Resolution of the Aerobic Respiratory System of the Thermoacidophilic Archaeon, Sulfolobus sp. Strain 7

II. CHARACTERIZATION OF THE ARCHAEAL TERMINAL OXIDASE SUBCOMPLEXES AND IMPLICATION FOR THE INTRAMOLECULAR ELECTRON TRANSFER*

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The terminal segment of the aerobic respiratory chain of the thermoacidophilic archaeon Sulfolobus sp. strain 7 is an unusual caldariellaquinol oxidase supercomplex, which contains at least one b-type and three spectroscopically distinguishable a-type cytochromes, one copper, and a Rieske-type FeS center. In this paper, we report the purification and characterization of two different forms of the archaeal a-type cytochromes, namely, a three-subunit cytochrome \( \text{aa}_3 \) subcomplex and a single-subunit cytochrome \( \text{aa}_3 \) derived from the cytochrome subcomplex, in order to facilitate further studies on the terminal oxidase segment of Sulfolobus. The optical and EPR spectroscopic analyses suggest the presence of two different low-spin heme centers and one high-spin heme center in the purified cytochrome \( \text{aa}_3 \) subcomplex, and one low-spin and one high-spin hemes in cytochrome \( \text{aa}_3 \), respectively. The Rieske-type FeS center detected in the purified cytochrome oxidase subcomplex was absent in two forms of the a-type cytochrome oxidase, indicating its association with cytochrome \( \text{bc}_{1\alpha} \). The crystal field parameters of the low-spin heme \( \text{aa}_3 \) center indicate that its axial ligands may be similar to those of cytochromes \( c \), rather than conventional bis-histidine ligation. In spite of the absence of any c-type cytochrome, a ferrocytochrome \( c \) oxidase activity was detected in the archaeal purified cytochrome \( \text{bc}_{1\alpha} \) subcomplex with no quinol oxidase activity, but not in the purified cytochrome oxidase supercomplex, which has been tentatively interpreted as a representative of electron transfer from the Rieske FeS center to cytochrome \( \text{aa}_3 \) in vivo. Thus, our results indicate the following scheme for the intramolecular electron transfer of the terminal oxidase supercomplex from Sulfolobus sp. strain 7: [caldariellaquinol → ] \( \text{bc}_{1\alpha} \) → Rieske FeS center → \( \text{aa}_3 \) → molecular oxygen.

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which grows optimally at 75-80 °C and at pH 2.5-3. As in the cases of other aerobic respiratory archaea such as S. acidocaldarius strain DSM 639 (20) and Halobacterium salinarium (22), it contains only the a- and b-type cytochromes, but no c-type cytochromes (21). Because of the unique phylogenetic status of Sulfolobus in the 16 S rRNA sequence tree (24, 25), the elucidation of the overview of the archaeal aerobic respiratory chain is a very challenging task. As described in the preceding paper (29), we have shown the presence of at least four distinct heme centers (b_{562}, a_{833}, and a_{a3}) in the membranes of Sulfolobus sp. strain 7, which have been copurified as a novel terminal oxidase supercomplex notably containing a Rieske FeS center. In addition, we have also shown that cytochrome b_{562}, which is probably in the upstream of the a-type cytochromes in the intramolecular electron transfer, is possibly equivalent to the heme b_{c}, center of the conventional cytochrome bc_{1} complex (30–32) in terms of the redox function (29). Thus, while the overall feature of the purified cytochrome supercomplex resembles S. acidocaldarius SoxM oxidase complex (27), our results are in sharp contrast to the previous proposal by Lübben et al. (10, 26) that the SoxC protein might contain two heme a_{a} centers (cytochrome a_{a3}) but no protoheme center. On the other hand, a functional account of a high-potential and unusual a-type cytochrome a_{833} of Sulfolobus sp. strain 7 (∆G, m = +270 mV (29)) remains to be studied, because of the overall complexity of the archaeal aerobic respiratory supercomplex.

One way to overcome a complexity of a multisubunit complex metalloprotein and to elucidate possible roles of its individual redox centers, may be to resolve it into much simpler but catalytically active subcomplexes and/or subunits in vitro, as performed in the mitochondrial NADH:quinone oxidoreductase complex (the respiratory complex I) (33, 34). In this paper, we report the purification and the spectroscopic and catalytic properties of two different forms of the a-type cytochrome oxidase from Sulfolobus sp. strain 7, in order to assign a possible function of the unusual cytochrome a_{833} in the archaeal aerobic respiratory chain. In addition, we report the EPR evidence for non-bis-histidine ligation of the low-spin heme a_{833} center. Furthermore, in conjunction with our data presented in the preceding paper (29), we propose a novel conserving mechanism for the terminal oxidase supercomplex from Sulfolobus sp. strain 7, which differs considerably from the recent proposals for S. acidocaldarius Sox oxidase complexes by Lübben et al. (10). A preliminary account of this work has been reported elsewhere (35).

**EXPERIMENTAL PROCEDURES**

**Materials**

MEGA-9 and MEGA-10 were purchased from Dojin (Kumamoto, J. apan), sodium cholate from Sigma, sucrose monopacrate and sucrose monolaurate from Mitsubishi Kasei Co. (Tokyo, Japan), and lauryl monolaurate from Mitsubishi Kasei Co. (Tokyo, Japan), sodium cholate from Sigma, sucrose monocaprate and sucrose-3-lactate from Sigma. Water was purified by the Milli-Q purification system (Millipore). The following abbreviations used are: MEGA-9, nanoyl-β-o-maltoside; MEGA-10, decanoyl-α-methylglucamide; HOOANO, 2-heptyl-4-hydroxyquinoline-N-oxide; PAGE, polyacrylamide gel electrophoresis; TPMPD, N,N,N’,N’-tetramethyl-p-phenylenediamine.

**Cell Culture and Membrane Preparation**

Sulfolobus sp. strain 7 was cultivated at pH 2.5-3 and at 75-80 °C as described previously (21, 40), and was harvested in the early to middle exponential phase of growth. The archael membranes were prepared as described in the preceding paper (29), and immediately used for the subsequent purification of the membrane-bound cytochromes (see below).

**Purification of the Terminal Oxidase Supercomplex**

The terminal oxidase supercomplex containing both a- and b-type cytochromes was solubilized and purified from the archael membranes as described in the preceding paper (29).

**Purification of the a-Type Cytochromes**

The following purification steps were carried out at 25-30 °C to avoid the precipitation of MEGA-10, by following the absorption at 290 and 422 nm and the reduced-minus-oxidized difference spectrum of each fraction at different purification steps, unless otherwise specified.

**Step 1: Cholate Treatment**—To the membranes were added a solution of 5% (w/v) cholate and 50 mM EDTA to final concentrations of 1% and 10 mM, respectively, and the mixture was gently stirred for 120 min at room temperature. While both the a- and b-type cytochromes were not solubilized at this stage, some loosely bound proteins (such as an NADH dehydrogenase (40)) were solubilized and removed by ultracentrifugation at 130,000 × g for 60 min at 20 °C.

**Step 2: Solubilization of the a-Type Cytochromes in the Presence of MEGA-9 and MEGA-10**—The washed membrane was made to 0.5% (w/v) MEGA-9, 1% (w/v) MEGA-10, 0.5 mM EDTA, and 20 mM sodium phosphate buffer, pH 6.5, and the resulting mixture was gently stirred for 120 min at 25-30 °C. Then, the MEGA-9/MEGA-10 extract containing the solubilized cytochromes were collected by ultracentrifugation at 130,000 × g for 60 min at 25 °C.

**Step 3: Hydroxylapatite Column Chromatography**—The MEGA-9/MEGA-10 extract was applied to a hydroxylapatite column (5.2 × 10 cm) equilibrated with distilled water. Then, the cytochromes were eluted with a 400-ml linear gradient of 80-400 mM sodium phosphate buffer, pH 6.5, containing 0.5% (w/v) MEGA-9 and 1% (w/v) MEGA-10. At this stage, two different forms of the a-type cytochrome oxidase were separately obtained, in addition to a partially-denatured cytochrome complex containing both the a- and b-type cytochromes (29); of these, only two a-type cytochromes were further purified separately hereafter. Each combined fraction was then rechromatographed in the same conditions.

**Step 4: Gel Filtration Column Chromatography**—The pooled fractions were brought to 15% with polyethylene glycol-6000, and the resulting precipitates were collected by centrifugation. They were dissolved in 20 mM potassium phosphate buffer, pH 6.8, containing 0.5% (w/v) MEGA-9, 1% (w/v) MEGA-10, 0.1 mM EDTA, and ~50% (w/v) glycerol, and then applied to a Sephacryl S-200 gel filtration column equilibrated with 100 mM potassium phosphate buffer, pH 6.8, containing 0.5% (w/v) MEGA-9, 1% (w/v) MEGA-10, and 0.1 mM EDTA. The cytochrome peak fractions were collected, and checked for purity by polyacrylamide gel electrophoresis in the presence of SDS. The purified a-type cytochromes were stored at ~80 °C until use.

**Purification of the Respiratory Complex II from Sulfolobus sp. Strain 7**

Solubilization and purification of the archael membrane-bound respiratory complex II (succinate:quinone oxidoreductase complex) was carried out as described in the accompanying paper (41).

**Measurements of Enzymatic Activities**

A ferrocytochrome c oxidase activity was measured spectrophotometrically at 50 °C according to Wakagi et al. (35) with horse heart ferrocytochrome c as an electron donor. A caldariellaquinol oxidase activity was measured spectrophotometrically at 50 °C according to Shafar et al. (42). The in vitro reconstitution of the Sulfolobus succinocinidase respiratory chain was monitored polargraphically at 50 °C with a Clark-type oxygen electrode (Oxygen analyzer MP-1000, Iizima Products, Tokyo, Japan) equipped with temperature-controlled cells. A preliminary test for a proton pumping activity of the purified cytochrome subcomplexes of Sulfolobus was performed according to Son and Yanagita (43), using ferrocytochrome c as an electron donor.

**Analytical Methods**

Absorption spectra were recorded with a Hitachi U-3210 spectrophotometer equipped with a thermodiologic cell holder, or with a Shimadzu.
RESULTS

Purification and Molecular Properties of Cytochrome aa₃ and Cytochrome a₅₈₃-a₃ Subcomplex—Fig. 1 shows a typical elution profile of the membrane-bound cytochromes of Sulfolobus sp. strain 7 solubilized in the presence of MEGA-9 plus MEGA-10, from a hydroxylapatite column. While the archaeal membrane exhibits four spectrophotometrically and potentiometrically distinct heme centers, i.e. b₅₆₂, a₅₈₃, and a₃ (29), this method allowed the decomposition of some amounts of the terminal cytochrome supercomplex into and the separation of two different forms of the a₅₈₃-type cytochromes from the rest of the cytochrome complex (Fig. 1, top). The a₅₈₃-type cytochrome with an absorption peak at 603 nm in the reduced minus oxidized difference spectrum is referred to as cytochrome a₅₈₃, and that with two absorption peaks at 583 and 604 nm in the reduced minus oxidized difference spectrum is referred to as cytochrome a₅₈₃-a₃ subcomplex, respectively. For efficient separation of these a₅₈₃-type cytochromes, the presence of relatively strong detergents such as MEGA-10 and the repetition of the hydroxylapatite column chromatography step appeared to be essential factors. Typical purification data of these a₅₈₃-type cytochromes are summarized in Table I. For the cytochrome a₅₈₃-a₃ subcomplex, an increase in the specific ferrocytochrome c oxidase activity was nicely accompanied by an enrichment of the specific heme A₅₈₃ content, as the purification proceeded. On the other hand, a ferrocytochrome c oxidase activity was abolished in purified cytochrome a₅₈₃ 

Fig. 2 shows the subunit compositions of highly purified cytochrome a₅₈₃ and cytochrome a₅₈₃-a₃ subcomplex from Sulfolobus sp. strain 7. Purified cytochrome a₅₈₃ was composed of a single subunit with an apparent molecular mass of 40 kDa on SDS-PAGE (Fig. 2, lane 1), while the cytochrome a₅₈₃-a₃ subcomplex was composed of three different subunits with apparent molecular masses of 40, 24, and 14 kDa on SDS-PAGE (Fig. 2, lane 4), respectively. Gel filtration analysis indicated that both purified a₅₈₃-type cytochromes have almost identical apparent molecular masses of ~130–150 kDa (data not shown).

Fig. 3 shows the optical properties of purified cytochrome a₅₈₃ and cytochrome a₅₈₃-a₃ subcomplex. Heme analysis of the purified a₅₈₃-type cytochromes using reverse-phase column chromatography and pyridine ferrohemochrome spectra suggested the presence of only heme A₅₈₃ as opposed to the case of the Sulfolobus membrane which contains both heme A₅₈₃ and protoheme (data not shown; cf. Ref. 38). The reduced minus oxidized difference spectrum of purified cytochrome a₅₈₃ exhibits maxima at 442 and 603 nm (Fig. 3A). Its CO-difference spectrum shows maxima at 429 and 595 nm and a minimum at 444 nm, which is characteristic of that of the a₅₈₃-type heme-copper oxidase (Fig. 3B). On the other hand, the reduced minus oxidized difference spectrum of purified cytochrome a₅₈₃-a₃ exhibits maxima at 438, 583, and 604 nm (Fig. 3C). At 77 K, the unusual ε-band at 583 nm splits into a prominent maximum at 582 nm with a small shoulder at ~570 nm (data not shown; cf. see Fig. 1 A in the preceding paper (Ref. 29)). In addition, the apparent intensity of the absorption peak at 604 nm of the latter subcomplex relative to that at 583 nm in the reduced
The EPR spectrum of purified cytochrome

15% SDS-PAGE was performed in the absence (lanes 2 and 3) and the presence (lanes 2 and 4) of 5% 2-mercaptoethanol, and proteins were visualized by silver staining (Wako Chemicals). Lanes 1 and 2, purified cytochrome aa₃ (subunit I); lanes 3 and 4, purified cytochrome a₅₈₃-a₃ subcomplex; Std, molecular size standards (Bio-Rad, low-range marker proteins).

minus oxidized difference spectrum varied slightly from preparation to preparation (data not shown). The CO-difference spectrum of purified cytochrome a₅₈₃-a₃ shows maxima at 433 and 598 nm and a minimum at 449 nm, which indicates that cytochrome a₅₈₃ is not CO-reactive (Fig. 3D). Small differences in the optical spectra of purified cytochrome aa₃ and cytochrome a₅₈₃-a₃ subcomplex probably reflect small alterations of the microenvironments around hemes a and a₃ centers during purification (see below).

EPR Properties of the Individual Heme Centers—Fig. 4 shows the comparative EPR spectra of the air-oxidized forms of the isolated Sulfolobus membranes (Fig. 4A), purified cytochrome aa₃ (Fig. 4B), purified cytochrome a₅₈₃-a₃ subcomplex (Fig. 4C), and purified terminal oxidase supercomplex containing all membrane-bound a- and b-type cytochromes (Fig. 4D; Ref. 29). The EPR spectrum of purified cytochrome aa₃ shows one high-spin heme component at g = 2.08, but notably, no Cu₄ signal (Fig. 4B). The EPR spectrum of purified cytochrome a₅₈₃-a₃ subcomplex shows one high-spin heme component at g = 2.08, and two distinct low-spin heme components at gₓᵧ = 2.90, 2.27, and 1.55, respectively (not detectable in the membrane-bound state, see Fig. 4A), and at gₓᵧ = 2.67, 2.38, and 1.64 (detectable in the membrane-bound state, see Fig. 4A), respectively; it also elicits additional resonances of a radical species at g = 2 and only traces of adventitious copper at g = 2.08, but notably, no Cu₄ signal (Fig. 4C). In conjunction with the optical properties of the purified a-type cytochromes (Fig. 3) and the membranes of Sulfolobus sp. strain 7 (21, 29, 35), these data are clearly consistent with the following EPR assignments for the low-spin heme centers in the archaeal terminal oxidase supercomplex. The heme a center in cytochrome aa₃, gₓᵧ = 2.90, 2.27, and 1.55; and heme a₅₈₃ center, gₓᵧ = 2.67, 2.38, and 1.64, respectively. In addition, the EPR properties of the low-spin heme a center in

| Step | Protein | Specific activity* (-fold) | Heme A₅ content (-fold) |
|------|---------|---------------------------|------------------------|
| 1. Washed membranes of Sulfolobus | 2310 | (0.5) | 0.75 |
| 2. MEGA-9/10 extract | 1470 | (1.0) | 1.0 |
| cytochrome a₅₈₃-a₃ subcomplex | | (1.0) | 1.1 |
| 3-1. Hydroxylapatite | 14.0 | (11.1) | 5.9 |
| 3-2. Hydroxylapatite | 32.2 | (16.5) | 13.1 |
| 4-1. Sephacryl S-200 | 0.37 | (28.0) | 15.8 |
| Cytochrome aa₃ (subunit I) | | | |
| 4-2. Sephacryl S-200 | 0.52 | (4.9) | 11.9 |

* Oxidation of 40 μM horse heart ferrocytochrome c at pH 6.8 and at 50 °C.
purified cytochrome a₅₈₃ aa₃ subcomplex (gₓᵧₓ = 3.02, 2.24, and 1.47) was slightly altered as compared to the membrane-bound state (gₓᵧₓ = 2.90, 2.27, and 1.55; Fig. 4, A and C), indicating a small change in the microenvironments around the heme a center during purification. This is consistent with its optical properties, where an a-band corresponding to cytochrome aa₃ was shifted from 603 to 604 nm in the purified subcomplex (Fig. 3). The EPR spectra of the membranes of Sulfolobus sp. strain 7 and the purified active terminal oxidase supercomplex are very similar, and show the presence of at least two different low-spin hemes in a ratio of 2:1 for the gₓ = 2.90 species and the gₓ = 2.67 species, respectively (Fig. 4, A and D). This suggests the presence of at least two gₓ = 2.90 species in the archaeal membranes and the terminal oxidase supercomplex, whose EPR line shapes are largely overlapping; on the basis of the presence of a non-CO-reactive heme b₅₆₂ center beside heme a, a₅₈₃, and a₃ centers in the membranes (29), the additional low-spin heme at gₓ = 2.90 probably corre-
sponds to cytochrome b$_{562}$. Thus, the EPR analysis indicates the presence of at least three low-spin heme centers and one high-spin heme center in the terminal oxidase supercomplex from Sulfolobus sp. strain 7 (Fig. 4D), and no additional heme centers could be detected in the archaeal membranes (Fig. 4A).

The EPR of a low-spin ferric heme species can be analyzed in terms of two crystal field parameters $V/\Delta$ and $\Delta/\lambda$, expressions for the rhombicity and the tetragonality of the heme, respectively (47–49). These two parameters obtained from the three g values, are very sensitive to the chemical nature of the axial ligands of the heme, to the geometrical arrangement of the ligand atoms, and to the $\pi$ bonds between the ligands and the iron. A two-dimensional plot of regions of crystal field parameters is useful for characterization and identification of various heme ligand combinations (47, 49), and most low-spin heme compounds fall into five relatively well defined groups, C type (e.g. cytochrome c), B type (e.g. cytochrome b$_{5}$, hemoglobin salicylate; the distal histidine with imidazole N as the fifth ligand and a methionine S as the sixth ligand, respectively. Which usually contain a histidine imidazole N as the fifth ligand and a methionine S as the sixth ligand, respectively.

$V$ and $\Delta$ (tetragonality) characteristic of a Rieske-type FeS cluster (g$_{xy}$ = 2.02, 1.89, and 1.79; cf. Fig. 8 in Ref. 29), detectable in the ascorbate-reduced terminal oxidase supercomplex (Fig. 5B), and both the succinate-reduced (Fig. 5C) and the ascorbate-reduced (not shown) membrane is clearly absent in purified cytochrome b$_{562}$-aa$_{3}$ subcomplex (Fig. 5A). It was not detected in purified cytochrome aa$_{3}$ either (data not shown). These data indicate that the archaeal Rieske FeS center probably associates more tightly with cytochrome b$_{562}$ than with the a-type cytochromes.

In the succinate-reduced membranes in the presence of 1 mM cyanide, a low-spin heme center at g$_{xy}$ = 2.90, and 2.27 partially remained in the oxidized state (Fig. 5C). Since the heme b$_{562}$ center (E$_{m}$ = +146 mV) and the heme a$_{583}$ center (E$_{m}$ = +270 mV) were almost fully reduced under the conditions (cf. Fig. 4 in Ref. 29), it might be attributable to the low-potential heme a center of cytochrome aa$_{3}$ portion (E$_{m}$ = +117 mV).

Catalytic Properties—In earlier studies, a ferrocytochrome c oxidase activity was found in the membranes of several different species of Sulfolobales (20, 21, 35). Because of the absence of any c-type cytochrome in the archaebacteria, this enzymatic activity has been merely considered as an artifact, and remains poorly characterized (cf. Ref. 52).

As summarized in Table I, a ferrocytochrome c oxidase activity was significantly copurified with the three-subunit cytochrome b$_{562}$-aa$_{3}$ subcomplex, but not with the single-subunit cytochrome aa$_{3}$. In the presence of 1% (w/v) cholate in the assay.

**Catalytic Properties**—In earlier studies, a ferrocytochrome c oxidase activity was found in the membranes of several different species of Sulfolobales (20, 21, 35). Because of the absence of any c-type cytochrome in the archaebacteria, this enzymatic activity has been merely considered as an artifact, and remains poorly characterized (cf. Ref. 52).
mixture, which stimulates a ferrocytochrome c oxidase activity (35), the following oxidase activities were observed at 50 °C for purified cytochrome aa3 subcomplex (expressed as per heme Aa3): –21 e/ with 40 μM horse heart ferrocytochrome c (Vmax = –76 e/ with –30 e/ with 100 μM TMPD, and –0.24 e/ with –100 μM caldariellaquinol. These activities were completely inhibited by –150 μM KCN. On the other hand, purified cytochrome aa3 showed the following oxidase activities (expressed as per heme Aa3): –0.49 e/ with 40 μM horse heart ferrocytochrome c and –11 e/ with –100 μM caldariellaquinol, which were also fully cyanide sensitive. These data indicate the requirement of cytochrome aa3 for the ferrocytochrome c oxidase activity in Sulfolobus sp. strain 7.

Nevertheless, the purified cytochrome supercomplex, which contains cytochrome b562 and a Rieske-type FeS cluster in addition to all of the a-type heme centers present in purified cytochrome aa3 +aa3 subcomplex (see Fig. 4 and 5) and functions as a true terminal oxidase in the in vitro succinate-oxidizing respiratory chain (–130 nmol of O2/min/nmol of heme Aa3 at 50 °C and pH 6.8) in the presence of the cognate respiratory complex II (succinatequinone oxidoreductase (41)) and caldariellaquinol (29), showed only negligible ferrocytochrome c oxidase activity at 50 °C, viz. –0.15 e/ with 60 μM horse heart ferrocytochrome c (expressed as per heme Aa3). In fact, in the elution profile shown in Fig. 1, the maximal level of a ferrocytochrome c oxidase activity was detected only in fractions enriched with cytochrome aa3 +aa3 subcomplex, but not in fractions containing both the a- and b-type cytochromes (data not shown).

Thus, a ferrocytochrome c oxidase activity of purified cytochrome aa3 +aa3 subcomplex may differ from those of typical cytochrome c oxidases from mitochondria and several bacteria. When incorporated into soybean phospholipid vesicles in vitro, it showed only lower ferrocytochrome c oxidase activity, and no ferrocytochrome c-dependent proton-pumping activity (data not shown). In addition, it did not reconstitute the in vitro succinoxidase respiratory chain in the presence of the cognate respiratory complex II and 7 μM caldariellaquinol at 50 °C and pH 6.8 (data not shown), as opposed to the case of the purified terminal oxidase supercomplex (29). Consequently, the ferrocytochrome c oxidase activity of Sulfolobus sp. strain 7 (21, 35) probably reflects the intramolecular electron transfer to the heme aa3 center, in the terminal oxidase supercomplex.

**DISCUSSION**

The a-Type Cytochromes of Sulfolobus sp. Strain 7—In the preceding paper (29), we have reported the detection of at least four distinct heme centers (b562, +146 mV; aa3, +270 mV; and aa3, +117 and +325 mV) in the membranes of Sulfolobus sp. strain 7, all of which are shown to be purified comitantly with a Rieske-type FeS cluster as a cytochrome supercomplex in the presence of a milder detergent mixture. Of these redox centers, inhibitor studies suggest that cytochrome b562 may be functionally equivalent to the heme b5 center of typical cytochrome bc2 complexes (30–32, 53), and that it is in the upstream of the a-type cytochromes in the archaeal respiratory chain. Thus, cytochrome b562 is probably a true site of reaction with the caldariellaquinone/quinol couple in the terminal oxidase supercomplex. In order to assign a possible function of the unusual heme aa3 center of Sulfolobus sp. strain 7, we applied a fairly stronger detergent mixture (MEGA-9 plus MEGA-10) to the archaeal membranes to partially decompose the terminal oxidase supercomplex and to purify its constituents in catalytically active forms. The methods employed in this study resulted in successful purification of the a-type cytochromes in two different forms, namely, cytochrome aa3 +aa3 subcomplex and cytochrome aa3 derived from the cytochrome subcomplex, respectively. On the other hand, we have so far failed to purify cytochrome b562 or aa3 as a single-subunit component, which indicates its strong association to other constituent(s).

Purified cytochrome aa3 is composed of a single 40-kDa subunit. Spectroscopic characterization suggests that it contains the oxygen reducing site of the terminal oxidase supercomplex, consisting of a high-spin heme a3 center and CuA, and an additional low spin heme a center (g = 2.90, 2.27 and 1.55). The rhombic and tetragonal crystal field parameters (V/Δ and Δ/Δ, respectively) of the EPR spectrum of the low-spin heme a center are characteristic of bis-histidine ligation (the H-type cytochrome oxidase superfamily, carrying cytochrome aa3). These properties are similar to those of cytochrome aa3 purified from S. acidocaldarius strain DSM 639 (54, 55).

Two additional smaller subunits (24 and 14 kDa) associate with the 40-kDa subunit I to form cytochrome aa3 +aa3 subcomplex (35). In addition to all metal centers bound to cytochrome aa3, it also contains another low-spin and high-potential heme a583 center, but no CuA center. Surprisingly, the rhombic and tetragonal crystal field parameters obtained from three g values of this unusual low-spin heme center (g = 2.67, 2.38, and 1.64) are found to fall into the C-type group, rather than B- or H-type, in the coordination system of Blumberg and Piesch (47). In spite of the presence of heme Aa3 (38), this implies that cytochrome aa3 may not contain bis-histidine ligation, but possibly histidine-methionine type axial ligation typically observed in cytochromes c. While further physicochemical evidence is required for confirmation, two lines of indirect evidence are consistent with this possibility. First, the midpoint redox potential at pH 6.8 of cytochrome aa3 is +270 mV (29), which is similar to those of some bacterial cytochromes c and cytochromes c1 of cytochrome bc1 complexes (31, 56, 57). Second, the three-subunit cytochrome aa3 +aa3 subcomplex showed a marked ferrocytochrome c oxidase and TMPD oxidase activity but no caldariellaquinol oxidase activity (35), while the single-subunit cytochrome aa3 (corresponding to the subunit I) showed a weak caldariellaquinol oxidase activity but no ferrocytochrome c oxidase activity. Thus, the weak quinol oxidase activity detected in purified subunit I probably represents an artificial electron transfer, reflecting the in vivo electron transfer from the heme aa3 center to the redox centers of subunit I carrying the heme a2–CuA binuclear center of the terminal oxidase supercomplex. Since the subunit composition of purified cytochrome aa3 +aa3 subcomplex is comparable to those of true cytochrome c oxidases from a variety of bacteria (3), we propose that cytochrome aa3 of Sulfolobus sp. strain 7 is functionally equivalent to the c-type cytochrome attached to a subunit (usually subunit II) of the caa3 or caO3-type respiratory heme-copper oxidases (1–4). It will be tempting to investigate in future studies whether the archaela cytochrome aa3 might be a structural homologue of mitochondrial cytochrome c, as in the cases of the membrane-bound cytochromes c from Bradyrhizobium japonicum (58) and Paracoccus denitrificans (59).

Possible Intramolecular Electron Transfer Scheme of the Terminal Oxidase Supercomplex from Sulfolobus sp. Strain 7—In several bacteria such as P. denitrificans (18), thermophilic Bacillus PS3 (19), and B. japonicum (60), the presence of a terminal oxidase supercomplex composed of cytochrome bc complex and cytochrome c-cytochrome c oxidase complex has been reported. Of these, the Paracoccus quinol oxidase supercomplex most closely resembles the mitochondrial respiratory chain. The electron transfer through the cytochrome bc complex segment of the supercomplex occurs by a proton-motive F cycle pathway originally proposed by Mitchell (61) and subse-
A Paracoccus denitrificans bc1 Cytochrome Super-complex

B Sulfolobus sp. Strain 7 Cytochrome Super-complex

FIG. 6. Possible intramolecular electron transfer schemes of the terminal oxidase supercomplexes from P. denitrificans (A) and Sulfolobus sp. strain 7 (B). A, the proton-motive Q cycle pathway of electron transfer from ubiquinol to molecular oxygen through different redox components in the cytochrome bc1-Qo supercomplex from P. denitrificans (18-30, 31, 51, 61). In this scheme, ubiquinol is oxidized at center Qo, which is adjacent to the heme bL center (cytochrome b660 (53, 63)). A first electron from ubiquinol is transferred to a Rieske-type [2Fe-2S] cluster in proximity to the center of bc1 and is subsequently transferred to cytochrome bc552 and further to molecular oxygen via cytochrome aa3. The second electron transferred from ubiquinol to the heme bH center (bH) recycles through cytochrome bc1 complex via the heme bL center (cytochrome bc566) and subsequently to ubiquinone at center Qo. These centers are apparently missing in the archaeal cytochrome supercomplex, and the redox functions of cytochrome aa3 are most likely replaced by cytochrome aa3. B, a model showing the tentative electron transfer scheme of the terminal oxidase supercomplex (cytochrome bc660-aa3-Qo supercomplex) from Sulfolobus sp. strain 7. The archaeal supercomplex contains one protocrome and three heme centers, bH, Ca, and bL, and a Rieske-type FeS cluster, which, as a whole, functions as a terminal cataldiiiaquinol oxidase complex. HQNO and antimycin A binds proximal to cytochrome bc660 which is most likely functionally equivalent to the heme bH center of cytochrome bc1 complex (29), while myxothiazol and stigmatellin were not inhibitory. In this model, we postulate that a low-spin high-potential cytochrome aa3 accepts electron transferred from cytochrome bc660 via the Rieske-type FeS center in vivo, as in the case of cytochrome bc1 complex (31). In the absence of cytochrome bc660, Sulfolobus FeS center complex, cytochrome aa3 undergoes an electron transfer from water-soluble electron donors such as ferrocyanochrome c and TMPD in vitro, but not from cataldiiiaquinol.

The 40-kDa subunit I of the cytochrome supercomplex carries the oxygen reducing site which is inhibited by cyanide and includes one low-spin heme center and three heme centers. Centers corresponding to the heme bH and Ca centers are apparently missing in the archaeal supercomplex, and the redox functions of the bacterial c-type cytochromes (cytochromes c1 and c552) are most likely replaced by cytochrome aa3.

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