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Surf1, Associated with Leigh Syndrome in Humans, Is a Heme-binding Protein in Bacterial Oxidase Biogenesis*

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Freya A. Bundschuh[‡], **Achim Hannappel**[‡], **Oliver Anderka**[‡1], **and Bernd Ludwig**[‡§2]

From the [‡]*Institute of Biochemistry, Molecular Genetics, Goethe University, D-60438 Frankfurt am Main and* [§]*Cluster of Excellence Macromolecular Complexes Frankfurt am Main (CEF), D-60438 Frankfurt am Main, Germany*

Biogenesis of mitochondrial cytochrome *c* oxidase (COX) relies on a large number of assembly factors, among them the transmembrane protein Surf1. The loss of human Surf1 function is associated with Leigh syndrome, a fatal neurodegenerative disorder caused by severe COX deficiency. In the bacterium *Paracoccus denitrificans*, two homologous proteins, Surf1c and Surf1q, were identified, which we characterize in the present study. When coexpressed in *Escherichia coli* together with enzymes for heme *a* synthesis, the bacterial Surf1 proteins bind heme *a in vivo*. Using redox difference spectroscopy and isothermal titration calorimetry, the binding of the heme cofactor to purified apo-Surf1c and apo-Surf1q is quantified: Each of the *Paracoccus* proteins binds heme *a* in a 1:1 stoichiometry and with K_d values in the submicromolar range. In addition, we identify a conserved histidine as a residue crucial for heme binding. Contrary to most earlier concepts, these data support a direct role of Surf1 in heme *a* cofactor insertion into COX subunit I by providing a protein-bound heme *a* pool.

Leigh syndrome (LS)[3] is an autosomal recessive inherited neurodegenerative disorder characterized by focal, bilateral lesions in one or more areas of the central nervous system (1). Symptoms start in early childhood, and the disease usually progresses rapidly. Although mutations in various mitochondrial enzymes can result in LS, its most frequent trigger is deficiency of cytochrome *c* oxidase (COX) caused by mutations in the *SURF1* gene, as identified in LS patients (2, 3). Human *SURF1*, the first gene of the *SURFEIT* gene locus on chromosome 9, encodes a 30-kDa protein related to COX assembly (2, 3).

Mitochondrial COX consists of up to 13 subunits (SU). The three core SU encoded by the mitochondrial genome carry all of the redox-active cofactors, two heme *a* moieties, and three copper ions. These three SU are highly conserved among different organisms and represent the main components of bacterial oxidase complexes as well (4, 5). The assembly process of mitochondrial COX is only marginally understood, involving the interplay of a large number of auxiliary proteins (6–9).

Despite intensive efforts over more than a decade to unravel Surf1 function, its exact role in COX assembly still remains unclear. Surf1 is not strictly essential for COX assembly because patients with LS have residuals of assembled oxidase with remaining activity of approximately 10–20% in all tissues (2, 3). Located in the inner mitochondrial membrane, Surf1 is predicted to form two transmembrane helices connected by a long loop facing the intermembrane space (10, 11). Sequence alignments confirm the presence of Surf1 homologs in many eukaryotes and prokaryotes (12).

One of the best studied Surf1 proteins is the yeast homolog Shy1p, which has been discovered and characterized in the context of *pet* mutants (10). Deletion of the gene leads to a strongly decreased COX level, although the residual enzyme appears fully functional. This points to a role of Shy1p in assembly or stabilization of COX (13), most likely during the formation of an early assembly intermediate consisting of the highly conserved core SU I and II (14).

So far, only three bacterial homologs have been inspected in closer detail (15, 16). In *Paracoccus denitrificans*, two Surf1 homologs were identified and named Surf1c and Surf1q for their specific role in serving a heme aa_3-type COX and a related heme ba_3-type quinol oxidase, respectively (15). With the function of Surf1 in COX assembly still being speculative, a role in heme *a* insertion into COX SU I seemed conceivable (15, 16).

Here we show that *P. denitrificans* Surf1c and Surf1q are able to bind heme *a* both *in vivo* and *in vitro*. This novel finding suggests that Surf1 proteins promote heme *a* insertion into SU I of either cytochrome *c* oxidase or quinol oxidase. In addition, Surf1 may modulate heme *a* synthase activity and provide a heme *a* cofactor pool in a safe, chelated form for COX SU I biogenesis.

EXPERIMENTAL PROCEDURES

Cloning of surf Expression Strains—The *surf1c* gene was obtained via PCR using *Paracoccus* genomic DNA as template with the forward primer (5′-TATAAGCTTCATATGGCC-CGG(CATCAC)$_5$ATGCGCCGTTACCTGTTCCC-3′) containing the sequence for an N-terminal His$_{10}$ tag and an NdeI site and the reverse primer (5′-ATGAGCTCTCTAGACTAG-AATTGCCGCTGCCTG-3′) specifying a SacI site. The product was NdeI- and SacI-digested and cloned into the *Escherichia coli* expression plasmid pET22b (Novagen), resulting in pFA48.

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[1] Present address: Novartis Pharma AG, Klybeckstr. 141, CH-4057 Basel, Switzerland.

[2] To whom correspondence should be addressed: Inst. of Biochemistry, Molecular Genetics, Goethe Univ., Max-von-Laue-Str. 9, 60438 Frankfurt am Main, Germany. Tel.: 49-69-7982-9237; Fax: 49-69-7982-9244; E-mail: ludwig@em.uni-frankfurt.de.

[3] The abbreviations used are: LS, Leigh syndrome; COX, cytochrome *c* oxidase; HPLC, high performance liquid chromatography; ITC, isothermal titration calorimetry; SU, subunit(s).

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The *surf1q* gene was obtained via PCR using *Paracoccus* genomic DNA as template with the forward primer (5′-TATAAGCTTCATATGGCCCGG(CATCAC)$_5$GTGACCCTGCGCCGGCTGG-3′) containing the sequence for an N-terminal His$_{10}$ tag and an NdeI site and the reverse primer (5′-ATGAGCTCTCTAGACCTTTGCGCCCGTCAGTCC-3′) adding a SacI site. The product was NdeI- and SacI-digested and cloned into the *E. coli* expression plasmid pET22b, generating pFA49. After sequencing, Surf1 expression plasmids pFA48 and pFA49 were transformed into *E. coli* C41(DE3) (17) competent cells, generating expression strains FA48 and FA49.

For cloning a heme *a* maturation plasmid, the pEC86 (18) derivative pGR50 was obtained via PCR using a forward primer (5′-ATGGAAGCCGGCGGCACCTC-3′) and reverse primer (5′-CTCGAGTATAGGGTACCACACGGTGCCTGACTGCGTT-3′), which introduces an Acc65I and an XhoI restriction site downstream of the *tet* promoter. The farnesyltransferase gene *ctaB* was amplified via PCR using *Paracoccus* genomic DNA as template with the forward primer (5′-TCAAGGTGTACAAAGGAGATACTCATGGCCGATATCAACGCATAT-3′) introducing a BsrGI restriction site and a ribosome-binding site and the reverse primer (5′-TAATAGCTCGAGATATGGGTACCTCACCATCCTCCGACCCAG-3′) adding an Acc65I and an XhoI site. The PCR product was BsrGI- and XhoI-digested and cloned into the Acc65I and XhoI sites of pGR50, resulting in the plasmid pGR51. In the next step, the heme *a* synthase gene *ctaA* was amplified via PCR using genomic *Paracoccus* DNA as template with the forward primer (5′-TCAAGGTGTACAAAGGAGATACTCATGTCGCGCCCGATCGAGAAG-3′) again introducing a BsrGI site and a ribosome-binding site and the reverse primer (5′-TAATAGCTCGAGATATGGGTACCTCATCGGACAGTTCCCCG-3′) adding an Acc65I and an XhoI site. This product was ligated into the Acc65I and XhoI sites of pGR51, generating the final heme *a* maturation plasmid pGR52. After sequencing, pGR52 was transformed into the Surf1 expression strains FA48 and FA49 (see above), resulting in FA48GR52 and FA49GR52, respectively.

Mutagenesis of surf1 Genes—To introduce the H193A mutation into Surf1c, the mutant primer (5′-GTCGCGGTCGAGGG**A**ATTCCGAACAAC**GCC**CTGAGCTATGCC-3′) specifying the amino acid exchange and an EcoRI site for screening purposes as well as the expression vector pFA48 (see above) as template were used in a QuikChange mutagenesis reaction (Stratagene). Clones were checked for the mutation via restriction analysis and sequencing and were subsequently transformed into *E. coli* C41(DE3) cells already containing the plasmid pGR52. For the Surf1q H202A mutation, a forward primer (5′-**GCG**CTGGGCTATGCGGCGACCTGG-3′) containing the sequence for the mutation that also introduces a BseYI site and a reverse primer (5′-GCTGTTGCGAAAACGGACCACGGTCA-3′) were used to amplify the expression plasmid pFA49 (see above) via PCR. After confirming the mutation, a plasmid was transformed into *E. coli* C41(DE3) cells that already contained the plasmid pGR52.

Growth Conditions and Membrane Preparation—Ten-liter *E. coli* cultures were grown on rich medium containing antibiotics at 32 °C in baffled flasks. Protein expression was induced with 1 mM final concentration isopropyl β-D-thiogalactopyranoside at A_{600} = 1.0, and cells were harvested after 3.5 h. Membranes were prepared by established methods, and protein concentration was determined using a modified Lowry protocol (19, 20).

Heme Extraction—Expression of heme *a*-synthesizing enzymes was checked by acidic acetone/ether extraction (21) of heme from a small portion of the *E. coli* culture. After evaporation of the ether, the heme preparation was dissolved in 200 μl of dimethyl sulfoxide and analyzed by subsequent pyridine spectra of the extract (see below). A heme *a* solution was prepared from purified COX (22) as mentioned above, and the concentration was determined spectroscopically.

Membrane Solubilization and Protein Purification—Membranes were solubilized in the presence of 3% (w/v) Triton X-100 in buffer (50 mM sodium phosphate, pH 8, 300 mM NaCl) at a final protein concentration of 10 mg/ml. The solubilisate was loaded on a nickel-nitrilotriacetic acid column (Qiagen), and Triton X-100 was exchanged against *n*-dodecyl-β-D-maltoside by washing with 10 column volumes of buffer (as above) containing 0.02% (w/v) *n*-dodecyl-β-D-maltoside and 20 mM imidazole. After washing the column with 4 column volumes of buffer containing 50 mM imidazole and 80 mM imidazole each, His-tagged Surf1 was eluted from the column by washing with 5 column volumes of buffer containing 200 mM imidazole. For isothermal titration calorimetry (ITC) measurements (see below), Surf1 proteins were purified further by size-exclusion chromatography on an Äkta purifier system (GE Healthcare) using a Superose 6 column equilibrated with 20 mM sodium phosphate, pH 8, 150 mM NaCl, 0.02% (w/v) *n*-dodecyl-β-D-maltoside.

Spectral Analysis—Redox difference spectra were recorded in the visible range on a Hitachi U-3000 spectrometer. Native and denaturing spectra were recorded by established methods (23), and heme *a* concentration was determined using the extinction coefficient $\epsilon_{587-620\ \mathrm{nm}} = 21.7\ \mathrm{cm}^{-1}\ \mathrm{M}^{-1}$ for heme *a* (24).

PAGE and Heme Stain—For SDS-PAGE, samples were denatured in SDS-containing buffer for 20 min at 37 °C. Electrophoresis was performed on 12% polyacrylamide gels according to Laemmli (25). For heme staining, samples were incubated with lithium dodecyl sulfate-PAGE sample buffer on ice. Lithium dodecyl sulfate electrophoresis was performed overnight at 4 °C on 12% polyacrylamide gels (26), and proteins were blotted on polyvinylidene fluoride membranes. After heme staining (27), membranes were destained with methanol, and proteins were stained with Ponceau S.

ITC—ITC measurements were carried out on a VP-ITC ultrasensitive titration calorimeter (MicroCal LLC, Northampton, MA). Protein samples were dialyzed twice for several hours at 4 °C against measuring buffer (see Table 1) and centrifuged (20,000 × g, 15 min, 4 °C) to remove insoluble material. ITC titrations and data analyses were performed as described (28).

RESULTS

In earlier studies of our group, we showed that the two *P. denitrificans* homologs Surf1c and Surf1q act exclusively on their cognate oxidase (15). Here, we focus on the heterologous

expression and characterization of the two Surf1 homologs to elucidate their exact role in oxidase biogenesis. Expression in *E. coli* was chosen for the fact that this bacterium neither carries a Surf1 homolog nor provides a heme *a*-synthesizing machinery and therefore allows a clear-cut analysis of potential interactions between these components.

Expression of Heme a-synthesizing Enzymes CtaA and CtaB—Heme *a* is synthesized from heme *b* in two steps. First, heme *b* is converted into heme *o* via farnesylation, which is catalyzed by the heme *o* synthase CtaB (29). The second step is catalyzed by heme *a* synthase CtaA not encoded in the *E. coli* genome. This latter reaction is oxygen-dependent because it involves conversion of a methyl group to a formyl group (29). Because the endogenous *E. coli* CtaB homolog CyoE may not interact with the *Paracoccus* CtaA, both *Paracoccus* enzymes CtaA and CtaB were expressed heterologously under high aeration (see "Experimental Procedures"), and heme *a* content of the *E. coli* cells was verified by pyridine redox spectra. In contrast to *E. coli* C41(DE3) cells or strains that expressed Surf1c or Surf1q alone, strains containing *Paracoccus* CtaA and CtaB in *trans* exhibited the characteristic heme *a* peak at 587 nm. Thus, the *Paracoccus* enzymes CtaA and CtaB were expressed in a functional state, enabling these *E. coli* strains to synthesize heme *a*.

Purification and Spectral Analysis of Surf1—*Paracoccus* Surf1 proteins were inducibly expressed in *E. coli* either in the absence or presence of constitutively expressed *Paracoccus* CtaA and CtaB. Both Surf1 proteins were incorporated into the *E. coli* cytoplasmic membrane as verified by cell fractionation and Western blot analysis. Membranes were solubilized, and the recombinant Surf1 proteins were purified by affinity chromatography via their N-terminal His_{10} tag; as shown previously, the tag does not interfere with Surf1 function (15). The resulting preparations contained no major impurities (Fig. 1*A*), and both proteins were produced with final yields between 1 and 2 mg of purified protein/liter of medium.

After expression in the absence of the heme *a* biosynthesis machinery, colorless preparations of Surf1 were obtained. However, when expressed in the presence of CtaA and CtaB, Surf1 displayed a greenish-brown color. Heme and protein staining after electrophoresis in semidenaturing gels (Fig. 1*B*) showed that heme was associated with both Surf1c and Surf1q and was lost after the addition of SDS.

Pyridine redox spectra demonstrated that purified Surf1c contained heme *a*, which was identified by a prominent peak at 587 nm (Fig. 2*A*). In addition, a minor peak at 552 nm was observed, which corresponds to heme *o* as verified by HPLC. In native redox spectra (Fig. 2*C*), the absorption maximum in the α region was at 595 nm for heme *a*, whereas heme *o* appeared as a shoulder around 560 nm. The molar heme *a*/protein ratio was 0.17 ± 0.05 (mean ± S.D. from four independent preparations). Pyridine redox spectra of purified Surf1c confirmed the consistent lack of heme *a* in protein preparations that were expressed in the absence of CtaA and CtaB (Fig. 2*A*).

Likewise, when expressed in the presence of CtaA and CtaB, Surf1q contained heme *a*, as indicated by a prominent peak at 587 nm under denaturing conditions (Fig. 2*B*), in addition to an absorption peak at around 552 nm identified as heme *o* by HPLC. In native redox spectra (Fig. 2*C*), the absorption maximum of the

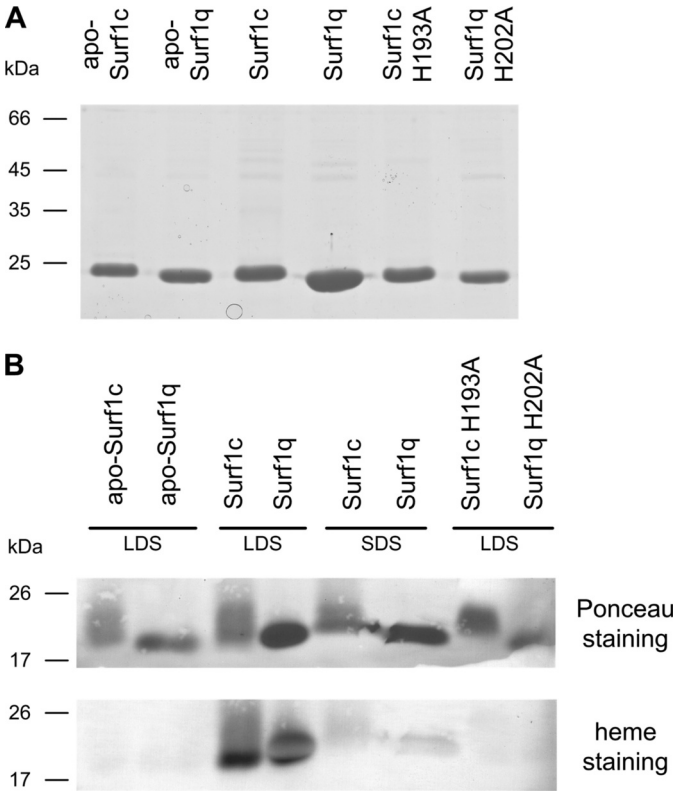

FIGURE 1. **Gel electrophoresis of purified Surf1 proteins.** *A*, Coomassie-stained SDS-polyacrylamide gel of Surf1 proteins after immobilized metal ion affinity chromatography purification. Approximately 3 μg of protein was loaded per lane. *B*, heme- and Ponceau-stained blot of a lithium dodecyl sulfate (*LDS*)-polyacrylamide gel of purified Surf1 proteins. Five micrograms were loaded per lane, and the sample buffer contained either lithium dodecyl sulfate or SDS as indicated under the *bars*. Apo-Surf1c and apo-Surf1q were expressed in *E. coli* in the absence of the heme *a*-synthesizing enzymes, while Surf1c and Surf1q as well as the respective mutants were produced in their presence.

detected heme *a* in the α region was at 600 nm, and the heme *o* peak appeared as a shoulder at 560 nm. The heme *a*/protein ratio was 0.09 ± 0.03 (mean ± S.D. from three independent preparations), only about half the value obtained for Surf1c. Spectra of Surf1q preparations expressed in the absence of CtaA and CtaB lacked a heme *a* signal, and only traces of heme *o* were detected (Fig. 2*B*).

Sequence alignments of a number of Surf1 homologs from different species show several conserved amino acid residues, most of which are clustered around the periplasmic sides of the two predicted transmembrane helices (15). To address the question of which residues of the protein are involved in heme *a* binding, we focused on a highly conserved histidine in the C-terminal helix and exchanged it for an alanine (Surf1c H193A and Surf1q H202A). The two mutant proteins were expressed in the presence of CtaA and CtaB and purified like their wild-type counterparts with similar expression rates and yields. Prior to purification, the cell cultures expressing the Surf1 mutants tested positive for functional heme *a* synthesis. After purification, heme staining (Fig. 1*B*) and spectral analysis (Fig. 2) demonstrated a lack of heme *a* in either variant, and only traces of heme *o* were detected in the Surf1c mutant protein. The complete

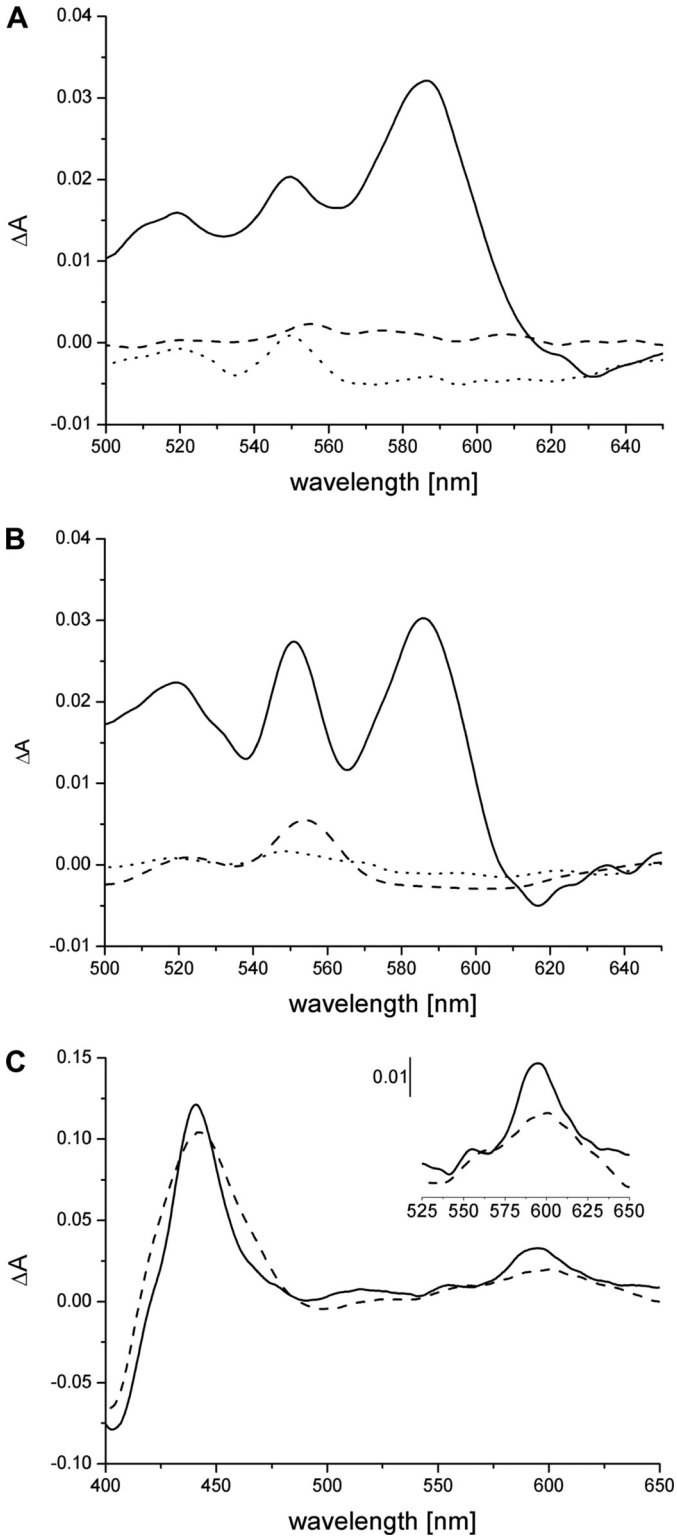

FIGURE 2. **Redox difference spectra of purified Surf1 proteins.** Shown are denaturing pyridine redox (reduced − oxidized) spectra of wild-type Surf1c (*A*, *solid line*), apo-Surf1c (*dashed line*), and Surf1c mutant H193A (*dotted line*) and wild-type Surf1q (*B*, *solid line*), apo-Surf1q (*dashed line*), and Surf1q mutant H202A (*dotted line*). *C*, shown are native redox spectra of Surf1c (*solid line*) and Surf1q (*dashed line*). The *inset* shows the enlarged α region of the spectra. Protein concentrations of purified Surf1 proteins were 20 μM throughout.

absence of heme *a* indicated that the conserved histidine residue is required for heme *a* binding of Surf1.

A heme *a* solution was titrated with either apo-Surf1c or apo-Surf1q, *i.e.* Surf1 proteins that had been expressed in the absence of CtaA and CtaB. The titration led to a distinct spectral red shift of the heme signal, most noticeable in its oxidized form, from 412 to 419 nm (Fig. 3). This shift was assigned to the specific binding of heme *a* to Surf1c and Surf1q because spectra of both Surf1 expressed in the presence of CtaA and CtaB also exhibited an absorption maximum at 419 nm in their oxidized state. However, this spectral shift did not occur with either of the histidine mutants of Surf1, suggesting that this side chain is indeed required to ligand the heme *a* metal ion (Fig. 3).

ITC Titrations of Surf1c and Surf1q with Heme a—The *in vitro* binding of heme *a* to apo-Surf1c and apo-Surf1q was quantified using ITC. All titrations yielded a binding stoichiometry near unity (Fig. 4 and Table 1). For heme binding to the wild-type proteins, submicromolar affinities were measured, with Surf1c (K_d = 303 nM) showing a higher affinity compared with Surf1q (K_d = 650 nM). Binding was strongly exothermic with an entropic cost. Remarkably, Surf1c exhibited a negative ΔH value approximately twice as large as observed for Surf1q (−21.1 *versus* −11.6 kcal/mol). This suggests that the superior heme *a*-binding affinity of Surf1c may be due to additional polar interactions that are reflected by the strongly increased binding enthalpy (30). Alanine substitution of the conserved histidine residue (see above) affected heme *a* binding, especially in the case of Surf1c (Fig. 4*B*). Here, binding affinity was reduced by a factor of ~11; for Surf1q, affinity was decreased by a factor of ~3. For Surf1c H193A, a strongly altered binding enthalpy was observed (−10.5 *versus* −21.1 kcal/mol for the wild type), which was in the same range as ΔH observed for the Surf1q protein. By contrast, the Surf1q H202A variant showed only minor changes in the thermodynamic profile relative to wild-type Surf1q. These observations suggest that the conserved histidine residue plays a more stringent role in heme *a* binding in the case of Surf1c. The strong change in ΔH for the H193A variant of Surf1c relative to the wild type can be tentatively explained by the loss of polar interactions of the heme ligand with the histidine residue.

DISCUSSION

Defects in the biogenesis pathway of COX are frequently associated with severe respiratory deficiencies. Mitochondrial COX is a multisubunit respiratory chain complex, and correct assembly of SU and redox centers is crucial for its function. More than 30 proteins are involved in the biogenesis of COX in eukaryotes, whereas only five assembly proteins are found in bacteria, all of the latter possibly involved in cofactor delivery (31). One of them is Surf1, which has been associated with LS, a fatal neurodegenerative disorder in humans.

In this study, we have focused on the characterization of the two homologous Surf1 proteins of *Paracoccus*, Surf1c and Surf1q, to gain insight into their role in oxidase assembly. To our knowledge, this is the first study to investigate

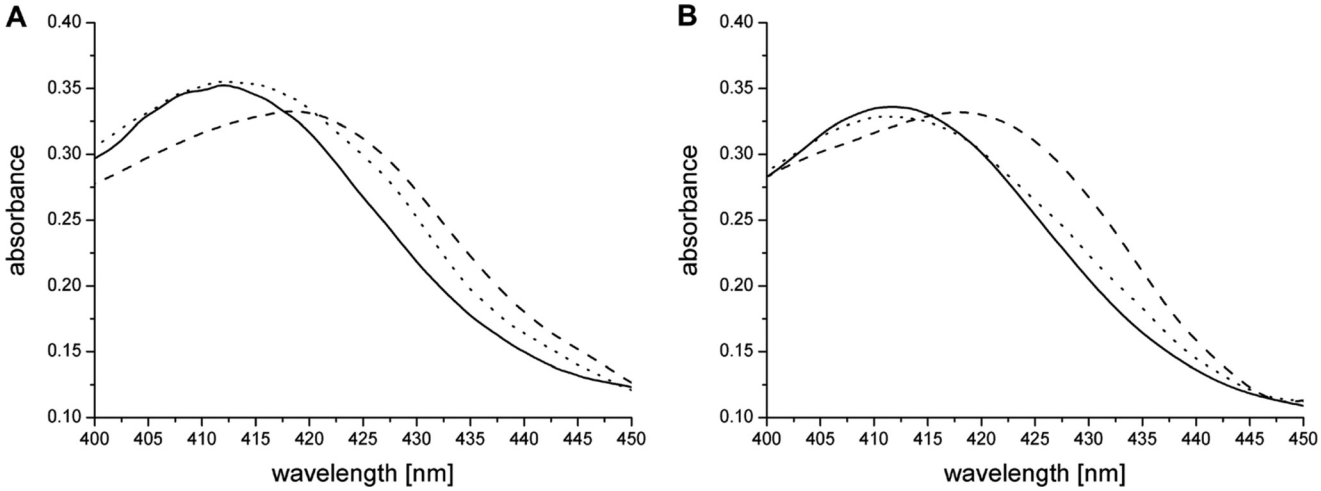

FIGURE 3. **Spectra of oxidized heme *a* in the absence and presence of Surf1.** Heme *a* spectra were recorded at a concentration of 3.75 μM in the Soret region in 20 mM phosphate, 0.02% *n*-dodecyl-β-D-maltoside, and 5% dimethyl sulfoxide. The following additions were made: *A*, buffer (*solid line*), apo-Surf1c (*dashed line*), and Surf1c mutant H193A (*dotted line*); *B*, buffer (*solid line*), apo-Surf1q (*dashed line*), and Surf1q mutant H202A (*dotted line*). Purified Surf1 proteins were added to a final concentration of 20 μM.

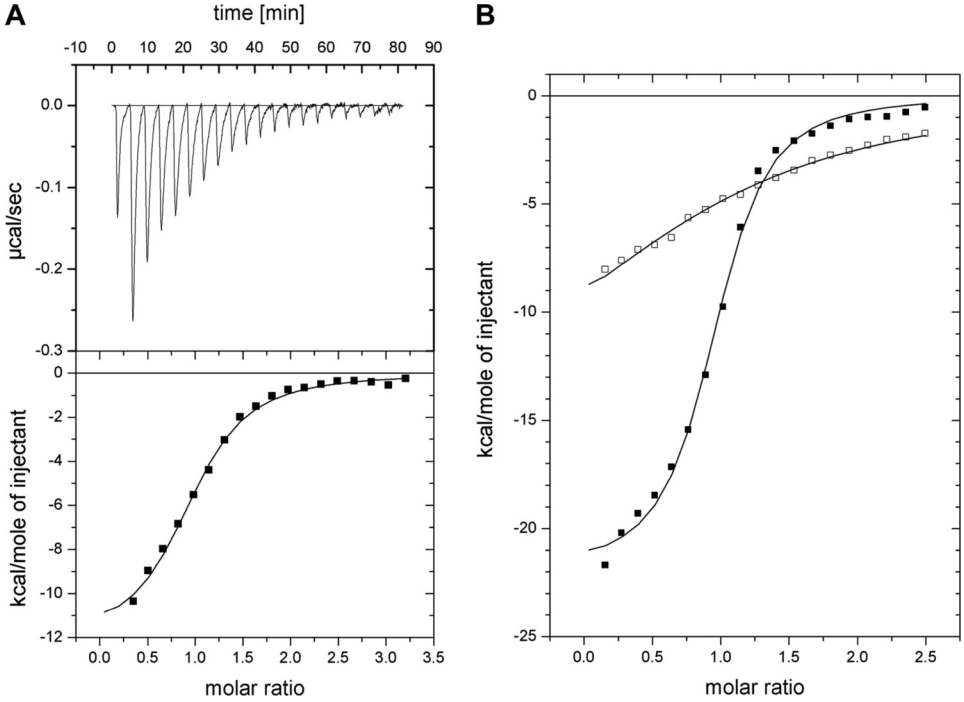

FIGURE 4. **ITC titrations of Surf1 proteins and heme *a*.** *A*, titration of heme *a* in the sample cell with wild-type Surf1q protein in the syringe. *Top panel*, raw heating power data. The first peak represents a small preinjection (5 μl) that is omitted in the integrated data. *Bottom panel*, data after peak integration and concentration normalization. *B*, isotherms for binding of heme *a* in the syringe to wild-type Surf1c (*filled squares*) or Surf1c H193A (*open squares*) in the sample cell. The *curve* is the fit of the data to a single-site binding model; for measurement conditions, see Table 1.

purified Surf1 proteins in detail. Contrary to concepts ranging from stabilizing mature SU I to an involvement in copper homeostasis (32, 33), both proteins were found to bind heme *a* at an equimolar ratio and with submicromolar affinities. Heme-binding affinities of a bacterial heme uptake machinery were reported to be in the same range (34, 35). Interestingly, the affinity of Surf1c for heme *a* is about twice as large as that of Surf1q (303 *versus* 650 nM). COX binds two heme *a* molecules, whereas the quinol oxidase accommodates one heme *b* and one heme *a*. Therefore, more heme *a* is needed for the assembly of COX. As Surf1c and Surf1q specifically supply heme *a* to their corresponding oxidases (15), we envisage this difference in heme *a* affinity as a potential regulatory mechanism to ensure proper heme *a* distribution between the two oxidases.

The two Surf1 proteins seem to provide slightly different binding sites for heme *a*, which is reflected both by the different absorption maxima in the α region of redox spectra (595 nm for Surf1c *versus* 600 nm for Surf1q; see Fig. 2*C*) and by the difference in ΔH values for the heme binding (-21.1 kcal/mol for Surf1c *versus* -11.6 kcal/mol for Surf1q). In addition, the substitution of the conserved histidine has a much stronger effect on heme binding for Surf1c than for Surf1q.

We used *E. coli* as a heterologous host and introduced the heme *a* biosynthesis machinery derived from *Paracoccus* CtaA (heme *a* synthase; COX15 in eukaryotes) and CtaB (heme *o* synthase; COX10 in eukaryotes). The *in vivo* level of heme binding of heterologously expressed Surf1 proteins in the presence of CtaA and CtaB changed with growth conditions. Aeration had an especially strong influence on heme content, which is easily explained by the oxygen dependence of CtaA (see above). Therefore, heme content varied with different preparations, although never reaching a 1:1 heme/protein ratio. In our experimental system, CtaA and CtaB were expressed constitutively in contrast to the inducible overexpression of the Surf1 proteins, and it may be assumed that heme *a* synthase activity

could not keep up with Surf1 expression rate. In this context, the observed heme *o* binding to the Surf1 proteins is probably unspecific and caused primarily by the abundance of heme *o* in the *E. coli* host, where heme *o* is required for the bo_3-type terminal oxidase. Preliminary experiments with both Surf1 proteins isolated from *Paracoccus* show that they exclusively bind heme *a* as ligand in the native host. This is possibly due to the low levels of the heme *o* intermediate in *Paracoccus* (36).

It is generally agreed that heme *a* does not occur in a protein-free form in the membrane because it is detrimental to the cell (37). Therefore, in eukaryotes, a direct heme *a* transfer from COX15 to COX SU I was assumed. COX15 activity and expression were found to be highly regulated in yeast, especially by heme *b*, which is not only a precursor of heme *a* but also the cofactor of heme *a* synthase (38). In the same study, it was proposed that heme *a* liberation from COX15 could be regulated by SU I or by COX assembly intermediates because this would prevent uncontrolled release of potentially toxic heme *a*. Our present data strongly suggest that Surf1 interacts with heme *a* synthase and may provide a heme *a* pool function, which directs heme *a* flux from heme *a* synthase to its final target SU I. As proposed in Fig. 5, membrane-integral Surf1 interacts with heme *a* synthase to take over the heme *a* product from its active site and then dissociates from the enzyme to allow continuation of heme *a* synthesis. Surf1 is suggested to interact directly with COX SU I to eventually transfer both heme groups present in the mature form of the enzyme.

At the moment, we can only speculate on the mechanism of heme *a* insertion into SU I. Studies on heme incorporation into four-helix bundles mimicking oxidase structure resulted in heme *a*-binding affinities in the low nanomolar K_d range (39); this high affinity would readily allow transfer of heme *a* because Surf1 binds heme *a* only with high nanomolar K_d values. We propose that heme *a* is inserted into SU I in a co-translational manner (Fig. 5). The 12-transmembrane helix bundle structure of SU I appears as a fairly rigid scaffold that, once folded, may not allow access to the rather bulky heme *a* moiety, as much as the fully assembled oxidase complex hardly loses either of its heme groups, not even on prolonged and excessive purification procedures.

The yeast homolog of Surf1, Shy1p, has been found to interact with Mss51p and Cox14p, two mitochondrial COX assembly factors involved in the regulation of SU I expression (40, 41). For the yeast system, it was suggested that Shy1p acts downstream of Mss51p and Cox14p, taking over and stabilizing fully assembled SU I, and thus allows its association with additional SU and assembly factors (42) and later the bc_1 complex to form supercomplexes (43). However, Mss51p and Cox14p are only found in fungi and lack counterparts both in higher eukaryotes and in bacteria; moreover, compared with other Surf1 homologs, Shy1p has a large additional loop not conserved in Surf1 proteins of other species. Because heme *a* incorporation into SU I is likely to occur co-translationally, an interaction of Shy1p with the translation machinery for SU I in yeast does not contradict its proposed role in heme incorporation. The observed association with SU I in later stages of the assembly process (13, 42, 43) hints at an additional role of Shy1p.

To summarize the possible functions of Surf1 in COX biogenesis, a 3-fold contribution to heme *a* insertion seems plausible. Surf1 (i) may modulate heme synthase activity by abstracting the product heme *a* from the active site of CtaA and thus allows continuing heme synthesis; (ii) avoids the presence and presumed detrimental action of free heme *a* in the membrane by providing a safe, yet readily available pool of this cofactor; and (iii) specifically chaperones heme *a* to its target sites in SU I of COX and positions the bulky cofactor for presumed co-translational insertion into both sites within the 12-transmembrane helix scaffold of COX SU I.

The importance of Surf1 in COX biogenesis is exemplified by the fact that its absence leads to severe COX deficiency in humans, which causes neurodegenerative LS. The elucidation of the exact function of Surf1 may spur the development of a treatment for LS. Our findings both *in vivo* and *in vitro* that Surf1 is a heme *a*-binding protein strongly indicate that Surf1 is more likely involved in direct cofactor incorporation into COX SU I rather than merely being a regulatory protein.

TABLE 1

Thermodynamic parameters for heme *a* binding to Surf1 proteins determined by ITC

Binding parameters were obtained from a fit of the calorimetric data to a single-site binding model. Values are given as means ± S.E. from two or three measurements, with two different preparations of protein used. All titrations were performed at 25 °C in 20 mM sodium phosphate, 150 mM NaCl, 0.02% (w/v) *n*-dodecyl-β-D-maltoside (pH 8). Titrations were performed starting with either the Surf1 protein or heme *a* at 10 μM in the sample cell and the respective binding partner in the syringe at 100 μM. No systematic variation of the results depending on the protein preparations or on the direction of titration was observed.

	N^a	K_d	ΔH	$T\Delta S$
		nM	*kcal/mol*	*kcal/mol*
Surf1c WT	0.9 ± 0.0	303 ± 28	−21.1 ± 0.1	−12.3 ± 0.1
Surf1q WT	0.9 ± 0.1	650 ± 40	−11.6 ± 0.2	−3.2 ± 0.3
Surf1c H193A	0.8 ± 0.2	3253 ± 1049	−10.5 ± 0.8	−2.9 ± 1.0
Surf1q H202A	0.9 ± 0.09	1650 ± 543	−11.9 ± 2.0	−4.0 ± 2.2

[a] Binding stoichiometry.

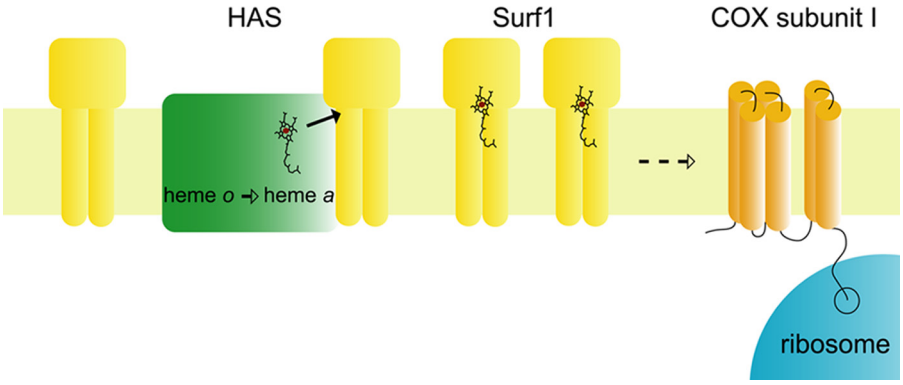

FIGURE 5. **Hypothetical role of Surf1 in co-translational incorporation of heme *a* into COX SU I.** Membrane-spanning Surf1 (*yellow*) in its unloaded apo form interacts with heme *a* synthase (*HAS*, green) to receive the heme group. Surf1 is suggested to interact directly with the nascent COX SU I polypeptide (*orange*) emerging from the ribosome (*blue*) eventually to receive both of its heme groups that are present in its fully folded form. Multiple copies of Surf1 depicted here denote its presumed heme *a* pool function.

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