The Naphthoquinol Oxidizing Cytochrome bc_1 Complex of the Hyperthermophilic Knallgasbacterium *Aquifex aeolicus*: Properties and Phylogenetic Relationships†

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Received March 19, 2003; Revised Manuscript Received July 10, 2003

ABSTRACT: Phylogenetic analysis of constituent proteins of Rieske/cytochrome *b* complexes [Schütz et al. (2000) *J. Mol. Biol. 300,* 663−675] indicated that the respective enzyme from the hyperthermophile *Aquifex* (*A.*) *aeolicus* is closely related to proteobacterial counterparts, in disagreement with positioning of its parent species on small subunit rRNA trees. An assessment of the details and possible reasons for this discrepancy necessitates a thorough understanding of the biochemical and biophysical properties of the enzyme in addition to the bioinformatic data. The cytochrome bc_1 complex from *A. aeolicus*, which is part of the "Knallgasreaction" pathway, was therefore studied in membranes and in detergent-solubilized, isolated complex. Hemes b_L ($E_{m,7} = -190$ mV; $g_z = 3.7$), b_H ($E_{m,7} = -60$ mV; $g_z = 3.45$), and c_1 ($E_{m,7} = +160$ mV; $g_z = 3.55$) were identified by EPR and optical spectroscopy in combination with electrochemical methods. Two electrochemically distinct ($E_{m,7} = +95$ mV; $E_{m,7} = +210$ mV) Rieske centers were detected in membranes, and the +210 mV species was shown to correspond to the Rieske center of the cyt bc_1 complex. The gene coding for this latter Rieske protein was heterologously expressed in *Escherichia coli*, and the resulting protein was characterized in detail. The pool quinone of *A. aeolicus* was determined to be naphthoquinone. The redox poises of the individual electron-transfer steps are compared to those of other Rieske/cyt *b* complexes. The *Aquifex* enzyme was found to represent the only extant naphthoquinol oxidizing true cyt bc_1 complex described so far. An improved scenario for the phylogenetic positioning of the *Aquifex* cyt bc_1 complex is proposed.

Aquificales are hyperthermophilic chemoautotrophic bacteria with optimal growth temperatures in the range of 85−95 °C. Representatives of this phylum are frequently found in geothermically heated habitats such as deep sea hydrothermal vents, solfatares, or hot ponds. Phylogenetic analysis based on small subunit r-RNA sequences features the Aquificales as the earliest branching phylum known so far of the domain Bacteria. Bioenergetic electron transfer in *Aquifex* is based on an uncommon and only scarcely studied metabolic pathway, the electron transfer from hydrogen to oxygen, earning the species the classification among the "Knallgasbacteria". Only a few extant bacteria utilize this reaction for their energy supply, and none of them have been studied in detail so far. We recently reported the characterization of the enzyme responsible for the initial reaction in this chain, i.e., electron transfer from hydrogen toward the quinone pool (*3*). The presence of a cytochrome *bc* complex in *Aquifex* indicates that the reduced quinone pool is reoxidized by this enzyme which may subsequently direct the electrons toward soluble periplasmic electron carriers. We have recently described the properties of a soluble and a membrane attached cyt c_{555} (*4*), which are probable candidates for accepting electrons from the cytochrome *bc* complex.

In 1998, the genome of a member of the Aquificales, *Aquifex* (*A.*) *aeolicus*, was published (*1*) rendering phylogenetic analyses of specific proteins possible. In the framework of such an approach, the phylogenetic position of genes coding for cytochrome *b* and the Rieske protein of the cytochrome *bc* complex was found to differ from that of their parent organism, *A. aeolicus*, as deduced from 16S r-RNA analysis (*2*). According to this study, the gene sequence of the cytochrome *bc* complex may have been acquired by lateral gene transfer most probably from an ϵ-proteobacterial donor. The purification and biochemical and biophysical characterization of the cytochrome *bc* complex from *A. aeolicus* reported in this article allow an in depth comparison of the *Aquifex* enzyme with other Rieske/cyt *b* complexes and provide further insights into the evolution of proteobacterial *bc* complexes.

† F.B. and M.S. were supported by postdoctoral fellowships from FEBS and the CNRS, respectively. The work of K.O.S. was financially supported by the Fonds der Chemischen Industrie, and the project on *Aquifex* at the BIP in Marseille benefitted from financial support from the PCV program of the CNRS.

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10.1021/bi034452a CCC: $25.00
Published on Web 08/16/2003

MATERIALS AND METHODS

Membrane Preparation. A. aeolicus was grown and harvested as described previously (*2*) and stored at −80 °C. Frozen cells from *A. aeolicus* were washed once in 35 mM MOPS buffer pH 7.8 and then broken by three passages through a French pressure cell (50 MPa). After centrifugation of the sample at 4300*g* for 10 min to remove unbroken cells, the pellet was washed once and recentrifuged at 9700*g* for 15 min. The supernatant was centrifuged at 200000*g* overnight in the presence of 5 mM EDTA. The resulting pellet was immediately used for further protein purification.

Proteomic Methods. N-terminal amino acid sequences were determined from the enzyme preparation after separation of the subunits by SDS−PAGE.[1] Upon electrophoresis on 10% SDS−PAGE, proteins were transferred onto a PVDF membrane for 40 min at a current intensity of 0.8 mA/cm^2 using a semidry electrophoretic transfer unit. Sequence determinations were carried out with an Applied Biosystems A470 gas-phase sequenator. Quantitative determination of phenylthiohydantoin derivatives was done by high-pressure liquid chromatography (Water Associates, Inc) monitored by a data and chromatography control station (Waters 840).

Excised gel plugs were proteolyzed in situ with trypsin by the following procedure. Bands were washed with H_2O, 100 mM CH_3CN, NH_4HCO_3, and subsequently with CH_3CN (3 times). Reductive alkylation was carried out in 100 mM NH_4HCO_3 with 10 mM DTT for 45 min at 56 °C followed by incubation in 100 mM NH_4HCO_3 with 55 mM Iodoacetamine for 30 min at 25 °C. Finally, two incubation steps in 100 mM NH_4HCO_3 for 5 min and in CH_3CN for 5 min were undertaken. For proteolysis, the bands were incubated in trypsin (Promega) 12.5 ng/μL in 25 mM NH_4HCO_3 for 45 min and subsequently washed (overnight) in 25 mM NH_4HCO_3. The supernatant was desalted and analysis of peptides was performed on MALDI-TOF Voyager DE-RP (Perceptice). Protein identification by mass spectrometry data was accomplished using Profound software against NCBI's nr database (http://prowl.rockefeller.edu/cgi-bin/ProFound).

Expression of pet A in Escherichia coli and Purification of the Gene Product. Two primers, petA1 (5′ gcggtaccttatatgagtttctttacaaa 3′) and petA2 (5′ gcgaattcatggccaaaacggaaatcgac 3′), were designed to PCR amplify a truncated version of *A. aeolicus petA* gene from nt 2362 to 2781 (*1*). Primer petA2 introduced an *Eco*RI site together with an initiation codon (ATG) immediately upstream from the alanine codon at position 2781, while primer petA1 introduced a *Kpn*I site downstream from the stop codon TAA at position 2362 in the genome.

The 437 bp amplicon was then digested by both *Eco*RI and *Kpn*I and subsequently cloned into plasmid pJF119EH (*5*) cut with the same two enzymes to give plasmid p119petA2. The sequence of the plasmid was verified by DNA sequencing.

E. coli TG1(p119petA2) was grown in 10 L of LB medium (ref *6*; supplemented with ampicillin (100 μg/μL)). At $OD_{600\,nm}$ = 0.6, IPTG (0.5 mM final concentration) was added and cells were cultured for 4 more hours. Cells were then pelleted, resuspended in 10 mM TRIS buffer (pH 7.6), and broken with a French press (50 MPa). This crude extract was then incubated for 40 min at 80 °C. The precipitated proteins were pelleted by ultracentrifugation (2 h at 170000 g) and the supernatant was loaded onto a carboxymethyl cellulose (CM52, Whatman) column, equilibrated with 10 mM TRIS buffer (pH 7.6). Proteins were eluted using a TRIS step-gradient ranging from 10 to 200 mM. Truncated PetA (tPetA) was eluted at 150 mM TRIS (pH 7.6).

Purity of the fraction was checked by SDS−PAGE (*7*). N-terminal sequence was determined on an Applied Biosystems A470 sequenator. The molecular mass of tPetA was determined by mass spectrometry on a Voyager DE-RP perceptive mass spectrometer.

Electrochemistry. Redox titrations of the purified cytochrome *bc* complex were performed electrochemically in a thinlayer cell in the visible spectral range (*8*, *9*). Mediators (diferrocene, monoferrocene, ferricyanide, *p*-benzoquinone, 2,5-dimethyl-*p*-benzoquinone, 1,2-naphthoquinone, 1,4-naphthoquinone, 2-methyl-1,2-naphthoquinone, 2,5-dihydroxybenzoquinone, anthraquinone, anthraquinone-2,6-disulfonate, anthraquinone-2-sulfonate) were present at a concentration of 75 μM each. Titrations were performed in oxidative and reductive directions in steps of 50 mV. Equilibration time was 5−20 min. Spectra were recorded on a Kontron/Uvikon spectrophotometer. The data were submitted to the global fit procedure mE_h-fit (Levenberg−Marquardt algorithm and general least-squares algorithm) (*10*) for the determination of redox midpoint potentials and corresponding amplitudes.

The redox midpoint potential of the Rieske protein expressed in *E. coli* was determined by cyclic voltammetry at different pH values. CVs were run at a scan rate of 20 mV s^{-1} on glassy carbon electrodes with 2 μL protein solution confined by a dialysis membrane to a uniform thin layer at the electrode surface (*11*). A mixed buffer consisting of 100 mM TRIS + 100 mM sodium acetate + 100 mM sodium borate was used and adjusted to the desired pH by adding sodium hydroxide or acetic acid. Potentials versus the standard hydrogen electrode have been obtained by adding 190 mV, as determined by calibrating the electrode versus saturated quinhydrone at pH 7.0.

EPR Spectroscopy. EPR spectra were obtained on a Bruker ESP300e X-band spectrometer fitted with an Oxford Instrument He-cryostat and temperature control system. Redox titrations on membrane fragments were performed as described by Dutton et al. (*12*) in the presence of mediators (100 μM each of neutral red, safranine T, anthraquinone-2-sulfonate, anthraquinone-2,6-disulfonate, indigocarmine, 2,5-dihydroxy-*p*-benzoquinone, methyleneblue, cresylblue, 1,2-naphthoquinone, 2,5-methyl-*p*-benzoquinone, 2,6-dichlorophenol, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine). Reductive titrations were carried out using sodium dithionite, oxidative titrations using ferricyanide and potassium hexachloroiridate (IV). Oriented membrane multilayers were produced as described by Rutherford and Sétif (*13*). Spectra in the

[1] Abbreviations: E_m, midpoint potential; E_h, ambient redox potential; EPR, electron paramagnetic resonance; EDTA, ethylenediaminetetraacetic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TRIS, tris[hydroxymethyl]aminomethane); AMPSO, (3-[1,1-dimethyl-2-hydroxy-ethyl)amino]-2-hydroxy-propanesulfonic acid; TMBZ, 3,3′,5,5′-tetramethylbenzidine; PVDF, polyvinylidene fluoride; DTT, dithiotreitol; PCR, polymerase chain reaction; OD, optical density; DEAE, diethylaminoethyl.

presence of stigmatellin were obtained by adding 100 μM of the inhibitor to the membranes before freezing.

RESULTS

Protein Purification. Membranes were prepared from 15 g (wet weight) of *A. aeolicus* cells as described in Materials and Methods. They were solubilized at 10 mg/mL of protein in 3% *n*-dodecyl β-D-maltoside for 1 h under continuous shaking at room temperature and subsequently centrifuged at 200000*g* for 1 h. The supernatant was loaded onto a DEAE Biogel A column, equilibrated with 35 mM MOPS pH 7.8, 0.01% *n*-dodecyl β-D-maltoside. The cytochome *bc* complex was followed by its optical absorption in the 400 to 600 nm range and was found to elute at 50 mM NaCl. The respective sample was concentrated on centriprep 30, washed with buffer (35 mM MOPS, pH 7.8, 0.01% *n*-dodecyl β-D-maltoside), and loaded onto a DEAE TRIS acryl (Biosepta) column equilibrated with the same buffer. The cytochrome *bc* complex came off this column at 50 mM NaCl. The respective fraction was concentrated on centriprep 30 and again washed with buffer (35 mM MOPS, pH 7.8, 0.01% *n*-dodecyl β-D-maltoside). This sample was then loaded onto an Ultragel ACA34 (Biosepta) column, equilibrated with the same buffer at a flow rate of 0.2 mL/min. Fractions containing the cytochrome *bc* complex were pooled as a function of the ratio of the amplitudes at 280 nm and at 430 nm. Four pools with ratios of 0.84, 0.86, 1.01, and above were obtained. The total amount of cells available for purification was limited due to the low yield and high cost of *A. aeolicus* cultures. The quantity of sample obtained with the highest purity index was consequently insufficient for optical redox and EPR experiments and was therefore dedicated to SDS gel electrophoresis and sequencing. Optical redox and EPR data were gathered on pools 2 and 3, respectively.

Subunit Composition. On SDS gels (not shown), the sample with $A_{420\ nm}/A_{280\ nm}$ of 0.84 reproducibly contained three major and three to four minor bands. The major bands were found to migrate at 21, 25, and 40 kDa, and minor bands stained at 14, 32, and above 55 kDa. The respective bands were subjected to N-terminal sequencing and mass spectrometry of trypsin proteolyzed fragments. An N-terminal sequence reading G-L-I-E-K-V-I-D-W-I was obtained for the protein migrating at 40 kDa. This sequence corresponds to the N-terminal sequence of cytochrome *b* (PetB) from the *A. aeolicus* cytochrome *bc*-operon. It is noteworthy that in the annotated genome, V and I in the K-V-I-D stretch are interchanged. Since the results of the N-terminal sequencing are unambiguous and since the used strain is identical to the sequenced one, we consider this interchange as probably due to an error during the sequencing procedure. The 21 kDa band was identified by mass spectroscopy on trypsin fragments (with a sequence coverage of 25%) as the Rieske subunit (the petA gene product). The band at 25 kDa was the only band heme-stained by TMBZ and was therefore assigned to cytochrome c_1. Unfortunately, no N-terminal sequences could be obtained for the Rieske protein (21 kDa band) and cytochrome c_1 (25 kDa band). The minor bands are due to contaminating oxidases which were also detected during redox titrations and in EPR spectra (see below). The intensity of the optical peaks observed in redox titrations demonstrates that the amount of oxidase is less than 20% of that of the cytochrome *bc* complex.

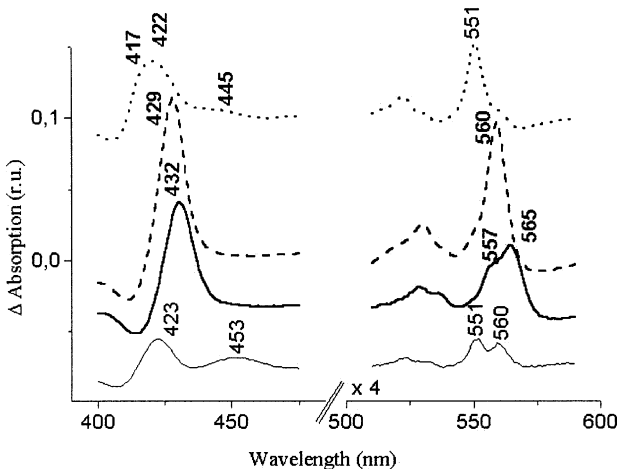


FIGURE 1: Redox-induced difference spectra of the cyt bc_1 complex from *A. aeolicus*. The spectra were obtained by a global fit to the data of a redox titration. Bold line: cytochrome b_L, $E_{m,7}$: −190 mV, dashed line: cytochrome b_H, $E_{m,7}$: −60 mV; dotted line: cytochrome c_1; $E_{m,7}$: +160 mV; continuous line: contaminating oxidase; $E_{m,7}$: +230 mV.

Biophysical Parameters of Observed Redox Centers. The sample showing an A_{430}/A_{280} ratio of 0.86 (pool 2) was used for electrochemical redox titrations in the visible spectral range from 400 to 650 nm. Deconvoluted spectra of the individual redox species were obtained by a global fit procedure (mE_h-fit, see Materials and Methods). Three major redox species with E_m values of −190, −60, and +160 mV were obtained from the global fit. Figure 1 shows the optical spectra corresponding to these three major redox centers. The −190 mV component is characterized by a split α-band (with a peak at 565 nm and a shoulder at 557 nm), a split β-band at 531/537 nm, and a Soret band at 432 nm. The −60 mV heme center peaks at 560, 531, and 429 nm, whereas the +160 mV heme has absorption maxima at 551 nm, at 523 nm, and at about 420 nm. On the basis of redox midpoint potentials and spectral parameters, the −190, −60, and +160 mV redox centers are attributed to cytochrome b_L, cytochrome b_H, and cytochrome c_1, respectively. The redox titration furthermore reveals that small amounts of oxidases were present in this sample as evidenced by the redox components at potentials above +240 mV (Figure 1, thin line, a detailed characterization of cytochrome oxidases from *A. aeolicus* will be reported elsewhere).

Figure 2 (top) shows EPR spectra obtained on isolated cytochrome bc_1 complex from *A. aeolicus* (pool 3) in the untreated (i.e., oxidized), ascorbate reduced and dithionite reduced states. The untreated sample featured a broad signal in the region of $g = 3.7$ to $g = 3.4$ (due to oxidized low spin heme species) as well as a relatively narrow peak at $g = 3$. Reduction by ascorbate strongly diminished the contribution of hemes with $g < 3.6$ revealing a well-resolved asymmetric peak at $g = 3.7$. The peak at $g = 3$, a contribution at $g = 3.4$ and the trough at $g = 1.76$ arise from the contaminating oxidase (a detailed description of the EPR parameters of *Aquifex* oxidases will be reported elsewhere). From the combination of the results of the optical redox titration on the isolated enzyme and of an EPR redox titration on membranes (see below), spectral species at $g =$

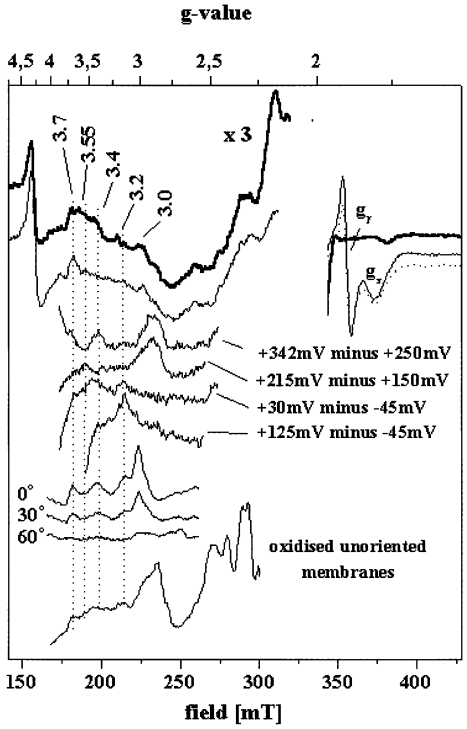


FIGURE 2: EPR spectra of paramagnetic redox species observed in isolated cyt bc_1 complex and in membrane fragments from *A. aeolicus*. The top spectra have been recorded on the isolated enzyme. Bold line: no addition; continuous line: ascorbate reduced; dotted line: dithionite reduced (only depicted for the right-hand field region of the Rieske center. The amplitude of the signals in the region above $g = 2.1$ was increased by a factor of 3 to better visualize the heme signals. The four spectra labeled as redox difference spectra correspond to redox differences calculated from the data obtained in an EPR redox titration on membranes. The three spectra labeled 0°, 30° and 60° have been recorded on partially ordered membrane multilayers in the oxidized state. The spectrum at 90° has been subtracted from absolute spectra at the indicated angles to yield the depicted spectra. The bottom spectrum represents the low spin heme region measured on nonoriented membrane fragments in the oxidized state. Instrument settings: temperature, 15 K; microwave frequency, 9.42 GHz; microwave power, 6.7 mW; modulation amplitude, 2.8 mT.

3.7, $g = 3.45$ and $g = 3.55$ (Figure 2, middle) were identified as arising from hemes b_L, b_H, and c_1, respectively. The spectral closeness of the three hemes' g_z-peaks rationalizes the broad, unresolved aspect of the g_z-region in the spectrum of the oxidized sample.

The maximal amplitude of the Rieske protein's EPR spectrum already appeared upon reduction by ascorbate. It showed the typical derivative-shaped g_y line at $g = 1.89$ and a g_x trough in the region of $g = 1.8$ (see also Figure 3). Upon reduction with dithionite, the signal amplitude slightly diminished, a feature reported previously for the Rieske protein from mitochondria (*14*). The fact that full reduction of the Rieske center was achieved by addition of ascorbate to an undegassed sample argues for an E_m value above +100 mV. To characterize the redox properties of the Rieske protein in more detail, the petA gene was expressed in *E. coli*, and the recombinant protein was purified and characterized (see below).

Identification of Low Spin Heme Species via EPR Titrations on Membrane Fragments. The bottom EPR spectrum in Figure 2 shows the heme region taken on untreated, i.e., relatively oxidized membrane fragments. In the field range

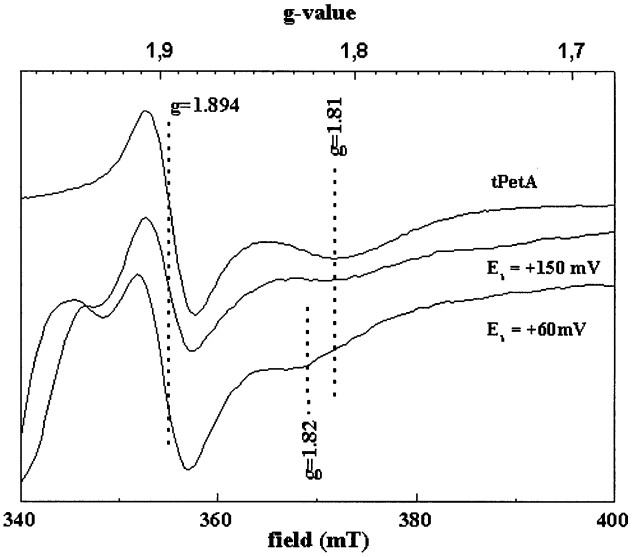


FIGURE 3: EPR spectra of Rieske-type paramagnetic centers in membrane fragments and in the *E. coli* expressed PetA protein. The lower two traces represent spectra obtained during a redox titration at pH 7 at the ambient potentials of +150 and +60 mV mV. The top spectrum was obtained on the heterologously expressed PetA Rieske protein in the asccorbate reduced state. Instrument settings: as for Figure 2, except for a modulation amplitude of 1.6 mT.

of low spin g_z-peaks, several paramagnetic species overlapped to yield a basically unresolved spectrum in which only a $g = 3.7$ and a $g = 2.9$ line could be clearly distinguished. The $g = 2.9$ peak is due to the previously characterized cytochrome c_{555}^m (*4*). To identify individual redox species, EPR titrations on membrane fragments were performed. Figure 2 (middle part) shows selected redox difference spectra identifying individual redox species. In the region between −100 and +100 mV, two different heme centers with g_z-peaks at $g = 3.45$ and 3.2 were seen to titrate. The $g = 3.2$ peak corresponds to that of the *b*-type cytochrome detected in hydrogenase 1 from *A. aeolicus* (*3*). It was found to titrate at slightly higher potential values than the g-3.45 peak (compare difference spectra +30 minus −45 mV and +125 minus −45 mV) which was therefore attributed to cyt b_H, for which an E_m value of −60 mV had been determined in optical redox titrations on the isolated enzyme (see above). For cyt c_1, the optical results yielded a midpoint potential of +160 mV. A corresponding redox cut in EPR titrations showed peaks at $g = 3.55$ and 2.9. As mentioned above, the $g = 2.9$ peak belongs to cyt c_{555}^m (E_m = +220 mV) and the $g_z = 3.55$ signal was consequently attributed to cyt c_1. This situation differs form that encountered in bc_1 complexes of proteobacteria where the g_Z signal of cytochrome c_1 is found on the high field side of the cytocrome b_H signal. At E_h values below −100 mV, only the asymmetric, ramp-shaped peak at $g = 3.7$ could be discerned (not shown). Both its low E_m value and its characteristic shape and *g*-value identify this signal as being due to heme b_L. The EPR signals of all three cytochromes from *Aquifex* bc_1 complex overlap in the spectrum of the isolated oxidized complex (Figure 2, top). At potentials above +250 mV, a $g_z = 3.4$ peak from one of the oxidases appeared, rationalizing the shoulder at this field position in the spectrum of the isolated complex in the oxidized state.

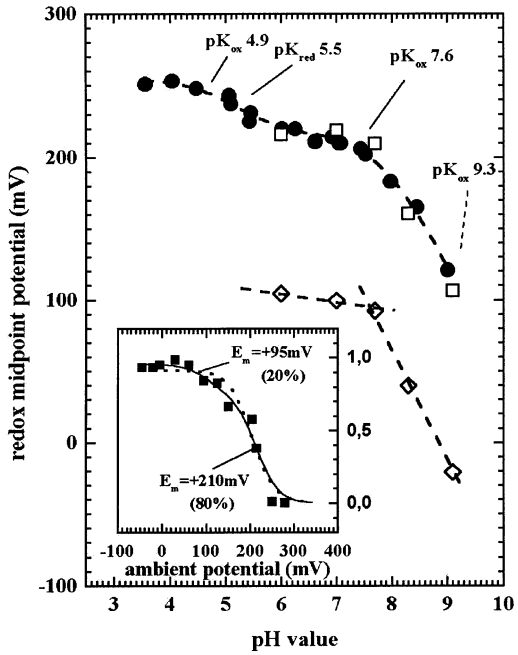


FIGURE 4: Electrochemical parameters of Rieske centers observed in membrane fragments and on the recombinant PetA protein. The main panel shows the pH-dependences of the redox midpoint potentials of the low (open diamonds) and high (open squares) potential components in membrane fragments (as obtained by chemical EPR titrations) and of the recombinant PetA protein (filled circles; measured by cyclic voltammetry). Cyclic voltammetry was performed in a buffer mixture of 100 mM sodium acetate/100mM TRIS/100 mM sodium borate. The data were fitted to two pK_{ox} values and one pK_{red} value (continuous line). The dashed line represents a fit to three pK_{ox} and one pK_{red} values. The inset shows $n = 1$/two-components (continuous line) and $n = 1$/one-component (dotted line) Nernst fits to data points corresponding to the signal amplitude of the $g = 1.89$ line in membrane fragments from *A. aeolicus.*

Two Electrochemically Distinct Rieske Centers Are Observed in Membrane Fragments. As can be seen from Figures 3 and 4 (inset), the spectral species showing the typical g values of a Rieske [2Fe−2S] center titrated as two electrochemically distinct entities, i.e., at +95 and at +210 mV (Figure 4, inset). In the sample used for the titrations shown in Figures 3 and 4, the higher potential redox species was present at a four times higher concentration than the lower potential center (Figure 4, inset). The stoichiometry of these two centers was observed to vary between different batches of cell material. The low potential form, however, was detected in redox titrations at all pH values. The pH dependences of the midpoint potentials of both redox species were found to be roughly similar with a pK value on the oxidized form of the redox center at about 7.6 (Figure 4), i.e., in line with those measured on typical Rieske centers from Rieske/cyt*b* complexes (*15*). This finding raised the question which of the two redox species (or both?) corresponded to the [2Fe−2S] protein of the cyt bc_1 complex.

Two arguments favor the higher potential redox species as the Rieske center belonging to the cyt bc_1 complex. (a) The Rieske center in the isolated complex is fully reduced by ascorbate in undegassed samples (Figure 2). (b) In Figure 3, a comparison of the detailed spectral parameters of the *E. coli* expressed PetA (see following section) and the different species observed during redox titrations is shown. The high potential species predominantly present in the spectrum at an ambient potential of +150 mV (Figure 3) displayed an EPR spectrum similar to that of PetA. When the potential was lowered so that both redox species were reduced (E_h = +60mV), both the g_y and (more markedly) the g_x peak shifted to lower magnetic fields demonstrating that the low potential component must be characterized by a spectrum distinctly different from that of the high potential cluster. The g values of both the high potential species and PetA correspond well to those observed on the Rieske protein in the isolated enzyme (Figure 2).

Characterization of the PetA Rieske Protein Expressed in E. coli. Our observation of two electrochemically distinct Rieske centers in *Aquifex* membranes is rationalized by the presence in the genome of *A. aeolicus* of two genes, soxF and petA, showing the characteristic Rieske protein sequence signatures. PetA is part of the petABC operon with petB and petC coding for cytochrome *b* and cytochrome c_1 of the *bc* complex, respectively. SoxF is located in a different position in the genome. The genes of both Rieske proteins were introduced into *E. coli* hosts, but only the petA gene product could be expressed in significant quantities so far.

PetA contains an N-terminal hydrophobic sequence that anchors the protein to the membrane. To produce a soluble form of the protein, a truncated version of the *petA* gene, in which the first 126 bases encoding the first 42 residues were deleted, was constructed by PCR using two specifically designed primers, petA1 and petA2, respectively. These primers were designed to introduce a methionine at position 43 in the wild-type sequence and unique restriction enzyme sites on both sides of the gene that were used to clone the amplicon into the expression vector, pJF119EH. A soluble form of *A. aeolicus* tPetA was thus produced in *E. coli*. The conditions we used allowed us to produce 20 mg of pure tPetA from 10 L culture of *E. coli* TG1 (p119petA). Because of its high thermal stability, tPetA was easily purified as *E. coli* proteins from the crude extract were precipitated by heating. Only one ion-exchange chromatography on CM-52 was necessary to obtain pure tPetA.

The N-terminal sequence of tPetA was found to be A-K-T-E-I-D-V-S-K-I which is identical to the sequence deduced from the *Aquifex* genome. It is noteworthy that the methionine residue which was introduced at the 5′-end of the truncated gene for translation was found to be cleaved in the mature tPetA. The molecular mass determined by mass spectrometry (15172.8) was very close to the expected mass (15176.89) calculated from the protein sequence.

The midpoint potential of tPetA at pH 7 was determined by equilibrium redox titrations and by cyclic voltammetry to +210 ± 20mV. The pH dependence of the midpoint potential was determined by cyclic voltammetry (Figure 4, full symbols). At pH 3 and pH 9, the peak separations of the cyclovoltammograms started to increase, indicating a slower electron exchange with the electrode. This phenomenon, however, was not due to irreversible denaturing of the sample since bringing the pH back to 7 restored the initial voltammogram. The obtained data points were fitted assuming a pK_{ox} value of 7.6. Toward higher pH values, the midpoint potential decreased by 60 mV per pH unit. Below pH 7, a less pronounced pH dependence of the midpoint potential was observed. The curve was fitted assuming a pK_{ox} value of 4.9 and a pK_{red} value of 5.5. The close vicinity of these two values results in the weak slope of the pH

dependence of the redox midpoint potential of only −25 mV per pH unit. Such a pH dependence was also observed for the Rieske protein from *Thermus thermophilus* where it was attributed to the effect of an ionizable amino acid residue, which is not in direct contact with the [2Fe−2S] cluster but still close enough to influence the Rieske E_m value via electrostatic interaction (*17*). Two distinct pK_{ox} values were reported for Rieske proteins in the alkaline pH range and assigned to sequential deprotonation of the cluster ligating histidine residues (*17*−*19*). Our data points are indeed slightly better fitted assuming two pK_{ox} values of 7.6 and 9.3. The poor reaction of the *Aquifex* PetA protein with the electrode at pH values above pH 9, however, precluded an unambiguous determination of the strongly alkaline pK_{ox} value.

A comparison of the pH dependences of the petA protein and the Rieske centers observed in membranes (Figure 4) unambiguously demonstrate that it is the +210 mV redox species that corresponds to the petA gene product.

The EPR spectrum of the ascorbate reduced tPetA protein is shown in Figure 3.

In Situ Stoichiometry between the cyt bc_1 Complex and Oxidases. Electrochemical redox titrations on membrane fractions were performed in an optical thin layer cell. The vast majority of the redox active spectral species in the visible region could be assigned to the cyt bc_1 complex, hydrogenase, cytochrome oxidase, and cytochrome $c_{555}{}^m$. Their midpoint potentials and spectra were very close to those found in the redox titrations of the isolated proteins. The experiments on membranes, however, additionally allowed us to estimate the stoichiometry of the two protein complexes in the membrane to approximately 1 oxidase per 2 cyt *bc* complexes.

EPR on Partially Ordered Membrane Multilayers. Figure 5 shows the orientation of the higher potential Rieske center's paramagnetic axes as deduced from partially ordered ascorbate reduced membrane fragments. The orientation of the g_y signal was found to be parallel to the membrane plane, whereas the g_x signal was oriented perpendicular to the membrane plane.

The lower part in Figure 2 shows representative spectra in the region of low spin hemes recorded on oxidized, partially ordered membranes. To eliminate the steep baseline due to the presence of a large (unoriented) rhombic iron signal at $g = 4.1$, the spectrum at 90° (magnetic field with respect to the membrane plane) was subtracted from those at 0°, 30°, and 60°. The dependence of signal amplitudes on angle indicate that all observed low spin heme species pointed their g_z direction parallel to the membrane, i.e., that the heme planes are all oriented roughly perpendicular with respect to the plane of the membrane. At the field position of cyt c_1's g_z signal, no peak was seen in the spectra taken on oriented membranes at low angles nor in difference spectra calculated between various pairs of angular orientations. Two explanations for this finding are possible. (a) The cyt c_1 heme is unoriented. Although we cannot formally exclude this possibility, it nevertheless seems unlikely to us. (b) The cyt c_1 g_z peak is indeed present (and maximal) at $g = 3.55$ in the spectrum at 0°. The fact that two signals (from heme b_H and from oxidase) overlap at $g = 3.4$ results in a substantial signal amplitude at this field position thereby masking the contribution of cyt c_1. The cyt c_1 heme thus seems to be oriented perpendicular to the membrane similar to its counterpart in the mitochondrial complex.

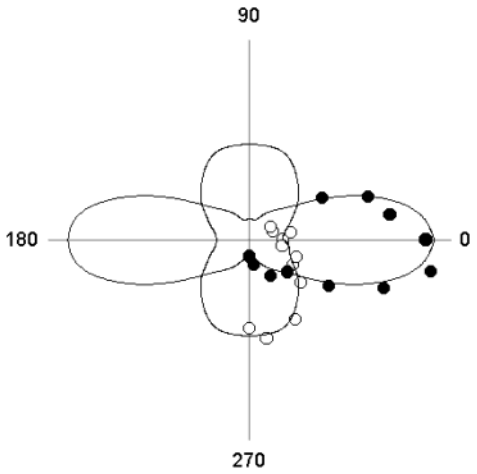


FIGURE 5: Orientations of the paramagnetic axes g_y and g_x of the +210 mV-Rieske center as measured on partially oriented ascorbate reduced membranes. The normalized amplitude of the signal at $g_y = 1.89$ (full circles) and $g_x = 1.83$ (open circles) versus the angle between membrane plane and magnetic field is depicted.

DISCUSSION

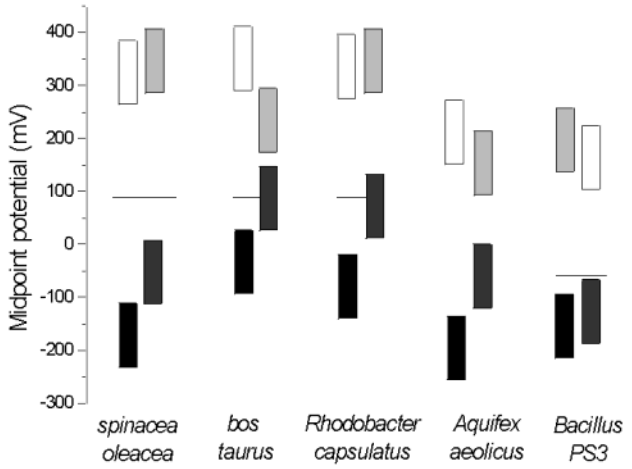
The Aquifex Enzyme Belongs to the bc_1 Subgroup of the Rieske/cytb Complexes. Rieske/cytochrome *b* complexes are found in almost all energy conserving electron-transfer chains of both Archaea and Bacteria. The biochemical characterization of the *Aquifex* enzyme reported in this work are strongly reminiscent of the proteobacterial cytochrome bc_1 complex. (a) Cytochrome b_L shows a distinctly split α-peak which is characteristic for proteobacterial cytochrome bc_1 complexes and is not seen in characterized Rieske/cytochrome *b* complexes from the other phylogenetic groups as characterized so far. (b) The approximate stoichiometry of redox cofactors determined in the isolated complex suggests the presence of only one heme *c* per enzyme. (c) The heme group of cytochrome c_1 in *Aquifex* appears to be oriented perpendicular to the membrane, i.e., similar to that of cytochrome bc_1 complexes and different from the case of the cytochrome b_6f complexes. For the other groups, respective data are not available so far. (d) The Rieske protein shows an orientation behavior strictly similar to that of cytochrome bc_1 complex (*21*).

Phylogenetic analyses of the petA and petB genes, encoding the Rieske and the cytochrome *b* subunits of the enzyme, respectively, position the *Aquifex* Rieske/cytochrome *b* complex close to its ϵ-proteobacterial counterparts. Although the Rieske and cytochrome *b* subunits of the enzymes found in ϵ-proteobacteria are phylogenetically close to those of the cytochrome bc_1 complexes present in α-, β-, and γ-proteobacteria, their cytochrome *c* subunit is a diheme protein similar to cytochrome *cc* in Rieske/cytochrome *b* complexes of high-GC Gram-positive bacteria (*20*). The ϵ-proteobacterial enzyme is therefore distinct from the "true" cytochrome bc_1 complexes (*2*). The cyc gene from *A. aeolicus*, however, clearly encodes a monoheme cytochrome unlike its corresponding gene in ϵ-proteobacteria. Despite its phylogenetic proximity to the ϵ-proteobacterial enzymes, the *Aquifex* complex thus appears to be a genuine cytochrome bc_1 complex.

Table 1: Summary of the Spectral and Electrochemical Parameters of the Redox Components Attributed to the cyt bc_1 complex as well as the SoxF-type Rieske Center Observed in Membranes

g value	λ (α-band)	$E_{m,7}$ (mV)	cofactor
3.7	565 nm/557 nm	−190	heme b_L
3.45	560 nm	−60	heme b_H
3.55	551 nm	+160	heme c_1
1.89		+210	Rieske (PetA)
1.89		+ 95	Rieske (SoxF)

Scheme 1 : Representation of the Relative Arrangement of Redox Cofactors in Rieske/cyt*b* Complexes from Different Organisms[a]



[a] Rectangles denote the productive reaction range (60 mV above and below the midpoint potential) of the cofactors on a redox scale. The line indicates the (two-electron transition) midpoint potential of the corresponding quinone. Values for Rieske/cytochrome *b* complexes were taken from the following references: spinach (*41*), bovine mitochondria (*41*), *Rhodobacter capsulatus* (*9*), *A. aeolicus* (this work), and *Bacillus PS3* (*27*, *24*). Values for quinones are from refs *24* and *42*.

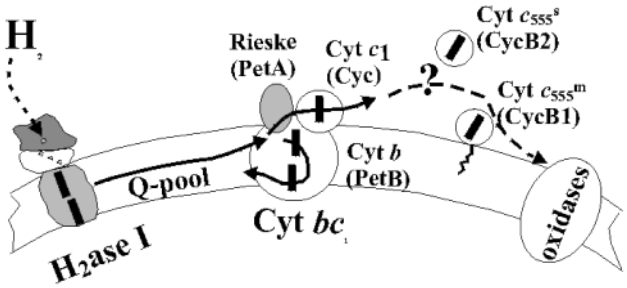
The Aquifex Enzyme Is a Naphthoquinol-Oxidizing Cytochrome bc_1 Complex. A major difference between the *Aquifex* enzyme and all other cytochrome bc_1 complexes studied so far consists in the redox midpoint potentials of the cofactors (see Table 1 and Scheme 1). The respective E_m values are significantly lower than in typical cytochrome bc_1 complexes but are strongly reminiscent of those found in menaquinol-oxidizing Rieske/cytochrome *b* enzymes (Scheme 1) (*2*, *23*−*28*). From an inspection of quinone synthesis genes in the *Aquifex* genome it was suggested that *Aquifex* possibly uses ubiquinone (UQ) as pool quinone (*29*), just as the α-, *β*-, and *γ*-proteobacteria do (*30*). Our analysis of the quinone content in *Aquifex* cells, however, clearly showed that the pool quinone in *Aquifex* is naphthoquinone (Baymann & Friedrich, manuscript in preparation), and the *Aquifex* Rieske/cytochrome *b* enzyme therefore represents the first cytochrome bc_1 complex oxidizing naphthoquinones.

Tracing down the Proteobacterial Donor for Lateral Gene Transfer. The above-described parameters allow one to obtain further information concerning the putative donor for lateral gene transfer into *Aquifex*. As already discussed in Schütz et al. (*2*), it appears very unlikely to us that the *Aquifex* cytochrome bc_1 complex was imported from an organism with a ubiquinone as pool quinone. The misfit in redox poises would have resulted in an only poorly functioning enzyme, and a considerable number of simultaneous mutations would have been necessary to render the imported complex useful. By contrast, extant *ϵ*-proteobacteria contain a menaquinone pool and thus seem better suited as potential donors for transfer of a functionally productive Rieske/cytochrome *b* complex into the Aquificales. Sequence analysis of cyt *b*, the Rieske protein (*2*), and the hydrogenases 1 and 2 from *Aquifex* (*3*) indicated that the respective protein subunits cluster together with their counterparts from either *ϵ*- or *δ*-proteobacteria. The transition from mena- to ubiquinone appears to have happened somewhere on the line between *ϵ*- and *δ*-proteobacteria on one side and the α-, *β*-, and *γ*-subdivisions on the other side (see (*2*)). Since the *Aquifex* enzyme contains a cytochrome c_1 rather than the diheme cytochrome of *ϵ*-proteobacteria, it seems likely to us that the donor organism corresponded to a hyperthermophilic ancestor of α-,*β*-,*γ*-proteobacteria close to the branching point of the *ϵ*-proteobacteria which already had substituted the diheme cytochrome for cyt c_1 but had not yet undergone the transition from the low potential (oxygen-labile) menaquinone to the high potential ubiquinone adapted to aerobic conditions. *ϵ*-Proteobacteria as well as so far uncharacterized clusters branching between the *ϵ*- and the α,*β*,*γ*,*δ*-groups were found in hyperthermophilic habitats which they share with Aquificales (*31*), a condition essential for lateral gene transfer to take place. Lateral gene transfer from a mesophilic organism indeed seems highly unlikely to us since a large number of mutational adaptations would be necessary before a thermolabile cytochrome bc_1 complex could function in *Aquifex* and thus confer an evolutionary advantage.

Two Rieske proteins in A. aeolicus. As mentioned above, in addition to petA, a second gene coding for a Rieske protein (annotated as soxF) is present in the genome of *A. aeolicus*. Correspondingly, two electrochemically distinct (E_m = +95 mV and E_m = +210 mV) EPR observable Rieske proteins were detected in membranes. Two key residues have previously been shown to influence the redox midpoint potential of Rieske proteins, i.e., a serine and a tyrosine residue located two and four residues downstream of the second cluster binding motif (*32*, *33*). Lack of hydrogen bond interactions from these two sequence positions to the cluster in the absence of one or both of these residues was shown to significantly decrease the E_m value of the [2Fe−2S] center. The serine residue was shown to confer a substantially larger effect than the tyrosine residue. Guided by these data, we previously (*2*) attributed the lower Rieske E_m value in *Aquifex* membranes to PetA, in which the serine is replaced by glycine, whereas the tyrosine is conserved. In SoxF, by contrast, the serine is conserved and a phenylalanine is present instead of tyrosine. The data detailed above obtained on the purified complex and on the PetA protein expressed in *E. coli* unambiguously demonstrate that the higher E_m of +210 mV belongs to the petA gene product present in the complex. This implies that parameters other than the mere replacement of the hydrogen bonding tyrosine residue must strongly contribute to the relatively low E_m value of +95 mV of SoxF.

For the cyanobacterium *Synechocystis* PCC6803, it has recently been proposed that the product of one of the additional Rieske genes present in the genome, called petC3, may replace the "genuine" subunit, the gene of which is associated to cytochrome *f* in an operon context (*34*). Similar

Scheme 2: Representation of Redox Complexes and their Hypothetical Interplay in the Electron Transfer Chain Linking the Oxidation of H_2 to the Reduction of O_2 in *A. aeolicus*



to the case of *Aquifex*, this putatively substituting Rieske protein has a significantly lower redox potential than the original one. We consider such a scenario as very unlikely for the case of *A. aeolicus* since no indication for the presence of a low potential component has been obtained in the purified enzyme. By contrast, significant amounts of the soxF gene product are present in membranes arguing for a functional role different from a Rieske/cytochrome *b* context.

The raison d'être of the second Rieske genes in a number of genomes sequenced so far indeed is an interesting question (see ref *2*). A subgroup of these additional Rieske proteins has recently been recognized as being part of the enzyme arsenite oxidase (*35*). SoxF in *Aquifex* clearly does not belong to this group (see ref *35*) and its functional role remains enigmatic at the present time.

Role of the Cytochrome bc_1 Complex in the Energy Conserving Electron Transport Chain of A. aeolicus. Aquifex is one of the few known organisms that obtain their energy from the so-called "Knallgas reaction", i.e., the electron transfer from hydrogen to oxygen. The bioenergetics of these organisms have not been characterized in detail so far. The biochemical characterization of the three hydrogenases present in *A. aeolicus* was reported recently (*3*). Two of them are membrane associated and are probably anchored to the membrane via a cytochrome *b* subunit. Interestingly, both of these hydrogenases belong to the proteobacterial heritage in the *Aquifex* genome. It has been proposed that the first step of the Knallgas reaction consists of an electron uptake from hydrogen by hydrogenase I and subsequent reduction of the quinone pool via the cytochrome *b* subunit (*3*). The cytochrome bc_1 complex most probably catalyzes the subsequent electron-transfer step from the quinone pool toward a periplasmic electron carrier. Two *c*-type cytochromes from *Aquifex* (*4*) are very likely candidates for accepting electrons from the cytochrome bc_1 complex. Both these cytochromes are of type I with 80% amino acid identity in the membrane-extrinsic, heme binding domain. One of them consists only in this domain and is soluble, whereas the other one is attached to the membrane by an N-terminal extension of 61 amino acids, keeping the heme binding domain mobile (*36*). There are several other examples where soluble or membrane attached type I cytochromes act as electron shuttles between the cytochrome *bc* complex and the oxidase (*37*−*40*), but in no case a second cytochrome with so similar properties has been described. Both proteins are located in the periplasm, and they appear to be genuine proteins from *Aquifex* in contrast to the imported cytochrome bc_1 complex and the hydrogenases I and II. Their redox midpoint potentials were determined to $+220 \pm 20$ mV, which makes them well-suited for accepting electrons from cytochrome c_1 in the cytochrome bc_1 complex. Scheme 2 summarizes the identity and the hypothetical arrangement of the components of the bioenergetic electron-transfer chain in *A. aeolicus*.

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BI034452A