The Biosynthesis of Heme O and Heme A Is Not Regulated by Copper†

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ABSTRACT: Heme A is an obligatory cofactor in all eukaryotic and many prokaryotic cytochrome *c* oxidase (C*c*O) enzymes. Despite its obvious importance to C*c*O and the electron transport pathway, essentially nothing is known concerning the regulation of heme A. Because C*c*O is the only natural target for heme A and copper is also required for C*c*O activity, it was postulated that copper might regulate heme A homeostasis. Work reported previously demonstrated that there is often a strong connection between copper and iron homeostasis in general, and circumstantial evidence pointed to a possible specific link between copper and heme A. To address this question, we conducted experiments to determine rigorously whether copper plays a role in heme A homeostasis. The two enzymes responsible for the conversion of heme B to heme A, heme O synthase (HOS) and heme A synthase (HAS), were separately genomically epitope-tagged in *Saccharomyces cerevisiae*, and their expression under various copper conditions was quantified by Western blot analysis. These results demonstrated that the sum of transcription, translation, and stability of HOS and HAS were independent of copper. Additionally, the effects of intracellular copper concentrations on the activity of HOS and HAS from *Bacillus subtilis* (expressed in *Escherichia coli*) and *Rhodobacter sphaeroides* were examined by analysis of cellular heme extracts. No trends with respect to intracellular copper were observed. In combination, our results demonstrate that intracellular copper levels do not affect the transcription, translation, stability, or activity of either HOS or HAS.

Cytochrome *c* oxidase (C*c*O)[1] is the terminal oxidase in all plants, animals, aerobic yeasts, and some bacteria (*1*−*5*). Catalyzing the reduction of molecular oxygen to water concomitant with the pumping of protons across the membrane (*6*−*11*), C*c*O generates a proton gradient that is responsible for approximately 50% of the ATP formed during aerobic metabolism in mammals. To accomplish this task, C*c*O requires a number of redox-active metal cofactors, including two hemes and three copper ions (*2*−*4*). A dinuclear Cu_A site accepts electrons from cytochrome *c* molecules and transfers them sequentially to the first heme, which in turn transfers the electrons to the active site, a novel heme−copper heterobimetallic center. Interestingly, the two hemes contained within all eukaryotic C*c*Os (and most bacterial C*c*Os) are not the typical B-type hemes (protohemes); rather, they are derivatized hemes known as heme A. Heme A is synthesized from heme B via two enzymatic reactions. The first reaction, catalyzed by heme O synthase (HOS), results in the conversion of the vinyl group on pyrrole ring A into a 17-hydroxyethylfarnesyl moiety in generating heme O (Scheme 1) (*12*−*15*). In the second transformation, heme A synthase (HAS) oxidizes the methyl group on pyrrole ring D of heme O into an aldehyde, thus generating heme A (*15*−*19*). The proper trafficking and assembly of these redox-active metal cofactors in C*c*O is a complex process that requires a number of different gene products working in concert (*20*, *21*).

Copper is obviously an obligatory cofactor in C*c*O, as well as in nearly 20 other mammalian enzymes (*22*). The copper needs of the cell, however, must be carefully balanced against its potential toxicity (*22*−*24*), and therefore, intricate homeostatic mechanisms for regulating the cellular concentration and localization of copper ions within the cell exist. In eukaryotic systems, copper uptake is mediated by the high- and low-affinity copper transporters Ctr1 and Ctr3, respectively (*25*, *26*). Once inside the cell, copper ions are trafficked and compartmentalized by a variety of "chaperones" (*27*, *28*). Although it is not clear at this time how copper is transported into the mitochondrion, a number of proteins within the mitochondrion are known to be important in inserting copper into C*c*O (*20*). For instance, experiments conducted using yeast proteins suggest that Cox17 is a copper chaperone within the intermembrane space, transferring copper to both Sco1 and Cox11 (*29*). Likewise, Cox11 is known to be essential for inserting copper into the Cu_B site of C*c*O in *Rhodobacter sphaeroides* (*30*) and is presumed to have the same function in eukaryotes, while Sco1 is proposed to insert copper into the dinuclear Cu_A site in both prokaryotes and eukaryotes (*31*, *32*). In addition, two other

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[1] Abbreviations: BCS, bathocuproinedisulfonic acid; C*c*O, cytochrome *c* oxidase; EDTA, ethylenediaminetetraacetic acid; HAS, heme A synthase; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HOS, heme O synthase; HPLC, high-performance liquid chromatography; ICP, inductively coupled plasma; IPTG, isopropyl β-D-1-thiogalactopyranoside; LB, Luria-Bertini medium; OD_{600}, optical density at 600 nm; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PEG, polyethylene glycol; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; TAP, tandem affinity purification epitope; YPD, yeast extract−peptone−dextrose medium; YPEG, yeast extract−peptone−ethanol−glycerol medium.

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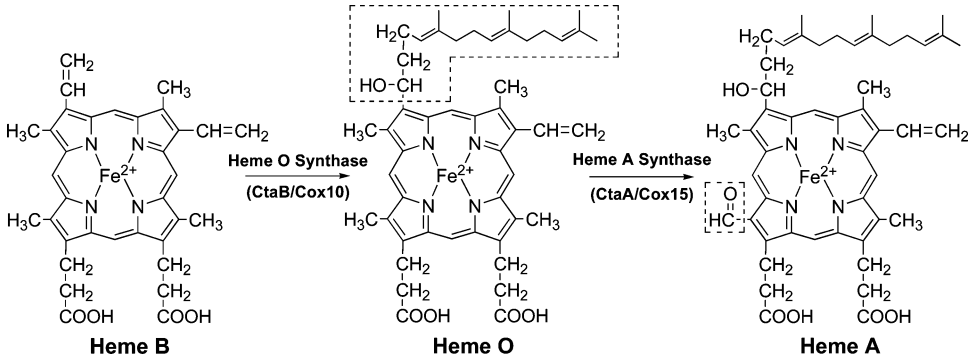
Scheme 1: Transformation of Heme B to Heme A Catalyzed by the Enzymes Heme O Synthase and Heme A Synthase



gene products in yeast, Cox19 (*33*) and Cox23 (*34*), have recently been implicated in the trafficking or insertion of copper into C*c*O, although their precise function remains unknown. Interestingly, a null *cox23* strain can be rescued by overexpression of Cox17 and high copper, suggesting that Cox23 may act upstream of Cox17 (*34*).

Like copper, free heme is toxic to cells (*5*, *35*, *36*), and the synthesis, transport, and ultimate insertion of heme A into C*c*O must be carefully controlled. Surprisingly, despite the importance of heme A to C*c*O and the electron transport pathway, much less is known concerning the homeostasis of heme A. The genes encoding HOS and HAS activity[2] were identified first in bacteria (*19*, *37*, *38*) and later in eukaryotes (*12*, *16*), and we and others have obtained mechanistic information concerning these enzymes (*14*, *15*, *17*, *39*−*41*). In addition, we recently demonstrated that HOS and HAS from both *R. sphaeroides* and *Bacillus subtilis* copurify when expressed in *Escherichia coli*, and that the activity of HOS appears to be reduced in the presence of HAS (*42*). From these data, we proposed that HOS and HAS form a physiologically relevant complex in vivo, and that heme O may be transferred directly between HOS and HAS. In this scenario, either the conversion of heme O to heme A or the release of the heme A product would be the rate-determining step, thus explaining the reduced activity of HOS in the presence of HAS. Exactly how heme A is ultimately transported to C*c*O is not known. Very recent evidence from *R. sphaeroides* suggests that Surf1 facilitates the insertion of heme A into the heme a_3−Cu_B active site, although it has no influence on the heme *a* center (*43*).

Another intriguing aspect of heme A biosynthesis concerns regulating the flux of heme through the HOS−HAS pathway. How is it that cells regulate this pathway such that there is adequate, but not excessive, heme A available? In *B. subtilis*, transcription of HOS and HAS is altered by the *resD* and *strC* gene products (*44*−*46*), although it is not clear how HOS and HAS are regulated once they are synthesized. In eukaryotes, very little is known about the regulation of the heme A biosynthetic pathway. Obvious factors that one might predict would affect the levels of proteins involved in C*c*O biosynthesis, such as O_2 concentrations (*47*), fermentable versus nonfermentable carbon sources (*48*, *49*), respiratory competency of the cell (*50*), and rich versus minimal media (*51*), do not affect the transcription of either HOS or HAS in *Saccharomyces cerevisiae*, and different levels of control must therefore be present. It has recently been proposed that HOS is positively regulated by a product from HAS, and that HAS itself may be regulated by an assembly intermediate of C*c*O (*52*).

It has also been suggested that copper may provide an additional layer of regulation in the heme A biosynthetic pathway. A regulatory role for copper in heme A biosynthesis would make intuitive sense because both copper and heme A are obligatory cofactors in C*c*O. Why should one synthesize heme A, which is only utilized in C*c*O, if there is not sufficient copper present? In addition, copper and iron (and heme) homeostasis are known to be intertwined in a variety of ways. For example, iron deficiency in yeast leads to the induction of Atx1 and Ccc2, two copper transporters responsible for delivering copper to Fet3, a multicopper oxidase involved in iron uptake (*53*). It is also known that the deletion of the ALA synthase gene in *S. cerevisiae* (*HEM1*), the first gene in the heme biosynthetic pathway, disrupts transcription of both iron and copper uptake genes (*54*). Finally, recent work with Long-Evans Cinnamon rats (an animal model of the copper toxicity disorder Wilson's disease) demonstrated that these animals had abnormal heme metabolism (*55*), providing further evidence for a link between copper and heme levels.

There is also circumstantial evidence that copper might directly affect heme A levels. Mitochondria isolated from copper-deficient swine cells were shown to have substantially lower levels of heme A (*56*), while copper-deficient yeast cells were shown to lack heme A completely (although heme B biosynthesis appeared to be normal) (*57*). Although this could partially be explained by heme A being degraded in the absence of C*c*O (eukaryotic C*c*O is often degraded in the absence of its copper cofactors), certain C*c*O mutants in *S. cerevisiae* completely lacking Cox1 (the C*c*O subunit containing the heme A cofactors) still contain some heme A (*52*). The presence of heme A in cells lacking Cox1 would seem to call this simplistic explanation into question, potentially providing further support for a role for copper. Interestingly, the copper-deficient yeast cells accumulated a heme-like product, proposed to be a heme A precursor, that disappeared when copper was returned to the growth media and heme A production commenced (*57*). More recently, it was shown in *S. cerevisiae* that deficiencies in many of the proteins involved in mitochondrial copper transport and/or

[2] In *B. subtilis* and many other bacteria, the genes encoding HOS and HAS are denoted *ctaB* and *ctaA*, respectively. In *R. sphaeroides* as well as in all eukaryotes, the genes encoding HOS and HAS are denoted *cox10* and *cox15*, respectively. In this paper, we will use the common names HOS and HAS to avoid confusion.

homeostasis lead to heme A deficiencies. For example, Δ*sco1*, Δ*cox11*, and Δ*cox17* mutants contain substantially lower heme A/heme B ratios than wild-type cells (*39*, *58*). Thus, there is substantial circumstantial evidence suggesting that copper levels might be important in the regulation of heme A biosynthesis.

In this paper, we test the hypothesis that the heme A biosynthetic pathway is partially regulated by copper. Utilizing HOS and HAS genes from *B. subtilis*, *R. sphaeroides*, and *S. cerevisiae*, we demonstrate that copper does not affect protein expression of either HOS or HAS, nor does copper affect the activity or stability of these enzymes. Thus, despite the earlier results described above, heme A biosynthesis must be regulated by some other mechanism.

EXPERIMENTAL PROCEDURES

Materials and General Procedures. Water was purified through a Millipore system and used throughout. To prepare metal-free water, 50 g of Chelex-100 (Bio-Rad) resin were added to 1 L of water and the mixture was gently stirred overnight. The Chelex was removed by sterile filtration of the water into acid-washed flasks. Metal-free flasks were prepared by soaking them in an acid bath overnight. The outside surfaces of the flasks were thoroughly rinsed using deionized water, while the inside surfaces of the flasks were rinsed with Chelex-treated water. The acid-washed flasks were autoclaved before they were used. Ampicillin and kanamycin were prepared using sterile Chelex-treated water and used at final concentrations of 50 and 30 mg/L, respectively. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was prepared with sterile Chelex-treated water and used at a final concentration of 75 mg/L to induce protein production in *E. coli*.

Cell Strains. The HOS and HAS genes (*cox10* and *cox15*, respectively) from *S. cerevisiae* were genomically modified at the C-terminus via homologous recombination to express the TAP tag and the c-Myc epitope tag, respectively, and were used to test the effect of copper on the expression and stability of HOS and HAS. The TAP tag containing the *TRP1* marker was amplified from pBS1479 (*59*) using the forward PCR primer 5′-GATTGGATATATCCTGGTGAAGCAA-AGCGACCACAGGAACGATTTTCCATGGAAAAGA-GAAG-3′ and the reverse primer 5′-GTTTAAATAT-TTATTTACAAGATTAATGACTGCCCTTTAAGC-GTTGTCTCTACGACTCACTATAGGG-3′ for addition to the HOS gene. The c-Myc epitope tag containing the *TRP1* marker was cloned from pFA6a-13Myc-TRP1 (*60*) obtained from D. Stillman (University of Utah) using the forward PCR primer 5′-AATTTTAAGTGAAGCGTCGAAGTTAGC-CTCGAAACCATTACGGATCCCCGGGTTAATTAA-3′ and 5′-GCGAGTATACTGTCAATTCTCATAAGAATACC-TTTATCCAGAATTCGAGCTCGTTTAAAC-3′ as the reverse primer. Homologous recombination was performed using PEG 4000 (Avocado Research Chemicals) and lithium acetate (Alfa Aesar) as previously described (*61*). Proper insertion of the epitope tag was confirmed with diagnostic PCR and DNA sequencing performed on an ABI 377 sequencer at the University of Utah's DNA Sequencing Core Facility.

To test the effect of copper on enzyme activity, HOS and HAS from *B. subtilis* were recombinantly expressed in *E. coli* strain BL21(DE3) cells containing plasmids pLysS, pET3a-CtaB, and pET9a-CtaA (*40*). The effect of copper on the total heme A biosynthetic pathway was studied by heme analysis of wild-type *R. sphaeroides* strain 2.4.1 cells (*62*) obtained from J. Hosler (University of Mississippi Medical Center).

Preparation of Media. Bacterial medium was prepared by dissolving 25 g of granulated Luria-Bertini (LB) media powder (Fischer Scientific) in 1 L of water. Yeast−peptone (YP) medium was made by mixing 20 g of tryptone and 10 g of yeast extract (Becton Dickinson) into 900 mL of water. Chelex-100 resin (20 g/L) was added to each solution, and the mixture was stirred gently for 2 h. The medium was filtered from the Chelex-100 resin into an acid-washed flask. The medium was autoclaved and allowed to cool. Either 100 mL of a 20% glucose solution in sterile Chelex-treated water (YPD) or 100 mL of 30% ethanol and 30% glycerol (YPEG) in sterile Chelex-treated water was added to the sterile Chelex-treated YP. Sistrom's medium (*63*) was prepared without modification except using Chelex-treated water.

High-purity metals were purchased from Alfa Aesar. A 5000-fold stock solution was prepared by dissolving $NiCl_2$ (41.6 mg), $MnCl_2$ (20.0 mg), $FeCl_2$ (9.9 mg), and $ZnCl_2$ (19.9 mg) in 10 mL of Chelex-treated water in an acid-washed flask. The metals were returned to the media by adding 200 μL of the stock mixture to 1 L of media. High-purity $CaCl_2$ (10 mg) and $MgCl_2$ (100 mg) were added directly to the media. An 8 mM stock of $CuSO_4$ was made by dissolving 17.9 mg in 10 mL of Chelex-treated water. Copper was returned to the media at various concentrations by adding different volumes from the $CuSO_4$ stock solution to final concentrations of 8, 2, 0.5, and 0.125 μM. No $CuSO_4$ was returned to the "No Copper" flask. In addition to the absence of $CuSO_4$, the "BCS" flask also contained the copper-specific chelator BCS (Sigma) at a final concentration of 100 μM.

Cellular Growth Conditions. Appropriate cell cultures were grown in No Copper conditions to stationary phase. The overnight culture was used to inoculate 50 mL cultures of Chelex-treated media (1/50) containing varying concentrations of copper. At an optical density of 0.6 at 600 nm (OD_{600}) measured on a Hewlett-Packard 8453 spectrophotometer, the *E. coli* cells were induced for 1 h with IPTG at a concentration of 75 mg/L. *S. cerevisae* strains were grown until the OD_{600} was between 1.3 and 1.5. *R. sphaeroides* were grown in either Sistrom's medium or LB medium until the OD_{600} was between 1.0 and 1.2. (*R. sphaeroides* cells grown in LB yielded considerably more heme for analysis, and therefore, most studies reported here used LB as the medium). The cells were collected in 50 mL centrifuge tubes that had been previously soaked in a 1 mM EDTA (Acros) solution to remove trace amounts of metals. The cell pellets were washed in a 0.25 M sucrose (Mallinckrodt) solution prepared from Chelex-treated water. Inductively coupled plasma (ICP) spectroscopy was performed to ascertain the metal content of the cells.

Metal Analysis via ICP. The cell pellet from 50 mL of culture was resuspended in 1 mL of a 0.25 M sucrose solution. The resuspended cells (150 μL) were digested in sealed Eppendorf tubes at 95 °C in 150 μL of metal-free 40% nitric acid (Optima) for 1 h. The samples were then diluted to 0.6 mL with double-distilled water for analysis on a PerkinElmer Optima 3100-XL inductively coupled

plasma-optical electronic spectrophotometer (ICP-OES). Commercially available mixed-metal standards were used to construct a standard curve. Blanks of nitric acid or sample buffer were also digested for comparison as controls.

Heme Extraction and Analysis. Cell pellets (50 mL culture from *E. coli* and *R. sphaeroides*) were resuspended in 0.5 mL of 30 mM Tris buffer (pH 8.0) containing 10 mM EDTA and 20% sucrose. The hemes from 200 μL of the cell resuspension were extracted with 600 μL of a 5% HCl/acetone mixture followed by sonication treatment. An additional 200 μL of Tris buffer was added to the extraction mixture before clarification by centrifugation. The supernatant was analyzed by reverse-phase HPLC to determine the total heme composition. Hemes were separated and analyzed on a Waters HPLC system equipped with a 600 Delta Pack pump and a model 996 photodiode array detector as described previously (*40*).

Mitochondrial Isolation. Mitochondria were isolated from *S. cerevisae* grown to an OD_{600} between 1.3 and 1.5. The cell pellet was resuspended in SHP buffer [0.6 M sorbitol, 30 mM HEPES (pH 7.4), and 1 mM phenylmethanesulfonyl fluoride (PMSF)] at a concentration of 2 mL/g of wet cell pellet. Approximately 500 μL of glass beads were added. The cell suspension was vortexed rapidly three times for 30 s. The broken cells were centrifuged for 5 min at 600*g* repeatedly until no pellet appeared. The supernatant was removed and centrifuged at 18000*g* for 10 min. The mitochondrial pellet was resuspended in fresh SHP buffer at 20 μL/g of original wet cell pellet.

Western Analysis. *S. cerevisae* mitochondrial proteins (5 μg) were analyzed by Western analysis. In short, proteins were separated via 12% SDS−PAGE, transferred to a nitrocellulose membrane (Bio-Rad), and probed using a mouse monoclonal antibody against the c-Myc epitope tag on Cox15 (Molecular Probes) or a rabbit monoclonal antibody against the TAP tag on Cox10 (Novagen) as the primary probes. Mitochondrial porin was probed with a mouse monoclonal porin antibody (Molecular Probes). The secondary antibody was a goat anti-mouse or goat anti-rabbit alkaline phosphatase-conjugated antibody (Southern Biotechnology Associates). Nitrocellulose membranes were then treated with ECF (Amersham) chemoluminescent substrate, and images were recorded on a Typhoon 9400 Variable Model Imager using ImageQuant 5.2 (Amersham).

Activity of CcO from S. cerevisiae and R. sphaeroides. Activity assays were performed using *S. cerevisiae* mitochondria or purified *R. sphaeroides* membrane extracts obtained by differential centrifugation. To obtain membrane fragments, a 50 mL culture of *R. sphaeroides* was centrifuged for 5 min at 7000*g* and resuspended in 50 mL of 50 mM K_2HPO_4 (pH 7.2) and 1 mM EDTA. The cells were passed three times through a French press (2000−2500 psi). The mixture was then centrifuged at 20000*g* for 20 min, and the supernatant was collected and centrifuged for an additional 90 min at 166000*g*. The membrane pellet was then resuspended in 0.5 mL of 50 mM K_2HPO_4 (pH 7.2) and 1 mM EDTA. The enzyme activity of C*c*O was determined by monitoring the oxidation of cytochrome *c* at 550 nm according to a literature procedure (*64*).

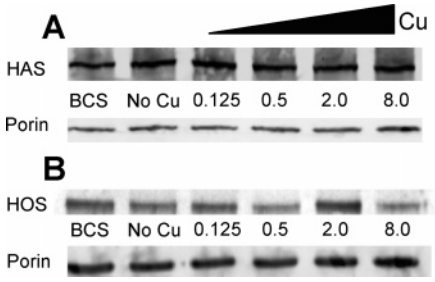


FIGURE 1: Western blot analysis of yeast mitochondria (5 μg of total protein per lane) grown in various medium copper concentrations with 2% glucose as the fermentable carbon source (YPD). HAS (A) and HOS (B) were probed by primary antibodies against the c-Myc and TAP epitopes, respectively. Porin was probed in each blot as a loading control. The numbers in each row represent the micromolar concentration of copper in the growth media, while "No Cu" represents the addition of no copper. "BCS" represents the addition of 100 μM copper chelator BCS to the media in addition to the absence of added copper.

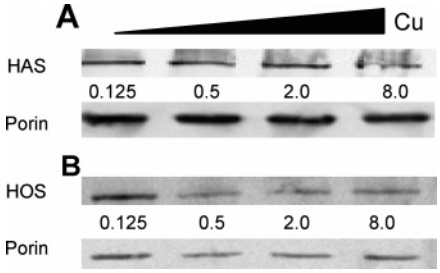


FIGURE 2: Western analysis of yeast mitochondria (5 μg of total protein) grown in various medium copper concentrations with 3% ethanol and 3% glycerol as the nonfermentable carbon sources (YPEG). HAS (A) and HOS (B) were probed by primary antibodies against the c-Myc and TAP epitopes, respectively. Porin was probed in each blot as a loading control. The numbers in each row represent the micromolar concentration of copper in the growth media. Note that *S. cerevisiae* cells do not grow in Chelex-treated YPEG in the absence of added copper.

RESULTS

Western Analysis of HAS and HOS from S. cerevisiae. To ascertain the effect of intracellular copper concentration on the protein levels of HOS and HAS, *S. cerevisiae* cells that contained either HOS (*cox10*) or HAS (*cox15*) genomically epitope-tagged were subjected to Western analysis. The cells were grown in YPD or YPEG medium with varying amounts of copper for approximately 40 h ($OD_{600} = 1.3-1.5$) before isolation of the mitochondria. The levels of HOS and HAS were determined by subjecting the mitochondrial proteins to SDS−PAGE and Western analysis, probing for either the TAP (HOS) or c-Myc (HAS) epitope tags. Mitochondrial porin was used as a loading control.

The results for these experiments are shown in Figures 1 and 2. The samples in the first lane of Figure 1 are from yeast cells that were grown in glucose with no copper added to the Chelex-treated media and in the presence of the copper chelator BCS, while the samples in the second lane contained mitochondria from cells that were also grown without added copper but in the absence of BCS. Subsequent lanes contained mitochondrial proteins obtained from cells grown in Chelex-treated media supplemented with increasing copper concentrations of 0.125, 0.5, 2.0, and 8.0 μM, respectively. These copper concentrations span a large range that is both well above and well below the value of 0.45 μM found in

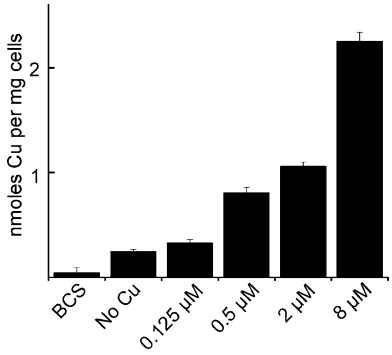


FIGURE 3: Estimated number of nanomoles of copper ions per milligram of *S. cerevisiae* cell pellet as determined by ICP analysis from a total of two trials. Cells were grown in media containing various amounts of copper. "No Cu" medium was Chelex-treated to remove the metals, while "BCS" medium was Chelex-treated and had the copper chelator BCS added. The error bars denote one standard deviation.

our YP medium as determined by ICP (data not shown). *S. cerevisiae* cells grown in media containing glucose as the carbon source have essentially identical levels of HOS and HAS regardless of the copper concentration of the media. Likewise, when the yeast cells were grown in ethanol and glycerol as the carbon sources (Figure 2), the levels of HOS and HAS were again independent of copper concentrations from 0.125 to 8.0 μM. As expected, yeast cells incubated in the absence of added copper are not able to grow on YPEG (ethanol and glycerol are nonfermentable carbon sources) because of the requirement of copper for respiration.

To verify that intracellular copper concentrations correlated with the copper concentrations of the growth media, metal analysis of whole cells was performed by ICP. These results (Figure 3) indicated that there is essentially no copper observed in yeast cells grown in glucose in the absence of added copper, and that the increase in growth medium copper concentration was mirrored by a corresponding increase in intracellular copper levels [see the Supporting Information for ICP results of iron and zinc (Figure S1)]. In addition, C*c*O activity was used as an indirect measurement of copper availability in the mitochondria. These results (Figure S4) are consistent with total intracellular copper levels.

Activity of HOS and HAS Expressed in E. coli. To ascertain whether copper directly interacts with either HOS or HAS to affect activity, HOS and HAS were cloned from *B. subtilis* and expressed in *E. coli*. HOS and HAS were coexpressed in *E. coli* on the IPTG-inducible plasmids pET3a and pET9a, respectively. One hour after induction, the cells were harvested, and the intracellular heme levels were analyzed by HPLC as described in Experimental Procedures. The resulting chromatograms (Figure 4A) reveal the presence of heme B, heme A, heme O, and two other hemes that coelute denoted I and II. Recently, we determined that the formation of heme I and heme II is dependent on HAS, and we identified these previously unknown hemes as the C8 alcohol and C8 carboxylate derivatives of heme O, respectively (*40*).

Our results reveal that copper has no direct effect on the activity of either HOS or HAS. As shown in Figure 4A, there is no effect on the relative amounts of heme I/II, heme A, or heme O observed when the cells were grown under varying copper conditions (note that retention times and peak areas have been normalized to heme B). Figure 4B summarizes these data along with the data from three additional and independent experiments. Because heme levels vary from growth set to growth set due to slight differences in induction times, etc., we report the average variability (or deviation from the arithmetic mean) of heme levels within individual growth sets. Each growth set consists of one cellular growth at each of the six copper concentrations from the same overnight culture and harvested on the same day. All values were calculated as a percentage of the total hemes. A variability of 1.0 indicates no difference in heme levels with varying copper conditions within a particular set. The columns represent the average of the variabilities of the hemes found in the four different growth sets, and the error bars denote the standard deviation. Once again, ICP data confirm that the intracellular copper content mirrors the copper concentration of the media, and that cells grown without added copper in the presence of BCS contain essentially no copper (Figure 5).

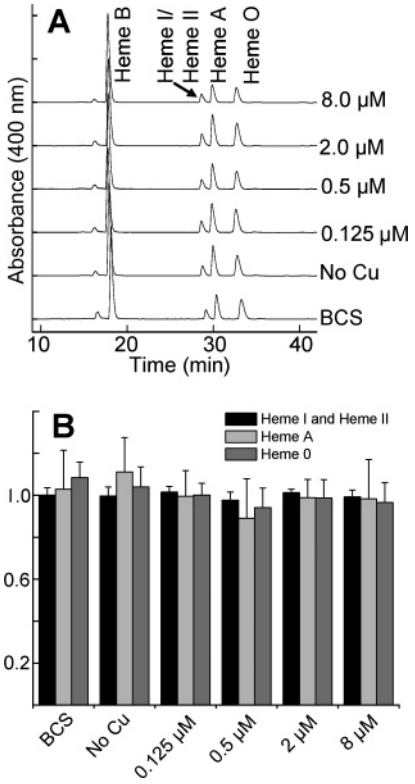


FIGURE 4: Heme analysis from *E. coli* cells. (A) HPLC chromatograms of hemes extracted from *E. coli*. The *E. coli* cells were grown with various concentrations of copper in the media. "No Cu" medium was Chelex-treated to remove the metals, while the "BCS" medium was Chelex-treated and had the copper chelator BCS added. Heme I and heme II were identified as the C8 alcohol derivative of heme O and the C8 carboxylate derivative of heme O, respectively (*40*). HPLC retention times were normalized to heme B; actual retention times varied due to slight variations in column conditions. (B) The average variability of the heme production in *E. coli* as a function of the copper concentration is reported. A value of 1.0 denotes a situation where the percentage of a particular heme isolated from cells grown at a specific copper concentration is identical to that same heme isolated from the other five copper concentrations in that particular growth set. The error bars denote one standard deviation from a total of four trials.

Analysis of Heme from Wild-Type R. sphaeroides. To examine the effect of copper on the total regulation of HOS and HAS under native conditions, wild-type *R. sphaeroides* 2.4.1 was grown under a variety of copper conditions in LB.

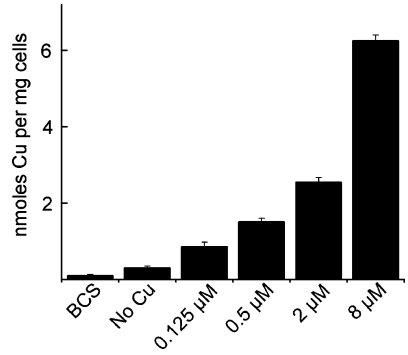


FIGURE 5: Estimated number of nanomoles of copper ions per milligram of *E. coli* cell pellet as determined by ICP analysis. Cells were grown in media containing various amounts of copper. "No Cu" medium was Chelex-treated to remove the metals, while "BCS" medium was Chelex-treated and had the copper chelator BCS added. The error bars denote one standard deviation from a total of four trials.

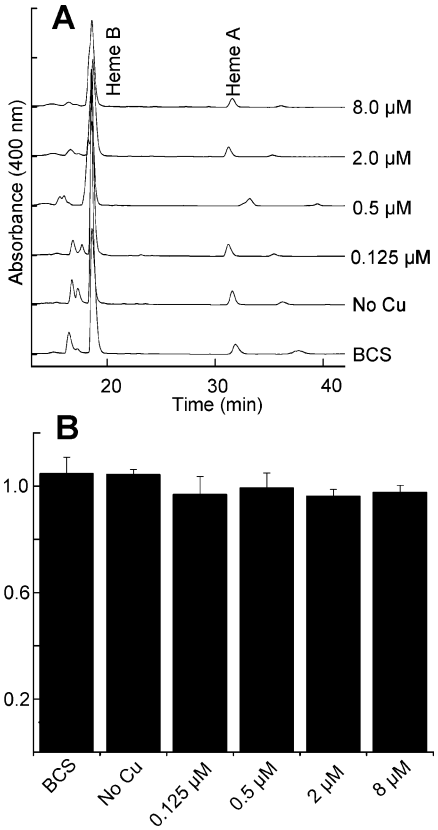


FIGURE 6: Heme A analysis from *R. sphaeroides* cells. (A) HPLC chromatograms of hemes extracted from *R. sphaeroides*. The cells were grown with various concentrations of copper in the media. "No Cu" medium was Chelex-treated to remove the metals, while "BCS" medium was Chelex-treated and had the copper chelator BCS added. HPLC retention times were normalized to heme B; actual retention times varied due to slight variations in column conditions. (B) The average variability of heme A production in *R. sphaeroides* as a function of the copper concentration is reported. A value of 1.0 denotes a situation where the percentage of heme A isolated from cells grown at a specific copper concentration is identical to the percentage of heme A isolated from the other five copper concentrations in that particular growth set. The error bars denote one standard deviation from a total of four trials.

Hemes were extracted from cells and analyzed by HPLC as described for the *E. coli* growths. Inspection of Figure 6A reveals the presence of heme B and heme A, as well as very small amounts of heme O. Both the alcohol and carboxylate

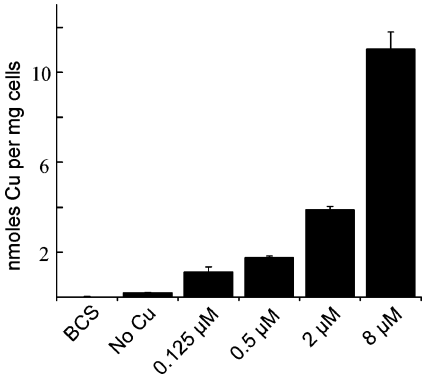


FIGURE 7: Estimated number of nanomoles of copper ions per milligram of *R. sphaeroides* cells as determined by ICP analysis. Cells were grown in media containing various amounts of copper. "No Cu" medium was Chelex-treated to remove the metals, while "BCS" medium was Chelex-treated and had the copper chelator BCS added. The error bars denote one standard deviation from a total of four trials.

derivatives of heme O are undetectable. This, however, is not surprising because neither heme A intermediates nor overoxidized products are expected to accumulate to any significant extent when HOS and HAS are expressed in their natural organism and under native control. As a result, only the heme A and heme B peaks could be accurately quantified (Figure 6B). Consistent with the coexpression of HOS and HAS from *B. subtilis* in *E. coli*, the copper concentration of the media had no effect on the accumulation of heme A in *R. sphaeroides*.

Control experiments were again performed to verify the expected intracellular copper levels. ICP analysis (Figure 7) confirmed that cells grown in a copper-starved state (i.e., in the presence of the copper chelator BCS) contained very little intracellular copper. In addition, we measured C*c*O activity on isolated membrane extracts from *R. sphaeroides* grown under varying copper conditions (Figure S5 of the Supporting Information). As expected, there was essentially no C*c*O activity observed in cells grown in LB in the absence of copper.[3] Together, the results from *R. sphaeroides* indicate that copper is not involved in any aspect of heme A biosynthesis or regulation.

DISCUSSION

Despite the obvious and critical importance of heme A to both C*c*O and the electron transport pathway, very little is known concerning its homeostasis. It was previously reported that HOS and HAS transcription levels in *S. cerevisiae* are not altered by any of the obvious conditions that affect the transcription of many of the other respiratory genes (*47*−*51*). We investigated copper as a potential regulator for heme A homeostasis because both are essential cofactors in eukaryotic C*c*O, and because copper and iron (and heme) levels appear to be linked in a number of different processes (*39*, *53*−*55*, *57*, *58*).

At first glance, two early in vitro studies seemed to indicate that copper was not required for the activity of either HOS (*14*) or HAS (*18*). In both of these studies, membrane

[3] Additional C*c*O activity assays performed on isolated membrane extracts from *R. sphaeroides* grown in Sistrom's medium yielded essentially identical results (data not shown).

fragments of *E. coli* cells overexpressing either HOS (from *E. coli*) or HAS (from *Bacillus stearothermophilus*) were incubated with the appropriate substrates to generate heme O and heme A, respectively, thus unambiguously establishing the role of each enzyme. Although additional copper was not needed for enzyme activity in these in vitro assays, it was present in the initial growth medium.[4] Therefore, it is theoretically possible that HOS and HAS contained copper during these in vitro assays. In another study, Svensson and Hederstedt reported only trace levels of copper in purified HAS from *B. subtilis*, although the enzyme was not reported to be active (*65*). It should also be noted that these studies do not address whether copper affects the transcription, translation, or in vivo stability of either HOS or HAS. Our objective was to test specifically and rigorously the potential importance of copper in heme A homeostasis, thus expanding on these important early works.

To test the hypothesis that copper modulates HOS and/or HAS levels, *S. cerevisiae* cells were grown under a variety of copper concentrations. The quantity of genomically epitope-tagged HOS and HAS present in the yeast mitochondria when the genes were under native control was determined by Western blot analysis. These experiments examine copper's cumulative effect on HOS and HAS transcription, translation, and stability. As shown in Figures 1 and 2, no discernible trend was detected in our Western analysis. Although it is theoretically possible that two or more processes could be altered by copper with equal but opposite effects, this seems to be exceedingly unlikely, and mRNA levels were therefore not independently probed. Thus, these results suggest that copper does not act as a regulator of either transcription or translation, nor does copper alter the stability of HOS or HAS. Because this experiment is examining protein levels, it does not address HOS and HAS activity. Unfortunately, both the C*c*O subunits and the heme cofactors are present at very low levels in the absence of copper in *S. cerevisiae*, and it would therefore be difficult to ascertain whether a decrease in the level of heme A was a direct result of a low level of copper, or an indirect result of poor subunit assembly.

To examine directly the effect of copper on the activity of HOS and HAS, we heterologously expressed HOS and HAS from *B. subtilis* in *E. coli*. This allowed us both to increase protein levels and to remove native transcriptional and translational control. It should be noted that this experiment could not be performed using the analogous genes from *S. cerevisiae* because expression of yeast HAS in *E. coli* led to an inactive protein. As shown in Figure 4, changing the intracellular copper concentrations did not alter the ratios of the heme products as determined by HPLC. This result strongly suggests that copper does not have any direct role in the activity of prokaryotic HOS and HAS. Because the proteins were heterologously expressed under non-native control, however, this experiment cannot detect any effect that copper might have on other proteins involved in heme A biosynthesis.

To assess the possibility that copper might be involved in some other unidentified control mechanism of heme A homeostasis, *R. sphaeroides* cells were grown under a variety of copper conditions. There are a number of features that make *R. sphaeroides* uniquely suited to address this question. The catalytic subunits of C*c*O in *R. sphaeroides* are very similar in sequence and structure to mitochondrial C*c*O (*66*−*68*), and *R. sphaeroides* is a member of the α subdivision of proteobacteria from which mitochondria are thought to have evolved. Therefore, what we learn about heme A biosynthesis and regulation in *R. sphaeroides* may very well be relevant to higher organisms. In addition, unlike C*c*O obtained from *S. cerevisiae*, C*c*O from *R. sphaeroides* is stable (but obviously not active) in the absence of either heme A (*69*) or Cu_B (*30*). Due to the stability of subunit I in the absence of its metal cofactors, any variation in the heme A level cannot be attributable to the absence of subunit I, and must therefore result from the direct or indirect regulation by copper. Significantly, Figure 6 clearly indicates that heme A is present at normal levels even in the complete absence of copper. This is a key result because HOS and HAS are being expressed in their natural organism under native control. Therefore, unlike the experiments in *E. coli* that only probed the direct effect of copper on HOS and HAS, the experiments in *R. sphaeroides* indicated additionally that there are no other proteins in the heme A biosynthetic pathway (e.g., a ferredoxin to HAS or a previously unidentified regulatory protein) that are affected by copper. Taken together, our results in *R. sphaeroides* indicate that copper does not affect the transcription, translation, activity, or stability of either HOS or HAS.

One possible explanation for our results is that the intracellular copper concentrations were not adequately controlled. It is well-known that copper is very difficult to remove completely from aqueous solutions, and that microorganisms can concentrate any residual copper found in the media and/or glassware. Several controls were designed and implemented to ensure that copper was actually controlled as we intended. All water and growth media were treated with Chelex-100 resin to remove divalent metal ions as described in Experimental Procedures. In addition, all of the glassware was acid-washed and rinsed with Chelex-treated water prior to use. ICP metal analysis of *S. cerevisiae*, *E. coli*, and *R. sphaeroides* (Figures 3, 5, and 7) confirmed that the intracellular copper concentrations were at appropriate levels.[5] In addition, C*c*O activity assays performed on *S. cerevisiae* mitochondria and *R. sphaeroides* membranes obtained from cells grown under rigorously copper-starved conditions revealed considerably less C*c*O activity compared to samples obtained from copper-replete cells (Figures S4 and S5 of the Supporting Information), thus corroborating the ICP results. Under these same conditions, however, HAS and heme A were detected at normal levels (Figures 1 and 6). These results further support our conclusion that copper is not involved in the regulation of the heme A biosynthetic pathway.

[4] HOS required the addition of either Mg^{2+} or Ca^{2+} to obtain maximal activity; the addition of $CuSO_4$ alone yielded minimal activity.

[5] Simply adding large concentrations of the copper chelator BCS to growth media that had not been treated with Chelex-100 yielded cells that contained intracellular copper concentrations identical to those of cells grown under normal growth conditions. In fact, using Millipore-purified water in lieu of Chelex-treated water to rinse the acid-washed glassware also resulted in cells with moderate residual intracellular copper concentrations and allowed *S. cerevisiae* to grow in Chelex-treated YPEG (albeit slowly) in the absence of added copper (data not shown).

If copper is not involved in heme A homeostasis, how is the production of heme A regulated? We recently reported that HOS and HAS from both *B. subtilis* and *R. sphaeroides* copurify when expressed in *E. coli*, and that the presence of HAS decreases the activity of HOS (*42*). We interpreted these results to suggest that, at least in prokaryotic organisms, HOS and HAS form a physiologically relevant complex with the possibility of direct heme O transfer between the two enzymes. The formation of a HOS−HAS complex would be an ideal location for regulation of heme A biosynthesis, with heme flux being controlled by product release. Very recently, it was shown that Surf1p is important for the insertion of heme into the heme a_3−Cu_B site (*43*). The fact that C*c*O in *surf1* deletion strains contained completely occupied low-spin heme *a* sites (*43*), however, argues against a role for Surf1 in heme A regulation, although this does not rule out the possibility that the release of heme A to some other protein might regulate the biosynthetic pathway. Alternatively, the fact that the presence of HAS alters the activity of HOS (*42*) is also consistent with regulation occurring via feedback inhibition, with a product of HAS inhibiting HOS.

Another possible mode of heme A regulation involves C*c*O, the only natural target of heme A. Barros et al. observed that most *S. cerevisiae* mutants deficient in C*c*O assembly have significantly less heme A than the corresponding parental strain (*52*). They proposed that the most likely explanation for these data is that heme A biosynthesis is positively regulated by either an assembly intermediate or a specific subunit of C*c*O. This could also explain the apparent correlation between copper and heme A levels (*57*); copper is required for the proper assembly of C*c*O in eukaryotes. (Alternatively, heme A levels could be reduced in yeast in the absence of copper because heme A is degraded when it is not associated with C*c*O.) It was further suggested by Barros et al. that the reaction catalyzed by HAS would likely be the point of regulation for this pathway because heme O oxidation and/or release of heme A appears to be the rate-limiting step (*52*).

In summary, our results demonstrate that despite previous circumstantial evidence to the contrary, copper does not regulate heme A homeostasis at the level of either HOS or HAS expression or stability in *S. cerevisiae*. Furthermore, we have also demonstrated that copper does not directly affect the activity of bacterial HOS or HAS, and our results in *R. sphaeroides* (considered an excellent model organism for studying eukaryotic C*c*O) showed that copper levels have no effect on heme A production or accumulation. From these results, we conclude that copper is not directly involved with heme A homeostasis. As discussed above, a number of possible modes exist to control the biosynthesis of heme A, and it is also possible that the heme A biosynthetic pathway is regulated by some still unknown influence. Finally, as is common in many biosynthetic pathways, it is quite possible that multiple regulatory strategies are utilized to control the biosynthesis of heme A. Although more work is clearly required to elucidate fully the regulatory mechanism, our work has allowed us to determine that copper is not directly involved in the regulation of heme A biosynthesis.

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SUPPORTING INFORMATION AVAILABLE

Figures S1−S3 show the intracellular levels of iron and zinc in *S. cerevisiae*, *E. coli*, and *R. sphaeroides*, respectively, grown at a variety of copper concentrations. Figures S4 and S5 provide the C*c*O activity data from isolated *S. cerevisiae* mitochondria and *R. sphaeroides* membranes, respectively, obtained from cells grown under two different copper conditions. This material is available free of charge via the Internet at http://pubs.acs.org.

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