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Mutations in *RCA1* and *AFG3* inhibit F_1-ATPase assembly in *Saccharomyces cerevisiae*

Marie-Françoise Paul, Alexander Tzagoloff*

Department of Biological Sciences, Columbia University, New York, NY 10027, USA

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Abstract The *RCA1* (*YTA12*) and *AFG3* (*YTA10*) genes of *Saccharomyces cerevisiae* code for homologous mitochondrial proteins that belong to the recently described AAA protein-family [Kunau et al. (1993) Biochimie 75, 209–224]. Mutations in either gene have been shown to induce a respiratory defect. In the case of *rca1* mutants this phenotype has been ascribed to defective assembly of cytochrome oxidase and ubiquinol-cytochrome *c* reductase. In the present study we show that the respiratory defect of *afg3* mutants, like that of *rca1* mutants, is also caused by an arrest in assembly of cytochrome oxidase and ubiquinol-cytochrome *c* reductase. In addition to the absence of the respiratory complexes, *rca1* and *afg3* mutants exhibit reduced mitochondrial ATPase activity. As a first step to an understanding of the biochemical basis for the ATPase defect we have examined the assembly of the F_1 and F_0 constituents of the ATPase complex. We present evidence that the ATPase lesion stems at least in part from the failure of *rca1* and *afg3* mutants to assemble F_1. Although the mutants also display lower steady-state concentrations of some F_0 subunits, this could be a secondary effect of defective F_1 assembly.

Key words: F_1-ATPase; ATPase assembly; *AFG3* (*YTA10*); *RCA1* (*YTA12*); *Saccharomyces cerevisiae*

1. Introduction

The AAA-protein family designation has been proposed [1] for the classification of a large number of proteins that share a common ATPase domain. This group of proteins, widely distributed in prokaryotic and eukaryotic organisms, play important roles in cell division [2], transcription [3], membrane fusion [4], and enzyme assembly [5]. At present, however, their precise functions/activities in these cellular processes are not known. *Saccharomyces cerevisiae* has at least 10 different proteins that are members of the AAA-protein family [6], and of these five have been localized to mitochondria. Two of the yeast mitochondrial proteins are encoded by nuclear genes *RCA1* (*YTA12*) and *AFG3* (*YTA10*) [5–7]. Mutations in each gene express a respiratory defect [5,7] which in the case of *rca1* mutants has been correlated with defects in the cytochrome oxidase, ubiquinol-cytochrome *c* reductase, and the F_0–F_1 ATPase complexes of the mitochondrial inner membrane [5]. This pleiotropic phenotype stems not from a failure of *rca1* mutants to synthesize either the nuclear or mitochondrially synthesized subunits of the respiratory and ATPase complexes, but rather from a block in assembly of the subunits into the functional enzymes [5].

In this study we have extended the characterization of *rca1* and *afg3* mutants. We present evidence showing that mutations in *RCA1* and *AFG3* express similar phenotypes and that the products of these genes are therefore likely to have related functions. Secondly we show that the earlier noted deficiency of mitochondrial ATPase activity in *rca1* mutants is caused by impaired assembly of F_1. A similar arrest of F_1 assembly is observed in *afg3* mutants. To our knowledge this is the first instance of nuclear encoded proteins that affect assembly of a broad range of mitochondrial enzyme including F_1.

2. Materials and methods

2.1. *Strains of* S. cerevisiae *and growth media*

The genotypes of the mutant and wild type yeast strains used in this study are listed in Table 1. The media used for the routine cultivation of yeast have been described previously [5].

2.2. *Cloning and disruption of* AFG3

The wild type *AFG3* gene was cloned by transformation of the mutant C125/LA1 (*α ade1-1 leu2-3,112 afg3-1*) with a yeast genomic DNA library. This respiratory deficient strain, along with 7 other independent isolates, was previously assigned to complementation group G73 of our collection of *pet* mutants [9]. The library used for the transformation of C125/LA1 was constructed by ligating partial *Sau*3A fragment of nuclear DNA from the respiratory competent strain D273-10B to the *Bam*H1 site of the yeast/*E. coli* shuttle vector YEp13 [10]. A disrupted allele of *RCA1* was introduced into respiratory proficient yeast by the one-step gene replacement procedure [11]. The mutant allele was constructed by substituting a 328 bp *Bgl*II fragment internal to *AFG3* with a 1.7 kb *Bam*H1 fragment containing the yeast *HIS3* gene. A linear fragment containing the resultant *afg3::HIS3* allele and flanking sequences was used to transform the respiratory competent strains of yeast W303-1A and W303-1B. Histidine prototropic and respiratory defective transformants were verified genetically and by Southern analysis of genomic DNA to have acquired the mutant allele.

2.3. *Preparation and assay of mitochondria*

Yeast were grown aerobically to early stationary phase in 2% galactose medium containing 1% yeast extract and 2% peptone and mitochondria were prepared by the method of Faye et al. [12] except for the use of Zymolyase 20,000 instead of Glusulase to prepare spheroplasts. Alternatively mitochondria were obtained from cells homogenized with glass beads [13].

Mitochondrial translation products were labeled in whole cells with [^{35}S]SO_4 (carrier free, ICN Biomedicals, Inc.) [14]. Proteins were separated by PAGE in 12.5% polyacrylamide gels prepared according to Laemmli [15] except for the addition of urea and glycerol to final concentrations of 4M and 30%, respectively. Proteins were detected immunologically, after electrophoretic transfer to nitrocellulose, by incubation of the blot with diluted antiserum, followed with a secondary reaction with [^{125}I]Protein A. Assay conditions for ATPase, cytochrome oxidase, NADH-cytochrome *c* reductase and spectral analysis of cytochromes were as described [13]. Protein was measured by the method of Lowry et al. [16].

*Corresponding author.

Abbreviations: AAA protein-family is defined as ATPase associated with diverse cellular activities [1].

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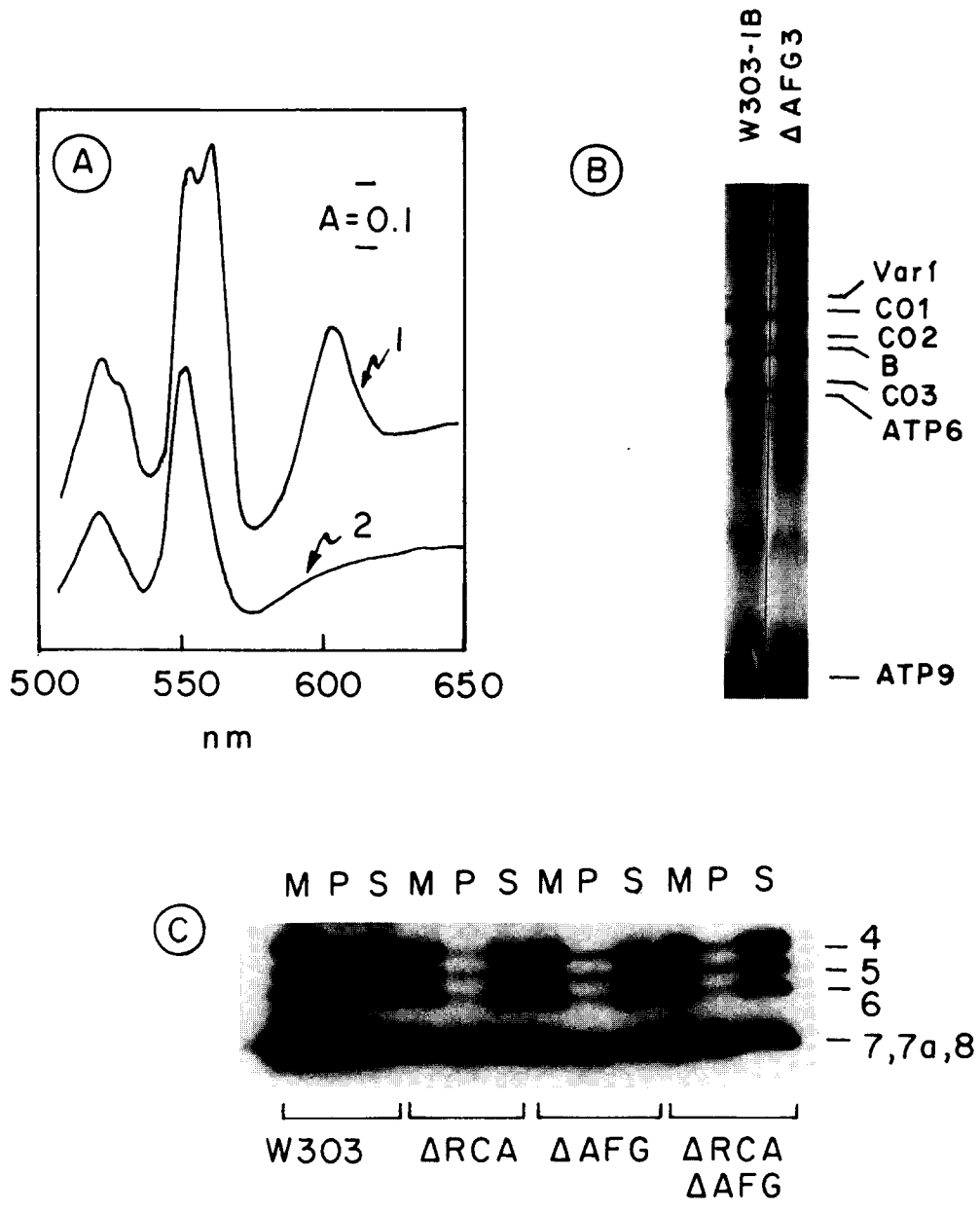

Fig. 1. (A) Spectra of mitochondrial cytochromes in the wild type strain W303-1B and in the *afg3* mutant W303ΔAFG3. Mitochondrial suspensions at a final concentration of (6 mg/ml) were extracted with deoxycholate under conditions that quantitatively solubilize cytochrome [13]. Absorption spectra of the extracts from the wild type (tracing 1) and mutant (tracing 2) strains were recorded as the difference between oxidized (ferricyanide) and reduced (dithionite) samples. (B) Mitochondrial translation products in wild type and in *afg3* mutants. The respiratory competent parental strain W303-1B and the *afg3* mutant W303ΔAFG3 (ΔAFG3) were grown in rich glucose medium to stationary phase and the mitochondrial translation products were labeled in vivo with ^{35}S in the presence of cycloheximide to inhibit cytoplasmic protein synthesis. Total mitochondrial proteins (50 μg) were separated in a 12.5% polyacrylamide gel. The gel was dried and exposed to Kodak XAR film. The mitochondrially translated subunits of cytochrome oxidase (CO1, CO2, CO3), cytochrome *b* (B), ribosomal subunit Var1 (Var1), and subunit 6 (ATP6) and 9 (ATP9) of the ATPase are identified in the right-hand margin. (C) Steady-state concentrations of nuclearly encoded subunits of cytochrome oxidase in mitochondria of wild type, and of *rca1* and *afg3* mutants. Mitochondria from the parental respiratory competent strain W303-1B (W303), from the single mutants W303ΔRCA1 (ΔRCA) and W303ΔAFG3 (ΔAFG), and the double mutant W303ΔRCA1/AFG3 (ΔRCA,ΔAFG) were extracted at a protein concentration of 12.5 mg/ml in the presence of 0.75% potassium deoxycholate and 0.5 M NaCl. The protein pellet obtained after centrifugation at 105,000 × g_{av} for 15 min was resuspended in the starting volume of buffer. Samples containing 10 μg of the starting mitochondria (M) and equivalent volumes of the extracts (S) and pellets (P) were separated by PAGE on a 16.5% polyacrylamide gel. The proteins were transferred to nitrocellulose and probed with a polyclonal antibody against all the nuclear subunits of cytochrome oxidase. The cytoplasmically synthesized subunits of cytochrome oxidase are identified in the right-hand margin. The unassembled subunits in the mutants are more readily extractable as evidenced by their relative distribution in the pellet and supernatant fractions.

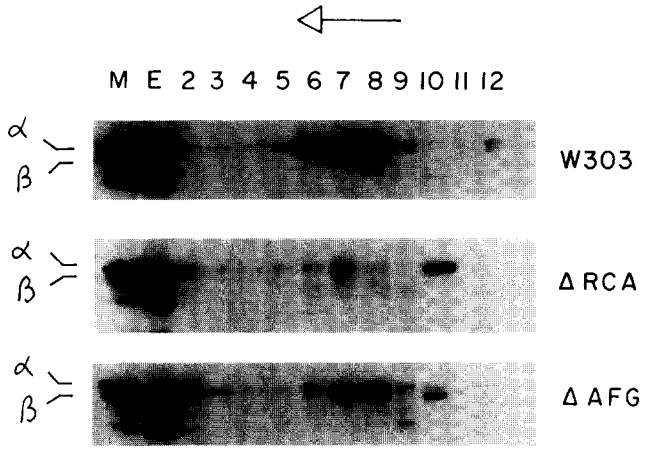

Fig. 2. Sedimentation of F_1 in sonic extracts of wild type and mutant mitochondria. Mitochondria were prepared from the parental strain W303-1B (W303), and from the mutants W303ΔRCA1 (ΔRCA) and W303ΔAFG3 (ΔAFG). They were suspended at a protein concentration of 6 mg/ml in buffer containing 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 2 mM ATP (TEA) and were sonically irradiated with the microprobe of a Branson Sonifier for 3 bursts of 10 s each. All subsequent steps were carried out at room temperature. The suspensions were centrifuged at $105{,}000 \times g_{av}$ for 20 min. The supernatants (0.5 ml) were collected and layered over a linear 6%–20% sucrose gradient built on top of a 0.7 ml cushion of 80% sucrose. Both the cushion and gradient contained TEA and 0.1% Triton X-100. The gradients were centrifuged in a Beckmann SW65 rotor at 65,000 rpm for three hours. The gradients were collected in twelve equal size fractions. Fraction 2 contains the material at the interface between the cushion and the gradient. The mitochondria (M), the sonic extract (E) and fractions 2–12 of the gradient were separated by PAGE on a 10% polyacrylamide gel. Proteins were transferred electrophoretically to nitrocellulose and reacted with a mixture of antibodies against the α and β subunits of F_1. The amount of extract and gradient fractions applied to the gel corresponded to two volume equivalents of the starting mitochondria. The positions of the α and β subunits are marked in the left-hand margin of each panel.

3. Results and discussion

3.1. *Mutations in* AFG3 *affect assembly of respiratory chain complexes and the proton translocating ATPase of mitochondria*

The mutant used to clone *AFG3* and the strains obtained by in situ disruption of the chromosomal copy of the gene are deficient in cytochrome oxidase, ubiquinol-cytochrome *c* reductase, and have reduced mitochondrial ATPase activity (data not shown). The respiratory chain defect is also manifested by the cytochrome composition of mutant mitochondria which exhibit the absence of both cytochrome *b* and cytochrome a,a_3 (Fig. 1A). This pleiotropic phenotype is generally observed in strains defective in mitochondrial protein synthesis because of the contribution by this translational system of a subset of the subunits of the respiratory and ATPase complexes. The results of in vivo labeling experiments, however, indicate that *afg3* mutants are capable of synthesizing all the mitochondrial gene products (Fig. 1B).

The deficiency of the respiratory and ATPase complexes in *afg3* mutants, even though they are able to express the mitochondrially encoded subunits of these enzymes, constitutes a very rare phenotype. Among more than one hundred different complementation groups representative of *pet* mutants, only mutations in *RCA1* have been noted to have this property. The absence of ubiquinol-cytochrome *c* reductase and cytochrome oxidase in *rca1* mutants was previously attributed to an assembly arrest at a post-translational step [5]. This appears to be true of *afg3* mutants as well. For example, the absence of cytochrome oxidase in *afg3* mutants is not due a block in either synthesis or processing of the mitochondrially or cytoplasmically synthesized subunits of the enzyme. Pulse-labeling data indicate normal synthesis of the mitochondrially encoded subunits 1–3 (Fig. 1B). Western analysis of total mitochondrial proteins also fail to disclose any significant differences in the concentration of the nuclear gene products subunits (Fig. 1C). Similar results were obtained when the immunological probings were extended to the subunits of the ubiquinol-cytochrome *c* complex (data not shown).

3.2. *Mutations in* RCA1 *and* AFG3 *retard assembly of* F_1

The mitochondrial ATPase complex of *Saccharomyces cerevisiae* is composed of at least 10 different subunits polypeptides [17]. Five subunits make up the F_1 ATPase that contains the nucleotide biding and catalytic sites of the complex [18]. The synthesis of F_1 subunits and their assembly into a catalytically active oligomer are not coupled to the synthesis of F_0. Strains unable to synthesize the mitochondrially or nuclearly encoded subunits of F_0 contain normal amounts of fully assembled and functional F_1 in mitochondria [19], although the enzyme is not linked to the inner membrane in any stable manner [20]. Until now, mutations impairing F_1 synthesis have been confined to the structural genes and to two other nuclear genes (*ATP11* and *ATP12*) which appear to be involved specifically in the assembly of this enzyme [21].

The effect of mutations in *RCA1* and *AFG3* on F_1 assembly was assessed in two ways. To determine whether the mutants synthesize and import the α and β subunit of F_1, the concentrations of these proteins in mitochondria was assayed immunologically by the Western method. Since the mutants were found to have normal concentrations of these subunits, the reduced ATPase activity did not appear to be related to inhibition of synthesis or import of the proteins.

An explanation for the ATPase defect emerged from a comparison of the sedimentation properties of the F_1 subunits in extracts of wild type and mutant mitochondria. The ATPase was extracted either by sonic disruption of mitochondria under

Table 1
Sources and genotypes of *S. cerevisiae* strains

Strain	Genotype	Source
D273-10B/A1	α *met6*	[8]
W303-1A	a *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1*	R. Rothstein[a]
W303-1B	α *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1*	R. Rothstein[a]
C125	α *met6 afg3-1*	this study
C125/U1	α *ura3-1 afg3-1*	C125 × W303-1A
aW303ΔAFG3	α *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 afg3::HIS3*	this study
W303ΔAFG3	α *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 afg3::HIS3*	this study
W303ΔRCA1	α *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 rca1::URA3*	[5]
W303ΔRCA1/AFG3	α *ade2-1 his3-1,15 leu2-3,112 trp1-1 ura3-1 rca1::URA3 afg3::HIS3*	W303ΔRCA1 × W303ΔAFG3

[a]Department of Human Genetics, Columbia University.

conditions known to severe linkage of F_1 to F_0 and also by solubilization of the F_0–F_1 complex with Triton X-100 [22]. Both type of extracts were centrifuged through sucrose gradients and the distribution of α and β subunits in the gradients was analyzed immunochemically. In confirmation of earlier studies, the F_1 subunits in the sonic extract of wild type mitochondria co-sediment as an F_1 oligomer of 350 kDa [22]. Only a fraction of the subunits in the extract of the *rca1* and *afg3* mutants, however, sedimented as F_1 (Fig. 2). The rest of the subunits either aggregated and collected at the cushion interface (α subunits) or, in the case of the β subunit, behaved as a monomer (based on its sedimentation for longer times in calibrated gradients). Since unassembled α and β subunits tend to aggregate [23], probably due to their somewhat hydrophobic properties [23], it is possible that the sonic treatment favored a preferential extraction of the oligomeric F_1. A more accurate indication of the relative proportion of subunits present in the oligomer was obtained from an analysis of the Triton X-100 extracts. As noted in previous studies, this detergent solubilizes the F_0–F_1 complex which sediments as a 550 kDa complex [22]. Because the F_0–F_1 complex has a larger mass than F_1, the α and β subunits penetrate deeper into the gradient (Fig. 3). Although a fraction representing approximately 20% of the α and β subunits in the mutant extracts have sedimentation properties similar to the F_0–F_1 ATPase, most of the remaining 80% are recovered in the fractions corresponding to the monomers subunits.

In vivo labeling of the *afg3* (Fig. 1B) and *rca1* mutants [5] indicate that the synthesis of the mitochondrially encoded subunits 6 and 9 of the ATPase is not affected in either strain. The steady-state abundance of the two proteins, however, is substantially lower in *rca1*, *afg3*, and in double mutants than in the parental respiratory competent strain (Fig. 4). In this experiment subunits 6 and 9 were extracted from mitochondria with chloroform/methanol to separate these hydrophobic components from the bulk of mitochondrial proteins [24]. The lower concentration of subunits 6 and 9 is probably a consequence of an increased turnover of these proteins in strains unable to

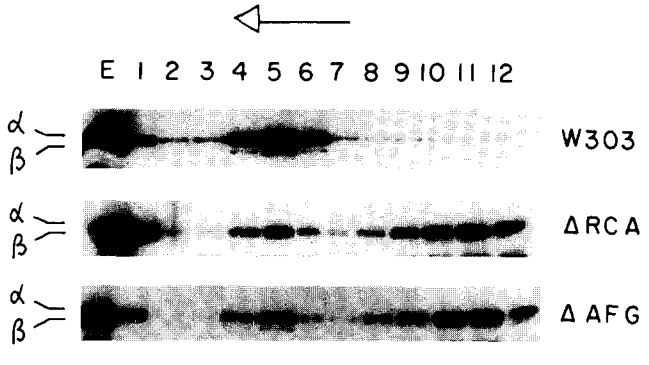

Fig. 3. Sedimentation of F_1 in detergent extracts of wild type and mutant mitochondria. Mitochondria from the parental respiratory competent yeast W303-1B (W303), and from *rca1* mutant W303ΔRCA1 (ΔRCA) and the *afg3* mutant W303ΔAFG3 (ΔAFG) were suspended in TEA and were extracted with 0.5% Triton X-100. This concentration of detergent has been shown to extract the F_1–F_0 complex [22]. Following centrifugation at $105{,}000 \times g_{av}$, the supernatants were applied to linear sucrose gradients prepared as in the legend to Fig. 2 except for an intervening layer of 0.5 ml of 50% sucrose between the cushion and the gradient. The conditions of centrifugation and Western blot analysis were identical to those described in the legend to Fig. 2. In this Western blot the β subunit was poorly detected.

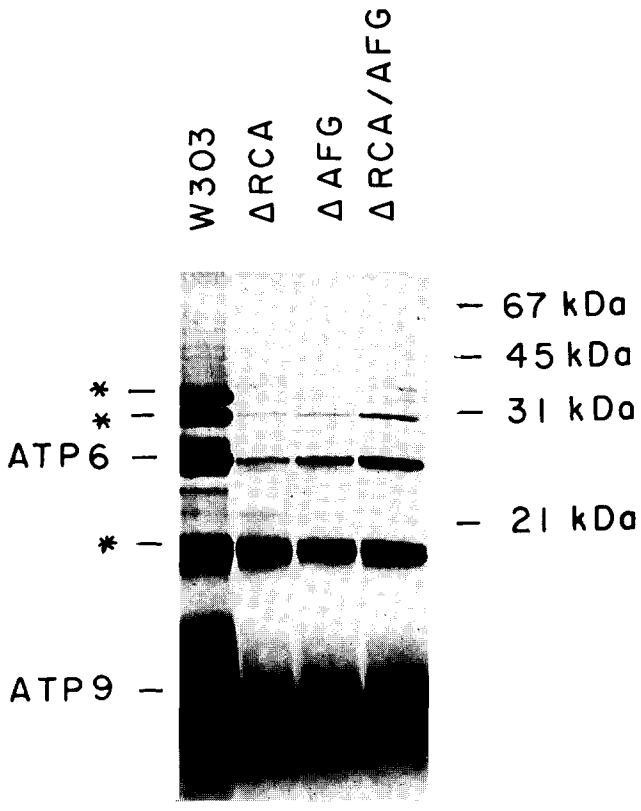

Fig. 4. Steady-state concentrations of ATPase subunits 6 and 9 in wild type and mutant mitochondria. Mitochondria (2 mg protein) were extracted with 1 ml of chloroform/methanol (2:1) as described by Michon et al. [24]. The crude proteolipid extracts were dried and dissolved in 5% SDS. One half of the samples were separated on a 15% polyacrylamide gel and proteins were visualized by silver staining. Extracts were obtained from the respiratory competent strains W303-1A, the single mutants W303ΔRCA1 (ΔRCA), W303ΔAFG3 (ΔAFG), and the double mutant W303ΔRCA1/AFG3 (ΔRCA/AFG). Subunits 6 (ATP6 and 9 (ATP9) are identified in the left-hand margin. The identities of the proteins marked with asterisks are not known. The migration of size standards is marked in the right-hand margin.

complete the assembly of F_0–F_1 because of mutations that block F_1 synthesis. We have noted a similar effect on subunits 6 and 9 in strains with mutations in the structural genes of F_1 (unpublished observations). Although an increased turnover of subunits 6 and 9 as a secondary effect of defective F_1 assembly is a reasonable explanation for the observed lower steady state concentrations of these proteins, a requirement of the *RCA1* and *AFG3* gene products for assembly of F_0 cannot be excluded. In contrast to subunits 6 and 9, subunit 4 and subunit d, two other constituents of F_0, are present in the mutants at near normal concentrations indicating that they are synthesized and are more resistant to proteolytic degradation (data not shown).

The details of the sequence of events leading to the formation of complex heteroligomeric enzymes such as cytochrome oxidase and the F_0–F_1 ATPase are still lacking. The fragmentary information available comes mostly from studies of F_1. Following its import into mitochondria, the β-subunit of F_1 interacts with the Hsp60p complex and is folded from a protease-sensitive into a native protease-resistant conformation [25]. Presumably Hsp60p is also involved in folding of the other F_1 subunits.

Events intervening between the release of the folded subunits from the Hsp60p complex and formation of final F_1 quaternary structure are not well understood at present. Two nuclear genes *ATP11* and *ATP12* have been implicated to function at a late stage of F_1 assembly [26,27]. Mutations in these genes elicit similar phenotypes, characterized by the absence of oligomeric F_1. In *atp11* and *atp12* mutants, the α and β subunits of F_1 are present as large intractable aggregates resistant to solubilization with non-denaturing detergents or by sonic disruption of mitochondria [21]. The extractability of the non-assembled F_1 subunits in *rca1* and *afg3* mutants by either procedure indicate that the chemical properties of the proteins in these strains are different from those of *atp11* and *atp12* mutants. These observations also imply that the reason for the F_1 assembly arrest is different in *rca1* and *afg3* than in *atp11* or *atp12* mutants.

Rca1p and Afg3p have been proposed to be ATP-dependent proteases based on the presence of a short sequence motif characteristic of Zn binding domain of certain proteases and reduced protein turnover rates in *afg3* strains [28,29]. Whether the Rca1p and Afg3p function in post-translational modification or provide a chaperonin-like function remains to be clarified.

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