Identification and Properties of a Quinol Oxidase Super-complex Composed of a bc₁ Complex and Cytochrome Oxidase in the Thermophilic Bacterium PS3*

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Evidence for the presence of a quinol oxidase super-complex composed of a cytochrome bc₁ complex and cytochrome oxidase in the respiratory chain of a Gram-positive thermophilic bacterium PS3 is reported.

On incubation with an octyl glucoside-solubilized fraction of the total membranes of PS3 anti-serum against PS3 cytochrome oxidase gave an immunoprecipitate that showed both quinol-cytochrome c reductase and cytochrome c oxidase activities. When the cholate-deoxycholate and LiCl-treated membranes of PS3 were solubilized and subjected to ion-exchange chromatography in the presence of octaethyleneglycol dodecyl ether, most of the A-, B-, and C-type cytochromes were copurified as a peak having both quinol-cytochrome c reductase and cytochrome oxidase activities. The immunoprecipitate and quinol oxidase preparation contained hemes a, b, and c in a ratio of about 2:2:3, indicating the presence of one-to-one complex of cytochrome oxidase containing 2 hemes a and one heme c, and a bc₁ complex containing 2 hemes b and 2 hemes c. Gel electrophoresis in the presence of dodecyl sulfate showed that the immunoprecipitate and quinol oxidase preparation were composed of seven subunits; those of 51 (56-kDa), 38, and 22 kDa for cytochrome oxidase and those of 29, 23, 21, and 14 kDa for the bc₁ complex. The 38-, 29-, and 21 kDa components possessed covalently bound heme c. The apparent molecular mass of the super complex was estimated to be as 380 kDa by gel filtration.

The Gram-positive spore-forming thermophilic bacterium PS3 contains A-, B-, and C-type cytochromes as components of its respiratory chain (1). The terminal oxidase of this organism, cytochrome oxidase containing cytochrome aa₃, cytochrome c, and Cu in three kinds of subunits (2), was shown to translocate protons in addition to carrying electrons (3). Recently Berry and Trumpower (6) reported evidence that a 3-subunit bc₁ complex and a 3-subunit aa₃ complex form a super-complex with ubiquinol oxidase activity in Paracoccus denitrificans. Since all the cytochromes c found in the thermophilic bacterium PS3 are hydrophlic, it seemed plausible that the cytochrome oxidase of PS3 also forms a super-complex with a bc₁ complex in the respiratory chain and that this might be the reason for the low yield of bc₁ cytochrome oxidase in the presence of a low concentration of Triton X-100 (7). By using polyclonal antibodies against the purified PS3 cytochrome oxidase and milder detergent treatment to avoid destroying the binding between the bc₁ complex and cytochrome oxidase, we found that the bc₁ complex and cytochrome oxidase form one-to-one super-complex in the thermophilic bacterium PS3.

EXPERIMENTAL PROCEDURES

Antibody—Antisera against the purified cytochrome oxidase from the thermophilic bacterium PS3 were prepared at the Medical School, University of Helsinki as follows. Two rabbits were immunized with PS3 cytochrome oxidase injected into popliteal lymph nodes, and given two boosters injections 2 and 6 weeks later. About 40 μg of enzyme protein was used for each injection. The rabbits were bled 10 days after the last injection.

Preparations—Cultivation of the thermophilic bacterium PS3, preparation of the membrane fraction, treatment of the membrane fraction with cholate plus deoxycholate and with 2.5 M LiCl to obtain "treated membranes," and purification of cytochrome oxidase from the treated membranes were as described previously (2, 5). The hydrophobic moiety of H⁺-ATP synthase was prepared from PS3 as described previously (8).

NAD-independent malate dehydrogenase was prepared from the supernatant fraction obtained by treatment of the PS3 membranes with cholate plus deoxycholate (5) as will be reported elsewhere.

Partially purified bc₁ complex was obtained from a DEAE-Toyopearl column (step 5 in Ref. 5) by raising the NaCl concentration to 40 mM, after extensive washing with 50 mM Tris-HCl (pH 8.0) containing 1.5% Triton X-100 and 10 mM NaCl. Quinol oxidase was prepared as follows. The treated membranes (120 mg of protein) were solubilized with 2.5% heptil thioglicoside (1) or MEGA 9 containing 10 mM Tricine-NaOH (pH 8.0) (10 ml). The mixture was centrifuged at 140,000 × g for 30 min and the supernatant was applied to a DEAE-Toyopearl column (1.2 × 5 cm) equilibrated with 50 mM Tris-HCl (pH 8.0) containing 0.5% C₁₂E₄. The column was then washed with 50 ml of the same solution containing 10 mM NaCl. Quinol oxidase was eluted by raising the NaCl concentration to 40 mM, and the reddish brown peak of material rich in cytochromes was collected.

Enzyme Assays—TMPD and cytochrome c oxidase activities were measured with a pH meter (Beckman 4500) with a combination pH electrode 39505 at 40 °C in reaction medium composed of 25 mM K₂SO₄, 2.5 mM MgSO₄, and 0.25 mM K-Mops buffer (pH 6.9-7.0), using ascorbate (5 mM) plus TMPD (50 μM) or ascorbate (5 mM) plus Candida kruase cytochrome c (10 μM) as described previously (9).

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Quinol-cytochrome c reductase activity was measured spectrophotometrically at 549 nm in reaction mixture (0.8 ml) containing 20 mM NaPi buffer (pH 6.8), 1 mM EDTA, 1 mM KCN, and 0.1 mg/ml sonicated soybean P-lipids at 40 °C. Duroquinol (6 mM) with d,L-malate (5 mM), PS3 malate dehydrogenase (0.02-0.05 units), and C. kruase cytochrome c (12-15 μM) were used as substrates and media tors, and the reaction was started by addition of the quinol oxidase preparation. Values were corrected for the slow nonenzymic reduction of cytochrome c by duroquinol. In the preliminary stage of this work, E. coli D-lactate dehydrogenase were used instead of PS3 malate dehydrogenase.

NAD-independent malate dehydrogenase was measured spectrophotometrically at 600 nm at 25 °C, in the same reaction mixture as for quinol reductase assay, with d,L-malate (5 mM) and 2,6-dichlorophenol indophenol (0.07 mM) as substrates. For the calculation, the ε m value of 2,6-dichlorophenol indophenol at 600 nm was taken as 20.6 and that of C. kruase cytochrome c at 549 nm as 24.3. Quinol oxidase activity was measured with an oxygen electrode (YSI 4001) in reaction mixture containing 20 mM NaPi buffer (pH 6.8), 1 mM EDTA, and 0.1 mg/ml soybean P-lipids at 38 °C. Durohydroquinone (25 μM) in the presence of D,L-malate (10 mM) and PS3 malate dehydrogenase (0.1-0.2 units) were used as substrates.

**Gel Filtration**—A G-4000 SW column (0.75 × 60 cm) of Toyo Soda was employed. Enzymes were assayed as described under "Experimental Procedures." M. lysodeikticus cytochrome c oxidase; TMPD oxidase; Δψ, quinol-cytochrome c reductase; Δψ, malate dehydrogenase.

**RESULTS**

Coprecipitation of the bc Complex with Antibody against Cytochrome Oxidase—An antiserum against purified cyt ochrome oxidase from the thermophilic bacterium PS3 immunoprecipitates PS3 cytochrome oxidase, although the cytochrome c oxidizing activity of the precipitate is only partially (about 50%) inhibited. This antiserum (315) was used to test for the presence of super-complex of c,a3-type cytochrome oxidase and the bc complex in PS3.

Fig. 1A shows the effect of addition of the antiserum on the electron transfer activities of the octyl glucoside-solubilized membrane fraction of PS3. Addition of 20 μl of the antiserum inhibited about 60% of the cytochrome c oxidizing activity (O), but scarcely affected the TMPD oxidizing activity (●) or quinol-cytochrome c (Δ) reductase activity. On the contrary, after centrifugation both the oxidases (O, ●) and most of the quinol-reductase (Δ) were no longer detectable in the supernatant fraction (Fig. 1B). NAD-independent malate dehydrogenase, which is also membrane-bound, remained in the supernatant fraction after centrifugation. Control rabbit serum did not precipitate the enzymes in the same conditions. These data exclude the possibility of nonspecific aggregation of the enzymes and suggest that the quinol-cytochrome reductase (bc, complex) present as a super-complex with cytochrome oxidase.

Concomitant Elution of bc and c,a3 Cytochromes—PS3 cytochrome oxidase has been purified from treated membranes (treated with cholate plus deoxycholate and then with 2.5 M LiCl) by ion-exchange chromatography in the presence of Triton X-100. Since these treated membranes are rich in quinol-cytochrome c reductase as well as cytochrome c oxidase (cf. Table I), they were solubilized with MEGA 9 to test for the presence of a “super complex” of bc, complex and cytochrome oxidase.

MEGA 9-solubilized fraction was added to a DEAE-Toyopearl column and materials was eluted with a gradient of NaCl in Tris-HCl buffer containing 0.5% C12Es and then with 2.5 M LiCl by ion-exchange chromatography in the presence of Triton X-100. Since these treated membranes are rich in quinol-cytochrome c reductase as well as cytochrome c oxidase, the fractions were used for measurements of activities without centrifugation. In the presence of a “super complex” of bc, complex and cytochrome oxidase.

**Fig. 1.** Titration of enzyme activities with antiserum against PS3 cytochrome oxidase. Antiserum 315 at the indicated volume was incubated with octyl glucoside-solubilized membranes (0.49 mg of protein) in 0.4 ml of 25 mM KPi buffer (pH 7.4) containing 0.5% octyl glucoside, and the immunoreaction was carried out at 38 °C for 30 min. A, 20 μl samples were used for measurements of activities without centrifugation. B, the mixtures were centrifuged at 15,000 rpm for 5 min in a microcentrifuge (Tomy MC-15A), and 20 μl samples of the supernatant were used for measurements of the activities. Enzyme activities were assayed as described under “Experimental Procedures.” A, cytochrome c oxidase; ●, TMPD oxidase; Δψ, quinol-cytochrome c reductase; Δψ, malate dehydrogenase.
branes (20 mg of protein) solubilized with 2.5% MEGA 9 as described under "Experimental Procedures" were diluted 3-fold with 20 mM Tris-HCl (pH 8.0) containing 0.5% C1zEs and NaCl developed with a linear gradient of up to 200 mM NaCl in the same buffer. The arrow shows the point where free PS3 cytochrome oxidase was eluted. Fractions of 2 ml were collected and their absorbance at 280 nm was measured. The absorption spectrum from 500 to 650 nm was measured after reduction by Na2SZO4.

In the next sections.

Subunit Composition and the Subunit Reacting with Antibody—Fig. 3A shows the subunit patterns of quinol oxidase fractionated with DEAE-Toyopearl (lane 1), the antibody-antigen complex precipitated by treating octyl glucoside-solubilized membrane fraction with 315 antiserum (lane 2) and extracted the antibody-antigen complex extracted with 3 M urea (lane 3) together with the partially purified bc1 (quinolcytochrome c reductase) complex (lane 4) and caa3-cytochrome oxidase (lane 5). Bands at 51, 38, 29, 23, 21-22, and 14 kDa are seen in both in lanes 1 and 2, suggesting that they may be subunits of quinol oxidase composed of the bc1 complex and cytochrome oxidase, if the fast migrating sharp band at the front is ignored. The peroxidase activity was also observed with o-tolidine as chromatolidine, which detects heme compounds (11); the bands at 38, 29, and 21 kDa turned green, indicating that they contained c-heme (not shown). The band at 90 kDa in lane 2, which was not seen in lanes 1 and 3, is probably due to antibody gamma-globulin. The band at 14 kDa was much weaker in lane 3 than in lanes 1 and 2, suggesting that materials of this band can be removed by urea treatment. Since cytochrome oxidase (lane 5) is composed of three subunits of 51 kDa (or 56 kDa by a Ferguson plot; cf. Ref. 2), 38, and 22 kDa, the remaining bands in lane 1 (28, 23, 21, and 14 kDa) are probably those of subunits of the bc1 complex. In fact the partially purified bc1 complex, separated from cytochrome oxidase on a column of DEAE-Toyopearl during purification of the latter (step 5 in Ref. 2), gave these four bands on SDS-gel electrophoresis, as shown in lane 4. Thus, we were able to prepare cytochrome oxidase, the bc1 complex, and a quinol oxidase super-complex composed of cytochrome oxidase and bc1 complex.

Fig. 3B shows the immunological detection of subunits of quinol oxidase transferred to a nitrocellulose filter and treated with antiserum 315 (10 pl) in 5 ml of 0.1 M Tris-HCl (pH 7.5) containing 0.1 M NaCl and 0.5% skimmed milk for 2 h with shaking, washed, and treated with 10 μl of 125I-protein D (Amersham Corp., 15 mCl/mg). Lane 1, quinol oxidase fraction from DEAE-Toyopearl (8 μg of protein). Lane 2, heptyl thioglucoside extract of the treated membranes (23 μg of protein).

TABLE I

<table>
<thead>
<tr>
<th>Step</th>
<th>Protein (mg)</th>
<th>Heme content (yield)</th>
<th>Activity (TN)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>a</td>
<td>b</td>
<td>c</td>
</tr>
<tr>
<td>Membranes</td>
<td>120</td>
<td>0.40 (100)</td>
<td>0.64 (100)</td>
</tr>
<tr>
<td>Treated membranes</td>
<td>18.2</td>
<td>2.4 (91)</td>
<td>3.1 (73)</td>
</tr>
<tr>
<td>Quinol oxidase</td>
<td>4.9</td>
<td>6.7 (68)</td>
<td>6.7 (43)</td>
</tr>
<tr>
<td>Immunoprecipitate</td>
<td>7.8</td>
<td>2.8 (80)</td>
<td>3.0 (54)</td>
</tr>
</tbody>
</table>

*The turnover number for TMPD oxidation is expressed as transferred electrons/aa s-1 and those for quinol oxidation and quinol-cytochrome c reduction are expressed as transferred electrons/2b s-1.

**Membranes (72 mg of protein) were treated with antiserum 315 (3 ml) under the same conditions as for Fig. 1 except that the incubation time was 60 min.
Quinol Oxidase from Thermophilic Bacterium PS3

A subunit II of cytochrome oxidase reacted with the antibody.

Absorption Spectra—Fig. 4 shows the absorption spectra of quinol oxidase prepared by ion-exchange chromatography as described under "Experimental Procedures." The spectrum of the reduced form (dotted line) shows the presence of A-, B-, and C-type cytochromes and is not much different from that of membranes (1). Fig. 5 shows the redox difference spectra of the immunoprecipitate of the super complex. The spectrum at room temperature was again not much different from that of membranes. The broad peak at about 550 nm suggests that this peak is composed of three different c-type cytochromes; i.e., c-547, c-549.5, and c-554. Cytochrome c-549.5 (550) has been shown to be a chromophore of subunit II of PS3 cytochrome oxidase (2). The presence of three c-type cytochromes was much clearer when the difference spectrum was measured at liquid N_2 temperature (dashed line). Splitting of the α-band of cytochrome b, on the other band, was not clear even at the low temperature, and thus we could not determine whether cytochrome b of PS3 split into two spectrally different species as in P. denitrificans (6, 14) and Rhodobacter sphaeroides (15). The difference spectrum of quinol oxidase prepared by chromatography (not shown) was very similar to those of the immunoprecipitates (Fig. 4). Using this preparation we reduced cytochrome b partially and cytochrome a, and C-type cytochromes almost completely by adding durohydroquinone and a small amount of Na_2S_2O_4 (partially reduced). The two difference spectra, fully reduced minus partially reduced and partially reduced minus oxidized (with reduced A-type and C-type cytochromes due to the presence of ascorbate), showed a peak at 561–562 nm. Thus, there is still no indication that there are two types of cytochrome b having different midpoint potentials.

Heme Contents and Enzymic Activities—The heme contents of membranes and quinol oxidase preparations were measured to quantify the super-complex. As summarized in Table I, quinol oxidase preparations prepared with antibody and by chromatography were both composed of 2 hemes a, 2 hemes b, and 3 hemes c, suggesting that cytochrome oxidase and the bc_1 complex form a one-to-one super-complex.

The TMPD oxidase and quinol-cytochrome c reductase activities of these preparations are also summarized in Table I. TMPD oxidase activity, which is not inhibited by the antibody, unlike the cytochrome c oxidase activity (Fig. 1), was concentrated almost in parallel with heme a content. Quinol-cytochrome c-reductase activity, however, was not so active as TMPD oxidase activity. Moreover the turnover number of the quinol-cytochrome c-reductase activity in the bc_1-oxidase super-complex prepared by DEAE-Toyopearl chromatography was about one-fifth of those of the other activities, for some unknown reason. Sonicated P-lipids of this bacterium were added. Without the addition the turnover number was about 1/5 of that of in the original membranes. Several inhibitors of bc_1 complex were examined. HQONO (1-heptyl, 4-hydroxyquinoline-N-oxide) caused strong inhibition of cytochrome c reduction with durohydroquinone by PS3 quinol oxidase (97% at 9.5 μM and 71% at 2.4 μM). Myxothiazol inhibited the activity (51% at 5 μM) also. On the other hand 2.3 μM antimycin A scarcely inhibited the activity.

The quinol oxidase activity was also measured with an oxygen electrode. The specific activity obtained was almost the same as that of quinol-cytochrome c reductase, indicating that the electron transfer reaction in the bc_1 complex is rate limiting. Although both quinol-cytochrome c reductase and cytochrome c oxidase activities are accelerated with C. krusei cytochrome c with a K_m of 12 μM for the reductase and 3.7 μM for the oxidase (9), C. krusei cytochrome c (0.2-10 μM) did not accelerate the quinol oxidase activity of octyl glucoside-solubilized membranes. A similar observation has been reported for the ubiquinol oxidase from P. denitrificans (6).

Gel Filtration Analysis—The apparent molecular weights of the quinol oxidase super-complex and its constituents were measured in a gel filtration column (TSK G-4000 SW, Toyo Soda) for high pressure liquid chromatography in the presence of Brij 35 (Fig. 6). The elution pattern of quinol oxidase (line...
A) cytochrome oxidase, and the
and the
protein); pH of 
if the detergent molecules are also stacked on the enzymes.
enzymes exist as oligomers in the non-ionic detergent, even
TFl incubated for 0.4% 
cytochrome oxidase 
(25 kDa). The mixture of the bcl complex 
was seen together with that of a mixture of the bc, complex, cytochrome oxidase (line B), cytochrome oxidase (line C), and the bc1 complex (line D). The mixture of the bc1 complex and cytochrome oxidase appeared to flow without interaction. The molecular mass of these samples were estimated roughly to be 650 kDa for bc1, 380 kDa for quinol oxidase, and 310 kDa for cytochrome oxidase, with thyroglobulin (669 kDa), TF (380 kDa), and aldolase (158 kDa) as molecular mass standards in the same buffer but without detergent. These values for apparent molecular weights suggest that these enzymes exist as oligomers in the non-ionic detergent, even if the detergent molecules are also stacked on the enzymes.

DISCUSSION

The present investigation showed that the bc1 complex of the thermophilic bacterium PS3 forms a super-complex with cytochrome oxidase and functions as quinol oxidase. Evidence for this as follows: 1) the oxidase and bc1 complex formed a one-to-one complex. 2) These two proteins were fractionated together even in the presence of a sufficient amount of detergents such as C12E8 and MEGA 9 on the occasions of the ion-exchange (Fig. 2) as well as gel filtration (Fig. 6A) chromatography. 3) The two enzymes, when prepared respectively, did not form the super complex again, although the elution medium was the same (Fig. 6B). If the interaction were nonspecific and hydrophobic in nature, the bc1-oxidase complex may be formed again. 4) The two enzymes seem to form an intimate electron transfer chain from cytochrome b to cytochrome aa3, since exogenously added yeast cytochrome c did not accelerate quinol-oxidizing activity, although exogenously added cytochrome c served as an electron acceptor for quinol oxidation and an electron donor for the cytochrome oxidase.

A quinol oxidase super-complex has been found in P. denitrificans (6). We were able to demonstrate the presence of this complex in the Gram-positive bacterium PS3 and also to show that the super-complex is composed of one bc1 complex and one aa3 oxidase (Table I), probably because of the high stability of these enzymes in this thermophile. At present we do not know whether this apparent one-to-one stoichiometry simply reflects the amounts of these enzymes synthesized in the membranes or whether it indicates specific interaction between them.

The cytochrome bc1 complex, or quinol cytochrome c reductase, has been prepared from several bacteria such as P. denitrificans (14), and R. sphaeroides (15, 16). The enzyme contains cytochrome b, cytochrome c1, and a high potential Fe-S center in three or four subunits, which are much simpler than those in the mitochondrial enzyme (but see Ref. 17). The enzyme from the thermophilic Bacillus PS3 contains cytochrome b, cytochrome c-553 probably corresponding to cytochrome c1, and a high potential Fe-S center in four subunits. The sum of these subunits is less than that of those in P. denitrificans (121 kDa) and R. sphaeroides (101 kDa). Sensitivity to inhibitors such as antianycin A and myxothiazol was different from the enzymes from those bacteria which were known to be rather closely related to the mitochondrial enzymes. We are now attempting to isolate and characterize the PS3 bc1 complex.

Combined analysis by potentiometric titration and low temperature spectroscopy of PS3 cytochromes has been reported by Poole et al. (18). They classified B- and C-type cytochromes into three groups; C-type cytochromes with a midpotential of about 210 mV, B-type cytochromes (cytochrome o) with a midpotential of 33 mV, and low potential B-type cytochromes with a midpotential of about −166 mV. They identified three C-type cytochromes with absorption peaks at 545, 547, and 552 nm, and two B-type cytochromes with absorption at 560.8 and 554.5 nm by fourth-order finite difference analysis of spectra at 77K, ignoring the minor component at 563 nm. All three C-type and two B-type cytochromes were detected from the difference spectra of quinol oxidase at 77K (Fig. 4, dotted line). We also found B-type cytochromes with absorption at 558 nm in the membrane fraction and in the treated membranes which were eluted at a higher salt concentration from a Toyopearl column (Fig. 2). This fraction has low TMPD oxidase activity, and thus the cytochrome is likely to be the cytochrome o reported by Poole et al. (19). Hence, almost all the cytochromes in the respiratory chain of PS3 are now assigned.

Cytochrome c-551 (about 12 kDa) is found in the cholate-deoxycholate soluble fraction. This cytochrome can be oxidized by PS3 cytochrome oxidase with a low KM (2). The role

2 N. Sone, unpublished observation.
of this relatively hydrophilic C-type cytochrome is not known at present. The chain of hydrophobic C-type cytochrome (c-547 and c-554 in the bc complex and c-549.5 in the oxidase) seems to be sufficient for the electron transfer in the respiratory chain, since quinol oxidase activity was comparable to quinol-cytochrome c reductase activity with a saturating amount of C. krusei cytochrome c.

de Vrij (20) identified the following five B- and C-type cytochromes in B. subtilis membranes: b-564, c-554, c-550, b-559, and b-556. Probably b-564 and c-554 are components of the B. subtilis bc1 complex, c-550 has affinity to aa3-type cytochrome oxidase of this bacterium (21), and the last two form cytochrome o as an alternative oxidase, as suggested by de Vrij (20). Thus the respiratory chains of two species of Bacillus would apparently be quite similar, except that cytochrome c-549.5 is fused with subunit II of a three subunit cytochrome oxidase in PS3.

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