Involvement of mitochondrial ferredoxin and Cox15p in hydroxylation of heme O

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Abstract Cox15p is essential for the biogenesis of cytochrome oxidase [Glerum et al., J. Biol. Chem. 272 (1997) 19088–19094]. We show here that *cox15* mutants are blocked in heme A but not heme O biosynthesis. In *Schizosaccharomyces pombe COX15* is fused to *YAH1*, the yeast gene for mitochondrial ferredoxin (adrenodoxin). A fusion of Cox15p and Yah1p in *Saccharomyces cerevisiae* rescued both *cox15* and *yah1* null mutants. This suggests that Yah1p functions in concert with Cox15p. We propose that Cox15p functions together with Yah1p and its putative reductase (Arh1p) in the hydroxylation of heme O.

Key words: *COX15*; Heme O; Hydroxylase; *YAH1*; Cytochrome oxidase; Mitochondrion

1. Introduction

The *COX15* gene of *Saccharomyces cerevisiae* was previously shown to be essential for expression of functional cytochrome oxidase [1]. Mutations in this gene, as in other nuclear genes that function in cytochrome oxidase assembly, lead to the loss of the visible absorption bands of cytochromes *a* and a_3, and promote increased turnover of the mitochondrial translated subunits of cytochrome oxidase. Cox15p is an intrinsic membrane protein with seven putative transmembrane domains. It is a low abundance protein of the mitochondrial inner membrane. Cox15p has homologs in fungal (GenBank accession number AF079448), mammalian [2], fly (GenBank accession number AE003462), and worm genomes (GenBank accession number Z49130). In *Schizosaccharomyces pombe*, *COX15* is fused to *YAH1* encoding mitochondrial ferredoxin (unpublished observation). Despite their marginal sequence similarity, the *ctaA* gene product of *Bacillus* [3] and other related bacterial proteins may also be homologs of mitochondrial Cox15p.

To learn more about the function of Cox15p, an improved method for separation of hemes A and O by HPLC was used to screen cytochrome oxidase mutants for their mitochondrial content of hemes. Only two mutants were found to lack heme A. One of the mutants had a mutation in *COX10*, which is known to encode heme A:farnesyl transferase that catalyzes the farnesylation of protoheme to heme O [4]. This mutant lacks both hemes A and O. The second heme A deficient mutant had a mutation in *COX15*. Unlike the *cox10* mutant, however, the *cox15* mutant displayed the presence of heme O. This phenotype is expected in a mutant blocked in the last step of heme A biosynthesis in which the methyl at position 8 of the porphyrin ring is converted to a formyl group. We propose that Cox15p is a monooxygenase which in conjunction with mitochondrial ferredoxin and ferredoxin reductase catalyzes the oxidation of the methyl to the corresponding alcohol.

2. Materials and methods

2.1. Yeast strains and media

The genotypes and sources of the strains of *S. cerevisiae* 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 [5].

2.2. Extraction and separation of mitochondrial hemes

Mitochondria, prepared as described previously [5], were suspended in 0.5 M sorbitol, 20 mM Tris–HCl, pH 7.5, 0.5 mM EDTA at a protein concentration of 15–20 mg/ml. Total heme was extracted from 3 mg of mitochondrial protein with 1 ml of acetone containing 2.5% HCl. The mixture was vortexed, clarified by centrifugation and mixed with an equal volume of 50% acetonitrile. Insoluble material was removed by a second centrifugation. The extract was adjusted to approximately pH 3.5 with 1.65 M ammonium hydroxide, clarified by centrifugation, and applied to a 3.9×300 mm C18 Bondclone column (Phenomenex, CA, USA). Hemes were eluted at a flow rate of 1 ml/min using a 30–50% acetonitrile gradient over the first 5 ml, followed by a 50–75% linear acetonitrile gradient over the subsequent 35 ml. The acetonitrile solutions contained 0.05% trifluoroacetic acid. The elution of heme compounds was monitored at 400 nm. The elution times are affected by the pH of the sample applied to the column.

2.3. Construction of a COX15–YAH1 fusion gene

YAH1 was copied from yeast nuclear DNA by polymerase chain reaction (PCR) with primers: 5′-ggcaagcttgggggcacaaatgaggg and 5′-ggcggatccatgcatccttttacgcacctct. The 0.5 kb product was digested with *Bam*HI and *Hin*dIII and ligated to pUC18. The *COX15* region was obtained by amplification of the gene in plasmid pG4/T1 [1] with primers 5′-agattccaaggatccacgatcgct and 5′-ggcatgcatggtaatgattacgtttctc. This 1.67 kb fragment was digested with *Nsi*I and *Bam*HI and ligated to the pUC18 construct with *YAH*. The fusion gene was transferred to the multicopy plasmids YEp352 (pYAH/ST8) and YEp351 (pYAH/ST9) and to the integrative plasmids YIp352 (pYAH/ST11) and YIp351 (pYAH/ST10) [6]. The one-step gene insertion method of Rothstein [7] was used to integrate the gene at the *LEU2* or *URA3* loci of yeast chromosomal DNA.

2.4. Miscellaneous procedures

Proteins were separated by PAGE in the buffer system of Laemmli [8]. Yah1p and Cox15p–Yah1p were visualized on Western blots with antibody against a short synthetic peptide with a Yah1p sequence [9]

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

Strain	Genotype	Source
W303-1A	a *ade2-1 his3-15 leu2-3,115 trp1-1 ura3-1*	R. Rothstein, Department of Human Genetics and Development, Columbia University
W303ρ°	α *ade2-1 his3-15 leu2-3,115 trp1-1 ura3-1ρ°*	
aW303ΔCOX15	a *ade2-1 his3-15 leu2-3,115 trp1-1 ura3-1 cox15::HIS3*	[1]
aW303ΔCOX15/ST10	a *ade2-1 his3-15 leu2-3,115 trp1-1 ura3-1 cox15::HIS3 leu2::pYAH/ST10*	This study
a/αW303ΔYAH1	a/α *ade2-1/ade2-1 his3-15/his3-15 leu2-3,115/leu2-3,115 trp1-1/trp1-1 ura3-1/ura3-1 yah1::URA3/YAH1*	[9]
W303ΔCOX15,YAH1/ST10	α *ade2-1 his3-15 leu2-3,115 trp1-1 ura3-1 cox15::HIS3 yah1::URA3 leu2::pYAH/ST10*	This study
W303ΔYAH1/ST1	a *ade2-1 his3-15 leu2-3,115 trp1-1 ura3-1 yah::URA3* plus pYAH1/ST1	[9]

followed by a secondary reaction with ^{125}I-protein A or with the Super Signal detection system (Pierce, Rockford, IL, USA).

3. Results and discussion

3.1. Heme A is absent in a cox15 null mutant

Cytochrome oxidase deficiency is one of the more common phenotypes encountered among nuclear *pet* mutants of *S. cerevisiae*. The collection that we have been studying contains at least three dozen complementation groups whose members display the absence of cytochrome oxidase, by both enzymatic and spectral criteria [10]. Approximately, a third of the genes defined by these mutants encode proteins that affect cytochrome oxidase at late stages of its assembly and include mutants that are defective in heme A biosynthesis [4] and copper homeostasis [11,12].

Improved conditions for the separation of heme components by reverse phase HPLC have allowed us to more accurately gauge the heme composition of mitochondria from cytochrome oxidase deficient mutants and to correct an earlier mistaken impression that the *cox15* mutant contained low but detectable amounts of heme A [1]. In the present study we have screened mutants from 13 different complementation groups including strains with lesions in structural subunits (subunits 4, 5, and 6) and in nuclear gene products required for cytochrome oxidase assembly. In 12 mutants the heme A was reduced to 4–12% of the wild type strain while the heme O concentration was increased by a factor of 2–8. These effects on the two hemes are illustrated by the chromatogram obtained with the *sco1* mutant (Fig. 1). A ρ° mutant which is unable to synthesize cytochrome oxidase because of a complete loss of its mitochondrial genome also had reduced amounts of hemes A and O. The two exceptions were the *cox10* and *cox15* mutants, both of which lacked heme A. In agreement with earlier results the *cox10* mutant also had no heme O [4]. This was not true of the *cox15* mutant which had a very low but detectable amount of heme O (Fig. 1). The absence of heme A but not heme O in the *cox15* null mutant suggests that Cox15p is involved in some aspect of the conversion of heme O to heme A.

A mutational block preventing hydroxylation of the methyl group in heme O as suggested here for the *cox15* mutant should result in an increased content of mitochondrial heme O. The fact that this is not observed suggests that for reasons we do not understand at present, the heme O intermediate is more unstable in the *cox15* than in other cytochrome oxidase mutants. As indicated above, the amount of heme O detected in the other mutants varied over a four-fold range indicating that the stability of this intermediate might depend on other factors. This is also true of the final heme A product. For example, all the mutant examined displayed significant reductions in heme A. This was also true of the ρ° strain (Fig. 1) in which heme A biosynthesis should not be affected.

3.2. Fusion of COX15 and YAH1

Protein homology searches revealed that *COX15* of *Schizosaccharomyces pombe* is fused at its 3′ end to another gene homologous to *YAH1*. The product of *YAH1* has been shown in *S. cerevisiae* to encode mitochondrial ferredoxin [9]. The fusion of the two genes implies that Cox15p and ferredoxin (Yah1p) function in a common pathway. To test whether a single protein expressed from the two *S. cerevisiae* genes would also be functional, *COX15* and *YAH1* were fused so as to create a continuous reading frame homologous to the naturally occurring gene of *S. pombe* (Fig. 2A). This construct lacked the sequences encoding the 24 C-terminal residues of Cox15p and the first 19 residues of Yah1p. The N-terminal sequence of Yah1p probably functions as a mitochondrial targeting signal. In addition the fusion protein had a methionine at the junction of Cox15p and Yah1p which does not occur in either protein.

The *S. cerevisiae* fusion gene was introduced into a *cox15* null mutant both on a multicopy episomal plasmid (pYAH/ST8) and in single copy (pYAH/ST11) by integration into chromosomal DNA. In both instances the respiratory defect of the mutant was complemented and the transformants were able to grow as well as the wild type parental strain on non-fermentable carbon sources (Fig. 2B). Since the null mutation in *YAH1* is lethal [9], the transformations with the fusion gene in the episomal and integrative plasmids were done with a heterozygous diploid strain containing only one copy of the *yah1* null allele. The transformants were sporulated and analyzed by tetrad dissections. This analysis showed that meiotic spore progeny with the *yah1* null allele were viable in the presence of the fusion gene and displayed wild type growth properties even when the gene was present in single copy. These results indicate that the functions of the *S. cerevisiae* Cox15p and Yah1p proteins are not compromised when they are incorporated into a single polypeptide modeled on the native protein of *S. pombe*.

Both transformants with the multicopy and integrated fusion gene had a mitochondrial protein estimated by SDS–PAGE to be approximately 67 kDa, a value in good agreement with the mass of 69 248 Da expected for the pri-

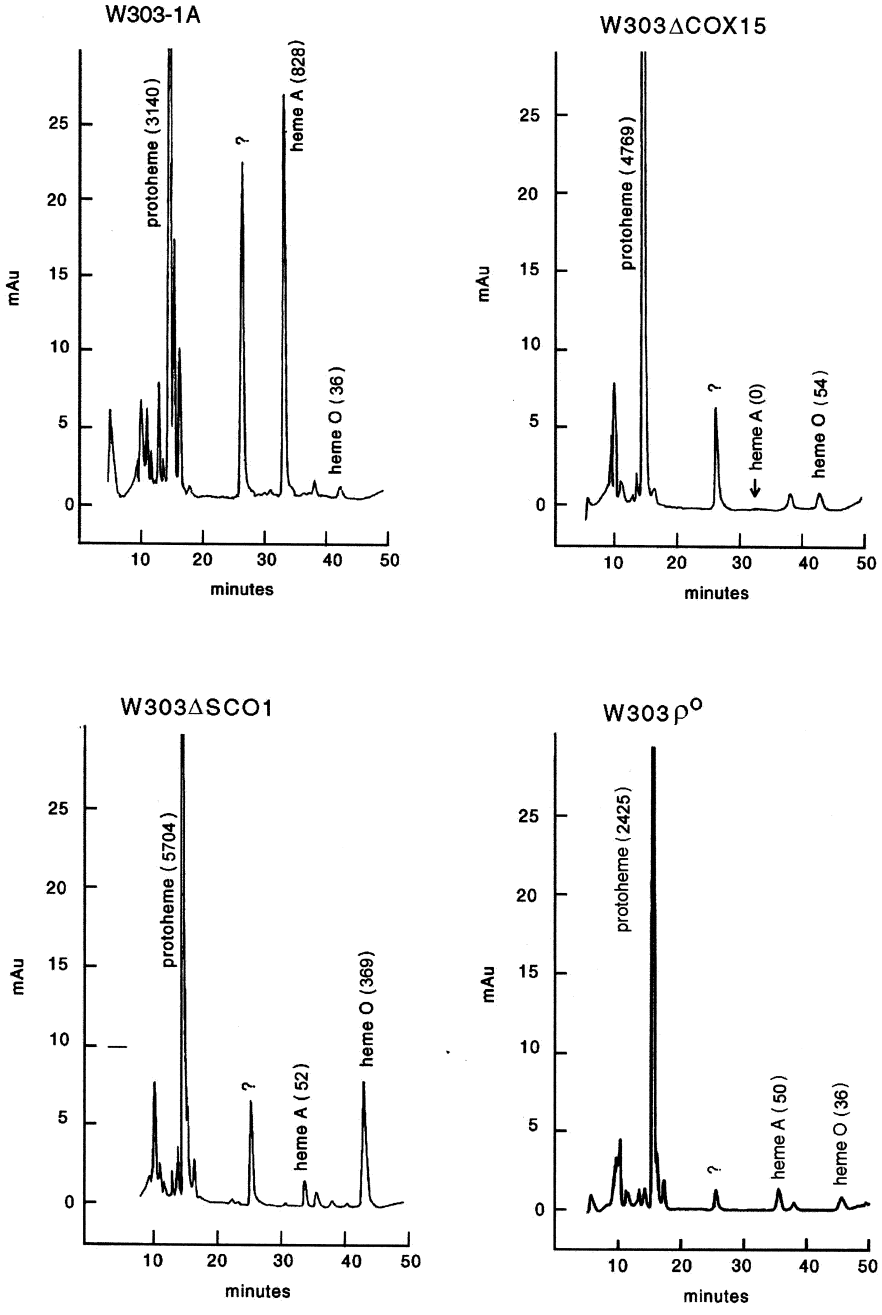

Fig. 1. Analysis of mitochondrial hemes in wild type and mutant mitochondria. Mitochondria (3 mg) of the wild type strain W303-1A, of aW303ΔCOX15 (*cox15* null mutant), aW303ΔSCO1 (*sco1* null mutant), and aW303ρ° (a ρ° derivative of W303-1A) were extracted with acidic acetone and separated by reverse phase chromatography. The protoheme, heme A, and heme O peaks were identified from the elution times of known standards. The peak eluting at 25 min has not been identified. It is unlikely to be related to heme A since it is present in a *cox10* null mutant that is blocked in farnesylation of protoheme (data not shown). The areas under the protoheme, heme A, and heme O peaks were quantitated and are reported in arbitrary units between the parentheses.

mary product expressed from the fusion gene (Fig. 2C). The small difference between the predicted and measured sizes could be due to loss of an N-terminal presequence in Cox15p. The identity of the 67 kDa band as the hybrid protein was supported by its higher concentration in mitochondria of cells transformed with the multicopy plasmid. These results provide strong evidence that complementation of the *cox15* and *yah1* mutants was effected by the hybrid protein rather than by a proteolytic product that might be derived from it.

3.3. Is Cox15p a monooxygenase?

The oxidation of the methyl group at position 8 of the porphyrin ring in heme O to a formyl group requires an initial hydroxylation of the methyl to the corresponding alcohol, followed by the further oxidation of the alcohol to the aldehyde. The initial hydroxylation reaction must be catalyzed by a monooxygenase while the final oxidation step involves a dehydrogenation.

The presence of a single *COX15–YAH1* gene in *S. pombe* and the demonstration that a similar fusion in *S. cerevisiae* is

able to complement mutations in the respective genes suggest that the two proteins function in a common step or pathway. The absence of heme A (but not heme O) in a *cox15* mutant indicates that Cox15p, and by inference Yah1p, catalyzes either the hydroxylation or the dehydrogenation step in the pathway. We propose that the two proteins are part of a mitochondrial (prokaryotic) type monooxygenase that hydroxylates the methyl group of heme O. Accordingly, the third component of this pathway is ferredoxin (adrenodoxin) dehydrogenase encoded by *ARH1* which has been localized in mitochondria of *S. cerevisiae* [13]. As depicted in Fig. 3, the function of Cox15p would be analogous to that of P450 in other three component monooxygenases [14].

3.4. Is Cox15p homologous to the bacterial ctaA product?

The *ctaA* product of *Bacillus subtilis* has been implicated in the hydroxylation or dehydrogenation of heme O [15]. Mutants in *ctaA* have been shown to contain heme O but not heme A [15]. Furthermore, *Escherichia coli* transformed with

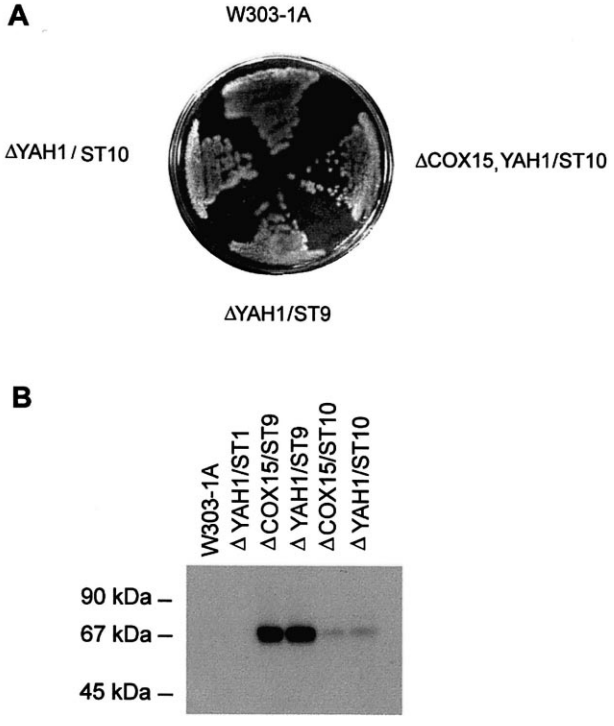

Fig. 2. Properties of yeast transformed with the *COX15–YAH1* fusion gene. A: Complementation of *cox15* and *yah1* null mutants by the *COX15–YAH1* fusion. The following strains were streaked on YPD medium, replicated on rich glycerol medium, and incubated overnight at 30°: W303-1A, parental wild type strain; aW303-ΔYAH1/ST9 (ΔYAH1/ST9), the *yah1* null mutant transformed with a multicopy plasmid containing *COX15-YAH1*; aW303ΔYAH1/ST10 (ΔYAH1/ST10), the *yah1* null mutant with a single integrated copy of *COX15–YAH1*; W303ΔCOX15,YAH1/ST10, the *cox15,yah1* double null mutant with a single chromosomally integrated copy of *COX15–YAH1*. B: Expression of the fusion protein in mutant strains harboring *COX15–YAH1* either on a multicopy plasmid or integrated into chromosomal DNA. Mitochondria (20 μg protein) were prepared from the indicated strains and were separated by PAGE on a 12% polyacrylamide gel. Proteins were transferred to nitrocellulose and probed with an antibody against Yah1p. aW303-ΔYAH1/ST1 (ΔYAH1/ST1) is the *yah1* null mutant transformed with *YAH1* on a multicopy plasmid and aW303ΔCOX15/ST9 (ΔCOX15/ST9) is a *cox15* null mutant with a chromosomally integrated copy of *COX15–YAH1*.

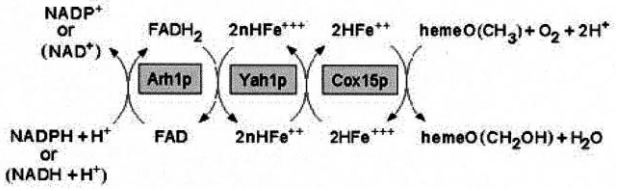

Fig. 3. Scheme showing reactions catalyzed by Arh1p, Yah1p, and Cox15p in hydroxylation of the methyl group in heme O. nHFe refers to the non-heme iron of ferredoxin and HFe to heme iron of Cox15p.

the *B. subtilis ctaA* gene converts heme O to heme A which is normally absent in this organism [3]. The product of *ctaA* has been shown to contain 0.2 mol of protoheme per mol of protein [16]. The substoichiometric amount of protoheme, however, could be a consequence of the fact that the protein was obtained from an overexpressing strain. When *ctaB*, encoding protoheme farnesyl transferase, and *ctaA* were both overexpressed, CtaA was found to contain equimolar amounts of protoheme and heme A [16]. The protoheme was suggested to be a prosthetic group of the enzyme involved in hydroxylation while the heme A was the product formed in the reaction. These findings suggested that in analogy to P450 in three component monooxygenases, CtaA is most likely involved in the reduction of molecular oxygen for the hydroxylation reaction [16].

An alignment of Cox15p with the *ctaA* products of *B. subtilis* and *Staphylococcus aureus* shows a very low but suggestive sequence similarity of the three proteins (Fig. 4). The bacterial proteins are shorter, lacking some 80 N-terminal residues and several shorter internal sequences that are present in Cox15p (Fig. 3). Despite this size difference, Cox15p and CtaA both have seven putative transmembrane domains (not shown). Taken together, the similarities in the primary sequence and domain structure suggest that the mitochondrial and present-day bacterial heme O monooxygenases are evolutionarily related but have undergone substantial divergence.

We were unsuccessful in detecting any sequence similarity between Cox15p and P450. The protoheme prosthetic group of bacterial, mitochondrial, and microsomal P450s is coordinated to the sulfur of cysteine [17]. There are three cysteines in the yeast Cox15p but none is conserved in either the mitochondrial or bacterial CtaA homologs. This is also true of the methionine residues. Three histidines (His-167, -243, and -366), however, are conserved among all the mitochondrial and bacterial proteins and therefore are the most likely candidates to be ligands for protoheme.

According to its proposed function in heme O hydroxylation, Cox15p catalyzes a reaction analogous to the reduction of molecular oxygen by P450. The lack of any sequence similarity between the two proteins suggests that Cox15p may be a novel type of heme monooxygenase.

Two plasmid constructs containing hybrid *COX15/CtaA* failed to rescue the respiratory defect of the *cox15* mutant. Similarly, *E. coli* harboring the yeast *COX15–YAH1* fusion gene did not produce detectable heme A. The inability of the yeast genes to convert heme O to heme A in *E. coli* and of the *Bacillus ctaA* gene to complement the yeast mutant is not too surprising in view of earlier findings that the human mitochondrial Cox15p, ferredoxin, and ferredoxin reduc-

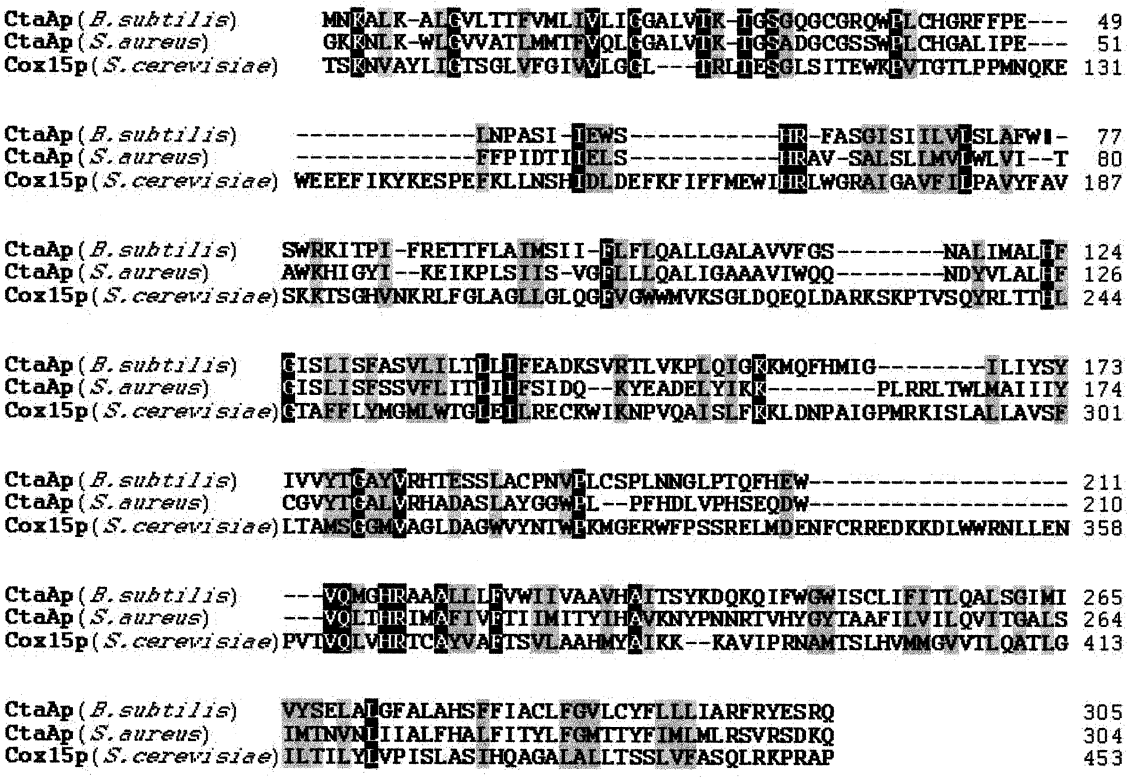

Fig. 4. Alignment of Cox15p with the *ctaA* products of *B. subtilis* and *S. aureus*. The alignment shows identical residues (darkened) and conservative substitutions (shadowed) shared by all three proteins.

tase are not interchangeable with the yeast proteins even though they share extensive sequence similarity (Glerum, personal observation; [8,9]). This apparently does not apply to bacteria where the *Bacillus* monooxygenase when introduced into *E. coli* is able to convert endogenous heme O to heme A [3].

3.5. Localization of Yah1p and Cox15–Yah1p

Cox15p was previously localized in the mitochondrial inner membrane but its topology was not determined [1]. Yah1p, on the other hand, is a soluble matrix protein (Fig. 5A) [9]. The Cox15–Yah1p fusion protein is an integral membrane protein with properties similar to Cox15p (data not shown). It is

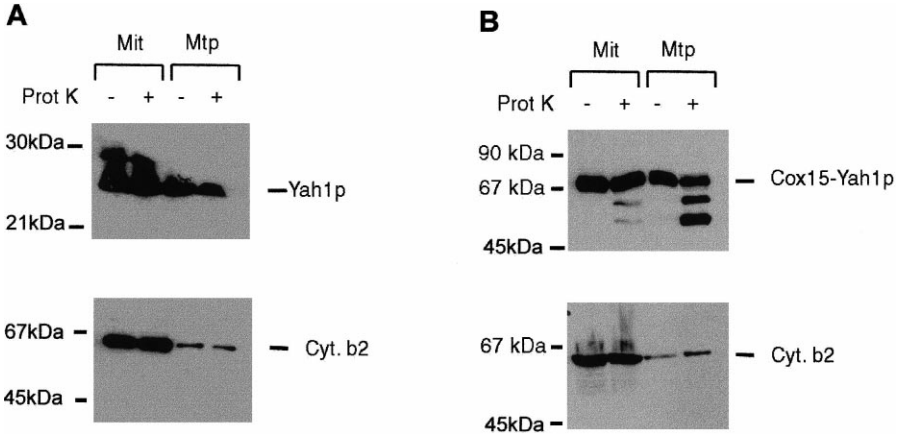

Fig. 5. Localization of Yah1p and the Cox15–Yah1p fusion protein. Mitochondria prepared by the method of Glick and Pon [20] were suspended at a protein concentration of 7 mg/ml and diluted either with seven volumes of 0.6 M sorbitol, 10 mM HEPES, pH 7.5 or with seven volumes of 10 mM HEPES, pH 7.5 buffer to rupture the outer membrane. To one half of each sample was added proteinase K at a final concentration of 40 μg/ml. After incubation at 4°C for 30 min, the mitochondria (Mit) and mitoplasts (Mtp) were collected by centrifugation, washed with 5% trichloroacetic acid and separated by PAGE on a 12% polyacrylamide gel. Proteins were transferred to nitrocellulose and reacted with antibody against Yah1p (upper panels) and separately against the intermembrane marker cytochrome b_2 (Cyt b2, lower panels). The higher molecular weight protein detected by the Yah1p antibody in the mitochondrial fraction is probably some Yah1p precursor that accumulates in the intermembrane space. It is protected against proteinase K in mitochondria and is lost during the conversion of mitochondria to mitoplasts. A: Mitochondria were prepared from the wild type strain W303-1A. B: Mitochondria from W303ΔCOX15,YAH1/ST10, a double mutant with null mutations in *cox15* and *yah1* and harboring a chromosomally integrated copy of *COX15–YAH1*.

present in the mitoplast fraction obtained by hypotonic swelling of mitochondria and is clipped by proteinase K to two smaller fragments (Fig. 5B). Since the antibody used in these experiments recognizes the Yah1p component, the proteinase-sensitive sites must be located in the Cox15p parts of the hybrid protein that are exposed to the intermembrane space. These results further indicate that the C-terminal end of Cox15p, together with Yah1p, is located in the matrix compartment. Yah1p fused to Cox15p is therefore in the same compartment as the native protein (Fig. 5B).

In addition to its proposed function in heme A synthesis, Yah1p has been shown to participate in biogenesis of the iron–sulfur cluster prosthetic groups of mitochondrial and cytoplasmic proteins [18,19]. Expression of Yah1p as part of a larger fusion protein did not affect the growth properties of *S. cerevisiae* on either fermentable or non-fermentable substrates indicating that it is able to support iron–sulfur cluster formation. Yah1p can therefore function with equal efficiency both as a peripheral component of the inner membrane and as a soluble matrix protein in both heme A synthesis and iron–sulfur cluster formation. The Arh1p (ferredoxin reductase) is tightly associated with the mitochondrial inner membrane even though it has no recognizable transmembrane domains [13]. It is possible that there is a further association of Arh1p with the Cox15–Yah1p fusion protein in the membrane that may be necessary for the functions of Yah1p in both the heme A and iron–sulfur cluster synthesis pathways.

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