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Heme O Synthase and Heme A Synthase from *Bacillus subtilis* and *Rhodobacter sphaeroides* Interact in *Escherichia coli*

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Abstract

Cytochrome *c* oxidase requires multiple heme and copper cofactors to catalyze the reduction of molecular oxygen to water. Although significant progress has been made in understanding the transport and incorporation of the copper ions, considerably less is known about the trafficking and insertion of the heme cofactors. Heme O synthase (HOS) and heme A synthase (HAS) from *Rhodobacter sphaeroides* (Cox10 and Cox15, respectively) and *Bacillus subtilis* (CtaB and CtaA, respectively) have been cloned and expressed in *Escherichia coli*. Our results demonstrate that HOS copurifies with HAS and that HAS copurifies with HOS, indicating that HOS and HAS interact and may form a physiologically relevant complex *in vivo*. Consistent with this hypothesis, the presence of HAS alters the total level of farnesylated hemes, providing further evidence that HOS and HAS interact. Our current working model is that HOS and HAS form a complex and that heme O is transferred directly from HOS to HAS. Because of the strong sequence similarity and evolutionary relationship between *R. sphaeroides* and mitochondria, our data suggest that this complex may form in eukaryotes as well.

Cytochrome *c* oxidase (CcO),¹ the terminal oxidase in all plants, animals, aerobic yeasts, and many bacteria, is a multicomponent complex localized in the inner membrane of prokaryotes and in the inner mitochondrial membrane of eukaryotes (2–4). CcO catalyzes the reduction of O₂ to H₂O concomitant with the translocation of up to four protons (eight charge equivalents) across the membrane, thus generating a considerable proton gradient. The membrane potential generated by CcO is ultimately utilized by ATP synthase and is responsible for nearly 50% of the ATP generated during aerobic metabolism (5–10). The malfunction or misassembly of CcO causes respiratory failure and results in the inability to grow on nonfermentable carbon sources in lower organisms and severe, usually lethal, health defects in higher organisms (11–13). Despite the importance of CcO in aerobic metabolism, the assembly of this intricate enzyme complex is still poorly understood.

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SUPPORTING INFORMATION AVAILABLE

Experimental details for the cloning of HOS and HAS from *R. sphaeroides* (Cox10 and Cox15, respectively) and the preparation of associated expression plasmids are provided on page S2. In addition, Table S1 is a summary of all plasmids, and Table S2 is a summary of all bacterial strains used in this paper. Figure S1 shows the alignment between Cox15 (the gene product from ORF RSP3831) in *R. sphaeroides* and Cox15p in *S. cerevisiae* as determined by ClustalW. Proof that Cox15 from *R. sphaeroides* produces heme A when expressed in *E. coli* and was afforded by UV-vis and mass spectrometries of the purified heme products (Figures S2 and S3, respectively). Figure S4 demonstrates that the same amount of HOS is present in cells expressing only HOS as in cells expressing both HOS and HAS. Figure S5 shows the copurification of HOS and HAS using *n*-dodecyl β -D-maltoside as the solubilizing detergent. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Eukaryotic CcO is composed of up to 13 subunits (14–16). The three largest are the “catalytic” subunits and are mitochondrially encoded, while the remainder are nuclearly encoded. In addition to these 13 subunits, eukaryotic CcO also contains a magnesium, calcium, and zinc ion as well as a number of redox active metal cofactors. The dinuclear Cu_A site in subunit II is responsible for accepting electrons from cytochrome *c* and transferring them to a heme *a* molecule buried in subunit I. This heme then transfers the electrons to a unique heme *a*₃-Cu_B heterobimetallic center (also located in subunit I), where O₂ is ultimately reduced to H₂O. Assembling the multiple cofactors and subunits in eukaryotic CcO requires over 30 accessory proteins (17), the exact function of which is still unknown in many cases.

The complexity of eukaryotic CcO assembly has led to the extensive use of prokaryotic CcOs as models for the eukaryotic system. *Bacillus subtilis* and *Rhodobacter sphaeroides* are two organisms that have been successfully exploited to increase our understanding of eukaryotic CcO assembly and function (18–20). Although CcO from these organisms contains only four subunits, both possess three catalytic orthologues (subunits I, II, and III) complete with the redox active metal cofactors, and X-ray crystallography reveals that the overall structure of subunits I, II, and III in *R. sphaeroides* (21) is essentially identical to the structure found in bovine CcO (22). Furthermore, there is very high sequence similarity between the catalytic subunits of mitochondrial CcO and those found in *R. sphaeroides*.

One of the more intriguing aspects of CcO assembly concerns the transport and insertion of the redox active metal cofactors. Because of the toxic effects of free copper (23), cells have developed specialized processes for the insertion of the Cu_A and Cu_B sites (24). At least three gene products are responsible for inserting copper into CcO. Substantial evidence suggests that Cox17p acts as a copper chaperone within the mitochondrial intermembrane space, directly transferring copper ions to both Sco1p and Cox11p (25). In turn, Sco1p is thought to insert the copper into the dinuclear Cu_A site in subunit II (24), while Cox11p is known to be essential for inserting copper into the Cu_B site of subunit I (26). It has also been suggested that two other gene products, Cox19p (27) and Cox23p (28), may be important for the transport and/or insertion of copper into CcO, but the precise function of these proteins remains to be elucidated.

While considerable progress has been made toward identifying the proteins responsible for the transport and insertion of the copper cofactors, relatively little is known about the trafficking of the heme A cofactors. Heme A is a derivative of heme B, and the two integral-membrane enzymes required for the conversion of heme B to heme A were initially identified in bacteria and later in eukaryotes. The first step, catalyzed by heme O synthase (HOS), adds a farnesyl tail to the vinyl group on pyrrole ring A (29–31), while the second reaction, catalyzed by heme A synthase (HAS), oxidizes the methyl substituent on pyrrole ring D to an aldehyde (1, 31–34). While considerable information is available concerning the enzymatic mechanisms of both HOS and HAS (1, 32, 34–36), less is known about how hemes are transported to or inserted into CcO. Similar to copper, free hemes are toxic to cells (3), and it is therefore likely that cells possess a controlled mechanism for heme transport. While proteins required for the transport, insertion, and covalent attachment of the heme to cytochrome *c* have been identified in *Escherichia coli* (37), no such heme chaperone

has been identified in the biosynthesis of C_αO in any organism. One intriguing possibility is that no chaperone exists and that HOS and HAS form a complex inside of the membrane to effect the biosynthesis and transport of heme A.

In this paper, we unequivocally identify ORF RSP3831 (38) as the gene responsible for HAS activity in *R. sphaeroides* and we suggest that it be named *cox15* [the eukaryotic HAS gene (1)] based on sequence similarity and function. Furthermore, by heterologously expressing HOS and HAS from *B. subtilis* (CtaB and CtaA) and *R. sphaeroides* (Cox10 and Cox15), we have determined that HOS and HAS form a complex *in vivo*, and we propose that this complex is physiologically relevant for the transfer of heme O. These results provide an important first step in understanding the regulation and trafficking of the heme cofactors during the biosynthesis of C_αO.

EXPERIMENTAL PROCEDURES

Materials and General Procedures.

Restriction enzymes were obtained from New England Biolabs (Beverly, MA) and Invitrogen (Carlsbad, CA). The *Taq* polymerase and pGEM vector were purchased from Promega (Madison, WI). The pET vectors were acquired from Novagen (Milwaukee, WI). All other chemicals were purchased from Sigma, VWR, or Fisher Scientific and used without further purification. The preparation of plasmids containing wild-type and epitope-tagged *ctaB* and *ctaA* as well as heme analysis via HPLC have all been previously described (34, 35). The pCF102 [containing *cox10* from *R. sphaeroides* 2.4.1 (38, 39)] and pUC18 plasmids were a kind gift from Professor Jonathon Hosler (University of Mississippi Medical Center). ORF RSP3831 was cloned from genomic *R. sphaeroides* 2.4.1 DNA (38) with the assistance of the Hosler lab as described in the Supporting Information. Sequencing was performed on an ABI 377 sequencer at the DNA Sequencing Core Facility of the University of Utah.

Transformation and Expression.

Expression plasmids (see Table S1 of the Supporting Information) containing the genes encoding HOS and HAS from *B. subtilis* and/or *R. sphaeroides* were transformed either singly or doubly into *E. coli* BL21(DE3) pLysS. (To insert two expression plasmids, cells were transformed with both plasmids simultaneously.) Transformants for heme analysis were grown with vigorous shaking at 37 °C in 250 mL of LB media in 2-L baffle flasks containing appropriate antibiotics (25 mg/L chloramphenicol, 50 mg/L ampicillin, and/or 35 mg/L kanamycin) until an OD₆₀₀ of approximately 0.6 was obtained. IPTG was added to a final concentration of 75 mg/L to induce expression. Cells were harvested after an induction for 2 h, washed with 0.25 M sucrose, and stored at -80 °C. Proteins were visualized via standard western analysis. In short, proteins were separated via 12% SDS-PAGE, transferred to a nitrocellulose membrane (Biorad), and probed using a mouse antibody against the T7 epitope tag (Novagen) or a mouse antibody against the 6× His epitope tag (Novagen) as the primary probes. The secondary antibody was a goat antimouse-alkaline phosphatase conjugated antibody (Southern Biotechnology Associates, Birmingham, AL). Nitrocellulose membranes were then incubated with ECF (Amersham) chemoluminescent substrate, and

images were recorded on a Typhoon 9400 Variable Model Imager using ImageQuant 5.2 (Amersham).

Protein Purification of Epitope-Tagged Proteins.

Cells were grown in 2 L of selective media in 6-L flasks, induced for 1 h, harvested, washed, and stored at -80°C . To purify T7 epitope-tagged proteins, cells were resuspended (15 mL/L cell culture) in 25 mM Tris buffer (pH 8.0), 20% sucrose, 50 mM NaCl, Triton X-100 (0.1% per 2 mg/mL total protein), and 125 units of Benzonase (Novagen) and then disrupted via sonication. Total cellular protein was determined by the Bradford assay using BSA as the standard. After the lysate was incubated on ice for 1 h, the cells were centrifuged at 37000g for 20 min. The supernatant was incubated with T7 antibody-conjugated agarose resin (Novagen) on a rocker at 20°C for 1 h. (In cases when the proteins were expressed in different cells, the detergent-solubilized supernatant from each cell pellet was combined and incubated on a rocker at 20°C for 1 h prior to incubation with the agarose beads.) The samples were then transferred to a chromatographic column to collect the resin. The resin was washed with at least 40 column volumes of wash buffer (25 mM Tris buffer at pH 8.0, 20% sucrose, 50 mM NaCl, and 0.1% Triton X-100), and the bound proteins were eluted with 1-mL aliquots of 0.1 M citric acid (pH 2.2) and neutralized with 150 μL of 2 M Tris buffer (pH 10.4). These samples were then analyzed via SDS-PAGE and western blot analysis as described above.

The purification of 6 \times His epitope-tagged proteins was accomplished in an identical manner except that 25 mM imidazole replaced the 50 mM NaCl in the resuspension and wash buffers. In addition, the agarose was derivatized with Ni-NTA, and the elution buffer was a mixture of 25 mM Tris buffer at pH 8.0, 20% sucrose, 500 mM imidazole, and 0.1% Triton X-100.

RESULTS

The Gene Product of ORF RSP3831 (cox15) from R. sphaeroides Is HAS. *R. sphaeroides* cells contain genes encoding a cytochrome aa_3 complex and a putative cytochrome caa_3 complex, yet the gene responsible for HAS activity has not been identified despite the fact that the three catalytic subunits of CcO from *R. sphaeroides* exhibit considerable sequence similarity with eukaryotic CcO. Sequence alignment reveals that there is 30% identity and 47% similarity between Cox15p (eukaryotic HAS) from *S. cerevisiae* and the expected gene product of ORF RSP3831 from *R. sphaeroides* (38) (see the Supporting Information for sequence alignment). On the basis of this sequence similarity, we hypothesized that ORF RSP3831 is the gene responsible for HAS activity in *R. sphaeroides*. To test this theory, ORF RSP3831 was cloned and expressed in *E. coli* BL21 cells using the pET family of vectors. The heme content of all transformed cells was analyzed via HPLC monitoring at 400 nm (Figure 1A). *E. coli* cells do not contain an *a*-type terminal oxidase (utilizing instead a cytochrome bo_3 complex and a cytochrome bd complex) and thus do not produce heme A. As expected, in the absence of cloned HOS or HAS, *E. coli* cells produce predominantly heme B and a slight amount of heme O (top trace). When *cox10* from *R. sphaeroides* is expressed, large quantities of heme O are produced (middle trace), verifying

that *cox10* (RSP1827) (38) is the gene responsible for HOS activity in *R. sphaeroides* and that at least a portion of Cox10 is properly folded and active. Significantly, when ORF RSP3831 is coexpressed with *cox10* (bottom trace), a small peak preceding heme O on the chromatogram is visible. The optical spectrum of the heme in this peak is identical to the spectrum of purified heme A obtained from bovine heart, and mass spectral analysis (852 amu) confirms that this unknown compound is heme A (see the Supporting Information for optical spectra and mass spectral data). Because *E. coli* does not naturally produce or utilize heme A, our data prove that ORF RSP3831 encodes for HAS.

On the basis of its sequence similarity to Cox15p (eukaryotic HAS from *S. cerevisiae*) and its proven catalytic function, we propose that ORF RSP3831 in *R. sphaeroides* should be known as *cox15* and that the gene product be named Cox15. We will use this new nomenclature for the remainder of the paper.

The Presence of HAS Affects Accumulation of Farnesylated Hemes.

An interesting feature of Figure 1A is that the presence of Cox15 reduces the total amount of farnesylated hemes present in cells expressing Cox10. This result is consistent with previous observations made in our lab (34), when we expressed HOS and HAS from *B. subtilis* (CtaB and CtaA, respectively) in *E. coli* (Figure 1B).² One possible explanation is that the activity of HOS is attenuated by a direct protein-protein interaction with HAS.

To pursue the idea that the presence of HAS alters the activity of HOS, the experiments were repeated such that each cell growth was performed under identical conditions (as described in the Experimental Procedures) to increase reproducibility for statistical analysis. It should be noted that cellular heme ratios are sensitive to growth flask conditions, and therefore these experiments are not directly comparable to our previous data that were obtained under slightly different conditions (34, 35), although the general results remain unchanged. The histograms in Figure 2 (averages of three experiments) summarize the total amount of farnesylated hemes produced by Cox10 (Figure 2A) or CtaB (Figure 2B) when expressed with different HAS proteins. The first column in each graph shows the amount of heme O observed (as a percentage of total extractable heme) in the presence of HOS only. Significantly, the total amount of farnesylated heme products observed decreases by approximately 30% when Cox10 is coexpressed with Cox15 or when CtaB is coexpressed with CtaA (column 3). When HOS and HAS from different organisms are coexpressed (column 4), the same effect is observed, although it is accentuated slightly in the case of the CtaA-Cox10 combination and less pronounced in the Cox15-CtaB combination. Together, these results confirm that expressing HOS in the presence of HAS decreases the amount of farnesylated hemes observed.

Three possible scenarios could explain the fact that the total amount of farnesylated hemes generated is depressed in the presence of HAS. The first possibility is that the presence of a second pET vector could alter the levels of the plasmid containing HOS. This scenario is

²The activity of Cox15 is considerably lower than that of CtaA. This is consistent with the high similarity observed between Cox15 from *R. sphaeroides* and Cox15p from *S. cerevisiae* and the fact that Cox15p from *S. cerevisiae* is apparently inactive when expressed in *E. coli* (1). Although the reason for the inactivity is unknown, one possibility is that, in *E. coli*, Cox15p cannot efficiently scavenge the electrons required for O₂ activation.

discounted, however, by the fact that coexpression of a second empty pET vector (the same vector used to express HAS) has no effect on the amount of farnesylated hemes accumulated in cells containing either Cox10 or CtaB (column 2). A second possibility is that the expression of the second integral-membrane protein alters the protein levels of HOS. A comparison of protein levels by western analysis, however, reveals constant levels of Cox10 and CtaB in both the presence and absence of Cox15 and CtaA, respectively (Figure S4 in the Supporting Information). Thus, this scenario is not supported by the results. The third and most intriguing explanation is that HOS and HAS interact and form a physiologically relevant complex.

HAS and HOS Copurify.

If HOS and HAS interact, then it is possible that this protein-protein interaction can be observed via standard biochemical techniques depending on the conditions used and the strength of the interaction. Unfortunately, both HOS and HAS are integral-membrane proteins with numerous predicted transmembrane helices. Furthermore, they are expressed at extremely low levels in the native organism, and we have not been able to obtain significant overexpression of these enzymes in *E. coli*, thus eliminating many biochemical techniques. Furthermore, CtaA and CtaB exhibit nearly identical electrophoretic mobility when analyzed via SDS-PAGE with an apparent molecular weight of approximately 33 000 Da. To overcome these challenges, HOS and HAS were modified with epitope sequences for purification and western analysis. We found that detergent concentration, salt concentration, and temperature were all very important to maintain these interactions *in vitro*. Scheme 1 summarizes the various interactions that we have observed.

CtaA (HAS) containing a C-terminal 6× His epitope tag was coexpressed in *E. coli* BL21 cells with CtaB (HOS) fused to an N-terminal T7 epitope tag. After solubilization in 0.3–0.5% Triton X-100, CtaA was purified on a Ni-NTA column and analyzed via western blot analysis. As can be seen in Figure 3A, when the blots are probed with an antibody against the 6× His epitope tag, the majority of CtaA elutes in fraction 2. Significantly, when these same fractions were analyzed with an antibody against the T7 epitope tag, we see that CtaB-nT7 also elutes from the Ni-NTA column in fraction 2 (Figure 3B), demonstrating that CtaB-nT7 copurifies with CtaA-cHis₆ from a Ni-NTA column. The reverse experiment was also performed. When CtaB was purified on T7 antibody-conjugated resin, it eluted in fractions 1–3, with the majority of the protein eluting in fraction 2 (Figure 3C). Again, when these same fractions were probed using a 6× His antibody, CtaA-cHis₆ was also observed (Figure 3D).

To obtain more stringent evidence of complex formation between CtaA and CtaB, similar experiments were performed in which CtaA and CtaB were expressed in different cells. In this experiment, CtaA-cHis₆ and CtaB-nT7 were expressed singly in *E. coli* BL21 cells and two separate detergent-solubilized cell lysates were prepared, one containing only CtaA-cHis₆ and a second containing only CtaB-nT7. The two cell lysates were mixed and incubated together for 1 h prior to protein purification using Ni-NTA resin. As previously observed, CtaB-nT7 coelutes with CtaA-cHis₆ from a Ni-NTA column, confirming that CtaB and CtaA interact (Figure 4).

Three separate controls support the hypothesis that CtaA and CtaB form a complex. First, Figure 5 confirms that CtaB-nT7 does not bind to a Ni-NTA column in the absence of CtaA-cHis₆, and that CtaA-cHis₆ does not bind to a T7 antibody-conjugated resin in the absence of CtaB-nT7. Second, CtaB-nT7 copurifies with CtaA-cHis₆ on a Ni-NTA column when the detergent is switched from Triton X-100 to *n*-dodecyl β -D-maltoside (see the Supporting Information), demonstrating that the two enzymes are not copurifying simply because they are trapped in the same micelle.³ Finally, to verify that CtaA and CtaB are not copurifying because of nonspecific interactions resulting from the coexpression of two integral-membrane proteins, CtaA-cHis₆ was coexpressed with an integral-membrane protein that it would not be expected to interact, Cox15 (HAS) from *R. sphaeroides*. No copurification is observed on a Ni-NTA column between the HAS proteins from these different organisms (Figure 6). Together, these results verify that the copurification of CtaA and CtaB is not an artifact because of nonspecific aggregation but rather the result of specific protein-protein interactions.

To ascertain whether the interaction between HOS and HAS is a general phenomenon or specific to CtaB and CtaA from *B. subtilis*, Cox10 (HOS) and Cox15 (HAS) from *R. sphaeroides* were epitope-tagged and coexpressed in *E. coli*. As shown in Figure 7A, Cox10-nHis₆ (approximately 34 000 Da) from detergent-solubilized fractions could be purified on a Ni-NTA column, eluting in fractions 1 and 2. When these same fractions were probed for the presence of Cox15-cT7 (approximately 43 000 Da) using T7 antibodies, Cox15 was found to elute in fractions 1 and 2 as well (Figure 7B). Control experiments verify that Cox15-cT7 does not bind to the Ni-NTA column in the absence of Cox10-nHis₆ (Figure 5), confirming the presence of protein-protein interactions between Cox10 and Cox15. When the reverse experiment was performed, however, Cox10-nHis₆ did *not* copurify with Cox15-cT7 from a column containing T7 antibody-conjugated resin (data not shown). This result highlights that, while HOS and HAS appear to form a stable and physiologically relevant complex *in vivo*, this interaction is tenuous in detergent-solubilized cell lysates. Interestingly, when Cox15-cHis₆ from *R. sphaeroides* is coexpressed with CtaB-nT7 from *B. subtilis*, HOS and HAS from different organisms still copurify (parts C and D of Figure 7). Thus, the interaction between HOS and HAS appears to be a general phenomenon.

DISCUSSION

CcO contains four redox active cofactors, a dinuclear Cu_A site, a Cu_B site, and two heme *a* molecules (24). Because free copper and free hemes are known to be toxic to cells (3, 23), nature utilizes complex mechanisms to safely transport these redox active cofactors. Considerable progress has been made in the last 5 years toward understanding the transport and insertion of copper into CcO, and we now know that Cox11p, Cox17p, Sco1p, and potentially Cox19p and Cox23p are all involved in either the transport of copper or its insertion into CcO in yeast (24, 26–28). During this same time, however, our understanding of the transport and insertion of the heme cofactors into CcO has remained quite limited.

³Both the micelle number and the critical micelle concentration (cmc) are smaller for *n*-dodecyl β -D-maltoside (78–92, 0.0087%) than for Triton X-100 (75–165, 0.015%).

The two heme *a* cofactors are located in the middle of subunit I and are buried within either the inner membrane of bacteria or the inner mitochondrial membrane of eukaryotes. On the basis of the available crystal structures of CcO (21, 22, 40), it appears likely that heme A is inserted very early in the assembly process. This hypothesis is supported by studies of CcO subassembly complexes formed in *R. sphaeroides* (19) and in patients carrying mutations in *COX10* and *COX15* (41–43). Relatively little data, however, exists concerning either the regulation or transport of heme O or heme A in any organism (44).

How might heme be transported through the HOS/HAS pathway? There are three limiting possibilities. The first possibility is that HOS freely releases heme O and that heme O diffuses through the membrane to HAS. Given the toxic nature of free hemes (3), however, this scenario does not seem plausible, and it is perhaps more likely that cells carefully control the transport of this redox active cofactor. The second possibility is that a “heme chaperone” exists to transport heme O from HOS to HAS. This would be consistent with the chaperones required to transport and insert the copper cofactors into CcO (24), and heme chaperones are known to be required in *E. coli* for the transport and covalent attachment of the heme cofactor during the biosynthesis cytochrome *c* (37). However, to date no heme O or heme A chaperone has been identified in any organism. The third limiting possibility is that HOS and HAS form a physiologically relevant complex and that heme O is transferred directly from HOS to HAS.

To address these and related questions, we have cloned the genes encoding HOS and HAS in *B. subtilis* (*ctaB* and *ctaA*, respectively) and expressed them in *E. coli*. This heterologous expression yielded the expected heme products, indicating that these proteins are properly translated, folded, and inserted into the bacterial membrane. Interestingly, our results demonstrate that the presence of CtaA (HAS) decreases the accumulation of farnesylated hemes produced by CtaB (HOS) by approximately 30%. This reduction occurs although protein levels of CtaB are not altered by the presence of CtaA as demonstrated by western blot analysis (Figure S4 in the Supporting Information). These data are consistent with the hypothesis that the activity of CtaB is attenuated by an interaction with CtaA. How can these results be explained? One hypothesis is that in the absence of its physiological partner, HOS freely releases heme O into the membrane. In the presence of HAS, however, HOS and HAS form a complex and heme O is transferred directly to HAS. In this complex, either the oxidation of heme O to heme A or the release of heme A is the rate-limiting step, and the activity of HOS is therefore decreased.

If this hypothesis is correct, then CtaA and CtaB might be expected to copurify. When CtaA-cHis₆ was purified on a Ni-NTA column, CtaB eluted from the column with CtaA. Likewise, when CtaB-nT7 was purified using a T7 antibody-conjugated resin, CtaA copurified with CtaB. These results are consistent regardless of whether CtaA and CtaB are coexpressed in the same cell or if they are expressed in separate cells with the detergent-solubilized lysates incubated prior to column purification. Furthermore, when CtaA is coexpressed with another integral-membrane protein with which it is not expected to interact, the two proteins do not copurify. The copurification of CtaB and CtaA provides additional evidence to support the hypothesis that these enzymes form a complex *in vivo*.

To ascertain if the interaction observed between CtaA and CtaB is specific to *B. subtilis* or a more general phenomenon, HOS (Cox10) and HAS (Cox15) from *R. sphaeroides* were expressed in *E. coli*. *R. sphaeroides* was chosen because, as a member of the R subdivision of the proteobacteria from which mitochondria are thought to have arisen, its CcO has very high sequence similarity with eukaryotic CcO. The 30% reduction of farnesylated hemes isolated from cells coexpressing Cox10 and Cox15 relative to cells expressing only Cox10 is consistent with the results obtained from the analogous experiments utilizing CtaB and CtaA. In addition, Cox15-cT7 copurifies with Cox10-nHis₆ on a Ni-NTA column, confirming that these two proteins interact in *E. coli*. Because of the high sequence similarity between HOS and HAS in *R. sphaeroides* and HOS and HAS in *S. cerevisiae*, it is possible that eukaryotic HOS and HAS form a physiologically relevant complex as well.

The possible existence of a heme A biosynthetic complex in *S. cerevisiae* has been explored by Barros et al. through the use of genomic deletions (44). Their data also suggest that HAS affects the activity of HOS, because *cox15* mutants accumulated considerably less heme O than other *cox* mutants. Because they were unable to observe a protein-protein interaction between Cox10p and Cox15p via analytical ultracentrifugation, however, they concluded that these two proteins were probably not part of a complex and instead proposed that the activity of Cox10p and Cox15p is regulated by some unidentified feedback mechanism during CcO assembly. Given the tenuous nature of the interaction of HOS and HAS from *B. subtilis* and *R. sphaeroides* *in vitro*, it is perhaps not surprising that Barros et al. did not observe an interaction between Cox10p and Cox15p, which leaves open the possibility of a physiologically relevant complex in yeast as well. Whether or not this putative interaction requires additional proteins or cofactors is unknown at this time.

In summary, our results demonstrate that HOS and HAS copurify and that the presence of HAS decreases the activity of HOS. We interpret these results as evidence that HOS and HAS form a “heme A biosynthetic complex” *in vivo*, and we suggest that heme O is transferred directly from HOS to HAS. Interestingly, this HOS-HAS complex could regulate the flux of hemes, and may be part of a larger protein complex required for the assembly of CcO. In this scenario, the heme flux is controlled by either the formation or release of heme A. Our data that prokaryotic HOS and HAS interact is not inconsistent with the hypothesis of Barros and Tzagoloff that in *S. cerevisiae* these enzymes are regulated by other external factors (44), nor is their hypothesis inconsistent with our proposal that HOS and HAS form a physiologically relevant complex in bacteria.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

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amu	atomic mass unit
BSA	bovine serum albumin
CcO	cytochrome <i>c</i> oxidase
ESI	electron spray ionization
HAS	heme A synthase
HOS	heme O synthase
HPLC	high-performance liquid chromatography
IPTG	isopropyl- β -D-1-thiogalactopyranoside
MS	mass spectrometry
NTA	nitriloacetic acid
PAGE	polyacrylamide gel electrophoresis
SDS	sodium dodecyl sulfate
TFA	trifluoroacetic acid

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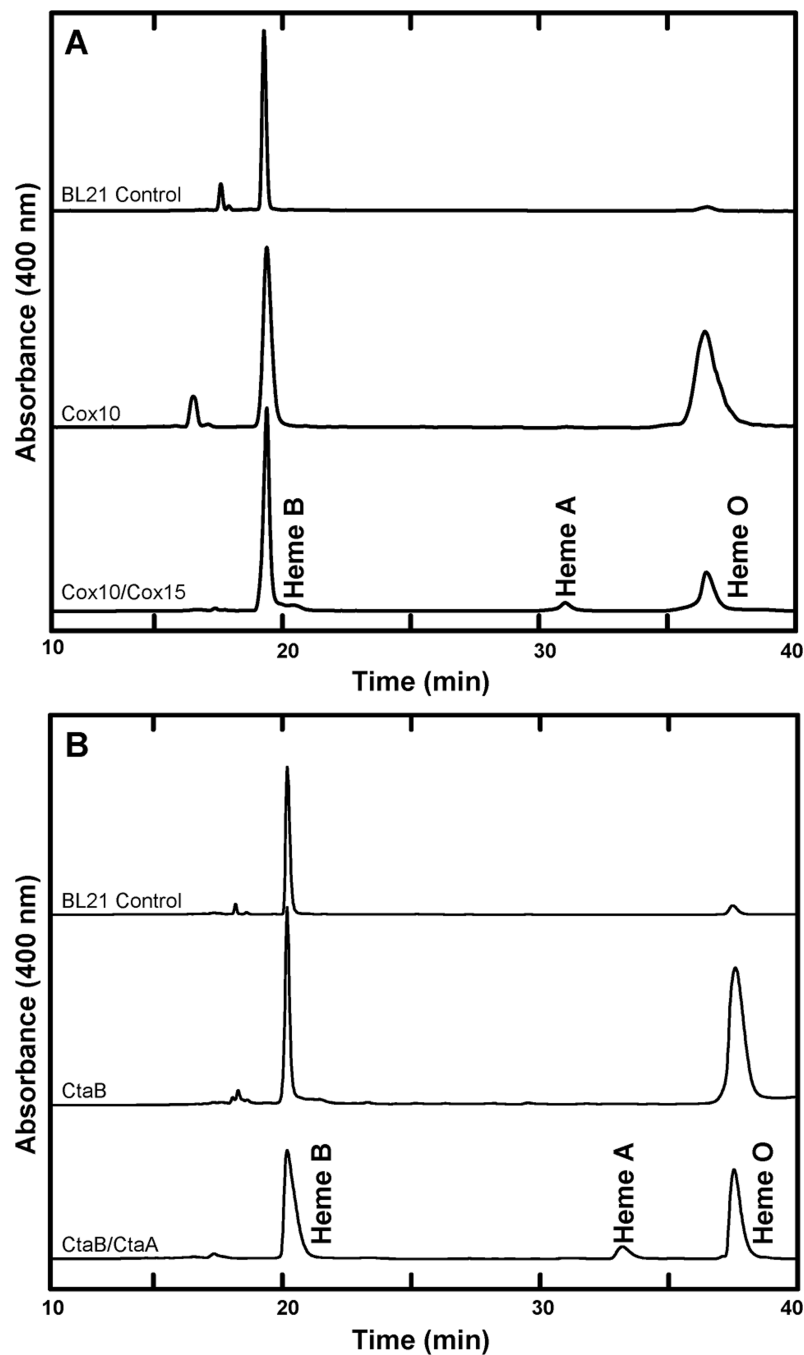


FIGURE 1: HPLC chromatogram showing heme profile of BL21 *E. coli* cells. The chromatograms represent control BL21 *E. coli* cells (top), cells expressing HOS only (middle), and cells expressing both HOS and HAS (bottom). Total heme and heme O retention times have been normalized within each set. (A) *E. coli* cells expressing HOS (pET29a(+)-Cox10) and HAS (pET21a(+)-Cox15) from *R. sphaeroides*. Note that Cox10 and Cox15 are the gene products from ORFs RSP1827 and RSP3831, respectively. (B) *E. coli* cells expressing HOS (pET29a(+)-CtaB) and HAS (pET3d-CtaA) from *B. subtilis*.

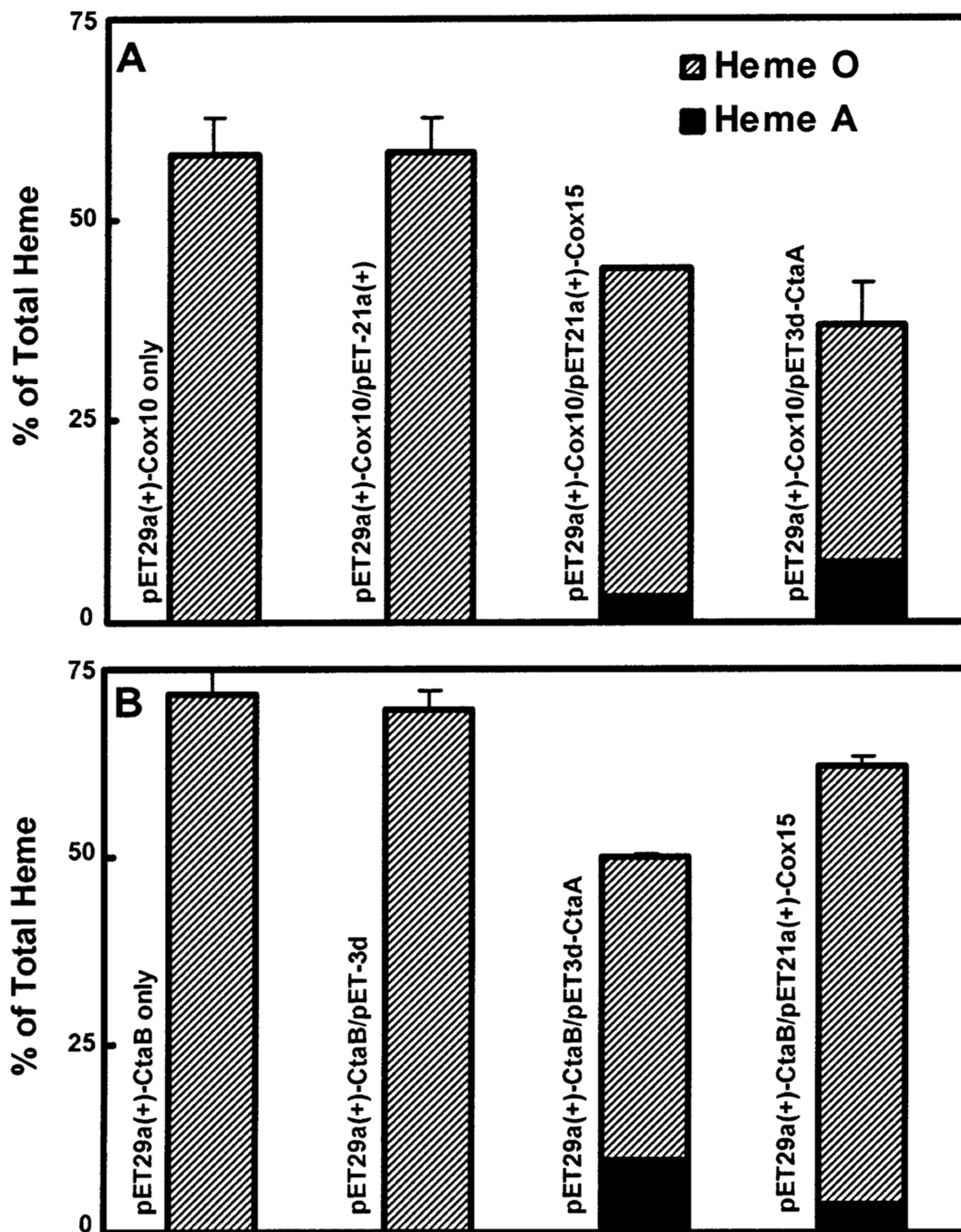
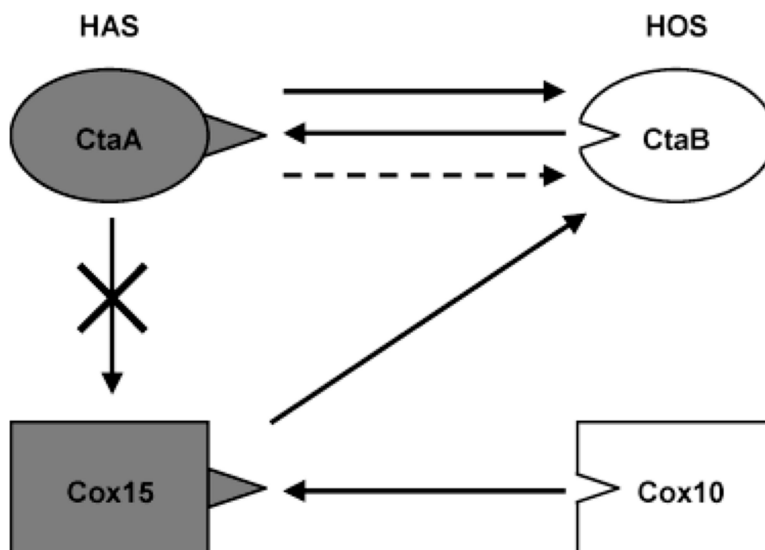
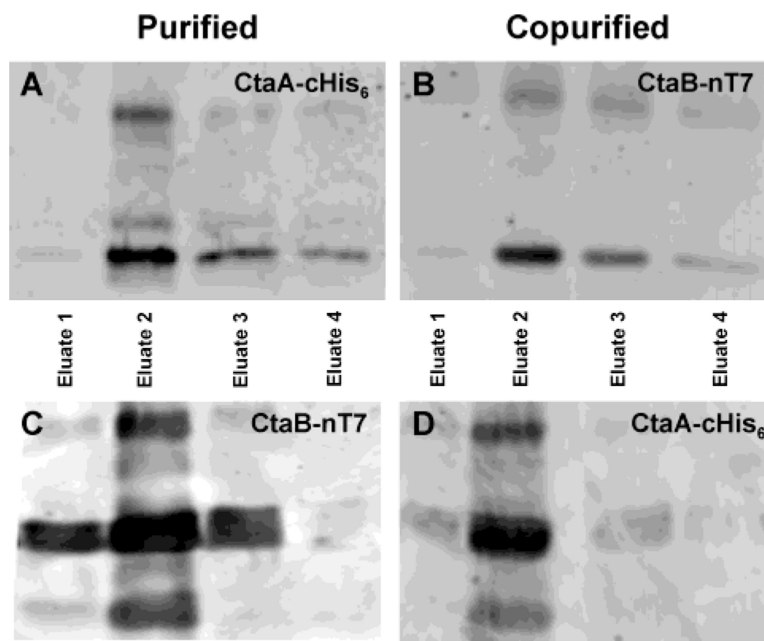


FIGURE 2: Quantification of farnesylated hemes produced in *E. coli* by HOS from (A) *R. sphaeroides* (Cox10, pET29a(+)-Cox10) and (B) *B. subtilis* (CtaB, pET29a(+)-CtaB) in the presence and absence of an empty pET vector, HAS from the same species, and HAS from the other species. The data are displayed as a percentage of the total extractable heme present in the cell. Each column represents the average of three separate experiments, and the error bars correspond to the standard deviation.

**Scheme 1:**

Summary of Protein-Protein Interactions Probed between HOS and HAS^a

^a CtaA and CtaB are the designations for HAS and HOS, respectively, in *B. subtilis* and many other prokaryotes, while Cox15 and Cox10 denote HAS and HOS in *R. sphaeroides*. The solid lines represent protein-protein interactions observed between HOS and HAS when the two enzymes were expressed in the same cell, while the dashed line indicates interactions observed between proteins grown in separate cells. The arrows point from the protein being purified to the protein that was copurified as a result of protein-protein interactions. No interaction was observed between CtaA and Cox15.

**FIGURE 3:**

Western blot analysis of proteins purified from *E. coli* cells coexpressing CtaA (pET9d-CtaA-cHis₆) and CtaB (pET3aCtaB-nT7). (A) CtaA-cHis₆ (bottom band) purified on a Ni-NTA column. (B) Same fractions as gel A probed with a T7 antibody demonstrate that CtaB-nT7 (bottom band) copurifies with CtaA-cHis₆. (C) CtaB-nT7 (middle band) purified on a T7 antibody-derivatized column. (D) Same fractions as gel C probed with a 6× His antibody demonstrate that CtaA-cHis₆ (middle band) copurifies with CtaB-nT7.

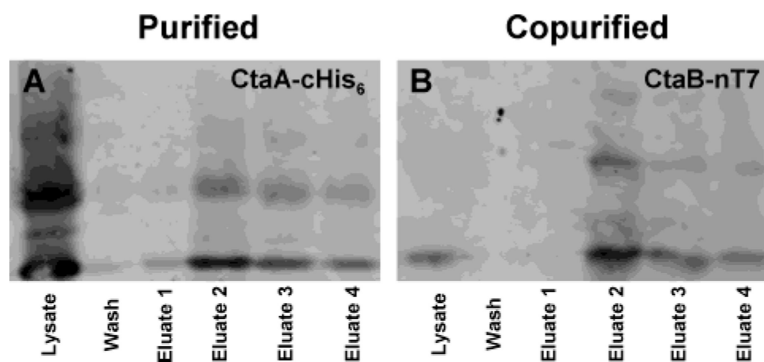


FIGURE 4: Western blot analysis of proteins purified from *E. coli*. CtaA (pET9d-CtaA-cHis₆) and CtaB (pET3a-CtaB-nT7) were expressed in different cells, and the detergent-solubilized lysates were incubated prior to protein purification. (A) CtaA-cHis₆ (bottom band) purified on a Ni-NTA column. (B) Same fractions probed with a T7 antibody demonstrate that CtaB-nT7 (bottom band) copurifies with CtaA-cHis₆.

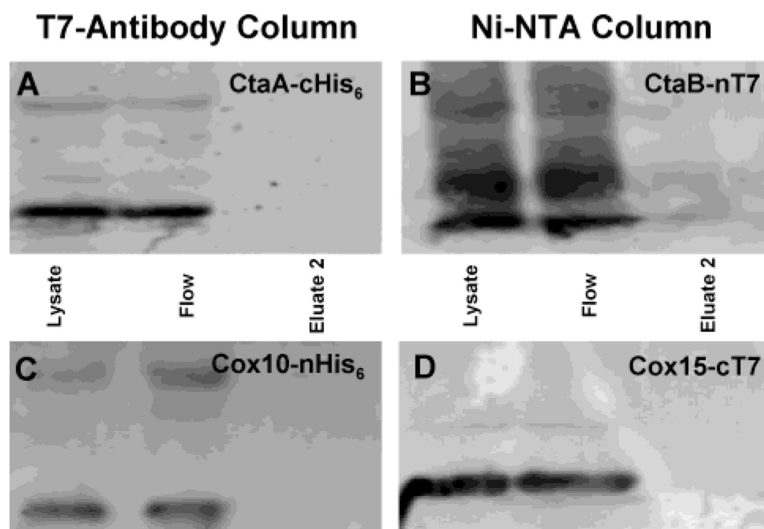
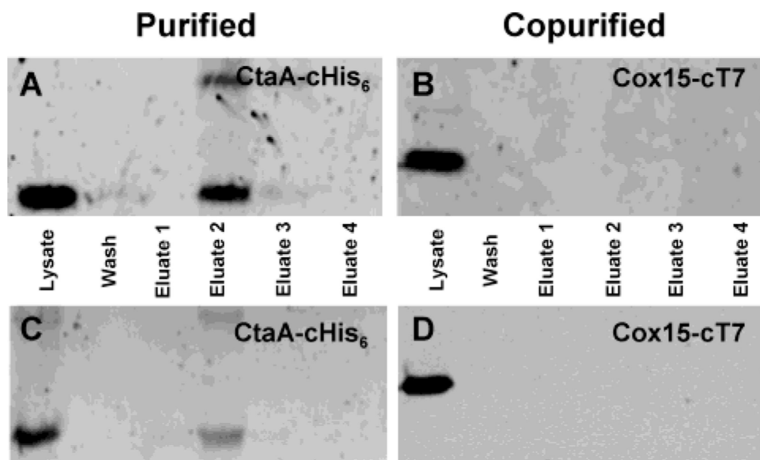


FIGURE 5: Western blot analysis of control reactions to demonstrate that copurification of HOS and HAS is not an artifact of adventitious binding to the column. (A) CtaA-cHis₆ (pET9d-CtaA-cHis₆) does not bind to a T7 antibody column in the absence of CtaB-nT7 (pET3a-CtaB-nT7). (B) CtaB-nT7 (middle band) does not bind to a Ni-NTA column in the absence of CtaA-cHis₆. (C) Cox10-nHis₆ (pET9d-Cox10-nHis₆) does not bind to a T7 antibody column in the absence of Cox15-cT7 (pET21a(+)-Cox15-cT7). (D) Cox15-cT7 does not bind to a Ni-NTA column in the absence of Cox10-nHis₆.

**FIGURE 6:**

Western blot analysis of control reactions showing that HAS from *R. sphaeroides* (Cox15, pET21a(+)-Cox15-cT7) does not interact with HAS from *B. subtilis* (CtaA, pET9d-CtaA-cHis₆) either when coexpressed in *E. coli* (gels A and B) or when expressed in separate cells with the solubilized cell lysates incubated for 1 h prior to purification (gels C and D). (A) CtaA-cHis₆ (bottom band) purified on a Ni-NTA column. (B) Same fractions from gel A probed with a T7 antibody. (C) CtaA-cHis₆ (bottom band) purified on a Ni-NTA column. (D) Same fractions from gel C probed with a T7 antibody.

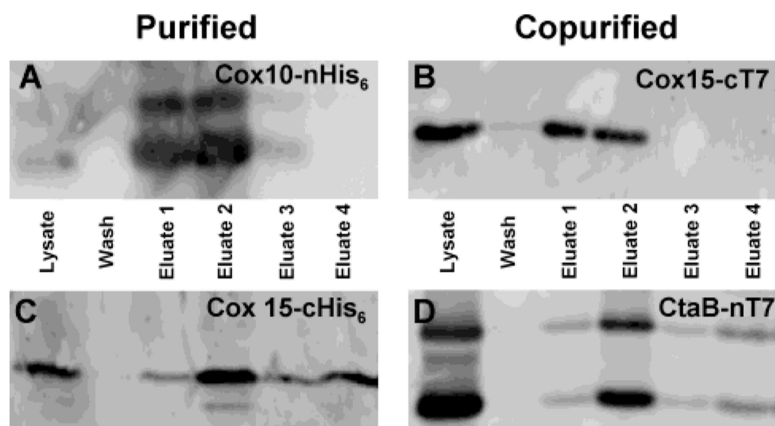


FIGURE 7: Western blot analysis of proteins purified from *E. coli* cells coexpressing HOS and HAS. (A) Cox10-nHis₆ (pET9d-Cox10-nHis₆, bottom band) purified on a Ni-NTA column. (B) Same fractions as gel A probed with a T7 antibody demonstrate that Cox15-cT7 (pET21a(+)-Cox15-cT7) copurifies with Cox10-nHis₆. (C) Cox15-cHis₆ (pET21a(+)-Cox15-cHis₆, top band) purified on a Ni-NTA column. (D) Same fractions as in gel C probed with a T7 antibody demonstrate that CtaB-nT7 (pET3a-CtaB-nT7, bottom band) copurifies with Cox15-cHis₆.