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Submitochondrial distributions and stabilities of subunits 4, 5, and 6 of yeast cytochrome oxidase in assembly defective mutants

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Abstract The concentration and submitochondrial distribution of the subunit polypeptides of cytochrome oxidase have been studied in wild type yeast and in different mutants impaired in assembly of this respiratory complex. All the subunit polypeptides of the enzyme are associated with mitochondrial membranes of wild type cells, except for a small fraction of subunits 4 and 6 that is recovered in the soluble protein fraction of mitochondria. Cytochrome oxidase mutants consistently display a severe reduction in the steady-state concentration of subunit 1 due to its increased turnover. As a consequence, most of subunit 4, which normally is associated with subunit 1, is found in the soluble fraction. A similar shift from membrane-bound to soluble subunit 6 is seen in mutants blocked in expression of subunit 5a. In contrast, null mutations in *COX6* coding for subunit 6 promote loss of subunit 5a. The absence of subunit 5a in the *cox6* mutant is the result of proteolytic degradation rather than regulation of its expression by subunit 6. The possible role of the ATP-dependent proteases Rca1p and Afg3p in proteolysis of subunits 1 and 5a has been assessed in strains with combined mutations in *COX6*, *RCA1*, and/or *AFG3*. Immunochemical assays indicate that another protease(s) must be responsible for most of the proteolytic loss of these proteins.

Key words: Yeast mitochondrion; Cytochrome oxidase; *RCA1*; *AFG3*

1. Introduction

Cytochrome oxidase mutants of *Saccharomyces cerevisiae* share a common phenotype, characterized by pronounced reductions in the steady-state concentrations of some constituent polypeptides, particularly hydrophobic products of the mitochondrial genetic system. The loss of these constituents is the result of proteolysis and occurs even in strains unable to complete assembly of the holoenzyme for lack of a prosthetic group [1–3]. These observations suggest that only the fully formed complex is protected from the action of endogenous proteases. The exceptions are mutations in subunits 6a, 6b, and 8 that do not affect the stability of the enzyme [4–6].

The X-ray crystallographic structure of bovine cytochrome oxidase has provided a wealth of information about the subunit interactions in the mitochondrial enzyme [7]. The yeast enzyme probably has a very similar structure since its subunit composition is almost identical to that of bovine cytochrome oxidase [8]. In the present study we attempt to explain the biochemical properties of different cytochrome oxidase mutants in the context of the recently reported structure.

2. Materials and methods

2.1. Yeast strains and growth media

The strains of *S. cerevisiae* used in this study are listed in Table 1. The compositions of the media used to grow yeast have been described [1]. Mitochondria were isolated by the method of Faye et al. [10] from yeast grown in 2% galactose, 1% yeast extract, and 1% peptone.

2.2. Strain constructions

Null alleles of *PET309*, *COX4*, *COX5*, and *COX6* were introduced in the respiratory competent strains *S. cerevisiae* W303-1A or W303-1B. *PET309* was disrupted by removing a 1.2 kb fragment between the two *Bam*HI sites in the gene and replacing it with a 1.8 kb *Bam*HI fragment containing *HIS3*. The *COX4* disrupted allele was made by deleting the internal 114 bp *Kpn*I fragment and inserting a 1 kb *Kpn*I fragment containing *URA3* in the gap. *COX5* was disrupted by removal of a 576 bp *Bgl*II–*Pst*I, of which 298 bp consisted of coding sequence and inserting the *HIS3* gene on a 1 kb *Bam*HI fragment. The *COX6* disruption was a simple insertion of a 1 kb *Hin*dIII fragment with *URA3* at the *Hin*dIII site in the *COX6* coding sequence.

To make the *lac*Z fusion, a 571 bp *Pst*I fragment (−273 to +298), containing the upstream and part of the coding region of *COX5*, was ligated to the *Pst*I site of the integrative plasmid YIp366 [11]. The resultant plasmid (pG46/ST20) with an in-frame fusion to *lac*Z was linearized at the unique *Bst*EII site in the *LEU2* gene of the plasmid. The linear plasmid was integrated at the *leu2* loci of W303-1A, W303ΔCOX5 and W303ΔCOX5ΔCOX6. Three independent transformants, selected for leucine prototrophy, were assayed for β-galactosidase activity [12].

2.3. Miscellaneous procedures

Standard procedures were used for the preparation and ligation of DNA fragments, and for transformation and recovery of plasmid DNA from *E. coli* [13]. Protein concentrations were determined by the method of Lowry et al. [14]. For Western blot analysis, proteins were separated either on 12% [15] or 16.5% acrylamide gels [16]. After transfer to nitrocellulose, the blots were reacted with antibodies specific for different cytochrome oxidase subunits, followed by exposure to ^{125}I-protein A, according to the protocol of Schmidt et al. [17].

3. Results and discussion

3.1. Submitochondrial distribution of 4, 5a, and 6 in cytochrome oxidase mutants

Subunits 4, 5a, and 6 of yeast cytochrome oxidase are encoded by the nuclear genes *COX4*, *COX5a*, and *COX6*, respectively [18–20]. These proteins are part of cytochrome oxidase and are present in a stoichiometry of 1 : 1 : 1 [21]. Subunit 4 is located on the matrix side of the inner membrane. It does not penetrate into the lipid bilayer and is associated

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Abbreviations: PET, yeast nuclear gene essential for mitochondrial function; kb and bp, kilobase pair and base pair, respectively; PAGE, polyacrylamide gel electrophoresis

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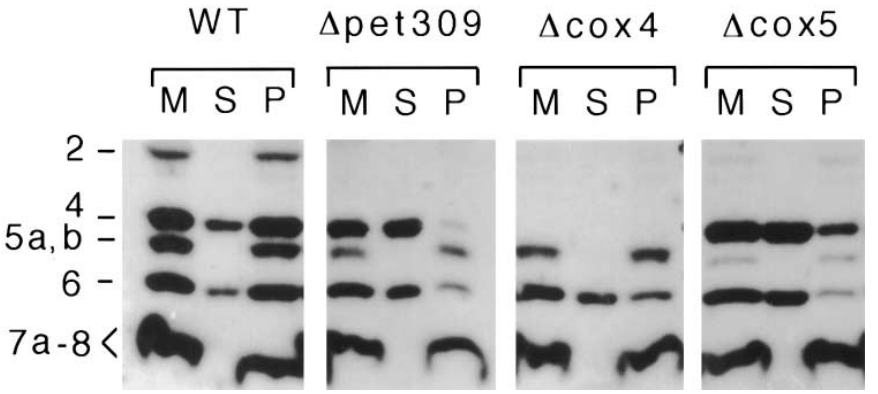

Fig. 1. Steady-state concentrations and distribution of subunits 2, 4, 5, and 6 in cytochrome oxidase mutants. Mitochondria were prepared from the respiratory competent haploid strain W303-1B (WT) and from mutants carrying disruptions in *PET309* (ΔPET309), *COX4* (ΔCOX4), and *COX5a* (ΔCOX5). Mitochondria suspended at protein concentrations of 10 mg/ml were sonically irradiated for 10 s with a Branson microprobe at a power output of 30 and centrifuged at $105\,000 \times g_{av}$ for 20 min. The supernatant was removed and the membrane pellet suspended in the starting volume of 20 mM Tris-HCl, pH 7.5. Equivalent volumes of mitochondria (M), of the supernatant (S) and membrane particles (P) were separated on a 16.5% polyacrylamide gel. The unfractionated mitochondria in all cases corresponded to 16 μg of protein. Following transfer to nitrocellulose, the cytochrome oxidase proteins were detected with an antibody against purified yeast cytochrome oxidase. The antibody detects subunits 2, 4, 5a, 5b, 6, and the smaller polypeptides of the complex. The identity of the cytochrome oxidase subunits is indicated in the left hand margin.

with subunits 1 and 3 of the complex [7]. This is also true of subunit 6, except that it interacts with subunits 5a and 7a, both of which span the membrane once and have domains extending into the matrix compartment that provide the anchoring sites for subunit 6. Subunit 1 and 5a are complexed through their hydrophobic transmembrane domains [7].

The distribution of subunits 4, 5a, and 6 between the soluble and membrane phases of mitochondria from respiratory competent yeast was examined by Western blot analysis using an antibody against the holoenzyme. Mitochondria of the respiratory competent haploid strain W303-1B were disrupted by a brief sonic treatment to release the soluble matrix proteins. Immunoblots of the starting mitochondria and of the membrane vesicles and soluble protein fraction show these constituents to be present largely in the membrane vesicles. A small percentage of subunits 4 and 6 (but not subunit 5a), however, is also detected in the soluble fraction (Fig. 1). These may represent a pool of unassembled subunits and/or subunits loosely bound to partially assembled complexes. Subunits 7a, 7, and 8 are not separated in the gel and are detected as a single composite band in the membrane fraction.

A similar analysis was carried out on several different mutants blocked in cytochrome oxidase assembly. Mutations in *PET309*, shown to be necessary for maturation/stability of the mitochondrial subunit 1 mRNA, lead to a deficiency of subunit 1 [22]. Western blot assays of cytochrome oxidase subunits in a *pet309* null mutant show that while the mitochondrial concentration of subunit 4 is comparable to that of wild type, it is recovered almost entirely in the soluble protein fraction (Fig. 1). The absence of membrane bound subunit 4 is consistent with its association with subunits 1 and 3. The inability of the *pet309* mutant to express subunit 1 also elicits a secondary deficiency in subunits 2 and 5a. The absence of subunit 2 must be the result of proteolysis since pulse-labeling experiments indicate that this constituent is synthesized in *pet309* [22]. The additional loss of subunit 5a in *pet309* probably stems from a higher turnover rate as well, but the effect is not as pronounced. Subunit 5a has a single transmembrane domain and therefore remains membrane bound in the mutants as well as wild type. Although the concentration of subunit 6 in the *pet309* mitochondria is not significantly different from the wild type, like subunit 4 most of it is present in the soluble fraction. A partial explanation for this may lie in the reduced level of subunit 5a with which it normally inter-

Table 1
Genotypes and sources of yeast 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
W303ΔCOX4	α *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 cox4::URA3*	This study
W303ΔCOX5	α *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 cox5::HIS3*	This study
W303ΔCOX6	α *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 cox6::URA3*	This study
W303ΔCOX5ΔCOX6	α *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 cox5::HIS3 cox6::URA3*	aW303ΔCOX5×W303ΔCOX6
aW303ΔRCA1	a *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 rca1::URA3*	[9]
aW303ΔAFG3	a *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 afg3::HIS3*	[9]
aW303ΔRCA1ΔAFG3	a *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 rca1::URA3 afg3::HIS3*	[9]
W303ΔRCA1ΔCOX6	α *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 rca1::URA3 cox6::URA3*	W303ΔCOX6×aW303ΔRCA1
W303ΔAFG3ΔCOX6	a *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 afg3::HIS3 cox6::URA3*	W303ΔCOX6×aW303ΔAFG3
W303ΔRCA1ΔAFG3ΔCOX6	a *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 rca1::URA3 afg3::HIS3 cox6::URA3*	W303ΔCOX6×aW303ΔRCA1-ΔAFG3
aW303ΔPET309	a *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 pet309::HIS3*	This study

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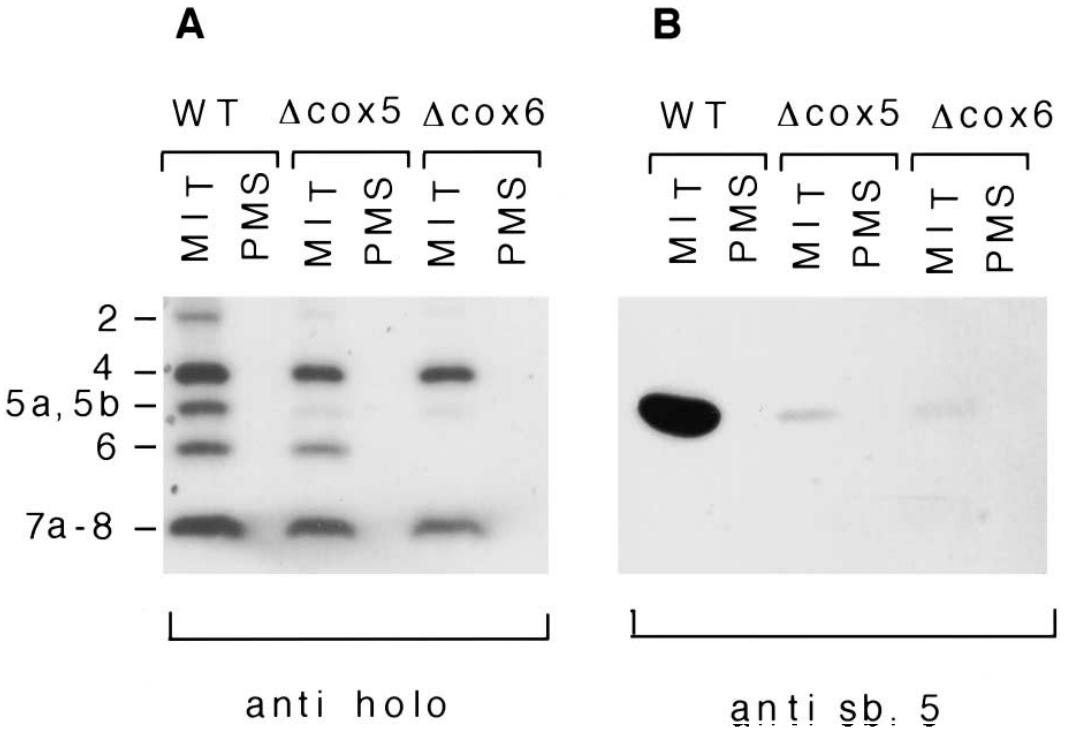

Fig. 2. Steady-state concentration of subunit 5 in a *cox6* null mutant. Panel A: Mitochondria and the post-mitochondrial supernatant fraction were prepared from W303-1B (WT), a respiratory competent haploid strain and from the cytochrome oxidase deficient mutants W303ΔCOX5 (ΔCOX5) and W303ΔCOX6 (ΔCOX6) having null mutations in *COX5* and *COX6*, respectively. Equivalent amounts of each fraction (20 μg protein) were separated on a 15% gel, transferred to nitrocellulose and reacted with antibody against native cytochrome oxidase. Panel B: The mitochondria and the post-mitochondrial supernatant fractions from the same strains of yeast were reacted with antibody against purified subunit 5. Cytochrome oxidase subunits are identified in the right hand margin.

acts. A similar phenotype is seen in the *cox4* null mutant, which also displays a somewhat lower concentration of subunit 5a and an absence of subunit 2 (Fig. 1).

Yeast have two isoforms of subunit 5 encoded by *COX5a* and *COX5b* [23]. Since subunit 5a, expressed from *COX5a*, is by far the more abundant of the two, mutations in this gene elicit a strong respiratory defect. Nonetheless, expression of the minor subunit 5b homolog from the second gene allows *cox5a* mutants to make a low but measurable level of cytochrome oxidase. This is also evident from the immunoblots of the mitochondrial fractions isolated from a *cox5a* null mutant (Fig. 1). In this mutant, the faint signal detected at the position of subunit 5 corresponds to the protein derived from *COX5b*. Aside from the absence of subunit 2 (for the same reason discussed above), the most striking feature of the *cox5a* mutant is the almost complete recovery of subunit 6 as a soluble protein. This observation suggests that either the interaction with subunit 7a is too weak to anchor subunit 6 to the membrane or that subunit 7a itself is also proteolytically degraded in this mutant. The fact that a low level of subunit 4 is present in the membrane fraction also indicates some cytochrome oxidase assembly in this mutant.

3.2. *Protection of subunit 5a by subunit 6*

The steady-state concentration of cytochrome oxidase subunits was also examined in the *cox6* null mutant W303ΔCOX6. Antibodies against either the holoenzyme or purified subunit 5 revealed that the *cox6* mutant had virtually undetectable levels of subunit 5a in mitochondria and in the post-mitochondrial supernatant fraction. The faint signal detected in mitochondria is probably subunit 5b, since a signal of equal intensity is seen in the *cox5a* null mutant W303ΔCOX5 (Fig. 2).

The severe depletion of subunit 5a in the *cox6* mutant background could be due to (1) failure of the protein to be imported, (2) decreased transcription or translation, (3) high rates of proteolytic turnover. The absence of subunit 5a in the post-mitochondrial supernatant fraction makes a transport defect improbable. Northern analysis of polyA-enriched RNA showed *COX5a* mRNA abundance to be approximately the same in the mutant and wild type, excluding a transcriptional defect (data not shown). The possibility that translation of the *COX5a* mRNA might be regulated by subunit 6 was tested with *lac*Z fusions. A *lac*Z construct containing 273 bp of the *COX5* 5′-untranslated region was integrated at the *leu2* locus of the respiratory competent haploid yeast W303-1A, and of the respiratory deficient mutants W303ΔCOX5 and W303ΔCOX5ΔCOX6. The β-galactosidase activities measured in the three different strains were not significantly different (data not shown), confirming that the *cox6* mutation does not affect transcription of *COX5a* and is also unlikely to exert a regulatory effect on translation of the mRNA.

The results of the Northern analysis and the *lac*Z fusion experiments argue strongly against a direct role of subunit 6 in expression of subunit 5a, but rather suggest that proteolysis of this protein is the most likely explanation for its absence in *cox6* mutants. Protection of subunit 5a by subunit 6 against endogenous proteases would imply that the two proteins are physically associated with one another, even in mutants unable to form the holoenzyme. In *pet309* and *cox4* mutants subunit 6 is found in the soluble protein fraction of disrupted mitochondria (Fig. 1). This indicates that the interaction of

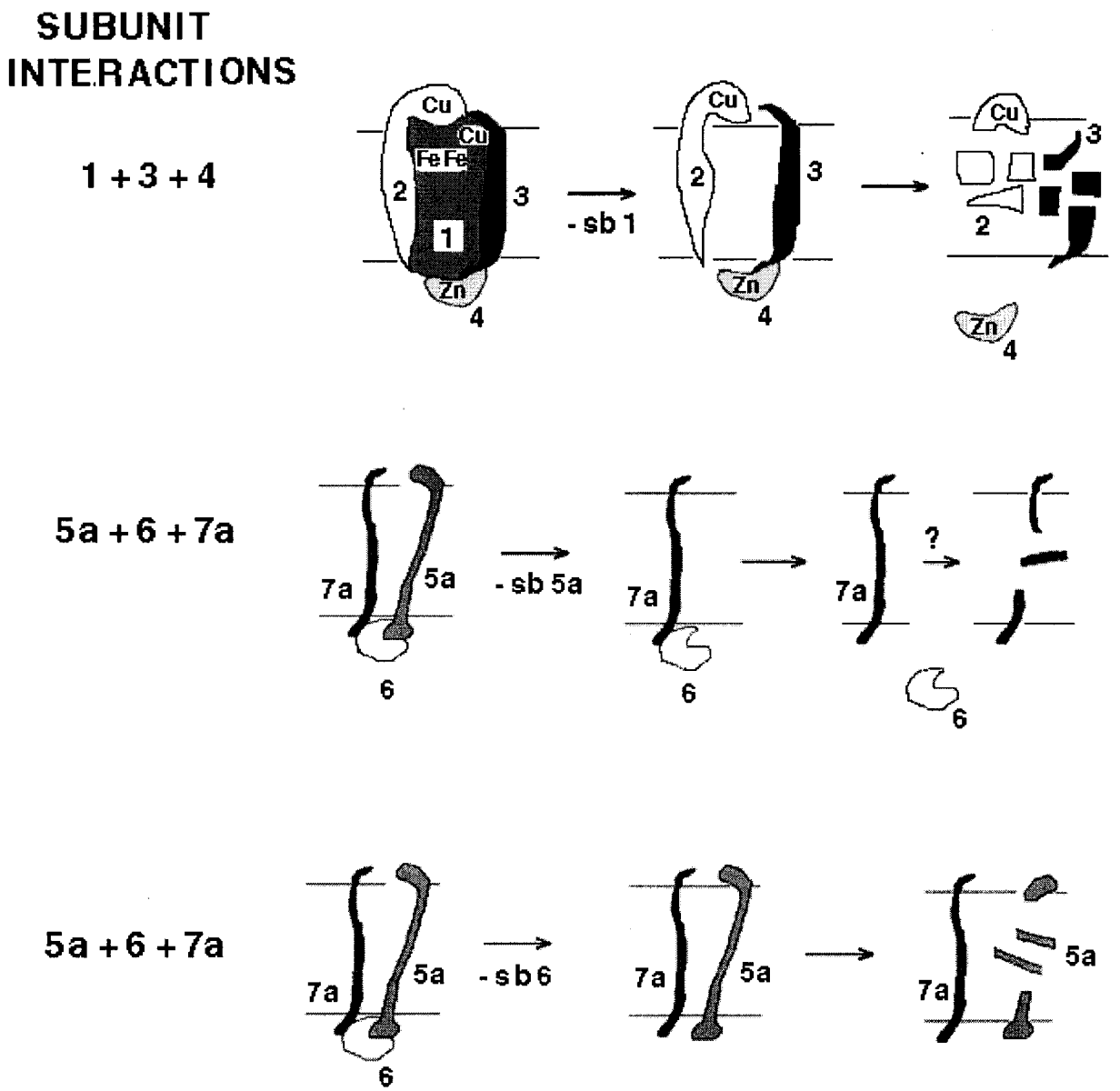

Fig. 3. Subunit interactions and proteolysis in mutants lacking subunits 1, 5a, and 6 of cytochrome oxidase. The subunit contacts are based on the structure of the bovine homologs [7]. In the top row, loss of subunit 1 results in proteolysis of subunits 2 and 3 (loss of subunit 3 is inferred but not experimentally verified in this study) and the dissociation of subunit 4 from the membrane. In the middle row the absence of subunit 5a labilizes the interaction of subunit 6 with the membrane. The loss of subunit 7a is hypothetical. The bottom row shows proteolysis of subunit 5a when subunit 6 is missing.

subunits 5a and 6 is too labile to be maintained even under the relatively mild sonic treatment used to fragment mitochondrial membranes. Attempts to detect such a complex by using detergents instead of physical means to disrupt mitochondria were also unsuccessful.

The results of the immunoblot assays are diagrammatically summarized in Fig. 3. It is of interest to note that the more hydrophobic constituents such as subunits 1, 2, 5a (and probably subunit 3) appear to be more prone to proteolytic degradation in assembly-arrested mutants. Subunits 4 and 6, whose associations with the inner membrane are entirely through contacts with integral membrane proteins, are released into the matrix and are relatively stable. This suggests that partially assembled hydrophobic proteins, perhaps because of their deleterious effect on membrane based processes, are rapidly removed by the action of local proteases.

3.3. *Is subunit 5a degradation prevented in rca1 and afg3 mutants?*

RCA1/YTA12 and *AFG3/YTA10* code for mitochondrial inner membrane proteins [24,25] that belong to the AAA-family [26]. Recent evidence indicates that Rca1p and Afg3p are subunits of an ATP-dependent protease [27] responsible for proteolytic clearing of improperly assembled proteins of mitochondria. Mutations in these genes have been shown to slow the rate of proteolytic degradation of some inner membrane proteins [28,29].

To further explore the biochemical basis for the subunit 5a

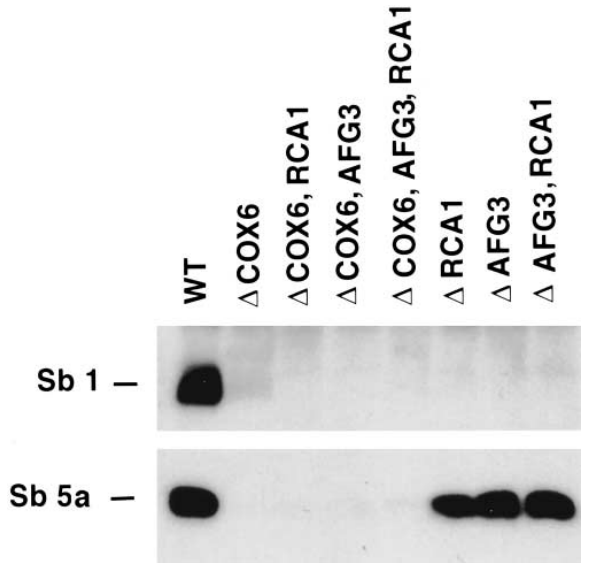

Fig. 4. Stability of subunits 1 and 5a in *afg3* and *rca1*. Mitochondria were prepared from the wild type haploid W303-1B (WT), the *cox6* null mutant W303ΔCOX6 (ΔCOX6) and from W303-ΔCOX6ΔRCA1 (ΔCOX6,RCA1), W303ΔCOX6ΔAFG3 (ΔCOX6, AFG3), W303ΔCOX6ΔRCA1ΔAFG3 (ΔCOX6,RCA1,AFG3), aW303ΔRCA1 (ΔRCA1), aW303ΔAFG3 (ΔAFG3), aW303ΔRCA1-ΔAFG3 (ΔRCA1,AFG3). Total mitochondrial proteins (12 μg) were separated on a 12% polyacrylamide gel, transferred to nitrocellulose, and probed with an antibody that recognizes subunit 1 (upper panel) and subunit 5a/5b (lower panel) of cytochrome oxidase.

deficiency, and in particular to assess the possible roles of Rca1p and/or Afg3p in degradation of this subunit, the *cox6* null mutation was introduced into strains with either single or double mutations in *RCA1* and *AFG3*. These strains were used to measure the steady-state concentration of subunit 5a in mitochondria. The results of the Western blot analyses indicate that mutations in *RCA1* or *AFG3* alone or in combination do not appear to spare subunit 5a (Fig. 4). In agreement with earlier results [9], mutations in the two ATP-dependent proteases by themselves do not appreciably affect the mitochondrial concentration of subunit 5a (Fig. 3).

Subunit 1 is not detected in the *rca1* and *afg3* single or double mutants. The absence of subunit 1 is very probably due to proteolysis, since pulse labeling experiments indicate that the *rca1* mutants synthesize this protein [24]. This is also true of *afg3* mutants, although in this case synthesis of subunit 1 is not as efficient as in wild type [28,30]. The proteolytic loss of subunit 1 in the *rca1* and *afg3* mutants independent of the *cox6* mutation is consistent with earlier evidence indicating that such mutants are defective in assembly of cytochrome oxidase [9,24]. The lack of a clear effect of the *rca1* or *afg3* mutations on the steady-state concentrations of subunits 1 or 5a in the *cox6* background suggests that a protease(s) other than Rca1p and Afg3p must be responsible for the increased turnover of these membrane constituents when assembly of cytochrome oxidase is blocked.

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