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Review

Heme A biosynthesis ☆

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ABSTRACT

Respiration in plants, most animals and many aerobic microbes is dependent on heme A. This is a highly specialized type of heme found as prosthetic group in cytochrome *a*-containing respiratory oxidases. Heme A differs structurally from heme B (protoheme IX) by the presence of a hydroxyethylfarnesyl group instead of a vinyl side group at the C2 position and a formyl group instead of a methyl side group at position C8 of the porphyrin macrocycle. Heme A synthase catalyzes the formation of the formyl side group and is a poorly understood heme-containing membrane bound atypical monooxygenase. This review presents our current understanding of heme A synthesis at the molecular level in mitochondria and aerobic bacteria. This article is part of a Special Issue entitled: Biogenesis/Assembly of Respiratory Enzyme Complexes.

1. Introduction

Heme A is essential for respiration in a wide range of different organisms including bacteria, archaea, plants and animals. This iron porphyrin compound is prosthetic group of cytochrome *a* in the terminal enzyme of the respiratory chain where dioxygen is reduced to water with the concomitant conservation of the energy released in the reaction to form an electrochemical transmembrane gradient. Biosynthesis of heme A comprises the chemically demanding conversion of one specific methyl side group, out of four possible ones, on heme, into a formyl group. This reaction is catalyzed by heme A synthase (HAS), an as yet little understood membrane-bound enzyme. Reasons for taking interest in heme A biosynthesis are the biological importance of heme A and the reaction mechanism of HAS. Detailed knowledge about heme A biosynthesis also contributes to our understanding of assembly of respiratory oxidases and of human diseases associated with mutations in the gene for HAS. In addition, heme A biosynthesis is a potential target for new antimicrobial drugs, e.g. against *Staphylococcus aureus* [1]. Finally, heme A and chlorophyll *b* biosynthesis show similar molecular features suggesting that insight into one of these processes will help to better understand also the other process.

This review first presents the molecular properties and biological function of heme A. Subsequently the heme A biosynthetic pathway and the central enzyme HAS are presented in the details. This includes properties of heme A deficient cells and organisms, the chemical composition of HAS and a compilation of experimental findings obtained so far with wild-type and mutant enzyme variants. Heme A and chlorophyllide *b* biosynthesis are then compared. Finally is presented some fundamental open questions concerning heme A and its biosynthesis.

2. Structure and properties of heme A

Heme A is a derivative of protoheme IX (heme B) with a formyl group at position C8 and a hydroxyethylfarnesyl (in most cases; see below) group at position C2 on the porphyrin macrocycle (Fig. 1). Heme B, the prosthetic group of most heme-proteins including for example cytochrome *b* and hemoglobins, has a C8 methyl group and a C2 vinyl group. The electron-withdrawing formyl group, as opposed to the methyl group, affects the redox, spectral and ligand-binding properties of the heme.

Heme O, present as prosthetic group in some terminal oxidases such as *Escherichia coli* cytochrome bo_3, is hydroxyethylprenylated like heme A but has, like heme B, a C8 methyl group. Variants of heme A and heme O with an ethenylfarnesyl, ethenylgeranylgeranyl or hydroxygeranylgeranyl C2 side chain are found in organisms belonging to the *Archaea* domain [2]. For example, the extremely thermophilic *Aeropyrum pernix* and species of *Sulfolobus* contain heme A (heme A_S) with a hydroxyethylgernaylgeranyl side group [2,3]. Except for the case of heme A, heme with a formyl group is rare in nature. Among the few examples are spirographis heme (2-formyl-4-vinyldeuteroheme) of unknown

Abbreviations: CAO, chlorophyllide *a* oxygenase; HAS, heme A synthase; HOS, heme O synthase; TM, transmembrane segment

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function found in *Spirograhis* annelid worms [4,5], and the formyl-substituted heme found in myeloperoxidase of polymorphogenic leukocytes [6].

Heme A, as compared to isolated heme B, has a higher midpoint redox potential and the light absorption maxima shifted to longer wavelengths. The reduced pyridine hemochromogen spectrum, for example, of heme B and heme A shows the alpha-band absorption peak at 556 and 585 nm, respectively [7]. The formyl group of heme A is prone to oxidation to a carboxyl group rendering heme A somewhat unstable after extraction from biological material. Heme B is an amphiphilic molecule, poorly soluble as monomer in aqueous solutions at neutral pH. The prenyl side chain of hemes A and O makes these heme variants even more lipophilic than heme B. Heme is rather toxic to cells due to its detergent-like property and because the iron atom of reduced heme in the presence of molecular oxygen can generate reactive oxygen species cf [8]. Inside the cells, heme must be transported and stored in such a way that toxic effects are minimized.

3. Heme A as prosthetic group

Heme A was discovered by Warburg and Gewitz [9] and first isolated in 1962 from bovine heart muscle [10]. Its chemical structure was established in 1975 [11] and confirmed by X-ray crystallography in 2005 when also the absolute configuration of the hydroxyethylfarnesyl group was determined [12]. Despite the vast importance and wide distribution among organisms, heme A is of low abundance in nature compared to many other types of heme. Heme A is only found in aerobic organisms and as prosthetic group in only one class of proteins, namely the cytochrome *a*-containing respiratory oxidases. Members of the family of heme–copper oxidases function as terminal enzyme in aerobic respiration in microbes, plants and animals. Cytochrome aa_3 of mammalian mitochondria (also known as cytochrome *c* oxidase and Complex IV) and that of the bacteria *Paracoccus denitrificans* and *Rhodobacter sphaeroides* is known in molecular details and serve well as paradigm for this family of respiratory oxidases [13]. The enzyme contains two heme A molecules as prosthetic groups designated heme *a* and heme a_3 by the cytochrome pioneers Keilin and Hartree based on the observed different reactivity of the two hemes to added ligands [14]. Heme a_3 and a copper atom (Cu_B) constitute part of the active site where the four electron reduction of dioxygen to water takes place. Heme *a* is the direct electron donor to the active site and is in turn reduced by a dicopper center (Cu_A). Heme-copper respiratory oxidases are multi-protein membrane bound enzymes with a variable number of polypeptides but have a conserved core of three different subunits. Both hemes and Cu_B are ligated to Subunit I whereas Cu_A is bound to Subunit II.

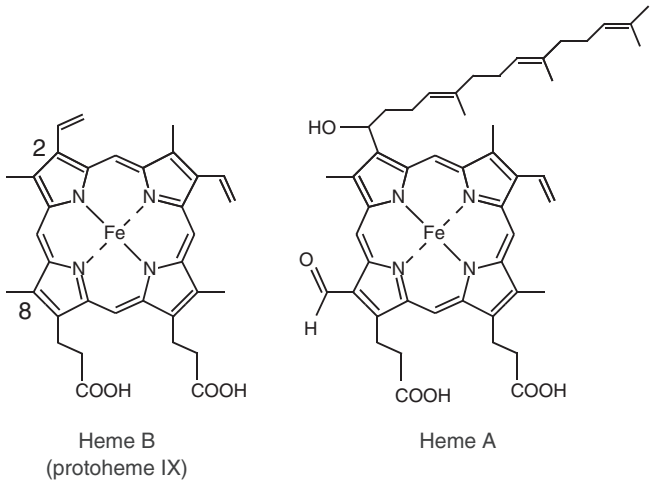

Fig. 1. Structure of heme B and heme A. Protoporphyrin IX carbon atoms 2 and 8 according to Fisher's nomenclature are indicated.

The 2.8 Å x-ray crystal structure of the 13 protein subunit bovine cytochrome *c* oxidase [15,16] shows the two heme A molecules positioned close to each other in the membrane spanning part of Subunit I. Both heme planes have a perpendicular orientation relative to the membrane plane and are in a 104° angle. The iron to iron distance between the hemes is about 14 Å. Heme *a* is hexa-coordinated with the imidazole groups of two histidine residues (His-61 on helix I and His-378 on helix X) as axial ligands to the iron atom. Heme a_3 is penta-coordinated (one coordination site is open for dioxygen binding) with His-376 (on helix X) as the distal ligand to the iron atom. The hydroxyethylfarnesyl chain of heme *a* has an extended conformation and is in contact with helices I, II, X, XI and XII of subunit I. The corresponding chain of heme a_3 is twisted and located between helices VIII and IX. All hydrophilic side groups of the two heme molecules are hydrogen-bonded to protein residues. The formyl group of heme *a* is hydrogen bonded to an arginine residue (Arg38) that is conserved in heme A-containing cytochrome *c* oxidases and has role in discrimination between heme A and heme O or heme B [17]. Interestingly, the hydroxyl group of the hydroxyethylfarnesyl moiety is hydrogen bonded to Ser328 in oxidized bovine cytochrome *c* oxidase but to Ser34 in the reduced enzyme [18].

The basic biophysical properties of heme A bound to protein have elegantly been investigated using synthetic four-helix bundle proteins, called maquettes [19,20]. Using such a cytochrome mimic, containing the histidine analog (3-methyl-L-histidine), and various hemes it was demonstrated that the formyl group of heme A results in a 179 mV positive shift in the redox potential due to destabilization of ferric heme binding to the maquette relative to ferrous heme binding. The hydroxyethylfarnesyl chain, in contrast, stabilizes the binding of both ferrous and ferric hemes to the maquette by at least 6.3 kcal/mol but does not significantly affect the redox potential of the heme compared to heme B. This increased binding due to hydrophobic interaction between the heme and protein seemingly counteracts the destabilizing effect of the formyl group [19].

Heme A is in principle not required for function of proton-pumping heme–copper respiratory oxidases. For example, in *E. coli* cytochrome bo_3 hemes B and O occupy the positions corresponding to those for heme *a* and heme a_3 in mammalian cytochrome aa_3 [21]. There are also bacterial cytochrome ba_3 enzymes containing heme B in the electron donor site [22] and heme A at the active site as well as cytochrome cbb_3 heme–copper oxidases [23]. The two heme groups are positioned similarly in Subunit I in these oxidases but there are structural differences depending on the type of heme in each site. At least some bacterial oxidases are promiscuous being able to incorporate heme B or heme O instead of heme A. Misincorporation of heme B or heme O instead of heme A into heme–copper oxidase is promoted by some mutations [17,24] and by oxygen-limitation during growth of the microorganism [25,26]. Reciprocally, *E. coli* cytochrome bo_3 has been observed to misincorporate heme A (into the heme O site) when expressed in *P. denitrificans* [27]. Heme-substitution in oxidases generally seems to negatively affect enzyme activity.

4. Overview of heme A biosynthesis

The heme A biosynthetic pathway from heme B involves two membrane bound enzymes and has heme O as one stable protein dissociable intermediate. The modification of the C2 vinyl group of heme B into the hydroxylethylfarnesyl group is catalyzed by heme O synthase (HOS) with farnesylpyrophosphate as co-substrate. The subsequent monooxygenation of the C8 methyl group to yield heme A is catalyzed by HAS and requires molecular oxygen (Fig. 2). This biosynthetic reaction and the enzyme protein involved were originally discovered by the use of metabolic labeling and mutants impaired in heme A synthesis [28]. In pioneering work, Sinclair et al.

demonstrated in 1967, using a *S. aureus* heme auxotrophic mutant, that heme A is synthesized from heme B [29]. This was subsequently confirmed using a *Bacillus subtilis* ferrochelatase-deficient mutant [30]. In 1974 Taber demonstrated that certain sporulation-deficient isolates of *B. subtilis*, with a mutation in a gene later identified and named *ctaA*, are cytochrome *a*-defective [31,32]. Viability of aerobic bacterial cytochrome *a*-deficient mutants is explained by the presence of a branched respiratory chain with a heme A-independent terminal oxidase, cytochrome *bd*. Heme O is normally not found at detectable levels in *B. subtilis* cells but can accumulate if the *ctaA* gene is inactivated. This finding by Svensson et al. in 1993, combined with their observation that *E. coli* cells containing the *B. subtilis* CtaA protein produce heme A (*E. coli* is able to synthesize heme O but not heme A), established CtaA as HAS [33]. The *cyoE* gene of the *cyoABCDE* operon in *E. coli* had previously been identified to encode HOS [34]. Based on sequence comparisons with CyoE and biochemical analysis of mutants, *B. subtilis* CtaB (as well as the paralogue CtaO [35]), *P. denitrificans* CtaB and *Saccharomyces cerevisiae* COX10p, were identified as HOS [28].

5. Heme A synthase (HAS)

5.1. Amino acid sequence and evolution

HAS in most organisms consists of a single polypeptide of some 300 amino acid residues. In silico primary sequence analysis [36,37] and topology studies with *B. subtilis* CtaA using fusions to alkaline phosphatase [38] indicate that the enzyme protein has 8 transmembrane segments (TM I–IIX) with both the N- and C-termini exposed on the negative side of the membrane (Fig. 3.), i.e., to the cytoplasm in bacteria and matrix in mitochondria. Some organisms belonging to the *Archaea* evolutionary domain contain a shorter HAS polypeptide variant of about 160 residues and only 4 TMs. This type, found for example in the hyperthermophile *A. pernix* and the halophile *Halobacterium salinarium*, is called compact CtaA (cCtaA) [39]. cCtaA polypeptides form functional homo-dimers corresponding in size and topology to the 8 TM HAS polypeptides. The 8 TM protein-type seems to originate from a primordial 4 TM variant by a tandem gene duplication event followed by gene fusion and subsequent divergent evolution of the N- and C-terminal halves of the polypeptide [39]. The amino acid sequences of HAS from distantly related organisms show relatively poor conservation but homology between the N- and C-terminal halves of the protein can generally be observed [36]. Possibly each half forms a four-helix bundle domain. Among the few conserved amino acid residues in HAS are four His (here denoted as H1, H2, H3 and H4; Fig. 3.). H1 is replaced by an Asp in bacteria belonging to Actinomycetales [37]. Two hydrophilic peptide loops, connecting TM I with II and TM V with VI, respectively, are predicted to be exposed on the positive side of the membrane. The sequence of the TM I–II loop is more conserved than that of the TM V–VI loop and contains in bacteria two invariant Cys residues here denoted as C1 and C2. HAS of *Bacillus* species contains two additional invariant Cys (C3 and C4) in the TM V–VI loop (Fig. 3).

5.2. Chemical composition of bacterial HAS

Published experimental data for HAS isolated from different bacteria is summarized in Table 1. The *B. subtilis* HAS (CtaA) has been studied most extensively and analyzed in situ in isolated membranes [36] and after solubilization and purification in the presence of non-ionic detergents. Wild-type, not tagged, as well as hexa-histidyl tagged (N- or C-terminal) CtaA variants has been isolated (Table 1). It is important to note that all published in vitro biochemical data concerning HAS is based on the protein produced by over-expression of the cloned structural gene in the homologous organism (the case of *B. subtilis*) or heterologously in *E. coli*.

Isolated bacterial HAS preparations contain heme B. Depending on the protein variant and the conditions under which the protein was synthesized before purification, preparations of HAS usually contain also heme O or derivatives of heme O including the enzyme product, heme A (Table 1). It is assumed that heme B acts as prosthetic group and that heme A, when present, is enzyme product remaining bound to HAS because a limiting number of acceptor proteins, such as heme A trafficking proteins or Subunit I apo-protein, is present. From available data it cannot be completely ruled out that the heme B found in HAS is an experimental artifact resulting from the overproduction of the protein. Metal and spectroscopic analyses of isolated HAS indicate heme as the single kind of prosthetic group; no significant amounts of non-heme iron nor copper have been detected in *B. subtilis* CtaA produced in *B. subtilis* [40].

B. subtilis HAS has been found to contain between 0.2 and 1.95 mol heme B per mol CtaA polypeptide and up to ≤0.2 mol heme A per mol CtaA (Table 1). The presence of heme A in CtaA is promoted by aeration of the culture and level of CtaB/heme O in the cell [40]. Low oxygen conditions cause accumulation on HAS of heme O (enzyme substrate) or heme I (first reaction intermediate) instead of heme A [41]. As judged from visible light absorption spectra of membrane bound and detergent-solubilized CtaA the heme binding properties are the same for *B. subtilis* HAS produced in *E. coli* as in *B. subtilis*. HAS of bacteria shows the features of a cytochrome of relatively high redox potential (Table 2). The iron atom of heme B, and also of heme A when present in HAS, is low spin and apparently hexa-coordinated [40]. EPR signals of heme A and heme B in oxidized isolated *B. subtilis* HAS are highly anisotropic with $g_{max} \geq 3.5$ (Table 2) suggesting bis-His or His-Met heme iron axial ligation [42].

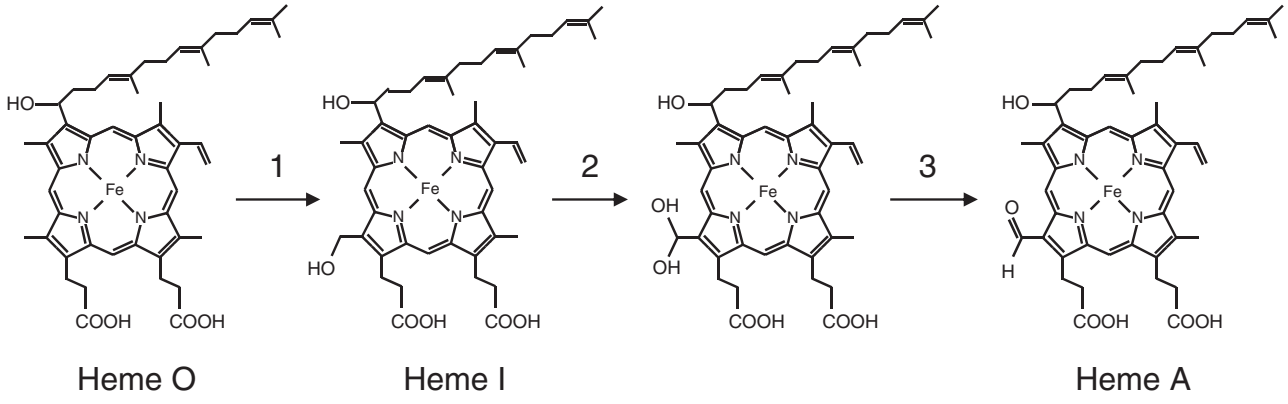

Fig. 2. Heme A biosynthetic pathway from heme O. All three reaction steps are proposed to occur on the HAS enzyme. Steps 1 and 2 are successive monooxygenase reactions resulting in a germinal diol that in step 3 is dehydrated resulting in the formyl group.

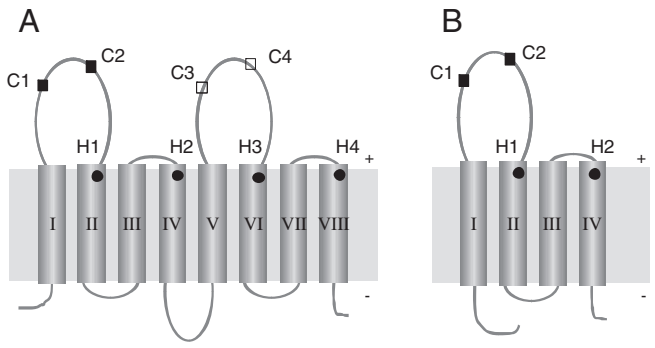

Fig. 3. Membrane topology models of CtaA from *B. subtilis* (A) and cCtaA from *A. pernix* (B). His residues conserved in HAS polypeptides are indicated by dots and denoted as H1, H2, H3 and H4. Cys residues conserved in bacterial HAS are indicated with filled squares and denoted as C1 and C2. Two Cys residues invariant only in CtaA of *Bacillus* species are indicated by open squares and denoted as C3 and C4. Residue numbers for conserved His and Cys in *B. subtilis* CtaA are provided in Table 3. The positive and negative sides of the membrane are indicated as + and −, respectively.

6. Mitochondrial HAS

Genes important for mitochondrial heme A synthesis were originally found by identifying mutations in collections of respiratory deficient mutants of baker's yeast, *S. cerevisiae* [43]. The gene for HAS, *COX15*, was identified by the phenotype (i.e., lack of cytochrome *a* and heme A), and weak sequence similarity of Cox15p to bacterial CtaA [44]. A striking difference in the primary sequence of mitochondrial and bacterial HAS proteins is that the former lacks the otherwise conserved C1 and C2 Cys residues. *COX15* in *Schizosaccharomyces pombe* is fused to the *YAH1* gene encoding a mitochondrial ferredoxin. This finding combined with a number of experimental results indicates that the heme O to heme A conversion in mitochondria involves Cox15p, Yah1p and the ferredoxin dehydrogenase Arh1p [44,45]. Recently the *Trypanosoma cruzi* gene for HAS was identified by sequence similarity to Cox15p and by the complementation of a *S. cerevisiae* COX15 mutant [46].

The human genome contains a gene encoding a yeast Cox15p homolog. Mutations in this gene have been found in patients with fatal infantile hypertrophic cardiomyopathy and cause mitochondrial heme A and cytochrome *c* oxidase deficiency [47]. Interestingly, mutations in the gene for HOS (*COX10*) and HAS, respectively, apparently result in very different clinical manifestations in humans [48,49]. This difference could indicate that the mutations in the two genes have different levels of penetrance, that heme O accumulation is toxic, or that heme O plays some function in addition of being substrate for HAS. Studies in *S. cerevisiae* indicate that heme O biosynthesis is positively regulated by heme A biosynthetic intermediates or HAS [50] and that transcription of *COX10* and *COX15* do not share the same regulatory mechanism [51]. Similar interdependence of HOS and HAS and differential gene regulation might occur also in human cells and contribute to different manifestation of disease depending on the mutation.

Table 1
Composition of isolated wild-type CtaA/Cox15 from different organisms.

Organism	Polypeptide; no. of residues	Production host	Heme content[a]	Refs.
A. pernix	cCtaA; 161	*E. coli*	Heme B, heme O, heme I	[39]
B. stearothermophilus	CtaA; 317	*E. coli*	Heme B, heme A	[62]
B. subtilis	CtaA; 306	*B. subtilis*	Heme B (≤0.4 mol/mol), heme A (≤0.2 mol/mol)	[37,40,52]
		B. subtilis[b]	Heme B, heme A	[52]
		E. coli[b]	Heme B, heme A	[36]
		E. coli[c]	Heme B (1.95 mol/mol)[d]	[37]
P. denitrificans	CtaA; 391	*E. coli*[b]	Heme B, heme A, heme O	[63]
Rh. sphaeroides	Cox15; 391	*E. coli*[b]	Not reported	[66]

[a] Indicated are major types of heme found in purified preparations of HAS. The relative amount of heme types present is influenced by genetic background and growth conditions.
[b] CtaA with a C-terminal hexa-histidyl tag.
[c] CtaA with a N-terminal hexa-histidyl tag.
[d] This reported stoichiometry is questionable since protein was determined using the BCA assay which probably underestimates the CtaA polypeptide content as discussed before [36].

7. Mutant HAS

7.1. His mutants

The spectroscopic features of bacterial HAS suggest bis-histidyl or perhaps His-Met axial ligation of heme A and heme B (see Section 5.2 and Table 2). The domain organization of the CtaA protein (Fig. 3), with homologous N- and C-terminal domains and *A. pernix* cCtaA being a homo-dimer, might reflect that HAS contain two heme binding sites perhaps with different heme specificities; one for the prosthetic group heme B, which presumably is permanently bound, and one for the substrate, heme O. From available spectroscopic and heme to protein stoichiometric data it cannot be decided whether HAS contains one or two heme binding sites [40]. Depending on the number of heme-binding sites, up to four His residues in the HAS protein could be involved as axial ligands to heme iron.

The importance of the four conserved His residues in HAS (H1, H2, H3 an H4) (see Fig. 3) for heme binding and enzyme activity has been investigated by site-specific mutagenesis with *B. subtilis* CtaA. The mutant variants were produced and studied in two widely different experimental settings; (i) after heterologous production in *E. coli* using a strongly inducible *ctaA* expression system [37] and (ii) after homologous overproduction in *B. subtilis* from *ctaA* with the native promoter region [52], respectively (Table 3). Another significant difference between the studies is the placement of the hexa-His tag, added to facilitate purification of protein variants, at opposite ends of the CtaA polypeptide. One major advantage with analysis in *B. subtilis*, compared to in *E. coli*, is that HAS activity of CtaA variants can be determined from the assembly of functional cytochrome aa_3 (quinol oxidase) and cytochrome caa_3 (cytochrome *c* oxidase) [52]. These enzymes can be assayed by spectroscopy and oxidase activity. Important to note is that this in vivo assay relies on both HAS activity and incorporation of heme A into oxidase. Activity of *B. subtilis* CtaA produced in *E. coli* can be estimated by the presence of heme I or heme A formed and remaining bound to the enzyme protein [41]. In the case of the *B. subtilis* CtaA mutant study in *E. coli* [37] wild-type CtaA contained for unexplained reasons only hemes B and O, suggesting no enzyme turnover (Table 3). Therefore, although some isolated mutant variants did contain heme I, the information from *E. coli* about HAS activity of several mutants is not conclusive.

Table 2
Biophysical features of hemes A and B in isolated *B. subtilis* CtaA.
Data from references [36,40,53].

	Heme A	Heme B	Refs.
Mid-point redox potential (mV)[a]	+230	+ 85 +168[d]	[40] [53]
EPR spectrum (g_{max})[b]	3.5	3.7	[40]
Alpha band absorption maximum (nm)[c]	585	559[e]	[36,40]

[a] At pH 7.
[b] Oxidized protein at 10 K.
[c] Reduced protein at room temperature.
[d] E_m of His_6-tagged CtaA.
[e] Split alpha-absorption peak at 77 K with maxima at 553 and 558 nm.

The results of the mutant studies combined indicate His residue H2 in *B. subtilis* CtaA as essential for HAS activity and H1 and H3 as being important but replaceable by Met without total loss of activity. HAS activity was found when H4 was replaced by Leu or Met. Since Met, in principle, can function in place of His as axial ligand, the combined findings indicate H1 and H3 as possible axial ligands to heme. Residues H1 and H3 have analogous positions in the two homologous halves of CtaA (Fig. 3). The functional importance of H3 is supported by the properties of an active *B. subtilis* CtaA variant lacking a segment comprising residues 211 to 217, which includes H3 (position 216). In this truncated variant a non-conserved His residue (at position 209 in wild-type CtaA) substitutes for the missing H3 [53].

The heme-binding properties of *B. subtilis* CtaA variants, mutated in residues that are conserved in HAS proteins, were analyzed by spectroscopy and heme analysis of the purified proteins produced in *E. coli* or *B. subtilis*. Results are presented in Table 3. All variants were found to bind heme B and, with only few exceptions, also heme O. The enzymatically inactive variants generally contained heme B and heme O showing that substrate binding and catalytic activity are separable. Remarkably, H1 + H2 (a His60Ala + His123Ala as well as a His60Gln + H123Gln) double mutants were found to bind heme B to the same extent as wild-type CtaA [37]. The findings are consistent with the presence of two heme binding sites in HAS. This is supported by the observation [54] that an *A. pernix* His75Leu mutant cCtaA (being equivalent to a H1 + H3 CtaA double mutant) was found to bind only heme B whereas a His136Met variant (equivalent to H2 + H4 double mutant) bound heme B and heme O. A His136Leu cCtaA mutant was found unstable and could therefore not be analysed.

CtaA mutant variants with low HAS activity and produced in *B. subtilis* contained, similar to wild-type and as expected, heme A. Some variants accumulated the mono-hydroxylated intermediate heme I (Fig. 2). This was pronounced in His216 (H3) and Arg217 mutant variants indicating defective enzyme turnover. At present, in the absence of information about the spatial organization of the eight TMs of CtaA, the available results (Table 3) [54] do not explain in any detail the roles of invariant His residues in HAS but suggest two separate heme bindings sites and that H3 functions as a heme ligand.

7.2. Cys mutant variants

The two conserved Cys (C1 and C2) in the N-terminal half of bacterial HAS appear important for enzyme function. Substitution of C1 (Cys35Ala) in *B. subtilis* resulted in very low enzyme activity whereas substitution of residue C2 (Cys42Ala) and also a C1 C2 double mutant, respectively, resulted in inactive enzyme as determined in vivo (Table 3). These three mutant variants were all found to bind heme B and heme O. The C1 mutant in addition contained a trace of heme A, consistent with low HAS activity. The two Cys residues in the C-terminal half of *B. subtilis* CtaA (C3 and C4) appear dispensable for function because HAS activity was fully retained in variants where both these Cys were replaced by Ala (Table 3).

Possible functions of the C1 C2 pair are: (i) As ligands to a hitherto unrecognized metal ion cofactor, or (ii) to form a disulfide bond to stabilize the enzyme or modulate enzyme activity, or (iii) to mediate interaction with other proteins of importance for e.g. heme A delivery to oxidase, or (iv) to have redox function in electron transfer to HAS similar to what has been observed for bacterial homologues of vitamin K epoxide reductase [55]. Our recent experiments with *A. pernix* wild-type, Cys49Ala, and Cys56Ala cCtaA mutant variants have demonstrated that an intra-molecular disulfide bond (between Cys49 and C56) as well as an inter-molecular disulfide bond (between Cys in the two cCtaA proteins in the homo-dimer) can form in bacterial HAS [54](A. Lewin and L. Hederstedt, unpublished data). The intermolecular disulfide cross-link shows proximity of the hydrophilic loops in the two protein domains in the folded enzyme protein (Fig. 3). The wild-type cCtaA as isolated contained predominantly intramolecular disulfide bonds. These results suggest the presence of a disulfide bond between C1 and C2 and between C3 and C4 in *B. subtilis* CtaA. A *B. subtilis* CtaA C1 + C2 double mutant was found unstable in *E. coli* [37] but this was not observed in *B. subtilis* [54]. The combined experimental results indicate that a disulfide bond between C1 and C2 is important for activity of bacterial HAS but is not required for binding heme O. The low activity of the C1 variant could be explained by the formation of a disulfide bond between C2 and C4 partly substituting for the missing bond between C1 to C2.

Table 3
Properties of mutant variants of *B. subtilis* CtaA overproduced in the native bacterium [52] or heterologously produced in *E. coli* [37].

CtaA variant		Produced in *B. subtilis*[a]		Produced in *E. coli*[b]
		Activity[c]	Heme content[d]	Heme content[d]
Wild-type		++	B, A	B, (O)
Trp39	→ Ala			B, (O)
Glu57	→ Ala			B, O
	→ Gln			B, O
His60 (H1)	→ Ala			B, O
	→ Leu	−	B, O	
	→ Met	+	B, (A)	
	→ Gln			B, O
Arg61	→ Ala			B, (O)
	→ Gln			B, (O)
Glu103	→ Ala			B, (O)
His123 (H2)	→ Ala			B, O
	→ Leu	−	B, O	
	→ Met	−	B, O	
	→ Gln			B, (O)
H216 (H3)	→ Ala[f]			B, O, I
	→ Leu[e]	−		
	→ Met	+	B, O, I, (A)	
	→ Gln[f]			B, O, I
R217	→ Ala			B, (O), I
	→ Gln			B, (O), I
Gln257	→ Ala			B, (O), (I)
H278 (H4)	→ Ala[f]			B, O
	→ Leu	+	(B), (A)	
	→ Met	+	(B), (A)	
	→ Gln[f]			B, O, I
Cys35 (C1)	→ Ala	+[g]	B, O, (I), (A)[g]	
Cys35 (C1) + Cys42 (C2)	→ Ala	−[g]	B, O[g]	B, O[f]
Cys191 (C3) + Cys197 (C4)	→ Ala	+[g]	B, I, A (O)[g]	B, O[f]

[a] CtaA with a C-terminal His_6-tag synthesized in *B. subtilis* strain LMT20R containing the *ctaA* gene with natural promoter on plasmid pHPKS. CtaA was solubilized from isolated membranes and purified in the presence of the detergent Thesit.
[b] CtaA with a N-terminal His_6-tag synthesized in *E. coli* strain BL21 (DE3)/pLys from *ctaA* under the IPTG-inducible T7 promoter in plasmid pET-15b. CtaA was solubilized from isolated cytoplasmic membranes and purified in the presence of sucrose monolaurate.
[c] Activity determined indirectly in vivo in *B. subtilis* as presence of functional cytochrome *c* oxidase (see text for details); ++, wild type activity, + low activity, − no detectable activity.
[d] Heme content of isolated protein variant determined using HPLC; small amounts of heme are indicated with brackets.
[e] CtaA polypeptide not detectable in membranes.
[f] CtaA polypeptide partly degraded.
[g] Results obtained in *B. subtilis;* A. Lewin and L. Hederstedt, unpublished data.

8. Chlorophyll *b* and heme A biosynthesis compared

Cyanobacteria, mosses, ferns and seed plants contain in their light-harvesting antenna complexes both chlorophyll *a* and *b* which only differ in structure at the C7 side group; chlorophyll *a* has a methyl group whereas chlorophyll *b* has a formyl group at this position. Biosynthesis of chlorophyll *b* shows similarities to heme A synthesis

by only occurring in aerobic organisms and comprising the conversion of a methyl group to a formyl group on a cyclic metallo-polypyrrole compound. It has been demonstrated by ^{18}O-labeling experiment that the oxygen atom of the chlorophyll *b* formyl group is directly derived from dioxygen [56,57]. Chlorophyllide *a* oxygenase (CAO) catalyzes monooxygenation of chlorophyllide *a* to yield chlorophyllide *b* which subsequently is converted to chlorophyll *b*. The CAO reaction is suggested to contain two reaction intermediates; a stable monohydroxy-methyl derivative and an unstable dihydroxy-methyl derivative which converts to a formyl group by the elimination of water [58]. CAO of *Arabidopsis thaliana* and *Chlamydomonas reinhardtii* is a 56 and 51 kDa, respectively, chloroplast membrane bound protein containing a Rieske-type [2Fe–2S] center and a non-heme iron center [59–61]. The amino acid sequences of CAO from cyanobacteria and plants are highly conserved [60] and show similarity to e.g. vanillate demethylase and *p*-toluenesulfonate monooxygenase which catalyze oxygenation of a methyl side chain of an aromatic ring converting it to a hydroxy-methyl group [59]. The electron donor to CAO is thought to be ferredoxin or possibly ferredoxin-NADPH reductase directly. Thioredoxin of 14 kDa has been found to be co-immunoprecipitated with *C. reinhardtii* CAO but the significance of this is unknown [61].

EPR studies with *A. thaliana* and *C. reinhardtii* CAO have shown the presence of a [2Fe–2S] center ($g = 2.057$ signal detectable at 15 K), a non-heme mono-nuclear iron ($g = 4.3$ signal detectable at 125 K) and a $g = 2.0042$ signal characteristic of an organic radical species [61]. The radical was found associated with a Tyr residue (Tyr422 in *C. reinhardtii* and Tyr518 in *A. thaliana*) present in a somewhat invariant sequence, -ValArg/AlaTyrArg-, close to the C-terminal end of the enzyme polypeptide. The EPR signal of the radical was found to form in the absence of cognate substrate and being stable, but quenched by addition of chlorophyll [61]. The findings together suggest that during the enzyme reaction the radical is transferred to the substrate and then reacts with an oxygen complex at the mono-nuclear iron center to generate the C7 hydroxy-methyl intermediate [61]. The structure and reaction mechanism of CAO are far from understood and physiological electron donors/interacting proteins remain to be identified.

9. On the possible enzyme mechanism of HAS

Studies aiming to elucidate the reaction mechanism of HAS are currently hampered by the lack of an in vitro enzyme assay system. So far, HAS activity has not been reported for any preparation of purified CtaA. As a lead in the search for assay conditions, HAS activity was detected in isolated *E. coli* membranes containing *B. stearothermophilus* CtaA [62]. Incubation of the membranes in the presence of added heme O and dithiothreitol yielded, apparently as the result of a single enzyme turnover, heme A bound to CtaA in the membrane. Under the same incubation conditions no activity was detected with *B. stearothermophilus* CtaA purified in the presence of the detergent Triton X-100.

In the absence of an in vitro activity assay for HAS it cannot be ruled out that one or more essential cofactors are missing in isolated CtaA. However, with heme B as prosthetic group and no other metal cofactor available, except the substrate heme O, the catalytic mechanism of bacterial HAS presumably involves heme iron. As discussed before [40], the enzyme protein does not show the features of a P450-type mixed function monooxygenase. Heme B or heme O, however, probably plays a key role in activation of oxygen for hydroxylation of the C8 methyl group of heme O. The heme A biosynthetic reaction comprises two enzyme bound hydroxylated intermediates; the C8 alcohol (heme I), which is stable, and the unstable putative C8 geminal diol that dehydrates to form heme A (Fig. 2). Molecular oxygen is required for HAS activity as concluded form a multitude of in vivo experiments using oxygen limitation, e.g., [41,63]. Under oxygen-limited conditions heme I accumulates and is, if oxygen is supplied, rapidly oxidized to heme A and can to some extent be over-oxidized to form heme II with a C8 carboxyl group [64]. Based on these experimental findings and comparison to chlorophyll *b* synthesis (see Section 8) one expects that the formyl group oxygen atom of heme A is directly derived from atmospheric molecular oxygen. Brown et al. [64] have, however, presented compelling evidence that HAS does not incorporate molecular oxygen into heme A. To rationalize their findings in the light of biochemical data with isolated HAS, the authors have proposed heme iron-dependent mechanisms for the heme O to heme A conversion step [40,64].

Assuming very similar reaction mechanisms of HAS and CAO it is interesting that the former is apparently dependent on heme and catalyses oxygenation of a methyl group on an iron-prophyrin whereas the latter is a non-heme enzyme catalyzing oxygenation of a methyl group on a magnesium-porphyrin. In both cases the reaction probably involves a radical but this has only been shown in the case of CAO. Generation of a radical in HAS is presumably connected to heme iron and binding of oxygen. In CAO the Rieske iron–sulfur center and mono-nuclear iron center are needed because the magnesium ion of the chlorophyllide is not useful for redox reactions. Ferredoxin (or ferredoxin reductase) is electron donor to CAO and apparently also to mitochondrial HAS. How electrons enter bacterial HAS is unknown but could involve Cys residues C1 and C2 (see Section 7.2). If so, an extra-cytoplasmic electron donor, such as a thioredoxin-like protein, would also be involved. TlpA (thioredoxin-like protein A) of *Bradyrhizobium japonicum* is required for cytochrome aa_3 maturation but it is not known if this membrane protein is important for heme A synthesis [65]. Menaquinol in the membrane can reduce heme B (and heme A) in membrane bound *B. subtilis* CtaA [36]. This presents quinol as a potential physiological electron donor to bacterial HAS. To substantiate this possibility, data regarding kinetics of electron transfer rates and binding of quinone to CtaA are desired.

10. Outlook — major unresolved questions regarding HAS

10.1. In vitro assay and protein structure

Critical to advances in HAS research is now to establish a defined in vitro system in which the cofactor dependency of the enzyme can be identified and the activity of isolated protein determined. It is relevant to mention that an in vitro system for CAO activity measurements is available based on ferredoxin and ferredoxin reductase as electron donors [58]. HAS research is also restricted by the lack of protein structural information beyond the trans-membrane topology level (Fig. 3.) and speculative models on how the TMs might be arranged relative one another [37,38]. With knowledge about the organization of TMs in the membrane and the number of heme binding sites, the available HAS mutant data (Table 3) would be more conclusive. Attempts in my laboratory to crystallize *B. subtilis* CtaA and *A. pernix* cCtaA have so far been unsuccessful. Besides the difficulty of crystallizing integral membrane proteins, inhomogeneity of HAS preparations with regard to heme content and composition poses a problem. Heme-free *A. pernix* cCtaA, produced by cell-free protein synthesis in the presence of detergent and purified [54], is potentially suitable for crystallization and useful for other types of experiments concerning HAS.

10.2. Heme trafficking and protein interactions

Only little is known about proteins interacting with HAS in the membrane and trafficking of heme O and heme A in cells. HOS and HAS appear to form a complex in the cytoplasmic membrane of bacteria [66] and probably also in the inner mitochondrial membrane. Consistent with the existence of such a complex, the HOS and HAS polypeptides in the bacterium *Thermus thermophilus* are fused into a 608 residues CtaA–CtaB protein. A complex does not seem important

for stability of the enzymes but thought to facilitate transfer of heme O from HOS to HAS. When heme A synthesis overrides the demand for heme A in the cell, i.e., when active HAS, and especially both HOS and HAS, is overproduced in the cell heme A remains bound to HAS. Thus, the release of heme A from HAS seemingly requires interaction with other proteins. Recently it was demonstrated in vitro with purified proteins that *P. denitrificans* Surf1 proteins interact with heme A-loaded HAS (CtaA) resulting in the transfer of heme A (but not heme B) to Surf1 [63]. *P. denitrificans* contains two Surf1 paralogues, Surf1c and Surf1q. Both variants showed heme A acceptor activity and mutation of a conserved Trp residue resulted in a shift in binding specificity from heme A to heme O. Surf1 in bacteria and yeast (Shy1p) are membrane proteins important for proper assembly of functional cytochrome a, especially the heme a_3 prosthetic group [67,68]. The findings suggest that binding of Surf1/Shy1p to HAS triggers release of heme A and that Surf1/Shy1p functions both as heme chaperone and cytochrome assembly factor. Some functional cytochrome *a* can assemble also in the absence of Surf1/Shy1p. This indicates the presence of additional heme A chaperones or that HAS is able to donate heme A directly to apo-cytochrome *a* [63]. Two heme A molecules are needed for assembly of each cytochrome oxidase molecule. The incorporation of these potentially harmful hemes and copper atoms has to be coordinated with the folding of the Subunit I polypeptide in the membrane and organized assembly with the other subunits in the membrane [69,70]. It remains to be investigated how and to what extent HAS interacts with structural polypeptides and specific assembly factors during biogenesis of respiratory cytochrome *a* oxidases. For example, during maturation of Subunit I are the two heme A molecules for heme *a* and heme a_3 assembly synthesized on the same HAS enzyme molecule or are they synthesized and delivered by separate HAS molecules?

10.3. Heme A degradation

Apparently completely unexplored aspects related to heme A synthesis is whether heme A and heme O are recycled in nature and how these hemes are degraded in biological systems. Is heme oxygenase, which normally uses heme B as both cofactor and substrate, active with modified hemes? Many bacteria can take up heme B from the environment and use it for heme protein synthesis or as an iron source. This could comprise also heme O and heme A. Information about transport of modified hemes across membranes and uptake by microorganisms is lacking. *B. subtilis* cells can take up heme B but a *ctaB* deletion mutant can apparently not use heme O in the growth medium for cytochrome *a* synthesis (L. Hederstedt, unpublished data). CAO plays a critical role in the so called “chlorophyll cycle” in plants by catalyzing oxygenation of chlorophyllide *a* to chlorophyllide *b* but also the reverse reaction. It remains to find out if HAS or some other enzyme in vivo can catalyze reduction of heme A to yield heme O. Several of these open questions can be experimentally addressed using heme A and heme O available as pure chemical compounds and suitable well defined microbial mutants.

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References

[1] M.O. Clements, S.P. Watson, R.K. Poole, S.J. Foster, CtaA of *Staphylococcus aureus* is required for starvation survival, recovery, and cytochrome biosynthesis, J. Bacteriol. 181 (1999) 501–507.
[2] M. Lubben, K. Morand, Novel prenylated hemes as cofactors of cytochrome oxidases. Archaea have modified hemes A and O, J. Biol. Chem. 269 (1994) 21473–21479.
[3] Y. Kabashima, J. Sakamoto, Purification and biochemical properties of a cytochrome bc complex from the aerobic hyperthermophilic archaeon *Aeropyrum pernix*, BMC Microbiol. 11 (2011) 52.
[4] H. Fisher, C. von Seeman, Die Konstitution des Spirographishämins, H.-S. Z. Physiol. Chem. 242 (1936) 133–157.
[5] M. Sono, T. Asakura, Separation and properties of spirographis and isospirographis porphyrin dimethyl esters, Biochemistry 13 (1974) 4386–4394.
[6] M. Sono, A.M. Bracete, A.M. Huff, M. Ikeda-Saito, J.H. Dawson, Evidence that a formyl-substituted iron porphyrin is the prosthetic group of myeloperoxidase: magnetic circular dichroism similarity of the peroxidase to *Spirographis* heme-reconstituted myoglobin, Proc. Natl. Acad. Sci. U. S. A. 88 (1991) 11148–11152.
[7] J.E. Falk, Haems, Porphyrins and Metalloporphyrins, vol. 2, Elsevier Publishing Company, Canberra, 1964, pp. 181–188, 240–241.
[8] I. Hamza, Intracellular trafficking of porphyrins, ACS Chem. Biol. 1 (2006) 627–629.
[9] O. Warburg, H.S. Gewitz, Cytohemin from the heart muscle, Z. Physiol. Chem. 288 (1951) 1–4.
[10] W.S. Caughey, J.L. York, Isolation and some properties of the green heme of cytochrome oxidase from beef heart muscle, J. Biol. Chem. 237 (1962) 2414–2416.
[11] W.S. Caughey, G.A. Smythe, D.H. O'Keeffe, J.E. Maskasky, M.L. Smith, Heme A of cytochrome *c* oxidase, J. Biol. Chem. 250 (1975) 7602–7622.
[12] E. Yamashita, H. Aoyama, K. Muramoto, K. Shinzawa-Itoh, S. Yoshikawa, T. Tsukihara, Absoulte configuration of the hydroxyfarnesylethyl group of haem A, determined by X-ray structural analysis of bovine heart cytochrome *c* oxidase using methods applicable at 2.8 Å resolution, Acta Crystallogr. Sect. D 61 (2005) 1373–1377.
[13] J. Abramson, T. Shimamura, S. Iwata, Structures of bacterial heme–copper oxidases: diversity of prokaryotic electron transport carriers, in: D. Zannoni (Ed.), Respiration in Archaea and Bacteria, vol. 1, Kluwer Academic Publishers, Dordrecht, 2004, pp. 115–128.
[14] D. Keilin, E.F. Hartree, Cytochrome and cytochrome oxidase, Proc. R. Soc. London B 127 (1939) 167–191.
[15] T. Tsukihara, H. Aoyama, E. Yamashita, T. Tomizaki, H. Yamaguchi, K. Shinzawa-Itoh, R. Nakashima, R. Yaono, S. Yoshikawa, Structures of metal sites of oxidized bovine heart cytochrome *c* oxidase at 2.8 Å, Science 269 (1995) 1069–1074.
[16] T. Tsukihara, H. Aoyama, E. Yamashita, T. Tomizaki, H. Yamaguchi, K. Shinzawa-Itoh, R. Nakashima, R. Yaono, S. Yoshikawa, The whole structure of the 13-subunit oxidized cytochrome *c* oxidase at 2.8 Å, Science 272 (1996) 11361144.
[17] A. Kannt, U. Pfitzner, M. Ruitenberg, P. Hellwig, B. Ludwig, W. Mäntele, K. Fendler, H. Michel, Mutation of Arg-54 strongly influences heme composition and rate and directionality of electron transfer in *Paracoccus denitrificans* cytochrome *c*oxidase, J. Biol. Chem. 274 (1999) 37974–37981.
[18] H. Yoshikawa, K. Muramoto, K. Shinzawa-Itoh, M. Mochizuki, Structural studies on bovine heart cytochrome *c* oxidase, Biochim. Biophys. Acta (2012), doi: 10.1016/j.bbabio.2011.12.012.
[19] J. Zhuang, A.R. Reddi, Z. Wang, B. Khodaverdian, E.L. Hegg, B.R. Gibney, Evaluating the roles of the heme a side chains in cytochrome C oxidase using designed heme proteins, Biochemistry 45 (2006) 12530–12538.
[20] B.R. Gibney, Y. Isogai, F. Rabanal, K.S. Reddy, A.M. Grosset, C.C. Moser, P.L. Dutton, Self-assembly of heme A and heme B in a designed four-helix bundle: implications for a cytochrome *c* oxidase maquette, Biochemistry 39 (2000) 11041–11049.
[21] J. Abramson, S. Riistama, G. Larsson, A. Jasaitis, M. Svensson-Ek, L. Laakkonen, A. Puustinen, S. Iwata, M. Wikström, The structure of the ubiquinol oxidase from *Escherichia coli* and its ubiquinone binding site, Nat. Struct. Biol. 7 (2000) 910–917.
[22] T. Soulimane, G. Buse, G.P. Bourenkov, H.D. Bartunik, R. Huber, M.E. Than, Structure and mechanism of the aberrant *ba*(3)-cytochrome c oxidase from *Thermus thermophilus*, EMBO J. 19 (2000) 1766–1776.
[23] S. Buschmann, E. Warkentin, H. Xie, J.D. Langer, U. Ermler, H. Michel, The structure of cbb_3 cytochrome oxidase provides insights into proton pumping, Science 329 (2010) 327–330.
[24] M. Contreras-Zentella, G. Mendoza, J. HMembrillo-Hernández, J.E. Escamilla, A novel double heme substitution produces a functional bo_3 variant of the quinol oxidase aa_3 of *Bacillus cereus*, J. Biol. Chem. 278 (2003) 31473–31478.
[25] N. Sone, Y. Fujiwara, Haem O can replace haem A in the active site of cytochrome *c* oxidase from thermophilic bacterium PS3, FEBS Lett. 288 (1991) 154–158.
[26] G.A. Peschek, D. Alge, S. Fromwald, B. Mayer, Transient accumulation of heme O (cytochrome *o*) in the cytoplasmic membrane of semi-anaerobic *ANacystis nidulans*, J. Biol. Chem. 270 (1995) 27937–27941.
[27] T. Schröter, C. Winterstein, B. Ludwig, O.-M.H. Richter, Expression of the *Escherichia coli cyo* operon in *Paracoccus denitrificans* results in a fully active quinol oxidase of unexpected heme composition, FEBS Lett. 432 (1998) 109–112.
[28] T. Mogi, K. Saiki, Y. Anraku, Biosynthesis and functional role of haem O and haem A, Mol. Microbiol. 14 (1994) 391–398.
[29] P. Sinclair, D.C. White, J. Barett, The conversion of protoheme to heme *a* in *Staphylococcus aureus*, Biochim. Biophys. Acta 143 (1967) 427–428.
[30] M. Hansson, C. von Wachenfeldt, Heme B (protoheme IX) is a precursor of heme A and heme D in *Bacillus subtilis*, FEMS Microbiol. Lett. 107 (1993) 121–125.
[31] H. Taber, Isolation and properties of cytochrome *a* deficient mutants of *Bacillus subtilis*, J. Gen. Microbiol. 81 (1974) 435–444.
[32] J.P. Mueller, H.W. Taber, Isolation and sequence of *ctaA*, a gene required for cytochrome aa_3 biosynthesis and sporulation in *Bacillus subtilis*, J. Bacteriol. 171 (1989) 4967–4978.
[33] B. Svensson, M. Lubben, L. Hederstedt, *Bacillus subtilis* CtaA and CtaB function in haem A biosynthesis, Mol. Microbiol. 10 (1993) 193–201.
[34] K. Saiki, T. Mogi, Y. Anraku, Heme O biosynthesis in *Escherichia coli:* the *cyoE* gene in the cytochrome *bo* operon encodes a protoheme IX farnesyltransferase, Biochem. Biophys. Res. Commun. 189 (1992) 1491–1497.

[35] M. Throne-Holst, L. Hederstedt, The *Bacillus subtilis ctaB* paralogue, *yjdK*, can complement the heme A synthesis deficiency of a CtaB-deficient mutant, FEMS Microbiol. Lett. 183 (2000) 247–251.
[36] B. Svensson, L. Hederstedt, *Bacillus subtilis* CtaA is a heme-containing membrane protein involved in heme A biosynthesis, J. Bacteriol. 176 (1994) 6663–6671.
[37] T. Mogi, Probing structure of heme A synthase from *Bacillus subtilis* by site-directed mutagenesis, J. Biochem. 145 (2009) 625–633.
[38] M. Throne-Holst, Synthesis of hemes found in heme-copper oxidases of *Bacillus subtilis*, Department of Microbiology, vol. PhD thesis, Lund University, Lund, 2001.
[39] A. Lewin, L. Hederstedt, Compact archaeal variant of heme A synthase, FEBS Lett. 580 (2006) 5351–5356.
[40] B. Svensson, K.K. Andersson, L. Hederstedt, Low-spin heme A in the heme A biosynthetic protein CtaA from *Bacillus subtilis*, Eur. J. Biochem. 238 (1996) 287–295.
[41] K.R. Brown, B.M. Allan, P. Do, E.L. Hegg, Identification of novel hemes generated by heme A synthase: evidence for two successive monooxygenase reactions, Biochemistry 41 (2002) 10906–10913.
[42] G. Zoppellaro, K.L. Bren, A.A. Ensign, E. Harbitz, R. Kaur, H.-P. Hersleth, U. Ryde, L. Hederstedt, K.K. Andersson, Studies of ferric heme proteins with highly anisotropic/highly axial low spin ($S = 1/2$) electron paramagnetic resonance signals with bis-histidine and histidine–methionine axial iron coordination, Biopolymers 91 (2009) 1064–1082.
[43] A. Tzagoloff, C.C. Dieckman, *PET* genes of *Saccharomyces cerevisiae*, Microbiol. Rev. 54 (1990) 211–225.
[44] M.H. Barros, C.G. Carlson, D.M. Glerum, A. Tzagoloff, Involvement of mitochondrial ferredoxin and Cox15p in hydroxylation of heme O, FEBS Lett. 492 (2001) 133–138.
[45] M.H. Barros, F.G. Nobrega, A. Tzagoloff, Mitochondrial ferredoxin is required for heme A synthesis in *Saccharomyces cerevisiae*, J. Biol. Chem. 277 (2002) 9997–10002.
[46] C. Buchensky, P. Almirón, B. Suarez Mantilla, A.M. Silber, J.A. Cricco, The *Trypanosoma cruzi* TcCox10 and TcCox15 catalyze the formation of heme A in the yeast *Saccharomyces cerevisiae*, FEMS Microbiol. Lett. 312 (2010) 133–141.
[47] H. Antonicka, A. Mattman, C.G. Carlson, D.M. Glerum, K.C. Hoffbuhr, S.C. Leary, N.G. Kennaway, E.A. Shoubridge, Mutations in COX15 produce a defect in the mitochondrial heme biosynthetic pathway, causing early-onset fatal hypertrophic cardiomyopathy, Am. J. Hum. Genet. 72 (2003) 101–114.
[48] A. Punter, D.M. Glerum, Defects in assembly of cytochrome oxidase: roles in mitochondrial disease, Top. Curr. Genet. 8 (2004) 123–148.
[49] E. Fernández-Vizarra, V. Tiranti, M. Zeviani, Assembly of the oxidative phosphorylation system in humans: what we have learned by studying its defects, Biochim. Biophys. Acta 1793 (2009) 200–211.
[50] M.H. Barros, A. Tzagoloff, Regulation of the heme A biosynthetic pathway in *Saccharomyces cerevisiae*, FEBS Lett. 516 (2002) 119–123.
[51] Z. Wang, Y. Wang, E.L. Hegg, Regulation of the heme A biosynthetic pathway: differential regulation of hem A synthase and heme O synthase in *Saccharomyces cerevisiae*, J. Biol. Chem. 284 (2009) 839–847.
[52] L. Hederstedt, A. Lewin, M. Throne-Holst, Heme A synthase enzyme functions dissected by mutagenesis of *Bacillus subtilis* CtaA, J. Bacteriol. 187 (2005) 8361–8369.
[53] A. Lewin, L. Hederstedt, Promoted evolution of a shortened variant of heme A synthase in the membrane of *Bacillus subtilis*, FEBS Lett. 582 (2008) 1330–1334.
[54] A. Lewin, Heme A synthase from *Bacillus subtilis* and *Aeropyrum pernix*, Department of Cell and Organism Biology, vol. PhD, Lund University, Lund, 2008.
[55] W. Li, S. Schulman, R.J. Dutton, D. Boyd, J. Beckwith, T.A. Rapoport, Structure of a bacterial homologue of vitamin K epoxide reductase, Nature 463 (2010) 507–512.
[56] M.A. Schneegurt, S.I. Beale, Origin of the chlorophyll *b* formyl oxygen in *Chlorella vulgaris*, Biochemistry 31 (1992) 11677–11683.
[57] R.J. Porra, W. Schafer, E. Cmiel, I. Katheder, H. Scheer, Derivation of the formyl-group oxygen of chlorophyll *b* from molecular oxygen in greening leaves of a higher plant (Zea mays), FEBS Lett. 323 (1993) 31–34.
[58] U. Oster, R. Tanaka, A. Tanaka, W. Rudiger, Cloning and functional expression of the gene encoding the key enzyme for chlorophyll *b* biosynthesis (CAO) from *Arabidopsis thaliana*, Plant J. 21 (2000) 305–310.
[59] A. Tanaka, H. Ito, R. Tanaka, N.K. Tanaka, K. Yoshida, K. Okada, Chlorophyll *a* oxygenase (CAO) is involved in chlorophyll *b* formation from chlorophyll *a*, Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 12719–12723.
[60] A. Tomitani, K. Okada, H. Miyashita, H.C.P. Matthijs, T. Ohno, A. Tanaka, Chlorophyll *b* and phycobilins in the common ancestor of cyanobacteria and chloroplasts, Nature 400 (1999) 159–162.
[61] L.L. Eggink, R. LoBrutto, D.C. Brune, J. Brusslan, A. Yamasato, A. Tanaka, J.K. Hoober, Synthesis of chlorophyll *b*: localization of chlorophyllide *a* oxygenase and discovery of a stable radical in the catalytic subunit, BMC Plant Biol. 4 (2004) 5.
[62] J. Sakamoto, A. Hayakawa, T. Uehara, S. Noguchi, N. Sone, Cloning of *Bacillus stearothermophilus ctaA* and heme A synthesis with the CtaA protein produced in *Escherichia coli*, Biosci. Biotechnol. Biochem. 63 (1999) 96–103.
[63] A. Hannappel, F.A. Bundschuh, B. Ludwig, Characterisation of heme binding properties of *Paracoccus denitrificans* Surf1 proteins, FEBS J. 278 (2011) 1769–1778.
[64] K.R. Brown, B.M. Brown, E. Hoagland, C.L. Mayne, E.L. Hegg, Heme A synthase does not incorporate molecular oxygen into the formyl group of heme A, Biochemistry 43 (2004) 8616–8624.
[65] H. Loferer, M. Bott, H. Hennecke, *Bradyrhizobium japonicum* TlpA, a novel membrane-anchored thioredoxin-like protein involved in the biogenesis of cytochrome aa_3 and development of symbiosis, EMBO J. 12 (1993) 3373–3383.
[66] B.M. Brown, Z. Wang, K.R. Brown, J.A. Cricco, E.L. Hegg, Heme O synthase and heme A synthase from *Bacillus subtilis* and *Rhodobacter sphaeroides* interact in *Escherichia coli*, Biochemistry 43 (2004) 13541–13548.
[67] D. Smith, J. Gray, L. Mitchell, W.E. Antholine, J.P. Hosler, Assembly of cytochrome-*c* oxidase in the absence of assembly protein Surf1p leads to loss of the active site heme, J. Biol. Chem. 280 (2005) 17652–17656.
[68] F.A. Bundschuh, K. Hoffmeier, B. Ludwig, Two variants of the assembly factor Surf1 target specific terminal oxidases in *Paracoccus denitrificans*, Biochim. Biophys. Acta 1777 (2008) 1336–1343.
[69] L. Hiser, J.P. Hosler, Heme A is not essential for assembly of the subunits of cytochrome *c* oxidase of *Rhodobacter sphaeroides*, J. Biol. Chem. 276 (2001) 45403–45407.
[70] I.C. Soto, F. Fontanesi, J.S. Liu, A. Barrientos, Biogenesis and assembly of eukaryotic cytochrome *c* oxidase catalytic core, Biochim. Biophys. Acta (2012), doi: 10.1016/j.bbabio.2011.09.005.