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Purification of a *ccb*-type quinol oxidase specifically induced in a deep-sea barophilic bacterium, *Shewanella* sp. strain DB-172F

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Abstract We investigated for the first time the respiratory chain system of a deep-sea barophilic bacterium, *Shewanella* sp. strain DB-172F. A membrane-bound *ccb*-type quinol oxidase, from cells grown at 60 MPa pressure, was purified to an electrophoretically homogeneous state. The purified enzyme complex consisted of four kinds of subunits with molecular masses of 98, 66, 18.5, and 15 kDa, and it contained 0.96 mol of protoheme and 1.95 mol of covalently bound heme *c* per mol of enzyme. Only protoheme in the enzyme reacted with CO and CN^-, and the catalytic activity of the enzyme was 50% inhibited by 4 μM CN^-. The isoelectric point of the native enzyme complex was determined to be 5.0. This enzyme was specifically induced only under conditions of elevated hydrostatic pressure, and high levels were expressed in cells grown at 60 MPa. The membranes isolated from cells grown at atmospheric pressure (0.1 MPa) exhibited high levels of both cytochrome *c* oxidase and *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPDH2)-oxidase activity. These results suggest the presence of two kinds of respiratory chains regulated in response to pressure in the deep-sea bacterium DB-172F.

Key words Deep-sea bacteria · *Shewanella benthica* · Hydrostatic pressure · Respiratory system · Pressure regulation

Introduction

The deep-sea environment is regarded as the most common habitat, covering about 78% of the total biosphere of the earth, and it is a special world with conditions of extremely high pressure and low temperature. Two barophilic bacterial strains, DB-172F and DB-172R, were isolated recently from a sample of deep-sea sediment collected from the Izu-Bonin trench, at a depth of 6500 m (Kato et al. 1996a). Both of these barophilic strains belong to the Proteobacteria gamma sub-group and are closely related to the species *Shewanella benthica* (Kato et al. 1997). The mechanisms of oxidative phosphorylation and respiration may play a central role in the baroadaptibility of deep-sea bacteria. Since strain DB-172F, at 4°C, can grow at atmospheric pressure (0.1 MPa) as well as at high hydrostatic pressure (60 MPa), it is expected that the respiratory chain components of this deep-sea bacterium may exhibit some novel characteristics.

Recent genetic studies revealed that barophilic bacteria generally contain several pressure-regulated gene clusters and promoters (Kato et al. 1997). Further, it has been observed that a barotolerant bacterium, *Shewanella* sp. strain DSS12, specifically synthesizes a membrane-bound *d*-type cytochrome when grown at high hydrostatic pressure (Tamegai et al. 1998). Genetic studies of strain DSS12 revealed the presence of a pressure-regulated open reading frame, which was homologous to that of the *cydD* gene of *Escherichia coli* (Kato et al. 1996b). The *cydD* gene in *E. coli* is reported to play a role in the stability of respiratory components (Poole et al. 1989, 1994). These reports suggest an intrinsic relationship between hydrostatic pressure and respiratory chain components.

Generally, the bacterial respiratory oxidases belong to a large superfamily of heme-copper oxidases, divided into two groups: the quinol oxidases and the cytochrome *c* oxidases (Chepuri et al. 1990b; Garcia-Horsman et al. 1994). These enzymes have in common a binuclear center consisting of a high-spin heme and copper (Cu_B). Quinol oxidases oxidize quinols such as ubiquinol, and they do not contain Cu_A, a redox center for accepting electrons from reduced cytochrome *c* in cytochrome *c* oxidases. Several bacterial cytochrome *c* oxidases have been reported that contain heme *c*, but no quinol oxidase containing heme *c* has so far been identified within the superfamily of oxidases.

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In the present study, we purified a membrane-bound *ccb*-type quinol oxidase enzyme complex to an electrophoretically homogeneous state from a deep-sea barophilic bacterium. Two kinds of respiratory chain systems in strain DB-172F, regulated by hydrostatic pressure, are discussed.

Materials and methods

Organism and culture conditions

The barophilic bacterium strain DB-172F was isolated from a sample of deep-sea sediment obtained from the Izu-Bonin trench at a depth of 6500 m as described previously (Kato et al. 1996a). The bacterium was cultivated in pre-autoclaved bags containing Marine Broth 2216 (Difco, Detroit, MI, USA) with or without the addition of oxygenated fluorinert (25% of total volume; FC-72, Sumitomo-3M, Tokyo). The bags containing the medium were placed in pressure vessels (titanium; HiP, Erie, PA, USA) kept at atmospheric pressure (0.1 MPa) or pressurized at 60 MPa. The vessels were placed at 4°C for a period of 6 days in the case of incubation at 60 MPa and for 12 days in the case of incubation at 0.1 MPa. Bacterial cell densities were determined by measuring the absorbance at 660 nm using a Beckman Model DU 7500 spectrophotometer. The cell yield from these cultures was 0.6 g/l (wet weight) in the case of growth at 60 MPa and 0.4 g/l (wet weight) in the case of growth at 0.1 MPa. The cells were harvested in the early exponential phase of growth by centrifugation at 10000 × *g* for 15 min and suspended in 10 mM Tris-HCl buffer (pH 8.0), containing 1 mM ethylenediaminetetraacetic acid (EDTA) and 10 μM phenylmethylsulfonyl fluoride (buffer A) and stored at −80°C until use.

Purification of membrane-bound quinol oxidase

Frozen cells (10 g wet weight) grown at 60 MPa were suspended in 100 ml of buffer A. The suspension was then treated with a sonic oscillator (20 kHz, 200 W) for 15 min at 4°C. Unbroken cells were removed by centrifugation (10000 × *g* for 15 min) and the cell-free extract was then centrifuged at 105000 × *g* for 1 h. The reddish pellets obtained were suspended in 50 ml of buffer A containing 0.3 M KCl and centrifuged at 105000 × *g* for 1 h. The washed membranes (2.3 mg of protein/ml) were suspended in buffer A and solubilized by treatment with 1.5% dodecyl maltoside. The suspension was gently stirred at 4°C for 2 h, and then centrifuged at 105000 × *g* for 1 h. The clear reddish supernatant was applied to ion-exchange chromatography on a diethylaminoethyl (DEAE)-Toyopearl column (1.5 × 9.5 cm) equilibrated with buffer A containing 0.5 g/l dodecyl maltoside. The adsorbed sample was washed with 3 void volumes of the equilibration buffer containing 0.1 M NaCl and the adsorbed enzyme was eluted with a linear gradient of NaCl (0.1–0.35 M) produced from 300 ml of the equilibration buffer. The fractions with quinol oxidase activity were pooled and ammonium sulfate was then added to 35% saturation. The suspension was gently stirred for 1 h at 4°C and then centrifuged at 10000 × *g* for 15 min. The pellets obtained were suspended in buffer A containing 1 g/l dodecyl maltoside and dialyzed for 6 h at 4°C against 2 l of buffer A containing 0.5 g/l dodecyl maltoside. The dialyzed sample was concentrated using an Amicon Centriplus-30 membrane (Amicon, Lexington, MA, USA) before being applied to gel filtration on a column of Sephacryl S-200 (2.3 × 162 cm) equilibrated with the same buffer as used for ion-exchange chromatography but containing 0.25 M NaCl. The fractions with high quinol oxidase activity were pooled, desalted, and concentrated using an Amicon Centriplus-30 membrane and used as the purified preparation.

Enzyme assay

Quinol oxidase activity was measured spectrophotometrically (Shimadzu UV-2400 PC, Shimadzu, Kyoto, Japan). The oxidation of quinol to quinone was determined by monitoring the absorbance at both 280 nm and 294 nm at room temperature. The standard reaction mixture for the assay contained 50 mM potassium phosphate buffer, pH 7.5, 1 mM EDTA, 1 g/l dodecyl maltoside, and 80 μM decylubiquinol [DBH; a synthetic analogue of ubiquinol-2, reduced according to Trumpower and Edwards (1979)] in a total volume of 1 ml. The reaction was initiated by addition of the enzyme preparation, and the millimolar extinction coefficient (ε_{mM}) used for DBH was 17 (Trumpower and Edwards 1979). KCN was used at 50 μM to inhibit the reaction. The activity of the amount of enzyme catalyzing oxidation of 1 μmol of DBH per min was defined as one unit. Alternatively, oxygen uptake was measured polarographically at 5°C using a Clark-type oxygen electrode (YSI model 5300, Yellow Springs Instrument Co., Inc., Ohio, USA). The reaction mixture contained 50 mM potassium phosphate, pH 7.5, 1 mM EDTA, 1 g/l dodecyl maltoside, 120 μM reduced DBH, and the appropriate amount of enzyme in a total volume of 2 ml. Analyses of the catalytic activity of washed membranes were performed spectrophotometrically. The cytochrome *c* oxidase activity was determined from the decrease in absorbance at 550 nm using the same reaction mixture except that DBH was replaced with horse heart ferrocytochrome *c*. *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPDH2)-oxidase activity was determined from the increase in absorbance at 610 nm in the presence of 1.2 mM TMPDH2.

Physical and chemical measurements

Absorption spectra were recorded with a Shimadzu model (UV-2400 PC) with 1-cm light path cuvettes at room temperature. The content of heme *b* and heme *c* was determined on the basis of millimolar extinction coefficients (ε_{mM}) of 34.4 $mM^{-1}cm^{-1}$ and 29.1 $mM^{-1}cm^{-1}$ at the α-peak of the pyridine ferrohemochromes of *b* (Morrison et al. 1960) and *c* (Drabkin et al. 1942), respectively. The content of heme *b* was determined with an HCl-acetone extract of the enzyme, while that of heme *c* was determined

with the residues obtained after the HCl-acetone extraction of the enzyme. Nondenaturing polyacrylamide gel electrophoresis (native-PAGE) was performed in the presence of 0.7 g/l dodecyl maltoside at 4°C, and polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) was performed by the method described by Schägger and Jagow (1987). The heme *b* and heme *c* in the native gel were detected by heme staining reagents (Connelly et al. 1958). Protein content was determined by the bicinchoninic acid (BCA) assay protocol (Pierce, Rockford, IL, USA) with bovine serum albumin as a standard. Isoelectric focusing was determined according to the protocol provided by Pharmacia (Uppsala, Sweden). All chemicals used were of analytical grade.

Quantitative analysis of membrane-bound quinol oxidase in strain DB-172F cultivated at various pressures

Strain DB-172F was cultured under various pressures ranging from 0.1 MPa to 80 MPa in pressurized vessels. Cells were collected in the early log phase of growth and washed membranes were prepared from each individual batch of cells as described previously (Qureshi et al. 1990). The membranes were solubilized with dodecyl maltoside and subjected to ion-exchange chromatography on a DEAE-Toyopearl column (under the experimental conditions already described). The difference spectra, dithionite-reduced minus ferricyanide-oxidized, were recorded at room temperature to estimate the cytochrome content.

Results

Purification of membrane-bound quinol oxidase from *Shewanella* sp. strain DB-172F

The membrane-bound quinol oxidase from cells grown at 60 MPa was solubilized with dodecyl maltoside, under conditions in which the enzyme retains its active form, and subsequently the solubilized enzyme was purified by ion-exchange chromatography and gel filtration chromatography in the presence of the detergent. Figure 1 shows the elution profile after ion-exchange chromatography. When the final preparation was subjected to PAGE in the presence of dodecyl maltoside at 4°C, one major band and a few faint bands were stained with Coomassie Brilliant Blue. The content of the faint bands seemed to amount to less than 1% of the intensity of the major band and these bands were not consistently found in the enzyme preparations. Only the major band was stained by the heme staining reagents (Fig. 2). This result shows that the enzyme preparation obtained in the present study was almost homogeneous. Representative results for each of the purification steps are summarized in Table 1.

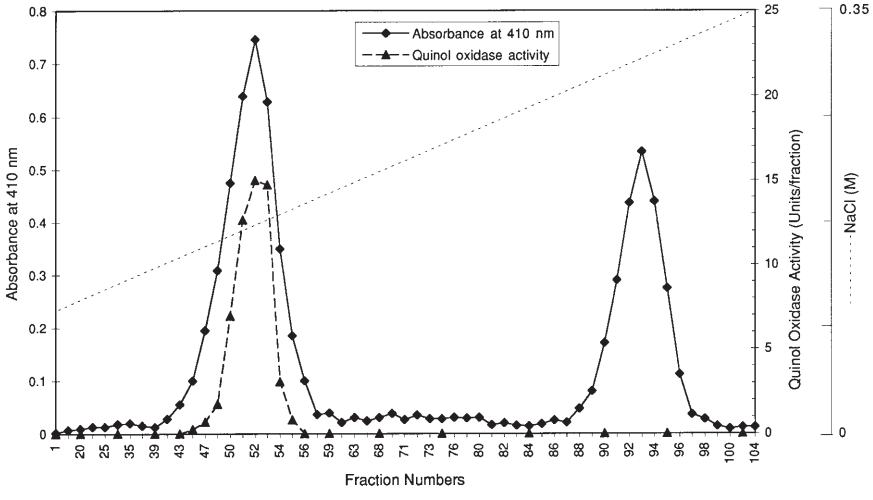

Fig. 1. Elution profile obtained upon ion-exchange chromatography of the solubilized membrane proteins from barophilic DB-172F cells grown at 60 MPa pressure. *Diamonds*, A_{410}; *triangles*, quinol oxidase activity

Table 1. Purification of quinol oxidase from barophilic DB-172F grown at 60 MPa pressure

Experimental steps	Total protein (mg)	Heme *c*		Protoheme		Quinol oxidase activity[a] ($\mu mol \cdot min^{-1} \cdot mg^{-1}$)
		(nmol)	(nmol/mg)	(nmol)	(nmol/mg)	
Washed membrane	71	262.7	3.7	69	0.97	–[b]
Membrane extract with dodecyl maltoside	64	243.2	3.8	65	1.01	1.4
DEAE-Toyopearl	26	171.6	6.6	52	4.0	5.5
Sephacryl S-200	1.1	10.8	9.9	5.4	4.9	25.4

[a] Quinol oxidase activity was measured using decylubiquinol as substrate.
[b] Since the typical activity assay is run under detergent conditions, no value is given here.

Spectral properties

The oxidized form of the enzyme showed an absorption peak at 409.7 nm and, after the addition of sodium dithionite, the reduced form showed peaks at 417, 522, and 552 nm and a shoulder at around 559 nm (Fig. 3A). The pyridine ferrohemochrome of the enzyme showed peaks at 414, 520, and 550 nm and a shoulder at around 560 nm (Fig. 3B). These spectral properties suggest that the enzyme contains heme *c* as well as heme *b*. Figure 4 illustrates the difference spectra of the enzyme complex bound to its ligands. The CO difference spectrum, (reduced + CO) minus reduced, of the enzyme showed a sharp peak at 415 nm and shoulders at around 442, 528, and 561 nm. Troughs were evident at 429, 522, and 552 nm. These spectral properties are somewhat similar to those of *co*-type cytochrome *c* oxidase (Tamegai and Fukumori 1994). The CN^- difference spectrum, (reduced + CN^-) minus reduced, showed peaks at 424, 442, 528, and 557 nm and troughs at 415, 429, 551, and 564 nm. These results seemed to indicate that protoheme rather than heme *c* in the enzyme combined with the ligands CO and CN^-.

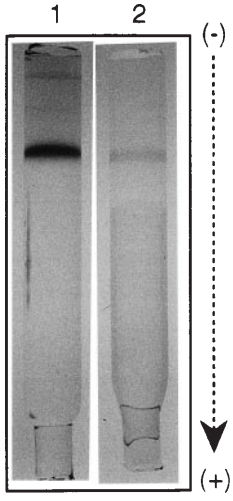

Fig. 2. Polyacrylamide gel electropharesis (PAGE) of the barophilic DB-172F quinol oxidase in the presence of detergent. The purified enzyme (58 μg of protein) was loaded on a 10% gel in the presence of dodecyl maltoside. After electrophoresis, the gel was stained with Coomassie Brilliant Blue (*lane 1*) or heme-staining reagents (*lane 2*)

Molecular and enzymatic properties

The subunit composition was analyzed by SDS-PAGE. When the purified enzyme was denatured at room temperature for 15 min in 2% SDS, four major protein bands appeared on the gel (Fig. 5). The molecular weights for the individual protein bands were estimated to be 98, 66, 18.5, and 15 kDa. When the same gel was soaked in the heme-staining reagents, one band corresponding to a molecular mass of 18.5 kDa was stained (Fig. 5). On the basis of the absorption spectrum of the pyridine ferrohemochrome, the heme *c* and heme *b* content was estimated to be about 9.9 and 4.9 nmol/mg of protein, respectively. These results indicate that the enzyme contains two heme *c* and one protoheme per minimal structural unit. When the absorption spectrum of the purified enzyme was measured between 700 and 980 nm, a broad peak appeared in the region of 830 nm, indicating the presence of a copper atom in the enzyme molecule (data not shown). The isoelectric point of

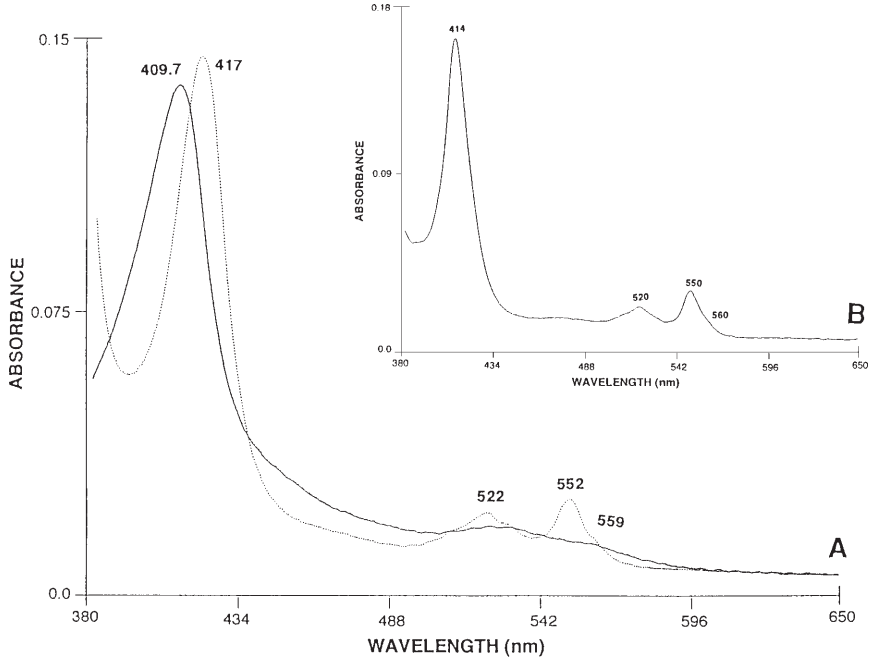

Fig. 3. Spectral properties of barophilic DB-172F quinol oxidase. **A** Absolute absorption spectrum at room temperature. The enzyme was suspended in 10 mM Tris-HCl (pH 8.0), containing 0.5 g/l dodecyl maltoside. *solid line*, oxidized state; *dotted line*, reduced with dithionite. **B** Pyridine ferrohemochrome spectrum. The enzyme was suspended in 0.2 N NaOH and 5% pyridine and reduced with a small amount of solid dithionite

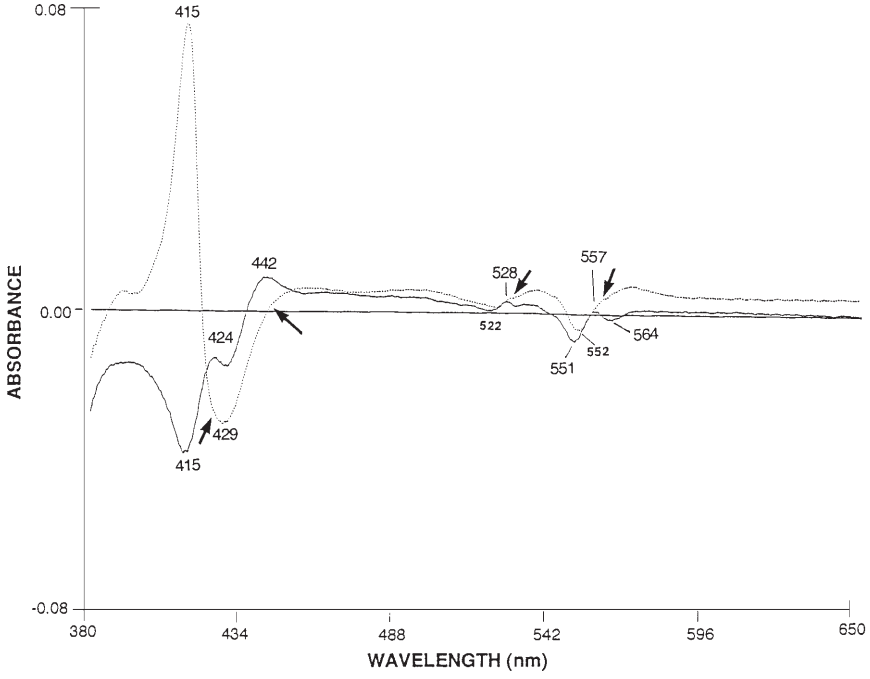

Fig. 4. Carbon-monoxide and cyanide binding spectra of the barophilic DB-172F quinol oxidase. The purified enzyme preparation (around 2.3 μM heme *c*) was dissolved in 10 mM Tris-HCl (pH 8.0) containing 1 mM ethylenediaminetetraacetic acid (EDTA) and 0.5 g/l dodecyl maltoside and was fully reduced by the addition of a small amount of solid dithionite. The reduced enzyme was incubated under a 100% CO atmosphere for 5 min, and the difference spectrum, (reduced + CO) minus reduced, was recorded (*dashed line*). *Arrows* indicate the shoulder points. The cyanide-difference spectrum, (reduced + CN^-) minus reduced, was recorded after incubating the reduced enzyme with 20 μM cyanide for 5 min (*solid line*)

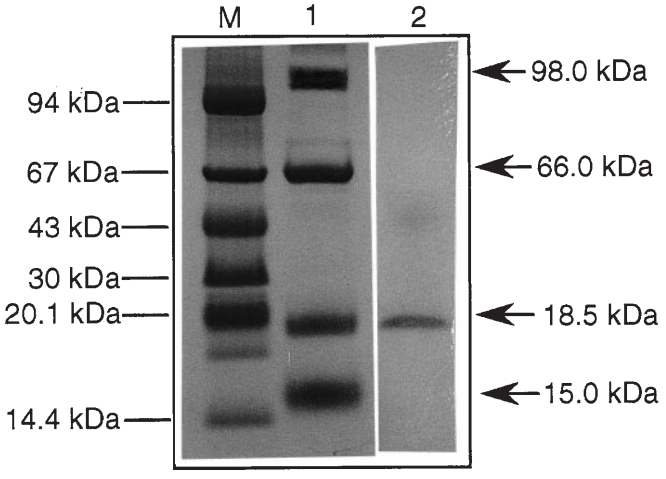

Fig. 5. Sodiumdodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of the barophilic DB-172F quinol oxidase enzyme. The purified enzyme (29 μg of protein) was treated with 3% SDS for 20 min at room temperature and then loaded on a 12% Tricine-SDS gel. *Lane 1*, stained with Coomassie Brilliant Blue; *lane 2*, stained with heme-staining reagents. *M*, molecular mass marker proteins [phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), α-lactalbumin (14.4 kDa)]. The molecular masses of the four subunits of DB-172F quinol oxidase were estimated to be 98, 66, 18.5, and 15 kDa, respectively

the purified enzyme was estimated to be 5.0 by isoelectric focusing.

The catalytic activity of DB-172F quinol oxidase was determined using reduced decylubiquinol (DBH) as substrate. The final preparation of the enzyme had a specific activity of approximately 25.4 units/mg of protein. The enzyme showed no oxidase activity when incubated with purified membrane-bound and cytoplasmic *c*-type cytochromes from DB-172F, yeast cytochrome *c*, or horse heart cytochrome *c*. A very low level of activity (0.8 units/mg of protein) was observed with *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPDH2) as an electron donor. Potent inhibitors of quinol-cytochrome *c* oxidoreductase (bc_1-complex), such as antimycin-A and 2-heptyl-4-hydroxyquinoline-*N*-oxide (HOQNO), did not show any inhibitory effect on the DB-172F quinol-oxidase activity. However, the activity was strongly inhibited by KCN, and 50% inhibition of the total catalytic activity was achieved with 4 μM CN^-.

Induction of quinol oxidase in response to a change in hydrostatic pressure

A quantitative evaluation of the membrane-bound quinol oxidase was carried out. The bacterium was cultivated at various pressures and the cells were collected in the early exponential phase of growth. Quinol oxidase was partially purified from the same wet weight of cells in each instance. Figure 6 shows the content of the membrane-bound enzyme complex in the cells grown at atmospheric pressure (0.1 MPa) and at four other hydrostatic pressures ranging from 20 MPa to 80 MPa. Cells grown at 60 MPa contained the highest amount of quinol oxidase, and this enzyme was not detected in cells grown at atmospheric pressure (0.1 MPa).

Discussion

In the present study, the deep-sea barophilic and psychrophilic bacterium, DB-172F, was grown at 60 MPa hydro-

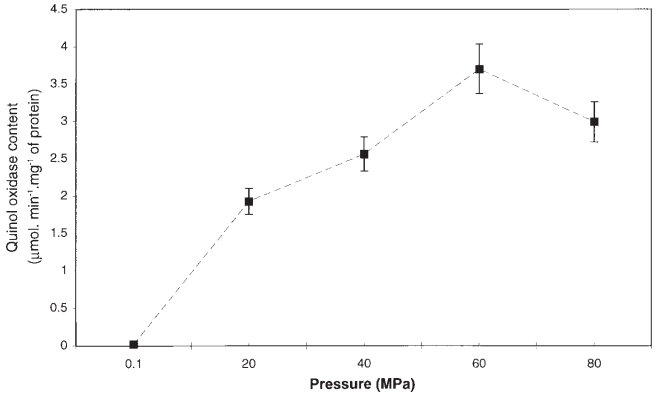

Fig. 6. Quantitative analysis of membrane-bound quinol oxidase in barophilic strain DB-172F cells grown at atmospheric pressure (0.1 MPa) and at various other hydrostatic pressures (from 20 MPa to 80 MPa)

static pressure. Initially, oxygenated fluorinert was added to the culture medium as an oxygen source. Because of the difficulty encountered in measurement of oxygen equilibrium between the fluorinert and medium phases under high-pressure conditions, the usual cultivation method was also employed, in which the medium was bubbled with pure oxygen but devoid of fluorinert. The bacterium grew equally well under such conditions. DB-172F can grow either at atmospheric pressure (0.1 MPa) or at elevated hydrostatic pressure (60 MPa), although the doubling time of the bacterium at 0.1 MPa pressure is comparatively longer than that at 60 MPa. This shows that this barophilic organism is well-adapted to high-pressure conditions. So far, no report has been published concerning purification from a deep-sea bacterium of enzymes or other proteins which participate in cellular respiration. Probably, the major problem in this regard is the limitation of available culturing techniques for aerobic growth of the organism under high-pressure conditions; this results in a very low growth yield.

The washed membranes from DB-172F cells (grown at 60 MPa) showed a high level of quinol oxidase activity, a very low level of TMPDH2-oxidase activity, and no cytochrome *c* oxidase activity. On the other hand, membranes from DB-172F cells grown at atmospheric pressure (0.1 MPa) showed high levels of both cytochrome *c* oxidase activity and TMPDH2-oxidase activity but no quinol oxidase activity, despite the fact that the same culture medium was employed for culturing the cells at 0.1 MPa and 60 MPa. Hence, it seems that this barophilic bacterium, DB-172F, can express two different respiratory chains depending on the pressure conditions. The existence of alternative respiratory chains has also been reported in other bacteria (Chepuri et al. 1990a).

The membranes of DB-172F cells grown at 60 MPa specifically displayed a high level of quinol oxidase. This enzyme was found to consist of four subunits which show almost equal staining intensities in SDS-PAGE. Quinol oxidases purified from *E. coli* (Matsushita et al. 1986), *Acetobacter aceti* (Fukaya et al. 1993), and *Paracoccus denitrificans* (Matthias et al. 1994) are also reported to consist of four subunits in each instance. The largest subunit of the DB-172F quinol oxidase showed a tendency to disintegrate slightly on SDS-PAGE but generally exhibited one major band. Such disintegration of a single subunit has also been reported in the case of quinol oxidase from *P. denitrificans* (Matthias et al. 1994). The molecular masses of subunit-III (18.5 kDa) and subunit-IV (15 kDa) of DB-172F quinol oxidase are similar in size to subunit-III (19.1 kDa) and subunit-IV (16.2 kDa) of the enzyme from *A. aceti* (Fukaya et al. 1993). This suggests that DB-172F quinol oxidase and those purified from other bacteria have some structural similarities.

Quinol oxidases purified from *E. coli* (Matsushita et al. 1986) and *Bacillus subtilis* (Santana et al. 1992) contain a single equivalent of copper, i.e., Cu_B, and do not contain Cu_A, which is generally present in cytochrome *c* oxidases and acts as an electron donor to the binuclear center. Spectroscopic scanning of the DB-172F quinol oxidase in the near-infrared region suggested the presence of copper (i.e., Cu_B) that may participate in the reduction of oxygen to water. However, further detailed analysis is needed to determine the content and role of copper in DB-172F quinol oxidase.

It has been established that quinol oxidase is a member of a superfamily of heme-copper oxidases (Chepuri et al. 1990b; Garcia-Horsman et al. 1994). Recently, a novel type of heme-copper oxidase has been discovered and classified as a cbb_3-type oxidase. Genes encoding this oxidase were isolated and called *fix*NOQP (Zufferey et al. 1996). However, cbb_3-type oxidases purified from several species act as cytochrome oxidases, not as quinol oxidases, and also lack the Cu_A which is generally present in other cytochrome oxidases. *Bradyrhizobium japonicum* cbb_3-type cytochrome oxidase is expressed only under microaerobic and anaerobic conditions, and the contribution of the individual subunits to the function and assembly of the membrane-bound complex was recently investigated (Zufferey et al. 1996). The primary nucleation step involves the insertion of polypeptides into the membrane encoded from the genes *fix*N and *fix*O, and is considered to be necessary for the stable assembly of the enzyme complex. In a phototrophic bacterium, *Rhodobacter capsulatus*, two terminal oxidases were reported to be present: a cytochrome *c* oxidase and a quinol oxidase. Only cytochrome *c* oxidase could be purified, and it was found to be a novel *cb*-type oxidase that lacks a Cu_A center (Gray et al. 1994).

The ubiquinol oxidase purified in the present study from the barophilic bacterium DB-172F was found to contain two mols of heme *c* and one mol of protoheme in the minimal structural unit (Mr 197 500), but it does not show any evidence of containing heme *a* or heme a_3. The lack of heme *a* and a_3 was also reported for ubiquinol oxidases from *A. aceti* (Fukaya et al. 1993) and *P. denitrificans* (Matthias et al. 1994). Heme staining confirmed the presence of heme *c* in subunit-III (18.5 kDa) of the DB-172F enzyme. In general, the spectral properties of this enzyme show some similari-

ties to those of *ccb*-type cytochrome *c* oxidases from other bacteria (Tamegai and Fukumori 1994; Gray et al. 1994; Zufferey et al. 1996). The absorption spectra of the carbonmonoxide and cyanide complexes of the enzyme show that heme other than heme *c* reacts with CO and CN^-. It seems that protoheme reacts with the ligands, as reported in the case of *cb*-type and cbb_3-type terminal oxidases (Gray et al. 1994; Garcia-Horsman et al. 1994); the absorption peak at 552 nm attributable to heme *c* is not affected by these ligands. This further supports the conclusion that the ubiquinol oxidase of strain DB-172F is a ccb_3-type cytochrome and belongs to the novel class of cb_3-type oxidases. Furthermore, to our knowledge, the DB-172F enzyme is the first quinol oxidase shown to contain heme *c* as a structural component.

The catalytic activity of the DB-172F quinol oxidase was measured in the presence of detergent and the enzyme showed no cytochrome *c* oxidase activity. Furthermore, the enzyme reduces oxygen to water with a reduced ubiquinol analogue (decylubiquinol) as substrate, and this activity was strongly inhibited by CN^-. A quantitative analysis revealed that the content of membrane-bound respiratory enzyme activity in strain DB-172F was significantly changeable depending on the hydrostatic pressure applied. This demonstrates an intrinsic relationship between the levels of respiratory components and the applied hydrostatic pressure (Fig. 6). Analysis of the catalytic activity of membranes isolated from strain DB-172F suggested the existence of at least two distinct respiratory chains which are regulated in response to changes in hydrostatic pressure. When DB-172F cells are grown at atmospheric pressure (0.1 MPa), a typical cytochrome *c* dependent oxidase mechanism seems to exist that involves a terminal oxidase for the reduction of oxygen, whereas at high hydrostatic pressure (60 MPa), a quinol oxidase enzyme complex seems to function for the direct reduction of oxygen with quinol as substrate.

Further studies are in progress to elucidate the regulatory mechanism controlling expression of the respiratory enzymes in bacterium DB-172F to clarify the relationship between the baroadaptibility of this deep-sea bacterium and the energy transduction mechanisms.

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