Mutaion Analysis of B-type Cytochrome *c* Oxidase Interacting with its Natural Substrate Cytochrome *c*-551

Running title: Interaction between Bacterial Oxidase and Cytochrome *c*

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Abbreviations: TMPD, *N*,*N*,*N'*,*N'*-tetramethyl-*p*-phenylenediamine; kDa, kilodalton; ORF, open reading frame, PCR. polymerase chain reaction; bp, base pair; MALDI-TOF mass, matrix-assisted laser desorption ionization time of flight; 3D, three dimension (steric)

The heme-copper oxidases in the respiratory chain can be classified into three subfamilies; A-, B- and C-types. The cytochrome bo_3-type cytochrome *c* oxidase from thermophilic *Bacillus*, one of the B-type oxidases, has been suggested to interact with the cytochrome *c* mainly with hydrophobic interactions, in contrast to A-type oxidases, which are bound to cytochromes *c* primarily with electrostatic forces between acidic residues in the oxidase subunit II and basic ones in the cytochromes. In order to investigate the substrate-binding site on cytochrome bo_3, eight acidic residues in subunit II were mutated to corresponding neutral amide ones and the enzyme activity was measured using cytochrome *c*-551 of closely related *Bacillus* PS3. The mutation of E116, which locates at the interface to subunit I, decreased the k_{cat} value most prominently without affecting the K_m value, indicating that the residue is important for electron transfer. The mutation of D99, located close to the Cu_A site, largely affected both the values, suggesting that it is important for both electron transfer and substrate binding. The mutations of D49 and E84 did not affect the kinetic values, whereas E64, E66 and E68 lowered the affinity to cytochrome *c*-551 without large effect on the k_{cat} value. These three residues are located at the side front of the hydrophilic globular domain of the subunit and distant from the Cu_A site, suggesting that they compose an acidic patch of the second substrate binding site. This is the first report on the site-directed mutation experiments of a B-type heme-copper oxidase.

Thermophilic *Bacillus* contains cytochrome bo_3-type cytochrome *c* oxidase as an alternative oxidase in the respiratory chain under air-limited conditions (1, 2), in addition to the main terminal oxidase, cytochrome caa_3 (3), and a menaquinol oxidase, cytochrome *bd* (4, 5). Both cytochromes bo_3 and caa_3 belong to the heme-copper oxidase superfamily, which can be classified into three subfamilies; A-, B- and C-types (formerly called SoxM-, SoxB-, FixN-types), based on

the amino acid sequence of their subunit I and the composition of their subunits and hemes (6). Cytochrome bo_3 is B-type, as cytochrome ba_3-type cytochrome *c* oxidase from *Thermus thermophilus* (7), while cytochrome caa_3 is A-type, as the mitochondrial oxidases. Although the content of cytochrome bo_3 in the *Bacillus* cells is low even under air-limited conditions, it is possible to detect and purify the enzyme from mutant strains of *Geobacillus thermodenitrificans*, such as K17 and K17q8, which lack cytochrome caa_3 (1, 8). We have also succeeded in cloning, sequencing and over-expression of cytochrome bo_3 in the cells of *G. thermodenitrificans*, which is a transformable thermophilic *Bacillus*, (2, 9). The B-type cytochrome *c* oxidase has been identified from some thermophilic bacteria. The enzymes of thermophile are very important not only for academic interest but also for engineering because of its stability. However, bioenergetics of thermophilic bacteria has been poorly characterized.

Subunit I of B-type enzymes contains six His residues as the ligands for the low spin heme and the binuclear center composed of the high spin heme plus Cu_B as A-type oxidases do, although it does not have the H^+ pathway, so-called D channel, and a Glu residue at the catalytic center conserved in A-type ones (2, see also for review). While subunit II of A-type oxidases contains two transmembrane helices at the N-terminus, that of B-type enzymes only has a single transmembrane helix. The subunit of the both types of cytochrome *c* oxidases contains the binuclear Cu_A center in the C-terminal hydrophilic domain. Cytochrome bo_3 from thermophilic *Bacillus* shows a biphasic dependency of cytochrome *c* oxidase activity on the substrate cytochrome *c*-551 concentration, as is well known for mitochondrial A-type cytochrome aa_3 oxidase with cytochrome *c* (10). The high affinity site, with the K_m value of about 10^{-8} M, is very specific for cytochrome *c*-551, while the low affinity site, with the value of about 10^{-5} M, shows broad substrate specificity and is replaceable by mitochondrial cytochromes *c* and even by the

artificial substrate *N*,*N*,*N'*,*N'*-tetramethyl-*p*-phenylenediamine (TMPD) (9). Another characteristics of the cytochrome *c*-551 oxidase is that the activity is optimal under high ionic strength conditions (1, 9), suggesting that hydrophobic interaction is important for the reaction between the substrate and the enzyme.

Cytochrome *c*-551 is a lipoprotein of 10.4 kDa, found in thermophilic *Bacillus* PS3 grown under air-limited conditions (11, 12), and plays a role as an electron carrier between quinol-cytochrome *c* oxidoreductase (b_6c complex) (13) and cytochrome bo_3 oxidase (1). Analyses of mature cytochrome *c*-551 have indicated that the signal peptide consisting of 18 amino-acid residues is cleaved off and the new N-terminal Cys residue is modified with a diacylated glycerol moiety at the sulfhydryl side chain and also with an acetyl group at the α-amino group (12,14). Cytochrome *c*-551 has been over-expressed in transformable *Geobacillus thermodenitrificans* K1041 by using the structural gene from *Bacillus* PS3 combined with a promoter for a neutral protease of this bacterium (14).

Early studies on the interaction of mitochondrial cytochrome *c* and cytochrome *c* oxidase have indicated that a basic cluster on cytochrome *c*, mostly composed of Lys residues around the heme C crevice, is responsible for its docking to the hydrophilic domain of subunit II of the mitochondrial oxidase (15). This view is strongly supported by the well-known observation that the interaction between the oxidase and cytochrome *c* becomes weaker under high ionic strength conditions. Interaction of cytochromes *c* and A-type oxidases has also been analyzed by site-directed mutations substituting negatively charged residues on subunit II by neutral residues. A study using soluble domains of mutated subunit II of the *Paracoccus denitrificans* enzyme supports the importance of ionic interaction between the oxidase and cytochrome *c* (16). More recent studies on the whole enzymes showed that the electrostatic forces are necessary for efficient

long-range docking, but hydrophobic interaction plays a role in the reorientation of the two redox partners in the second step (17, 18). It was also shown that Trp121, but not other aromatic residues, is important to the electron transfer from cytochrome *c* to Cu_A (19-22).

In our previous study, several mutants of cytochrome *c*-551 were prepared by site-directed mutagenesis in the expression plasmid containing the structural gene *cccA* for cytochrome *c*-551 from *Bacillus* PS3 and by their expression in the *G. thermodenitrificans* host cells (23). In most of the mutant cytochromes, one of several Lys residues were substituted to Ala or Ser and we found that these mutant cytochromes kept their activity as the substrate, although the K_m values were varied between 0.04-0.12 mM, dependent on the site replaced. On the contrary, cytochrome *c*-551 delipidated by lipases only showed very low ability as the substrate. Furthermore, the C19A mutant cytochrome, which was produced in *Brevibacillus choshinensis* as a secretary protein, almost completely lost the ability. These results suggest that fatty acyl-glycerol residue covalently bound to cytochrome *c*-551 plays an essential role for the ability. Some of the acidic residues in subunit II of the A-type oxidases are also conserved in B-type oxidases (2). Recently, atomic structure of cytochrome ba_3 from *T. thermophilus* become available (24), although no over-expression systems have been reported for B-type oxidases, except of that for *G. thermodenitrificans* cytochrome bo_3 (9). Here, we constructed the expression system of site-directed mutants of cytochrome bo_3, and carried out kinetic experiments of the oxidase with mutated subunit II. Some of the mutant enzymes had higher K_m values than the wild type, while an enzyme with mutation at an amino-acid residue facing to subunit I showed a lower V_{max} value. We also propose a new binding site on subunit II to cytochrome *c*-551, which is side front of the hydrophilic domain of the subunit, but not at the top surface right above the Cu_A site demonstrated in the case of mitochondrial and proteobacterial A-type oxidases.

MATERIALS AND METHODS

Materials A transformable strain *Geobacillus thermodenitrificans* K1041 (formerly *Bacillus stearothermophilus* K1041) was donated by Dr. Narumi (25). A expression plasmid for this bacterium, pSTE12 (14), was used for the construction of the plasmids for over-production of *G. thermodenitrificans* K1041 cytochrome bo_3, and the enzyme was purified as described previously (9). Cytochrome *c*-551 of *Bacillus* PS3 was over-expressed in K1041 and purified as described (14). DEAE-Toyopearl anion exchange gel, hydroxyapatite, octyl-cellulose and TMPD were obtained from Tosoh (Tokyo, Japan), Bio-Rad (Hercules, United States), Chisso (Tokyo, Japan) and Wako (Kyoto, Japan), respectively. Proteins used as molecular mass standards and cytochromes *c* of yeast and horse heart were purchased from Sigma (St. Louise, United States). Other chemicals were obtained as described previously (9).

Site-directed Mutagenesis for Cytochrome bo_3 Mutants were constructed as follows. Part of the *G. thermodenitrificans* bo_3 gene was amplified by PCR using F-*Cla* (:5'-CTGACGACAATCGATCC 3') and R-*Kas* (:5'-ATGATGGCCGGCGCCGC-3'), as the primer set, and pUCcba2 (9), as the template. Resulting 321-bp DNA fragment containing subunit II region (810 to 1130, see Fig.1A) was purified and ligated into *Sma*I site of pUC118 to formed pUCcbasub2. Mutagenesis was performed by QuickChange™ Site-Directed Mutagenesis Kit (Stratagene), using pUCcbas2 as the template. Primers used for subcloning and mutagenesis are shown in Table 1. Obtained mutants were sequenced to confirm the mutation. The 296 bp mutated subunit II genes excised by *Cla*I and *Kas*I digestion were ligated into 6.1-kb fragment of pUCcba2 generated by *Cla*I and *Kas*I digestion, to restore full-length bo_3 gene containing subunit II mutation. Then the mutant bo_3 genes were digested with *Eco*RI and *Pst*I, purified, and ligated into the same site of the shuttle vector, pSTE12, to construct expression plasmid in *G.*

thermodenitrificans. Transformation of these plasmids to *G. thermodenitrificans*, cell culture and purification procedure of the oxidase were as described previously (9).

Purification of Mutated Oxidases Cytochrome bo_3 and its mutants were purified as described previously for the wild type enzyme (9). In brief, the membrane fraction was treated with sodium cholate to remove peripheral membrane proteins and intrinsic membrane proteins was solubilized with 1% (w/v) of a 1:1 mixture of *n*-nonanoyl *N*-metylglucamide (MEGA9) and *n*-decanoyl *N* methylglucamide (MEGA10) (MEGA9+10). Cytochrome bo_3 oxidase was purified by three step column chromatography in the presence of 0.5% (w/v) MEGA9+10. The supernatant was applied to a DEAE-Toyopearl column in the presence of 100 mM NaCl to remove many proteins including cytochrome b_6c-type reductase and caa_3-type oxidase. The flow-through, containing cytochrome bo_3-type oxidase, was dialyzed, applied to a second DEAE-Toyopearl column, and eluted by increasing the NaCl concentration. The oxidase was further purified with a hydroxyapatite column.

Measurement of Oxidase Activity Cytochrome *c* oxidase activity of the purified enzyme was continuously monitored with a pH electrode (Beckman No.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 acceptor ascorbate, as described previously (3), AscorbateH^- + H^+ + 1/2O_2 --> Dehydroascorbate + H_2O. The reaction was carried out with 0.25-0.5 nmol cytochrome bo_3 oxidase at 40°C in the high ionic concentration medium (2.5 ml) composed of 200 mM KCl, 1 mM $MgSO_4$, 1 mM Na-P_i buffer, pH 6.8, supplied with 10 mM sodium ascorbate and 0.075 mM TMPD as the substrate. An aliquot of *c*-551 was added and pH change due to H_2O formation with H^+ was measured. The data were plotted in [S]/*v* vs. [S] fashion, and V_{max} and K_m were obtained graphically. H^+ absorption was titrated by adding

adequate volumes of 10 mM HCl to each assay.

Measurements of Optical Spectrum Contents of cytochromes bo_3 and *c*-551 were determined from reduced minus oxidized difference spectra using millimolar extinction coefficient differences of 21.3 at 604 nm minus 630 nm (3), and 24.3 at 551 nm minus 538 nm (11), respectively.

Others Protein concentration was determined as a modified Lowry method (26). Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (27) with acrylamide concentrations were usually 13.5% (w/v) and sometimes 16% (w/v), except that boiling of protein samples was omitted. For sequence analysis, the proteins were separated by SDS-PAGE with 16% (w/v) acrylamide gel and electro-transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was washed thoroughly with water to remove glycine, treated with 0.6 N HCl at 37°C for 12 h to release a possible N-terminal formyl group, and applied to a pulse-liquid peptide sequencer (Applied Biosystems, model 477A) for Edman degradation. To estimate the molecular masses of subunits which compose cytochrome bo_3, mass analysis of purified wild type enzyme was performed. Matrix-assisted laser desorption ionization (MALDI) mass spectrometry of proteins was performed using 2- (4-hydroxyphenylazo) benzoic acid (HABA) as the matrix, as described by Ghaim *et al*. (28). First, trichloroacetic acid (TCA) precipitation of purified enzyme was dissolved in 99% formic acid, and then mixed at a 1:5 ratio with 50% acetonitrile solution of 1.3 mg HABA ml^{-1} and 0.1% trifluoroacetic acid. The mixture was spotted onto a sample plate and analyzed using MALDI mass spectrometer. The modeling of the 3D-structure of the cytochrome bo_3 subunit II was performed by homology modeling programs, Modeller (9v7) (http://www.salilab.org/modeller/).

RESULTS

Expression and Purification of Mutant Cytochrome *bo*$_3$ Mutations were introduced in the expression plasmids for cytochrome *bo*$_3$ to change Glu and Asp residues to Gln and Asn, respectively. The wild-type cells of *G. thermodenitrificans* K1041 scarcely produce cytochrome *bo*$_3$ without a plasmid, but when used as the host cells, they produced the wild type (9) and mutant oxidases in large amounts, as the result of the plasmid introduction. Table 1 lists the set of primers used for introducing the mutations, and Table 2 summarizes the total amounts of *b*-type cytochromes in the membrane fractions. The amount of *b*-type cytochromes in the control cells without the plasmids was about 0.5 nmol/mg membrane protein, and those with one of the plasmids were mostly between 1.2-2.0 nmol/mg. The *G. thermodenitrificans* cells contain cytochrome b_6c_1 complex, cytochrome *bd*-type quinol oxidase and succinate dehydrogenase as the other *b*-type cytochromes (29). It is thus likely that the increment of 0.7-1.5 nmol/mg protein, may be due to cytochrome *bo*$_3$ expressed with the plasmids, unless the production of those other *b*-type cytochromes is somehow enhanced. The TMPD oxidase activities of the membrane fractions prepared from transformed cells were partially inhibited by 5 μM cyanide, while no portion of the activity in the wild-type membranes was inhibited by cyanide at such a low concentration (30). Since cytochrome *bo*$_3$ has higher sensitivity to cyanide than cytochrome *caa*$_3$ and cytochrome *bd*, the observation supports the presence of cytochrome *bo*$_3$ in the transformant cells. The mutated oxidases, in which acidic residues had been replaced by neutral ones, were eluted at 30, 40 or 70 mM NaCl, while the wild enzyme was eluted at 100 mM (1). Figure 2 shows elution pattern of the second DEAE-Toyopearl chromatography for the E64Q and E68Q mutants, as well as that for the wild-type enzyme overexpressed in the original plasmids. Subunit composition of purified enzyme was investigated by CBB and heme stainings after SDS-PAGE,

and it was found that all purified enzymes had subunit I and II but not other oxidases or cytochrome, which would be influential to the activity assay (data not shown).

Mass spectral analysis of cytochrome bo_3 indicated that the purified cytochrome consisted of three major polypeptides. The observed mass values of the wild type enzyme are 60,371 ± 117, 17,074 ± 20 and 5,976 ± 41. The molecular masses of subunits I and II, estimated from the nucleotide sequences, are 60,647 and 17,016, respectively, based on the finding that the initial Met of subunit I is cleaved off while that of subunit II is maintained (1, 2). The two observed values are consistent to the calculated ones, although the accuracy of the measurement was not sufficient to detect the differences due to the mutations. SDS-PAGE analysis at a high gel concentration also indicated that the purified enzyme contained subunits I and II, and a third polypeptide with apparent molecular mass of 6 kDa. Edman degradation of the 6 kDa novel component, extracted from the gel at a high concentration, showed that the N-terminal four amino-acid residues were AKPE. A short open reading frame (ORF), starting with MAKPE, is found at the upstream region of the subunit II gene, *cbaB* (Fig. 1A). The calculated molecular weight of the ORF product is 5920 when the initial Met is omitted. This value is close to the observed mass of the third polypeptide, suggesting that this is a third subunit of cytochrome bo_3. The deduced amino acid sequence has a low but apparent similarity to that of subunit IIa found in cytochrome ba_3 from *T. thermophilus* (24). The gene for *T. thermophilus* subunit IIa, named *cbaD*, is also located upstream of the subunit II gene, *cbaB* (31), suggesting that they are homologues.

Effects of Mutations on the Cytochrome *c*-551 Oxidase Activity The cytochrome *c* oxidase activity of the mutant enzymes were measured with various concentrations of wild type cytochrome *c*-551 to clarify the effects of the mutations on the substrate concentration-dependence

of the activity. The reaction mixture contained TMPD, which effectively mediates electron transfer from the reductant ascorbate to cytochrome *c*-551 bound at the high affinity-substrate site, by binding to the low affinity-substrate site with a K_m value of about 0.1 mM. Under these experimental conditions, the obtained K_m and the V_{max} values should be those for the high affinity-binding site for the substrate (7, 24). Table 3 summarizes these kinetic parameters of partially purified mutant oxidases. The most effective change was observed in the D99N mutant, which had a 6-fold larger K_m value for cytochrome *c*-551 with a partially reduced k_{cat} value. The decrease in k_{cat} value is most prominent in the E116Q mutant oxidase. The K_m values were also increased by the mutations of Glu residues at 64, 66, 68 and 139 to Gln residues. The extents were smaller than that for the D99N mutation, but still significant. The mutations of acidic residues to neutral ones at the other locations are less effective than those.

Effects of Mutations on the Optical Spectra To clarify the perturbation of redox metal centers by the mutations, optical spectra of the purified enzyme were measured. No significant changes were detected in the redox difference spectra of the mutant enzymes at the α-band region, indicating that the amino-acid substitutions perturb neither the low spin heme *b* nor the high spin heme o_3 in subunit I (data not shown). To know the perturbation of the Cu_A center in subunit II, optical spectra at 500-900 nm were measured for the oxidized form of the purified oxidases. Figure 3 shows the spectra of the wild-type oxidase and some of the mutant enzymes. In all the enzymes, the peak at 530 nm due to β-band region was observed even in the oxidized form. A weak absorption due to Cu_A is detectable at 830 nm in oxidized form of many A-type oxidases such as cytochrome aa_3 from beef heart and *Paracoccus denitrificans* (32), whereas cytochrome caa_3 from the thermophilic *Bacillus* showed the absorption peak at 780 nm (Sone and Chance, unpublished observation). The B-type cytochrome bo_3 showed a low absorption peak at 810-820

nm, in addition to the high peak around 530 nm and shoulders at 565 nm and 665 nm due to the hemes. None of the mutant oxidases showed significant differences, except E139Q, which gave a small but clear red shift to 830 nm, although this enzyme showed a normal activity with the similar K_m value for cytochrome *c*-551.

DISCUSSION

The kinetic data of the mutant B-type oxidases reported here can be summarized as follows; 1) the affinity for cytochrome *c*-551 decreased mildly without changing k_{cat} much in the E64Q, E66Q and E68Q mutants, 2) both the affinity for the substrate and the k_{cat} value decreased largely in the D99N enzyme, and 3) the k_{cat} value of the E116Q mutant decreased with keeping the affinity to cytochrome *c*-551.

There are 14 acidic amino-acid residues in subunit II of cytochrome bo_3 oxidase from *G. thermodenitrificans*. D99 is conserved both in A- and B-type cytochrome *c* oxidases. Mutation of this residue of the *Paracoccus* enzyme expressed in *E. coli* host cells brought the largest effect on the k_{on} value between cytochrome *c* and Cu_A domain of subunit II (16). Large increase in K_m to horse heart cytochrome *c* and decrease in k_{cat} was also observed using the whole *Paracoccus* enzyme with mutation of the residue (17, 18). This residue is located close to the Cu_A-binding site in the atomic structure (33). It seems therefore likely that this Asp residue is important both for the cytochrome *c* binding and the electron transfer.

The E116 is not strictly conserved but an acidic residue is present at the vicinity in both A- and B-type enzymes. This residue is located at the interface to subunit I both in *Paracoccus* aa_3 (33) and *Thermus* ba_3 oxidases (24). Large decrease in the k_{cat} value was observed in the E116Q enzymes without change in the K_m value to cytochrome *c*-551. Lappalainen *et al*. reported that

the *on* constant and the *off* constant with horse heart cytochrome *c* of the *Paracoccus* Cu_A-domain with the corresponding D221N mutation were almost the same as those of the wild-type domain (16). It seems likely that E116 affects the binding of subunit II to subunit I, and mutations of this residue reduce the rate of electron transfer from Cu_A in subunit II to heme *b* in subunit I. On the contrary to the case of E116Q, the k_{cat} for *c*-551 of the E139Q mutant were almost the same as those of the wild type enzyme. This result is consistent with the facts that this Glu residue is not conserved and substituted to Gln in *T. thermophilus* cytochrome ba_3 (7), and that the presence of the residue itself is important since the α-carbonyl group of this locus is one of the ligands for Cu_A atom, and the γ-carbonyl group is a ligand for the Mg atom (24, 33).

It has been repeatedly demonstrated that electrostatic interaction is important in the reaction between A-type cytochrome aa_3 oxidase and cytochrome *c* from mitochondria and proteobacteria (15-20). In the previous report using several mutant cytochromes *c*-551, it is revealed that the interaction of the cytochrome *c* and the B-type oxidase is unique in the thermophilic *Bacillus* (23). The interaction is mainly hydrophobic, since replacement of Lys/Arg residues in cytochrome *c*-551 by Ala did not weaken the interaction, and the oxidase activity was highly stimulated under high ionic strength conditions (1, 9). The present data shows that many of amide substitution of carboxyl groups did not affect the substrate binding to the oxidase to high extents, though the E64Q, E66Q and E68Q mutation resulted in lowering affinity to *c*-551. These residues seem to compose an acidic patch corresponding to what has been found in *Paracoccus* enzyme (22), however, its location is much different and at the side front of the hydrophilic globular domain of subunit II, far from the Cu_A site (Fig. 1B). In conclusion, the hydrophobic interactions are critical for the cytochrome *c*-551 binding to the B-type oxidase, and the electrostatic interactions are subordinate for the substrate binding in the second binding site.

X-ray crystallography of *T. thermophilus* cytochrome ba_3 revealed that the oxidase is composed of subunits I, II and IIa (24), the last of which is a short polypeptide with one hydrophobic helix (31) and is located at the position equivalent to that the N-terminal transmembrane helix of subunit II of A-type oxidases (24). The present experiment showed that *G. thermodenitrificans* cytochrome bo_3 also contains a polypeptide homologous to subunit IIa. In spite of the structural similarity of these two B-type oxidases, the enzyme-substrate interactions are not very similar between them. It was reported that the interaction between *T. thermophilus* cytochrome ba_3 and cytochrome *c*-552 is prevented by the presence of salts (34). The sharp difference is not very surprising because of the structural difference of the substrate cytochromes *c*. Cytochrome *c*-552 is not a lipoprotein and its amino-acid sequence is much different from that of cytochrome *c*-551 (35, 36).

The absorption band of the oxidized cytochrome *c* oxidase at around 800 nm is known to be due to Cu_A (31). The peak band is usually at 830 nm in the case of A-type oxidases from mitochondria and Gram-negative bacteria, but that of cytochrome caa_3 of the thermophilic *Bacillus* PS3 is at 780 nm. The peak of B-type cytochrome bo_3 was at 810-820 nm as that of the mitochondrial cytochrome *c* oxidases (Fig. 3). The E139Q mutant oxidase showed a small red shift of the Cu_A peak. The effect of polypeptide environment on the Cu_A center has not yet been elucidated well, but these differences in absorption spectra would be a useful clue to investigate it.

The B-type cytochrome *c* oxidase is also important for bio engineering, since this type enzyme is identified from some thermophilic bacteria and the respiratory chain is essential for survive. Further work is needed to solve the mechanism of this type oxidase. We are presently attempting to solve the interaction between B-type bo_3 oxidase and its cytochrome *c* in more detail, using the intrinsic substrate cytochrome *c*-551 of *G. thermodenitrificans* K1041.

REFERENCES

1. **Sakamoto, J., Handa, Y., and Sone, N.**: A Novel Cytochrome *b*(*o*/*a*)$_3$-Type Oxidase from *Bacillus stearothermophilus* Catalyzes Cytochrome *c*-551 Oxidation. J. Biochem., **122**, 764-771 (1997).

2. **Nikaido, K., Sakamoto, J., Handa, Y., and Sone, N.**: The *cbaAB* genes for bo3-type cytochrome *c* oxidase in *Bacillus stearothermophilus*. Biochim. Biophys. Acta, **1397**, 262-267 (1998).

3. **Sone, N. and Yanagita, Y.**: A cytochrome *aa*$_3$-type terminal oxidase of a thermophilic bacterium. Biochim. Biophys. Acta **682**, 216-226 (1982).

4. **Sakamoto, J., Matsumoto, A., Oobuchi, K., and Sone, N.**: Cytochrome *bd*-type quinol oxidase in a mutant of *Bacillus stearothermophilus* deficient in *caa*$_3$-type cytochrome *c* oxidase. FEMS Microbiol. Lett., **143**, 151-158 (1996).

5. **Sakamoto, J., Hayakawa, A., Uehara. T., Noguchi, S., and Sone, N.**: Cloning of *Bacillus stearothermophilus ctaA* and Heme A Synthesis with the CtaA Protein Produced in *Escherichia coli*. Biosci. Biotechnol. Biochem. **63**, 96-103 (1999).

6. **Castresana, J. and Saraste, M.**: Evolution of energetic metabolism: the respiration-early hypothesis. Trends Biochem. Sci., **20**, 443-448 (1995).

7. **Keightley, J.A., Zimmermann, B.H., Mather, M.W., Springer, P., Pastuszyn, A., Lawrence, D.M., and Fee, J.A.**: Molecular genetic and protein chemical characterization of the cytochrome *ba*$_3$ from *Thermus thermophilus* HB8. J. Biol. Chem., **270**, 20345-20358 (1995).

8. **Sone, N., Koyanagi, S., and Sakamoto, J.**: Energy-yielding properties of SoxB-type cytochrome *bo*(3) terminal oxidase: analyses involving *Bacillus stearothermophilus* K1041 and its mutant strains. J. Biochem., 127, 551-557 (2000).

9. **Nikaido, K., Sakamoto, J., Noguchi, S., and Sone, N.**: Over expression of *cbaAB* genes of *Bacillus stearothermophilus* produces a two-subunit SoxB-type cytochrome *c* oxidase with proton pumping activity. Biochim. Biophys. Acta, **1456**, 35-44 (2000).

10. **Ferguson-Miller, S., Brautigan, D.L., and Margoliash, E.**: Correlation of the kinetics of electron transfer activity of various eukaryotic cytochromes *c* with binding to mitochondrial cytochrome *c* oxidase. J. Biol. Chem., **251**, 1104-1115 (1976).

11. **Sone, N., Kutoh, E., and Yanagita, Y.**: Cytochrome *c*-551 from the thermophilic bacterium PS3 grown under air-limited conditions. Biochim. Biophys. Acta, **1977**, 329-334 (1989).

12. **Fujiwara, Y., Oka, M., Hamamoto, T., and Sone, N.**: Cytochrome *c*-551 of the thermophilic bacterium PS3, DNA sequence and analysis of the mature cytochrome. Biochim. Biophys. Acta, **1144**, 213-219 (1993).

13. **Kutoh, E. and Sone, N.**: Quinol-cytochrome *c* oxidoreductase from the thermophilic bacterium PS3. Purification and properties of a cytochrome bc_1(b_6f) complex. J. Biol. Chem., **263**, 9020-9026 (1988).

14. **Noguchi, S., Yamazaki, T., Yaginuma, A., Sakamoto, J., and Sone, N.**: Over-expression of membrane-bound cytochrome *c*-551 from thermophilic Bacillus PS3 in *Bacillus stearothermophilus* K1041. Biochim. Biophys, Acta, **1188**, 302-310 (1994).

15. **Ferguson-Miller, S., Brautigan, D., and Margoliash, E.**: Definition of cytochrome *c* binding domains by chemical modification. III. Kinetics of reaction of carboxydinitrophenyl cytochromes *c* with cytochrome c oxidase. J. Biol. Chem., **253**, 149-159 (1978).

16. **Lappalainen, P., Watmough, N.J., Greenwood, C., and Saraste, M.**: Electron transfer between cytochrome *c* and the isolated Cu_A domain: identification of substrate-binding residues in cytochrome *c* oxidase. Biochemistry, **34**, 5824-5830 (1995).

17. **Witt, H., Malatesta, F., Nicoletti, F., Brunori, M., and Ludwig, B.**: Cytochrome-*c*-binding site on cytochrome oxidase in *Paracoccus denitrificans*. Eur. J. Biochem., **251**, 367-373 (1998).

18. **Witt, H., Malatesta, F., Nicoletti, F., Brunori, M., and Ludwig, B.**: Tryptophan 121 of subunit II is the electron entry site to cytochrome-*c* oxidase in *Paracoccus denitrificans*. Involvement of a hydrophobic patch in the docking reaction. J. Biol. Chem., **273**, 5132-5136 (1998).

19. **Zhen, Y., Hoganson, C.W., Babcock, G.T., and Ferguson-Miller, S.**: Definition of the interaction domain for cytochrome *c* on cytochrome *c* oxidase. I. Biochemical, spectral, and kinetic characterization of surface mutants in subunit ii of *Rhodobacter sphaeroides* cytochrome *aa*(3). J. Biol. Chem., **274**, 38032-38041 (1999).

20. **Wang, K., Zhen, Y., Sadoski ,R., Grinnell, S., Geren, L., Ferguson-Miller ,S., Durham, B., and Millett, F.**: Definition of the interaction domain for cytochrome *c* on cytochrome c oxidase. Ii. Rapid kinetic analysis of electron transfer from cytochrome *c* to *Rhodobacter sphaeroides* cytochrome oxidase surface mutants. J. Biol. Chem., **274**, 38042-38050 (1999).

21. **Drosou, V., Malatesta, F., and Ludwig, B.**: Mutations in the docking site for cytochrome *c* on the *Paracoccus* heme aa_3 oxidase. Electron entry and kinetic phases of the reaction. Eur. J. Biochem., **269**, 2980-2988 (2002).

22. **Drosou V., Reincke B., Schneider M., and Ludwig, B.**: Specificity of the interaction between the *Paracoccus denitrificans* oxidase and its substrate cytochrome *c*: comparing the mitochondrial to the homologous bacterial cytochrome *c*(552), and its truncated and site-directed mutants. Biochemistry, **41**, 10629-10634 (2002).

23. **Kagekawa, S., Mizukami, M., Noguchi, S. Sakamoto, J. and Sone, N.**: Importance of hydrophobic interaction between a SoxB-type cytochrome *c* oxidase with its natural substrate

cytochrome *c*-551 and its mutants. J. Biochem., **129**, 189-195 (2002).

24. **Soulimane, T., Buse, G., Bourenkov, G.P., Bartunik, H.D., Huber, R., and Than, M.E.**: Structure and mechanism of the aberrant *ba*(3)-cytochrome *c* oxidase from *Thermus thermophilus*. EMBO J., **19**, 1766-1776 (2000).

25. **Narumi, I., Sawakami, K., Nakamoto, S., Nakayama, N., Yanagisawa, T, Takahashi, N., and Kihara, H.**: A newly isolated *Bacillus stearothermophilus* K1041 and its tranformation by electroporation. Biotechnol. Biotechniques, **6**, 83-86 (1992).

26. **Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J.**: Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**, 265-275 (1951).

27. **Laemmli, U.K.**: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227**, 680-685 (1970).

28. **Ghaim, J.B., Tsatsos, P.H., Katsonouri, A., Mitchell, D.M., Salcedo-Hernandez, R. and Gennis, R.B.**: Matrix-assisted laser desorption ionization mass spectrometry of membrane proteins : demonstration of a simple method to determine subunit molecular weights of hydrophobic subunits. Biochim Biophys Acta **1330**, 113-120 (1997).

29. **Sakamoto, J. and Sone, N.**: In Zannoni, D. (ed.), Respiration in Archaea and Bacteria, vol.I: Diversity of Prokaryotic Electron Transport Carriers (series: Advances in Photosynthesis & Respiration, Vol. 15), pp. 87-113, Kluwer Academic Publishers, The Netherlands (2004).

30. **Nicholls, P. and Sone, N.**: Kinetics of cytochrome *c* and TMPD oxidation by cytochrome *c* oxidase from the thermophilic bacterium PS3. Biochim. Biophys. Acta, **767**, 240-247 (1984)

31. **Soulimane, T., Than, M.E., Dewor, M., Huber, R., and Buse, G.**: Primary structure of a novel subunit in ba_3-cytochrome oxidase from *Thermus thermophilus*. Protein Science **9**,

2068-2073 (2000).

32. **Sone, N**.: Respiration-Driven Proton Pumps, p. 1-32, In Krulwich, T.A. (ed.), The Bacteria, vol. 12. Academic Press, New York (1990).

33. **Iwata, S., Ostermeier, C., Ludwig, B., and Michel, H.**: Structure at 2.8 Å resolution of cytochrome *c* oxidase from *Paracoccus denitrificans*. (1995) Nature, **376**, 660-669

34. **Giuffre, A., Forte, E., Antonini, G., D'Itri, E., Brunori, M., Soulimane, T., and Buse, G.**: Kinetic properties of ba_3 oxidase from *Thermus thermophilus*: effect of temperature. Biochemistry, **38**, 1057-1065 (1999).

35. **Titani, K., Ericsson, L.H., Hon-nami, K., and Miyazawa, T.**: Amino acid sequence of cytochrome *c*-552 from *Thermus thermophilus* HB8. Biochem. Biophys. Res. Commun., **128**, 781-787 (1985).

36. **Soulimane, T., von Walter, M., Hof, P., Than, M.E., Huber, R., and Buse, G.**: Cytochrome-*c*552 from *Thermus thermophilus*: a functional and crystallographic investigation. Biochem. Biophys. Res. Commun., **237**, 572-576 (1997).

FIGURE LEGENDS

FIG. 1. Sequences and Structural model of cytochrome bo_3 subunit II of *G. thermodenitrificans*. **A** nucleotide and amino acid sequences of the wild-type cytchrome bo_3 subunit II. The white letters on closed squares indicate the mutation residues. **B** 3D-structure prediction of the subunit II of cytochrome bo_3 (stereo view). The cytochrome ba_3 from *T. thermophilus* (PDB ID: 1EHK) was used as a template. The hydrophilic domain of the subunit II and mutated residue was showed. 3D-structure modeling was performed by the Modeller (http://www.salilab.org/medeller/), and cartoon drawings of the structures were obtained using PyMOL (http://pymol.sourceforge.net). The subunit II is in light grey and the mutation sites are in black.

FIG. 2. Elution profiles of the wild type and mutant cytochromes bo_3 from anion exchange chromatography columns. The membrane proteins of the transformed *G. thermodenitrificans* cells were solubilized with the detergent, flowed through the first DEAE-Toyopearl column to remove cytochromes other than cytochrome bo_3, dialyzed and then applied to the second DEAE column. Absorbed proteins were eluted by stepwise increase in the NaCl concentration. The amounts of *b*-type cytochrome were estimated with redox difference spectra of the eluate fractions. closed circle, wild type; open square, E64Q; closed triangle, E84Q.

FIG. 3. Absorption spectra of the purified cytochrome bo_3-type oxidases. The absolute absorption spectra of the purified oxidases were measured at 2 μg protein/ml in the air-oxidized form. *a*, the wild type; *b*, E66A; *c*, E116Q; *d*, E139Q. *Inset*, the spectra, in the wavelength range from 700 to 900 nm, were enlarged as indicated with the scale bar.