The Largest Mitochondrial Translation Product Copurifying with the Mitochondrial Adenosine Triphosphatase of \textit{Saccharomyces cerevisiae} Is Not a Subunit of the Enzyme Complex

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Mitochondrial adenosine triphosphatase isolated from a double mutant of \textit{Saccharomyces cerevisiae} lacking cytochrome \textit{b} apoprotein and subunit II of cytochrome oxidase does not contain the mitochondrial translation product (approximate molecular weight, 32,000) previously suggested to be a subunit of the enzyme complex.

The membrane sector of the mitochondrial oligomycin-sensitive ATPase complex (designated \(F_0\)) is known to contain several key components required for the coupling of oxidative phosphorylation. In comparison to the \(F_1\) sector, which contains the catalytic site of the enzyme complex, however, the subunit structure of the \(F_0\) sector has not been well characterized (for reviews, see references 3, 11, and 14). It was initially suggested that in the yeast \textit{Saccharomyces cerevisiae}, the \(F_0\) sector contains four products of mitochondrial protein synthesis, with estimated molecular weights of 29,000, 22,000, 12,000, and 7,500 (designated subunits 5, 6, 8, and 9 respectively [13]). However, we have recently proposed that two of these polypeptides (subunits 5 and 8; apparent molecular weights, 32,000 and 10,000 in our gel system) are not components of the mitochondrial ATPase. In particular, we suggested that the largest of these polypeptides is a contaminant consisting of cytochrome \textit{b} apoprotein and subunit II of cytochrome oxidase, which copurify with the enzyme complex (7). This conclusion was based on the following observations. (i) Analyses of mitochondrial translation products in \textit{mit}⁻ mutants of \textit{S. cerevisiae} (10), which lack cytochrome \textit{b} apoprotein or subunit II of cytochrome oxidase, indicate that these proteins are the only mitochondrial translation products that have a molecular weight of approximately 32,000. (ii) A mitochondrial translation product of molecular weight 32,000 could be precipitated from a purified preparation of mitochondrial ATPase by using rabbit monospecific antisera against the cytochrome \textit{b} apoprotein or subunit II of cytochrome oxidase. The 32,000-dalton mitochondrial translation product(s) copurifying with the mitochondrial ATPase could not be removed completely from the preparation by using either one of the above antisera singly, but it completely disappeared from the preparation when a combination of the two antisera was used. (iii) Mitochondrial ATPase isolated from \textit{mit}⁻ strains lacking subunit II of cytochrome oxidase or cytochrome \textit{b} apoprotein both contain a 32,000-dalton band when analyzed on sodium dodecyl sulfate-polyacrylamide gels. The 32,000-dalton band was presumably cytochrome \textit{b} apoprotein (the electrophoretic mobility of which has apparently been slightly altered during isolation of the mitochondrial ATPase) in the first mutant and cytochrome oxidase subunit II in the second mutant.

More definitive evidence that subunit 5 is not an authentic subunit of the mitochondrial ATPase complex would be obtained if a mutant strain lacking both cytochrome \textit{b} apoprotein and subunit II of cytochrome oxidase could be shown not to contain the largest mitochondrial translation product of the ATPase complex. We have constructed such a strain from two \textit{mit}⁻ mutants having lesions in the \textit{cyb-box} or the \textit{oxi1} regions of the mitochondrial genome (4, 6). The two \textit{mit}⁻ strains used have previously been shown to lack either cytochrome \textit{b} apoprotein (strain 41-2-4 (1), \textit{a adel [cyb]} [9]) or subunit II of cytochrome oxidase (strain 1203, \textit{a adel his [oxi1]} [7]).

To ensure that the double mutants constructed were isonuclear to the wild-type parent and to avoid the difficulties in the detection of the presence of the two \textit{mit}⁻ mutations in a diploid strain, a mitochondrial DNA-less (\textit{rho}⁻) strain of yeast carrying the \textit{karl-1} mutation (strain BT2-1, \textit{a karl leu [rho]}) was employed.
in the construction of the double mit\textsuperscript{−} mutants. 

Haploid cells carrying the karl-1 mutation form diploids at only a low frequency (2). The majority of cells resulting from such a cross are heterokaryons which subsequently segregate haploid cells containing one of the parental nuclei with the mixed cytoplasm from both parents. The mitochondrial DNA carrying the oxil mutation was first transferred from strain 1203 to the mitochondrial DNA-less karl-1 strain BT2-1 by crossing the two haploid strains as described previously (4). Colonies having the BT2-1 nuclearly determined auxotrophic growth requirement (leu) were first selected. These were then screened for the presence of mitochondrial DNA by crossing to a cyb mit\textsuperscript{−} strain (2223, a ade1 his [cyb\textsuperscript{−}]); colonies that produced respiratory-competent cells in these crosses contained the BT2-1 nucleus and strain 1203 mitochondria. One such strain [denoted BT2-1(1203); Table 1] was used in the subsequent cross with strain 41-2-4 (1), which carries a cyb mutation, to construct the double mit\textsuperscript{−} strain. In this cross, strains having the parental 41-2-4(1) nuclear genetic marker (ade) were first selected. These strains were analyzed by marker rescue analysis (4, 6), using the tester strains shown in Table 1 to detect the presence of the oxil and cyb mutations. Strains containing the cyb-box and oxil mutations showed no segregation of respiratory-competent cells when crossed with petite deletion mutants which retained only the cyb region or the oxil region of the mitochondrial genome, but did produce respiratory-competent diploid cells when crossed with tester strains which retained both of these regions. 

To confirm biochemically that the constructed strains contained both the oxil and cyb mutations, one of the double mutants (strain JO1-2; Table 1) was further analyzed. The products of mitochondrial protein synthesis of the double mit\textsuperscript{−} strain were labeled with [\textsuperscript{35}S]sulfate in the presence of cycloheximide (5), which allows the incorporation of the isotope only into the mitochondrial translation products. These products were analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (5). The double mit\textsuperscript{−} strain lacked both subunit II of cytochrome oxidase (32,000 daltons) and cytochrome b apoprotein (30,000 daltons) (Fig. 1). All other mitochondrial translation products were present in normal amounts except for subunit 1 of cytochrome oxidase, the synthesis of which appeared to be reduced in the constructed strain. This phenomenon is frequently observed in mit\textsuperscript{−} mutants of S. cerevisiae (see reference 5 for example) and is presumably due to the effect of catabolite repression.

The mitochondrial ATPase was then isolated from the double mit\textsuperscript{−} strain by centrifugation of a Triton extract of the above mitochondria on glycerol gradients, essentially as described by Tzagoloff and Meagher (12). The mitochondrial translation products associated with the mitochondrial ATPase were analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (5). The 32,000-dalton polypeptide(s) which was associated with the mitochondrial ATPase isolated from a wild-type strain could not be observed in the mitochondrial ATPase isolated from the double mit\textsuperscript{−} strain (Fig. 1).

The ATPase activity of the purified enzyme preparation was assayed (8) and was found to be high, although slightly reduced compared with that of the wild type (70% of the wild type). The sensitivity of the isolated enzyme preparation to oligomycin, which indicates whether the activity is of the F_{1}-ATPase complex (14), was also assayed and was shown to be similar to that of the wild type. These data show that the ATPase complex isolated from the mutant strain contains all of the subunits required for the activity and proper assembly of the complex.

It was shown earlier that a 32,000-dalton mitochondrial translation product is associated with the mitochondrial ATPase isolated from the parent mit\textsuperscript{−} strain (7). Therefore, the absence of the 32,000-dalton polypeptide in the double mit\textsuperscript{−} strain strongly substantiates our earlier suggestions that this polypeptide is a contaminant consisting of cytochrome oxidase subunit II and apocytochrome b and that the 32,000-dalton polypeptide observed in the parental mit\textsuperscript{−} strain was cytochrome b apoprotein in the case of strain 1203 and subunit II of cytochrome oxidase in the case of strain 41-2-4(1).

Table 1. Strains used in the construction of the double mit\textsuperscript{−} strain JO1-2

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Segregation of respiratory competent cells in crosses with a mit\textsuperscript{−} line</th>
</tr>
</thead>
<tbody>
<tr>
<td>BT2-1</td>
<td>a leu1 karl-1 [rho\textsuperscript{−}]</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>ade1 his [oxil]</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>a ade1 [cyb]</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>oxil [oxil]</td>
<td>+</td>
</tr>
</tbody>
</table>

\* Strains used in gene rescue analyses to determine the presence of the cob-box or oxil mutations in the constructed strains were 2223 (a ade1 his [cyb\textsuperscript{−}]), 409-8 (a ade1 lys2 trpl [rho\textsuperscript{−} cyb\textsuperscript{−}]), 424-1 (a ade1 lys2 trpl [rho\textsuperscript{−} oxil\textsuperscript{−}]), 2422m (a lys2 [oxil mit\textsuperscript{−}]), and Ma30e (a lys2 oxy2 mit\textsuperscript{−})). Only appropriate crosses were performed between the tester strains and the strains used in the construction of the double mit\textsuperscript{−} mutant.

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