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Review

Role of Surf1 in heme recruitment for bacterial COX biogenesis ☆

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ABSTRACT

Biogenesis of the mitochondrial cytochrome *c* oxidase (COX) is a highly complex process involving subunits encoded both in the nuclear and the organellar genome; in addition, a large number of assembly factors participate in this process. The soil bacterium *Paracoccus denitrificans* is an interesting alternative model for the study of COX biogenesis events because the number of chaperones involved is restricted to an essential set acting in the metal centre formation of oxidase, and the high degree of sequence homology suggests the same basic mechanisms during early COX assembly. Over the last years, studies on the *P. denitrificans* Surf1 protein shed some light on this important assembly factor as a heme *a* binding protein associated with Leigh syndrome in humans. Here, we summarise our current knowledge about Surf1 and its role in heme *a* incorporation events during bacterial COX biogenesis. This article is part of a Special Issue entitled: Biogenesis/Assembly of Respiratory Enzyme Complexes.

1. Introduction

Cytochrome *c* oxidase (COX) is the pivotal enzyme of the respiratory chain. As the terminal electron acceptor it reduces molecular oxygen to water and couples the free energy of this reaction to the generation of a proton gradient across the membrane. COX belongs to the superfamily of heme/copper oxidases and in mitochondria consists of up to 13 subunits. The three core subunits are encoded by the organellar genome and are highly conserved among different organisms, whereas the genes for the accessory subunits are spread across the nuclear genome, with only low levels of conservation on the protein level [1].

The correct assembly of this enzyme is essential for its function, and defects in COX biogenesis lead to severe respiratory deficiencies [2,3]. On elucidating the COX biogenesis pathway a large number of assembly chaperones has been identified so far [4,5]. For the eukaryotic model organism *Saccharomyces cerevisiae* more than 30 such protein factors have been described. However, many of these are not conserved among higher eukaryotes [5,6]. Therefore, the biogenesis of COX in yeast seems to be regulated more strictly, enabling this organism to adapt rapidly to the availability of oxygen or different carbon sources. In fact, most of the non-conserved assembly factors regulate transcription or translation of COX subunits or are involved in early assembly steps, e.g. membrane incorporation [5,6].

Bacterial cytochrome *c* oxidases typically consist of 2–3 subunits corresponding to the mitochondrially encoded polypeptides, making the assembly of bacterial oxidases a priori a less complex system to study. Yet, the close endosymbiontic relationship between bacteria and mitochondria suggests that both share essential steps of COX biogenesis especially in the assembly of the core subunits. Among the multitude of assembly factors found in yeast and higher eukaryotes only five are conserved in the soil bacterium *Paracoccus denitrificans*, all of them involved in redox centre assembly [6].

Here, our focus is on the assembly factor Surf1 that has been associated with Leigh syndrome in humans [7-9].

In the following, we will summarise the present information on Surf1 function in mitochondria and bacteria and together with new data discuss a model for the role of Surf1 during COX biogenesis in *P. denitrificans*.

2. The COX biogenesis factor Surf1

2.1. Surf1 in mitochondrial COX biogenesis

The human *surf1*, the first gene of the *surfeit* gene locus, codes for a 30 kDa protein located in the inner mitochondrial membrane. Surf1 contains two transmembrane helices connected by a large loop facing the intermembrane space [8,10]. Mutations in the *surf1* gene leading to a functional loss of the protein are a frequent cause of Leigh syndrome [7,9], a fatal neurological disorder associated with severe COX deficiency [11]. All tissues show a loss of COX activity between 80 and 90%; yet, Surf1 is not strictly essential for COX assembly since patients

Abbreviations: COX, cytochrome *c* oxidase; QOX, quinol oxidase; ROS, reactive oxygen species; SU, subunit

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with Leigh syndrome have residuals of fully assembled and therefore active oxidase [7,9]. Sequence alignments reveal many Surf1 homologues in eukaryotes and prokaryotes showing a striking degree of sequence identity and the same characteristic transmembrane helix topology [12]. The yeast homologue Shy1p has been discovered and characterised in connection with yeast *pet* mutants [10], and deletions of the gene also lead to reduced oxidase activity as is observed for the human case. The role of Surf1 in mitochondrial COX biogenesis still remains unknown, and a variety of functions has been proposed, ranging from stabilisation of subunit I to an involvement in copper homeostasis [13,14].

For the yeast homologue Shy1p, interactions with a large number of assembly factors and oxidase subunits were shown. Early studies revealed the presence of Shy1p, as well as human Surf1, in a high-molecular weight complex containing also subunit II, possibly constituting a transient interaction intermediate [15]. Therefore a role of Surf1 in stabilisation of the subunit association seems plausible. An additional role as part of a control mechanism may be envisaged where Surf1 ensures that only correctly assembled subunits I and II associate. In addition to that, Shy1p interacts with the assembly factors Mss51 and Cox14, both involved in expression regulation of subunit I [16,17]. Mss51 is a translational activator whereas Cox14 negatively influences subunit I synthesis, and both proteins form a stringent regulation system for the translation of subunit I, binding the polypeptide during its synthesis. Mss51 is then released from the complex to further initiate a new round of subunit I translation [16], whereas Cox14 seems to remain with COX subunit I [18]. In case the assembly is stalled, Mss51 is not released and cannot interact with the ribosome. Shy1p is thought to act downstream of Mss51 and Cox14, stabilising fully assembled subunit I and promoting the release of Mss51, followed by further subunits and assembly factors binding to the nascent complex [18]. Since Shy1p remains bound within the assembly complex and is found in high-molecular weight complexes also comprising subunits of respiratory complex III, it is also believed to promote the formation of supercomplexes [18].

It is worth mentioning that the assembly factors Mss51 and Cox14 have only been described in fungi, with no homologues identified in higher eukaryotes or in bacteria. However, the findings in yeast that Shy1p interacts with regulators of COX subunit I translation clearly indicate an early involvement of Surf1 in the biogenesis process. The yeast Shy1p contains a large internal region within the periplasmatic loop of the protein not conserved in other Surf1 homologues. The observed association of Shy1p with late assembly intermediates suggests an additional function of the protein in *S. cerevisiae* accounting for its larger size. One could imagine Shy1p forming a platform where COX assembly starts and is further orchestrated.

In yeast the *Δshy1* phenotype can be partially compensated by the overexpression of both the farnesyl transferase Cox10 and the translational activator Mss51 (see Table 1 for heme specific biogenesis factor terminology) [19]. Thus, not only the availability of heme *a* is indirectly increased in the membrane and the probability of correct heme insertion is improved, but also a higher COXI level was detected explaining the compensating effect. The overexpression of another assembly factor, Coa2, can also complement the *Δshy1* effect [20]. Shy1p and Coa2 act in a parallel manner during COX biogenesis, and Coa2 seems to exert a stabilising role on subunit I upon cofactor insertion. A similar effect is proposed for the association of the structural subunits Va and VI to subunit I, and in both the yeast and the human system, overexpression of these subunits can at least partially restore the *Δshy1/surf1* phenotype [21]. All the above mentioned compensatory effects lead to a higher level of subunit I by an increase of either expression or stabilisation and therefore protection against proteolytic degradation. The elevated availability of subunit I for further assembly steps may counterbalance the loss of Surf1, especially in the light of the fact that this assembly factor is not strictly essential for the overall process. Though so far the role of Shy1p in the assembly line of COX is not finally clarified, studies in yeast could resolve several individual steps and intermediates of eukaryotic COX biogenesis.

Table 1
Biogenesis factors involved in heme incorporation into COX.

Biogenesis factor	Bacterial homologue (*P. denitrificans*)	Eukaryotic homologue (*S. cerevisiae*)
Heme *a* synthase	*ctaA*	*COX15*
Heme *o* synthase	*ctaB*	*COX10*
Surf1	*surf1c*, *surf1q*	*shy1*

2.2. Surf1 in bacteria

Next to *S. cerevisiae*, also bacterial systems such as *Rhodobacter sphaeroides* and *Paracoccus denitrificans* contributed to the understanding of fundamental aspects COX biogenesis. The bacterial versions of assembly factors are very similar to their eukaryotic counterparts, pointing to comparable assembly mechanisms.

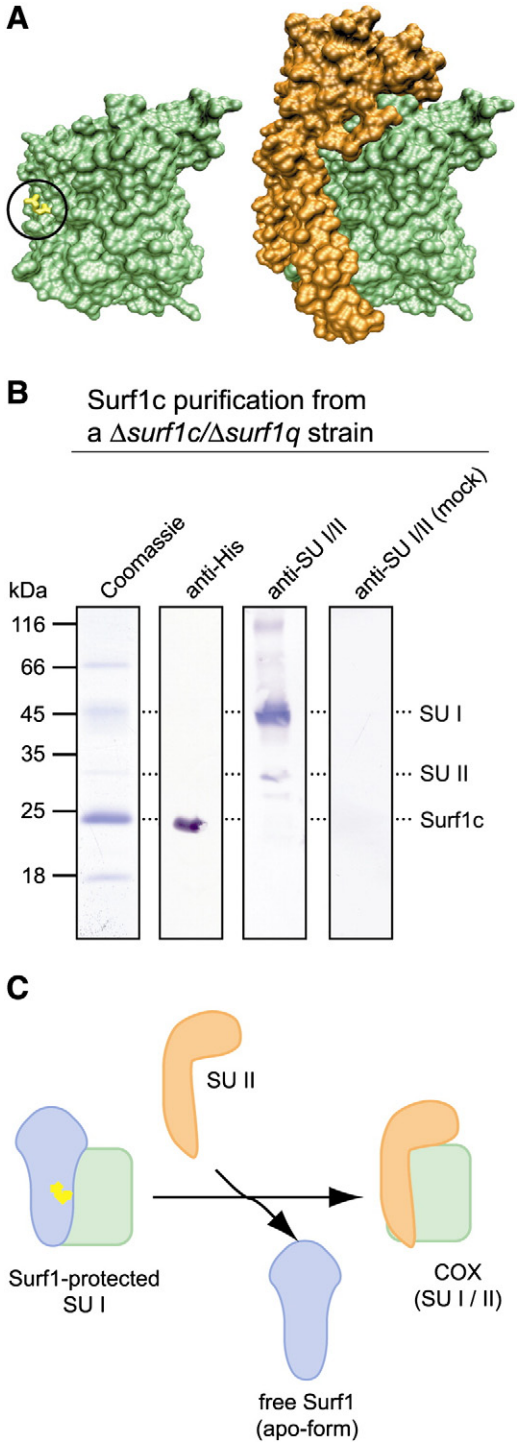
In *Paracoccus* only those assembly factors are conserved that participate in the incorporation of the copper and heme metal centres [6]: these are the heme *a* biosynthesis factors CtaB, CtaA, Surf1c and ferredoxin, and the copper chaperones CtaG for subunit I and Sco1/Sco2 for subunit II (see Table 1 for heme specific biogenesis factors terminology). In the past, we used the heterologous host *Escherichia coli* to investigate the individual events in COX assembly, especially subunit I maturation, to take advantage of the fact that *E. coli* does not contain a heme aa_3-type cytochrome *c* oxidase and therefore lacks any COX-specific assembly factors. For this reason we do not expect any interference from *E. coli*-derived proteins during expression of the heterologous *Paracoccus* chaperones. In addition to that, this system may allow accumulation of assembly intermediates present only transiently in the native host.

In the *Paracoccus* genome, two homologues of the *surf1* gene are found. The gene *surf1c* is the last of the *cta* operon which encodes not only the aa_3-COX subunits II and III, CtaC and CtaE, but also the farnesyl transferase, CtaB, and the copper chaperon for subunit I, CtaG. This co-localization of the *surf1c* gene with structural subunits and other assembly factors suggests an important role of the Surf1 protein in fundamental assembly steps of COX. The same situation is encountered for *surf1q*, associated with the *qox* locus which encodes the subunits of the ba_3-quinol oxidase, another terminal oxidase of *Paracoccus*.

Both Surf1 proteins have been shown to exclusively serve their corresponding oxidases without overlapping functions in the assembly process [22]. Deletions of either *surf1* gene leads to an approximately 60% loss of its corresponding oxidase activity in membranes, and previous analysis of the purified aa_3-COX revealed a specific decrease of the heme *a* cofactor. Together with similar findings in the bacterium *R. sphaeroides* these were the first indications that Surf1 contributes to the incorporation of the heme *a* cofactors. Because Surf1 is not strictly indispensable in COX biogenesis, it is thought to facilitate heme insertion that, due to the amphiphilic nature of the heme *a* moieties, should occur in a cotranslational manner. Subunit I consists of 12 transmembrane helices organised in three bundles of four helices each. The final structure of the subunit may be achieved more or less spontaneously by the folding of the three packages of helices around the heme *a* molecules. Specific interactions between Surf1 and apo-subunit I may stabilise a transient complex between heme *a* synthase and subunit I while heme *a* is transferred as discussed below. It was shown earlier that exogenous incubation with heme *a* promotes the association of subunit I with subunits II and III [23,24], which would make heme incorporation an early step, prior to the interaction with other COX subunits. However, an oxidase variant in which the interaction between subunits I and II is disrupted due to mutation lacks the

heme a_3 [25], and data from *R. sphaeroides* suggest that only the binding of subunit II leads to a stable formation of the binuclear heme a_3–Cu_B centre [26]. The crystal structure of the *Paracoccus* aa_3-COX reveals that the hydrophobic farnesyl side chain of the heme a_3 molecule protrudes from subunit I exactly at the contact surface with subunit II (Fig. 1A).

Although the chronological order of redox centre insertion steps still remains open, heme incorporation prior to copper insertion seems likely. In *R. sphaeroides* an oxidase subpopulation has been identified which harbours heme *a* but lacks Cu_B [27], and in *E. coli* heme insertion during biogenesis of the bo_3-quinol oxidase has a much stronger effect on the assembly of the enzyme than has copper insertion [28].



Recently, Surf1 was expressed in *E. coli* for in vivo studies, and for examining potential heme binding properties, the heme *a* synthesising machinery of *Paracoccus* was introduced into the host system as well, comprising the farnesyl transferase CtaB and the heme *a* synthase CtaA (see table 1). This approach clearly demonstrated that both *Paracoccus* Surf1 homologues are heme-binding proteins. Cofactor binding of Surf1 was strongly dependent on growth conditions due to oxygen dependency of CtaA, and the heme content of both Surf1 proteins varied greatly among different preparation conditions, affecting the total heme to protein ratio as well as the type of heme bound (heme *a* and contaminating traces of heme *o*) [29]. This situation was greatly improved by changing expression conditions to an autoinductive medium [30]. However, when purified from the homologous host system, Surf1 exclusively binds heme *a* ([29] and Table 2). Additionally, small amounts of COX subunits I and to a minor extent subunit II are copurified with the Surf1c protein (Fig. 1B). Interestingly, when purified from an oxidase deletion strain, the heme content of the Surf1 preparations drops dramatically (Table 2), a fact that cannot satisfactorily be explained by the mere loss of potentially heme harbouring subunit I. Rather, it appears that the availability of heme *a* for Surf1 drops considerably when the final acceptor subunit I is not present. This may be due to a kind of feed-back mechanism with a decrease in protein expression of heme *a* synthase or a decrease of heme *a* synthase and/or heme *o* synthase activity. It is generally agreed that within the cell heme *a* is only present in a protein-bound form, since free heme is considered toxic for the cell due to its high redox potential [31]. Since Surf1 binds heme *a* when coexpressed with CtaA, an in vivo interaction between the two proteins seems mandatory.

2.3. Heme binding properties of Surf1

Isothermal calorimetry measurements on purified Surf1 proteins showed that both *Paracoccus* homologues bind heme *a* with a 1:1 stoichiometry and sub-micromolar affinities [29]. Recently we could also identify several conserved amino acid residues involved in heme *a* binding to the proteins [30]. These data clearly assign a major role to Surf1 in heme incorporation rather than merely being a generic stabilising factor in subunit I assembly.

In eukaryotes, a direct heme transfer from Cox15 to subunit I was assumed. Both the expression rate and activity of Cox15 are strongly regulated in yeast, especially *via* the concentration of heme *b* [32] which is not only a precursor molecule of heme *a*, but most likely also a cofactor of heme *a* synthase [33]. The presence of COX subunit I could trigger the transfer of heme *a* from Cox15, preventing an uncontrolled release of this potentially toxic cofactor. The interaction of Surf1 with heme *a* synthase and subunit I as well as its heme

Fig. 1. Interaction of Surf1c and COX subunit I in *P. denitrificans*. A: Structure surface presentation of *Paracoccus* cytochrome *c* oxidase (pdb code 1QLE) created with visual molecular dynamics (VMD, [76]). Left: the farnesyl sidechain of the heme a_3 molecule (yellow, encircled) protrudes from subunit I (mint). Right: subunit II (orange) covers subunit I and interacts with the farnesyl sidechain of the heme. B: Polyacrylamide gel electrophoresis of a Surf1c preparation from *P. denitrificans*. Membranes of a Δ*surf1c*/Δ*surf1q* double deletion strain [22] complemented with *surf1c in trans* were solubilised with 2% (w/v) DDM at a final protein concentration of 10 mg/ml. Purification was done *via* the N-terminal deka-histidine tag on a Ni^{2+}-NTA column (Qiagen, Hilden) with three imidazole washing steps (20 mM, 50 mM and 80 mM) in 50 mM NaP_i pH 8 (4 °C), 300 mM NaCl, 0.02% (w/v) DDM prior to elution with 200 mM imidazole in the same buffer. After concentration of the elution fraction, 5 µg protein was loaded on a 12% Laemmli polyacrylamide gel and analysed *via* Western blot. The Coomassie staining shows several bands coeluting with Surf1, the most prominent band identified by a Western blot against the histidine-tag (lane 2). Subunit I and to lesser extent subunit II could be identified using a polyclonal antibody directed against *Paracoccus* COX subunits I and II (lane 3). In a mock-preparation from a Δ*surf1c*/Δ*surf1q* strain lacking the *surf1c* complementation plasmid neither subunit I nor II is detected (lane 4), providing evidence for a specific interaction of Surf1 with either subunit. C: Schematic presentation of the interaction of Surf1 with COX subunit I. Surf1 (blue) binds subunit I (mint) and protects the heme molecule *via* interaction with the farnesyl sidechain (yellow). Surf1 is then replaced by subunit II and dissociates from the structural subunits.

Table 2
Heme content of Surf1 proteins after purification from different *P. denitrificans* strains.

Strain	Heme *a*-containing oxidase	Purified Surf1	Heme *a* : surf1 ratio
FA3.61[a]	aa_3 and ba_3	Surf1c	0.47
FA3.62	aa_3 and ba_3	Surf1q	0.77
TSP24.61[b]	–	Surf1c	0.14
TSP24.62	–	Surf1q	0.10

[a] Double deletion strain for Δ*surf1c* and Δ*surf1q*, complemented with *surf1c* [22].
[b] Double deletion strain for Δaa_3-cytochrome *c* oxidase and Δba_3-quinol oxidase [72], complemented with *surf1c*.

binding capacities suggest a reservoir function of Surf1 that controls the flux of heme *a* from its site of biosynthesis to its final target in oxidase subunit I. As mentioned above, Surf1c preparations from *P. denitrificans* contain, to a small extent, COX subunits I and II (Fig. 1B). Therefore one may speculate that during affinity purification of Surf1c, differently assembled intermediates have been trapped: most of the Surf1c corresponds to the free form of the protein, a minor fraction is associated with subunit I and in some cases an intermediate is purified in which subunit II is about to replace Surf1c and assembles with subunit I (Fig. 1C).

The findings in yeast suggest a role for Surf1 as a chaperon that cotranslationally recruits de-novo synthesised helices of subunit I to keep them in an open conformation, thus enabling insertion of its heme cofactors. Once the hemes have been transferred, COXI is released and further assembly steps ensue. This specific interaction of Surf1 with apo-subunit I would also explain why in *Paracoccus* the ba_3-quinol oxidase requires a Surf1 protein of its own for correct recognition and heme loading [22].

At the moment the analysis of bacterial Surf1 homologues is limited to those of *R. sphaeroides* and *P. denitrificans*. Deletion studies clearly showed that the heme content of COX purified from these strains is diminished, a fact that was interpreted as an involvement in heme *a* insertion into COX subunit I [22,27,29]. The two *Paracoccus* homologues Surf1c and Surf1q are the only ones being characterised on a protein biochemical level. As the two proteins bind heme *a*, first steps in the characterisation of the cofactor binding in Surf1 were taken by site-directed mutagenesis of a conserved histidine residue near the N-terminal end of the predicted transmembrane helix 2 (H193, Surf1c nomenclature). Histidine residues are by far the most abundant axial ligands for the iron central atom of heme moieties [34]. Indeed a replacement by alanine reduced the in vitro heme *a* binding affinity for both *Paracoccus* proteins, and spectral analyses indicated that this residue ligands the heme iron [29]: ligand binding spectra of both *Paracoccus* Surf1 proteins show a shift of the α-absorbance band upon CO-binding which suggests that the histidine is the only axial ligand and that the central iron of the heme is only five-fold coordinated (Fig. 2A).

Further strictly conserved residues (W24, Q25, Y196 and W200; Surf1c nomenclature, see Fig. 2B) were mutated and variant proteins heterologously expressed in *E. coli* together with the *Paracoccus* heme *a* synthesising enzymes CtaB and CtaA [30]. In all mutants tested, heme binding was severely affected and mutations in W24, Q25, H193 and Y196 completely lost any cofactor binding properties. For the tyrosine residue located in TMH 2 it has been known that a mutation to aspartate causes Leigh syndrome in humans [35]. It can easily be envisaged that the introduction of a negative charge within the transmembrane part strongly interferes with any heme *a* binding and therefore disturbs Surf1 function during COX biogenesis. Two independent studies in yeast tried to mimick this clinically relevant mutation in Shy1p (Y344D) [36,37]. Cells expressing Shy1p Y344D do not show any growth impairment on non-fermentable carbon sources [36]. The mutant protein is stably expressed and imported into mitochondria, but COX biogenesis is stalled at a 200 kDa assembly intermediate explaining the fact that patients develop Leigh syndrome [37]. The above mentioned histidine residue was also analysed in the yeast system, but only modest growth impairment on non-fermentable carbon sources was detected [36]. It is still unknown whether eukaryotic Surf1 proteins also bind heme *a*, but it seems most likely because of the high degree of sequence conservation between pro- and eukaryotic homologues.

A conserved tryptophan in transmembrane helix II (W200, see Fig. 2B) takes an exceptional position among all *Paracoccus* mutant versions tested so far. A replacement by phenylalanine not only causes a drop of heme *a* binding to the protein but now favours binding of the precursor molecule heme *o*, a fact that can be interpreted such that this residue is involved in the recognition of the formyl group of the A-ring of the heme *a* moiety [30], and W200 may act as a gatekeeper responsible for the delivery of the physiologically correct heme type towards subunit I. The exact nature of this interaction will, however, only be revealed by future structural studies.

Our current binding model of heme *a* to Surf1 favours the porphyrin ring being positioned within one third of the lipid bilayer, in analogy to the positions of both hemes in COX. The conserved histidine residue acts as an axial ligand for the heme iron whereas W200 recognises the formyl group of ring A of the porphyrin ring system (Fig. 2B). This arrangement would precisely orient the cofactor within the membrane, readily positioned for later integration into COX subunit I.

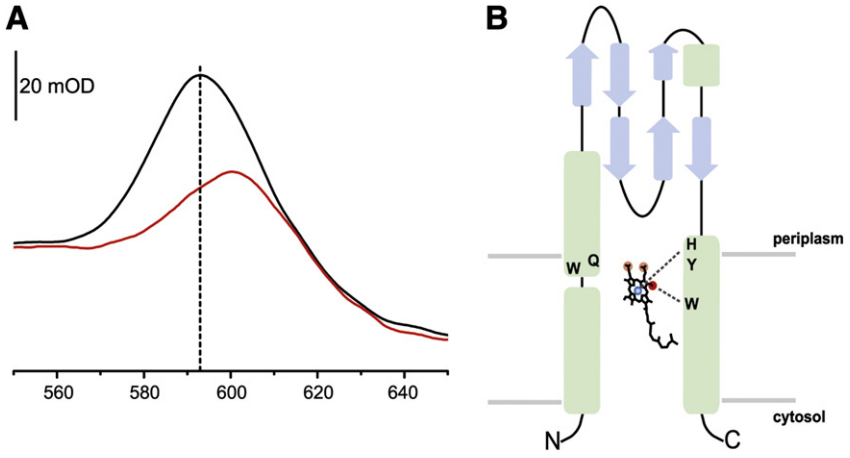


Fig. 2. Heme binding characteristics of Surf1. A: Absorbance spectra of Surf1c. A 20 μM protein sample, purified as described in Ref. [29], was reduced with sodium dithionite and a spectrum recorded before (black trace) and after sparging with carbon monoxide (CO) for 1 min (red trace). The peak of the reduced spectra (vertical dotted line) is shifted upon CO binding. B: Schematic representation of heme *a* binding to Surf1, with a conserved histidine coordinating the heme iron (blue sphere) and a critical tryptophan residue recognising the heme *a* formyl group (red sphere).

Most likely, additional residues participate in heme binding besides the amino acids mentioned above. Since heme *a* is, in contrast to hemes *b* and *c*, only present in terminal oxidases, binding modes based on structural data are very limited to date. In the *Paracoccus* cytochrome *c* oxidase the formyl group of heme a_3 is bound by an arginine residue and the propionate side chains by an arginine and a tryptophan [38]. A systematic analysis of the binding characteristics of heme cofactors (mainly *b*- and *c*-type) to their surrounding protein scaffold reveals, next to histidine and methionine as axial ligands, the importance of the aromatic residues phenylalanine, tyrosine and tryptophan that stabilise the heme moiety via π-stacking [34,39]. The propionate side groups are often liganded by arginines as in cytochrome *c* oxidase or by lysines. A conserved arginine residue can also be found in the periplasmic loop region of the Surf1 proteins but whether this residue contributes to the heme binding remains unsolved so far.

Considering the function of Surf1 during COX biogenesis, only a transient binding of heme *a* to the protein is required, calling for a stable folding of both the apo- and the holo-form of the protein, a fact regularly observed for heme proteins that deliver the cofactor to other proteins [34]. Another indication for the transient nature of the heme binding to Surf1 is the observation of a penta-coordinated iron central atom (Fig. 2A and B), with the above mentioned histidine as single protein-derived ligand [29].

3. Heme *a* biogenesis and regulation in *P. denitrificans*

Heme *a* biosynthesis is a prerequisite for the assembly, and mutations in the two executive enzymes Cox10 and Cox15 lead to oxidase deficiencies [40-46]. However, only little is known on the transfer of heme *a* from its site of formation within Cox15 to its final destination in subunit I. Free heme is potentially toxic

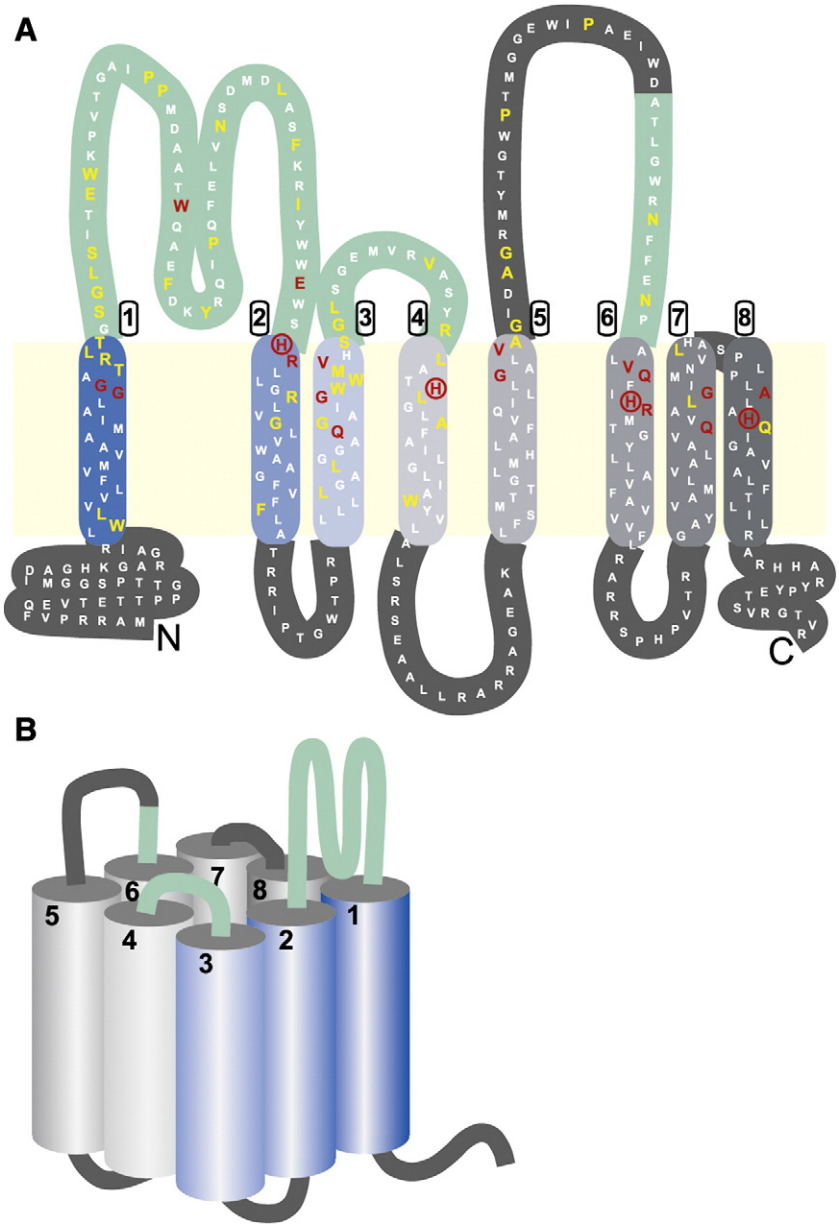


Fig. 3. Toplogy model of CtaA. A: The topology model highlights conserved amino acid residues in red that are found in all Cox15/CtaA sequences used in the sequence alignment shown in Supplement Fig. S1. Presumed heme liganding histidines are encircled, transmembrane helices are annotated by 1–8. Amino acid residues exclusively conserved in organisms that encode a Surf1 homologue such as *P. denitrificans*, *H. sapiens* and *S. cerevisiae* are highlighted in yellow. Additional or strongly deviating loop regions (compared to *Bacillus*-like CtaA variants) that can be found in this subgroup of Cox15/CtaA proteins may provide interaction sites for COX biogenesis factors like Surf1 and are presented as a mint ribbon. B: Projection of *Paracoccus* CtaA protein with the altered loop regions coloured in mint on a CtaA topology model developed by T. Mogi [67].

for the cell [47,48], because due to its redox activity, the central iron ion may stimulate ROS production in the Fenton reaction catalysed by reduced transition metals [49,50]. Especially copper (I) ions are thought to be detrimental for the living cell [51]. Therefore numerous copper binding proteins exist in eukaryotes that chaperone this metal to its target sites, and virtually no free copper is detectable [52]. In case of the cytochrome *c* oxidase, copper is required for the Cu_A centre in subunit II and the binuclear heme a_3-Cu_B centre in subunit I. The chaperone Cox17 is assumed to deliver copper to the mitochondrium and transfer copper to Sco1 and Cox11 [53], which then hand over the metal ion to subunits II and I respectively [4,54]. In contrast, knowledge about the heme transfer and incorporation into COX is very limited. Due to its toxicity, protein shuttles for the heme cofactors are likely to ensure safe transport and insertion. In *E. coli* a series of proteins required for the transport and covalent linkage of heme *c* into cytochrome *c* has been identified [55-57], but for heme *a* insertion into COX, so far, no comparable chaperone system had been described. However, due to its heme binding properties, Surf1 can now be regarded as an established candidate for a specific heme *a* shuttle molecule.

The bacterial homologues of Cox15 and Cox10 (CtaA and CtaB) from *R. sphaeroides* and *Bacillus subtilis* form a 1:1 complex [58]. Due to the close relationship to *R. sphaeroides* such a complex can also be assumed for *P. denitrificans*, whereas in yeast no such interaction has been detected so far [59], and the molar ratio between Cox15 and Cox10 was reported to be rather 8:1 than 1:1 [32]. Also the expression and activity of Cox15 are modulated by the concentration of heme *b*, a precursor of its substrate and a likely cofactor [32]. In the absence of Cox15 no accumulation of heme *o* is detected in the cell [60], and it is assumed that Cox15 or its product positively affect the activity of the farnesyl transferase Cox10 [59]. An additional regulatory mechanism seems to be COX assembly itself. In COX-deficient cells the level of heme *a* drops dramatically [59]. The low heme content in Surf1 preparations from COX deletion strains in *Paracoccus* (see Table 2) indirectly supports this assumption, where assembly intermediates and/or subunit I influence the controlled release of heme *a* from CtaA.

It is worth mentioning that in *B. subtilis* no Surf1 homologue is found even though this bacterium has two heme *a*-containing terminal oxidases [61,62]. Also, thermophilic bacteria such as *Thermus thermophilus* have terminal oxidases with *a*-type hemes [63], but do not have Surf1 homologues. For these oxidases a different mechanism of heme insertion may be assumed, and indeed a protein factor named CbaX was recently characterised, possibly involved in heme incorporation into the ba_3-COX in *T. thermophilus* [64]. Although CbaX does not share any sequence homology with Surf1, its described effects on the *Thermus* ba_3-COX biogenesis suggest a functional relation to Surf1, indicating different evolutionary solutions to the heme incorporation challenge. In line with the endosymbiontic theory it is assumed that mitochondria originated from the α-proteobacteria [65,66], and the presence of *surf1* genes in representatives of this subgroup such as *P. denitrificans* and *R. sphaeroides* may point to an origin of this gene late in evolution to organise heme transfer more efficiently. A detailed characterisation of heme *a* synthase so far is limited to the CtaA homologue from *B. subtilis*. However, a sequence alignment reveals the existence of at least two subgroups for this enzyme [60] (see also Supplement. Fig. S1 and S2). CtaA from *B. subtilis* and other Gram-positive bacteria differs remarkably compared to homologues from other species, including the human, yeast and *Paracoccus* enzymes. The highest sequence discrepancies lie in the loop regions between transmembrane helices 1 and 2 and between helices 3 and 4, while the catalytic amino acid residues are not affected (Fig. 3A). Condensing these differences into the structural model for the *Bacillus* CtaA [67] reveals a potential contact surface for protein partners such as Surf1 that may interact with the *Paracoccus* heme *a* synthase (Fig. 3B). Even though highly speculative, the existence of sequence differences in CtaA homologues may explain why some organisms expressing heme *a*-type oxidases like *B. subtilis* lack a Surf1 homologue. Heme *a* synthases of the *Paracoccus* type offer the advantage that the transfer of its enzymatic product to an additional protein factor such as Surf1 enables the cell to further regulate the biosynthesis of this cofactor. Furthermore, this arrangement allows a clear discrimination of heme *a* over its precursor molecule heme *o* and the correct positioning of the heme molecule for insertion into subunit I.

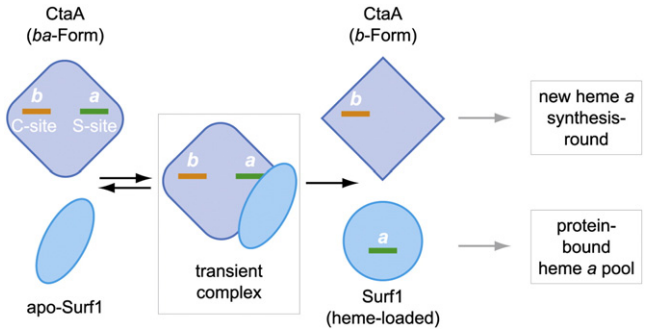


Fig. 4. Model of the interaction of CtaA with Surf1. CtaA (in its *ba*-form with a heme *b* in its catalytic C-site and a heme *a* in its substrate S-site) interacts with apo-Surf1 and the heme *a* molecule is transferred. Surf1 may reversibly bind to CtaA as indicated by the formation of a transient complex. After cofactor transfer CtaA is available for a new round of heme *a* synthesis while the heme-loaded Surf1 presents a protein-bound heme pool for cofactor insertion into subunit I.

Analysis of *E. coli* strains expressing *Paracoccus* CtaB and CtaA under different growth conditions showed that heme *a* synthesis is only successful under highly aerobic growth conditions [30]. Heme *a* synthase seems to be strictly regulated in *Paracoccus*, and the *E. coli* system provides the experimental opportunity to control the heme content of cells (and of CtaA) merely by the modification of growth conditions.

For the *Bacillus* CtaA contradictory results are discussed for the heme binding sites [33,67,68]. In the case of the *Paracoccus* protein we propose two binding sites: a C-site (catalytic) where the heme *b* is bound and an S-site (substrate) where the heme *o* is converted to heme *a* which is abstracted from the protein by Surf1c (Fig. 4). This is supported by the fact that upon mixing purified *Paracoccus* CtaA with Surf1c in vitro, exclusively heme *a* gets transferred to Surf1c [30].

When *Paracoccus* CtaA is expressed in *E. coli* the formation of heme *a* is strictly oxygen dependent as mentioned above. The same behaviour seems to be true for *Bacillus*, where heme *a* can also only be efficiently produced under high aeration [69]. Whatever the enzymatic mechanism of the enzyme is, two key points should be noted.

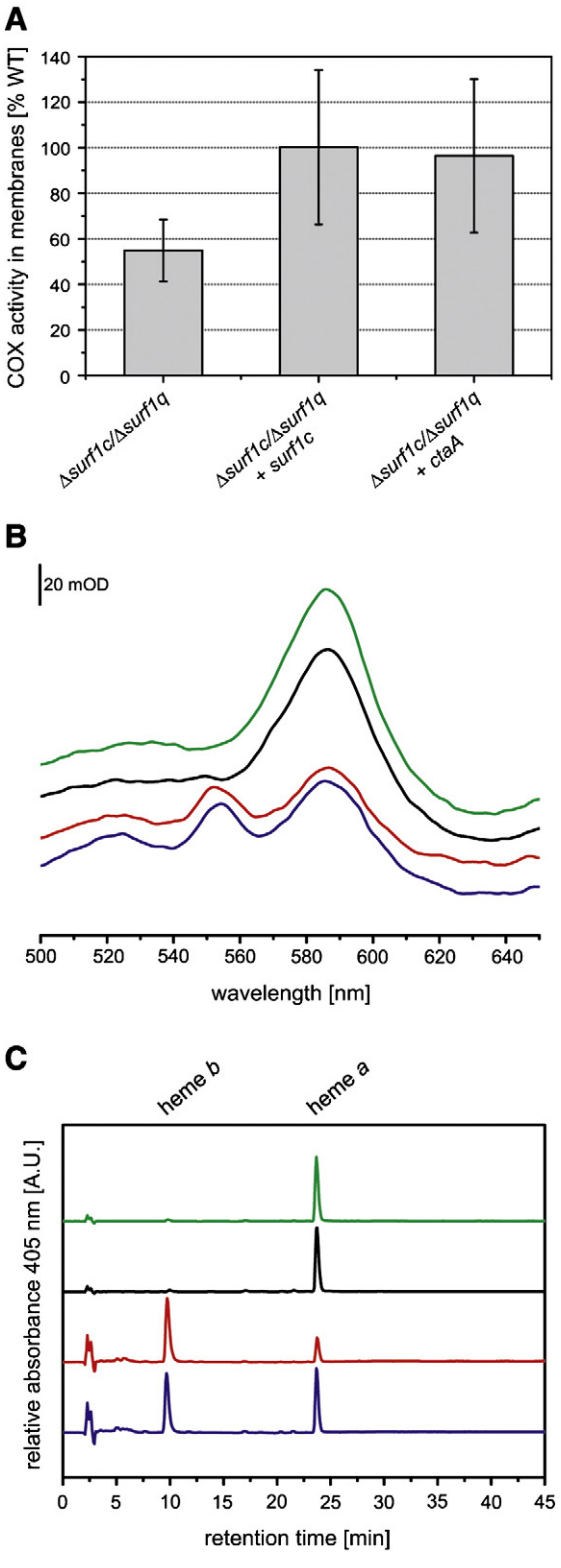
Table 3
Heme composition of purified *P. denitrificans* CtaA.

CtaA purification from strain	Strain description	Strain background	Heme types present in CtaA
Heterologous expression (E. coli), see Ref. [30]:			
HA23N[a]	*ctaA in trans*	DH5-α	*b*
HA02N	*ctaB-ctaA in trans*	DH5-α	*b*, *o*
HA02S[b]	*ctaB-ctaA in trans*	DH5-α	*b*, *a*, (*o*)
Homologous expression (P. denitrificans):			
Pd1222.HA21	Pd1222 derivative, *ctaA in trans*	Wildtype	*b*
MR31.HA21	MR31 derivative (Δ*cta*DI::Km^r Δ*cta*DII::Tet^r), *ctaA in trans*	Deletion of COX SU I	*b*
ST4.HA21	ST4 derivative (Δ*cta*CBGE::Km^r), *ctaA in trans*	Deletion of *cta*B	*b*
FA3.HA21	FA3 derivative (Δ*surf1c*::Km^r, Δ*surf1q*::Gm^r), *ctaA in trans*	Deletion of *surf1* genes	*b*

[a]N refers to expression under low aeration, [b]S refers to expression under high aeration. (For strain background see: Pd1222 [73], MR31 [74], ST4 [75], FA3 [22]).

Paracoccus CtaA is only enzymatically active in *E. coli* under high aeration conditions. In the native system, however, the formation of heme *a* is not as strictly controlled, because here the cofactor can be produced in sufficient amounts under limited oxygen concentrations. This is of little surprise, since the soil bacterium *Paracoccus* is well adopted to low oxygen availability.

Under physiological conditions a heme *a* biosynthesis complex consisting of CtaB and CtaA [58] is important as mentioned above.



In contrast to that study, copurification of *Paracoccus* CtaB with CtaA from *E. coli* was not observed, probably being due to the stringent purification protocol including relatively harsh solubilisation conditions [30]. But it should be noted that on expression in *E. coli*, the *Paracoccus* CtaA protein can be obtained in three spectroscopically distinct forms: a heme *b*-, *bo*- and a *ba*-form [30]. These data also support the presence of two distinct heme binding sites within CtaA. Purifications from the native organism only result in the *b*-form (Table 3), thus avoiding that neither heme *o* nor heme *a* accumulate at the level of CtaA. This fact indicates that the binding of the substrate heme *o* and its conversion into heme *a* is directly coupled to its immediate usage within oxidase biogenesis.

As mentioned earlier there are clear indications for an interaction of *Paracoccus* Surf1 and heme *a* synthase in vivo as well as in vitro. Further evidence for this interaction was also found in yeast, where Cox15 was identified by mass spectrometry to coelute in an affinity purification of Shy1p [18]. Though the interaction seems to be transient in *Paracoccus*, to date, only speculations can be made on the mode of interaction between Surf1 and CtaA. Surf1 in its apo form may have a certain affinity towards CtaA. The directionality of the heme transfer may be either conformationally driven or simply follow a gradient in binding affinities (Fig. 4).

As discussed earlier, the activity of heme *a* synthase seems to be regulated by the precursor molecule heme *b*, as heme *a* synthesis and transfer to subunit I are coupled. In what way the gene expression of *Paracoccus ctaA* is controlled by heme, has not been investigated yet; however the isolation of CtaA from deletion strains exclusively in its *b*-form leads to the conclusion that heme *a* synthase activity is linked to the availability of a heme *a* consumer, i.e. COX subunit I.

4. Heme *a* incorporation into cytochrome *c* oxidase

One can envisage several functions for Surf1 during heme transfer and COX assembly. The examination of purified COX from Surf1 deletion strains showing a heme *a* deficiency [22,27] and the ability of Surf1 to bind heme *a* [29,30] point to a direct involvement in heme *a* transfer. For now, only speculations on the mechanism of heme incorporation into subunit I are feasible. Studies on heme insertion into four-helix bundles mimicking COX structure, so called protein maquettes, revealed heme affinities in the low nano-molar range [31]. Heme affinities of Surf1 are in the high nano-molar range (303 nM for Surf1c and 650 nM for Surf1q [29]) and therefore a directional, thermodynamically driven transfer seems plausible. Surf1 could be an additional, transient protein constituent within the heme *a* biosynthesis complex CtaB/CtaA, directly interacting with heme *a* synthase and abstracting heme *a*. Newly synthesised subunit I could also bind to this complex leading to a Surf1-mediated heme transfer that is either repeated for the second heme by the same Surf1 protein or orchestrated sequentially by two different Surf1 molecules. Surf1 may then leave together with subunit

Fig. 5. Characterisation of cytochrome *c* oxidase from a Surf1c deletion strain. A: Cytochrome *c* oxidase activity measured in *P. denitrificans* membranes derived from a Δ*surf1c*/Δ*surf1q* strain and complementation strains with either *surf1c* or *ctaA*, compared to wildtype membranes (Pd1222). Membrane isolation and activity measurements were done as described in Ref. [22]. Mean values from three independent membrane preparations are given. B: Denaturing pyridine redox spectra of 2.5 μM *Paracoccus* COX purified from a Δ*surf1c*/Δ*surf1q* strain. Whereas only the typical heme *a* absorbance peak around 587 nm is found in COX from the wild type control (black line) and the *surf1c* complementation strain (green line), an additional heme species around 550 nm is found in the COX preparations from the Δ*surf1c*/Δ*surf1q* strain (red line) and its complementation with *ctaA* (blue line). C: HPLC analysis of the heme content of purified *Paracoccus* oxidase. After acidic heme extraction from 3 to 6 mg of purified COX, extracts were separated on a μRPC-C2/C18 column (GE Healthcare) in a 50–100% acetonitrile gradient. Oxidase preparations from the Δ*surf1c*/Δ*surf1q* strain (red line) and its complementation with *ctaA* (blue line) show, to varying extent, an additional heme peak corresponding to heme *b*, not observed for the wildtype control (black line) or the *surf1c* complementation strain (green line) samples.

I once heme incorporation is completed. The transient nature of interaction with the heme *a* biosynthesis machinery enables both *Paracoccus* Surf1 proteins (Surf1c and Surf1q) to organise the heme incorporation events into subunits I of both their cognate terminal oxidases (COX and QOX).

Deletion mutants of *surf1* in *Paracoccus* lead to a partial loss of heme *a* and a concomitant decrease of COX activity [22]. Further analysis of the oxidase phenotype in *Paracoccus* revealed an increased expression of CtaA in the Δ*surf1* strain, assuming that a higher biosynthesis rate of heme *a* may overcome the lack of Surf1. Upon expression of CtaA, activity of COX when measured in isolated membranes is restored to levels comparable to a *surf1c*-complementation strain (Fig. 5A); however, COX purified from this deletion strain still exhibits the characteristics of a Δ*surf1* phenotype with low heme content and activity. The phenotype observed in membranes is therefore probably only due to an increased production level of oxidase. Spectral analysis of COX preparations from the Δ*surf1* strain revealed an additional heme type not detected earlier in native redox difference spectra [22], only observed in denaturing pyridine redox difference spectra and clearly identified as heme *b* by HPLC analysis (Fig. 5B, C). The fact that this non-physiological heme type is not prominent in native spectra points to a heterogenous coordination in the binding site accompanied by the loss of oxidase activity. It has long been known that incorrect heme types do not support activity of terminal oxidases, as has been shown for a ao_3-variant of *Paracoccus* COX and a bb_3-variant of *E. coli* quinol oxidase [70,71]. We can only speculate on the insertion pathway of heme *b* into subunit I. Despite its postulated high affinity for heme groups, it appears unlikely that subunit I acquires heme *b* directly from the cellular environment, especially since the final enzyme of heme *b* biosynthesis, the ferrochelatase, is predicted to be located in the cytoplasm of prokaryotic organisms; therefore any interaction with COX subunit I seems unlikely.

A more plausible explanation might be the presence of a Surf1-mediated interaction between subunit I and the heme *a* biosynthesis complex of CtaA and CtaB as indicated above. Surf1 extracts the heme *a* molecule from CtaA and directly transfers the cofactor to subunit I acting as a filter for the incorporation of the correct heme type and facilitating heme insertion. It is likely that CtaA recognises subunit I also in the absence of Surf1, but this direct interaction appears less efficient, both in terms of transfer kinetics and heme specificity, so that fewer heme molecules are inserted and extenuated discrimination for the correct cofactor is exerted (Fig. 6). Due to the proposed high affinity of subunit I for heme, it is more likely that heme *b* is directly extracted from the heme *a* biosynthesis complex CtaB/CtaA, since heme *a* production in *P. denitrificans* is regulated on the level of heme *b*, thus strictly coupling synthesis to the incorporation of heme *a* into COX.

5. Conclusions

In this review we have summarised current Surf1 research, and presented additional data on the role of this assembly factor in *P. denitrificans*. We can now delineate a model, in parts still

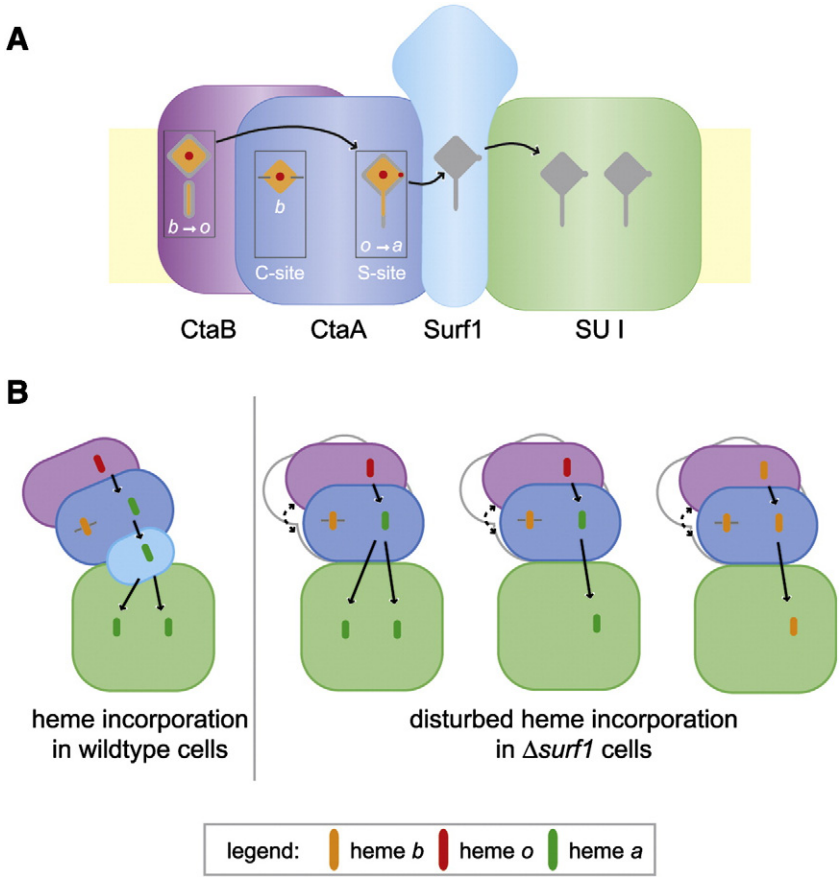


Fig. 6. Proposed steps of heme insertion into COX subunit I of *P. denitrificans*. A: Side view. Under normal conditions Surf1 (light blue) mediates the coordinated heme *a* flux from heme *a* biosynthesis complex CtaA/CtaB to subunit I (mint) and acts as a molecular filter for the transfer of the correct heme type. B: Top view. In the wild type situation (left panel) Surf1 controls the interaction of the heme *a* biosynthesis machinery CtaB/CtaA with subunit I. In the absence of Surf1, this interaction is disturbed (dotted arrow) lacking stringent heme-type discrimination. This results in only approximately 30% active oxidase complexes carrying the proper heme *a* cofactors, while several dysfunctional subpopulations with no heme, only a single heme *a* or a heme *b* molecule accumulate in the membrane.

speculative, on the multi-functional role of Surf1 during COX biogenesis in this bacterium:

(i) Surf1 protects fully assembled subunit I until subunit II is bound (Fig. 1).
(ii) Surf1 modulates the activity of CtaA by withdrawing heme *a* from the active site of the enzyme to generate a protected, yet readily available protein-bound heme *a* pool for oxidase biogenesis (Fig. 4).
(iii) Surf1 is directly involved in heme *a* insertion into COX subunit I and acts as a molecular quality control element, ensuring on the one hand that only the physiologically relevant heme *a* is transferred and on the other that the cofactor is precisely positioned for insertion into COX subunit I (Fig. 6).

Bacteria offer an interesting model system for the study of cofactor incorporation events into cytochrome *c* oxidases. The high degree of sequence conservation in chaperones mediating redox cofactor delivery point at basically similar mechanisms in early biogenesis steps of both bacterial and mitochondrial COX. In studying factors involved in the COX biogenesis in *P. denitrificans* we have recently characterised Surf1 as a heme binding protein and shown a direct interaction with the heme *a* synthase CtaA. The bacterial system provides easy access on purified chaperone components and allows to put forward a model for the pivotal heme incorporation steps into COX that may also give important clues for the mitochondrial system.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.bbabio.2011.09.007.

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