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Acidianus ambivalens Complex II Typifies a Novel Family of Succinate Dehydrogenases

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Complex II from the thermoacidophilic archaeon *Acidianus ambivalens,* an archetype of an emerging class of succinate dehydrogenases (SDH), was extracted from intact membranes and purified to homogeneity. The complex contains one molecule of covalently bound FAD and 10 Fe atoms. EPR studies showed that the complex contains the canonical centres S1 ($[2Fe\text{-}2S]^{2+/1+}$) and S2 ($[4Fe\text{-}4S]^{+2/+1}$) but lacks centre S3 ($[3Fe\text{-}4S]^{+1/0}$); these observations agree with the fact that the iron-sulfur subunit contains an extra cysteine that may allow the binding of a new centre, most probably a tetranuclear one. Succinate-driven oxygen consumption is observed in intact membranes indicating that *in vivo,* complex II operates as a succinate:quinone oxidoreductase, despite missing the typical anchor domain subunits. The pure complex was found to contain bound caldariella quinone, the enzyme physiological partner. An alternative membrane anchoring for this new type of SDHs, based on the amphipathic nature of the putative helices found in SdhC, is suggested.

Key Words: **archaea; thermophile; succinate dehydrogenase; iron-sulfur.**

Complex II, or succinate dehydrogenase (SDH)—succinate:quinone oxidoreductase—is a membrane bound complex that is simultaneously involved in the respiratory chain and in the citric acid cycle, catalysing the oxidation of succinate to fumarate with the concomitant electron transfer to quinones. Two distinct domains compose canonical complex II. A catalytic domain, which is an hydrophilic heterodimer formed by a flavoprotein (64–79 kDa) with a covalently bound FAD, and an iron-sulfur protein (27–31 kDa) which accommodates three clusters: centre S1, $[2Fe\text{-}2S]^{2+/1+}$, centre S2, $[4Fe\text{-}4S]^{+2/+1}$, and centre S3, $[3Fe\text{-}4S]^{+1/0}$. The anchor domain that attaches the whole complex to the membrane and provides quinone binding sites is composed by one large (23–30 kDa) or two smaller (11–16 and 13–18 kDa) transmembrane polypeptides that may contain two, one or none B-type hemes (see (1) for a review).

In recent years, evidence has accumulated showing that several complex II enzymes, mostly from archaeal sources, have atypical features and may constitute a new class of enzymes. Its members are distinguished by the fact that they have an unusual composition of iron sulfur clusters and lack the usual membrane anchoring subunits. The first examples of such a succinate dehydrogenase were found in the archaea *Acidianus (A.) ambivalens* and *Sulfolobus (S.) acidocaldarius,* thermoacidophilic organisms that live optimally at 80°C and pH 2.0–4.0. A preliminary report of EPR measurements performed in intact membranes of *A. ambivalens* accounted for the possibility that centre S3 was absent (2), a finding later suggested by spectroscopic data in intact membranes and genetic studies (3, 4). In both archaea, it was found that the cysteine cluster of the SdhB subunit (the iron-sulfur protein) contains an additional cysteine, in a position apparently adequate to enable the coordination of a $[4Fe\text{-}4S]^{2+/1+}$ centre in replacement of the usual centre S3, thus corroborating the spectroscopic observations (3, 4). Another interesting finding in these complex II concerns the putative membrane anchor polypeptides, SdhC and SdhD, which are completely distinct from the usual ones, being mainly hydrophilic and missing any unequivocal transmembrane helical segments.

This new type of complex II enzymes has been essentially found in thermophilic archaea belonging to the Sulfolobales order, namely in *A. ambivalens, A. infernus, S. solfataricus* (3), *S. acidocaldarius* (4), and *Sulfolobus* strain 7 (5). Nevertheless, this subclass is not common to all archaea. For example, *S. metallicus,* also a member of the Sulfolobales, appears to contain a regular complex II (6), as well as the thermophile *Thermoplasma acidophilum* (7) the halophile *Natronobac-*

Abbreviations used: dichlorophenolindophenol, DCPIP; dithiothreitol, DTT; iodoacetoamide, IAA; potassium phosphate, PP; phenazine methosulphate, PMS; 2,6-; Tetramethyl-*p*-phenyldiamine, TMPD.

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terium pharaonis (8) and the hyperthermophile *Aeropyrum pernix* (9).

The purification, biochemical, and spectroscopic properties of *A. ambivalens* complex II reported here, contribute to clarifying several aspects of this new type of succinate dehydrogenases which remain to be addressed, namely (i) the evidence that, in the pure protein, the trinuclear FeS cluster is absent; (ii) the lack of additional FeS clusters in the cysteine-rich subunit; and (iii) the interaction with quinone. Furthermore, analyses of the predicted helical structure of the putative membrane anchor subunits (SdhC and SdhD) suggest that these new SDHs are monotopic. Extensive comparison with SdhC homologous found in other membrane bound proteins lacking predicted transmembrane domains suggests that this may be a common membrane attaching mode in a quite large number of proteins.

MATERIALS AND METHODS

Cell growth and detergent extract preparation. *A. ambivalens* cells were grown under aerobic conditions as in (10, 11). Membranes were prepared as in (11). The solubilised membrane extract (DM extract) was prepared by addition of β-dodecyl-maltoside (DM) to the resupended membranes in a ratio of 2 g DM/g protein; the suspension was stirred for 30 min at 4°C.

Protein purification. Purification steps were performed on a Pharmacia HiLoad system, at 4°C, and all buffers were adjusted to pH 6.5 and contained 0.1% DM. DM extract was applied to a DEAE-Sepharose column equilibrated with 40 mM potassium phosphate (PP) buffer. SDH eluted at 500 mM NaCl in a linear gradient of 0 to 1 M NaCl in the equilibrating buffer. The enzyme-containing fraction was loaded into a HTP Ceramics column equilibrated with 40mM PP buffer. A 40 to 1000 mM linear PP gradient was applied to the column and the SDH activity eluted at about 500 mM PP. The fraction was injected onto a Superdex 200 column and eluted with 40 mM PP buffer/150 mM NaCl. Succinate dehydrogenase activity was followed throughout the purification steps by its succinate:DCPIP oxidoreductase activity. Enzyme purity was assessed by gradient SDS–PAGE (10 to 15% acrylamide/0 to 15% sucrose). Prior to the application on the gel, samples were incubated for 30 min in loading buffer containing 8 M urea. Proteins were stained with Coomassie brilliant blue (12).

Spectroscopic methods. EPR spectra were recorded on a Bruker ESP 380 spectrometer, equipped with an ESR900 continuos-flow helium cryostat from Oxford Instruments. Parallel mode EPR was performed using a Bruker dual mode cavity.

Ultraviolet-visible spectra and kinetical assays were recorded on a Shimadzu UV-1630 spectrophotometer, equipped with a temperature controller and a stirring system.

Biochemical procedures. Succinate:DCPIP oxidoreductase was monitored by following the PMS coupled reduction of DCIP at 578 nm ($\varepsilon = 21 \times 10^3\ M^{-1}cm^{-1}$) (13). The reaction mixture contained 100 mM PP buffer pH 6.5, 0.1 mM PMS and 0.2 mM DCPIP. Prior to the assay, all the samples containing quinol oxidase were incubated for 3 min with 10 mM KCN (stock solution: 2M KCN in 1M PP buffer pH 8) in order to block this enzyme activity. Assays were performed at 70°C. One unit (U) is defined as the consumption of 1 μmol of succinate per min. The rate of decylubiquinone reduction was accessed by measuring the absorbance decrease rate at 278 nm ($\varepsilon = 19230\ M^{-1}cm^{-1}$) (14). Protein concentration was determined by the modified microbiuret method (15). Labile iron content was chemically determined by the 2,4,6-tripyridyl-*s*-triazine (TPTZ) method (16).

Quinone extraction. Quinone was extracted by adding 1 ml of 50% (v/v) methanol/acetone to the sample (50 μl); after 30 min 750 μl of petroleumbenzin (Riedel-deHaën No. 24541) were added to the mixture. The upper petroleum phases were collected, evaporated and the dried residue was re-suspended in methanol (17). Stock caldariella quinone was purified from *A. ambivalens* as described in (18).

Alkylation of sulfhydryl groups. The protein was incubated in 50 mM Tris–HCl pH8/2% SDS/8 M urea/20 mM DTT for 30 min at 37°C. Sulfhydryl groups were alkylated by a 30 min incubation in 100 mM iodoacetoamide (IAA) at 37°C (19).

Hydroxylamine treatment. The SDH sample was incubated overnight with an equal volume of hydroxylamine 1 M pH 8, at room temperature (20). Remaining sulfhydryl groups were alkylated as described above. The samples were analysed by gradient SDS–PAGE (10 to 15% acrylamide/0 to 15% sucrose).

Redox titrations. The iron-sulfur clusters reduction potentials were determined by redox titrations monitored by EPR spectroscopy. The enzyme was titrated under anaerobic conditions, at room temperature and pH 6.5 in the presence of suitable redox mediators as described in (21). Reduction potentials are quoted in respect to the standard hydrogen electrode.

Sequence analysis tools. The sequences used in multiple alignment comparisons were retrieved from protein databases using the NCBI Entrez protein sequence search. Multiple alignments were performed using Clustal W version 1.6 (22). Protein secondary structure predictions were generated in PSIPred (23). Helical wheel projections were generated in Win Pep 2.11.

RESULTS

Purification and Biochemical Properties

Complex II from *A. ambivalens* is clearly a membrane bound protein, as the membranes comprise 80% of total SDH activity (3). The purification procedure resulted in a 70-fold enrichment of the complex with a yield of 11% and a final specific activity of 1.15 U/mg of protein at 70°C (Table 1). The protein preparation was found to be pure by gradient SDS–PAGE (Fig. 1, lane B). The gel shows that the isolated complex consists of four different subunits with apparent molecular masses of 67, 33, 28, and 14 kDa in agreement to those predicted from the genomic sequence of the *sdhABCD* operon (3).

Iron determination showed that *A. ambivalens* complex II contains 10 ± 1 iron atoms per molecule. This is consistent with the presence of one dinuclear [2Fe-2S]$^{2+/1+}$ cluster (S1), one tetranuclear [4Fe-4S]$^{+2/+1}$ cluster (S2) and one additional tetranuclear cluster, which is replacing centre S3. This determination also excludes the presence of any additional iron centres in the cysteine-rich SdhC subunit.

Spectroscopic Properties

The UV-Visible spectrum of the as purified (oxidised) protein exhibits a broad band, centred at around 450 nm, typical of flavoproteins, and additional features from 300 to 600 nm, attributable to the iron-sulfur

TABLE 1

Purification Table for *A. ambivalens* Complex II

Purification step	Protein Mg	Total act. U	Sp. act. U/mg	Yield (%)	Enrichment-fold
Membranes	16,450	270	0.02	100	1
DM Extract	7000	276	0.04	102	2
Q-Sepharose	960	209	0.22	78	13
HTP	168	181	1.08	67	66
S-200	25	29	1.15	11	70

clusters (Fig. 2). The flavin is completely succinate-reducible, as 5 min incubation with succinate, at 60°C under anaerobic conditions, resulted in the disappearance of the 450 nm band (data not shown). Flavin quantification, using $\epsilon^{450} = 11300\ M^{-1}.cm^{-1}$ (13), from the succinate-reduced redox spectra, showed that the complex contains one FAD per molecule (0.75 FAD/mol). The flavin cofactor could not be extracted by 20% TCA, thus indicating that it is covalently bound.

The EPR spectrum of the native, oxidised protein, shows a minor resonance centred at $g = 2.00$, typical of a radical, lacking any additional features (Fig. 3A, trace a). This unequivocally indicates that the trinuclear centre S3, which under these conditions exhibits an intense signal at g = 2.02, is absent from *A. ambivalens* complex II. Even upon incubation with potassium hexacyanoferrate (III), no additional resonances were detected (data not shown). Incubation with 20 mM succinate at 70°C for 20 min resulted in the appearance of a rhombic type signal with $g_{max} = 2.034$, $g_{med} = 1.937$, and $g_{min} = 1.911$ (Fig. 3A, trace b). Reduction with sodium dithionite led to the full development of this signal (Fig. 3A, trace c). No other features were detected in the dithionite or deazoflavin reduced enzyme (Fig. 3A, trace d) at either higher (up to 50K) or lower temperatures, neither by changing the microwave power, indicating that the tetranuclear centres are likely in close spatial contact and under magnetic interaction, thus preventing their observation. In particular, no resonances at low magnetic field, typical of reduced iron-tetranuclear clusters with a spin ground state higher than one-half, could be detected. Most important, parallel experiments performed with an intact membrane extract and with a partially purified protein, did not show any additional resonances indicating the integrity of the purified enzyme. The microwave power saturation behaviour of the $[2Fe\text{-}2S]^{1+}$ cluster in the enzyme samples reduced by succinate or dithionite suggests a magnetic interaction of this centre with another centre, most probably the reduced cluster S2, $[4Fe\text{-}4S]^{+1}$ (Fig. 3C), as generally observed for succinate dehydrogenases (1).

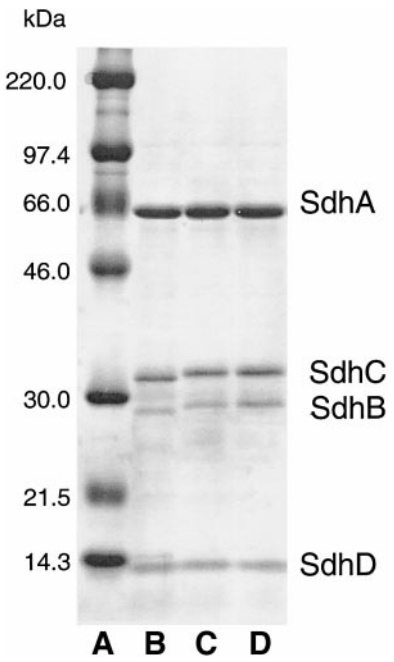

FIG. 1. SDS–PAGE (10 to 15% acrylamide and 0 to 15% sucrose gradient) of the purified *A. ambivalens* complex II. Lane A, molecular mass marker proteins; lane B, 3 μg of pure protein; lane C, 3 μg of pure protein incubated with IAA; and lane D, 3 μg of pure protein incubated with hydroxylamine prior to incubation with IAA.

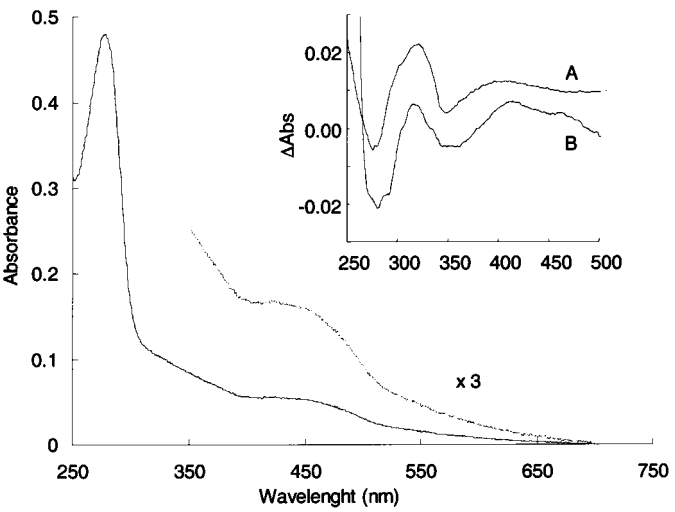

FIG. 2. UV-visible spectrum of native *A. ambivalens* complex II at room temperature. Inset: UV-Visible spectra of caldariella quinone, in methanol. Trace A, extracted from *A. ambivalens* membranes; Trace B, extracted from the purified complex II.

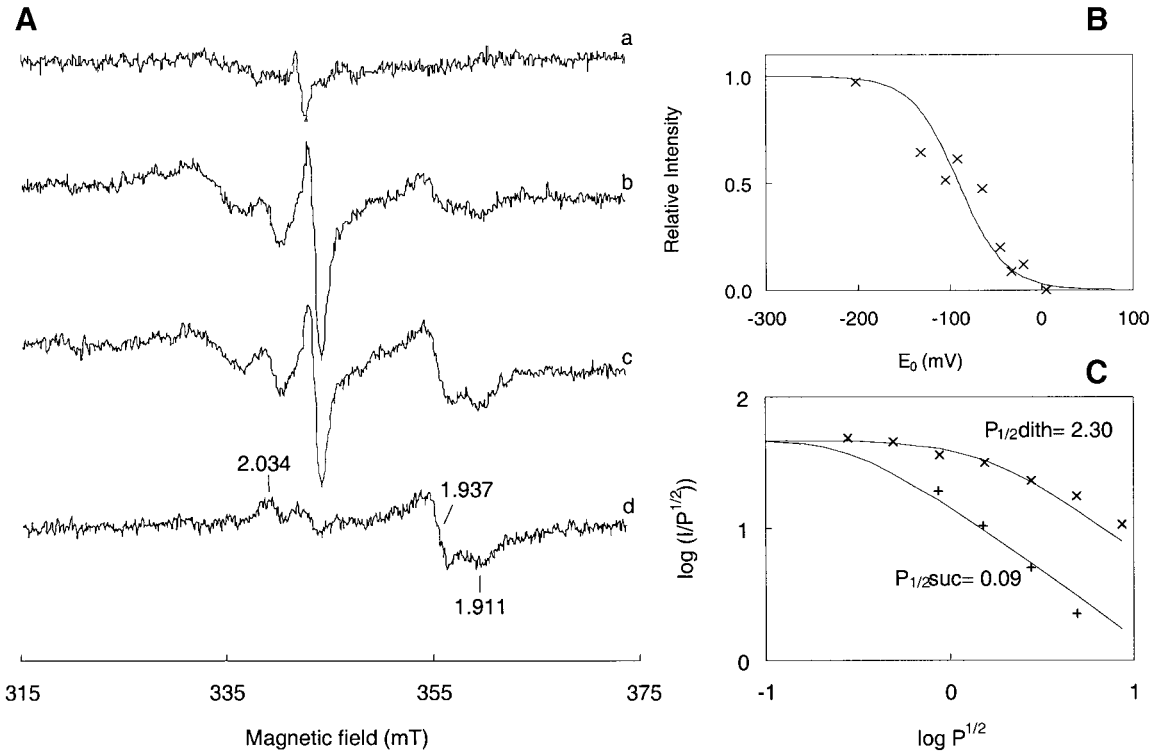

FIG. 3. (A) EPR spectra of *A. ambivalens* complex II at 10K in different redox conditions. Traces: a, native protein as isolated; b, succinate-reduced; c, dithionite reduced; and d, deazoflavin reduced. Temperature 14 K; microwave power 2.4 mW, microwave frequency 9.643 GHz. Protein concentration 1.5 mg.ml^{-1} in 50 mM potassium phosphate buffer pH 6.5/0.1% DM. The succinate reduced form was obtained by a 20 min incubation, at 70°C, with 20 mM of succinate, under a nitrogen atmosphere. The deazoflavin reduced form was obtained by a 30 min light irradiation, under a nitrogen atmosphere, in the presence of EDTA. (B) EPR redox titration curve for *A. ambivalens* complex II cluster S1. The mean of the heights of the $g = 1.94$ and $g = 1.91$ resonances was plotted against each potential and normalised in respect to the fully reduced form. The solid line was calculated from a Nernst equation with $n = 1$ and $E^0 = -90$ mV. (C) Microwave power saturation behaviour of the means of the $g = 1.94$ and 1.91 EPR signals of *A. ambivalens* complex II at 14 K. + succinate-reduced (sample as used in A b) and × dithionite reduced (sample as used in A c). The solid lines were calculated using 2.30 mW and 0.09 mW as the microwave power of half saturation.

Redox Potentiometry

The reduction potential of the dinuclear centre was determined by an EPR monitored redox titration, performed on the pure enzyme complex. The intensities of $g = 1.937$ and $g = 1.911$ resonances were measured as a function of the redox potential of the solution. The mean of the experimental data points were fitted to a Nernst curve with E = −90 mV and $n = 1$ (Fig. 3B).

Bound Caldariella Quinone

The purified succinate-reduced complex II is capable of reducing decylubiquinone, a synthetic quinone, at a rate of 1.2 U.mg^{-1} at 70°C and pH 6.5, under anaerobic conditions. This finding suggests that, although *A. ambivalens* complex II does not contain canonical membrane anchors, it must have quinone-binding site(s). The enzyme was tested for the presence of quinones by extraction with methanol/acetone. The ethanolic extract, upon reduction with borohydride, in anaerobic conditions, displayed a reduced-oxidised UV-Visible spectrum that is identical to that of caldariella quinone isolated from *A. ambivalens* membranes (Fig. 2, inset). This demonstrates that the pure protein contains bound caldariella quinone, similarly to what was recently found from the crystal structure of fumarate reductase from *E. coli*, which contains bound quinone molecules (24).

Membrane Anchoring

No unequivocal transmembrane helices are predicted in the entire *sdhABCD* operon; an alternative could be the association to the membrane through fatty acyl groups (25). The protein was tested for the presence of fatty acids, which could bind to the protein through the several cysteine residues present in the SdhC subunit (3). This possibility was investigated by SDS–PAGE: a first incubation of the protein with iodoacetoamide (IAA) in the presence of SDS and DTT revealed that the SdhB subunit, containing 10 cysteine residues, reacted with IAA, observable by an increase on its molecular mass (Fig. 1, lane C). The SdhC sub-

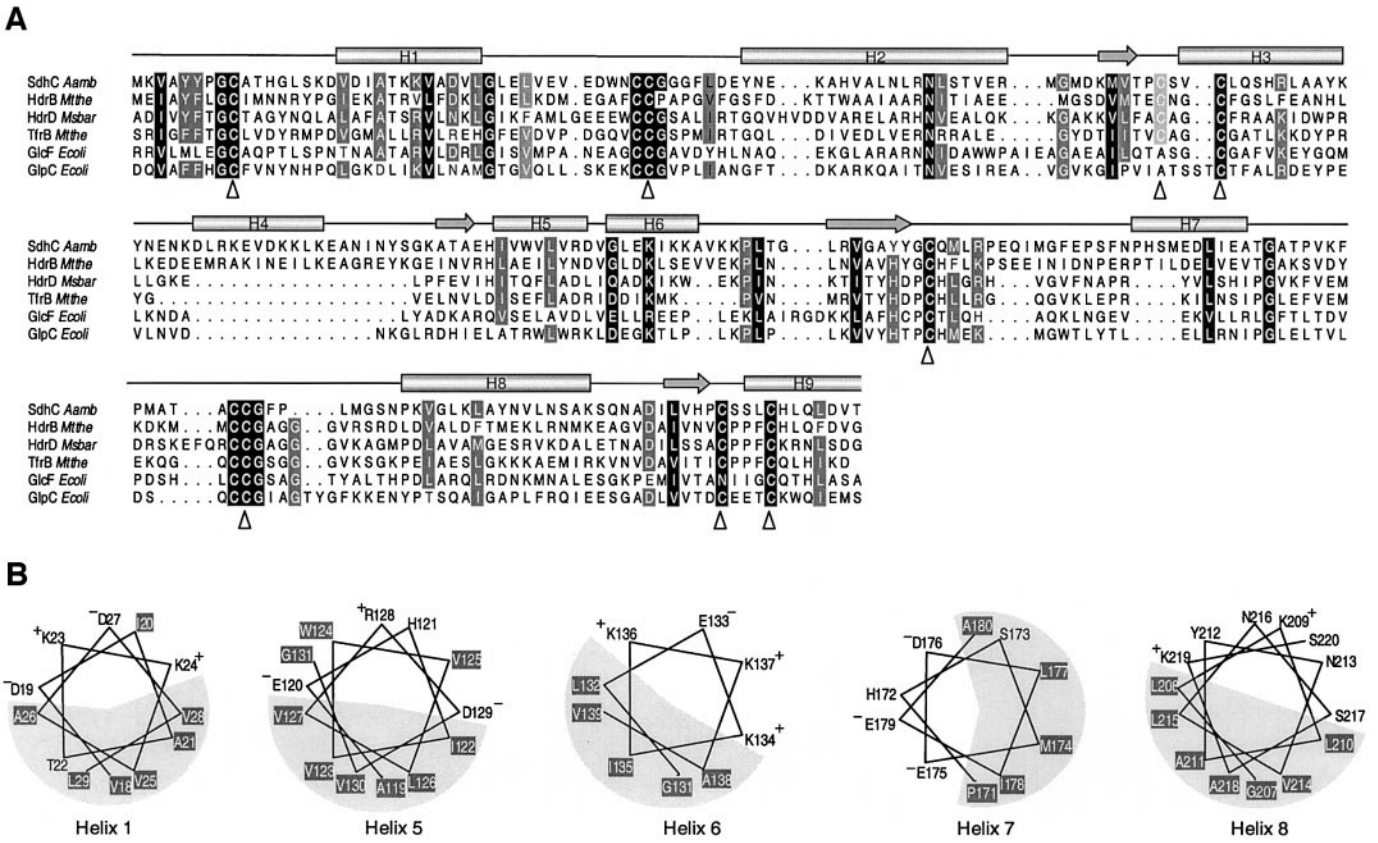

FIG. 4. (A) Multiple aminoacids sequence alignment of *A. ambivalens* complex II SdhC subunit with homologous peptides. △ indicates the cysteine motif. SdhC*Aamb,* succinate dehydrogenase from *A. ambivalens* (CAA06782); Hdr*Mttherm,* heterodissulfide reductase from *Methanobacterium thermoautotrophicum* (ABB86345); Hdr*Msbar,* heterodissulfide reductase from *Methanosarcina barkeri* (CAA70997), Tfr*Mttherm,* thiol:fumarate reductase from *Methanobacterium thermoautotrophicum* (CAA04399); GlcF*Ecoli, g*lycolate oxidase from *Escherichia coli* (P52074) and GlpC*Ecoli* anaerobic sn-glycerol-3-phosphate dehydrogenase from *Escherichia coli* (P13034). (B) Wheel projections of helices 1, 5, 6, 7, and 8 from SdhC of *A. ambivalens.* Shaded boxes indicate hydrophobic side chains and the hydrophobic part of the helices are shaded.

unit, also containing 10 cysteine residues, reacted with IAA, affecting the protein mobility with the same increment (Fig. 1, lane C).

If fatty acids were present in the SdhC subunit, a hydroxylamine treatment would generate extra alkylation sites. To confirm this, prior to the DTT/IAA incubation, the protein was incubated with hydroxylamine; the treatment had no extra effect on the SdhC mobility (Fig. 1, lane D) indicating that indeed the protein does not bind fatty acids.

DISCUSSION

The archaeon *Acidianus ambivalens* has a very simple respiratory system. It is composed by a type-II NADH dehydrogenase, solely containing a covalently bound flavin as cofactor (26); a complex II with a different subunit and cofactor composition (this work); a pool of caldariella quinone (18), which carries electrons between the dehydrogenases and a single type of complex IV, an aa_3-type quinol oxidase (27–30).

A New Class of Succinate Dehydrogenases

The *A. ambivalens* complex II is an excellent example of an emerging new class of succinate dehydrogenases found both in Archaea and Bacteria, which have features distinct from those of the canonical complexes. The evidence for these proteins came either from direct spectroscopic evidence (like for those from *A. infernus, S. solfataricus* and *Sulfolobus sp.* strain 7 (3)), from comparative genomics (*Synechocystis* (31) and *Aquifex aeolicus* (32)), or a combination of both (*A. ambivalens* (3) and *S. acidocaldarius* ((4)). Interestingly, among this new class of SDHs, there is a difference between Archaea and Bacteria concerning the organisation of the gene locus encoding for these new family of succinate dehydrogenases: in Archaea (*A. ambivalens* and *S. acidocaldarius*) a single operon contains the four structural genes, while in Bacteria (*Synechocystis* and *Aquifex aeolicus*) the iron-sulfur and the flavoprotein encoding genes are located in distinct regions of the genome.

TABLE 2

Proteins Containing the $CXnCCGXnCX_2C$ Cysteine Motif

Protein	Organism	Domain	Reference
Succinate dehydrogenase (SdhC)	*Acidianus ambivalens*	A	(3)
	Sulfolobus acidocaldarius	A	(4)
Heterodisulfide reductase (HdrB)	*Methanobacterium thermoautotrophicum*	A	(46)
	Aquifex aeolicus[b]	A	(32)
	Methanococcus jannaschii[b]	A	(47)
	Synechocystis sp[b]	B	(31)
	Campylobacter jejuni[b]	B	a
Heterodisulfide reductase (HdrD)	*Methanosarcina barkeri*	A	(48)
	Archaeoglobus fulgidus[b]	A	(49)
Thiol:fumarate reductase (TfrB)	*Methanobacterium thermoautotrophicum*	A	(43)
	Methanococcus jannaschii[b]	A	(47)
Glycolate oxidase (GlcF)	*Escherichia coli*	B	(45)
	Bacillus subtilis[b]	B	(50)
	Deinococcus radiodurans[b]	B	(51)
	Synechocystis sp[b]	B	(31)
Anaerobic sn-glycerol-3-phosphate dehydrogenase (GlpC)	*Escherichia coli*	B	(41)
	Haemophilus influenzae[b]	B	(52)
	Aquifex aeolicus[b]	B	(32)
	Helicobacter pylori[b]	B	(53)
High Molecular weight cytochrome complex (Hmc orf6)	*Desulfovibrio vulgaris*	B	(54)

Note. Domain, Archaea (A) and Bacteria (B).

[a] Preliminary sequence data was obtained from the Institute for Genomic Research website at http://www.tigr.org.

[b] For these organisms only the gene is known.

Iron-Sulfur Centres Composition

The most prominent divergent feature of this new class of succinate dehydrogenases is a distinct composition of iron-sulfur centres, being the trinuclear iron-sulfur cluster absent. The extra cysteine found in the iron sulfur subunit, SdhB (3) could be the additional ligand to a new tetranuclear cluster, providing it would have an adequate spatial position. In fact, this is the case as shown by homology modelling of the sdhB from *A. ambivalens* using the structure of FrdB from *Escherichia coli* fumarate reductase (38% similarity between sequences) as a template (24) (data not shown). Interestingly, site directed mutagenesis studies in *E. coli* complex II have shown that by adding an extra cysteine, the trinuclear cluster can be converted to a $[4Fe\text{-}4S]^{2+/1+}$ one (33). Thus, in *A. ambivalens*, the likely electron entry point from quinols is the tetranuclear centre providing that it has the adequate redox properties. Since the direct observation of the $[4Fe\text{-}4S]^{2+/1+}$ centres was not possible, probably due to magnetic interactions with neighbouring centres, its reduction potentials were not determined. This magnetic interaction is reminiscent of that occurring on [NiFe] hydrogenases (34), in which the distances between the Fe/S centres is ~10Å (35), close to that found in SDHs (24). Also, it becomes clear, that subunit C, which contains a high number of cysteines, does not contain additional Fe clusters. The reduction potential of the dinuclear centre is slightly lower (−90 mV, pH 6.5) than usual (range from −80 to +80 mV, reviewed in (1)).

Alternative Anchor

A. ambivalens complex II is clearly a membrane anchored protein (3), but only a small putative transmembrane segment in the C terminus can be predicted for the SdhC subunit, whereas the hydropathy profile of SdhD indicates that it is mainly hydrophilic thus, an alternative membrane anchoring containing a quinone-binding site must be present in this complex. Monotopic proteins achieve an alternative anchoring trough amphipathic α-helices that embedded the protein within one of the two leaflets of the membrane (36–39). Sequence analysis of SdhC shows that it contains nine putative helices plus a putative transmembrane helix in the C terminus and two tandem sequences of a $CX_{31-35}CCGX_{38-39}CX_2C$ motif—the cysteine motif—suggesting a gene duplication event (Fig. 4). Conserved helices and similar cysteine motifs are also found in several other proteins (see Table 2 and Fig. 4). All these proteins are found to be membrane associated but they all lack obvious transmembrane helices. For the anaerobic *sn*-glycerol-3-phosphate dehydrogenase

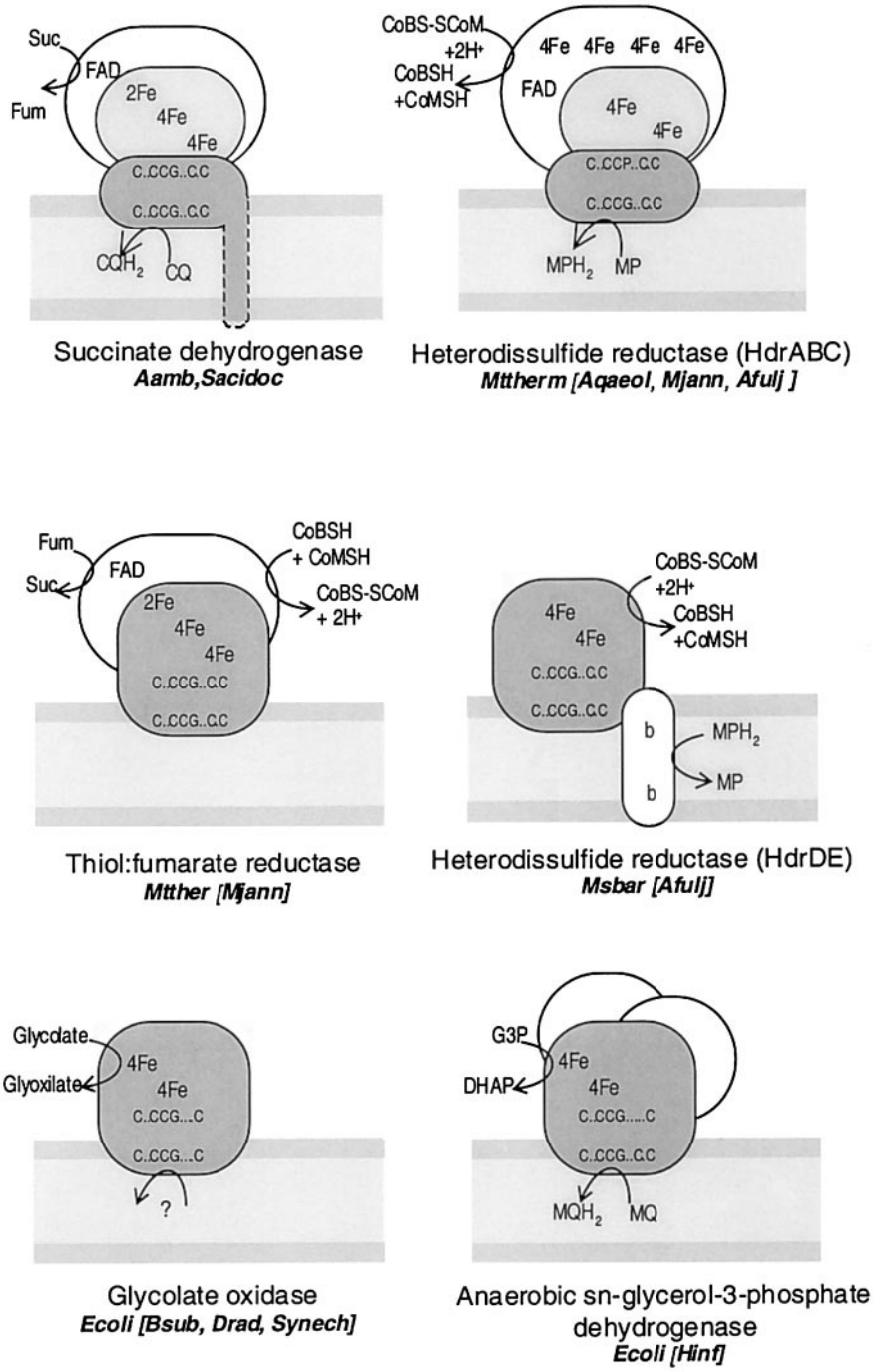

FIG. 5. Schematic representation of several proteins containing SdhC-like domain highlighting the independent module combination hypothesis. CQ, caldariella quinone; CQH_2 reduced caldariella quinone; CoBSH; 7-mercaptoheptanoylthreonine phosphate (coenzyme B) CoMSH, 7-mercaptohethanesulfonate (coenzyme M); CoBS-ScoM, heterodisulfide of coenzyme B and coenzyme M; DHAP, dihydroxyacetone phosphate; Fum, fumarate; MP, methanophenazine; MPH_2, reduced methanophenazine; G3P, glycerol-3-phosphate; Suc, Succinate; *Aamb, A. ambivalens; Sacidoc, S. acidocaldarius; Synech, Synechocytis sp; Aqaeol, Aquifex aeolicus; Mttherm, Methanobacterium thermoautotrophicum; Mjann, Methanoccocus jannaschii; Afulj, Archaeoglobus fulgidus; Msbar, Methanosarcina barkeri; Ecoli, Escherichia coli; Hinf, Haemophilus influenzae; Bsub, Bacillus subtilis; Drad, Deinococcus radiodurans* R1. For the organisms indicated between brackets only the gene is known.

from *E. coli* it was shown that the subunit containing the cysteine motif is essential for membrane anchoring and for interaction with menaquinone (40, 41). Wheel projections of the conserved helices from *A. ambivalens* SdhC and from HdrB*Mtther*, HdrD*Msbar*, TfrB*Mtther*, GlcF*Ecoli*, and GlpC*Ecoli* predict that several amphi-

pathic α-helices can be formed (Fig. 4) leading to the hypothesis that the SdhC peptide from *A. ambivalens* anchors to the protein monotopically, similarly to what may also happen in the other presented proteins (Fig. 5). A characteristic of some amphipathic helices is that the correct helical fold is only achieved in the presence of anionic lipids (37); this might explain why, *in vitro*, the purified *A. ambivalens* complex II is not capable of reducing caldariella quinone: this slight misfold would prevent adequate caldariella quinone binding. Database searching for analogues of SdhD result in no significant matches, with the exception of SdhD from *S. acidocaldarius* (4), but the peptide also contains amphipathic α-helices suggesting that it may also be involved in membrane attachment by a similar strategy (data not shown).

The observed sequence similarities between different proteins further extends the recent suggestion that complex II enzymes have a modular architecture (42). In fact, these protein complexes are apparently a combination of independent modules: flavoprotein, iron-sulfur and cysteine-motif containing (Fig. 5). In the case of the *A. ambivalens* SDH, these modules remained unassembled but for instance, in Thiol:fumarate reductase from *M. thermoautotrophicum* Marburg (43) and anaerobic sn-glycerol-3-phosphate dehydrogenase and Glicolate oxidase from *E. coli* (40, 41, 44, 45), two of the modules fused and a single peptide holds the cysteine-motif as well as the iron sulfur clusters. In the case of the Hdr from *M. thermoautotrophicum* Marburg (46), the flavinic peptide bears additional iron-sulfur clusters while maintaining separated the remaining modules. This denotes that these proteins share a common ancestry in respect to their composing domains, whose combination was imposed by the fine tuning of the different catalytic functions they perform. Undoubtedly, the elucidation of the molecular phylogeny of these proteins and the clarification of the function of the cysteine-rich subunit in these complexes, is a challenging task for the future.

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