Electron Transfer Proteins from the Haloalkaliphilic Archaeon *Natronobacterium pharaonis:* Possible Components of the Respiratory Chain Include Cytochrome *bc* and a Terminal Oxidase Cytochrome ba_3[†]

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ABSTRACT: *Natronobacterium pharaonis,* an aerobic haloalkaliphilic archaebacterium, expresses high concentrations of redox proteins as do alkaliphilic eubacteria. The first redox protein characterized from *N. pharaonis* was halocyanin [Scharf, B., & Engelhard, M. (1993) *Biochemistry 32*, 12894−12900], a small blue copper protein. It is a peripheral membrane protein and is conjectured to function in a manner similar to plastocyanin. In the present work, the respiratory chain is further elucidated and the purification and characterization of the most abundant components cytochrome *bc* and cytochrome ba_3 from the membrane fraction are described. The cytochrome *bc* complex consists of a 14 and an 18 kDa subunit in a 1:1 ratio, with heme *c* bound to the larger polypeptide. An Fe-S subunit similar to that found in eukaryotic *bc* complexes has not yet been identified. The second membrane complex carries two different heme groups of the ba_3-type as well as copper. It contains two subunits of 36 and 40 kDa. This cytochrome ba_3 binds carbon monoxide, a feature common to terminal oxidases. There is no spectroscopic evidence for a second terminal oxidase; hence, under the growth conditions chosen the respiratory chain of *N. pharaonis* appears to be unbranched. In addition to these cytochromes, a succinate dehydrogenase which is solubilized from the membrane by detergents was isolated. A cytochrome *c* which was isolated from the cytosol has an unusually high molecular weight and a redox potential of −142 mV. A second cytosolic protein, ferredoxin, was purified to homogeneity. A comparison of the redox potentials of the isolated proteins with those obtained from the native membrane allows the construction of a possible electron transfer chain.

The respiratory chains of archaea have only recently been investigated in more detail although they deserve extended attention. Most of the presently available data have been obtained from the halophilic species *Halobacterium salinarium* and more recently from the thermoacidophilic *Sulfolobus acidocaldarius* [for reviews see Schäfer et al. (1990) and Lübben (1995)].

The thermoacidophilic *S. acidocaldarius* co-expresses two terminal oxidases during heterotrophic growth. The primary sequences of these multi-subunit complexes have been determined (Lübben et al., 1992, 1994a). Both oxidases (SoxABCD and SoxCM) use caldariella-quinol as their natural substrate (Anemüller & Schäfer, 1990) and are present in nearly equal amounts during all growth conditions tested. No *bc*-type complex or cytochrome *c* is present in the membrane (Anemüller et al., 1985). Apparently, electrons derived from NADH and/or succinate are funneled via caldariella-quinol to the terminal oxidases. Other substrates, e.g. small blue copper proteins (Castresana et al., 1995) might also be involved in the electron transfer.

Cytochromes of the halophilic species *Halobacterium cutirubrum* have already been studied quite early by Lanyi (1968) and Cheah (1969). In another closely related halobacterial species, *H. salinarium*, the knowledge about the electron transfer chain is only rudimentary, although much scientific effort was spent on this species because of its unique photophosphorylation and phototaxis system which is based on bacterial rhodopsins [reviewed in Oesterhelt and Tittor (1989)]. Cheah (1970a,b) described that *H. salinarium* membranes contain mostly *b*-type cytochromes. Two CO reactive components and one cytochrome *c* were also identified. Hallberg Gradin and Colmsjö (1989) characterized four different cytochrome *b* proteins by redox titrations and proposed a scheme for the electron transport. A terminal oxidase, cytochrome aa_3, has been described and purified (Fujiwara et al., 1989). Its amino acid sequence is now available (Denda et al., 1991). Recently, two *b*-type cytochromes, *b*-558 and *b*-562, were isolated (Fujiwara et al., 1993). Cytochrome *b*-558 consisted of two subunits with molecular masses around 15.4 and 11.7 kDa. The larger subunit contained the heme and could still be stained by TMBZ[1] even after treatment with SDS. These authors also note that cytochome *c* contrary to Cheah (1970a) is found neither in the cytoplasm nor in the membrane. Menaquinone was isolated from membranes of *H. salinarium* (Collins et al., 1981) and might function as mobile carrier of the electron transfer chain. Interestingly, a small blue copper protein has been isolated and spectroscopically characterized (Steiner, 1983).

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[1] Abbreviations: CD, circular dichroism; DM, dodecyl maltoside; PMSF, phenylmethanesulfonyl fluoride; V_{SHE}, standard hydrogen electrode; SDS, sodium dodecyl sulfate; SDS−PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TMPD, tetramethyl-*p*-phenylendiamine; TMBZ, 3,3′,5,5′-tetramethylbenzidine.

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As noted earlier *S. acidocaldarius* thrives at the acidic pH of 2. Another archaeal haloalkaliphilic species *Natronobacterium pharaonis* finds optimal conditions for growth at the other end of the pH scale at a pH of about 9.5 (Soliman & Trüper, 1982; Tindall et al., 1984). Like other alkaliphilic bacteria, the content of cytochromes is increased as compared to neutrophiles and presumably compensates the inversed pH gradient with a cytoplasmic pH around two units below the pH of the external medium (Lewis et al., 1980). The electron transfer chain of *N. pharaonis is* not yet very well characterized, although all three types of cytochromes (*a*, *b*, and *c*) are expressed (Scharf, 1992). Furthermore, a small blue copper protein, halocyanin, has been isolated (Scharf & Engelhard, 1993) and subsequently sequenced (Mattar et al., 1994). Halocyanin is a peripheral membrane protein with a pH-dependent midpoint potential of +183 mV (vs SHE) at a pH 7.3 (Brischwein et al., 1993) and might serve as mobile carrier in electron transfer reactions taking over the role of cytochrome *c*.

In the present work redox proteins of the natronobacterial membrane and cytosol are characterized. In the course of the experiments a cytochrome *bc* and a terminal oxidase of the ba_3-type as well as ferredoxin have been isolated. Furthermore, a membrane-bound succinate dehydrogenase (SDH) was isolated and a cytoplasmic cytochrome *c* was detected. Their spectroscopic and physiological data are discussed in the frame of a provisional electron transfer chain.

MATERIALS AND METHODS

Materials. Cytochrome *c* (horse heart) and Triton X-100 for membrane extraction were purchased from Boehringer (Mannheim, Germany). Triton X-100 used for purification of SDH was a product from Fisher Scientific (U.S.A.). Sodium deoxycholate, β-D-dodecyl maltoside (DM), and *N*,*N*-dimethyldodecylamine-*N*-oxide (LDAO) were obtained from Fluka (Neu-Ulm, Germany). DEAE-Sepharose, QAE-Sephadex, and Superose 12 prep-grade were products from Pharmacia (Freiburg, Germany). Hydroxyapatite Bio-Gel HT was purchased from Bio-Rad (München, Germany). All chemicals used were of analytical grade.

Strain and Cell Culture. N. pharaonis strain SP1(28) was grown aerobically as described in Lanyi et al. (1990) in a synthetic growth medium (Scharf & Engelhard, 1994). The cells were harvested by centrifugation and washed three times with 4 M NaCl.

Spectroscopy. The visible spectra were recorded on a Perkin Elmer Lambda 9 double-beam spectrophotometer. The low-temperature spectrum of cytochrome *bc* in 60% glycerol (path length, 2 mm) was taken at 77 K on a Perkin Elmer 320 spectrophotometer. Midpoint potentials were determined by electrochemical redox titration on a Cary 219 UV/vis spectrophotometer as described in Baymann et al. (1991).

The circular dichroism (CD) spectra were recorded on a Jobin-Yvon MIII dichrograph. The raw data were averaged and stored on a Nicolet 1074 (Nicolet, Madison, WI) transient recorder and transformed to ellipticity (Θ) using a home-made program.

The iron and copper contents were determined after dialysis of the samples against 20 mM EDTA on a Hitachi Z-8000 absorption spectrometer by calibrating the peak height with Fe and Cu standards.

Purification of Redox Proteins. In the first step of the purification protocol the cell membrane (the cytoplasmic supernatant was used for the purification of cytochrome *c* and ferredoxin) was treated with Triton X-100 to remove peripherally bound membrane proteins including halocyanin (Scharf & Engelhard, 1993). The residual membrane was subsequently solubilized with dodecyl maltoside (DM). The use of DM at this point is indispensable for the integrity and functionality of the cytochromes. In all further steps DM can be exchanged against LDAO. The first DEAE-Sepharose chromatography removes 90−95% of the original protein material. It also separates cytochrome *bc* from cytochrome ba_3.

Triton-extracted membranes from 40 L of cell culture were suspended in 20 mM NaCl, 10 mM Tris, pH 8.0, to a protein concentration of about 9 mg/mL and stirred for 14 h in the dark with 2% DM. The mixture was centrifuged for 60 min at 360000*g*. The supernatant containing cytochrome *bc* and cytochrome ba_3 was applied to a DEAE-Sepharose CL-6B column (5.0 × 4.0 cm) which had been pre-equilibrated with 0.1 M NaCl, 0.2% DM, 10 mM Tris, pH 8.0, at a flow rate of 25 mL/h. After application of the sample the column was washed with five bed volumes of 0.1 M NaCl, 0.2% DM, 10 mM Tris, pH 8.0, then 0.15 M NaCl, 0.2% DM, 10 mM Tris, pH 8.0, at a flow rate of 40 mL/h. At an ionic strength of 0.2 M NaCl cytochrome *bc* eluted in a volume of about 350−500 mL (fraction A). After the column had been washed with two bed volumes of 0.3 M NaCl buffer, cytochrome ba_3 could be eluted with buffer containing 0.4 M NaCl in a volume of about 100−300 mL (fraction B).

Cytochrome bc. For further purification the yellow-orange fraction A was diluted with one volume of 20 mM NaCl, 10 mM Tris, pH 8.0, and then applied to a second DEAE-Sepharose CL-6B column (2.6 × 4.0 cm), which had been prewashed (25 mL/h) with two bed volumes of 0.1 M NaCl, 0.2% DM, 10 mM Tris, pH 8.0. When the ionic strength was increased to 0.2 M NaCl, cytochrome *bc* was obtained in a volume of about 100 mL.

The cytochrome *bc* fraction was concentrated to a volume of about 8 mL and diluted as described above. The fraction was applied to a QAE-Sephadex column (2.6 × 3 cm) pre-equilibrated with 50 mM NaCl, 0.2% DM, 10 mM Tris, pH 8.0. Cytochrome *bc* was eluted with 300 mL of a linear gradient from 0.1 to 0.2 M NaCl, 0.2% DM, 10 mM Tris, pH 8.0.

At this stage of the purification protocol an additional chromatography on hydroxyapatite was sometimes necessary. For this purpose the concentrated QAE-Sephadex pool fractions were adjusted to 150 mM NaCl, 2 mM $MgCl_2$, 0.2% DM, 20 mM Na_2HPO_4, pH 8.0, and to a protein concentration of about 8 mg/mL. This sample was divided into two halves, and each portion was applied to two separate hydroxyapatite columns (2.0 × 2.0 cm) which were equilibrated with the above buffer. Most of the protein impurities and a minor part of the cytochrome *bc* eluted without retention. After the column had been washed with ten bed volumes of the equilibration buffer the cytochrome *bc* could be desorbed from the matrix with 0.2% DM and 200 mM Na_2HPO_4, pH 8.0.

Fractions from the QAE-Sephadex or hydroxyapatite chromatography were concentrated in Centriprep CP10 and Centricon C10 to a protein concentration of 5 mg/mL and were subjected in 0.5 mL portions to FPLC gel filtration (Superdex 200, HR16/60 column). The column was equilibrated and developed in 150 mM NaCl, 0.2% DM, 10 mM Tris, pH 8.0, at 1 mL/min.

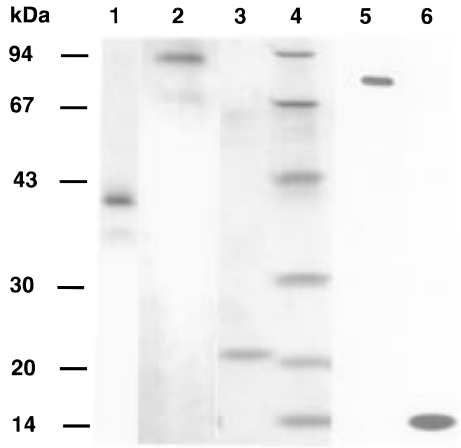

FIGURE 1: SDS gel of cytochrome ba_3 (lane 1), SDH (lane 2), ferredoxin (lane 3), and molecular weight standards (lane 4) stained with Coomassie Blue, cytochrome *c* stained with TMBZ (lane 5), and horse heart cytochrome *c* (lane 6).

Cytochrome ba_3. The yellow-brown fraction B was diluted with one volume of 20 mM NaCl, 10 mM Tris, pH 8.0, and applied to a second DEAE-Sepharose column (2.6 × 3.0 cm) equilibrated with 0.1 M NaCl, 0.2% DM, 10 mM Tris, pH 8.0. The column was washed with two column volumes of the equilibration buffer. Cytochrome ba_3 was eluted with 300 mL of a linear gradient from 0.1 to 0.4 M NaCl in 0.2% DM, 10 mM Tris, pH 8.0.

The pooled fractions of the second DEAE-Sepharose chromatography were applied to a hydroxyapatite column (2.6 × 3 cm) which had been equilibrated with 0.1 M Na_2HPO_4, 0.4% sodium cholate, pH 8.0. The column was washed with two bed volumes of equilibration buffer, and the cytochrome was eluted with 300 mL of a linear gradient from 0.1 to 0.4 M Na_2HPO_4, 0.4% sodium cholate, pH 8.0, at a flow rate of 24 mL/h. Cytochrome ba_3 eluted between a phosphate concentration of 0.15−0.25 M.

The cytochrome ba_3 fractions were concentrated to a protein content of about 9 mg/mL and purified by FPLC gel filtration as described for cytochrome *bc*.

Cytosolic Cytochrome c. The cytoplasmic supernatant of the membrane preparation was applied to a DEAE-Sepharose column equilibrated with 20 mM TAPS, pH 8.5, at 4 °C. One part of the cytochrome *c* fraction was adsorbed by the column, while another part eluted in the void volume and was used for SDS−PAGE.

Succinate Dehydrogenase (SDH). The whole purification procedure was performed at 4 °C. Membranes from 4 L of cell culture were suspended in 50 mL of 20 mM NaCl, 20 mM Tris, pH 8.0, and solubilized for 14 h in 3% (w/v) Triton X-100. The mixture was centrifuged for 60 min at 360000*g*, the supernatant was diluted with one volume of 0.1 M NaCl, 0.5% Triton X-100, 1 mM PMSF, 20 mM Tris, pH 8.0, and applied to a DEAE-Sepharose CL-6B column (2.6 × 7 cm) equilibrated with the same buffer. The column was washed with equilibration buffer, followed by buffer containing 0.2 and 0.25 M NaCl. SDH was eluted by increasing the ionic strength of the buffer to 0.3 M NaCl. SDH-containing fractions were pooled, diluted 1:1 with equilibration buffer, and applied to a second DEAE-Sepharose column (1.6 × 6 cm). After the column was washed with 0.25 M NaCl, 0.5% Triton X-100, 1 mM PMSF, 20 mM Tris, pH 8.0, SDH was eluted with 300 mL of a linear gradient from 0.25 to 0.4 M NaCl, 0.5% Triton X-100, 1 mM PMSF, 10 mM Tris, pH 8.0, in the first third of the gradient.

Ferredoxin. Ferredoxin was purified from the cytoplasm of 20 L of cell culture according to Kerscher et al. (1976).

Analytical Procedures. Gel electrophoresis was performed essentially using the procedure of Laemmli (1970) in a 2 mm flat gel in a linear gradient from 10% to 17.5% acrylamide. Heme staining of SDS gels was done according to Thomas et al. (1976). Extraction of covalently bound FAD from SDS gels was performed according to Singer and Edmondson (1978). Fluorescence of extracted FAD was measured with an Aminco Bowman series 2 luminescence spectrometer.

Amino acid analysis was performed on a Biotronik automatic analyzer (model LC 7000) after total hydrolysis of the samples in 6 N HCl with 0.1% phenol at 110 °C for 24, 48, or 72 h. For the determination of cysteine and methionine, protein samples were first oxidized according to Hirs (1967).

Determination of NADH and succinate dehydrogenase activity in membrane suspensions was done as described in Singer (1991).

Samples were reduced by the addition of a few grains of sodium dithionite to the sample. The reduction of cytochrome ba_3 with ascorbate in the presence of TMPD was done as described by Cheah (1970a). Protoheme and heme *a* were determined from the pyridine hemochrome spectra according to Falk (1964). Hemes extracted with HCl−acetone were analyzed by reversed-phase HPLC (Lübben & Morand, 1994).

RESULTS

Ferredoxin

The first cytoplasmic redox active component was identified as ferredoxin. Ferredoxins are iron−sulfur electron transfer proteins which are ubiquitously distributed among bacteria, algae, higher plants, and animals. *H. salinarium* contains a ferredoxin characterized by a 2Fe-2S cluster which resembles those from plants more than those from other bacteria (Kerscher et al., 1976). This protein was found to be a coenzyme of α-ketoacid oxidoreductase (Kerscher & Oesterhelt, 1977). The natronobacterial ferredoxin was purified under almost identical conditions to those described for the halobacterial protein (Kerscher et al., 1976).

Purified ferredoxin (yield: 20 mg of ferredoxin/20 L of cell culture) showed a single band on SDS−PAGE at an apparent molecular mass of 22 kDa (Figure 1, lane 3). The amino acid composition (data not shown) is characteristic for a soluble protein from halophiles with about 42% acidic amino acids or their amides. The absorption spectrum of oxidized ferredoxin displayed three maxima at 329 nm (ϵ = 13600 M^{-1} cm^{-1}), 421 nm (ϵ = 9950 M^{-1} cm^{-1}), and 467 nm (ϵ = 8960 M^{-1} cm^{-1}). The latter extinction coefficients decreased upon reduction by about 55% and 60%, respectively.

Purified ferredoxin is reduced in cell free bacterial extracts in the presence of α-ketoglutarate and coenzyme A (data not shown), indicating that it might function as the coenzyme of α-ketoacid oxidoreductase, as was proven for the halobacterial ferredoxin (Kerscher & Oesterhelt, 1977). Another similarity between the two ferredoxins is the occurrence of only four Cys, likely to be involved in Fe binding. Two Fe/molecule were determined, proving the iron cluster to be of the 2Fe-2S type. The absorption spectra of the ferredoxins

from both species are nearly indistinguishable, and even the redox potentials with −345 mV (*H. salinarium*) and −342 mV (*N. pharaonis)* are almost identical. The homologies between the archaeal ferredoxins on the one hand and the chloroplast-type ferredoxins on the other hand imply a common ancestor as was already proposed by Hase et al. (1977).

Succinate Dehydrogenase (SDH)

SDH catalyzes the reduction of fumarate from succinate and transfers the reducing equivalents to the respiratory chain. It usually contains a cytochrome *b*, FAD, and Fe-S centers. Purified SDH from *N. pharaonis* showed a major band at 94 kDa in Coomassie-stained SDS gels (Figure 1, lane 2) which is a typical size for this class of proteins. FAD could be extracted from this band and identified by its emission at 530 nm after excitation at 450 nm. The absorption spectrum of reduced SDH exhibits one maximum at 425 nm with a shoulder at 430 nm and a second maximum at 551 nm. The maxima clearly indicate the presence of cytochrome *b*, whereas the shoulder can be assigned to FAD. An iron−sulfur center has not yet been experimentally characterized, although measurements using electron paramagnetic resonance (EPR) indicate their presence in membrane preparations (data not shown). The purified protein showed SDH activity, which could be inhibited with malonate. However, the enzymatic activity is decreased by a factor of 5 compared to the protein in membrane suspensions.

Cytochrome c

The cytoplasm of *N. pharaonis* contained a cytochrome *c* which could be partially purified by chromatography on DEAE-Sepharose although its susceptibility to proteolysis impeded further purification and analysis. The absorption spectrum of the reduced cytochrome *c* fraction exhibited an α-band at 550 nm as typical for *c*-type cytochromes. The Soret band had its maximum at 408 nm in the oxidized form and at 417 nm in the reduced form. Heme could also be detected by TMBZ staining of the gel after SDS−PAGE (Figure 1, lane 5), a property which is typical for *c*-type cytochromes. Both cytosol and the cytochrome *c* fraction showed a single band at an apparent molecular mass of about 75 kDa. Cytochrome *c* exhibits one reduction potential at −142 mV which was determined in whole cytosol preparation as well.

Cytochrome bc

Purification. The general scheme for the purification of cytochrome *bc* has been described in Materials and Methods. In some cases an additional purification step using hydroxyapatite was necessary to separate from minor protein impurities. Cytochrome *bc* binds to hydroxyapatite in the presence of 2 mM Mg^{2+}, whereas most of the protein impurities and minor amounts of cytochrome *bc* were not retarded under these conditions. Cytochrome *bc* can then be recovered by increasing the phosphate concentration in the buffer to 200 mM. After a final gelfiltration step a fraction is recovered which exhibits two bands after SDS−PAGE with apparent masses of 14 and 18 kDa (Figure 2, lane 2). From 40 L of cell culture an average yield of 29 mg of cytochrome *bc* could be obtained.

Spectral Classification. The absorption spectrum of oxidized cytochrome *bc* showed typical absorption maxima

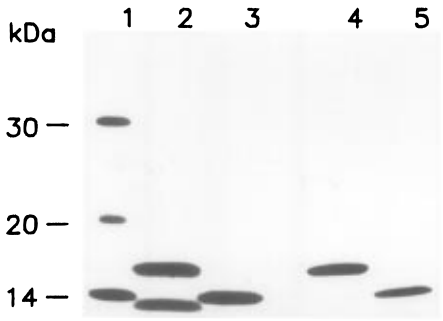

FIGURE 2: SDS gel of cytochrome *bc*. Lanes 1−3 (Coomassie stain): (1) molecular weight markers; (2) cytochrome *bc*; (3) horse heart cytochrome *c*. Lanes 4 and 5 (TMBZ stain): (4) cytochrome *bc*; (5) horse heart cytochrome *c*.

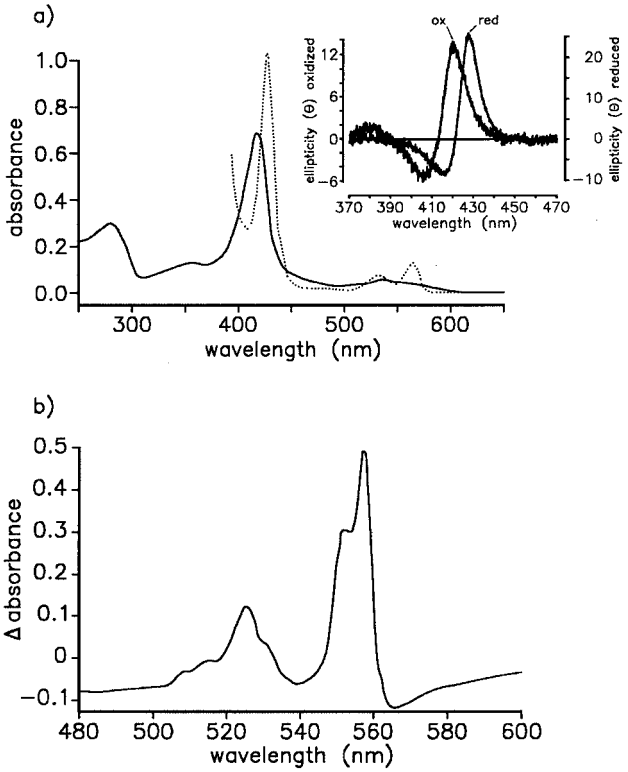

FIGURE 3: Absorption spectra and difference spectra of *N. pharaonis* cytochrome *bc*. (a) absorption spectrum of oxidized (straight line) and reduced (with a few grains of sodium dithionite) (broken line); (b) reduced cytochrome *bc* in 60% glycerol at 77 K in the region of α- and β-bands; (pathlength of the cell, 2 mm). A spectrum of the oxidized protein was used as base line. Insert: CD spectrum of oxidized and reduced cytochrome *bc*. 0.19 mg/mL in 150 mM NaCl, 0.2% DM, 10 mM HEPES, pH 8.0 (path length of the cell, 1 cm); average of four scans. After the spectrum of the oxidized sample was recorded, 10 μL of 1 M sodium dithionite was added and the spectrum of the reduced form was recorded.

at 280 and 413 nm (Figure 3a). The Soret band at 413 nm is 2.5 times more intense than the protein absorption. The low absorption in the aromatic region indicates the presence of only a few Trp and Tyr residues. On reduction with dithionite, the Soret band shifts to 423 nm which is accompanied by an increase of its extinction coefficient. Additionally, the β-band at 525 nm and the α-band at 558 nm can now be recognized. The α-band exhibits a shoulder around 552 nm. To resolve the components of the α-band, a low-temperature spectrum of the reduced cytochrome was taken (Figure 3b) where two peaks with maxima at 556 and 551 nm are discernible. The α-band at about 556 nm is typical for a *b*-type cytochrome, whereas that at about 550

nm is characteristic for a *c*-type cytochrome. This indicates that the preparation consists of type-*c* and type-*b* cytochromes.

The circular dichroic spectrum of cytochrome *bc* has been recorded in the Soret region between 370 and 470 nm (Figure 3a, insert). The spectrum of the oxidized sample exhibits a minimum at 406 nm and a maximum at 420 nm with the zero transition at 413 nm. For the reduced sample the corresponding values are 416, 427, and 421 nm, respectively. It should be noted that upon reduction the ellipticity increases. The zero transitions correlate well with the maxima of the Soret band.

Biochemical Characterization. Treatment of *b*-type cytochromes with acid−acetone allows the release of the prosthetic group as hemin, whereas the covalently bound chromphore in *c*-type cytochromes remains bound to the protein. The supernatant of the acid−acetone extract of the cytochrome *bc* preparation was analyzed by reversed-phase HPLC. Only one product absorbing at 400 nm could be detected which shows the same elution behavior as protoheme proving it to be heme B. The pyridine ferrohemochrome spectrum of the precipitate shows a 550 nm band typical for *c*-type cytochromes (data not shown).

Cytochrome *bc* was further analyzed by SDS gel electrophoresis and the heme-staining reagent TMBZ. Since in *c*-type cytochromes the heme is covalently bound via two thioether bridges to the cysteine residues, only the heme of *b*-type cytochromes should be lost during separation by SDS gel electrophoresis. As shown in Figure 2 (lane 4) the 18 kDa band was stained exclusively by TMBZ; therefore, this band is assigned to cytochrome *c*.

The iron content as determined by atomic absorption spectroscopy correlates well with the heme content determined as pyridine ferrohemochrome. 2 mol each of heme and iron could be found per 36.4 kDa protein. This is about 10% higher than the additive molecular mass (32 kDa) estimated from SDS−PAGE. Copper was only detected in substoichiometric amounts (0.7% of the Fe content).

No Cys could be determined during the analysis of the amino acid composition after performic acid oxidation (data not shown), but since heme C is covalently bound via two cysteine residues to the protein the hydrolysis product would escape the detection by amino acid analysis. This would argue for only two Cys per cytochrome *bc*. Similarly, under the same conditions of analysis the two Cys heme ligands of horse heart cytochrome *c* could not be detected. The high amount of hydrophobic amino acids determined for cytochrome *bc* is characteristic of a membrane protein. As is typical for proteins of halophilic archaea, acidic amino acids outnumber basic residues (Eisenberg et al., 1992).

Cytochrome ba₃

Purification. After cytochrome ba_3 had been separated from cytochrome *bc* it was applied to further chromatographic steps including rechromatography on DEAE-Sepharose, hydroxyapatite chromatography, and FPLC gel-filtration. The yield of cytochrome ba_3 was about 40 mg from 40 L of culture. An SDS−PAGE analysis of the final product showed one major band with an apparent mass of about 40 kDa and one minor band of about 36 kDa (Figure 1, lane 1). Recent work (Mattar, 1996) has shown that these two proteins correspond to subunits I and II of bacterial cytochrome *c* oxidases, respectively. A large portion of the smaller subunit, only present in substoichiometric amounts, was apparently lost during the purification procedure. Especially the solubilization contributed to the desintegration of the complex as proven by SDS−PAGE (data not shown) which might also be a reason for the partial loss of redox activity (see below).

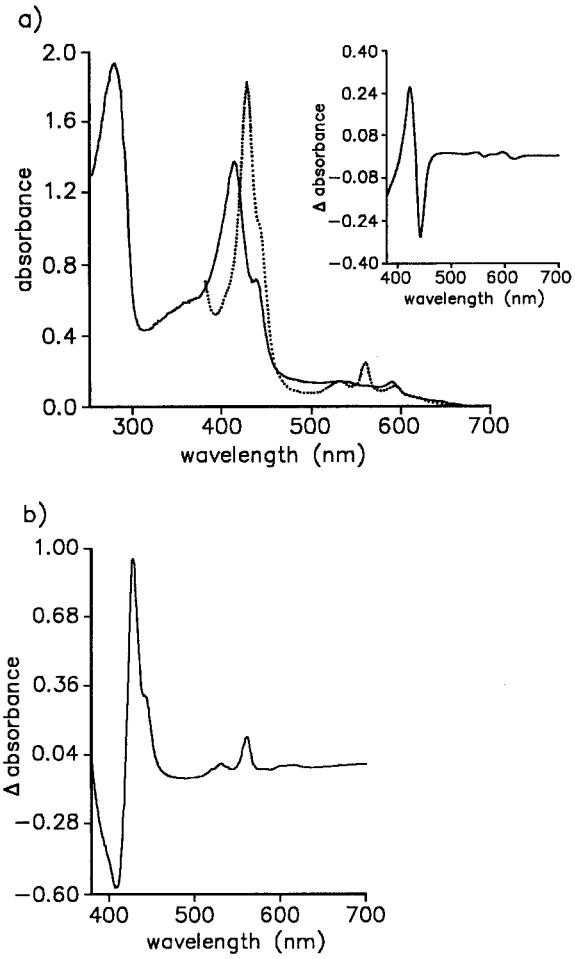

FIGURE 4: Absorption spectra and difference spectrum of *N. pharaonis* cytochrome ba_3. (a) Oxidized (straight line) and reduced (with a few grains of sodium dithionite) (broken line); (b) difference spectrum between reduced and oxidized cytochrome ba_3. Insert: C═O difference spectrum of cytochrome ba_3 (reduced + C═O) minus reduced cytochrome ba_3.

Spectral Classification. In Figure 4 the absorption spectrum of the final preparation of cytochrome ba_3 is shown. The Soret band has its maximum at 414 nm with a shoulder at 440 nm. The maximum at 592 nm can be assigned to the α-band. The two latter bands are typical for a cytochrome *a* in its reduced state. The absorption of these bands could only be slightly increased upon reduction with dithionite indicating that heme A is already partially reduced under the conditions of purification. It was also not possible to fully reoxidize the heme A with oxygen. However, in the electrochemical experiments the redox reactions of the heme components were fully reversible. The enzyme was still reactive toward CO (Figure 4, insert). Upon binding of CO the shoulder at 440 nm disappears and concomitantly the extinction at 414 nm increases. The shoulder at 440 nm caused by the cytochrome *a* component is shifted hypsochromically to 426 nm. These results are evidence for its function as a terminal oxidase.

Upon reduction by sodium dithionite the γ-band at 414 nm undergoes a bathochromic shift to 426 nm. In the region

Table 1: Compilation of Biochemical and Spectroscopic Data of the Purified Redox Proteins[a]

protein	localization	M_r	cofactor	absorption maxima (nm) ox.	red.	redox potential (mV)
				329		
ferredoxin	cytoplasm	15 200	2Fe-2S	420	decrease	−342
				470	decrease	
			FAD	412	430	+8
SDH	membrane	90 000	Fe-S			
			heme B	412	425	
					551	−312 (−340)
cytochrome *c*	cytoplasm	75 000	heme C	408	422	
					524	−142[b]
					550	
				413	423	
cytochrome *bc*	membrane	14 000	heme B	413	423	
					525	−117 (−112)
					558	
		18 000	heme C	413	423	
					525	−44 (−42)
					552	
halocyanin	membrane	15 456[c]	copper	600	colorless	+128
			heme B	414	426	
					532	+268 (+268)
					560	
cytochrome ba_3	membrane	40 000	heme A_S[d]	414	440	
					532	+358 (+358)
					592	
			Cu_B[e]	nd	nd	nd
		36 000[e]	Cu_A[e]	nd	nd	nd

[a] The redox potentials were measured at pH 8. The values given in parenthesis denote the redox potentials in membrane preparations. [b] Potential measured in cytosol. [c] Data taken from Mattar et al. (1994). [d] Personal communication by M. Lübben. [e] Personal communication by S. Mattar.

of the α- and β-bands new absorption maxima at 560 nm (α-band) and at 532 nm (β-band) appear (for the difference spectrum see Figure 4b). These three bands can be assigned to cytochrome *b*. It is functionally active since it can also be reduced by ascorbate in the presence of TMPD (data not shown).

Biochemical Characterization. The ratio of the optical density at 280 nm (protein) to that at 414 nm (Soret) is 1.4, which is considerably higher than that of cytochrome *bc* and indicates the presence of Trp and Tyr. The ratio of heme A to heme B as determined by analysis of the pyridine hemochromogens always gave higher values for heme B (heme A:B = 1:1.5) pointing to the loss of heme A during purification of the protein. This is also reflected in the ratio of Fe:Cu which was about 1:2. The presence of hemes A and B could also be shown by reversed-phase HPLC of acid acetone extracts. Heme B eluted at the same retardation time as the heme B from cytochrome *bc* indicating their identity. It should be noted that the side chain of the heme A extracted from cytochrome ba_3 preparations has been determined to be a hydroxylethylgeranylgeranyl group (heme A_S) (Lübben & Morand, 1994). Heme A_S has also been detected in other archaeal species like *S. acidocaldarius* as well as in the eubacterial *Thermus thermophilus* (Lübben et al., 1994b; Lübben & Morand, 1994).

Redox Potential Measurements in Membranes and Purified Proteins

The redox potentials of the purified proteins and the membrane fraction were measured electrochemically (Baymann et al., 1991) and are summarized in Table 1. The membrane was treated with Triton X-100 prior to the measurements to remove halocyanin which obscured the spectral identification of the α- and β-bands of the cytochromes. Due to the low signal to noise ratio in this spectral region even after removal of halocyanin, the exact determination of the redox potentials was done by analyzing the absorption changes at the Soret band of the cytochromes during titration. The titration curve displays at least three distinct steps (Figure 5) with calculated potentials at −340, −108, and +268 mV. The absorption changes leading to the −108 mV step can be equally well fitted by two midpoint potentials at −112 and −42 mV. These values agree with those determined from the isolated cytochrome *bc* complex (see below). On the basis of these absorption changes and those in the α-band region (data not shown), the redox potentials were assigned to three *b*-type and one *c*-type cytochrome. Additionally, an *a*-type cytochrome with its Soret band at 436 nm and a midpoint potential at +358 mV was identified (see inset of Figure 5).

The redox potentials of the isolated cytochromes and the SDH are consistent with the values gained from the membrane fraction. The lowest potential at −340 mV in the membrane titration were assigned to cytochrome *b* of SDH (−312 mV). The values at −112 and −40 mV correspond to cytochrome *bc*, for which −117 mV (heme B) and −44 mV (heme C) were measured. The positive values of +268 and +358 mV are characteristic for a terminal oxidase and are identical to those found for purified cytochrome ba_3.

DISCUSSION

The main emphasis of this work has been the purification and biophysical characterization of redox proteins from *N. pharaonis.* In the following the molecular data of the purified and partially purified redox proteins will be discussed (for a compilation of the biochemical and spectroscopic data, see Table 1). These data combined with the

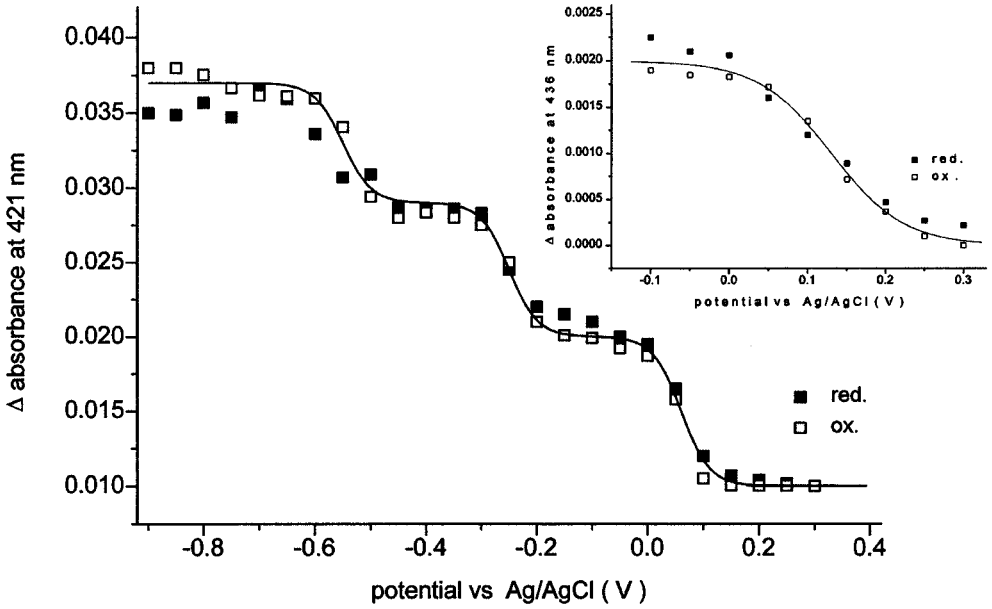

FIGURE 5: Electrochemical titration curve of *N. pharaonis* membranes. Prior to the measurements at pH 7 the membranes were extracted with Triton to remove endogeneous halocyanin. Open and filled sqares represent oxidative and reductive titration, respectively. The insert shows the change of absorbance during titration at 436 nm.

redox potentials determined in membranes as well as those obtained from the isolated proteins imply a possible model of the respiratory chain of *N. pharaonis*.

It should be noted, that the determination of the catalytic activities of the redox proteins were attempted; however, only in the case of ferredoxin and cytochrome ba_3 could preliminary data be obtained. The purified SDH and cytochrome *bc* are presumably not isolated in a native-like structure, because possible functional subunits are missing in the final preparation. In the case of cytochrome *bc* the native substrates are unknown. Unfortunately, experiments failed in which membrane preparations were treated with reduced menaquinone which might donate electrons to cytochrome *bc*. The activity of cytochrome ba_3 dropped considerably during solubilization of the membrane and remained almost constant at a low level during the following steps of purification. This observation indicates that during this step either functionally important subunits are lost or the treatment with detergents inactivates the protein.

Succinate Debydrogenase (SDH). Mitochondrial SDH is composed of two hydrophilic subunits and two integral membrane proteins. The cofactors FAD and the three Fe-S centers are bound to the two cytoplasmic proteins, respectively, whereas the two hydrophobic membrane-spanning domains harbor the quinone binding site and a cytochrome *b*. SDH has also been detected in *H. salinarium* where it was found not only in the membrane fraction but also as a soluble component in the cytosol (Hallberg Gradin et al., 1985). Its molecular mass was determined to be 90 kDa, which is similar to the natronobacterial enzyme; however, the latter protein could only be solubilized by treating the membrane fraction with Triton X-100. It is noteworthy that the isolated natronobacterial SDH contains both FAD and cytochrome *b*. This indicates that at least two different subunits with different hydrophobicity were copurified, possibly due to a very close binding of the smaller hydrophobic cytochrome *b*-containing subunit to the hydrophilic FAD subunit. There is no evidence for Fe-S centers in this preparation of SDH, which might be reasoned in the loss of the other hydrophilic subunit during the purification. As mentioned earlier, Fe-S centers could be verified in the membrane fraction by EPR, indicating that this archaebacterial SDH might possess the second hydrophilic subunit harboring the three Fe-S centers.

Cytoplasmic Cytochrome c. The soluble cytochrome *c* from *N. pharaonis* displays some unusual features which are different to other known cytochrome *c* proteins. Mitochondrial and eubacterial cytochromes *c* are small soluble proteins with a molecular mass of 12−14 kDa. The natronobacterial protein, however, according to SDS gel electrophoresis, has a very high molecular mass of 75 kDa. It is noteworthy that a comparable value (M_r = 67 000) was described for a *c*-type cytochrome (Hmc) from *Desulfovibrio gigas* (Chen et al., 1994) which was proposed to function as an electron carrier between hydrogenases and other redox proteins. Whereas this protein possesses multiple heme binding sites with distinct reduction potentials, preparations of cytochrome *c* from *N. pharaonis* exhibit only a single reduction potential at −142 mV. Therefore, the structure and function of this cytosolic cytochrome *c* might be different than those of Hmc. Another unusual feature of the natronobacterial cytochrome *c* concerns its relatively negative reduction potential which, together with the location in the cytosol, excludes its function as electron donor to a terminal oxidase. If this soluble cytochrome *c* is a member of the respiratory chain it must be involved in electron transfer reactions occurring upstream of the cytochrome *bc* complex (complex III). It should be noted that a soluble cytochrome *c* has also been identified in *H. salinarium* by its spectroscopic data (Fujiwara et al., 1987). Its function, however, has not yet been identified.

Cytochrome bc. The mitochondrial or bacterial cytochrome bc_1 or complex III generally consists of several hydrophobic subunits with two distinct hemes B, one heme C, a binuclear Fe-S center (also called Rieske protein), and two different quinone binding sites (Trumpower & Gennis, 1994). Purified natronobacterial cytochrome *bc* consists of two heme proteins with heme C and heme B, respectively. A couple of observations suggest these two proteins to be subunits of a functional complex. First, the two protein bands in the SDS gel (Figure 2) occur in a ratio of 1:1

throughout the whole purification (data not shown). Second, both proteins eluted during gel filtration at the same time at 67 mL and the native molecular mass could be estimated to about 40 kDa (for comparison cytochrome ba_3 eluted at 63 mL under the same conditions). Third, the respiration could be inhibited to about 50% and 60% by stigmatellin and funiculosin, respectively (Wittenberg, 1995), which are typical inhibitors of the cytochrome *bc* complex. Fourth, the redox potentials of cytochrome *bc* are almost identical to those measured in whole membrane fractions. Finally, the difference of the reduction potentials of the two proteins (see below) is such that it would be compatible with that for a cytochrome *bc* complex. However, it cannot be excluded that the copurified cytochromes are not functionally related.

Although the difference in redox potentials between cytochrome *b* and cytochrome *c* ($\Delta = -73$ mV) would be energetically sufficient for vectorial transfer of protons across the membrane, two observations are not compatible with the properties of a classic complex III. The purified protein dimer does not contain an iron−sulfur center, although it could have been lost during purification. The other observation concerns the ratio between B and C hemes. Whereas complex III always has a ratio of 2:1, in the natronobacterial cytochrome *bc* the ratio is 1:1. This ratio did not change during the last steps of purification, therefore a stoichiometry of 1:1 for the natronobacterial cytochrome *bc* seems possible. Since both the iron−sulfur centers and the second heme B are essential for a functional Q-cycle, it is not clear whether the natronobacterial cytochrome *bc* represents an unusual non-proton-pumping complex III or whether the isolation of only two subunits with *b*:*c* stoichiometry of 1:1 represents an artefact. On the other hand, the inhibition of the respiration by stigmatellin indicates the presence of a normal complex III. Sequence analysis of the corresponding gene should clarify this question.

Cytochrome ba_3. The final acceptor of the respiratory chain is the terminal oxidase or complex IV. According to the electron donor the terminal oxidases are divided into quinol oxidases and cytochrome *c* oxidases. Quinol oxidases contain one copper and two hemes as prosthetic groups whereas the cytochrome *c* oxidases contain a second copper Cu_A which accepts electrons from the soluble cytochrome *c*. In mitochondria this protein complex consists of 13 different subunits with the two major large subunits I and II. Bacterial terminal oxidases are generally composed of at least subunits I and II. The function of additional subunits is not known. Moreover, it has been shown that subunits I and II are sufficient for a fully functional complex.

The absolute and relative concentrations of cytochrome ba_3 as well as the other redox proteins do not vary with oxygen tension. A second terminal oxidase, if it is expressed in *N. pharaonis*, must be synthesized at concentrations that are orders of magnitude lower than that of cytochrome ba_3. Therefore, it is probable that the respiratory chain of *N. pharaonis* unlike the one of *H. salinarium* (Fujiwara et al., 1987) is unbranched.

The spectroscopic data of the purified cytochrome ba_3 show that heme A is partially reduced possibly with a ligand attached to it. It was not possible to chemically reoxidize the heme A component. Reasons for the lack of total reversibilty under these conditions could be due to a loss of essential subunits during purification or a partial denaturation by the low salt concentrations as well as by the treatment with detergents. On the other hand, it was possible to reversibly oxidize and reduce both heme A and heme B during the redox titration of the purified protein. The resulting midpoint potentials are almost identical to those measured in the intact native membrane. Furthermore, the binding of C═O to the reduced protein clearly shows that the protein is in its native state.

From the biochemical data of the natronobacterial cytochrome ba_3 it can be concluded that this enzyme belongs to the group of the cytochrome *c* oxidases. It consists of at least two subunits which might represent the cofactor containing proteins I and II. The high Fe/Cu ratio indicates that cytochrome ba_3 might be related to the cytochrome *c* oxidase family which has a Fe:Cu ratio of 2:3 (example given for cytochrome ba_3 from *Thermus thermophilus;* Fee et al., 1995). However, it cannot be excluded that the 2:3 ratio of the Fe:Cu couple might also be due to a partial loss of heme A during preparation, although recent results from the sequence of the cytochrome ba_3 gene suggest that the smaller subunit possesses the ligands for a Cu_A site (S. Mattar, personal communication). These data indicate that the terminal oxidase of *N. pharaonis* is a cytochrome *c*-type oxidase. Since only a cytosolic cytochrome *c* has been identified which, as discussed above, cannot be the electron donor to the cytochrome ba_3, the question arises which redox protein can overtake the role of the electron donor. The small blue copper protein halocyanin might have these properties. Halocyanin is presumably attached by its hydrophobic anchor to the periplasmic side of the plasma membrane (Mattar et al., 1994). Furthermore its redox potential of +128 mV at pH 8 would allow an electron transfer to cytochrome ba_3.

Redox Potentials. The redox potentials determined for the purified proteins and measured in the membrane fraction suggest a possible scheme for the respiratory chain. The sequence of electron transfer steps is either initiated by oxidation of succinate to fumarate or by dehydrogenation of NADH. The reactions are catalyzed by SDH or by NADH dehydrogenase, respectively. Both enzymes have been identified in *N. pharaonis* although NADH dehydrogenase has only been characterized enzymatically and by inhibition experiments (capsaicin blocks the respiration to about 50%; Wittenberg, 1995). The existence of SDH in *N. pharaonis* indicates that electrons from succinate are funneled via SDH into the respiratory chain. Indeed, succinate increases oxygen consumption (R. Wittenberg, M. Lübben, and M. Engelhard, unpublished results).

In the next steps the reduction equivalents could be transferred from SDH and/or NADH dehydrogenase to the cytochrome *bc* complex with menaquinone as mobile carrier, which has been identified in natronobacterial membranes (Wittenberg, 1995). An alternative possibility is that the cytoplasmic cytochrome *c* functions as electron acceptor. Its reduction potential of −142 mV places cytochrome *c* upstream of cytochrome *bc* which is compatible with its role as electron carrier between SDH and cytochrome *bc*. It should be noted that SDH could be oxidized by the cytosol, which includes cytochrome *c* as a possible substrate (data not shown).

The link between cytochrome *bc* and the terminal oxidase might be taken over by halocyanin. The redox potential of halocyanin (+128 mV) would allow for this role as well as its possible location at the extracellular side of the membrane. Future work must determine the sidedness of halocyanin as

well as its physiological interaction with cytochrome *bc* and cytochrome ba_3.

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