQKTKLAAIEAEWETQPA-----PAAFTLFGIPDQRR-RRTFAIQIPFALGIIATRS--VDKQVTGLKALMVQH 313
Avi.CydA 234 LSVIVLGDESGYEVGEVQKAKLAAIEAEWETHPA-----PASFTLIGFPNEEEQRTDFAVKIPWVLGIIATRS--LDEQVIGIKDLIADH 316
Eco.CbdA 232 IGTLQLGDSSAYEVAQVQPVKLAAMEGEWQTEPA-----PAPFHVVAWPEQDQERNAFALKIPALLGILATHS--LDKPVPGLKNLMAET 314
                   *          *  * *  *                                    *
               V

Bst.CbdA 313 RPP------------------------------------------------------------------LYIHYLFDVMVTIGVFLMVVA 336
Bsu.CbdA 309 WPP------------------------------------------------------------------LFIHTLFNAMVGVGMLLILYS 332
Bsu.CydA 312 EKIYGKG--------------------------------------------------------DYIPPVKTTFWSFRIMVGAG-VVMILA 344
S68.CydA 321 RPRM----------------------------------------------------------------VGLIYYSFRIMVAIG-LFFAAL 345
Pae.CioA 316 RPNS-----------------------------------------------------------------TVVFWSFRIMAGLG-MLMILV 339
HNR.CydA 337 PP------------------------------------------------------------------VALVFWSFRFMVGLG-FLFIGL 359
Eco.CydA 315 EERIRNGMKAYSLLEQLRSGSTDQAVRDQFNSMKKDLGYGLLLKRYTPNVADATEAQIQQATKDSIPRVAPLYFAFRIMVACG-FLLLAI 403
Kpn.CydA 314 EERIRNGMKAYALLEQLRAGSTDQAVRDRFNDVKKDLGYGLLLKRYTDNVLDATEEQIALAAKDSIPAVAPLYFAFRIMVACG-VLMLLI 402
Avi.CydA 317 EARIRNGMVRYGLLEELRAGNKSPEKIAAFNEVKDDLGYGLLLKKYTPNVVDASEEQIKQAAKDTIPSVASMFWSFRAMVGAG-FAMLIL 405
Eco.CbdA 315 YPRLQRGRMAWLLMQEISQGNREPHVLQAFRGLEGDLGYGMLLSRYAPDMNHVTAAQYQAAMRGAIPQVAPVFWSFRIMVGCG-SLLLLV 403
                                                                                        *  *   *
                                                                                         b558     VI
```

C. Region around helices I and II of subunit II

```
Bst.CbdB  21 VASIDFGAGFFSVYSHWANQQHILHRIIQRYLSPVWEVTNVFLVFFFVGIVGFFPKTAYYYGSILLVPASIAIVLLAIRGSYYAFHTYGE 110
Bsu.CbdB  24 MATMDFGAGFWSMIYLNKEHMK-ATDIANRFLSPTWEVTNVFIVAIVVALFSFFPGATFVLGTVLLIPGSMILLLLAIRSGFLVFSNTAK 112
Bsu.CydB  22 LEGFDFGVGMATRFLGHNELE---RRVLINTIGPFWDANEVWLLTGAGAIFAAFPNWYATMLSGYYIPFVIVLLALMGRGVAFEFRGKVD 108
S68.CydB  29 LDGFDLGVGILSLTA-SSEER---RSILMTSLGNVWDANETWLVLMGGSLFGAFPLAYATILNALYLPAVIMVVGLILRAVSFEFRENAN 114
Pae.CioB  23 MDGFDLGIGMLYPFF-KDSGD---RDVMMNTVAPVWDGNETWLVLGGAALFGAFPLAYAVVLSALYLPLIFMLVGLIFRGVAFEFRFKAK 108
HNR.CydB  34 LDGFDFGIGMLYATR-TDEHE---RETFLAAFGPVWDANEVWIVAFGTMLLAAFPRVYSRLLADNYLLALGFVVALVFRGLGPELREQRE 119
Eco.CydB  25 TDGFDMGVGMLTRFLGRNDTE---RRIMINSIAPHWDGNQVWLITAGGALFAAWPMVYAAAFSGFYVAMILVLASLFFRPVGFDYRSKIE 111
Kpn.CydB  25 ADGFDMGVGMLTRFLGRNDTE---RRIMINAIAPHWDGNQVWLITAGGALFAAWPMVYAAAFSGFYVAMILVLASLFFRPVGFDYRSKIE 111
Avi.CydB  25 TDGFDMGAMALMPFIAKTDNE---RRVAINTVAPHWDGNQVWLITAGGALFAAWPLVYATAFSGMYWALLLVLFGLFFRPVGFDYRSKLE 111
Eco.CbdB  25 SDGFDMGIGCLLPLVARNDDE---RRIVINSVGAHWEGNQVWLILAGGALFAAWPRVYAAAFSGFYVAMILVLCSLFFRPLAFDYRGKIA 111
                 * *                            *                  *                    *  *
                  I                                                             II
```

Fig. 6. Multiple sequence alignment of cytochromes *bd* and their homologs. Three selected regions of *B. stearothermophilus* (*Bst*) CbdAB are compared with their counterparts of *B. subtilis* (*Bsu*) CbdAB (YthAB) and CydAB [35], *Synechocystis* sp. PCC6803 (*S*68) CydAB [42], *Pseudomonas aeruginosa* (*Pse*) CioAB [41], *Halobacterium* sp. NRC-1 (*H*NR) CydAB (GenBank accession number AF029621), *E. coli* (*Eco*) CydAB [6] and CbdAB (AppcB) [48], *Klebsiella pneumoniae* (*Kph*) CydAB [55] and *A. vinelandii* (*Avi*) CydAB [10], by using Clustal W [25] with manual adjustments. Asterisks, residues conserved completely; boxes, two out of three axial ligands suggested for hemes *b*-595 and *b*-558; solid underlines accompanied with Roman numbers, transmembrane segments suggested previously [6]; a dashed underline with 'Ib', an additional transmembrane segment suggested in this paper.

3.5. Characteristics of CbdB

The hydropathy plot of *B. stearothermophilus* subunit II indicates that the subunit is likely to contain 8 membrane-spanning helices like its counterparts of proteobacteria (data not shown). *B. stearothermophilus* subunit II is smaller than proteobacterial ones, except *P. aeruginosa* CioB. This is mainly due to deletion of about 30 residues in loop V/VI. Subunit II gave an essentially similar phylogenetic tree as

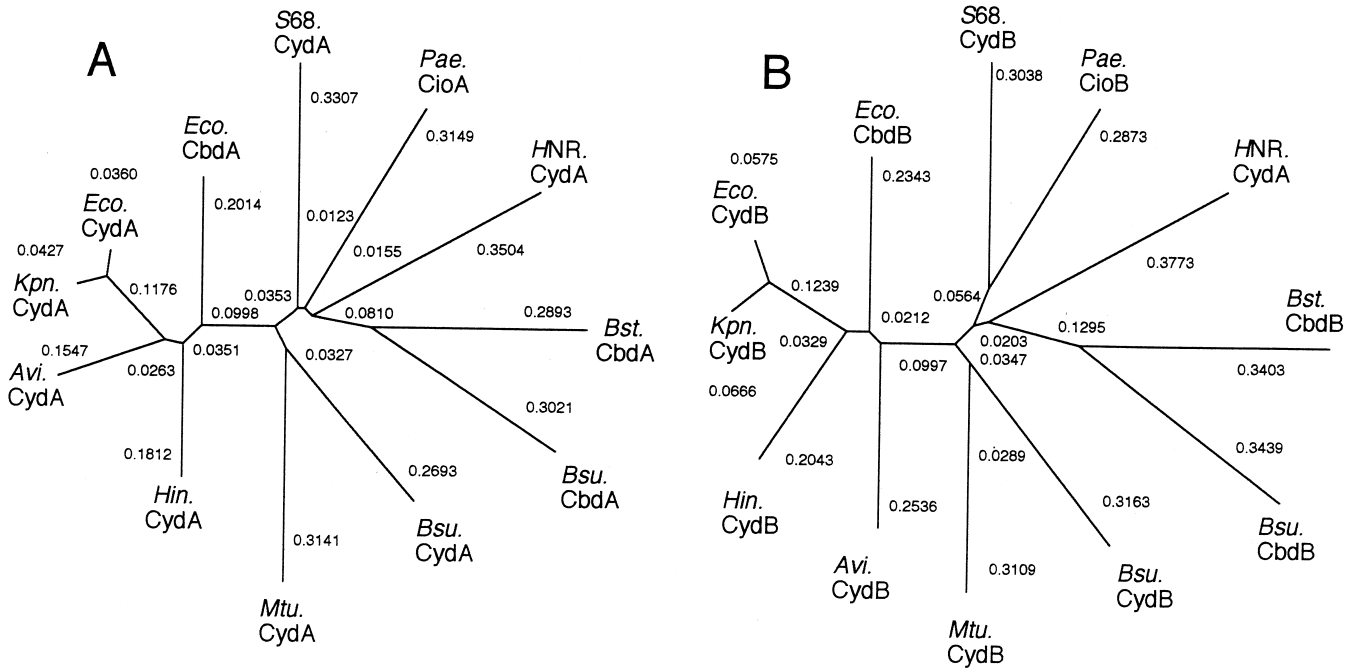

Fig. 7. Phylogenetic trees of the cytochrome *bd* subunits and their homologs. Phylogenetic trees for subunits I (A) and II (B) were constructed with Clustal W as described for Fig. 6. Origins of sequences and abbreviations for the names of organisms are as described in Fig. 6 in addition to *Haemophilus influenzae* (*Hin*) CydAB [56] and *Mycobacterium tuberculosis* (*Mtu*) CydAB [57]. The numbers indicate the branch lengths corresponding to amino acid substitutions per site.

subunit I, although there are some differences in the relative positions of closely located branches (Fig. 7B). Subunit II has been suggested to be necessary for the *E. coli* enzyme to bind heme *b*-595 and heme *d* [7,43,44], although only a few amino acid residues are completely conserved in the region around segments I and II (Fig. 6C), and no residues in the rest of the structure. No methionine or histidine residues are conserved, whereas an aspartate in helix I, a tryptophan in loop I/II and an arginine at the C-terminal end of helix II are preserved in addition to a couple of other neutral residues.

4. Discussion

The apparent differences in the sizes of subunits between *B. stearothermophilus* and *E. coli* cytochrome *bd* (Fig. 3) were confirmed by the DNA sequences. The size of *B. stearothermophilus* subunit I was estimated to be 50.2 kDa with the deduced amino acid sequence, while *E. coli* CydA is reported to be 58.3 kDa [6]. The difference in the overall size is mainly due to the length of loop V/VI. The hydropathy analysis showed that *B. stearothermophilus* CbdA has an additional hydrophobic segment between the putative transmembrane segments I and II (Fig. 5). Although the hydropathic index is much smaller in the corresponding region of *E. coli* CydA, subunit I from many organisms including these two species show appreciable sequence similarity around the region, suggesting that their membrane topology should be common. The topological folding of the entire *E. coli* cytochrome *bd* was excellently analyzed with β-galactosidase- and alkaline phosphatase-fusion techniques [45,46], however, it is not very clear whether segments I and II are parallel or anti-parallel. If there is indeed an additional transmembrane segment between them, both heme *b*-558, which is ligated by His-186 and Met-325, and heme *b*-595, which might be ligated by His-21 and composes the binuclear center with heme *d*, should locate on the same extracellular side of the cell membrane. If not, heme *b*-595 should exist on the opposite cytoplasmic side of the membrane. Accurate determination of the heme-heme distance may judge the membrane topology in the future. Hill et al. [47] showed that electron transfer from heme *b*-558 to the binuclear center

is not slower than that from heme *b*-595 to heme *d* with in the center. Their observation seems to prefer the former membrane topology.

The genome of *B. subtilis* contains two sets of cytochrome *bd* genes. *CydAB* has been identified as homologs to proteobacterial *cydAB* encoding cytochrome *bd* [35], whereas *ythAB* was not clearly identified in the first place but was found to be the most similar to the novel cytochrome *bd* genes of *B. stearothermophilus* in this study. *E. coli* genome also has two sets of genes for cytochromes *bd*. In addition to *cydAB* encoding cytochrome *bd*-I [6], *appCB* is recently shown to encode a second cytochrome *bd* and therefore is proposed to be renamed *cbdAB* [48,49]. We now propose to rename the *ythAB* genes *cbdAB*, as the second set of cytochrome *bd* genes of *B. subtilis*, and consequently to name our novel *B. stearothermophilus* genes as *cbdAB* based on its highest similarity to the *B. subtilis* genes. It is reported that *B. subtilis* mutant cells defective in caa_3-type oxidase show two absorption peaks at 617 and 627 nm [18]. The former peak may be due to CbdAB (YthAB) and the latter to CydAB, although no direct evidence has been presented.

The purified enzyme showed only a low duroquinol oxidase activity [22]. The absorption spectrum of the reduced enzyme did not clearly show the α-band peak at 595 nm or γ-band shoulder at 437 nm, which are suggested to be due to high-spin heme *b* in the case of the *E. coli* enzyme [50]. Similar enzymatic and spectral features are observed for *B. firmus* cytochrome *bd* [23]. An explanation of these features is that these *Bacillus* cytochromes *bd* may easily lose the high-spin heme *b* during preparation and resulted in the decrease in enzyme activity.

The present study showed that preincubation with MK largely stimulated the quinol oxidase activity (Fig. 3), indicating that the cytochrome *bd* is in fact a quinol oxidase. Furthermore, this observation also suggests that the enzyme contains a second quinone binding site besides a catalytic site. The possible explanation is that cytochrome *bd* physiologically contains a quinone bound to the second site which is essential for the catalytic activity but is apt to be decomposed or released during enzyme purification. *Bacillus* cells contain MK-7 as their physiological quinone [13], although MKs with shorter side chains were more effective in the enzyme activation (Fig. 3). This may be due to experimental difficulty for highly hydrophobic quinones to be dissolved in the reaction mixture and then to be restored to the proper site. A similar order of MK potency in activation effect was also observed for *Bacillus* PS3 quinol-cytochrome *c* oxidoreductase [33]. In contrast to *Bacillus bd*, proteobacterial *bd* maintains its high enzymatic activity during purification [5,31]. This difference may be due to the fact that MK is more labile than UQ and decomposed more rapidly under ultraviolet light. A similar second quinone-binding site has been proposed for a heme-copper oxidase, cytochrome *bo*-type ubiquinol oxidase [51]. An alternative explanation for the activation effect is that the preincubated quinone would be bound to the empty binding site for heme *b*-595 and partially mimic the heme's function as a redox center, although a difficulty of this assumption is the deep structural difference between protoheme IX and quinones.

The difference in peak wavelength due to cytochrome *d*, 618 nm for *B. stearothermophilus* vs. 628 nm for proteobacteria, might be attributed to the difference of the heme environments. Since hydrophobic environments generally cause blue shift of absorption due to $\pi \rightarrow \pi^*$ transition relative to polar ones [52], heme *d* in *B. stearothermophilus* oxidase may exist in a more hydrophobic environment than the heme in proteobacterial enzymes. It is also interesting that the air-oxidized enzyme as prepared did not show a dominant α peak in the long wavelength region (Fig. 1), in contrast to proteobacterial *bd* and in agreement with the *B. firmus* enzyme. In *E. coli*, the peak is demonstrated to be due to oxygenated ferrous heme *d* [53]. It is likely that the oxygenated species is more labile in the *Bacillus bd* than in the proteobacterial enzymes. Furthermore, the *B. stearothermophilus* oxidase is more sensitive than *E. coli* enzyme to anionic inhibitors such as cyanide and azide (Table 1), which inhibit oxidases by binding to the heme-heme binuclear center [3,4]. These findings suggest that the redox binuclear center of the *Bacillus bd* is more 'open' or accessible than that of *E. coli bd* to protons and/or electrons, resulting in easier decomposition of the oxygenated intermediate, and to those inhibitors, resulting in higher susceptibility to them. Alternatively, these differences could be caused by the absence of heme *b*-595 in the *Bacillus* oxidase.

Phylogenetic trees based on the sequence data (Fig. 7) demonstrate that cytochromes *bd* and their homologs cloned so far can be classified into two groups; cytochromes *bd* with the C-terminal half of the Q loop, including most of the γ proteobacteria, and those without that region, including all the Gram-positive bacteria, the cyanobacterium, the archaeon, and the γ-proteobacterium *P. aeruginosa*. Since the latter type of cytochrome *bd* is distributed more widely in taxonomic groups, it is plausible that original cytochrome *bd* was without the C-terminal region of the Q loop and acquired it by proteobacteria after they diverged from the other organisms. *Bacillus* cytochrome *bd* is suggested to operate as an alternative oxidase having high affinity to dioxygen to support energy-transducing respiration under microaerobic conditions and to reduce oxygen toxicity as in the case of *E. coli bd*, however, little is known about the relationship between the protein structure and physiological function. Identification and characterization of the cytochrome *bd* proteins in various organisms and extensive site-directed mutagenesis experiments may help us to understand this unique enzyme family.

Acknowledgements

We would like to thank Dr. Tatsushi Mogi and Dr. Yasuhiro Anraku of The University of Tokyo for their gift of *Escherichia coli* cytochrome *bd* and inhibitors, and Eisai for giving us several MKs and UQs. We also wish to acknowledge Dr. Lars Hederstedt and Dr. Claes von Wachenfeldt of Lund University and Dr. T. Mogi and Dr. Takashi Ogura of The University of Tokyo for their valuable suggestions. This study was supported by Grants-in-Aid for Scientific Research (B) (07459018), (C) (10680617) and that on Priority Areas (08249233) from the Ministry of Education, Science, Sports, and Culture of Japan.

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