A Succinate Dehydrogenase with Novel Structure and Properties from the Hyperthermophilic Archaeon *Sulfolobus acidocaldarius*: Genetic and Biophysical Characterization

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The *sdh* operon of *Sulfolobus acidocaldarius* DSM 639 is composed of four genes coding for the 63.1-kDa flavoprotein (SdhA), the 36.5-kDa iron-sulfur protein (SdhB), and the 32.1-kDa SdhC and 14.1-kDa SdhD subunits. The four structural genes of the *sdhABCD* operon are transcribed into one polycistronic mRNA of 4.2 kb, and the transcription start was determined by the primer extension method to correspond with the first base of the ATG start codon of the *sdhA* gene. The *S. acidocaldarius* SdhA and SdhB subunits show characteristic sequence similarities to the succinate dehydrogenases and fumarate reductases of other organisms, while the SdhC and SdhD subunits, thought to form the membrane-anchoring domain, lack typical transmembrane α-helical regions present in all other succinate:quinone reductases (SQRs) and quinol:flavoprotein reductases (QFRs) so far examined. Moreover, the SdhC subunit reveals remarkable 30% sequence similarity to the heterodisulfide reductase B subunit of *Methanobacterium thermoautotrophicum* and *Methanococcus jan-nashii*, containing all 10 conserved cysteine residues. Electron paramagnetic resonance (EPR) spectroscopic studies of the purified enzyme as well as of membranes revealed the presence of typical S1 [2Fe2S] and S2 [4Fe4S] clusters, congruent with the deduced amino acid sequences. In contrast, EPR signals for a typical S3 [3Fe4S] cluster were not detected. However, EPR data together with sequence information implicate the existence of a second [4Fe4S] cluster in *S. acidocaldarius* rather than a typical [3Fe4S] cluster. These results and the fact that the *S. acidocaldarius* succinate dehydrogenase complex reveals only poor activity with caldarriella quinone clearly suggest a unique structure for the SQR of *S. acidocaldarius*, possibly involving an electron transport pathway from the enzyme complex into the respiratory chain different from those for known SQRs and QFRs.

Succinate:quinone reductase (SQR), a membrane-bound enzyme complex and component of the respiratory chain (complex II) of all aerobic organisms, catalyzes the oxidation of succinate to fumarate in the tricarboxylic acid cycle and transfers the electrons directly to quinones in the membrane. The reverse reaction, the reduction of fumarate, which is the last step in fumarate respiration of anaerobic or facultative anaerobic organisms, is catalyzed by quinol:flavoprotein reductase (QFR). The two enzyme complexes are very similar with regard to composition of subunits, prosthetic groups, and probably structure (2, 24). SQRs and QFRs are composed of three or four subunits of which the largest protein is a flavoprotein (*M* of 65,000 to 79,000) carrying covalently bound 8-α-N(3)-histidyl-flavin adenine dinucleotide (FAD). An iron-sulfur protein (*M* of 25,000 to 37,000) together with this flavoprotein subunit represents the peripheral part of the enzyme complex. The iron-sulfur protein is suggested to contain three different types of iron-sulfur clusters, each equimolar with FAD. Magnetic circular dichroism and electron paramagnetic resonance (EPR) spectroscopy investigations identified the iron-sulfur clusters as a binuclear center 1 [2Fe2S], a tetranuclear center 2 [4Fe4S], and a trinuclear center 3 [3Fe4S]. These different types of iron-sulfur centers are displayed in the amino acid sequence of the iron-sulfur protein containing 11 conserved cysteine residues (10 in *Escherichia coli* SdhB) which are supposed to function as ligands of the metal clusters. Four cysteine residues (three in *E. coli*) are arranged in a cluster close to the N terminus and ligand center 1, as deduced from studies of mutant enzymes with truncated iron-sulfur subunits (3, 27, 36), as well as from site-directed mutagenesis (62). The tetranuclear center 2 and trinuclear center 3 are supposed to be ligated by cysteine residues arranged in two clusters near the C terminus. Very likely, the trinuclear center 3 is directly involved in electron transfer to quinones, which was demonstrated by site-directed mutagenesis studies of *E. coli* QFR and *Bacillus subtilis* SQR (10, 20, 37).

Depending on the organism, the membrane-spanning domain of each of the SQRs and QFRs is composed of one or two hydrophobic subunits anchoring the peripheral part to the membrane and is involved in oxidation or reduction of quinones (2, 19, 24, 64). Furthermore, the hydrophobic subunits of many organisms contain one (e.g., *E. coli* SQR [44]) or two (e.g., *B. subtilis* SQR [18]) b-type cytochromes, whereas other enzymes (e.g., *E. coli* QFR [13]) do not contain any b-type cytochromes. The function of prosthetic heme groups, particularly in internal electron transfer, has not been determined, but they are suggested to play a structural role in stabilizing the transmembrane helices and to be important in assembly of the enzyme complex (19, 43). In contrast to SQRs and QFRs of eubacteria and eukaryotic cells, only little information is available about the composition and properties of these enzymes in archaea. Remarkable enzyme activities of succinate oxidation in the cytoplasm as well as in plasma membranes had been demonstrated previously for the halophilic *Halobacterium halobium* (16) and for the thermoacidophile *Sulfolobus acidocaldarius* (39). A putative succinate...
dehydrogenase, deduced from the nucleotide sequence of the iron-sulfur subunit and of the two hydrophobic subunits containing two hemes, was identified in *Thermoplasma acidophilum* (5). EPR spectroscopy of membranes from *T. acidophilum* clearly revealed the characteristic signals of S1, S2 and S3 centers (4). Recently, the nucleotide sequence of the succinate dehydrogenase complex from *Natronobacterium pharaonis* became available and displayed a typical four-subunit diheme complex. An unusual thiol-driven fumarate reductase from *Methanobacterium thermoautotrophicum* subsequently was purified and quite recently was sequenced (7, 21).

The succinate dehydrogenase complex from *S. acidocaldarius* was purified and characterized previously (40). It consists of four subunits (66, 31, 28, and 12.8 kDa), of which the largest was identified as the flavoprotein. The *S. acidocaldarius* SDR complex contains no heme and showed, in contrast to electron-accepting dyes, poor activity with caldariella quinone, which is supposed to be the physiological electron mediator in the *Sulfolobus* respiratory chain. A similar composition of subunits (66, 37, 33, and 12 kDa) was reported for the SDR of *Sulfolobus* sp. strain 7 (26). This complex also was shown to lack any heme group, but in contrast to *S. acidocaldarius* SDR, it revealed considerable activity with caldariella quinone.

To date, there is only incomplete information about archaeal SQRs or QFRs comprising both protein data and genetic data. In the present work, we report on the isolation, sequencing, and analysis of the *S. acidocaldarius* *sdh* genes and on EPR spectroscopic studies of the purified enzyme. Considering these and previously obtained results, a novel type of succinate dehydrogenase seems to be present in the crenarchaeon *S. acidocaldarius*.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, and culture conditions.** *S. acidocaldarius* DSM 639 was grown aerobically at 75 to 80°C and pH 2.5 as described previously (40). Cells used for enzyme purification were harvested in the late logarithmic growth phase at an absorbance at 546 nm of 1.5 to 1.7; cells used for DNA or RNA extraction were harvested at an absorbance at 546 nm of 0.8 to 1.0. *E. coli* XL2-blue (Stratagene) was grown at 37°C in Luria-Bertani medium supplemented with 50 μg of ampicillin per ml. The plasmid pBluescript II KS was purchased from Stratagene, and pSP64 was purchased from Serva.

**Isolation of the Sdh protein complex.** The Sdh complex was isolated from plasma membranes of somatocytes of *S. acidocaldarius* by solubilization with Chaps and subsequent hydroxypapit and DEAE chromatography as described previously (40).

**Determination of amino acid sequences.** For determination of internal and external sequences, the Sdh complex was loaded onto a preparative SDS–15% polyacrylamide gel and stained with Coomassie brilliant blue. The protein bands corresponding to the subunits SdhA and SdhB were cut out and electroeluted in 50 mM NH₄HCO₃–0.1% (wt/vol) SDS for 4 h (Bio-Rad model 422 electroluter). The eluted Sdh protein subunits were concentrated in a SpeedVac, precipitated with ice-cold ethanol, and resuspended in 50 mM NH₄HCO₃ (pH 7.8). The samples were then incubated with endoproteinase Glu-C at a protease/protein ratio of 1:20 for 18 h at 25°C. Resulting fragments were further separated by Tricine-SDS-PAGE as described by Schägger and von Jagow (51), followed by blotting onto a polyvinylidene difluoride membrane. The N-terminal sequences of several fragments and of the Sdh subunits were determined by Edman degradation, using an Applied Biosystems model 473A protein sequencer.

**DNA manipulation.** Standard cloning and DNA analysis procedures were performed as described by Sambrook et al. (48). Genomic DNA of *S. acidocaldarius* was prepared by the method of Marmur (38); plasmid DNA propagated in *E. coli* was isolated on small scale by a modification of the method of Birnboim and Doly (6) or in large scale by anion-exchange chromatography (Qiagen). Genomic DNA of *S. acidocaldarius* was digested with various restriction enzymes, separated on agarose gels, and blotted onto a nylon membrane (Ny-Lyn, Schleicher & Schuell) in 0.5 M NaOH–1.5 M NaCl for 1 h at room temperature. Nonradioactive labeling of oligonucleotide probes with digoxigen}

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tained with the probe derived from a 10.2-kDa fragment of SdhB digested with endoproteinase Glu-C. This probe hybridized to a 1.1-kb BamHI fragment and a 3.7-kb PstI fragment, while other probes or digests did not show unambiguous hybridization signals in Southern blot analysis. The 1.1-kb BamHI fragment was isolated and further cloned into the pSP64 vector, yielding the derivative pSJS1. Sequence analysis of this plasmid revealed a part of the coding region of the Sdh flavoprotein and of the iron-sulfur protein. To obtain complete information about the Sdh genomic region, the 3.7-kb PstI fragment containing the major part of the sdhA gene, all of the sdhB and sdhC genes, and the beginning of the sdhD gene was isolated and cloned (pSJS2). Adjacent genomic regions were cloned and further screened, using digested genomic DNA with probes derived by PCR with primers located close to the 5' or 3' region of pSJS2. A 900-bp AccI fragment and a 2-kb EcoRI fragment were cloned for isolation of the missing parts of the sdhD and sdhA genes, respectively. Figure 1 shows a restriction map of the DNA regions sequenced as well as the sequencing strategy.

**Gene organization.** As shown in Fig. 1, analysis of the sequenced DNA region revealed the presence of four open reading frames which are closely linked: an open reading frame from nt 416 to 2116 encoding SdhA, followed by an open reading frame from nt 2118 to 3071 containing sdhB, directly followed by sdhC (nt 3075 to 3947) and sdhD (nt 3947 to 4315). This kind of clustering of bacterial Sdh or Frd structural genes is typical in all cases examined so far (2, 24), whereas the arrangement of the individual genes within the operon can differ. A fifth open reading frame (orf1) from nt 7 to 378 is located upstream of the sdhA gene.

**Amino acid sequence and composition of the flavoprotein subunit, SdhA.** The sdhA gene encodes a product with 566 amino acids and a predicted molecular mass of 63,080 Da. SDS-PAGE of the SdhA subunit revealed an Mr of 66,000 (40). The identity of the protein was confirmed by comparison with the N-terminal amino acid sequence and with the sequence of a 26-kDa internal peptide (Table 1). The deduced amino acid sequence of the Sdh flavoprotein of *S. acidocaldarius* was compared to the sequences of flavoprotein subunits of several succinate dehydrogenases as well as fumarate reductases. The *S. acidocaldarius* flavoprotein displays considerable sequence similarity to other flavoprotein subunits (31% with SdhA of *B. subtilis*, 36% with SdhA of *Bos taurus*, 38% with human SdhA, 39% with SdhA and FrdA of *E. coli*, 41% with *Rickettsia prowazekii* flavoprotein, 35% with *M. jannaschii*, and 40% with the SdhA subunit of *N. pharaonis*). Unexpectedly, there is no closer relation to euryarchaeal homologs of *Methanococcus* and *Natronobacterium* than to the flavoproteins of eubacteria.

TABLE 1. N-terminal amino acid sequences of internal peptides of the subunits of succinate dehydrogenase from *S. acidocaldarius*

<table>
<thead>
<tr>
<th>Subunit molecular mass</th>
<th>Peptide</th>
<th>Amino acid sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>31 kDa (SdhB)</td>
<td>N terminus</td>
<td>Blocked</td>
</tr>
<tr>
<td>28 kDa (SdhC)</td>
<td>N terminus</td>
<td>Blocked</td>
</tr>
<tr>
<td>12.8 kDa (SdhD)</td>
<td></td>
<td>Blocked</td>
</tr>
</tbody>
</table>

* Internal peptides were obtained by endoproteinase Glu-C digestion. The subunit molecular masses given are the apparent values determined by SDS-PAGE (34).
or eukaryotes; the levels of sequence similarity are about the same throughout all three kingdoms.

Within the SdhA polypeptide are several clusters of highly conserved residues as described for other flavoprotein subunits (2, 24, 35, 42). Close to the N terminus, there is the characteristic nucleotide-binding βαβ-fold which is considered to be important in noncovalent binding of the AMP moiety of the FAD molecule (65). It consists of a stretch of four hydrophobic residues (Ala-8 to Ile-11) forming the first β-sheet, followed by two invariant glycine residues (Gly-12 and Gly-14). These are followed by a conserved stretch (α-helix) of residues with small side chains (residues 15 to 27) and a second hydrophobic segment (residues 31 to 34). Another highly conserved region, in which His-43 is supposed to be involved in a covalent α-N-(3)-histidyl linkage with the FAD cofactor (29, 60, 61), is located only a few residues away. An internal segment which forms the top of the AMP binding domain of the flavoprotein is conserved in all succinate dehydrogenases and fumarate reductases (13, 24) as well as in glutathione reductases and lipoamide dehydrogenases (47). This holds true also for the flavoprotein, in which residues 346 to 372 might contribute the AMP binding domain.

The S. acidocaldarius SdhA sequence displays also a highly conserved His-Pro-Thr triplet at positions 235 to 237. These residues contribute to the dicarboxylic binding site, suggesting that the imidazole moiety of the histidine functions as a general acid-base catalyst (53, 58). Nearby there are further conserved residues (248 to 255) involved in substrate binding. The conserved arginine at position 251 possibly provides a strong two-point interaction with the substrate carboxylates (53). The sequence of this cluster is -Cys-X4-Cys-X2-Cys-X11-Cys-, with the imidazole moiety of the histidine functions as a general acid-base catalyst (53, 58). Nearby there are further conserved residues (248 to 255) involved in substrate binding. The conserved arginine at position 251 possibly provides a strong two-point interaction with the substrate carboxylates (53). The sequence of this cluster is -Cys-X4-Cys-X2-Cys-X11-Cys-, with the imidazole moiety of the histidine functions as a general acid-base catalyst (53, 58).

Amino acid sequence and composition of the iron-sulfur protein subunit, SdhB. The predicted sdhB gene product contains 317 amino acids and has a molecular mass of 36,475 Da. Via SDS-PAGE, the apparent molecular mass was determined to be 31,000 Da (40). The identity of the protein was confirmed by comparison with the amino acid sequence derived from the 10.2-kDa internal peptide fragment of the 31-kDa subunit (SdhB) (Table 1) as well as by sequence comparison with iron-sulfur proteins of other succinate dehydrogenases and fumarate reductases. The S. acidocaldarius flavoprotein lacks in this active-site region (residue 250) a putative reactive cysteine residue (59) which is conserved in sequences of S. taurus SdhA, E. coli SdhA and FrdA, and other flavoproteins. However, recent studies disproved that this cysteine residue is essential for catalysis (23, 35, 53, 58).

The S. acidocaldarius SdhB sequence displays also a highly conserved His-Pro-Thr triplet at positions 235 to 237. These residues contribute to the dicarboxylic binding site, suggesting that the imidazole moiety of the histidine functions as a general acid-base catalyst (53, 58). Nearby there are further conserved residues (248 to 255) involved in substrate binding. The conserved arginine at position 251 possibly provides a strong two-point interaction with the substrate carboxylates (53). The sequence of this cluster is -Cys-X4-Cys-X2-Cys-X11-Cys-, with the imidazole moiety of the histidine functions as a general acid-base catalyst (53, 58). Nearby there are further conserved residues (248 to 255) involved in substrate binding. The conserved arginine at position 251 possibly provides a strong two-point interaction with the substrate carboxylates (53). The sequence of this cluster is -Cys-X4-Cys-X2-Cys-X11-Cys-, with the imidazole moiety of the histidine functions as a general acid-base catalyst (53, 58).

Comparing the complete SdhB and FrdB sequences of several organisms, we found that the S. acidocaldarius sequence contains a C-terminal extension of about 60 amino acid residues which is also present in the putative Synechocystis iron-sulfur protein. Only the sequence of the suggested M. jannaschii FrdB subunit containing a C-terminal extension of about 270 amino acid residues appears more striking.

Hydropophicity analysis of the S. acidocaldarius SdhB protein revealed no significant hydrophobic stretches functioning as possible transmembrane segments.

Amino acid sequences and compositions of the SdhC and SdhD subunits. The SdhC subunit consists of 289 amino acid residues with a predicted molecular mass of 32,110 Da, whereas SDS-PAGE of SdhC gave an M, of 28,000 (40). The sequence of SdhD indicates that the SdhD subunit is composed of 122 amino acids and has a molecular mass of 14,082 Da. The experimentally determined value was 12.8 kDa (40). The identity of the SdhD protein was confirmed by comparison with the N-terminal amino acid sequence. The initiating methionine was missed in the protein sequence, perhaps due to posttranslational processing of this subunit.

The membrane-integral domains of the enzyme complexes of SOX or QFR are usually composed of one larger hydrophobic subunit (SdhC/FrdC), as in B. subtilis and Wolinella

![FIG. 2. Alignments of cysteine clusters I, II, and III of the SdhB and FrdB subunits from S. acidocaldarius (S.ac), M. jannaschii (M.jan), Synechocystis sp. (Syn.sp), N. pharaonis (N.ph), T. acidophilum (T.ac), B. subtilis (B.sub), S. cerevisiae (S.cer), and E. coli.](http://jb.asm.org/)
succinogenes, or of two smaller subunits (SdhC and SdhD/FrdC and FrdD), as in E. coli SQR and QFR (2, 24). They anchor the soluble complex containing the flavoprotein and iron-sulfur subunit to the membrane. Furthermore, they are involved in quinone reduction or oxidation. In contrast to subunits A and B, they display generally little sequence conservation except for the pattern of hydrophobic segments (19). The so-called one-polypeptide anchors, e.g., those of B. subtilis SQR (18) and W. succinogenes QFR (32), show a common motif of five predicted transmembrane α-helical segments, whereas both polypeptides of two-polypeptide anchors, e.g., E. coli SdhC/D and FrdC/D, contain three predicted transmembrane segments (44). Hydrophobicity analysis of the S. acidocaldarius SdhC and SdhD subunits is depicted in Fig. 3. Surprisingly, the SdhD subunit proves to be very hydrophilic and exhibits no putative transmembrane segments, making its function as a membrane-anchoring subunit uncertain. Also, the SdhC subunit does not fit the scheme of the anchor polypeptides mentioned above. However, there is one small putative membrane-spanning segment at the C terminus of SdhC comprising residues 253 to 273. Databank searches unambiguously revealed overall similarities of the SdhC polypeptide of 30% with HrdB of M. thermoautotrophicum (22) and M. jannaschii (two copies) and 15% with the C terminus of the putative FrdD subunit of M. jannaschii. It also shows 31% sequence similarity with a hypothetical protein from Synechocystis. An alignment of these sequences is shown in Fig. 4. Conspicious within this alignment is the existence of 10 conserved cysteine residues which are scattered over the whole sequence. A possible function of these residues is unknown. Recently, two additional genes containing these 10 conserved cysteine residues were sequenced and found to encode a subunit of an unusual thiol-driven fumarate reductase (TfrB) from M. thermoautotrophicum (21) as well as a heterodisulfide reductase subunit (HrdE) from Methanosarcina Barkeri (33). Corresponding to the S. acidocaldarius SdhC polypeptide, HrdB from M. thermoautotrophicum revealed a putative transmembrane segment at its C terminus, as determined by hydrophobicity analysis; for this reason, it has been proposed to function as a membrane anchor of the heterodisulfide reductase complex and to possibly operate in energy transduction (22). As described above, the SdhD subunit shows no putative transmembrane segments, nor does it reveal any sequence similarity to known proteins.

Transcription. Northern blot analysis using a homologous probe derived by in vitro transcription of a region comprising sdhA and sdhB sequences revealed a single mRNA transcript (data not shown). The length of the transcript of 4.2 kb corresponds well with the complete sequence size of the sdhABCD genes (3,899 bp). In addition, the 4.2-kb transcript could include the sequence of orf1 (371 bp), which is located directly upstream from the sdhA gene. Databank searches revealed 37% sequence similarity of the orf1 product with a hypothetical protein from Streptococcus aureus. There is no open reading frame downstream of the sdhD gene. However, Northern blot analysis using a probe derived from a region within orf1 gave no positive signal, indicating that orf1 is not transcribed together with the sdh genes (result not shown). In contrast, analysis by the primer extension method as shown in Fig. 5 gave one band on the gel, clearly suggesting that the transcription start coincides with the first base of the ATG start codon of the sdhA gene. Thus, the four S. acidocaldarius sdh genes are transcribed into one polycistronic message. Archaeal promoter sequences thus far described consist of two consensus regions which have been defined by comparison: box A [TTTA(A/T)A], centered about 27 bases upstream of the transcription start site, and box B [(T/A)TG(A/C)] at the start site (46, 56). There are similar sequences at the −28 and +1 regions of the S. acidocaldarius sequence, each differing by one nucleotide from the conserved motifs described above.

The determined transcription start of the S. acidocaldarius genes being congruent with the first base of the ATG start codon is not unusual for archaea. Although there are archaeobacterial mRNAs consisting of a leader sequence with a ribosome binding site motif similar to that of bacteria (9), a large number of archaeabacterial messages with very short leaders or no leader have been identified (14, 28, 30, 31). From this finding arises the question of how translation initiation and interaction with ribosomes take place. To account for this, it has been suggested that ribosome binding may occur downstream of the start codon within the coding region itself (14, 28). Little information about translation initiation in archaea is available, but it is possible that there are different mechanisms. Furthermore, it could be demonstrated by Northern blot analysis comparing RNA extracted from cells grown on medium with sucrose and glutamate and from cells grown on minimal medium supplemented only with glutamate that the sdh transcript is expressed constitutively (data not shown).

EPR characterization. The EPR spectrum of the air-oxidized Sulfolobus enzyme (Fig. 6) displays a nearly isotropic resonance with a maximum g value at 2.02 that does not increase after addition of potassium ferricyanide. This resonance with an optimum amplitude at 20 K was already observable at...
temperatures around 50 K. To further characterize this resonance, power saturation studies were performed at two different temperatures. At 15 K, a half-saturation power of 3.7 mW was obtained, whereas at 6.7 K, the resonance was saturated at 0.2 mW. This kind of relaxation behavior is very atypical for an expected [3Fe4S] cluster normally relaxing so rapidly that it is clearly observable only below 20 K. Another difference from typical [3Fe4S] clusters of succinate dehydrogenases or fumarate reductases was revealed by spin quantitation of this resonance, performed under nonsaturating conditions (50 mW and 15 K), relative to the below-described [2Fe2S] cluster yielding a substoichiometric ratio of 1:7.

Reduction of the purified *Sulfolobus* enzyme with succinate produced a different EPR spectrum (Fig. 7, upper trace) displaying a rhombic resonance with values of $g_z = 2.025$, $g_y = 1.935$, and $g_x = 1.904$. This resonance was observed optimally at temperatures around 20 K and was still detectable at temperatures around 70 K, a behavior typical for reduced [2Fe2S] clusters (Fig. 7, lower trace).

Indirect evidence for the presence of a magnetically coupled [4Fe4S] cluster, the canonical S2 center, was obtained by full reduction of the Sdh with dithionite, revealing a dramatic enhancement of the spin relaxation properties of the [2Fe2S] cluster indicated by power saturation behavior: the half-saturation power in the dithionite-reduced state at 20 K was determined as 36 mW, whereas in the succinate-reduced state, this value was calculated as 4.3 mW. Moreover, the EPR spectrum of the dithionite-reduced sample at 25 K (Fig. 8, trace b) exhibited a strong, nearly axial resonance, whereas the EPR spectrum at 70 K displayed the rhombic resonance of the [2Fe2S] (Fig. 8, trace c). Comparison of these EPR spectra at 25 and 70 K clearly shows that an additional component, most probably of the [4Fe4S] type normally not observed in EPR spectra of known succinate dehydrogenases, contributes to the

![FIG. 5. Sequence alignment of SdhC from *S. acidocaldarius* to HdrB of *M. jannaschii* (Mc.jan.) and *M. thermoautotrophicum* (Mb.th.), to the C-terminal sequence (253 amino acid residues) of FrdB from *M. jannaschii*, and to a hypothetical protein from *Synechocystis* sp. (Syn.). Asterisks indicate identical residues between all sequences; dots indicate conservative replacements. Conserved cysteine residues are in boldface.]
spectrum at 25 K. To substantiate the indications for a novel [4Fe4S] cluster, the spectrum at 70 K was subtracted from the spectrum of the dithionite-reduced sample at 25 K. The resulting difference spectrum (Fig. 8, trace c) clearly displays a new axial EPR spectrum with $g_z = 2.020$ and $g_y = 1.930$. The integration of this new resonance yielded a value of 22% relative to the S1 cluster.

To detect a [3Fe4S] resonance, which often gets lost during the purification procedure, EPR spectra were obtained at different microwave power and temperatures in the most integral, native membraneous state. In the $g_z = 2$ region, no resonance at $g = 2.021$, as displayed in the air-oxidized spectrum of the purified enzyme, was observed in membranes. However, two resonances with $g_{\text{max}}$ values of 2.027 and 2.018 could be detected. To test whether one of these might be due to an S3 cluster, the relaxation behaviors of both resonances were investigated. Both resonances could still be detected at temperatures higher than 30 K, an unusually high value for [3Fe4S] clusters. Furthermore, both resonances disappeared at very low temperatures around 5 K, where [3Fe4S] resonances normally can be detected as sharp resonance lines. Therefore, both resonances are obviously not due to a [3Fe4S] cluster. However, addition of succinate to the membranes in the presence of cyanide revealed the typical resonance of the [2Fe2S] cluster still observable at 70 K. In addition, the low-spin heme resonances disappeared, due to their reduction, and the Rieske iron-sulfur resonance could be detected. The succinate-induced reduction of the membrane-bound Rieske iron-sulfur center could be inhibited by malonate. These results clearly indicate an electron transfer via the succinate dehydrogenase in the respiratory chain of S. acidocaldarius.

DISCUSSION

Sequence analysis of the four S. acidocaldarius sdh genes and EPR spectroscopy data revealed for S. acidocaldarius SQR some characteristic features of known conventional SQRs but also displayed some novel properties diverging from those of all SQRs and QFRs examined so far. The S. acidocaldarius 63-kDa flavoprotein exhibits all typical features with regard to conserved sequence motifs for the substrate binding site and for covalent flavin attachment. This is in agreement with our previous results revealing an equimolar content of acid-non-extractable flavin in the purified S. acidocaldarius Sdh complex (40).

Another typical aspect is the arrangement of the four Sulfolobus sdh genes in an operon (sdhABC), which is a common motif in all bacterial SQRs and QFRs. Only the order of the hydrophobic subunit(s) relative to the genes of subunits A and B is variable (2, 24). Exceptions are the genes of the SQR from the halophile archaeon N. pharaonis, grouped as an sdhCDBA operon, and the genes for putative flavoproteins and putative iron-sulfur subunits in the methanogen archaeon M. jannaschii as well as in the cyanobacterium Synechocystis, which are scattered over the genome. However, it is not clear whether these genes are expressed.

The S. acidocaldarius SdhB sequence shows 21 to 34% sequence similarity to other Sdh or Frd iron-sulfur proteins; a really striking difference is evident within the third conserved cysteine cluster, very likely representing the ligands of the trinuclear [3Fe4S] cluster in other iron-sulfur subunits examined so far (2, 24, 37). As shown in Fig. 2, the S. acidocaldarius sequence contains an additional cysteine residue (Cys-213), suggesting a second [4Fe4S] cluster rather than a [3Fe4S] cluster. EPR spectroscopy of the purified Sdh complex and of membranes confirms that a typical S3 center in S. acidocaldarius appears to be absent. The resonance at $g = 2.021$ in the oxidized state of the purified enzyme showed a temperature and relaxation behavior very atypical for [3Fe4S] clusters and was present only substoichiometrically (1:7). The classical [3Fe4S] clusters from E. coli fumarate reductase and Thermus
EPR spectra of dithionite-reduced *S. acidocaldarius* Sdh (1.5 mg/ml) at 25 K (trace a) and 70 K (trace b) and the resulting difference spectrum (trace c; spectrum at 25 K minus the spectrum at 70 K). Samples were reduced with a few grains of dithionite for 5 min. Spectra were recorded at 9.6426 GHz with a modulation amplitude of 5 G and microwave power of 20 mW. The multiplica-
tion factor indicates relative spectrometer gains for the two spectra of each sample.

**FIG. 8.** EPR spectra of dithionite-reduced *S. acidocaldarius* Sdh (1.5 mg/ml) at 25 K (trace a) and 70 K (trace b) and the resulting difference spectrum (trace c; spectrum at 25 K minus the spectrum at 70 K). Samples were reduced with a few grains of dithionite for 5 min. Spectra were recorded at 9.6426 GHz with a modulation amplitude of 5 G and microwave power of 20 mW. The multiplication factor indicates relative spectrometer gains for the two spectra of each sample.

Thermophilus ferredoxin display EPR resonances which can be observed only at temperatures below 30 K and which are fast-
relaxing systems not saturated at microwave powers of up to 50 mW at 13 K (41). In contrast to this behavior of a classical [3Fe4S] cluster, the g = 2.02 resonance of the *Sulfobolus* Sdh could be observed at temperatures below 50 K and possessed a half-saturation power of 3.2 mW at 15 K, drastically lower than the typical value given above. As this resonance at g = 2.021 could not be detected in oxidized membranes, it probably represents an artifact arising during the course of preparation. EPR spectra of the purified *S. acidocaldarius* enzyme in succi-
nate- and dithionite-reduced states give evidence for typical S1 and S2 centers but also contain features suggesting an addi-
tional component. EPR data combined with the present se-
quence information suggest that there is an additional [4Fe4S] cluster in *S. acidocaldarius* Sdh instead of the typical [3Fe4S] cluster. Indications for the presence of this new [4Fe4S] cluster were obtained from the EPR difference spectrum. Although the EPR difference spectrum does not display a pure axial spectrum, it must be kept in mind that the resonances of the [2Fe2S] clusters broaden at 25 K, compared to 70 K, due to relaxation with another paramagnetic cluster, most probably a reduced [4Fe4S] cluster. This broadening effect might induce the relative impure difference spectrum. The low stoichiometry of the novel [4Fe4S] cluster of about 22% may be explained by a partial reduction of this center.

Interestingly, mutants of *E. coli* fumarate reductase exhib-
ted a [3Fe4S]-to-[4Fe4S] cluster conversion when a fourth cysteine residue (Val 207→Cys) was introduced into the puta-
tive F3 binding motif (37). Although the midpoint potential of the newly assembled [4Fe4S] cluster decreased to −350 mV, compared to the usually high potential F3-cluster (Em = −70 mV), and the catalytic activity with quinones decreased by more than 80%, growth of *E. coli* was still possible under con-
ditions requiring a physiologically competent fumarate reduc-
tase and/or succinate dehydrogenase. However, the same mu-
tation in *B. subtilis* SQR affected the stability of the enzyme and also the EPR properties of cluster S3 without generating a cluster conversion (20). The quinone reductase activity was lowered to 35%. These observations clearly support the imp-
ortant function of the trinuclear cluster for stability and in-
teraction with quinones. The purified *S. acidocaldarius* enzyme showed only weak SQR activity with the physiological acceptor caldariella quinone (Em = 100 mV) (40), which may be attribut-
ed to the observed alterations in regular S3 cluster proper-
ties. In this regard, the SQR from *Sulfobolus* sp. strain 7 re-
vealed considerable activity with caldariella quinone, displaying typical S1, S2, and S3 signals in EPR spectroscopy (26). Nota-
ably, a number of further differences observed with this strain may be explained by its likely assignment to the species *S. solfataricus* (49).

In contrast to *T. acidophilum* SQR, which in membranes exhibits more than 10-times-higher Sdh activity than *S. acidocal-
darius* (4), further characterization of the different *Sulfobol-
us* iron-sulfur centers by EPR redox titration was not possible due to very low EPR signal intensity in native membranes.

An important difference regarding SdhC and SdhD becomes apparent. At first sight, these 31- and 14-kDa subunits appear to be the membrane-anchoring subunits of the complex, in analogy to SQRs and QFRs examined thus far. The membrane anchor domains of SQRs have been classified into four groups based on differences in polypeptide and heme composition (17, 19). Sdh or Frd complexes containing two membrane-anchoring polypeptides with three membrane-spanning segments each and with two hemes (e.g., *T. acidophilum* SQR) are of type A. Type B anchors consist of one polypeptide with five predicted transmembrane helices and two hemes (e.g., *B. sub-
tilis* SQR), whereas type C anchors have two polypeptides with three membrane-spanning segments each and only one heme (e.g., *E. coli* SQR). Type D anchors possess two membrane-anchoring polypeptides each with three transmembrane heli-
ces, but they do not contain any heme (e.g., *E. coli* QFR). A proposed structure, common to all four types, is that four transmembrane helices are arranged as an antiparallel bundle which is possibly stabilized by heme (if present). In contrast, *S. acidocaldarius* SdhD is rather hydrophilic, and SdhC displays only one putative hydrophobic stretch at its C terminus (Fig. 3). This totally different structure in *S. acidocaldarius* implica-
tes a likely peripheral and looser attachment of the Sdh complex to the membranes rather than a transmembrane-
spanning anchoring. This coincides with the observation that only 10 to 30% of total *Sulfobolus* Sdh activity was found in the membrane fraction (39).

Also, there is a remarkable 30% sequence similarity of *S. acidocaldarius* SdhC to HdrB of *M. thermoautotrophicum* and *M. jannaschii* and to a hypothetical protein of the cyanobacte-
rium *Synechocystis*, each showing an accumulation of 10 highly conserved cysteine residues. HdrB from *M. thermoautotrophi-


cum is a component of the three-subunit-comprising heterodisulfide reductase complex forming together with a hydrogenase the H₂-heterodisulfide oxidoreductase complex (22, 54).HdrB, showing a C-terminal hydrophobic stretch analogous to that of SdhC, is suggested to be physically anchored to the membrane via this hydrophobic α-helix. Nevertheless, this complex was recovered from the soluble cell fraction, indicating thenly a loose association to integral membrane components possibly involved in energy transduction. The same sequence similarity to the HdrB subunit is exhibited in the C-terminal extension of the gene-derived iron-sulfur protein sequence from M. jannaschii. Additionally, this putative FrdB subunit of M. jannaschii displays high sequence similarity to the recently purified and sequenced thiold: fumarate reductase B (TrfB) subunit of M. thermoautotrophicum; at its C terminus it also contains sequence motifs for one [2Fe2S] and two [4Fe4S] clusters, as well as the 10 highly conserved N-terminal cysteine residues. The thiold: fumarate reductase from M. thermoautotrophicum, suggested to play a role in abiotic reactions, represents an unusual cytoplasmic fumarate reductase which catalyzes the irreversible reduction of fumarate with coenzyme M (CoM-S-H) and coenzyme B (CoB-S-H) as electron donors to succinate and CoM-S-CoB. It is assumed that the 10 conserved cysteine residues may be involved in the catalytic mechanism of CoM-S-H and CoB-S-H oxidation (21).

In any case, the physiological reaction of the S. acidocaldarius SQR is succinate oxidation and not fumarate reduction. Therefore, due to the very negative redox potential of thiols, an involvement of these components in succinate oxidation appears impossible. A further conceivable function of the S. acidocaldarius SdhC subunit, if not a direct involvement in electron transfer, may be that it plays a structural role in the Sdh complex; alternatively, it may simply be a relict of an anaerobic ancestor. Another function, aside from membrane anchoring, of typical hydrophobic SQR and QFR subunits is interaction with quinones. Based on mutational studies with E. coli QFR (10, 63, 64) and inhibitor studies with 2-n-heptyl 4-hydroxyquinoline N-oxide in B. subtilis SQR (55), it is suggested that there are two different transmembranously located quinone binding sites. Considering the differing and rather hydrophilic character of S. acidocaldarius SdhC and SdhD subunits, the question arises of how quinone interaction and electron transfer from the S. acidocaldarius SQR to caldariella quinone may occur. The low quinone reductase activity as well as the significant similarity to the unusual fumarate reductases of M. thermoautotrophicum and M. jannaschii which catalyze an irreversible thiold-driven fumarate reduction in the absence of quinones casts doubt on a direct electron transfer from S. acidocaldarius SQR to caldariella quinone. However, it was demonstrated by EPR spectroscopy that succinate oxidation in membrane fractions resulted in electron transfer to Rieske iron-sulfur proteins and low-spin hemes which are components in the S. acidocaldarius respiratory chain (50), demonstrating the physiological reaction of the S. acidocaldarius SQR. Although the reaction is reversible, as has been confirmed also for the S. acidocaldarius enzyme (data not shown), fumarate reduction is not likely to occur under physiological conditions in S. acidocaldarius, because (i) S. acidocaldarius is an obligate aerobic organism, obviously excluding an anaerobic fumarate respiration pathway, and (ii) autotrophic growth implicating a reductive tricarboxylic acid cycle could not be observed in the strain of S. acidocaldarius, DSM 639, used in this work. Therefore, it might be envisaged that electron transfer from the loosely membrane associated Sulfolobus SQR occurs either via an unknown membrane integral component to caldariella quinone or directly to the high-potential Rieske iron-sulfur protein. Alternatively, electrons may be transferred via another, hitherto unidentified redox carrier. From this aspect, the functional role of the 10 conserved cysteine residues accumulated in SdhC becomes an interesting issue for further studies of the S. acidocaldarius succinate dehydrogenase complex.

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