Regulation of the heme A biosynthetic pathway in *Saccharomyces cerevisiae*

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Abstract Biosynthesis of heme A, a prosthetic group of cytochrome oxidase (COX), involves an initial farnesylation of heme B. The heme O product formed in this reaction is modified by hydroxylation of the methyl group at carbon C-8 of the porphyrin ring. This reaction was proposed to be catalyzed by Cox15p, ferredoxin, and ferredoxin reductase. Oxidation of the alcohol to the corresponding aldehyde yields heme A. In the present study we have assayed heme A and heme O in yeast COX mutants. The steady state concentrations of the two hemes in the different strains studied indicate that hydroxylation of heme O, catalyzed by Cox15p, is regulated either by a subunit or assembly intermediate of COX. The heme profiles of the mutants also suggest positive regulation of heme B farnesylation by the hydroxylated intermediate formed at the subsequent step or by Cox15p itself. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Biosysnthesis; Heme A; Cytochrome oxidase; *Saccharomyces cerevisiae*

1. Introduction

Heme A is an important electron carrier of mitochondrial and some bacterial cytochrome oxidases (COXs). The synthesis of heme A begins with farnesylation of a vinyl group at carbon C-2 of the porphyrin ring of heme B (protoheme) by the product of the *COX10* gene. [1,2]. Conversion of the resultant heme O to heme A requires a further oxidation of the methyl group at C-8 to a formyl group [3]. We have previously proposed that the C-8 methyl is first hydroxylated by a three-component monooxygenase consisting of Cox15p, ferredoxin (Yah1p), and ferredoxin reductase (Arh1p) [4]. According to this scheme the last step of the pathway involves oxidation of the C-8 methanol group to a formyl group. The enzyme responsible for this reaction has not been identified.

The requirement of Cox15p, Yah1p and Arh1p for heme A synthesis is supported in part by studies on the heme compositions of mitochondria in different COX[1] mutants [5]. In the course of these studies, we noted that most COX mutants are grossly deficient in heme A but accumulate variable amounts of heme O. A notable exception are *cox15* mutants as they lack heme A altogether and have only negligible amounts of heme O [4]. The failure of *cox15* mutants to accumulate heme O was unexpected in view of the proposed role of Cox15p in hydroxylation of heme O. The generally low levels of heme A in mutants completely blocked in COX assembly and the equally low amount of heme O in *cox15* mutants, suggested that either conversion of heme B to heme A is regulated or that hemes A and O are unstable when COX assembly is blocked.

To examine the relationship of heme A to COX synthesis we have extended the heme analyses to mutants with null mutations in *COX15* and other genes previously implicated in COX assembly [6]. The heme compositions of the single and double mutants and of mutants overexpressing *COX10* [2] are most compatible with several regulatory steps in heme A synthesis and are discussed in light of what is currently know about this pathway.

2. Materials and methods

2.1. Yeast strains and media

The genotypes and sources of the *Saccharomyces cerevisiae* strains used in this study are listed in Table 1. The compositions of YPD (rich glucose), YEPG (rich glycerol), and YPGal (rich galactose) used to grow yeast have been described elsewhere [15].

2.2. Construction of imp2 and pet117 null alleles

A null allele of *IMP2* [16] was constructed by replacing the sequence between *Kpn*I and *Msc*I spanning most of the gene with a 1 kb fragment containing *URA3*. A clean deletion of *PET117* [17] was obtained by polymerase chain reaction (PCR) amplification of the 5′- and 3′-non-coding regions with bi-directional primers. The gene was replaced with a 1 kb fragment of DNA containing *HIS3*. The null alleles, isolated as linear fragments of DNA, were substituted for the native genes in W303-1A and W303-1B by the one-step gene replacement method [18]

2.3. Heme analysis

Mitochondria were prepared from yeast grown in YPGal to early stationary phase by the method of Faye et al. [19] using Zymolyase 20 000 (ICN, CA, USA) to convert cells to spheroplasts. The spheroplasts were suspended in 0.5 M sorbitol, 20 mM Tris–HCl, pH 7.5, 0.5 mM EDTA at a protein concentration of 20 mg/ml and hemes were extracted by mixing 200 μl of the mitochondrial suspension with 1 ml of acetone containing 3% HCl. The conditions for separation of hemes on a 3.9×300 mm C18 Bondclone column (Phenomenex, CA, USA) have been described [4]. Fluorescent porphyrin compounds were monitored fluorometrically with an excitation wavelength of 400 nm and emission wavelength of 600 nm. The heme B standard was purchased from Sigma (St. Louis, MO, USA). Heme A was extracted from partially purified COX, and heme O from *Escherichia coli* cells. The concentrations of heme were calculated from the integrated areas under the peaks corresponding to hemes A, B and O. Since the concentration of heme B was not significantly affected in

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Abbreviations: ρ^0 mutant, a strain of yeast lacking mitochondrial DNA; COX, cytochrome oxidase; Cox1p and Cox2p, subunits 1 and 2, respectively, of cytochrome oxidase

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Table 1
Genotypes and sources of *S. cerevisiae* strains

Strain	Genotype	Source
W303-1A	a *ade2-1 his3-1,15 leu2-3,112 trp1-1, ura3-1*	[a]
W303-1B	α *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1*	[a]
C261	α *met6 cyc3*	[7]
WC261	α *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 cyc3*	C261×W303-1A
WC261-iCOX15	α *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 cyc3 trp1::pGCOX15-3*	this study
WC261ΔYAH1/ST1-iCOX15	α *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 cyc3 yah1::URA3 trp1::pGCOX15-3*+pYAH1/ST1	this study
WC261ΔYAH1/ST109-iCOX15	α *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 cyc3 yah1::URA3 trp1::pGCOX15-3*+pYAH1/ST109	this study
aW303ΔCOX11	a *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 cox11::HIS3*	[8]
aW303ΔCOX14	*a ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 cox14::HIS3*	[9]
aW303ΔCOX15	a *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 cox15::HIS3*	[10]
aW303ΔCOX18	a *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 cox18::URA3*	[11]
aW303ΔCOX20	a *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 cox20::URA3*	[12]
W303ΔIMP2	α *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 imp2::URA3*	[5]
aW303ΔSCO1	a *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1sco1::URA3*	[13]
aW303ΔSHY1	a *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1shy1::URA3*	[14]
aW303ΔPET111	a *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1pet111::HIS3*	this study
aW303ΔPET117	a *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1pet117::HIS3*	this study
aW303ΔCOX15/eCOX10	a *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 cox15::HIS3*+pG19/T4	this study
W303-1A/eCOX10	a *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1* + pG19/T4	this study
aW303ΔCOX14/eCOX10	*a ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 cox14::HIS3*+pG19/T4	this study
aW303ΔCOX11/eCOX10	a *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 cox11::his3*+ pG19/T4	this study
W303ΔSCO1/eCOX10	α *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 sco1::URA3*+ pG19/T4	this study
aW303ΔIMP2/eCOX10	a *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 imp2::URA3*+ pG19/T4	this study
W303ΔIMP2ΔCOX15	α *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 imp2::URA3 cox15::HIS3*	this study
W303ΔSCO1ΔCOX15	α *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 sco1::URA3 cox15::HIS3*	this study
aW303ΔPET117ΔCOX15	a *ade2-1 his3-1,15 leu2-3,112, trp1-1 ura3-1 pet117::HIS3 cox15::HIS3*	this study
aWC261ΔCOX15	a *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 cyc3 cox15::HIS3*	this study

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The multicopy plasmids pG19/T4 and pYAH1/ST1, and the integrative plasmid pGCOX15-3 and have been described in [28], [4], and [5], respectively.

COX mutants, the heme A and heme O values were normalized to heme B to correct for errors introduced by heme extraction and sample handling. The heme values reported in Figs. 3 and 4 were obtained from a single analysis. Some mutants (*imp2*, *sco1*, *cox11*, *cyc3*, *pet117*, *pet111*, *shy1*, *cox15*) were analyzed 2–3 times and the results obtained were similar to those reported.

2.4. Miscellaneous procedures

Standard methods were used for the preparation and ligation of DNA fragments and for transformation and recovery of plasmid DNA from *E. coli* [20]. Yeast was transformed by the method of Schiestl and Gietz [21]. Proteins were separated on a 12% polyacrylamide gel by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) [22]. Western blots were treated with a monoclonal antibody against yeast Cox1p (Molecular Probes, OR, USA) followed by a second reaction with anti-mouse or anti-rabbit IgG conjugated to horseradish peroxidase (Sigma, MO, USA). Antibody–antigen complexes were visualized with the SuperSignal chemiluminescent substrate kit (Pierce, IL, USA). Mitochondrial spectra were recorded on deoxycholate extracts at room temperature [23]. Protein concentrations were determined by the method of Lowry et al. [24].

3. Results and discussion

3.1. COX mutants have depressed levels of heme A

S. cerevisiae mutants blocked in COX assembly have 30–300 times less heme A than the parental respiratory competent strain (Table 2). The exceptions are *shy1*, *cox20*, and *cox5a*

Table 2
Heme A and heme O levels in COX mutants

Strain	Gene	Function	Heme A/B	Heme O/B	Heme A/O
W303-1A	–	–	0.29	0.0037	∼80
aW303ΔSCO1	SCO1	copper delivery to Cox2p	0.001	0.045	0.022
aW303ΔCOX10	COX10	heme:farnesyl transferase	0	0	–
aW303ΔCOX11	*COX11*	copper delivery to Cox1p	0.001	0.076	0.013
aW303ΔCOX14	*COX14*	assembly of COX	0.005	0.1	0.05
aW303ΔCOX15	*COX15*	heme O hydroxylation	0	0.025	–
aW303ΔCOX18	*COX18*	insertion of Cox2p	0.01	0.05	0.2
aW303ΔCOX20	*COX20*	chaperone of Cox2p	0.078	0.042	1.86
aW303ΔPET111	*PET111*	translation of Cox2p	0.006	0.07	0.086
aW303ΔPET117	*PET117*	assembly of COX	0.004	0.094	0.043
aW303ΔSHY1	*SHY1*	assembly of COX	0.04	0.003	13.3
aW303ΔIMP1	*IMP1*	processing of Cox2p	0.006	0.15	0.04
aW303ΔIMP2	*IMP2*	processing of Cox2p	0.002	0.17	0.011
WC261	*CYC3*	cytochrome *c* heme lyase	0.002	0.05	0.04

Mitochondrial hemes were extracted and separated by reverse phase chromatography as described in Section 2. The values reported in the table are calculated from the areas under the peaks corresponding to heme A, B (protoheme) and heme O. Since the extinction coefficient for each heme at 400 nm is different, these values should not be interpreted to correspond to molar ratios.

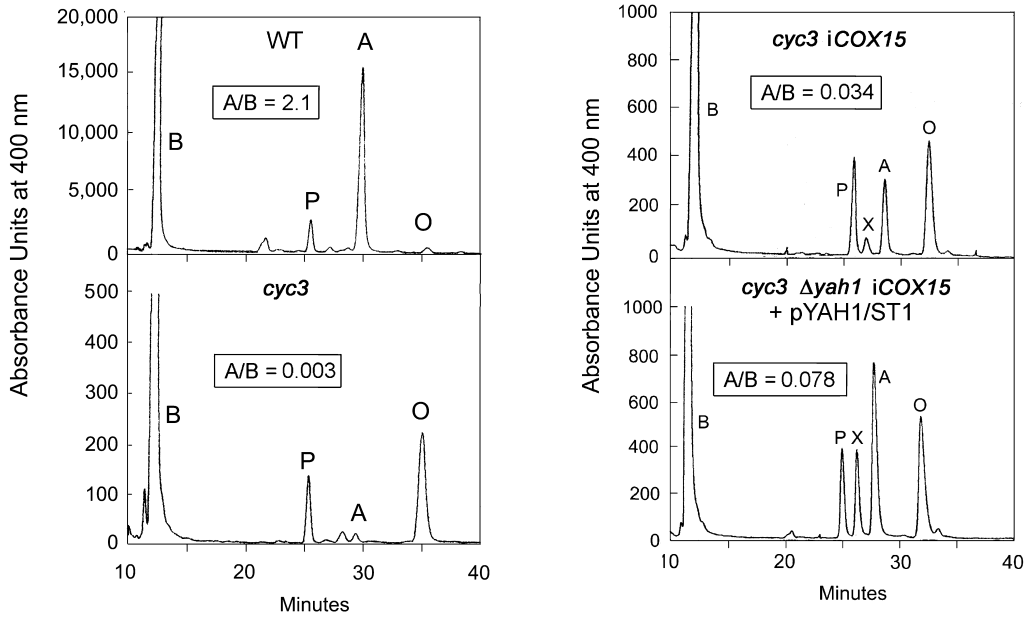

Fig. 1. Mitochondrial hemes in wild type yeast and in *cyc3* mutants. Mitochondria were prepared from yeast grown in 2% rich galactose medium. The following strains were analyzed: W303-1A (WT); WC261 (*cyc3*) a respiratory deficient strain with a point mutation in *CYC3*; WC261-iCOX15 (*cyc3* i*COX15*), the *cyc3* mutant with the *GAL10–COX15* fusion gene integrated at *trp1*; WC261ΔYAH1/ST1-iCOX15 (*cyc3* Δ*yah1* i*COX15*+pYAH1/ST1): a *cyc3*, *yah1* double mutant containing chromosomally integrated *GAL10–COX15* fusion gene and *YAH1* on a high copy episomal plasmid (pYAH1/ST1). Because Yah1p is an essential protein, the *yah1* null allele allows selection of pYAH1/ST1. Total hemes were extracted and analyzed on a reverse phase C18 column as described in Section 2. The peaks corresponding to heme B (B), heme A (A), heme O (O), and a porphyrin compound (P) are marked. The peak labeled X has not been identified but could be the hydroxylated intermediate. The ratios of heme A to heme B (A/B) were calculated from the areas under the peaks.

(not shown) mutants that retain 10–25% heme A. The heme A is probably associated with the residual COX present in these strains [25,26].

Since COX deficient mutants have low ($<10\%$) concentrations of Cox1p, the heme-bearing component of the enzyme, the decrease in heme A could be due to turnover of heme A not bound to Cox1p. This explanation is incompatible with the ability of some mutants to maintain high concentrations of heme A, even when Cox1p is nearly completely absent. CYC3 codes for the lyase that catalyzes a covalent linkage of heme to apocytochrome *c* [27]. For reasons that are not clear at present, mutants lacking cytochrome *c*, including *cyc3* mutants, do not assemble COX [28]. Predictably, *cyc3* mutants, like all other COX assembly defective mutants, accumulate heme O and have very low concentrations of heme A (Table 2, Fig. 1). Heme A is substantially increased in *cyc3* mutants overexpressing Cox15p (Fig. 1). The increase in heme A is even more pronounced in *cyc3* mutants overexpressing both Cox15p and Yah1p encoded by *YAH1* (Fig. 1). The heme A in such strains is also detected spectrally in mitochondria (Fig. 2A). Unlike cytochromes *a* and a_3, which have α absorption bands at 605 nm, the α absorption band of heme A in the *cyc3* transformant peaks at 595 nm, indicative of a non-native environment. The almost complete absence of Cox1p in the transformant (Fig. 2B) confirms that the heme A cannot be associated with its usual protein partner. The *cyc3* mutant overexpressing *Cox15p* and Yah1p accumulates a new compound (labeled X in Fig. 1). This peak is also seen in other mutants transformed with *COX15* and *YAH1* but its concentration is much lower. This compound has not been identified but could be the hydroxylated intermediate.

The relatively high concentrations of heme A in *cyc3* and in other COX mutants transformed with *COX15* and *YAH1* [5] argues against rapid turnover of heme as a plausible explanation for its almost complete absence in such strains. The presence of heme A in mutants lacking Cox1p also makes it unlikely that heme A synthesis depends on an interaction of heme O with this subunit. Instead, our data suggest that conversion of heme O to heme A is coupled to some event or step in COX assembly. The coordinate production of heme A and COX could involve positive regulation of its synthesis by a subunit/intermediate (e.g. Cox1p or holoenzyme) of the enzyme. The absence or reduced levels of this subunit/intermediate in mutants defective in COX assembly could account for their repressed heme A synthesis. Alternatively, heme A production could be negatively regulated by an assembly intermediate. Increased concentrations of such an intermediate in assembly-arrested mutants could also act to inhibit heme A synthesis. Although less attractive, this explanation can also account for the low concentration of heme A in the mutants.

The accumulation of heme O in most mutants (Table 2) indicates that hydroxylation of heme O, catalyzed by Cox15p, is the rate-limiting step in the pathway and is the most likely target for either mode of regulation.

2.2. *Mutations in COX15 repress heme O synthesis and have a dominant effect when combined with other mutations*

The accumulation of heme O in COX mutants (Table 2) indicates that this intermediate is stable. Based on the proposed function of Cox15p in heme O hydroxylation [4,5] *cox15* mutants should have high concentrations of this heme. The fact that they have very low levels of heme O implies additional regulation of the pathway. To determine if the *cox15* phenotype is dominant, heme O was analyzed in double mutants, in which the *cox15* null allele was combined with mutations in other COX-specific genes. These as-

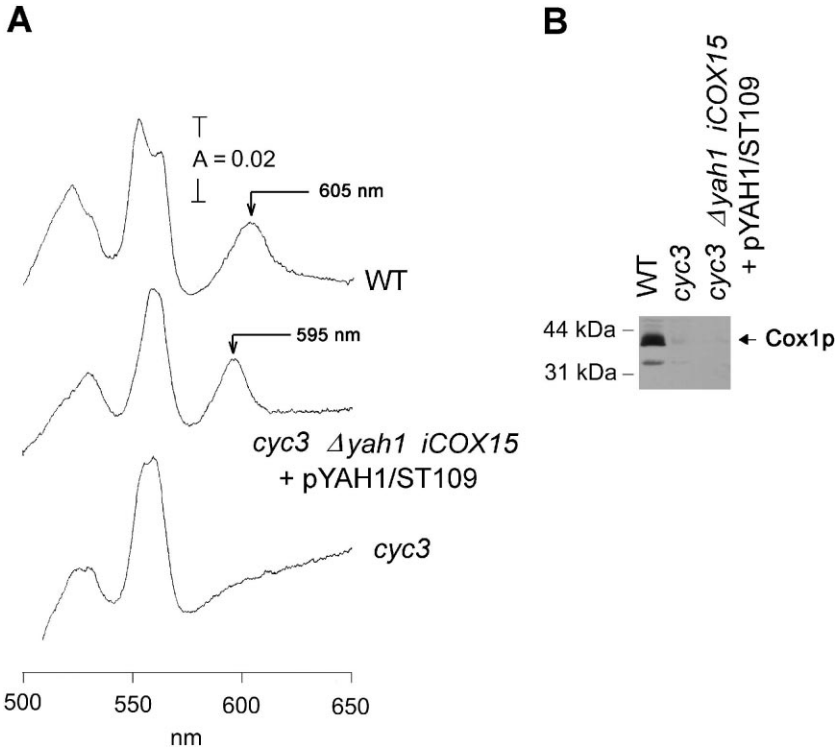

Fig. 2. Spectra and steady state concentration of Cox1p in wild type and *cyc3* mutants. A: Mitochondria were prepared from the respiratory competent haploid strain W303-1A (WT), from the cytochrome *c* lyase mutant WC261 (*cyc3*), and from WC261ΔYAH1/ST109-iCOX15 (*cyc3* Δ*yah1* i*COX15*+pYAH/ST109), a *cyc3*, *yah1* double mutant with a chromosomally integrated copy of the *GAL10–COX15* fusion and *yah1* on the high copy plasmid pGYAH1/ST109. The *yah1* gene in pYAH/ST109 has two mutations (H108S and Y134C) that partially impair the activity of Yah1p (Barros, unpublished). Mutations in the corresponding residues of human Yah1p have been reported to affect its interaction with redox partners [29]. The reduced (dithionite) versus oxidized (ferricyanide) spectrum of the *cyc3* mutant shows a reduction in absorbance at 550 nm due to the absence of cytochrome *c* but not c_1. This strain also lacks the 605 nm α absorption band corresponding to cytochromes *a* and a_3. The spectrum of the *cyc3*, *yah1* double mutant harboring the *GAL10–COX15* fusion and pYAH/ST109 is identical to that of the *cyc3* mutant except for the appearance of a new band with a maximum at 595 nm. B: Western analysis of Cox1p. Mitochondria (20 μg protein) from the same strains used for the spectra shown in A were separated by SDS–PAGE on a 12% polyacrylamide gel. Following transfer to nitrocellulose, the blot was probed with a monoclonal antibody against yeast Cox1p (arrow in margin).

says indicate that introduction of the *cox15* mutation, in each case, causes a reduction in heme O (Fig. 3). With the exception of the *cox15*, *imp2* mutant, the heme O concentration of the other double mutants was similar to that of the *cox15* mutant. Introduction of the *cox15* allele into the *imp2* background, nonetheless, caused a two-fold decrease in heme O.

As a further test of Cox15p-dependent regulation of heme O synthesis, we examined the effect of overexpression of the farnesyl transferase on heme O accumulation in a panel of COX mutants. The mutants were transformed with pG19/T4, a high copy plasmid containing *COX10* [30]. All the COX deficient strains, except the *cox15* mutant have at least two times more heme O when they harbor *COX10* on a high copy plasmid (Fig. 4). The failure of farnesyl transferase overexpression to raise the heme O level in the *cox15* mutant is consistent with a Cox15p-dependent activation of the first step in the heme A biosynthetic pathway.

The farnesyl transferase activity of Cox10p could require the presence of Cox15p if the two proteins are part of a complex. This explanation, however, is unlikely in view of the identical sedimentation property of Cox15 extracted from wild type and from a *cox10* null mutant (data not shown). The dependence of heme farnesylation on Cox15p, therefore, is likely to involve some other mechanism such as activation the farnesyl transferase by the hydroxylated product.

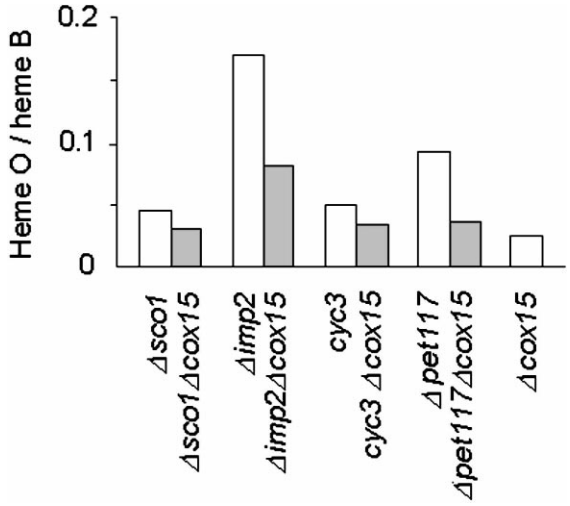

Fig. 3. Ratios of heme O to heme B in single and double mutants. Mitochondria were prepared from the following mutants grown in rich galactose medium: aW303ΔSCO1 (*Δsco1*), aW303ΔIMP2 (*Δimp2*), WC261 (*cyc3*), aW303ΔPET117 (*Δpet117*) and aW303Δ COX15 (*Δcox15*). Strains containing the same mutations combined with the *cox15* null allele were obtained by crosses of the single mutants to W303ΔCOX15. Hemes were analyzed in the single mutants (clear bars) and double mutants (filled bars) as described in Table 2.

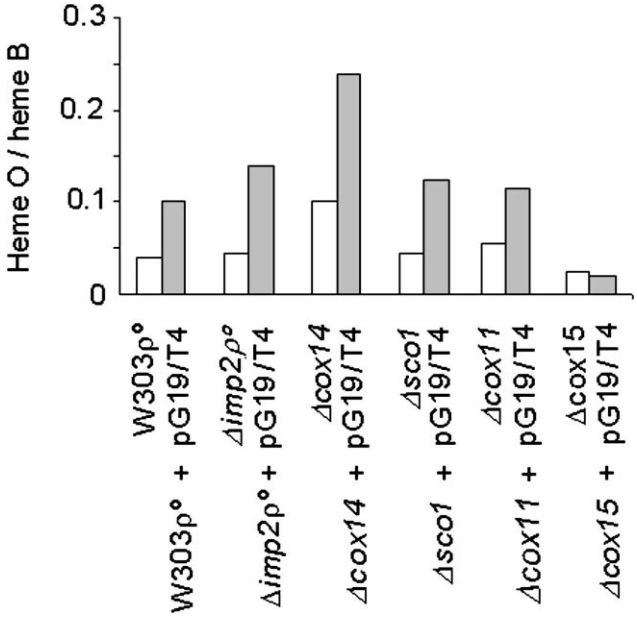

Fig. 4. Ratios of heme O to heme B in mutants overexpressing heme :farnesyl transferase. Mitochondria were prepared from a ρ^0 derivative of the wild type strain W303-1A (W303 ρ^0) and from the COX deficient mutants aW303ΔIMP2ρ^0 (*Δimp2* ρ^0), aW303ΔCOX11 (*Δcox11*), aW303ΔCOX14 (*Δcox14*), aW303ΔSCO1 (*Δsco1*), aW303ΔCOX15 (*Δcox15*). The mutants were also transformed with COX10 on a high copy plasmid (pG19/T4). Hemes were analyzed in the mutants (clear bars) and transformants (filled bars) as described in the legend of Table 2.

The heme A biosynthetic pathway may be subject to still other regulatory influences. For example, deletion of mitochondrial DNA in the *imp2* mutant elicits a four-fold decrease in the steady state concentration of heme O (compare Table 2 and Fig. 4). The present studies point to heme O hydroxylation, catalyzed by Cox15p, as an important regulatory step. As indicated in Fig. 5, activation of heme A synthesis by downstream assembly events may be the mechanism for coordinating the rate of heme A synthesis with its utilization for COX maturation.

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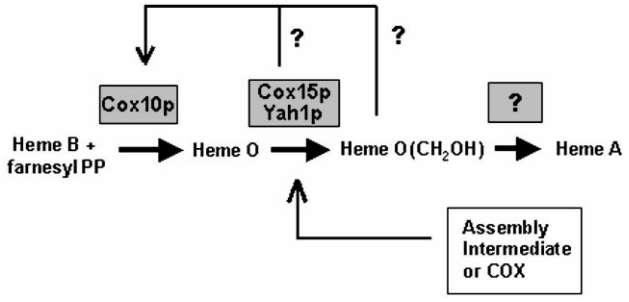

Fig. 5. Proposed regulation of heme A synthesis. Hydroxylation of heme O is positively regulated by a downstream assembly intermediate or subunit of COX (arrows). Farnesylation of heme B is also shown to be positively regulated (arrow) either by Cox15p or the hydroxylated intermediate.

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