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Structural Organization of Essential Iron-Sulfur Clusters in the Evolutionarily Highly Conserved ATP-binding Cassette Protein ABCE1*

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The ABC protein ABCE1, formerly named RNase L inhibitor RLI1, is one of the most conserved proteins in evolution and is expressed in all organisms except eubacteria. Because of its fundamental role in translation initiation and/or ribosome biosynthesis, ABCE1 is essential for life. Its molecular mechanism has, however, not been elucidated. In addition to two ABC ATPase domains, ABCE1 contains a unique N-terminal region with eight conserved cysteines, predicted to coordinate iron-sulfur clusters. Here we present detailed information on the type and on the structural organization of the Fe-S clusters in ABCE1. Based on biophysical, biochemical, and yeast genetic analyses, ABCE1 harbors two essential diamagnetic [4Fe-4S]²⁺ clusters with different electronic environments, one ferredoxin-like (CPX₂C₅₋₅₋₅₋₅; Cys at positions 4–7) and one unique ABCE1-type cluster (CXX₄C₅₋₅₋₅₋₅₇₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋₅₋¢-

Iron-sulfur (Fe-S)² clusters constitute an ancient prosthetic group, which can be found in proteins from all living organisms. They are only composed of the inorganic components sulfur and iron. In the most common cluster variants, [2Fe-2S], [3Fe-4S], and [4Fe-4S], the metal ions are directly coordinated by the protein backbone (1). Nevertheless, amino acids like histidine can also contribute to the iron coordination, as known for [2Fe-2S] Rieske clusters. Additionally, much more complicated structural arrangements can be found. The complex FeMoco and P-cluster of nitrogenase are just one example of clusters with higher nuclearity, arising from smaller substructures and clusters containing additional metal atoms like molybdenum (2). Furthermore, interconversion of Fe-S clusters is a widely distributed phenomenon, reflecting the dynamic arrangement and behavior (3). Despite their relative simple composition of only iron and sulfur (in most cases), Fe-S clusters are often essential components for the enzymatic function and are thereby involved in a vast variety of cellular processes. Beside their obvious role in electron transport, they operate as sensors for iron, for example, modulate protein stability, and play a role in nucleic acid binding and modification (1, 4).

Although Fe-S clusters can be synthesized in vitro (5), their assembly and maturation in vivo requires a highly complex and regulated machinery (6). Mitochondria are the central compartment in Fe-S cluster biogenesis, which is also the only essential function of this organelle known to date (6, 7). The underlying concept is not understood so far, because mitochondrial Fe-S cluster proteins are not essential for cell viability. The cytosolic, essential Fe-S protein ABCE1 could explain this phenomenon, because of the fact that Fe-S clusters can be transported from mitochondria to the cytosol (8, 9), where they are incorporated into the protein moiety.

ABCE1 is found evolutionarily conserved in all Archaea and Eukaryota, where it is essential for life (9–13). ABCE1 belongs to the superfamily of ATP-binding cassette (ABC) proteins with twin ABC ATPase domains, which are arranged in a head-to-tail orientation via a flexible linker and hinge region (14). Most of these members constitute membrane proteins that mediate ATP-driven unidirectional transport of a variety of molecules across biological membranes. Because ABCE1 does not contain any transmembrane domain, its function cannot be related to a membrane transport process. The protein was orig-
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Expression of S. solfataricus ABCE1 in Escherichia coli—The open reading frame SSO0287 of S. solfataricus ABCE1 was amplified by PCR using genomic DNA of S. solfataricus and the primers, P1f 5’-CCATATCCATGTTGAGATTGTC-3’ and P1r 5’-CCATATGGATCCCTGGAATGGAACAGAGGAG-3’, and cloned into the NcoI and BamHI sites of the pSA4 expression vector (22). The resulting plasmid (pSD1) codes for wild type ABCE1 of S. solfataricus with a C-terminal His tag. The two conserved cysteines, Cys-24 and Cys-54, were individually exchanged for serine residues (C24S and C54S) by QuikChange site-directed mutagenesis (Stratagene). PCR and mutagenesis products were confirmed by DNA sequencing. The E. coli strain BL21(DE3) (Novagen) was co-transformed with either of the ABCE1 constructs and the pRARE plasmid (Novagen) coding for rare tRNAs and grown in LB medium supplemented with 100 μg/ml ampicillin and 25 μg/ml chloramphenicol at 37 °C. Expression was induced at an OD_{600} of 0.6 for 3 h at 30°C by adding 0.2 mM isopropyl β-D-thiogalactopyranoside.

Purification of ABCE1—All purification steps and experimental analysis were carried out under an argon atmosphere or in an anaerobic chamber containing 95% N_2, 5% H_2 (Coy Laboratories). Before use, buffers were degassed and equilibrated inside the anaerobic chamber for several days. Cell pellets of S. solfataricus were resuspended in lysis buffer A (20 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 7.4), disrupted by using a Branson Sonifier 250 at 60% output in eight pulses of 30 s on ice, and centrifuged for 30 min at 114,000 × g. Afterward, ABCE1 was purified to homogeneity via metal affinity chromatography (HisSelect, Sigma) by washing/elution with 20 mM, 300 mM imidazole. For purification of ABCE1 expressed in E. coli, frozen cells were thawed, resuspended in lysis buffer B (20 mM Tris-HCl, 100 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, pH 7.4), and disrupted by sonication as described before. After centrifugation at 114,000 × g for 30 min, the supernatant was heated at 70°C for 10 min and additionally centrifuged for 1 h at 114,000 × g. The solution was subsequently applied to metal affinity chromatography (HisTrap, GE Healthcare). ABCE1 was purified by washing/elution with 60 mM, 200 mM imidazole. Protein fractions were exchanged to buffer C (20 mM Tris-HCl, 100 mM NaCl, pH 7.4) using a Centricon device (Millipore). The protein concentration was determined by the Coomassie Plus™ Bradford assay (Pierce) using bovine serum albumin as a standard.

Determination of Iron and Sulfur—Total reflection x-ray fluorescence (TXRF) as a trace multielement analytical method was applied to quantify the iron and sulfur content of purified ABCE1. The measurement was carried out using an EXTRA IIA spectrometer (Atomica Instruments) with a sample volume of 4 μl in 50 mM Tris acetate buffer, pH 7.5. 20 μl of protein solution were placed onto siliconized quartz carrier plates and evaporated to dryness. By excitation with the Mo(Kα) line for 1000 s, a multielement fluorescence spectrum was obtained. The intensities of the sulfur and iron peaks were related to a rubidium peak as an internal standard. The iron content of ABCE1 was further determined colorimetrically by the method of...
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Fish (23), and the inorganic sulfur was quantified according to Beinert (24).

UV-visible Spectroscopy—Spectra of wild type and ABCE1 mutants were recorded on a Cary 50 spectrophotometer (Varian) in buffer C. ABCE1 was titrated with sodium dithionite or freshly prepared potassium ferricyanide (both in buffer C). The spectra of the oxidant and reductant solutions alone were subtracted from the corresponding curves.

EPR Spectroscopy—ABCE1 was analyzed by continuous wave EPR, either untreated, reduced with sodium dithionite or ascorbate, or oxidized with potassium ferricyanide in buffer C. X-band EPR spectra were measured on a Bruker E-500 elexsys spectrometer using a standard rectangular Bruker EPR cavity equipped with an ESR900 helium flow cryostat (Oxford Instruments). The spectra were recorded under the following experimental conditions: microwave frequency, 9.424 GHz; microwave power, 8 milliwatts; field modulation frequency, 100 kHz; field modulation depth, 5 G (peak to peak); temperature, 10 K.

To study the immediate environment around the Fe-S cluster, pulsed EPR was performed on a Bruker E-580 spectrometer using a Bruker EPR cavity (MD5-W1) equipped with a helium flow cryostat (CF935, Oxford Instruments). The pulses were amplified using a 1-kilowatt pulsed traveling wave tube amplifier. A conventional two-pulse ($\pi$/2-\(\tau\)-\(\pi\)) sequence was used. A two-pulse, echo modulation experiment (ESEEM) was performed by integrating the area under the Hahn echo as a function of time between the two microwave pulses. The frequency domain spectrum was obtained by Fourier transformation of the time domain trace after subtraction of a mono-exponential decay function. This measurement was performed at 10 K at a microwave frequency of 9.745 GHz using a $\pi$/2 pulse length of 8 ns, a starting $\tau$ of 132 ns, and a short repetition rate of 1 ms.

Mössbauer Spectroscopy—For preparation of the Mössbauer sample, metallic $^{57}$Fe (96% enrichment; Chemotrade) was dissolved in 37% (v/v) HCl at 80 °C for several days. The obtained $^{57}$FeCl$_3$ solution was directly added to the S. solfataricus medium (without FeCl$_3$) to a final concentration of 41 $\mu$M. Mössbauer spectra of purified ABCE1 were recorded in buffer C by using a conventional spectrometer in the constant acceleration mode. Isomer shifts are given relative to $\alpha$-Fe at room temperature. Zero-field spectra were measured in bath cryostats (Oxford Instruments), whereas for the high field spectra (4 T $\perp \gamma$), a cryostat equipped with a superconducting magnet was used (Oxford Instruments). Magnetically split spectra were simulated within the spin Hamilton formalism (25); otherwise, spectra were analyzed by least square fits using Lorentzian line shape.

X-ray Absorption Spectroscopy (XAS)—ABCE1 was concentrated to 1.2 $\mu$M in iron. Afterward, XAS sample was filled into the 25-$\mu$l plastic XAS cuvettes and stored at cryogenic temperatures. The K-edge iron x-ray absorption spectrum was recorded at the beam line D2 of the EMBL Outstation Hamburg at DESY (Germany). The DORIS storage ring operated at 4.5 GeV with the positron beam current ranging from 145 to 80 mA. A $^{111}$Si double-crystal monochromator scanned x-ray energies around the iron K-edge (6.9–8.1 keV). Harmonic rejection was achieved by a focusing mirror (cut-off energy at 20.5 keV) and a monochromator detuning to 50% of its peak intensity. The sample cells were mounted in a two-stage Displex cryostat and kept at about 20 K. The x-ray absorption spectra were recorded as iron $K_{\alpha}$ fluorescence spectra with a Canberra 13-element germanium solid-state detector. Data reduction, such as background removal, normalization, and extraction of the fine structure, was performed with KEMP (26) assuming a threshold energy $E_{\text{th}} = 7120$ eV. Sample integrity during exposure to synchrotron radiation was checked by monitoring the position and shape of the absorption edge on sequential scans. No changes were detectable. The extracted iron K-edge (25–860 eV) EXAFS data were analyzed as reported previously (27).

Functional Analysis of ABCE1 Mutants in Yeast—To investigate various cysteine mutants of ABCE1, we used a yeast strain, in which the endogenous promoter was replaced by a tetracycline-regulated (28). The cells were transformed with the multicopy plasmid pRS423 harboring the wild type or ABCE1 mutants. A plasmid coding for $abce1$ from Saccharomyces cerevisiae was generated by PCR using the primers P2f $5'\text{-ATGGTCAGCCTCTCGTCTGCAAGCG-3'}$ and P2r $5'\text{-ATACCCCCGATCGCCGATCACGGAGG-3'}$ with chromosomal DNA as a template. The amplified construct was inserted into the Sall and Smal restriction sites of the vector pRS423. ABCE1 was expressed under the control of the endogenous promoter. The highly conserved N-terminal cysteine residues Cys-16, Cys-21, Cys-25, Cys-29, Cys-55, Cys-58, Cys-61, and Cys-65 as well as the cysteine Cys-38 were exchanged to serines or to alanines by site-directed mutagenesis. Single, double, and triple mutations were created and transformed into yeast cells. Following doxycycline treatment, the chromosomal expression of ABCE1 was repressed and the ABCE1 mutants substituted for the endogenous protein in the cells. The pRS423 vector containing the wild type ABCE1 gene and the empty vector served as control plasmids.

RESULTS

Expression and Isolation of ABCE1—To analyze the structural organization and function of Fe-S clusters in ABCE1, we used a double tracked approach. (i) Functional studies of each cysteine in Fe-S cluster assembly of ABCE1 were performed in yeast. (ii) For biophysical and biochemical analyses of the Fe-S cluster, we chose ABCE1 from the hyperthermophilic crenarchaeote S. solfataricus. Notably, this thermostable protein contains only the eight conserved cysteines putatively coordinating Fe-S clusters. The yeast and archaeal protein are highly homologous (43% identity and 66% similarity, see also alignment of the N-terminal domain in Fig. 7B).

Based on a newly developed inducible expression system in S. solfataricus (18), ABCE1 was overexpressed and purified to homogeneity in quantities sufficient for biophysical studies (Fig. 1A). 0.5 mg of protein (68 kDa) was obtained from 1 liter of culture. Because of the unexpected finding of viable ABCE1 cysteine mutants, we transferred the corresponding mutations into ABCE1 from S. solfataricus for further analysis. Genetic manipulations in Archaea are still time-consuming and by far not a routine method. Therefore, wild type and the ABCE1 mutants were heterologously expressed in E. coli and purified to homogeneity as described above. In this case, 1–2 mg of...
protein was obtained from 1 liter of E. coli culture. Interestingly, some degradation products were copurified with the C24S mutant (Fig. 1B). This behavior is typical for Fe-S proteins, in which clusters are absent or not correctly assembled.

All ABCE1 preparations, except the C24S mutant, showed a brownish color, typical for Fe-S cluster proteins (Fig. 1C). After exposure to oxygen, the C54S mutant rapidly lost its color, whereas WT ABCE1 isolated either from S. solfataricus or E. coli remained brownish for at least several hours (data not shown).

Assembly of Two Fe-S Clusters in ABCE1—ABCE1 isolated from S. solfataricus exhibits a characteristic UV-visible spectrum with a maximum at 280 nm, a shoulder at 320 nm, and a broad peak around 410 nm (Fig. 2A), indicative for cubane [4Fe-4S]- or cuboidal [3Fe-4S]-type clusters (29). The molar extinction coefficient ε_{410} of 29,000 M\(^{-1}\) cm\(^{-1}\) is in the range of proteins containing two of these clusters (30). The absorption spectrum of WT ABCE1 isolated from E. coli differed only by the extinction coefficient ε_{410} of 24,000 M\(^{-1}\) cm\(^{-1}\) (17% reduction compared with ABCE1 isolated from S. solfataricus). Surprisingly, the C54S mutant showed an absorption spectrum very similar to WT ABCE1 purified from E. coli (ε_{410} = 22,500 M\(^{-1}\) cm\(^{-1}\)), demonstrating that the assembly of the Fe-S clusters is comparable in both proteins. In contrast, the C24S mutant showed no specific absorption at 410 nm revealing a defect in Fe-S cluster assembly. As addressed below, the functional consequences of these mutations have been examined in yeast.

Titration of WT ABCE1 with the reductant sodium dithionite (Fig. 2B) or ascorbate (not shown) did not significantly change the UV-visible spectra. This demonstrates the stability of the Fe-S cluster at low redox potential. In contrast, titration with the oxidant potassium ferricyanide resulted in loss of the
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410-nm peak and the appearance of new absorption bands at 340 and 450 nm (Fig. 2C). Remarkably, the C54S mutant exhibited a greater sensitivity to oxidation by ferricyanide as compared with WT ABCE1, resulting in an immediate loss of the absorption at 410 nm (data not shown).

We next quantified the amount of incorporated iron and sulfur in the WT and ABCE1 mutants by TXRF spectroscopy and colorimetric assays. WT ABCE1 (1 nmol) isolated from S. solfataricus contains 7.0 nmol of iron and 6.1 nmol of acid-labile sulfur per nmol of protein (Table 1). In comparison, WT ABCE1 purified from E. coli harbors 13% less iron (6.1 nmol) and 10% less acid-labile sulfur (5.5 nmol). Considering a small amount of impurities and the intrinsic error in protein quantification, it seems likely that the iron and sulfur content are slightly underestimated. Especially for the protein isolated from E. coli, a small population of nonassembled Fe-S cluster can be found.

Together with the UV-visible data, these findings clearly demonstrate the presence of two cubane or one cubane and one cuboidal Fe-S clusters in the ABCE1. The C54S mutant showed also incorporation of iron and acid-labile sulfur but with decreased values compared with WT ABCE1. It is worth mentioning that the Fe-S clusters in the C54S mutant were extremely labile, resulting in a loss of iron and sulfur during buffer exchange (e.g. dialysis). In the C24S mutant, no significant iron and acid-labile sulfur were detected, consistent with the colorless protein solution (Fig. 1C) and the UV-visible spectra (Fig. 2A). Importantly, no other metal ions such as copper, nickel, zinc, or molybdenum were found in all ABCE1 proteins by TXRF analysis.

ABCE1 Harbors Diamagnetic Fe-S Clusters—We next examined the Fe-S clusters in ABCE1 by EPR spectroscopy. The two Fe-S clusters found in ABCE1 are EPR-silent and therefore in a diamagnetic state upon oxidation or cysteine mutagenesis. Two [4Fe-4S]^{2+} Clusters of ABCE1 Exist in Different Electronic Environments—To finally confirm the type of the two diamagnetic Fe-S clusters, WT ABCE1 was analyzed by Mössbauer spectroscopy. ABCE1 was labeled with ^{57}Fe in S. solfataricus and purified as described under the “Experimental Procedures.” The Mössbauer spectrum of ABCE1 obtained at 77 K has been analyzed with three quadrupole doublets (Fig. 4A). Species 1 has an isomer shift of δ1 = 0.43 mm/s, a quadrupole splitting of ΔE_{Q1} = 1.32 mm/s, and a relative contribution of 44%. Species 2 exhibits an isomer shift of δ2 = 0.42 mm/s, a quadrupole splitting of ΔE_{Q2} = 0.86 mm/s, and also a relative contribution of 44%. The negative sign of ΔE_{Q2} has been determined by the analysis of the high field Mössbauer spectra (see Fig. 4B). The isomer shifts of species 1 and 2 are characteristic for Fe^{2+} pairs of [4Fe-4S]^{2+} clusters (25, 33).

Fig. 4B shows a Mössbauer spectrum of ABCE1 taken at 4.2 K in a field of 4 T perpendicular to the γ-beam. The observed magnetic splitting was successfully reproduced by the simula-
Coordination of Iron in the Clusters—The edge shape of the x-ray absorption near-edge structure spectra (Fig. 5A), which can be used as a fingerprint for the electronic structure of the metal ions, resembles the one reported for oxidized hydrogenase II from Clostridium pasteurianum, which harbors \([4\text{Fe}-4\text{S}]^{2+}\) clusters (34). Typical features are the resonance in the rising edge and the rather flat maximum followed by a sharp minimum. The extracted fine structure (EXAFS) is dominated by a single frequency with a shift at about 8 Å\(^{-1}\). The high intensity at wave numbers larger than 10 Å\(^{-1}\) in biological samples indicates the presence of a multinuclear metal center. This is consistent with the peak at about 2.8 Å in the corresponding Fourier transformation (FT). The high intensity of the main peak at about 2.3 Å in the FT points toward a homogeneous first coordination sphere formed by sulfur ligands. The FT is very similar to the one observed for the \([4\text{Fe}-4\text{S}]^{2+}\) clusters mentioned above (34). Models based on the assumption of \([4\text{Fe}-4\text{S}]^{2+}\) clusters resemble the data very well (Fig. 5, B and C). All attempts to replace one of the sulfur ligands in the fit by light atoms such as oxygen or nitrogen lead to artificially long Fe-S distances. Thus, recognizable contributions above 10% of the average ligand sphere are excluded. The Fe-S distance of 2.74 Å and the Fe-3Fe signal at 2.29 Å match the literature values for oxidized \([4\text{Fe}-4\text{S}]^{2+}\) clusters very well (35).

**Essential Function of the Fe-S Clusters in ABCE1**—By means of biophysical and biochemical analyses, we resolved that ABCE1 from *S. solfataricus* contains two diamagnetic \([4\text{Fe}-4\text{S}]^{2+}\) clusters. However, the functional impact of the two different Fe-S clusters cannot easily be addressed in Archaea. Therefore, we initiated a genetic analysis in *S. cerevisiae*, where ABCE1 has been shown to be essential for viability because of its fundamental role in translation initiation and ribosome biosynthesis (9, 12, 17). By systematic mutation of all conserved cysteine residues, individually or in combination, we addressed...
ABCE1 Contains Two Essential [4Fe-4S]$_2^{2+}$ Clusters

**A**

![Diagram A](image1)

**B**

![Diagram B](image2)

**C**

![Diagram C](image3)

**D**

<table>
<thead>
<tr>
<th>single mutants</th>
<th>double mutants</th>
<th>triple mutants</th>
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<td>C61S non viable</td>
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**FIGURE 6.** Functional analysis of ABCE1 in *S. cerevisiae*. Plasmids of ABCE1 harboring the indicated mutations were transformed into a yeast strain, in which the endogenous promoter was replaced by a tetracycline-repressible promoter and plated on minimal agar using histidine as a selection marker. Single colonies were then spread on minimal agar plates containing doxycycline. The plasmid containing WT ABCE1 served as a positive control (wt), and the empty vector as a negative control (−K). A, mutation of the eight conserved cysteine residues to serine. B, single cysteine-to-alanine mutations of the viable mutants from the first screen, as well as the double mutant S28A/C29A. C, mutation of the extra cysteine residue Cys-38 to alanine individually and in combination with selected conserved residues. Double and triple mutants of the viable mutants from the first screen are lethal. D, schematic overview of the cysteine mutation analysis in yeast.

Remarkably, only five of the eight conserved cysteines (Cys-16, Cys-25, Cys-55, Cys-61, and Cys-65; at positions 1, 3, 5, 7, and 8) were found to be essential for cell survival (Fig. 6A). To our surprise the C21S, C29S, and C58S mutants (at positions 2, 4, and 6) are still viable (Fig. 6A). It should be mentioned that the C21S mutant shows a slow growth phenotype. Because it is known that serine residues can coordinate Fe-S clusters in certain cases, we subsequently mutated each of these three cysteine residues to alanine. Here, C29A and C58A (at positions 4 and 6) were still viable, but the C21A mutant (at position 2) was lethal (Fig. 6B). In conclusion, six coordinating cysteines, of which serine at position 2 (C21S) can partially be accepted, are strictly required for the formation of the Fe-S clusters and cell survival. The inviability of the C25S and C61S mutant is in agreement with previous studies, which further demonstrated that substitution of these cysteines had no effect on the expression level and stability of the protein (9).

Interestingly, the viable yeast Cys-58 mutant corresponds to the C54S mutant in *S. solfataricus*. Biological and biochemical analyses indicate that this mutant contains a [4Fe-4S]$^{2+}$ and a paramagnetic [3Fe-4S]$^{2+}$ center. In conclusion, the cysteine at position 6 is not essential for the assembly of the Fe-S cluster and vital function of ABCE1.

The viability of the Cys-29 mutants remained enigmatic, because the corresponding mutant in ABCE1 from *S. solfataricus* (C24S) showed a defect in Fe-S cluster assembly (see Figs. 1 and 2). We excluded that the adjacent Ser-28 rescues the Cys-29 mutation (Fig. 6B). However, by *in silico* analysis, we noticed that, in contrast to most archaeal homologues, all ABCE1 in Eukarya carry an extra cysteine in close proximity to the iron-sulfur centers. This corresponds to Cys-38 in *S. cerevisiae* ABCE1. In a process called ligand swapping, such a cysteine, originally not involved in Fe-S cluster coordination, can take over the function of a missing or mutated cysteine residue. To test this hypothesis, we generated the double C29A/C38A mutant. Indeed, this double mutation was lethal, whereas cells with the double mutation C58A/C38A as a control grew normally, demonstrating a site-specific ligand swapping between Cys-29 and Cys-38 (Fig. 6B).

Finally, double and triple mutants comprising the dispensable cysteines were generated. Notably, any combination of the otherwise viable mutations shows an additive effect, leading to a lethal phenotype (Fig. 6C).

**DISCUSSION**

The presence of Fe-S clusters discriminates the evolutionarily highly conserved protein ABCE1 from all other members of the ABC superfamily. In this study, we determined the type, coordination, and functional relevance of the Fe-S clusters in ABCE1 of Archaea and Eukarya. For detailed biophysical and biochemical analyses, we used ABCE1 from the hyperthermophilic crenarchaeote *S. solfataricus*, which contains only the eight conserved cysteine residues, putatively coordinating Fe-S clusters. By means of a novel expression system, ABCE1 was overexpressed and isolated from the homologous host, which ensured the complete machinery for Fe-S cluster assembly. Expression in *E. coli* was used to examine various mutants efficiently. The functional role of the Fe-S clusters was, however,
ABCE1 Contains Two Essential \([4\text{Fe}-4\text{S}]^{2+}\) Clusters

The combination of structural and functional analyses clearly demonstrates the presence of two diamagnetic \([4\text{Fe}-4\text{S}]^{2+}\) clusters in ABCE1 and further highlights the essential role of the conserved cysteines for Fe-S cluster assembly. Sequence comparison shows that the Fe-S cluster coordination in ABCE1 (Fig. 7A) partially resembles those of eight iron ferredoxins, e.g., in *Desulfovibrio africanus* ferredoxin III or *Azotobacter vinelandii* ferredoxin I (36, 37). We therefore conclude that ABCE1 contains one ferredoxin-like \([4\text{Fe}-4\text{S}]^{2+}\) cluster formed by the cysteines at positions 4–7. Indeed, this cluster perfectly matches the ferredoxin-type consensus sequence CP\(X_n\)CP\(X_n\)CP\(X_n\)CP (Fig. 7B). The coordination of the second Fe-S cluster in ABCE1 (cysteines at positions 1–3 and 8) has not been described in any other protein. Hence, we propose a unique ABCE1-type \([4\text{Fe}-4\text{S}]^{2+}\) cluster formed by the cysteines at positions 1–3 and 8.

addressed in yeast, where ABCE1 is essential for cell viability (9, 12, 17).

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Both clusters are equally present but have a slightly different electronic environment as demonstrated by Mössbauer spectroscopy (see Fig. 4). Based on the quadrupole splitting, species 1 (\(\delta = 0.43 \text{ mm/s}, \Delta E_{Q1} = 1.32 \text{ mm/s}\)) is typical for a ferredoxin-like cluster (38, 39), whereas species 2 (\(\delta = 0.42 \text{ mm/s}, \Delta E_{Q2} = -0.86 \text{ mm/s}\)) should reflect the ABCE1-type cluster. Additional atoms, such as oxygen or nitrogen from aspartate, histi-
ABCE1 Contains Two Essential [4Fe-4S]2+ Clusters

TABLE 1
Iron and sulfur determination from ABCE1 (1 nmol)

<table>
<thead>
<tr>
<th></th>
<th>Ferrozine</th>
<th>Inorganic sulfur, methylene blue</th>
<th>Total sulfur, TXRF</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>7.2 ± 0.5</td>
<td>6.8 ± 0.3</td>
<td>6.1 ± 0.2</td>
</tr>
<tr>
<td>WT</td>
<td>6.2 ± 0.2</td>
<td>6.0 ± 0.2</td>
<td>5.5 ± 0.1</td>
</tr>
<tr>
<td>C54S</td>
<td>4.4 ± 0.1</td>
<td>ND</td>
<td>3.1 ± 0.1</td>
</tr>
<tr>
<td>C24S</td>
<td>ND</td>
<td>4.0 ± 0.1</td>
<td>0.0 ± 0.0</td>
</tr>
</tbody>
</table>

* ND indicates not done.

Iron and sulfur determination from ABCE1 (1 nmol)

ABCE1 contains two essential [4Fe-4S]2+ clusters, which can exist in a [3Fe-4S] cluster that is dispensable for the essential ABCE1 function. Strikingly, this cluster can also exist in a [3Fe-4S] cluster, is dispensable for the essential ABCE1 function. Strikingly, all eukaryotic ABCE1 proteins contain a conserved extra cysteine within the N-terminal Fe-S cluster domain, which can rescue Cys-29 (position 4) by ligand swapping. This extra cysteine, absent in most Archaea, explains why the mutation at position 4 (C24S) in S. solfataricus ABCE1 showed no assembled Fe-S clusters. Noteworthy, mutation of Cys-38 (extra cysteine) has no effect on ABCE1 function in yeast. It is therefore questionable if ligand swapping occurs in vivo.

ABC-type proteins are evolutionarily highly conserved molecular machines, coupling ATP binding and hydrolysis to conformational changes (43, 44). The smallest functional unit appears to be an ABC dimer, which operates in a processive engagement/disengagement cycle (44–46). These chemomechanical engines drive not only membrane translocation but also a variety of other crucial biological processes, such as DNA repair and chromosome segregation. The fundamental role of ABCE1 in RNase L inhibition, human immunodeficiency virus capsid maturation, translation initiation, and ribosome biosynthesis (8, 12, 13, 15–17) suggests that the two essential diamagnetic [4Fe-4S]2+ clusters identified in this study are involved in recognition and modification (chemical or conformational) of RNA assemblies.

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REFERENCES

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