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Generation of proton-motive force by an archaeal terminal quinol oxidase from *Sulfolobus acidocaldarius*

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The terminal quinol oxidase of the cytochrome aa_3 type was isolated from the extreme thermoacidophilic archaeon *Sulfolobus acidocaldarius*. In micellar solution, the enzyme oxidized various quinols and exerted the highest activity with the physiological substrate caldariella quinol. The enzyme was functionally reconstituted into monolayer liposomes composed of archaeal tetraether lipids also derived from *S. acidocaldarius*. With the electron donor system ascorbate and *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine, the reconstituted enzyme was more active in the archaeal lipids as compared to lipids derived from *Escherichia coli* at temperatures above 50°C. Due to the low proton permeability of the tetraether lipids, it was possible to generate a steady-state transmembrane electrical potential ($\Delta\Psi$, interior negative), and transmembrane pH gradient (ΔpH, interior alkaline) at temperatures up to 70°C. The successful functional reconstitution of the cytochrome aa_3-type quinol oxidase from *Sulfolobus* identifies it as the key energy converter in the respiratory system of this hyperthermophilic archaeon.

The study of the bioenergetic system of aerobic, extremely thermoacidophilic archaea is of special interest for three reasons. First, archaea appeared very early in evolution as anaerobic species and were harshly exposed to the drastic change in the atmosphere from reducing to oxidizing conditions. Secondly, the evolutionary rates in the archaeal kingdom are very low suggesting the presence of still very simple structures of the energy-converting system. Thirdly, the drastic thermal and acidic conditions in their habitat imply very stable structures for their proteins and membrane architecture [1–3].

The terminal quinol oxidase of the thermoacidophilic archaeon *Sulfolobus acidocaldarius* has been studied extensively by various biophysical methods. This enzyme was the first to be shown to react as a quinol oxidase of the cytochrome aa_3 type [4]. Subsequently, bacterial cytochrome aa_3 was also found to act as a quinol oxidase [5]. Biophysical characterization by EPR spectroscopy indicated a high-spin and a low-spin heme center, which could be attributed to the heme a_3 and heme *a* moieties. In addition, the presence of a binuclear center, composed of heme a_3 and copper B could be demonstrated [6]. Furthermore, resonance-Raman spectroscopy suggested an unusual heme environment in the enzyme showing the lack of significant hydrogen bonds for the formyl group of heme *a* [71], in contrast to the situation of both cytochrome c oxidase [8] and bacterial quinol oxidases [9]. In addition, evidence for two forms of heme a_3 was presented [7]. Recent genetic evidence suggests in intact membranes the enzyme consists of at least three subunits, i.e., soxA–C [10]. The *soxA* and *soxB* genes code for proteins related to the cytochrome-*c* oxidase subunits II and I, respectively. *soxC* encodes a protein homologous to cytochrome *b*.

The quinol oxidase acts as a true terminal oxidase as it reduces the terminal electron acceptor oxygen using four electrons [4]. In addition, the, measurements of the H^+/O ratio in a whole-cell system has yielded a value greater than or equal to 4, suggesting the presence of at least one coupling site [11]. Among other quinol oxidases, proton-pumping activity could be demonstrated only for the cytochrome-*bo*-type enzyme of *Escherichia coli* [12]. To investigate the proposed proton-pumping activity of the *S. acidocaldarius* quinol oxidase, it is necessary to reconstitute the protein in a functional state into liposomes. The lipid composition of the cytoplasmic membrane of *S. acidocaldarius* is, however, distinctly different from that of bacteria. The archaeal membrane almost ecclusively contains tetraether lipids [13]. Unlike conventional phospholipid bilayers, tetraether lipids assemble as a monolayer. Closed and stable monolayer liposomes can be formed from a tetraether lipid fraction derived from a total lipid extract of *S. acidocaldarius*. These liposomes permit the functional reconstitution of different integral membrane enzymes [14–16], and exhibit an extraordinary temperature stability and low proton permeability. In this study, we demonstrate the functional reconstitution of the archaeal terminal oxidase of the cytochrome aa_3 type in bacterial and archaeal lipids. When reconstituted into archaeal lipids, the quinol oxidase is able to generate and main-

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Abbreviations. $Ph(NMe_2)_2$, *N,N,N′,N*-tetramethyl-*p*-phenylenediamine; Q-0, 2,3-methoxy-5-methyl-*p*-benzoquinol; Q-1, ubiquinol; diaminodurol, 2,3,4,5-tetramethyl-*p*-phenylenediamine; duroquinol, 2,3,4,5-tetramethyl-*p*-benzoquinol; Δp, proton-motive force; ΔpH, transmembrane pH gradient; $\Delta\Psi$, transmembrane electrical potential; Ph_4P^+, tetraphenylphosphonium ion.

Note. This study is dedicated to Klaus Lacher.

tain a proton-motive force at temperatures approximately equivalent to the physiological growth conditions of this organism.

MATERIALS AND METHODS

Cell culture and membrane preparation

S. acidocaldarius (DSM 639) was grown in a mineral salt medium [17] as described in [4]. Cells were suspended in 50 mM Mes pH 5.5, at 4°C, centrifuged for 15 min at 7800×*g*, and suspended in the same buffer. Membranes were isolated as described previously [4].

Materials

The fluorescent dyes 3′,3′-dipropylthiadicarbocyanine iodide and pyranine were from Molecular Probes Inc. Caldariella quinol was isolated as described in [18].

Purification of cytochrome aa_3

Cytochrome aa_3 from. *S. acidocaldarius* (DSM 639) was isolated essentially as described by Anemüller and Schäfer [4, 19], with the modification that 30 mM $Na_4P_2O_7$ was used for chaotropic preextraction of the membranes instead of 1 M KSCN. The purified enzyme was stored at −70°C at 35−40 nmol heme *a*/ml 0.8 M KH_2PO_4, 0.05% (mass/vol.) sarcosyl, pH 7.4. Protein was determined by the Lowry method in the presence of SDS [20] using bovine serum albumin as a standard. The heme *a* content was determined using an absorption coefficient of 10.4 mM^{-1} cm^{-1} for reduced-minus-oxidized heme at the wavelength couple 604−630 nm. This absorption coefficient is based upon pyridine hemochrome determinations [21] as a reference for the reduced-minus-oxidized difference spectra. The quality of the preparations was further examined by the A_{278}/A_{426} ratio which was exactly in agreement with the range given in [22] for aa_3 type oxidases.

Reconstitution of quinol oxidase into proteoliposomes

Tetraetherlipids from *S. acidocaldarius* were isolated and fractionated from freeze-dried cells as described by Elferink et al. [14, 15]. *E. coli* total lipid extract (PE, Sigma, type IX) was purified by acetone/ether extraction as described [23]. Both lipid preparations were dried by rotary evaporation and dispersed in 50 mM potassium phosphate, pH 6.5, at 14 mg/ml in the presence of 45% (by mass) *n*-octyl-β-d-glucopyranoside. The suspension was sonicated using a probe-type sonicator employing five pulses of 15 s with 45-s rest intervals. Sonication was performed at 0°C and the sample was flushed with nitrogen. Cytochrome aa_3 (9 nmol heme *a*) was added to 2 ml lipid suspension and mixed extensively. The suspension was dialysed four times at room temperature against a 500-fold volume of 50 mM potassium phosphate, pH 6.5. Proteoliposomes were stored in liquid nitrogen. Before use, the suspension was slowly thawed at room temperature and pulse sonicated (5-s intervals) on ice until turbidity vanished completely.

Analytical procedures

Cytochrome aa_3 activity was routinely measured spectrophotometrically at various temperatures by oxidation of

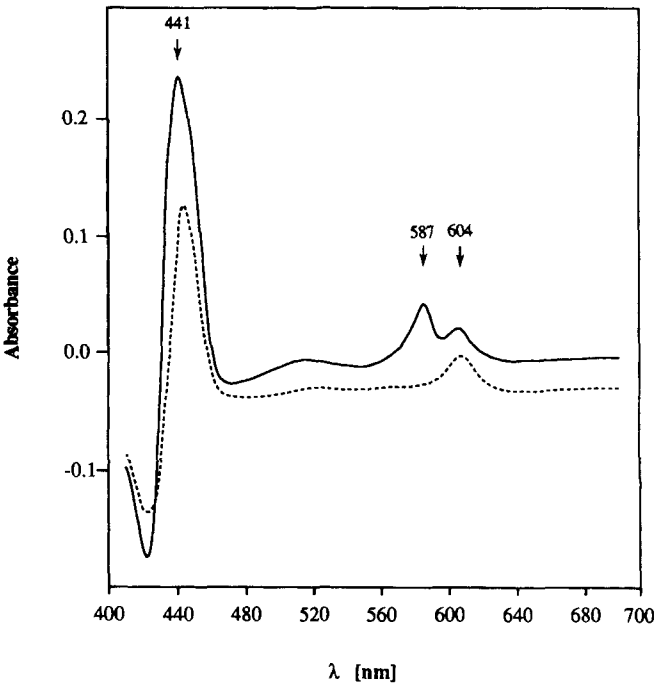

Fig. 1. Reduced-minus-oxidized difference spectra of terminal oxidase complex (–) and 'single entity' cytochrome aa_3 (- -) from *S. acidocaldarius*. The spectra are normalized at the wavelength couple 604−630 nm. The concentration of both enzymes was 1.44 μM, determined using a differential absorption coefficient of 20.8 mM^{-1} cm^{-1}.

N,N,N′,N′-tetramethyl-*p*-phenylenediamine [$Ph(NMe_2)_2$)] using an $\varepsilon_{546\,nm}$ value of 8 mM^{-1} cm^{-1} in a buffer containing 10 mM citric acid, pH 6.5. The final concentration of $Ph(NMe_2)_2$) was 100 μM. The substrate specifcity was measured by continuous measurement of the oxidation of reduced quinones using a Hewlett Packard HP 8450-A diode-array spectrophotometer. The activity was determined using differential absorption coefficients between absorption maxima and the isosbestic point as reference wavelength, respectively.

Oxygen consumption was determined polarographically at 25°C in a 2.84-ml reaction vessel using a Clark-type oxygen electrode designed by Eschweiler (Kiel, Germany). All assays were carried out in air-saturated 50 mM potassium phosphate, pH 6.5.

Measurement of the transmembrane electrical potential and pH gradient

The transmembrane electrical potential ($\Delta\Psi$, interior negative) was determined from the distribution of the lipophilic cation tetraphenylphosphonium (Ph_4P^+) using a Ph_4P^+-sensitive electrode. A correction for concentration-dependent Ph_4P^+ binding was applied [24, 25] assuming symmetric binding of Ph_4P^+ to both membrane surfaces. For the $\Delta\Psi$ measurements, proteoliposomes were diluted 20-fold in 50 mM potassium phosphate, 5 mM $MgSO_4$, pH 6.5, and energized by the addition of ascorbate and different quinols at 30°C. A Ph_4P^+ concentration of 4 μM was used. At temperatures above 30°C, the fluorescent probe cyanine 3′,3′-dipropylthiadicarbocyanine iodide (3 μM) was used as a qualitative assay for the generation of a $\Delta\Psi$, interior nega-

Table 1. Substrate specificity of detergent-solubilized cytochrome aa_3 from *S. acidocaldarius*. The activity was tested by oxidation of quinol. All experiments were performed in air-saturated 50 mM potassium phosphate, pH 6.5, at 25 °C. The substrate concentration was kept at 50 μM. The respective wavelengths or wavelength couples used for the measurements are indicated in brackets.

Quinol	ε	Turnover	Activity
	M^{-1} cm^{-1}	s^{-1}	%
Q-0	–	0	0
Q-1	12500 (275–237 nm)	7.6	26.3
Tetrachloro-*p*-benzoquinol	5835 (326–363 nm)	0.6	2.1
Tetrachloro-*p*-benzoquinol (0.03% Triton X-100)	5835 (326–363 nm)	1.8	6.2
Duroquinol	13069 (275–234 nm)	0.8	2.9
$Ph(NMe_2)_2$	8000 (546 nm)	6.8	23.4
Diaminodurol	19335 (283–241 nm)	0.2	0.7
Caldariella quinol	1778 (341–351 nm)	29	100

tive. Excitation and emission wavelengths were 643 nm and 666 mn, respectively [26].

The transmembrane pH gradient (ΔpH, inside alkaline) was monitored by following the fluorescence of liposome-entrapped pyranine [27]. Pyranine (100 μM) was incorporated into the proteoliposomes during the sonication step, and the external pyranine was removed by chromatography on a Sephadex G-25 column (5 cm×1 cm). Proteoliposomes were diluted 40-fold in 50 mM potassium phosphate, pH 6.5, and energized by the addition of the electron-donor system ascorbate (10 mM) and $Ph(NMe_2)_2$ (100 μM). Fluorescence measurements were performed at varying temperature using a fluorometer type SLM 4800S. The excitation and emission wavelengths were 450 and 508 nm, respectively. At each temperature tested, fluorescence levels were calibrated by titration with aliquots of HCl and KOH in the presence of nigericin (400 nM) and valinomycin (500 nM). ΔpH was calculated from the difference between the external and internal pH.

Electron microscopy

Freeze-fracture electron micrographs were prepared from proteoliposomes frozen in liquid nitrogen ('N_2 slush') in 50 mM potassium phosphate, pH 7.0. Replicas were obtained using an etching time of 0 s and 100 s, respectively. For etching, a temperature difference of 50 °C was applied. The replicas were examined in a Philips EM-300 electron microscope.

RESULTS

Catalytic properties of the enzyme in detergent solution

The quinol oxidase of the cytochrome aa_3 type isolated from *S. acidocaldarius* was spectroscopically pure (Fig. 1). This enzyme preparation differs from the entire terminal oxidase complex in that the latter contains two additional hemes *a* contributing at 587 nm to the α-band absorption spectrum and at 441 nm to the Soret-band intensity. Using SDS/PAGE, only one protein was visible [4], representing soxB (cyt-a_{604}), a polypeptide homologous to subunit I of the mitochondrial cytochrome-*c* oxidase. Western-blot analysis of the purified enzyme using antibodies against the soxA protein [10] revealed the presence of minor amounts of the subunit-II homologous polypeptide. In the context of this study, we assign this purified enzyme as a 'single entity' oxidase, in contrast to the entire terminal oxidase complex which besides soxB also contains the soxA and soxC (cyt-a_{587}) polypeptides [10]. An improved purification procedure for the entire terminal oxidase complex has been achieved (Gleißner, M. and Schäffer, G., unpublished results).

The substrate specifity of 'single entity' oxidase was tested with several quinols (Table 1). The highest activity was observed with the physiological substrate caldariella quinol, a benzothiophene quinol. Significant lower activities were observed with benzoquinol derivatives. Ubiquinol^{-1} (Q-1) was the second-best substrate, whereas the enzyme showed no activity with 2,3-methoxy-5-methyl-*p*-benzoquinol (Q-0). Interestingly, tetrachloro-*p*-benzoquinol, a halogenated benzoquinol, is oxidized at a three times higher rate in the presence of 0.03% (by vol) Triton X-100. The benzoquinol analog $Ph(NMe_2)_2$, a single electron donor, proved to be a good substrate for the enzyme. In contrast to caldariella quinol, $Ph(NMe_2)_2$ is water soluble and therefore a suitable electron donor for reconstitution studies.

To determine if the 'single entity' oxidase catalyzes the complete reduction of oxygen to water, quantitative oxygen consumption measurements were performed by adding known amounts of ascorbate in the presence of catalytic amounts of tetrachloro-*p*-benzoquinol to the enzyme in micellar solution (Fig. 2). Quantification of the oxygen consumption yielded a stoichiometry of $4e^-/O_2$ for each cycle. This ratio did not change upon addition of catalase indicating that H_2O was formed and not H_2O_2. Cyanide (2.5 mM) completely inhibited this reaction (data not shown). These data demonstrate that the cytochrome aa_3 from *S. acidocaldarius* is a true terminal oxidase reducing oxygen to water.

Catalytic properties of the reconstituted enzyme

The enzyme was reconstituted into liposomes composed of either *S. acidocaldarius* or *E. coli* lipids. Reconstitution was achieved by dialysis and subsequent freezing, thawing and sonication. This technique normally produces a random orientation of enzymes in the membrane [28]. Proteoliposomes composed of *E. coli* lipids were studied by freeze-fracture electron microscopy. Proteoliposomes had a diameter of 300–500 nm, and the incorporated enzyme was visible as minute particles on the replicas of the two fracture faces, i.e. convex (Fig. 3A) and concave half (Fig. 3B). In the ab-

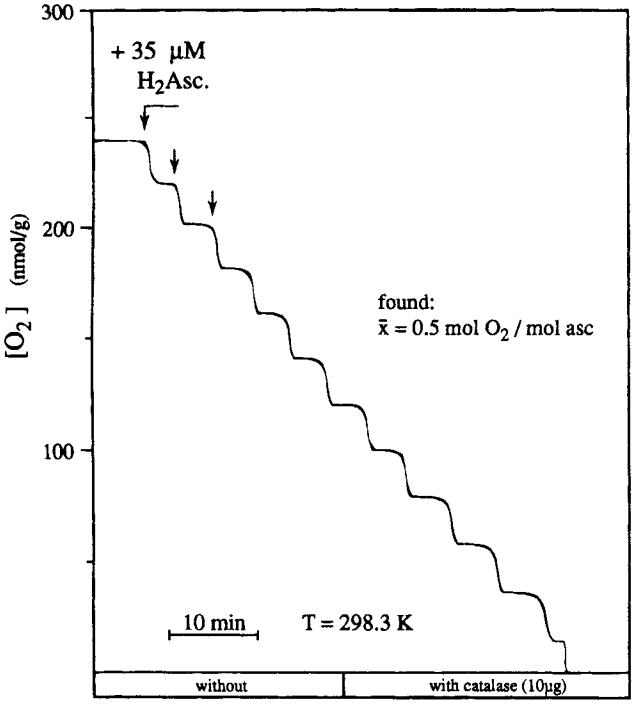

Fig. 2. Oxygen uptake by a solution of *S. acidocaldarius* cytochrome *aa*$_3$. Ascorbate aliquots (35 µM) were added successively to a detergent solution of cytochrome *aa*$_3$ (75 nM) in presence of 3.5 µM tetrachloro-*p*-benzoquinol. The air-saturated buffer contained 50 mM potassium phosphate, pH 6.5, 0.03% (by vol.) Triton X-100. The temperature was 25°C. The reaction scheme is as follows:

$$H_2Asc + 2\,TCBQ \rightarrow Asc + 2\,TCBQ^{\cdot} + 2\,H^+, \quad (I)$$

$$2\,TCBQ^{\cdot} + 1/2\,O_2 + 2\,H^+ \xrightarrow{aa_3} 2\,TCBQ + H_2O, \quad (II)$$

$$H_2Asc + 1/2\,O_2 \xrightarrow{aa_3} Asc + H_2O. \quad (I + II)$$

TCBQ, tetrachloro-*p*-benzoquinol

sence of the oxidase, only smooth surfaces were observed (data not shown).

Proteoliposomes prepared from *S. acidocaldarius* tetraetherlipids were not further investigated by freeze-fracture electron microscopy as the membrane-spanning character of these lipids prevents them from splitting along their half diameter shell [14].

The oxidase reconstituted into *S. acidocaldarius* lipids (0.32 nmol heme *a*/mg lipid) showed a maximal turnover with the electron donor $Ph(NMe_2)_2$ of approximately 530 s^{-1} at 65°C (Fig. 4). This activity was several-fold higher than the activity found for the enzyme solubilized in the detergent 0.05% (mass/vol.) *N*-lauroyl sarcosine, i.e. a V_{max} value of 80 s^{-1}. The activity of the enzyme reconstituted in *S. acidocaldarius* (Fig. 4) and *E. coli* lipid were measured as a function of temperature. The insert of Fig. 4 shows the corresponding Arrhenius plots. The activation energy for the enzyme reconstituted in *S. acidocaldarius* and *E. coli* lipids was 54.9 kJ/mol and 40.3 kJ/mol, respectively. Above 40°C, highest activities were obtained with the enzyme reconstituted into *S. acidocaldarius* lipids. These data indicate that, within a broad temperature range, the quinol oxidase is more active when reconstituted into *S. acidocaldarius* lipid as compared to *E. coli* lipid.

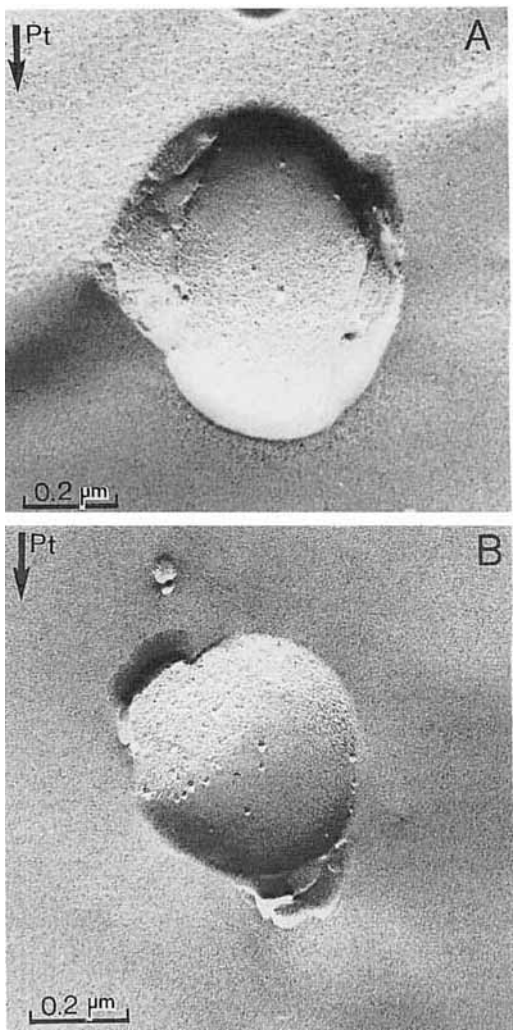

Fig. 3. Freeze-fracture replicas of *S. acidocaldarius* cytochrome *aa*$_3$-containing proteoliposomes composed of *E. coli* lipid. (A) Convex half. (B) Concave half. The direction of shadowing is indicated (⬇). Bar = 200 nm.

Generation of a proton-motive force

To determine the ability of the reconstituted oxidase to generate a proton-motive force, $\Delta\Psi$ (interior negative) measurements were performed utilizing various reduced quinones (Table 2). The $\Delta\Psi$ was calculated from the transmembrane distribution of Ph_4P^+ and corrected for the non-specific binding of Ph_4P^+ to the membranes (Table 2). Symmetry of Ph_4P^+ binding to both membrane surfaces was assumed. $\Delta\Psi$ generation was monitored with an ion-selective electrode that senses the external concentration of the lipophilic cation. After the addition of ascorbate and $Ph(NMe_2)_2$, uptake of Ph_4P^+ was observed for both membrane systems (Fig. 5). The ionophore nigericin, that collapses the pH gradient through the electroneutral exchange of protons against potassium ions, increased $\Delta\Psi$ in the proteoliposomes composed *of S. acidocaldarius* lipid (Fig. 5A and Table 2). This effect of nigericin was not observed when *E. coli* lipids were used. The K^+ ionophore valinomycin caused the dissipation of the $\Delta\Psi$ and the level of Ph_4P^+ uptake returned to the basal level (Fig. 5). The reconstitution procedure was optimized with respect to $\Delta\Psi$ generation. The optimum protein/lipid ratio for reconstitution was 0.32 nmol heme *a*/mg *S. acidocaldarius* lipid

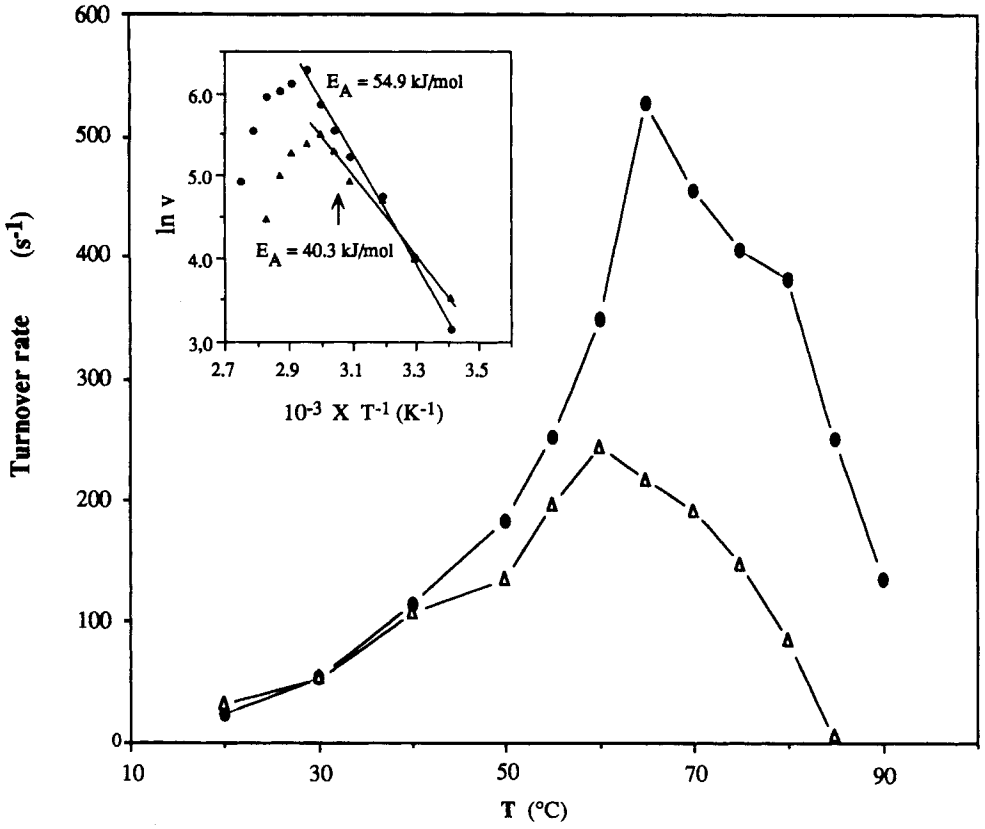

Fig. 4. Temperature dependence of cytochrome aa_3 activity in liposomes composed of *S. acidocaldarius* (●) or *E. coli* lipid (△). The maximal oxidase activity was determined by oxidation of $Ph(NMe_2)_2$ (100 μM) in the presence of nigericin (500 nM) and valinomycin (400 nM). Other details are given in the Materials and Methods. The inset shows an Arrhenius plot and apparent energies of activation for temperatures up to 65 °C.

Table 2. Generation of $\Delta\Psi$, interior negative, by the reconstituted cytochrome aa_3. Cytochrome aa_3 was reconstituted with *S. acidocaldarius* or *E. coli* lipid at 0.32 nmol heme *a*/mg lipid. Ph_4P^+ uptake was measured at 30 °C in a 1-ml vessel, containing 50 mM potassium phosphate, pH 6.5, 5 mM $MgSO_4$ and 4 μM Ph_4P^+. Each reaction contained 10 mM ascorbate as oxidant. Nigericin and valinomycin were used at 500 nM and 400 nM, respectively.

Electrondonor	Concentration	Ionophore	$\Delta\Psi$ with	
			S. acidocaldarius lipid	*E. coli* lipid
	μM		mV	
N-Methyldibenzopyrazine methyl sulfate	20	–	−27	−100
		nigericin	−49	−100
$Ph(NMe_2)_2$	200	–	−52	−95
		nigericin	−64	−95
2,3-Methoxy-5-methyl-6-decyl-*p*benzoquinol	250	–	−29	−67
		nigericin	−33	−67
2,3-Methoxy-5-methyl-6-decyl-*p*-benzoquinol +	215	–	−62	−104
N-methyldibenzopyrazine methyl sulfate	1.2	nigericin	−72	−104
Any donor		valinomycin	0	0

(data not shown). The experimentally determined correction factor [24, 25] differed for both types of lipids (148 for *S. acidocaldarius* and 55 for *E. coli* lipids). The highest $\Delta\Psi$ value, i.e. −104 mV, was observed with proteoliposomes composed of *E. coli* lipid using ascorbate (10 mM) and decyl-benzoquinol (215 μM) as electron donor pair. *N*-Methyldibenzopyrazine methyl sulfate (1.2 μM) was added to accelerate electron wmsfer from ascorbate to decyl-benzoquinol. At higher concentration, *N*-methyldibenzopyrazine methyl sulfate also functioned directly as an electron donor. With $Ph(NMe_2)_2$, a slightly lower value was found, i.e. −95 mV. In general, the calculated values for $\Delta\Psi$ in the proteoliposomes

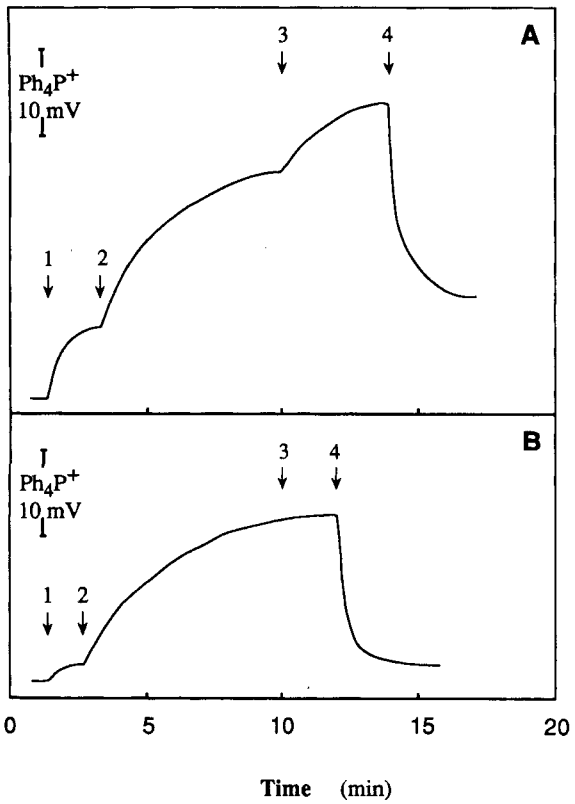

Fig. 5. Tetraphenylphosphonium uptake by cytochrome aa_3-containing proteoliposomes composed of *S. acidocaldarius* (A) and *E. coli* lipid (B). Uptake of Ph_4P^+ was monitored with an ion-selective electrode using a Ph_4P^+ concentration of 4 μM at 30°C. Proteoliposomes (0.32 heme *a*/mg lipid) were prepared as described in the Materials and Methods section and diluted 20-fold into 50 mM potassium phosphate, pH 6.54 (total volume 1 ml). The arrows indicate the following additions: 1, membranes; 2, ascorbate (10 mM) and $Ph(NMe_2)_2$ (200 μM); 3, nigericin (500 nM); 4, valinomycin (400 nM).

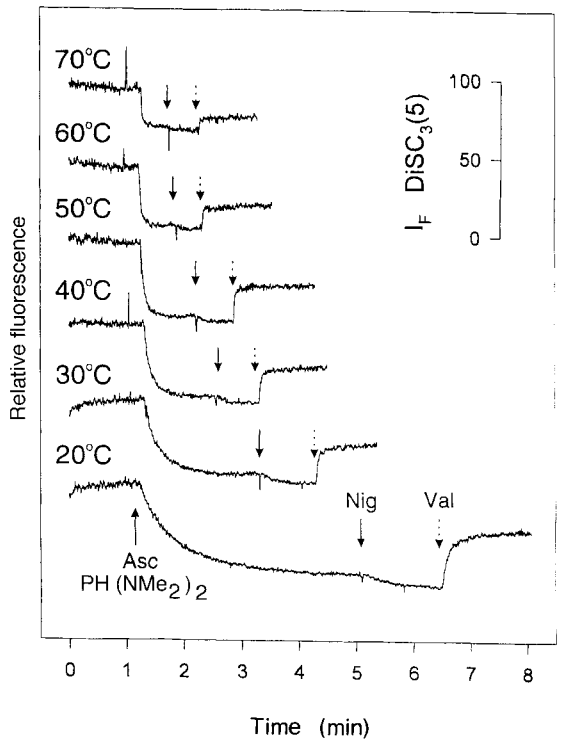

Fig. 6. Generation of a $\Delta\Psi$ in cytochrome aa_3 proteoliposomes composed of *S. acidocaldarius* lipid. $\Delta\Psi$ was monitored with the fluorescent probe 3′,3′-dipropylthiadicarbocyanine iodide (3 μM, $DiSC_3(5)$). Membranes were energized by the addition of ascorbate (5 nM) and $Ph(NMe_2)_2$ (100 μM). The final concentration of nigericin and valinomycin was 1 μM and 0.2 μM, respectively. Other conditions are given in Fig. 5.

composed of *S. acidocaldarius* lipid were somewhat lower. Since the Ph_4P^+ electrode is not stable at temperatures above 30°C, further $\Delta\Psi$ measurements were performed with the fluorescent cyanine dye 3′,3′-diprophylthiadicarbocyanine iodide. With this qualitative technique it was possible to record a $\Delta\Psi$ in the *S. acidocaldarius* proteoliposomes up to 70°C with $Ph(NMe_2)_2$ as electron donor (Fig. 6). Though a recent study describes quantitative evaluation of this fluorescence based potential indication [29], only qualitative measurements with the dye indicator were possible for the following reasons. First, quantitative evaluation would require an individual calibration curve for each temperature and each vesicle preparation which is limited by the amount of available material. Secondly, and more importantly, the use of $Ph(NMe_2)_2$ interferes strongly with quantification of the fluorescence quenching, introducing an error that cannot be calculated.

For measurements of the ΔpH, the fluorescent pH indicator pyranine was trapped in the liposomal lumen. The oxidase reconstituted into archaeal tetraether lipids generated a ΔpH when activated by ascorbate and $Ph(NMe_2)_2$ (Fig. 7). Upon the addition of $Ph(NMe_2)_2$, a rapid initial decrease in the fluorescence signal was recorded. This effect was caused by the accumulation of the oxidized form of $Ph(NMe_2)_2$ (data not shown). At low temperatures, the internal pH (fluorescence level) slowly increased upon energization. A considerable increase of the internal pH was observed after the addition of valinomycin (200 nM) reaching a steady-state ΔpH value of 1.2 (Δp value of −79 mV) at 50°C. ΔpH was completely dissipated by nigericin. The rate by which ΔpH generation increased with temperature is shown in Fig. 7B. A steady-state ΔpH value of 0.8 was generated at 60°C. When the oxidase was reconstituted into *E. coli* lipids, it was not possible to detect the generation of any significant ΔpH level (data not shown). These data demonstrate that the quinol oxidase reconstituted into *S. acidocaidarius* lipids is able to maintain a proton-motive force up to 70°C.

DISCUSSION

The quinol-oxidase of the cytochrome aa_3 type functions as the terminal oxidase in the electron-transfer chain of the extreme thermoacidophilic archaeon *S. acidocaldarius*. The purified enzyme showed a high specificity for various quinols. Optimal activity was found with caldariella quinol, which most likely acts as the physiological substrate. The ubiquinol analogue Q-1 showed only 26% of the activity, while Q-0 was not a substrate. Apparently, the presence of an isoprenoid side chain is an important attribute for specific binding of quinols. To reduce the caldariella quinol, a strong reducing agent is needed such as borohydride. Therefore, we also tested the activity of the quinol oxidase with various water-soluble artificial electron donors that could be of a more practical use in a reconstituted system. Tetrachloro-*p*-

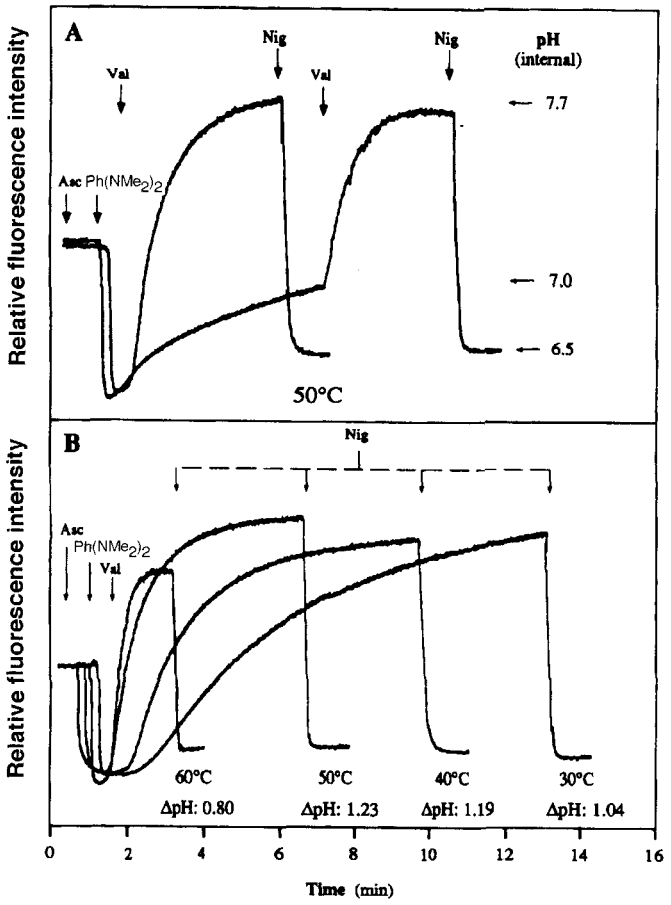

Fig. 7. Generation of a ΔpH in cytochrome aa_3 proteoliposomes composed of *S. acidocaldarius* lipid. Proteoliposomes (0.32 heme *a*/m lipid) were diluted 20-fold into 50 mM potassium phosphate, pH 6.5; and energized by the addition of ascorbate and $Ph(NMe_2)_2$. ΔpH was measured with the fluorescent dye pyranine. At the arrows, the following compounds were added in sequential order: ascorbate (5 mM), $Ph(NMe_2)_2$ (100 µM), valinomycin (200 nM) and nigericin (1 µM).

benzoquinol, 2,3,4,5-tetramethyl-*p*-phenylenediamine (diaminodurol) and 2,3,4,5-tetramethyl-*p*-benzoquinol (durominol) showed a relatively low activity. Under special conditions, tetrachloro-*p*-benzoquinol can also be oxidized by cytochrome-*c* oxidase [30] suggesting that both the quinol and cytochrome-*c* oxidases of the aa_3 type harbour sites accessible to this substrate analog. Since the *S. acidocaldarius* oxidase does not contain Cu_A^{2+}, a direct reduction of the heme centre by tetrachloro-*p*-benzoquinol may be assumed. The single electron donor $Ph(NMe_2)_2$ proved to be a suitable substrate for the quinol oxidase and was used further in the reconstitution experiments.

The enzyme preparation used in this study contains spectroscopically pure cytochrome aa_3 (soxB), with variable amounts of soxA, while it completely lacks the soxC protein. The enzyme, however, represents a minimal functional form of the quinol oxidase as it reduces O_2 to H_2O. The addition of exactly defined amounts of reducing equivalents, i.e reduced tetrachloro-*p*-benzoquinol, allowed the repetitive stoichiometric consumption of O_2. If H_2O_2 instead of water was formed as the terminal reaction product, the amount of O_2 consumption/titration step should have been half of the amount observed. When reconstituted into proteoliposomes, the oxidase showed a significantly higher activity than in the detergent-solubilized state. The reconstituted system more closely resembles the natural environment; this was further substantiated by the observation that the enzyme shows a higher turn-over number when reconstituted in *S. acidocaldarius* lipids as compared to *E. coli* lipids (Fig. 4). The activity peaks at a temperature approximately 15–20°C lower than the optimum growth temperature of the organism. The explanation for this observation could be that the *S. acidocaldarius* lipid fraction used does not represent the complete set of lipids needed to stabilize/activate the enzyme. Alternatively, the use of $Ph(NMe_2)_2$ as an electron donor instead of the caldariella quinol might produce this effect.

The present study demonstrates the functional reconstitution of an archaeal terminal oxidase into liposomes, and the ability of this system to generate and maintain a proton-motive force at high temperatures. The overall proton-motive force generated by the oxidase when reconstituted into *S. acidocaldarius* lipids was only approximately half of that observed with intact cells [11, 31]. This may be caused by several factors. First, a significant fraction of the oxidase may have been reconstituted in the wrong orientation, and these inside-out molecules may be activated by $Ph(NMe_2)_2$ which is slightly membrane permeable. Secondly, $Ph(NMe_2)_2$ may not be the optimal substrate and the turn-over that is measured may thus be suboptimal. Thirdly, *in vivo*, at a low external pH, ΔpH is the most prominent component of the proton-motive force. The experiments with the reconstituted system have been performed at pH 6.5. Fourthly, $\Delta\Psi$ values may be overcorrected for non-specific probe binding. For instance, a subfraction of the liposomes may not contain oxidase and thus does not contribute to $\Delta\Psi$ generation whereas it will bind Ph_4P^+.

We were unable to measure a ΔpH when the oxidase was reconstituted into liposomes composed of *E. coli* lipid. This was also evident from the lack of a stimulatory effect of nigericin on $\Delta\Psi$ generation. The proton permeability of liposomes composed of *S. acidocaldarius* lipid is two-orders of magnitude lower as compared to *E. coli* lipids over a broad temperature range (Elferink, M. G. L., de Wit, J. G., Driessen, A. J. M., and Konings, W. N., unpublished results). The proton permeability of the *E. coli* liposomes may be too high to detect proton-pumping activity of the quinol oxidase at ambient temperatures. In contrast, the system reconstituted in the *S. acidocaldarius* lipids appears to be sealed more tightly and allows the determination of proton-pumping activity of the quinol oxidase. Though the determination of exact H^+/e ratios is still hampered by the inherent properties of the system, especially the lack of an absolutely assymmetrically acting reductant, the above results clearly show that this archaeal terminal oxidase is capable of energizing the membrane by a large proton-motive force. Similar energy-transducing quinol oxidases are known from the mesophilic bacteria *E. coli* [12, 32] and *Bacillus subtilis* [33]. In the thermoacidophile *Sulfolobus*, however, this may even be the only coupling site for conservation of respiratory energy since neither a bc_1 complex is present [34], nor a proton-pumping complex I equivalent could be detected in its plasma membrane. Due to its extremely acidic physiological environment, *Sulfolobus* needs an effective proton-translocating device, and respiration-driven proton extrusion with a H^+/O ratio greater than 3 has been shown previously with whole cells [11]. Whether or not the stoichiometry of proton translocation may be enhanced in the terminal oxidase complex by integration of the unusual cytochrome *a*-586 (puta-

tive *soxC* gene product), as has been speculated elsewhere [10], remains to be demonstrated.

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