J. Biochem. 132, 189–195 (2002)

Importance of Hydrophobic Interaction between a SoxB-Type Cytochrome *c* Oxidase with Its Natural Substrate Cytochrome *c*-551 and Its Mutants

Sayaka Kagekawa,* Makoto Mizukami,† Shunsuke Noguchi,* Junshi Sakamoto,* and Nobuhito Sone*,[1]

**Department of Biochemical Engineering and Science, Kyushu Institute of Technology, 680-4 Kawazu, Iizuka, Fukuoka 820-8502; and †Department of Research and Development, Higeta Shoyu Co., Choshi, Chiba 288-0041*

Received March 11, 2002; accepted May 13, 2002

Cytochrome *c*-551, the electron donor of SoxB-type cytochrome *c* oxidase in thermophilic bacilli, can be over-expressed in *Bacillus thermodenitrificans* cells by tranformation with pSTEc551. Several mutant cytochromes *c*-551 were prepared by site-directed mutagenesis to this expression plasmid. Among them, several Lys residues were changed to Ala/Ser, and we found that these mutant cytochromes retained their activity as substrates, although their K_m values were 0.04–0.12 μM, depending on the site replaced. In contrast, the C19A mutant cytochrome, which was produced in *Brevibacillus choshinensis* as a secretion protein, lost its activity as a substrate, suggesting that the fatty acyl-glyceryl residue covalently bound to the cysteine residue of the wild-type *c*-551 plays a very important role in the activity. The importance of the hydrophobic fatty acid residue for the binding of cytochrome *c*-551 to the oxidase was also shown by the loss of substrate activity in deacylated cytochrome *c*-551. These results show the importance of the hydrophobic interaction between this cytochrome and SoxB-type oxidase, despite the fact that the importance of an electrostatic interaction between cytochrome *c* and mitochondrial cytochrome aa_3 oxidase has already been established.

Key words: cytochrome bo_3, cytochrome *c* oxidase, cytochrome *c*-551, respiratory chain, thermophilic *Bacillus*.

Cytochrome *c*-551 is a lipoprotein of 10.4 kDa found in thermophilic *Bacillus* PS3 grown under air-limited conditions (*1, 2*). It is likely to play a role as an electron carrier between quinol-cytochrome *c* reductase (b_6c_1 complex) and an alternative $b(a/o)_3$-type cytochrome *c* oxidase other than the caa_3-type oxidase (*3, 4*). The thermophilic *Bacillus* cells grown under aerobic conditions show a typical *abc*-type cytochrome pattern (*5, 6*) and use the b_6c_1 complex (*7, 8*) and caa_3-type cytochrome oxidase (*9, 10*) as the main respiratory chain components (*6*), while cells grown under air-limited conditions show a somewhat different cytochrome pattern, with a decreased amount of *a*-type cytochrome and much higher levels of *b*- and *c*-type cytochromes (*5*).

Analyses of mature cytochrome *c*-551 from PS3 have indicated that the signal peptide of 18 amino acids is cut off and the new N-terminal Cys residue is modified to possess a diacylated glycerol moiety at the sulfhydryl side chain and an acetyl group at the alpha amino group (*2, 11*). This cytochrome *c* was over-expressed in transformable *Bacillus thermodenitrificans* K1041[2] using the structure gene for PS3 cytochrome *c*-551, named cccA (*11*), with a promotor designed for the expression of neutral proteases of this bacterium (*12*). The characteristics of cytochrome $b(a/o)_3$ oxidase were not elucidated until recently, since its level in membranes is low even in cells grown under air-limited conditions. The oxidase was prepared for the first time from the K17 mutant of *B. thermodenitrificans* K1041, in which cytochrome caa_3 is no longer expressed (*3, 13*). The oxidase is composed of two subunits with apparent molecular masses of 56 and 19 kDa, determined by Ferguson plot in SDS-PAGE, and contains protoheme IX, heme O, heme A, and Cu in a ratio of 1:0.7:0.2:3 (*3*). We then cloned the *cbaAB* gene for this enzyme, and found from its deduced amino acid sequence that the oxidase does not belong to SoxM-type, but to SoxB-type (*14*), as in the case of cytochrome ba_3 oxidase from *Thermus thermophilus* (*15*). Most of the heme-copper oxidases in mitochondria and bacteria are SoxM-type (*16, 17*).

Using a plasmid with *cbaAB*, we succeeded in over-expressing cytochrome $b(a/o)_3$ in K1041 cultured under air-limited conditions. The expressed protein contains 1 heme B and 1 heme O, and was thus called cytochrome bo_3 (*18*). An enzyme kinetic study of the purified cytochrome bo_3

[1] To whom correspondence should be addressed. Tel: +81-948-29-7813, Fax: +81-0948-29-7801, E-mail: sone@bse.kyutech.ac.jp

[2] *B. thermodenitrificans* K1041 was formerly named *B. stearothermophilus* K1041 by the finders (*25*). However, this strain was recently assigned as a strain of the former as a result of 16 S r-RNA analysis [Studholme, D.J., Jackson, R.A., and Leak, D.J. (1999) Phylogenetic analysis of transformable strains of thermophilic *Bacillus* species. *FEMS Microbiol. Lett.* **172**, 85–90].

Abbreviations: SDS-PAGE, sodium dodecylsulfate–polyacrylamide gel electrophoresis; 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).

indicated that the oxidase activity shows a biphasic dependency on cytochrome *c*-551 concentration, as is well known for mitochondrial cytochrome *c* with cytochrome aa_3 (*18*). The high affinity site (about 10^{-7} M) is very specific for cytochrome *c*-551, while the low affinity site (about 10^{-5} M) is relaxed, and available to mitochondrial cytochromes *c* and even TMPD (*18*). Another characteristic of this cytochrome *c*-551 oxidase is that the activity is optimal under high ionic strength conditions (*3, 18*), suggesting the importance of a hydrophobic interaction between the substrate and the enzyme. In the case of mitochondrial SoxM-type cytochrome aa_3 and cytochrome *c*, ionic interactions between cytochrome *c* and SoxM-type cytochrome *c* oxidase are known to be important (*19–21*). Since cytochrome *c*-551 is also rich in lysine (*2*), an ionic interaction between cytochrome *c*-551 and cytochrome bo_3 may be also important, even if the interaction is only be very local. Although neither cytochrome *c*-551 nor cytochrome bo_3 has been crystalized, their 3D-structures can be assumed based on the structures of their homologues. Cytochrome ba_3 from *T. thermophilus*, a SoxB-type cytochrome *c* oxidase homologous to the thermophilic *Bacillus* cytochrome bo_3, has been crystalized and its atomic structure was reported (*22*). The 3D-structure of cytochrome *c*-553 from *Bacillus pasteurii*, another *Bacillus* small *c* (*23*) with a protein sequence very similar to cytochrome *c*-551 (46% identical) has been reported (*24*).

We report here studies measuring oxidase activity using several genetically and biochemically altered cytochromes *c*-551 as substrates for the high affinity site, and showing the importance of hydrophobic interactions of these cytochromes, and the specific role of the diacyl-glyceryl moiety of *c*-551 in bo_3-type oxidase activity.

MATERIALS AND METHODS

Materials—A transformable strain of *B. thermodenitrificans* K1041 (formerly *B. stearothermophilus* K1041)[2] was donated by Dr. Narumi (*25*). *Brevibacillus choshinensis* (formerly *Bacillus brevis* HPD31) was isolated in Ciba by Takagi *et al.* (*26, 27*). An expression plasmid for this bacterium, pSTE12 (*11*), was used to construct plasmids for the over-production of *B. thermodenitrificans* K1041 cytochrome bo_3, and the enzyme was purified as described previously (*18*). Cytochrome *c*-551 of *Bacillus* PS3 was overexpressed in K1041 and purified as described (*11*). Porcine pancreas lipase, *Candida cylindracea* lipase and *Rhizopus delemar* lipase were purchased from Elastic Products (Owenceville, MI), Nacalai Tesque (Kyoto), and Seikagaku Kogyou (Tokyo), respectively. DEAE-Toyopearl anion exchange gel, hydroxyapatite, octyl-cellulose, and TMPD were obtained from Tosoh (Tokyo), Bio-Rad (Hercules), Chisso (Tokyo), and Wako (Kyoto), respectively. Proteins used as molecular mass standards and cytochromes *c* from yeast and horse heart were purchased from Sigma. Other chemicals were obtained as described previously (*12*).

Site-Directed Mutagenesis for Cytochrome c-551—Most mutants were constructed for expression in *B. thermodenitrificans* K1041 as follows. A cytochrome *c*-551, expression plasmid, pSTEc551, was digested with *Eco*RI and *Bam*HI. The resulting 751 bp fragment containing the *c*-551 expression cassette was purified and introduced into the multicloning site of pUC119 to obtain pUCc551E. For mutants R87A, K96A, K102S, W106A, K110* (K110 and K111 deletion), and K111* (K111 deletion), to prepare single strand templates by *in vitro* mutagenesis, *E. coli* XL-1B containing pUCc551 were cultured overnight in 2xTY medium (6 ml) with helper phage M13KO7 (about 10^9 pfu). The phage particles were harvested from the culture medium and single stranded pUCc551 was purified. *In vitro* mutagenesis was performed by the Sculptor™ *in vitro* mutagenesis system (Amersham) according to the manufacturer's instructions. For the preparation of mutated *c*-551, such as K69A, K73A, K80A, W106Y, and W106F, mutagenesis was performed with a QuickChange™ site-directed mutagenesis kit (Stratagene) using double stranded pUCc551 as the template. The primers used for mutagenesis are shown in Fig. 1. The obtained plasmids were sequenced to confirm the mutation. The 751 bp mutated *c*-551 expression cassettes excised by *Eco*RI plus *Bam*HI digestion were ligated into the multicloning site of pSTE12 for construction of the mutant *c*-551 expression plasmids. Transformation of these plasmids to *B. thermodenitrificans*, and the cell culture and purification procedures for mutated *c*-551 have been described previously (*11*).

Production of c-551 Using B. choshinensis as a Host to Prepare C19A—Brevibacillus choshinensis was used as a host. The expression plasmid (named pNUC19A) used in the production of *c*-551 was constructed by ligating the PCR product to *Pst*I and *Hin*dIII-cut pNU212 (*28*) to prepare a chimeric protein with the signal sequences of *B. choshinensis* cell wall protein and *c*-551 with Ala instead of Cys at 19th position. PCR was performed using pUCc551 as a template and the following primer sets: 5′-AAACTG-CAGGCGGTGGCGGGGACAAT-3′ changing the codon for Cys to Ala, and introducing a *Pst*I site, and 5′-AAAAAGCT-TATTTTTTCGCAGCCAGCC-3′ for the anti-sense primer introducing a *Hin*dIII site into the C terminal part of the coding region. The resultant plasmids were introduced into *B. choshinensis* by electroporation. Transformants harboring the expression plasmids were cultured for 3 days at 30°C in T2M medium (*29*) supplemented with erythromycin (10 mg/liter).

Purification of Mutated c-551—Membrane-bound *c*-551 and its mutants were purified as described previously as the wild-type (*11*). Essentially, the cholate-extract of the membrane fraction containing *c*-551 was purified and concentrated on two DEAE-Toyopearl columns in the presence of 0.1% Triton X-100. C19A excreted in the culture medium was purified as follows: After the cells were centrifuged down, solid ammonium sulfate was added to the culture medium (2 liters) to 70% saturation. After 20 min the medium was centrifuged and the supernatant was applied to an octyl cellulose column (2 × 10 cm). Two-thirds of the column turned red, and it was washed with 100 ml 50% sat. ammonium sulfate containing 10 mM Tris-HCl buffer, pH 7.2. The red band was then eluted by lowering the ammonium sulfate concentration to 30% saturation. The red eluate was dialyzed against 5 mM Tris-HCl buffer, and then applied to a DEAE-Toyopearl column (1.6 × 8 cm). The resultant red band was eluted with 15 mM Tris-HCl after washing with 5 mM Tris-HCl, pH 7.2.

Preparation of Delipidated c-551—Mono-deacylated *c*-551: The wild-type *c*-551 (0.18 mM) was incubated in 20 mM Tris-HCl buffer, pH 7.5, with purified pancreatic lipase (0.01 mg/ml) for 120 min. This treatment gave a derived *c*-

551 with a mass number of 10,198. Di-deacylated *c*-551: The wild-type *c*-551 (0.18 mM) was incubated in 20 mM Tris-HCl buffer, pH 7.5, with *C. cylindriacea* lipase (500 units/ml) for 20 h.

Measurement of Oxidase Activity—Cytochrome *c* oxidase activity of the purified enzyme was monitored continuously with a pH electrode (Beckman No. 39030) as the increase in pH 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 (*30*), Ascorbate H^- + H^+ + 1/$2O_2$ → Dehydroascorbate +H_2O. The reaction was carried out with 0.25–0.05 nmol cytochrome bo_3 oxidase at 40°C in 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 substrate. An aliquot of *c*-551 was added and the 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. Turnover number was expressed as mol of electrons transferred per mol of heme in the enzyme per s.

Measurements of the Optical Spectrum and Redox Potential—The contents of cytochromes aa_3 and *c*-551 were determined from the reduced minus oxidized difference spectra using millimolar extinction coefficient differences of 21.3 at 604 nm minus 630 nm (*4*), and 24.3 at 551 nm minus 538 nm (*10*), respectively. The redox potentials of *c*-551 were obtained by measuring the percentage of reduced cyt. *c*-551 equilibrated with 5 μM ferricyanide and 2 mM ferrocyanide (initial concentration).

MALDI-TOF mass spectrometry was done in a Voyger™ Linear-DE/K model (PerSeptive Biosystems) using sinapinic acid in acetonitrile–0.1% trifluoric acid as the matrix.

Others—The hydrophobicities of *c*-551 derivatives were monitored by high-pressure liquid chromatography (HPLC) on a C4 column (Waters microbondasphere N10036) as described previously (*11, 31*).

Protein concentration was determined by a modified Lowry method (*13*). Sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli except that boiling of the protein samples was omitted (*13*).

RESULTS

Preparation of Mutant Cytochromes c-551—Despite the acidity of the whole protein, *c*-551 is rich in Lys, with 10 Lys in the 72-residue mature protein. We prepared 8 Ala or Ser substitutes of *c*-551. In addition to these mutants, Trp106 near the C-terminus was changed to Phe/Tyr, as well as R87 was changed to Ala. Although the transformed cells with the plasmids that should produce W106F or K110* (K110- and K111-deleted *c*-551) did not over-produce the cytochrome, the other transformants produced the mutant cytochromes *c*-551 well, so that colonies of transformed cells were clearly reddish. From these cells, the respective

```
AAGAGGACCATGGCATGGAAATTGGCTGCGATGTTCCTTGGCGTTTCGCTCGCGCTTGCC
          M  A  W  K  L  A  A  M  F  L  G  V  S  L  A  L  A   17

GCGTGCGGCGGTGGCGGGGACAATGCCGGGGAGAAAAACGGCGGCAGCAACGGCGGAGGG
A  C  G  G  G  G  D  N  A  G  E  K  N  G  G  S  N  G  G  G   37

GATACAGCAGCGGCTGCCGAGCAAATTTTTAAACAAAACTGTGCGTCCTGTCATGGACAA
D  T  A  A  A  A  E  Q  I  F  K  Q  N  C  A  S  C  H  G  Q   57

GACCTGTCGGGCGGGGTCGGCCCGAACTTGCAAAAGGTTGGAAGCAAGTATTCCAAAGAT
D  L  S  G  G  V  G  P  N  L  Q  K  V  G  S  K  Y  S  K  D   77

LGAAATTAAAAACATTATCGCCAACGGCCGCGGCGCGATGCCGGCAGGAATCATTAAAGG
E  I  K  N  I  I  A  N  G  R  G  A  M  P  A  G  I  I  K  G   97

GGAAGACGCCGACAAAGTGGCGGAATGGCTGGCTGCGAAAAAATAATGGACGGATGTTTC
E  D  A  D  K  V  A  E  W  L  A  A  K  K  *                 111

--------------------------------------------------
primer R87A   : 3'-GCGGTTGCCGCGGCCGCGCTAC-5'
primer K96A   : 3'-TCCTTAGTAACGTCCCCTTCTG-5'
primer K102S  : 3'-TCTGCGGCTGAGTCACCGCCTT-5'
primer W106A  : 3'-TCTCCGCCTTCGCGACCGACGC-5'
primer K110*  : 3'-CGACCGACGCATTTTTATTAC-5'
primer K111*  : 3'-CCGACGCTTTATTATTACCTG-5'
primer K69A-F : 5'-GCCCGAACTTGCAAGCGGTTGGAAGCAAGT-3'
primer K69A-R : 5'-ACTTGCTTCCAACCGCTTGCAAGTTCGGGC-3'
primer K73A-F : 5'-AAAAGGTTGGAAGCGCGTATTCCAAAGATG-3'
primer K73A-R : 5'-CATCTTTGGAATACGCGCTTCCAACCTTTT-3'
primer K80A-F : 5'-CCAAAGATGAAATTGCAAACATTATCGCCA-3'
primer K80A-R : 5'-TGGCGATAATGTTTGCAATTTCATCTTTGG-3'
primer W106Y-F: 5'-CAAAGTGGCGGAATACCTGGCTGCGAAAAA-3'
primer W106Y-R: 5'-TTTTTCGCAGCCAGGTATTCCGCCACTTTG-3'
primer W106F-F: 5'-CAAAGTGGCGGAATTTCTGGCTGCGAAAAA-3'
primer W106F-R: 5'-TTTTTCGCAGCCAGAAATTCCGCCACTTTG-3'
--------------------------------------------------
```

Fig. 1. **Sequences and primers for site-directed mutagenesis.** Upper: DNA and protein sequences of wild-type *B. thermodenitrificans* *c*-551. Lower: primers used for mutagenesis.

mutant cytochromes *c*-551 were purified by the method used for purification of the wild-type (*11*). In addition, mutant C19A, in which the diacyl-glyceryl residue is omitted, was also expressed and purified. This C19A mutant was designed to be expressed in *Brevibacillus choshinensis* with the signal sequence of the bacterium (*29*). The transformed cells excreted soluble *c*-551 with Ala as the N-terminus into the culture medium at levels as high as 3.8 μM (about 40 mg/liter), indicating that the signal sequence of 18 residues was cut out. From this culture medium, C19A was purified as described in "MATERIALS AND METHODS" with a yield of about 50%.

Table I summarizes the contents of *c*-type cytochromes in the membrane fractions and the mass numbers obtained. Most of these mutant *c*-551s were expressed as well as the wild-type cytochrome, and most of the total *c*-type cytochromes in these transformants were *c*-551. In the case of W106A, W106Y, and K110*, very small amounts of *c*-551, if any, were found, since the values tabulated were the total amount of *c*-type cytochromes and control cells without plasmids contained 0.5–0.7 nmol *c*-type cytochromes per mg membrane protein. In contrast, W106F and K111* were produced well. The mass numbers of these purified mutant cytochromes were measured with a MALDI-TOF mass spectrometer. The values of most species were in accord with the expected values, although in the case of K111*, the difference was exceptionally large, but still 18. It is not known whether this is due to occasional error, or due to some unknown modification(s).

Effects of Mutation on Cytochrome c-551 Oxidase Activity—The effects of mutations on the *c*-551 concentration–dependence of the oxidase activity were measured in the presence of TMPD, which effectively mediates electron transfer from ascorbate to *c*-551 at the high affinity site by binding to the low affinity site. Under these experimental conditions, the K_m of *c*-551 for the high affinity site of the oxidase and V_{max} should be obtained (*18*). Table II summarizes these kinetic parameters with these cytochrome *c*-551 as substrates of the oxidase. Although C19A produced in *Br. choshinensis* did not serve as an effective substrate, the other mutant *c*-551s were, more or less, active as substrates of the oxidase. Especially, the K_m values of K69A, K73A, R87A, and K96A were smaller than that of the wild-type. On the contrary, only negligible or slight changes were found in the case of K80A, K102S, and W106F. The 106th W is conserved among the *Bacillus* small *c* group (*23*), but the change to Phe did not affect electron transfer, except for a slight increase in K_m, with no change in the V_{max}. However, K102S and K111* were not good substrates, as judged from their larger K_m and smaller V_{max} values. C19A, which was expressed in *Br. choshinensis* as a secretion protein, was not oxidized well, suggesting the importance of diacylglycerate at the Cys residues. All these cytochromes, including C19A, showed indistinguishable visible absorption spectra with the alpha band of the reduced form at 551 nm. Even the membrane fractions of these transformants were red and showed a prominent peak at 551 nm upon reduction. We also measured the redox potentials of wild and mutant cytochromes *c*-551 such as C19A, K96A, K102S, W106F, and K111*, and found them to be almost the same (165 + 5 mV), indicating that mutation does not affect the electron stability of the heme.

TABLE I. **Expressed amounts of mutated cytochromes *c*-551 and their mass numbers.**

c-551 mutated	Found in membranes[a] (nmol/mg protein)	Mass number	
		Observed	Expected
Wild-type	6.3	10,442.5	10,440
K69A	5.6	10,386.3	10,385.3
K73A	4.8	10,387.7	10,385.3
K80A	5.7	10,386.4	10,385.3
R87A	5.6	10,358.0	10,357.3
K96A	4.2	10,382.0	10,385.3
K102S	2.1	10,404.3	10,401.3
W106A	0.7	–	–
W106Y	0.7	–	–
W106F	5.3	10,404.7	10,403.4
K110*	0.8	–	–
K111*	3.8	10,314.4	10,296.2
C19A	3.8[b]	9,814.4	9,818.4

[a]Total amount of *c*-type cytochromes measured at 551–533 nm. [b]Concentration in the culture medium in μM.

TABLE II. **Summary of the kinetic constants of mutated cytochrome *c*-551.**

c-551 mutation	K_m (nM)	V_{max} (s^{-1})
Wild-type	74	270
K69A	40	190
K73A	45	230
K80A	97	220
R87A	56	220
K96A	45	220
K102S	140	140
W106F	82	220
K111*	270	110
C19A	n.d[a]	n.d

[a]Not detectable.

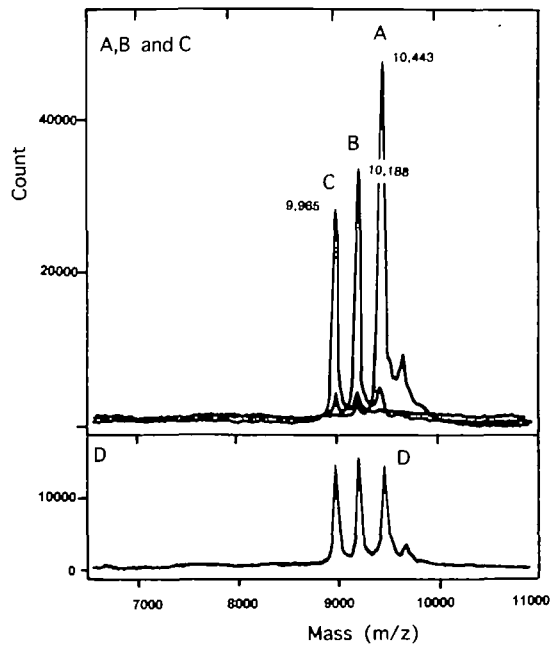

Fig. 2. **Mass spectra of cyt. *c*-551 and its hydrolysed derivatives.** An aliquot of *c*-551 (0.1 ml; *ca.* 0.1 mM) was incubated with pancreatic lipase (2.0 μg) or *C. cylindracea* lipase (25 units) in 5 mM Tris-HCl containing 0.5 mM EDTA at 37°C. The hydrolysis was monitored with a MALDI TOF mass spectrometer. A: *c*-551 before incubation. B: After 3 h with pancreatic lipase. C: After 20 h with *C. cylindracea* lipase. D: After 1 h with *C. cylindracea* lipase.

TABLE III. **Effects of fatty acids at the glyceryl residues of c-551.**

Sample	without Tween 20		with Tween 20	
	K_m (nM)	V_{max} (s^{-1})	K_m (nM)	V_{max} (s^{-1})
c-551, wild	78	230	88	160
Mono-delipidated	90	200	210	78
Di-delipidate	n.d[a]	n.d	580	32
C19A	n.d	n.d	620	9

The reaction medium contained 0.08% (w/v) Tween 20 as indicated.
[a]Not detectable.

It is thus not likely that any lysine residue is important for the binding of oxidase and cytochrome c-551.

Role of the Diacylglyceryl Moiety—Since C19A without the diacylglyceryl moiety did not act as a substrate for the oxidase, we prepared mono-deacyl- and di-deacyl derivatives of wild-type c-551 and measured their kinetic parameters. Figure 2 shows the mass spectra of c-551 hydrolysed by two types of lipases. The wild-type c-551 gave a peak at 10,443 (line A). Upon hydrolysis with pancreatic lipase, one new peak appeared at 10,188 in addition to the original peak (not shown). After 3 h, the peak at 10,188 was the only peak (line B). This sample was used as mono-deacyl c-551. Treatment with *C. cylindracea* lipase gave a third peak at a mass of 9,965 after overnight incubation (line C). At an early stage of hydrolysis by *C. cylindracea* lipase, all three peaks found in lines A, B, and C were observed (line D), indicating that the two fatty acids were hydrolysed by the lipase one by one. Since most lipases are known to hydrolyse ester bonds at position-1 more easily than those at position-2, the 10,188 compound is the 1-deacyl derivative and the 9,965 compound is the di-deacyl derivative (glyceryl c-551). On the other hand, the mass number of 245 at position-1 is between those of palmitate and stearate residues. We believe that the acyl group at position-1 is an iso- or anteiso-derivative of palmitate, which are 14 mass units larger than palmitate. It is thus likely that the derivative of c-551 with a mass number of 9,965 is di-deacyl c-551 (*11*). This di-deacyl derivative is also produced by *R. delemar* lipase (*11*). Reverse-phase chromatography of these esterase adducts showed that the treatment induced a great loss of hydrophobicity, as expected by the release of fatty acids. It was also observed that deacylated c-551 became soluble without any detergent.

In order to clarify the role of fatty acid moieties at the N-terminal cysteine residue, mono-deacyl- or dideacyl-derivatives of native c-551 were used as substrates for the oxidase. As summarized in Table III, neither the dideacyl derivative of c-551 nor C19A served as an electron donor, showing the importance of diacyl groups bound to Cys at the N-terminus. On the other hand, the mono-deacyl derivative was oxidized with a V_{max} value even lower than for the wild type. The C19A mutant and the dideacyl derivative, however, showed electron-donating activity when low concentrations of a non-ionic detergent were present. In the presence of 0.05% Tween 20, even C19A operated as a substrate, showing a K_m of 0.62 μM and V_{max} of 9.0 s^{-1}, and cytochrome c-551 with glycerated Cys at the N-terminus also acted as a substrate (Table III). The K_m and V_{max} values of the wild-type and mono-deacyl derivative changed only slightly in the presence of Tween 20.

Figure 3 shows the effect of C19A on cytochrome c oxidase as a competetive inhibitor. The K_i of about 1 μM shows the affinity of C19A for the oxidase, which is comparative to the K_m value for the substrate in the presence of Tween 20. These results indicate the importance of diacyl groups for the binding of cytochrome c-551 to the oxidase.

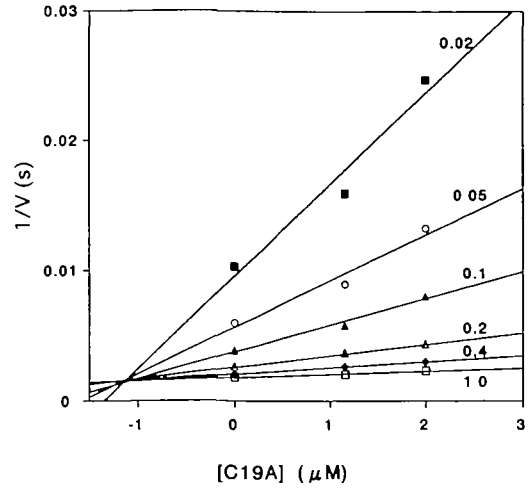

Fig. 3. **Dixon plot for measuring K_i of C19A.** *B. thermodenitrificans* cytochrome bo_3 (45 pmol) was used to oxidize cytochrome c-551. The concentrations of c-551 (shown in μM on the each slope) and C19A (horisontal axis) as the competitive inhibitor were as indicated in the figure. Other reaction conditions and measurement conditions are described in "MATERIALS AND METHODS."

DISCUSSION

The data report here reveal the presence of a unique substrate-enzyme interaction between c-551 and the bo_3-type oxidase of a thermophilic *Bacillus*. The interaction is mainly hydrophobic, since replacement of Lys/Arg by Ala did not weaken the interaction. In addition, the oxidase activity was highly stimulated under high ionic strength conditions (*3, 18*). These characteristics are thus quite different from those well known for SoxM-type mitochondrial cytochrome aa_3 and cytochrome c (*19–21*). The homologous enzyme, cytochrome ba_3, in *T. thermophilus* is also a Cu_A-containing cytochrome c oxidase of the SoxB-type. Cytochrome ba_3 was recently crystalized and its 3D-structure shows it to be composed of subunits I, II, and IIa. Subunit IIa is a polypeptide with one hydrophobic helix (*22*) as is also found in cytochrome bo_3 from *B. thermodenitrificans* (Sakamoto *et al.*, unpublished observations). In spite of this structural similarity, the interaction between *T. thermophilus* cytochrome ba_3 and its substrate cytochrome c-552 does not appear to be similar to that between cytochrome bo_3 and c-551 of thermophilic *Bacillus*, because c-552 itself is not a lipoprotein and shows little similarity in its protein sequence to that of c-551 (*32, 33*). Also, the interaction between cytochrome ba_3 and c-552 has not been reported to be prevented by the presence of salts (*22*).

Bacillus small cytochromes c, including the present cytochrome c-551, form a distinct group among class-1 cytochromes c (*23*). They are membrane-bound due either to their uncleaved signal peptide-like hydrophobic N-terminal helix, as in the case of CccA of *B. subtilis* (*34, 35*), or to a diacyl-glycerol moiety at a Cys close to the N-terminus, as

in CccB of *B. subtilis* and *c*-551 of the thermophilic bacilli (*2, 36*). Cytochromes *c* in this group show close similarities in protein sequence to cytochromes c_6 and c_8, of which several members have been crystalized. Recently, cytochrome *c*-553 from *Bacillus pasteurii* was crystalized as a soluble cytochrome containing only 71 of the 92 expected residues, produced by some nonphysiological protease cleavage after physiological processing to obtain the 92-amino acid polypeptide with the diacyl-glyceryl N-terminal Cys (*24, 37*). The 3D-structure of the crystal shows the following characteristics probably common in *Bacillus* small *c*: (i) a largely exposed C-heme surrounded by three alpha-helices in a compact manner as in cytochromes c_6 and c_8, (ii) inter-helix loop parts that are also short, the shortest among known class-I cytochromes *c*, and (iii) a charge distribution that is very asymmetric, so that the heme surface patch lacks net charges (*24*). These characteristics might be common in *c*-551, since the sequences of *c*-553 and *c*-551 are very similar, 46% identity without any insertions or deletions, and most of the hydrophobic residues and Gly and Pro residues are conserved. In light of the above structural considerations, the effect of mutations, such as K73A, K80A, R87A, and K96A, near the heme surface is interpreted as the result of strengthening the substrate–oxidase hydrophobic interaction due to the loss of a positive-charged Lys. On the other hand, mutational changes in residues distant from the heme edge do not affect the affinity. While the role of Lys at the C-terminus is not known, its deletion may induce some unfavorable change on the structure, since K110* was not produced and K111* was not oxidized well (Table II).

The mature cytochrome *c*-551 from thermophilic bacilli has a diacyl-glyceryl Cys19 residue as the new N-terminal residue after processing and can be purified without further proteolysis. The precise structure of the *c*-551 N-terminal region containing diacyl glycerol is not known at present, because it lacks the N-terminal 21 residues in the *B. pasteurii* crystal *c*-553. However, it seems reasonable that the moiety plays a role as the anchor at the bottom of the N-terminal helix, since the N-terminal part of c_6 and c_8 forms an alpha helix toward the heme surface from the bottom, and the hydrophobic acyl-glycerol residue should locate in the lipid bilayer.

Enzyme kinetics indicated that the fatty acyl group of this *c*-551 is important not only for binding to the oxidase but also for the catalytic activity of the oxidase itself. Neither C19A nor deacylated *c*-551 was oxidized unless a small amount of Tween 20 was added (Table III). C19A was confirmed to act as a competitive inhibitor for *c*-551 oxidation with a K_i of 1.1 μM (Fig. 3). Thus the oxidase needs a long hydrocarbon group, either on the *c*-551 or as Tween 20, for activity. Our basal oxidase assay medium contains neither P-lipid nor detergent, but the oxidase and *c*-551 contain small amounts of detergent. The concentration of the detergent (below 0.0005%), however, seems insufficient for the oxidase to show activity. Cytochrome bo_3 is probably partially surrounded by a detergent micelle, but its concentration is still too low to contact the specific site of the oxidase and does not activate the oxidase. In contrast, in the presence of 0.02% Tween 20, activation of the oxidase may occur but less compared with the activation by the fatty acyl group of *c*-551. We also tried high concentrations of detergent and P-lipids but recovery of the activity was less than in the case of 0.02% Tween 20. It seems likely that the relative loss of oxidase concentration in the micelle upon detergent addition, and liposomal vesicle formation in the case of P-lipid addition prevent *c*-551 from attaching to the specific site of the oxidase due to a dilution effect. Although it is also known that SoxM-type oxidases, for example cytochrome caa_3 from thermophilic *Bacillus* PS3, require much higher concentrations of P-lipid or a detergent such as Triton X-100 or Tween 20 for the expression of full activity (*9*), the present finding that cytochrome bo_3 requires the fatty acyl group of *c*-551 for full activity is worth clarifying by chemical and physical methods as the next step.

It is also noteworthy that fatty acids found at positions-1 and -2 of glyceryl cysteine are of the iso- or anteiso-type. Lipase treatment showed that the difference in the mass number is 255 for pancreatic lipase treatment or the first step of *C. cylindriacea* lipase treatment, and 223 for the second step of *C. cylindriacea* lipase treatment (Fig. 2). Since the mass of $C_{15}H_{31}CO$ is 239, the fatty acid at position-1 is likely to be an iso- or anteiso-derivative of palmitic acid, and that at position-2 an iso- or anteiso derivative of myristic acid. The production of branched fatty acids is a characteristic of thermophilic bacteria, as reported previously (*38*). We formerly expected incorrectly that two palmitic acids esterified glycerol residues, without hydrolysing the fatty acid residues one by one (*11*).

We found a new role for the fatty acid moiety of the substrate lipoprotein in the activity of the terminal oxidase, and confirmed that the interaction between *c*-551 and bo_3-type oxidase is mainly hydrophobic. This characteristic is not found for *T. thermus* *c*-552 and ba_3-type oxidase, although both oxidases are closely related SoxB-type cytochrome *c* oxidases. SoxB-type oxidases are also distributed in Archaea, but most of them are a quinol oxidases (*39*). The *Natronobacter* enzyme is not a quinol oxidase, but its substrate is reported to be a blue copper electron carrier (*40*). However, the whole genome sequence of alkalophilic *Bacillus halodurans* shows the presence of a SoxB-type enzyme as one of five terminal oxidases, as well as CccB for a small *c*-type cytochrome (*41*), homologous to thermophilic *Bacillus* *c*-551. In *B. subtilis* one of two membrane-bound small cytochromes *c*, named cytochrome *c*-551 (*cccB* product), is the homologous protein of *c*-551 with diacyl-glyceryl Cys (*36*). Transformable *B. thermodenitrificans* K1041 also produced *c*-551. It is thus likely that this unique division of the respiratory chain is distributed at least among *Bacillus* genuses.

REFERENCES

1. Sone, N., Kutoh, E., and Yanagita, Y. (1989) Cytochrome *c*-551 from the thermophilic bacterium PS3 grown under air-limited conditions. *Biochim. Biophys. Acta* **977**, 329–334
2. Fujiwara, Y., Oka, M., Hamamoto, T., and Sone, N. (1993) Cytochrome *c*-551 from the thermophilic bacterium PS3, DNA sequence and analysis of mature cytochrome. *Biochim. Biophys. Acta* **1144**, 213–219
3. Sakamoto, J., Handa, Y., and Sone, N. (1997) A novel Cytochrome $b(o/a)_3$-type oxidase from *Bacillus stearothermophilus* catalyzes cytochrome c-551 oxidation. *J. Biochem.* **122**, 764–771
4. Sone, N., Koyanagi, S., and Sakamoto, J. (2000) Energy-yielding properties of SoxB-type cytochrome bo_3 terminal oxidase: Analyses using *Bacillus stearothermophilus* K1041 and its mutant strain. *J. Biochem.* **127**, 551–557
5. Sone, N., Kagawa, Y., and Orii, Y. (1983) Carbon monooxide-

binding cytochromes in the respiratory chain of thermophilic bacterium PS3 grown with sufficient or limited aeration. *J. Biochem.* **93**, 1329–1336
6. Sone, N., Sekimachi, M., and Kutoh, E. (1987) Identification and properties of a quinol oxidase super complex composed of a bc_1 complex and cytochrome oxidase in the thermophilic bacterium PS3. *J. Biol. Chem.* **262**, 15386–15391
7. Kutoh, E. and Sone, N. (1987) Quinol-cytochrome *c* oxidoreductase from the thermophilic bacterium PS3. *J. Biol. Chem.* **263**, 9020–9026
8. Sone, N., Tsuchiya, N., Inoue, M., and Noguchi, S. (1996) *Bacillus stearothermophilus qcr* operon encoding Rieske FeS protein, cytochrome b_6 and a novel-type cytochrome c_1 of quinol-cytochrome *c* reductase. *J. Biol. Chem.* **271**, 12457–12462
9. Sone, N. and Yanagita, Y. (1982) A cytochrome aa_3-type terminal oxidase of a thermophilic bacterium. *Biochim. Biophys. Acta* **682**, 216–226
10. Ishizuka, M., Machida, K., Shimada, S., Mogi, A., Tsuchiya, T., Ohmori, T., Souma, Y., Gonda, M., and Sone, N. (1990) Nucleotide sequence of the gene coding for four subunits of cytochrome *c* oxidase from the thermophilic bacterium PS3. *J. Biochem.* **108**, 866–873
11. Noguchi, S., Yamazaki, T., Yaginuma, A., Sakamoto, J., and Sone, N. (1994) Over-expression of membrane-bound cytochrome *c*-551 from thermophilic *Bacillus* PS3 in *Bacillus stearothermophilus* K1041. *Biochim. Biophys. Acta* **1188**, 302–310
12. Narumi, I., Sawakami, K., Kimura, T., Nakamoto, S., Nakayama, N., Yanagisawa, T., Takahashi, N., and Kihara, H. (1992) A novel oligonucleotide cassette for the overproduction of *Escherichia coli* aspartate transcarbamylase in *Bacillus stearothermophilus*. *Biotechnol. Lett.* **14**, 759–764
13. Sakamoto, J., Matsumoto, A., Oobuchi, K., and Sone, N. (1996) 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
14. Nikaido, K., Sakamoto, J., Handa, Y., and Sone, N. (1998) The *cbaAB* genes for bo_3-type cytochrome *c* oxidase in *Bacillus stearothermophilus*. *Biochim. Biophys. Acta* **1397**, 262–267
15. Keightley, J.A., Zimmermann, B.H., Mather, M.W., Springer, P., Pastuszyn, A., Lawrence, D.M., and Fee, J.A. (1995) Molecular genetic and protein chemical characterization of the cytochrome ba_3 from *Thermus thermophillus* HB8. *J. Biol. Chem.* **270**, 20345–20358
16. Garcia-Horsman, J.A., Barquera, B., Rumbley, J., Ma, J., and Gennis, R.B. (1994) The superfamily of heme-copper respiratory oxidase. *J. Bacteriol.* **176**, 5587–5600
17. Castresana, J., Lubben, M., Saraste, M., and Higgins, D.G. (1994) Evolution of cytochrome oxidase, an enzyme older than atmospheric oxygen. *EMBO J.* **13**, 2516–2525
18. Nikaido, K., Sakamoto, J., and Sone, N. (2000) Over-expression of *cba-AB* genes and proving proton pomp activity of two-subunit bo_3-type cytochrome *c* oxidase in *Bacillus stearothermophilus*. *Biochim. Biophys. Acta* **1456**, 35–44
19. Ferguson-Miller, S., Brautigan, D., and Margoliash, E. (1978) Definition of cytochrome *c* binding domains by chemical modification. *J. Biol. Chem.* **253**, 149–159
20. Witt, H., Malastesta, F., Nicolettei, F., Brunori, M., and Ludwig, B. (1998) Cytochrome *c*-binding site on cytochrome oxidase in *Paracoccus denutrificans*. *Eur. J. Biochem.* **251**, 367–373
21. Dopner, S., Hildebrandt, P., Rosell, F., Mauk, A.G., von Walter, M., Buse, G., and Soulimane, T. (1999) The structural and functional role of lysine residues in the binding domain of cytochrome *c* in the electron transfer to cytochrome *c* oxidase. *Eur. J. Biochem.* **261**, 379–391
22. Saulimane, T., Buse, G., Bourenkov, G.P., Bartunik, H.D., Huber, R., and Than, M.E. (2000) Structure and mechanism of the aberrant ba_3-cytochrome *c* oxidase from *Thermus thermophilus*. *EMBO J.* **19**, 1766–1776
23. Sone, N. and Toh, H. (1994) Membrane-bound *Bacillus* cytochromes *c* and their phylogenetic position among bacterial class I cytochromes. *FEMS Microbiol. Lett.* **122**, 203–210
24. Benini, S., Gonzalez, A., Rypniewski, W.R., Wilson, K., Van Beeumen, J., and Ciurli, S. (2000) Cristal structure of oxidized *Bacillus pasteurii* cytochrome *c*-553 at 0.97 Å resolution. *Biochemistry* **93**, 13115–13125
25. Narumi, I., Sawakami, K., Nakamoto, S., Nakayama, N., Yanagisawa, T., Takahashi, N., and Kihara, H. (1992) A newly isolated *Bacillus stearothermophilus* K1041 and its tranformation by electroporation. *Biotechnol. Biotechniques* **6**, 83–86
26. Takagi, H., Kadowaki, K., and Udaka, S. (1989) Screening and characterization of protein hyper-producing bacteria without detectable exoprotease activity. *Agric. Biol. Chem.* **53**, 691–699
27. Shida, O., Takagi, H., Kadowaki, K., and Komagata, K. (1996) Proposal for two new genera, *Brevibacillus* gen. nov. and *Aneurinibacillus* gen. nov. *Int. J. Syst. Bacteriol.* **46**, 939–946
28. Ichikawa, Y., Yamagata, N., Tochikubo, K., and Udaka, S. (1993) Very efficient extracellular production of cholera toxin B subunit using *Bacillus brevis*. *FEMS Microbiol. Lett.* **111**, 219–224
29. Tokunaga, H., Yamakawa, M , Mizukami, M., Takagi, H., and Tokunaga, M. (1998) Molecular cloning of the *dnaK* locus, and purification and characterization of a DnaK protein from *Bacillus brevis* HPD31. *Biochim. Biophys. Acta* **1387**, 65–79
30. Nicholls, P. and Sone, N. (1984) Kinetics of cytochrome *c* and TMPD oxidation by cytochrome *c* oxidase from the thermophilic bacterium PS3. *Biochim. Biophys. Acta* **767**, 240–247
31. Kai, K., Noguchi, S., and Sone, N. (1997) Over-expression and post-translational modification of thermophilic *Bacillus* cytochrome *c*-551 in *Bacillus subtilis*. *J. Ferm. Bioeng.* **84**, 190–194
32. Titani, K., Ericsson, L.H., Hon-nami, K., and Miyazawa, T. (1985) Amino acid sequence of cytochrome *c*-552 from *Thermus thermophilus* HB8. *Biochem. Biophys. Res. Commun.* **128**, 781–787
33. Than, M.E., Hof., P., Huber, R., Bourenkov, G.P., Bartunik, H.D., Buse, G., and Soulimane, T. (1997) *Thermus thermophilus* cytochrome *c*-552: A new highly thermostable cytochrome *c* structure obtained by MAD phasing. *J. Mol. Biol.* **271**, 629–644
34. Von Wachenfeldt, C. and Hederstedt, L. (1990) *Bacillus subtilis* 13-kDa cytochrome *c*-550 encoded by *cccA* consists of a membrane-anchor and a heme domain. *J. Biol. Chem.* **265**, 13939–13948
35. David, P.S., Dutt, P.S., Wathen, B., Jia, Z., and Hills, B.C. (2000) Characterization of a structural model of membrane bound cytochrome *c*-550 from *Bacillus subtilis*. *Arch. Biochem. Biophys.* **377**, 22–30
36. Bengtsson, J., Rivolta, C., Hederstedt, L., and Karamata, D. (1999) *Bacillus subtilis* contains two small *c*-type cytochromes with homologous heme domains but different types of membrane anchors. *J. Biol. Chem.* **274**, 26179–26184
37. Vandenberghe, I.H., Guisez, Y., Ciurli, S., Benini, S., and Van Beeumen, J. (1999) Cytochrome *c*-553 from the alkalophilic bacterium *Bacillus pasteurii* has a primary structure characteristics of a lipoprotein. *Biochem. Biophys. Res. Commun.* **264**, 380–387
38. Kagawa, Y. and Ariga, T. (1977) Determination of molecular species of phospholipids of thermophilic bacterium PS3 using mass-chromatography. *J. Biochem.* **81**, 1161–1165
39. Luebben, M. (1995) Cytochromes of archaeal electron transfer chains. *Biochim. Biophys. Acta* **1229**, 1–22
40. Scharf, B., Wittenberg, R., and Engelhard, M. (1997) Cytochrome ba_3 from *Natronobacterium pharaonis*, am archaeal four-subunit cytochrome-*c*-type oxidase. *Biochemistry* **36**, 4471–4479
41. Takami, H., Nakasone, K., Takaki, Y., Maeno, G., Sasaki, R., Masui, N., Fuji, F., Hirama, C., Nakamura, Y., Ogasawara, N., Kuhara, S., and Horikoshi, K. (2000) Complete genome sequence of the alkaliphilic bacterium *Bacillus halodurans* and genomic sequence comparison with *Bacillus subtilis*. *Nucleic Acids Res.* **28**, 4317–4331