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Studies of the Electron Transport Chain of the Euryarcheon *Halobacterium salinarum*: Indications for a Type II NADH Dehydrogenase and a Complex III Analog

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The components involved in the respiratory system of the euryarcheon *Halobacterium salinarum* were investigated by spectroscopic and polarographic techniques. Previous results about the cytochrome composition could be verified. However, under low oxygen tension, the expression of a *d*-type cytochrome was detected. Membranes exerted an NADH- and succinate-cytochrome-*c* oxidoreductase as well as an NADH and succinate oxidase activity. These activities could be blocked by the following inhibitors: 7-jodocarboxylic acid, giving evidence for the presence of a type II NADH dehydrogenase, antimycin A, and myxothiazol, indicating the presence of a complex III analog, and the typical succinate dehydrogenase (SDH) and terminal oxidase inhibitors. Complex I inhibitors like rotenone and annonine were inactive, clearly excluding the presence of a coupled NADH dehydrogenase. In addition, no [Fe-S] resonances in the region of the NADH dehydrogenase (NDH) clusters could be observed after NADH addition. One of the terminal oxidases could be shown to act as a cytochrome-*c* oxidase with a K_m value of 37 μM and an activation energy of 23.7 kJ/mol. The relative molecular mass of the endogenous *c*-type cytochrome could be determined as 14.1 kD. The complex III analog could be enriched after detergent extraction with Triton X-100 and hydroxylapatite (HTP) chromatography. The partially purified complex contained a Rieske iron–sulfur cluster, *b*- and *c*-type cytochromes, and was catalytically active in the decylubiquinone–cytochrome-*c* oxidoreductase assay.

KEY WORDS: Archea; inhibitors; NADH dehydrogenase; complex III; cytochromes; Rieske iron–sulfur cluster; terminal oxidase.

INTRODUCTION

The aerobic, extremely halophilic euryarcheon *Halobacterium salinarum* grows on salted fish products, causes pinkeye, and exhibits unusual physiological and biochemical properties, such as its capability to thrive in extremely salty biotops with salt concentrations up to 4 M NaCl (Cheah, 1970a). Knowledge about its electron transport chain is only rudimentary, whereas its unique photophosphorylation and phototaxis system has been widely elucidated (Oesterhelt and Tittor, 1989).

In the euryarcheotic branch of the archeal urkingdom, an NADH oxidase activity has already been reported for *H. salinarum* (Hochstein and Dalton, 1968), whereas an NADH dehydrogenase activity has been described in *H. cutirubrum* (Lanyi and Stevenson, 1970). The former enzyme activity was due to a soluble, cytosolic enzyme and was studied with respect to its cation and anion specificity. Its cytosolic location implies that this enzyme most probably was a member of type II NDH. However, this was not investigated, because at that time no inhibitors of type II NDH were known. Furthermore, nothing was reported with

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respect to the presence of a membrane-bound, proton-pumping NDH.

Euryarcheotic succinate–quinone–reductase (SQRs) have been studied in the species *Thermoplasma acidophilum*, *Natrono bacterium pharaonis*, *H. halobium*, and *H. salinarum*. In *H. halobium*, the SDH activity was predominantly found in the cytosolic fraction (Hallberg-Gradin *et al.*, 1985). Because of its low K_m value for succinate and its typical inhibitor sensitivity for malonate and oxaloacetate, it was characterized as an SQR and not as a fumarate reductase. In *H. salinarum*, an SDH activity was described 30 years ago (Aitken and Brown, 1969). In contrast to the situation in *H. halobium*, the specific enzyme activity as determined by the reduction of phenazine methosulfate was mainly found in the membrane fraction.

Cheah (1970a, b) reported that *H. salinarum* membranes contained mostly *b*-type cytochromes. In addition, two CO-reactive hemoproteins, identified as cytochrome *o* and a_1, as well as one cytochrome *c* could be detected. Four different membrane-bound *b*-type cytochromes could be distinguished in *H. halobium* by potentiometric titrations (Hallberg–Gradin and Colmsjö, 1989). In addition, a terminal oxidase of the cytochrome aa_3-type was purified in an inactive form and its amino acid sequence analyzed (Fujiwara *et al.*, 1989; Denda *et al.*, 1991). As yet, its functional classification as a quinol or cytochrome-*c* oxidase has not been performed, because the gene of subunit II was not found.

Evidence for the presence of a cytochrome bc_1 complex in the archeal urkingdom was presented first by Cheah (1970c), who observed a partially antimycin A-sensitive respiration of *H. halobium* membranes. Hallberg and Baltscheffsky (1981) later succeeded in purifying an ascorbate reducible cytochrome *b* complex, which they suggested was a part of an ancestral cytochrome bc_1 complex. However, the partially purified complex exhibited no catalytic activity and no evidence for the presence of a Rieske iron–sulfur cluster was provided. Recently, the purification of a cytochrome *bc* complex was reported from the haloalkalophilic euryarcheon *N. pharaonis* (Scharf *et al.*, 1997). This complex consisted of one *b*- and one *c*-type cytochrome. As in the case of the *H. halobium* enzyme, no indications for the presence of a Rieske iron–sulfur cluster have been found thus far. Furthermore, no catalytic activity was reported for the *N. pharaonis* complex. In the crenarcheotic branch of the archel urkingdom, however, the presence of a typical Rieske iron–sulfur center could be demonstrated unequivocally in the thermoacidophilic species *Sulfolobus acidocaldarius* (Anemüller *et al.*, 1993, 1994).

In the present work we report on different proteins involved in the respiratory chain of *H. salinarum*. For our experiments, we used the mutant strain *H. salinarum* JW5, which lacks carotenoids and rhodopsins. Therefore, investigations by optical spectroscopy of the cytochromes could be performed without interference. Our data give evidence for the presence of an type II NADH dehydrogenase, whereas the presence of a coupled type I NADH dehydrogenase can be excluded. Furthermore, we give experimental support for the first euryarcheotic Rieske iron–sulfur cluster, associated with *b*- and *c*-type cytochromes. Finally, we supply some indications for the terminal oxidase to function as a cytochrome-*c* oxidase.

MATERIALS AND METHODS

Halobacterium salinarum (DSM 3754), strain JW 5, was a kind gift from Dr. Peter Palm (Max Planck Institut für Biochemie, Martinsried). The strain, devoid of carotenoids and rhodopsins, was grown aerobically either in 5-L Erlenmeyer flasks in a gyratory shaker at 120 rpm or in a 50-L fermenter with a flow rate of air of 37 L/h at 37°C for 72 h. The growth medium contained (in grams) 250 NaCl, 20 $MgSO_4$, 3 Na-citrate, 2 KCl, and 10 peptone per liter and was adjusted to pH 7.2. Cells were harvested in the stationary phase by continuous-flow centrifugation with a Westphalia separator at room temperature. The cells were suspended in basal salt medium (growth medium without peptone) and centrifuged for 10 min at 7500 × *g* at room temperature.

Membranes were prepared by ultrasonification with a Branson sonifier, equipped with a large tip. The cells were resuspended in maleate buffer (3.4 M NaCl, 50 mM Tris–maleate, pH 7.2) to a final concentration of 20 g wet cells/100 ml and sonified for 20 min in the pulsed mode (50% duty cycle) at 4°C. The resulting homogenate was centrifuged for 15 min at 18,500 × *g* at room temperature to remove cells and large debris. The supernatant was spun at 100,000 × *g* for 1 h at 4°C. The pellet was washed once with maleate buffer (see above) and spun again as before. The final pellet was resuspended to a final protein concentration of 45–60 mg/ml and stored at −20°C until further use.

Protein concentration was determined by the biuret method in the presence of Triton X-100, as described by Watters (1978)

Respiration of membranes was measured polarographically with a Clark-type oyxgen electrode at 37°C. NADH oxidase activity was measured in a buffer containing 3.5 M NaCl, 50 mM maleate, pH 7.2, in a volume of 2.84 ml. Membranes (3 mg) were added to the vessel, which was preincubated for 4 min, and then NADH was added to a final concentration of 1 mM. The succinate oxidase assay was performed accordingly using 6 mg of membrane protein instead of 3 mg, with a final succinate concentration of 5 mM. The respective inhibitors were added prior to the substrates.

For the spectrophotometric assays, cytochrome *c* from bovine heart was used. NADH–cytochrome-*c* oxidoreductase activity was tested in a buffer containing 3.4 M NaCl, 50 mM Tris-maleate, pH 7.2, 1 mM KCN, and 50 μM cytochrome *c* in a volume of 1 ml. After addition of 0.3 mg membrane protein, the reaction was started by NADH with a final concentration of 0.2 mM. Different concentrations of inhibitors were added prior to the addition of substrate. Succinate–cytochrome-*c* oxidoreductase activity was followed in a similar manner, using 0.6 mg membrane protein and a final succinate concentration of 1 mM. Cytochrome-*c* oxidase activity was determined as described (Smith and Conrad, 1956). Ubiquinol–cytochrome-*c* oxidoreductase activity was measured in the same Tris-maleate buffer as described above, using 3,5-dimethoxy-5-methyl-1,4-benzoquinol (Q-1) or *n*-decylubiquinol (Q-10) as substrates. The final concentrations were about 25 μM for both substrates. All assays were performed at 37°C in an Eppendorf photometer 1101 M, using a 546 nm filter.

For the solubilization of the membranes, *H. salinarum* membranes were first diluted to half the protein concentration with 50 mM MOPS, 3.4 M NaCl, pH 7.2, yielding a final protein concentration from 22.5 to 30 mg/ml. Subsequently, an equal amount of extraction buffer, consisting of 1% Triton X-100, 50 mM MOPS, pH 7.2, was added to the diluted membrane fraction and the mixture incubated for 1 h at room temperature. Then, the extraction mixture was centrifuged at 100,000 × *g* for 1 h at 4°C. The resulting pellet was discarded and the supernatant used for the further purification of the solubilized cytochromes.

For the partial purification of the complex III analog, a hydroxyapatite (HTP) chromatography at room temperature was used. The detergent extract was loaded onto a HTP column (2.5 × 10 cm), equilibrated with a buffer containing 10 mM NaP_i, 0.1% Triton X–100, pH 7.2, with a flow rate of 30 ml/h. Subsequently, the column was washed with about 40 ml equilibration buffer. A gradient from 10 to 100 mM NaP_i in 0.1% Triton, pH 7.2, was then applied to the column (V = 400 ml). The complex III analog was then eluted with 0.2 M NaP_i in 0.1% Triton, pH 7.2. All reddish fractions were pooled and concentrated by ultrafiltration with an Amicon PM-30 membrane.

Reduced–minus–oxidized difference spectra as well as pyridine hemochrome spectra were recorded with a HP 8450 diode array spectrometer. Reduced samples were obtained by adding a few grains of solid sodium dithionite to the samples. The as-prepared samples were considered as oxidized, as addition of ferricyanide did not decrease the apparent absorption bands of the cytochromes. Specific heme *b* content was determined by pyridine hemochromogen as described (Williams, 1964).

Electron paramagnetic resonance (EPR) spectra were recorded with an X-band Bruker ER 200 D-SRC spectrometer, equipped with an ESR 910 continuous-flow helium cryostat from Oxford Instruments.

Polyacrylamide gel electrophoresis in the presence of SDS was carried out by the Laemmli procedure (Laemmli, 1970) on 15% gel with an acrylamide to bisacrylamide ratio of 100:1; proteins were visualized by Coomassie brilliant blue staining. For the detection of heme-containing proteins, gels were also stained for peroxidase activity (Thomas *et al.*, 1976).

Antimycin A, myxothiazol, and stigmatellin were obtained from Sigma Chemical Co., USA. 2-Methyl-3-dodecylquinolone, 7-jodoacridone-4-carboxylic acid, 2-dodecylquinolone, and dodecyl-*N*-hydroxyquinolone (aurachin C) were a kind gift by Prof. Walter Oettmeier (Ruhr Universität, Bochum, Germany). Annonine was a kind gift by Prof. Hans Weiss (Universität Düsseldorf, Germany). MOA-stilbene was a kind of gift of Prof. U. Brandt (Universität Frankfurt, Germany).

RESULTS

NADH–Dehydrogenases (NDH)

Membranes of *H. salinarum* exhibited an NADH oxidase activity, which was followed polarographically, as well as an NADH–cytochrome-*c* oxidoreductase activity, which was followed spectrophotometrically. As shown in Table I, typical respiratory rates for *H. salinarum* membranes were about 10.3 (nmol O_2/min mg) at 37°C. To elucidate which class of NDH was responsible for this activity, the effect of

Table I. Effect of Inhibitors on Respiration of *H. salinarum* Membranes

Inhibitor	NADH oxidase	Inhibition (%)	Succinate oxidase	Inhibition (%)
None	10.3[a]	—	5.6	—
Rotenone (25 μM)	10.3	—	5.6	—
Annonine (12 μM)	10.3	—	5.6	—
7-Jodoacridone-4-carboxylic acid (180 μM)	1.3	87	2.2	39.3
Antimycin A (500 μM)	5.1	50	4.2	25
Myxothiazol (0.26 μM)	5.1	50	5.3	5
Aurachin C (80 μM)	0.5	95	0.85	85
2-Dodecyl-quinolone (80 μM)	2.7	74	0.55	90
MOA-stilbene (200 μM)	7.1	33	3.9	30

[a] Activity in nmol O_2/min/mg.

various inhibitors on the NADH oxidase activity was studied. The classical inhibitor of class I NDH, rotenone, had no effect on the NADH respiration. Even annonine, the strongest inhibitor of class I NDH known so far with a pK_i value of 9, did not influence the activity. On the other hand, 7-jodoacridone-4-carboxylic acid, a typical inhibitor of class II NDH, turned out to be a very potent inhibitor of NADH respiration. Its inhibitory effect on the respiration was titrated. At 10 μM concentration, only 40% of the respiration could be blocked. The maximal inhibition was obtained with 180 μM of the inhibitor, leaving only about 10% residual respiratory activity. Furthermore, the NADH respiration was influenced by quinone analogs. The strongest inhibitor at all turned out to be aurachin C, which nearly totally suppressed the respiration. Still 75% inhibition was observed after addition of 2-dodecylquinolone. Only about 50% inhibition could be achieved by 2-methyl-3-dodecylquinolone.

In the NADH–cytochrome-*c* oxidoreductase assay system, a maximal rate of 9.8 (nmol cyt *c* red./min/mg) was observed at 37°C. The activation energy for this reaction could be determined as 32.3 kJ/mol, thus falling into the typical range for enzymatic reactions. The inhibitor sensitivity for this reaction was the same as for the NADH oxidase system, as shown in Table II.

Finally, the effect of NADH addition to *H. salinarum* membranes was investigated by EPR spectroscopy. After addition of the substrate, no resonances from iron-sulfur centers could be detected in the EPR spectrum, thus clearly excluding the presence of the characteristic NDH clusters of the class I NDH.

Succinate–Quinone–Reductase (SQR)

The activity of SQR was also assayed by means of polarography and optical spectroscopy. The typical respiratory rate of *H. salinarum* membranes with succinate as substrate could be determined as 5.6 (nmol O_2/min mg) at 37°C, which was only half of the value determined for the NADH oxidase activity (as shown in Table I). As in the case of the NADH oxidase activity, the effect of various inhibitors was screened. As expected, rotenone and annonine had no inhibitory effect on succinate oxidation. However, 7-jodoacridone-4-carboxylic acid, the typical inhibitor of external NDH, caused a significant, but weak, reduction in the respiratory rate also for succinate oxidation. Furthermore, the influence of the quinone analogs, studied above, could also be observed on succinate respiration. Whereas the inhibitory effect of aurachin C was expressed less on succinate respiration as compared to NADH respiration, the former respiration was remarkably more blocked by 2-dodecylquinolone than the latter. The influence of 2-methyl-3-dodecylquinolone on succinate respiration was comparable to its effect on NADH respiration.

In the succinate–cytochrome-*c* oxidoreductase system, the results of the polarographic experiments could be confirmed (as shown in Table II). In addition, the effects of malonate as a classical inhibitor and of tetrachlorobenzoquinone, an inhibitor of archeal SQR in *S. acidocaldarius* (Moll and Schäfer, 1991), were tested. Whereas malonate was not very effective and inhibited totally at 20 mM, the latter caused total inhibition at 0.3 mM. Interestingly, with 2-dodecylquinolone, a total block of the upper activity could be obtained, whereas, in the polarographic test system, 10% residual activity persisted after addition of the inhibitor.

Complex III Analog

For the characterization of the complex in the membrane-bound state, indirect studies were performed using the NADH and succinate oxidizing assays described above employing both polarography and spectroscopy. As shown in Tables I and II, the typical cytochrome bc_1 inhibitors antimycin A, myxo-

Table II. Effect of Inhibitors on NADH- or Succinate-Cytochrome-*c* Reductase Activity of *H. salinarum* Membranes

Inhibitor	NADH–cytochrome-*c* reductase	Inhibition (%)	Succinate–cytochrome-*c* reductase	Inhibition (%)
None	2.57[a]	—	0.31	—
Rotenone (25 μM)	2.57	—	0.31	—
Annonine (12 μM)	2.57	—	0.31	—
7-Jodoacridone-4-carboxylic acid (80 μM)	0.64	75	0.27	14
Antimycin A (300 μM)	1.28	50	0.12	60
Myxothiazol (0.37 μM)	1.28	50	0.14	55
Aurachin C (80 μM)	0.12	95	0.05	85
2-Dodecylquinolone (80 μM)	0.42	83	0.01	99

[a] Activity in nmol cytochrome *c* reduced/min/mg.

thiazol, and MOA-stilbene remarkably slowed down both catalytic activities. As an example for a Q_i site inhibitor, the effect of antimycin A was titrated in both test systems with NADH and succinate. In the polarographic assay, a $K_{i(50\%)}$ value of 500 μM was determined for NADH, whereas the respective value for succinate was 680 μM. In parallel, the inhibitory effect in the spectroscopic assay was evaluated. The respective $K_{i(50\%)}$ values were measured as 300 μM for the NADH–cytochrome-*c* oxidoreductase system and 230 μM for the succinate–cytochrome-*c* reductase system. As an example for a Q_0 site inhibitor, the effect of myxothiazol was also titrated in both test systems with NADH and succinate. In the polarographic system, a $K_{i(50\%)}$ value of 0.26 μM was determined for NADH, whereas the respective value for succinate was about 0.8 μM. The respective $K_{i(50\%)}$ values in the spectroscopic assay system were 0.37 μM for NADH and 0.35 μM for succinate.

For further characterization the complex was partially enriched by hydroxylapatite chromatography as described earlier. With respect to the membranes, an up to sevenfold increase of the specific heme *b* content could be obtained. The partially purified complex III analog was catalytically active as a decylubiquinone–cytochrome-*c* reductase with a specific activity of 54 (nmol/min/mg) at 37°C. With respect to the NADH–cytochrome-*c* oxidoreductase activity, taken as the corresponding activity in the membranes, an up to sixfold increase in catalytic activity was obtained. A reduced–minus–oxidized difference spectrum of a typical preparation is shown in Fig. 1 (upper spectrum). Clearly, a broad maximum at 560 nm, due to a *b*-type cytochrome, can be detected. In addition, a shoulder at 552 nm is displayed, which can be attributed to a *c*-type cytochrome. The presence of the *c*-type cytochrome is better resolved in the first derivative spectrum displaying a maximum at 552 nm (Fig. 1, lower spectrum). The latter cytochrome, however, was present in varying amounts, depending on the preparation. In addition, the apparent molecular mass of the *c*-type cytochrome could be determined by heme staining after SDS gel electrophoresis, yielding a value of about

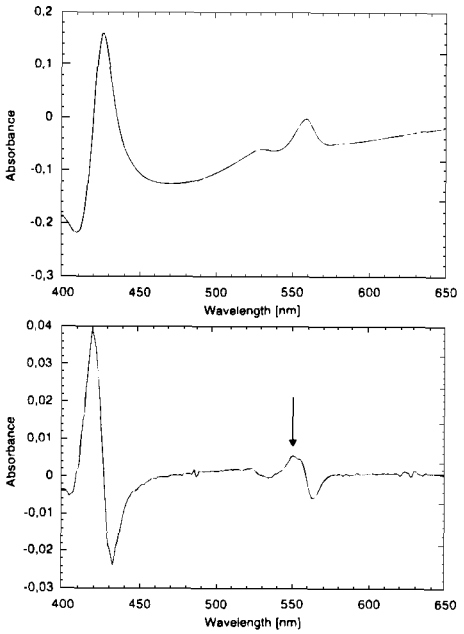

Fig. 1. Dithionite reduced–minus–oxidized difference spectrum of the partially enriched complex III analog (upper spectrum) and first derivative spectrum (lower spectrum). The arrow indicates the absorption of the *c*-type cytochrome. Protein concentration was 10.7 mg/ml in a buffer containing 0.2 M NaP_i, 0.1% Triton, pH 7.2. Reduction was obtained by adding a few grains of solid sodium dithionite to the sample. The as-prepared sample was considered as oxidized, since addition of ferricyanide did not decrease the apparent absorption bands of the cytochromes.

14 kD. The EPR spectrum of the ascorbate reduced partially purified fraction is displayed in Fig. 2. It unequivocally shows a prominent g value at 1.89, which is the characteristic g_y value of a Rieske iron–sulfur center. The g_z value would be expected to be around 2.025, but is obviously superimposed by another resonance, thereby impeding direct determination.

Cytochrome *c* Oxidase

Halobacterium salinarum membranes possessed a cytochrome-*c* oxidase activity with bovine heart cytochrome *c* as substrate, which could be totally inhibited by cyanide. The pH optimum for this activity was in the range 6–7.5. At salt concentrations from 0.5 to 6 M NaCl, the activity was nearly constant at about 7 (nmol cytochromes *c* oxidized/min/mg) at 37°C. Surprisingly, in the absence of salt during the assay, a drastic transitory increase of this activity with values up to 80 (nmol cytochromes *c* oxidized/min/mg) could be registered. This very high activity, however, decreased after preincubation of the membranes without salt, yielding 25% of the initial activity after 1 h incubation. The K_m - value for the cytochrome-*c* oxidase activity was determined as 37 μM for the substrate cytochrome *c* at a salt concentration of 3.4 M NaCl (as shown in Fig. 3). From the temperature dependence of this reaction, the activation energy could be calculated as 23.7 kJ/mol.

Cytochrome *d*

When *H. salinarum* cells were grown with constant aeration in a 50-L fermenter instead of 5-L Erlenmeyer flasks, the cytochrome pattern of the membranes

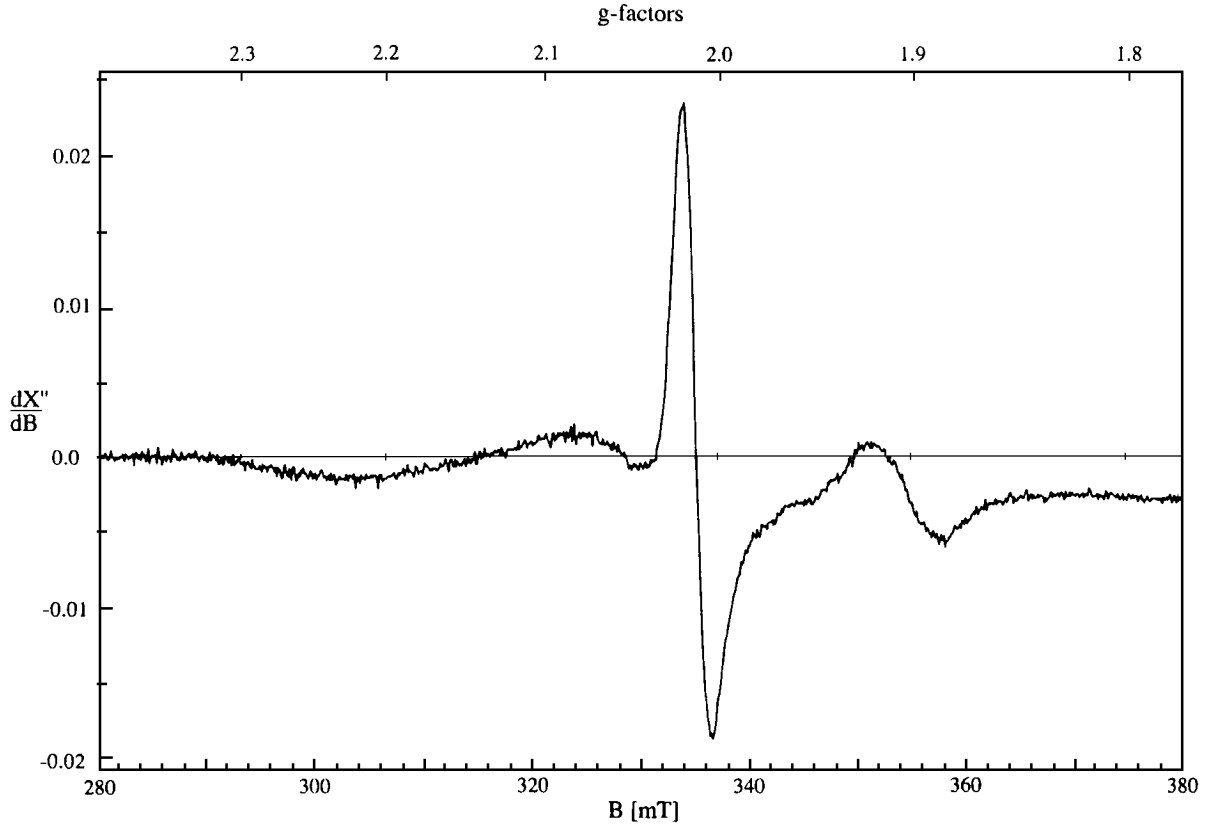

Fig. 2. EPR spectrum of the partially enriched complex III analog. The sample was reduced with 5 mM ascorbate for 10 min at room temperature, subsequently poured in an EPR tube and frozen in liquid nitrogen. Protein concentration was 8.5 mg/ml in a buffer containing 0.2 M NaP_i, 0.1% Triton, pH 7.2. EPR conditions: microwave frequency; 9.432 GHz; modulation frequency; 100 kHz; modulation amplitude; 0.5 mT; microwave power; 2 mW; temperature; 20 K. In this figure, the assay was carried out with 50 μg of solubilized plasma membrane.

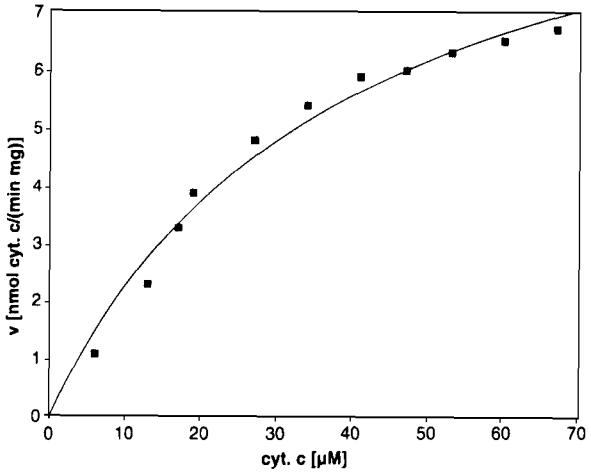

Fig. 3. Michaelis Menten presentation of the cytochrome-*c* oxidase activity of *H. salinarum* membranes.

changed. The α range of the dithionite reduced–minus–oxidized difference spectrum of a Triton X-100 detergent extract of membranes, obtained from the above cells, is displayed in Fig. 4. Evidently, a broad peak with a maximum at 625 nm can be detected, which is typical for a *d*-type cytochrome. To further characterize this pigment, CO was added to the dithionite-reduced detergent extract, which induced a typical red shift of about 5 nm in the dithionite-reduced plus CO minus oxidized difference spectrum.

DISCUSSION

NADH Dehydrogenases (NDH)

In the archeal urkingdom, only few species have been studied for NDH activity. In the case of the thermoacidophilic crenarcheon *Sulfolobus* sp. strain 7, an NDH was purified and characterized with artificial dyes as electron acceptors (Wakao *et al.*, 1987). However, this activity was only loosely attached to the plasma membrane. The purified protein was a homodimer with a molecular mass of 95 kD and characterized as a flavoprotein with about 2 moles of FAD/mol

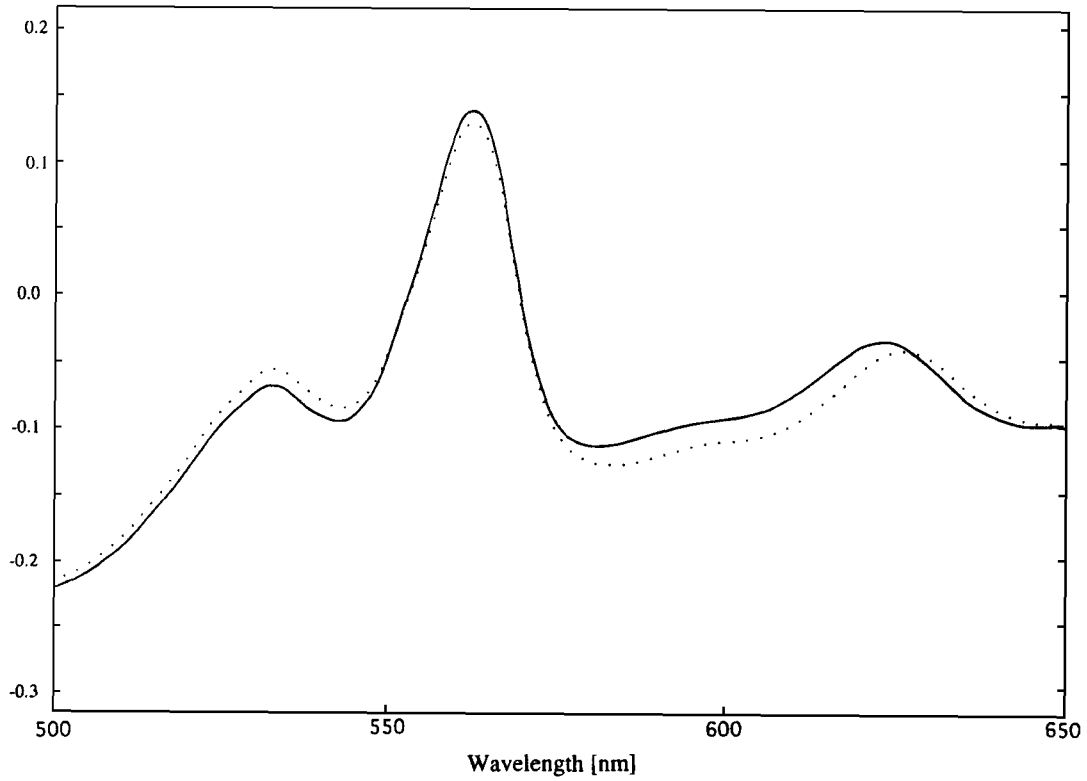

Fig. 4. Dithionite reduced minus oxidized difference spectrum of a Triton extract of cytochrome *d*-containing *H. salinarum* membranes in the α range of the spectrum (full line). Dotted line; dithionite reduced + CO minus oxidized difference spectrum of the detergent extract. To obtain the CO form, CO was bubbled through the dithionite-reduced sample. Protein concentration was 29.9 mg/ml in a buffer containing 50 mM maleate, 1% Triton, pH 7.2.

enzyme. Although its activity with the endogenous caldariellaquinone was only very low, the enzyme might serve as an entry point of the reducing equivalents from NADH to the electron transport chain. Evidence for the presence of iron–sulfur clusters, the prerequisite for a coupled, proton-pumping NDH, NDH type I, was not shown.

In the case of *S. acidocaldarius* (DSM 639), no evidence for the presence of a coupled NDH could be found either. First, the NADH oxidase activity of *S. acidocaldarius* membranes was not influenced by the classical NDH type I inhibitors rotenone and piericidin A (Anemüller *et al.*, 1985). In addition, after reduction of the membranes with NADH, all EPR detectable resonances for the classical [Fe–S] clusters of NDH type I complexes were absent (Anemüller, 1995, unpublished observation). On the other hand, cell respiration could be slowed down by acridone carboxyl acid derivatives, the typical inhibitors of type II NDH (Oettmeier *et al.*, 1994). Therefore, in both *Sulfolobus* strains, a coupled, proton-pumping NDH is obviously absent, whereas a nonpumping enzyme (NDH type II) seems to be the NADH oxidizing component in both species.

Recently, an NADH oxidase was isolated from the thermoacidophilic archeon *Acidianus ambivalens* (Gomez and Teixeira, 1998). It was also characterized as a soluble protein with a molecular mass of 70 kDa hosting one FAD molecule as single cofactor. As typical for type II NDH, it was devoid of iron–sulfur clusters.

In the present studies, we could show that inhibitors of the coupled, proton-pumping NDH neither influenced the NADH oxidase nor the NADH–cytochrome-*c* oxidoreductase activity. In addition, the total lack of EPR - detectable NDH [Fe–S] clusters was demonstrated. Therefore, it can be concluded, that an type I NDH is obviously absent in *H. salinarum*. On the other hand, the typical inhibitor of type II NDH, 7-jodoacridone-4-carboxylic acid, exerted a very strong inhibitory effect on both the NADH oxidase and the NADH–cytochrome-*c* oxidoreductase activity. From the titration data of this inhibitor, a $K_{i(50\%)}$ value of 20 μM could be estimated, corresponding to a pIC_{50} value of 4.7. This value corresponds to the value of 5.1 determined for the type II NDH of the eukaryote *Saccharomyces cerevisiae* (Oettmeier *et al.*, 1994). In conclusion, in the euryarcheotic branch of the archea, only external NDHs seem to be present, whereas coupled, proton-pumping NDHs are obviously missing. As to whether or not the absence of type I NDH may be considered as a general rule in the whole archeal urkingdom, appears a likely possibility, but remains to be validated by investigation of other species.

Succinate–Quinone–Reductase (SQR)

In contrast to NDH, SQR have been studied in various archea on both the molecular and the genetic level. In the crenarcheotic branch, the enzyme has been characterized in the species *S. acidocaldarius* (DSM 639), *Sulfolobus* sp. strain 7, and *Acidianus ambivalens*. Whereas the enzyme from *Sulfolobus* sp. strain 7 was described to be a classical enzyme with the three canonical iron–sulfur clusters S1([2Fe–2S]), S2 ([4Fe–4S]), and S3 ([3Fe–4S]) (Iwasaki *et al.*, 1995), in both other crenarcheotic species strong evidence could be provided for the presence of a novel type of SQR with a different iron–sulfur cluster composition (Janssen *et al.*, 1997; Anemüller *et al.*, 1996). Although the typical [2Fe–2S] and [4Fe–4S] clusters could be shown to be present in the enzymes, the characteristic [3Fe–4S] cluster, however, was obviously lacking. Instead, a new, second [4Fe–4S] cluster was detected, of which the functional role is still enigmatic.

In the euryarcheotic branch, the enzyme from *Th. acidophilum* could be characterized in the most integral, membraneous state due to its extremely high abundance (Anemüller *et al.*, 1995). It turned out to be a classical SQR with the typical iron–sulfur centers S1, S2, and S3. In *N. pharaonis*, the SQR was purified as a single-subunit enzyme and was shown to contain cytochrome *b* and FAD (Scharf *et al.*, 1997). The presence of the respective iron–sulfur centers was first postulated from EPR spectra of the membranes. From DNA sequence data of the B-subunit of the enzyme, however, it could later be concluded, that the *N. pharaonis* enzyme also hosts the three classical iron–sulfur centers S1, S2, and S3.

In this study we could unequivocally verify a membrane-associated SQR activity. The catalytic activity could be totally blocked by the classical inhibitor malonate and the inhibitor of both the *Th. acidophilum* and *S. acidocaldarius* (DSM 639) enzyme, tetrachlorobenzoquinone (Anemüller *et al.*, 1995; Moll and Schäfer, 1991). Interestingly, its activity could also be suppressed by the typical inhibitor of type II NDH, 7-jodoacridone-4-carboxylic acid. This might indicate, that the inhibitor rather interferes with the quinone binding site than with the NADH binding site of type II NDH. In addition, the SQR activity was remarkably

more influenced by 2-dodecylquinolone than the NDH activity, also indicating a stronger interaction of the quinone analog with the quinol binding site of the former enzyme.

Complex III Analog

The classical mitochondrial, plastidic, or bacterial complex III or cytochrome bc_1/b_6f complex serves as one coupling site of the respiratory chain and hosts two different *b*-type cytochromes, one *c*-type cytochrome (cytochrome c_1 or *f*), and a binuclear iron–sulfur cluster, also called Rieske iron–sulfur center (Trumpower and Gennis, 1994). In addition, two different quinone binding sites can be differentiated. In the archeal urkingdom, an integral complex III has not been found thus far.

In the present study, however, we present some evidence for the existence of a complex III ancestor or analog in the membranes of *H. salinarum* by functional and structural studies. From the functional aspect, our inhibitor studies of membranes strongly indicate the presence of a Q_i as well as of Q_o site. Whereas the effect of the Q_i site inhibitor antimycin A on halobacterial membranes was already known for a long time (Cheah, 1970c), nothing was reported thus far about the influence of Q_o site inhibitors. Our titration data of both, antimycin A as a Q_i site inhibitor and myxothiazol as a Q_o site inhibitor, clearly show for the first time evidence for the presence of both quinone sites in an archeal complex III analog. Surprisingly, the strength of these inhibitory effects differed remarkably. Whereas the pIC_{50} values for antimycin A (8.38) and myxothiazol (8.51) were reported to be nearly identical with complex III of the bacterium *Rhodospirillum rubrum* (Oettmeier *et al.*, 1994), the respective values for the membrane-bound complex III analog of *H. salinarum* were in the range pIC_{50} 3.15–3.63 for antimycin A and 6.3 to 6.58 for myxothiazol, depending on the assay system used. As to whether or not this behavior is due to an accessibility and/or a specificity problem, cannot be clarified thus far.

The partially enriched fraction described here was shown to contain all components for a typical complex III, *b*- and *c*-type cytochromes and a Rieske iron–sulfur cluster. This was achieved for the first time with an archeal organism. However, the *c*-type cytochrome was present in varying amounts, depending on the preparation. Even more strikingly, its molecular mass of 14.1 kDa is by far below that of a cytochrome c_1 of cytochrome bc_1 complexes, which usually range from 32 to 47 kD, as, for example, in complex III from *Rhodobacter sphaeroides* (Gabellini and Hauska, 1983) and *Paracoccus denitrificans* (Kurowski and Ludwig, 1987). The copurification of the *c*-type cytochrome with the *b*-type cytochrome and the Rieske iron–sulfur cluster may be caused by its strong binding to the halobacterial membrane. Therefore, the endogenous *c*-type cytochrome of *H. salinarum* is probably not a equivalent to cytochrome c_1, but rather serves as the electron carrier between the complex III analog and the terminal oxidase, as discussed below.

The presence of a *b*-type cytochrome was shown by optical spectroscopy, whereas the Rieske iron–sulfur cluster was detected by EPR spectroscopy in the ascorbate reduced state. The halobacterial protein, therefore, represents the second example of an archeal Rieske iron–sulfur cluster. The other example, the Rieske iron–sulfur cluster from *S. acidocaldarius*, has already been characterized intensively. The purified protein, with an apparent molecular mass of 32 kDa, contained one [2Fe–2S] cluster and exhibited the characteristic rhombic EPR spectrum with an average *g* value of 1.902, indicating nitrogen ligation of the cluster (Schmidt *et al.*, 1995). In addition, the isolated protein showed a ubiquinol–cytochrome-*c* oxidoreductase activity. However, the presence of a cytochrome bc_1 or b_6f complex could be excluded in *S. acidocaldarius*, because neither membrane-bound nor soluble *c*-type cytochrome were detected in this species (Anemüller *et al.*, 1985). Surprisingly, the situation in *S. acidocaldarius* became even more interesting by the observation, that two different Rieske iron–sulfur proteins were simultaneously expressed (Schmidt *et al.*, 1996).

The partially enriched complex from *H. salinarum* was capable of interacting with both quinones and cytochrome *c*, as demonstrated by the decylubiquinone–cytochrome-*c* oxidoreductase activity. The actual activity, however, was remarkably lower than that determined for the Rieske iron–sulfur cluster of *S. acidocaldarius* (Schmidt *et al.*, 1995). This may be due to the assay conditions, where neither the endogenous quinone nor the endogenous *c*-type cytochrome was used.

The composition of the cytochrome *bc* complex from *N. pharaonis* (Scharf *et al.*, 1997) is also unique, as compared to the *H. salinarum* and *S. acidocaldarius* complexes. The purified complex was demonstated to host only one *b*-type and one *c*-type heme per enzyme

molecule. However, this complex was devoid of a Rieske iron–sulfur center and exhibited no catalytic activity. The lack of activity can be explained by the presence of only one *b*-type heme and the absence of the Rieske [Fe–S] cluster. Either the second *b*-type heme or the Rieske center are prerequisites for a classical Q cycle.

Therefore, a complex III seems also to be missing in the euryarcheotic branch of the archea. The functional capability of the halobacterial complex III analog still remains to be elucidated, and the respective investigations are currently under study.

Terminal Oxidases

Terminal oxidases are widespread in the archeal urkingdom. The most thoroughly studied archeon, with respect to its cytochrome oxidases, is *S. acidocaldarius* (as reviewed in Schäfer, 1996; Lübben, 1995). From the aspect of catalysis, all crenarcheotic oxidases most probably work as quinol or sulfocyanine oxidases. Thus far, no true cytochrome-*c* oxidase has been found in the crenarcheotic branch. However, this is not surprising, since *c*-type cytochromes, in general, seem to be absent in this branch of the archea.

In contrast to the situation in the crenarcheotic branch, some aerobic euryarcheotes have been described to contain *c*-type cytochromes, like *Th. acidophilum* (Belly *et al.*, 1973), *H. salinarum* (Hallberg–Gradin and Colmsjö, 1989), and *H. cutirubrum* (Lanyi, 1968). Although a cytochrome-*c* oxidase activity with horse heart cytochrome *c* as substrate has already been described for *H. salinarum* (Fujiwara *et al.*, 1987), the authors did not classify the terminal oxidase as a cytochrome-*c* oxidase, but assumed a *b*-type cytochrome to be the natural electron donor (Fujiwara *et al.*, 1987), because an *H. salinarum* strain devoid of *c*-type cytochromes was used in their studies. In membranes of the *H. salinarum* mutant used in the present study, we also observed a cytochrome-*c* oxidase activity at high ionic strength, which was remarkably lower than the one at low ionic strength. However, this residual activity is comparable to the respiratory rates observed with NADH or succinate as substrates and to the NADH– or succinate–cytochrome-*c* oxidoreductase rates. Taken into account that not the endogenous but a eukaryotic *c*-type cytochrome was used as substrate in the assay system, an even higher rate would presumably be measured with the endogenous cytochrome *c*. The function of the endogenous *c*-type cytochrome would nicely be fulfilled by the *c*-type cytochrome described here with a molecular mass exactly in the range for typical *c*-type cytochromes as substrates for terminal cytochrome-*c* oxidases. Therefore, in contrast to previous conclusions (Fujiwara *et al.*, 1987), our data suggests that the terminal cytochrome oxidase of the *H. salinarum* mutant used in this study serves as a true cytochrome-*c* oxidase.

In the archeal urkingdom, the presence of *d*-type cytochromes has only been reported for the euryarcheote *Th. acidophilum* (Holländer, 1978; Gärtner, 1991). Inhibitor studies as well as spectroscopic data of *Th. acidophilum* membranes and of detergent extracts gave evidence for the existence of a terminal oxidase complex of the cytochrome *bd*-type (Holländer, 1978). For the first time, our observations show *d*-type cytochromes to be present in a halobacterial species. Both the redox as well as the CO difference spectra, are characteristic for cytochrome *d*. Therefore, the complicated models on electron transport chains in halophiles have to be extended for an additional member of the terminal oxidase family.

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