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Level of ATP synthase activity required for yeast *Saccharomyces cerevisiae* to grow on glycerol media

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Abstract

Two independent cold-sensitive *pet* mutants in the gene (ATP5) coding for the oligomycin sensitivity conferring protein (OSCP) have been isolated in the yeast *Saccharomyces cerevisiae*. The mutations in both strains alter the initiating methionine codon in the ATP5 gene: ATG to ATA (Ile) and AAG (Lys). Western blot analysis of total yeast protein after the cells were grown at 18°C, 30°C, and 37°C, indicates that the level of OSCP decreased 80% relative to the wild type strain. In addition, the level of the oligomycin-sensitive ATPase decreased 85% relative to the wild type strain, after growth at 30°C. These findings indicate that for *S. cerevisiae*, the level of oxidative phosphorylation can decrease 85% without showing a large growth defect on media containing glycerol at 30°C, but not at 18°C.

Key words: Oxidative phosphorylation; *pet* mutant; Mitochondrion; ATP synthase; *Saccharomyces cerevisiae*

1. Introduction

The yeast *Saccharomyces cerevisiae* is an excellent model organism, and it has been used extensively, to study the biogenesis, function/structure, and regulation of the genes and proteins of the mitochondrion and those involved in oxidative phosphorylation. A large number of complementation groups that have *pet* phenotypes have been isolated from various laboratories [1–3] and these have been used to isolate genes involved in the regulation or function of oxidative phosphorylation. Cells that exhibit a *pet* phenotype are unable to grow on media that contains a nonfermentable carbon source, generally glycerol. Despite the wide use of this *pet* phenotype to identify defects in oxidative phosphorylation, there has been no report on the level of oxidative phosphorylation required to support growth on glycerol medium. In this study, two independent mutations in the gene (ATP5) coding for the oligomycin sensitivity conferring protein (OSCP) have been isolated and these mutant stains have been used to directly address this question.

The yeast mitochondrial ATP synthase is composed of a membrane portion, F_0, a hydrophilic region, F_1, and the stalk, which is minimally composed of OSCP. The F_1 contains the catalytic site for ATP synthesis, it has a molecular weight of 360,000, and it is composed of $\alpha_3\beta_3\gamma\delta\varepsilon$ [4–8]. The β-subunit is thought to constitute the catalytic site, but other subunits, especially the α-subunit, probably contribute to the active site. The F_0 is a hydrophobic membrane complex composed of $a_1b_1c_{6-10}$. Minimally, F_0 is responsible for proton translocation to F_1, but it may also act as an energy transducer changing the energy of the proton gradient to high energy conformational states.

The mitochondrial ATP synthase catalyzes the synthesis of ATP during oxidative phosphorylation. However, the ATP synthase can also run in reverse as an ATPase and this activity, like ATP synthesis, is sensitive to oligomycin. OSCP has been shown to be required both in vitro and in vivo [9,10], for the formation of the oligomycin sensitive ATPase. As such, the level of OSCP can dictate the level of the functional ATP synthase.

This study provides a upper limit for the relative level of oxidative phosphorylation necessary for growth of yeast on glycerol medium. This study also indicates that this upper level is higher for cells that are growing at 18°C than those growing at 30°C. Finally, this study suggests the reason why no dominant negative *pet* mutations have been isolated in the β-subunit of the ATP synthase and proposes a method to identify biochemically dominate negative mutants in the β-subunit.

2. Materials and methods

2.1. Yeast strains

The yeast *Saccharomyces cerevisiae* strain, MLY007 (MATa ura3–52, lys2–801, ade2–101, trp1-Δ63, his3-Δ200, leu2-Δ1, atp5::LEU2) was used throughout this study. This strain has a null mutation in the ATP5

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that is identical to the null mutation in strain MLY001 described earlier [10]. The parental strain of MLY007 is YPH499 [11]. The gene coding for yeast OSCP, ATP5 [10], was cloned into the *E. coli*–yeast shuttle vector, pRS316 [11], and transformed into yeast MLY007. The ATP5 gene altered for expression of OSCP in *E. coli* [12] was cloned into the single copy *E. coli*–yeast shuttle vector, pRS314 [11], mutated by a misincorporation scheme [13], the mutant library DNA was amplified, isolated, and transformed into MLY007 with the wild type ATP5 gene on pRS316. The wild type ATP5 gene was cured by selection on 5-fluoro-orotic acid and the resulting transformants were screened for the *pet* phenotype at 18°C, 30°C, and 37°C. Details of the mutagenesis and selection will be provided in a later manuscript. From the selection scheme, two cold sensitive mutants were isolated, OM1G and OM2A3, the plasmids were isolated and the entire ATP5 gene was sequenced by the dideoxy method using Sequenase (United States Biochemicals).

2.2. Western blot analysis

For Western blot analysis of OSCP in yeast, cells were grown at 18°C, 30°C, and 37°C, in minimal medium containing all the auxotrophic requirements and 2% glucose. The yeast cells were grown to stationary phase and until the glucose level dropped to less than 0.1%. Under these conditions, the cells are not glucose repressed, similar to that of cells grown on medium containing glycerol as the carbon source (reviewed in [14,15]). Total yeast protein was extracted [16], separated by SDS-gel electrophoresis, blotted to nitrocellulose, reacted with antibody against yeast OSCP, as described previously [10].

2.3. Assay of ATPase activity

For the isolation of mitochondria, yeast were grown at 30°C in semisynthetic medium containing 1% galactose as the carbon source [17]. Cells grown in galactose are not glucose repressed (reviewed in [14,15]). ATPase activity was determined by the coupled enzyme method [18] with the addition of 5 μg of oligomycin, as indicated. Protein was determined by the bicinchoninic acid method as described [19].

3. Results

This laboratory has initiated an analysis of the structure and function of OSCP using, in part, random mutagenesis of the gene coding for OSCP, ATP5. In the process of this analysis, two cold-sensitive mutants in OSCP were isolated, OM1G and OM2A3. Fig. 1 shows the growth phenotype of the mutants, the wild type cells and a negative control. The conditional mutant cells grew well at 30°C and 37°C, but grew very slowly, or not at all, at 18°C, as compared to the wild type cells.

The plasmids containing the mutant ATP5 genes were isolated and the entire sequence of the ATP5 gene was determined. The result of the sequencing is shown in Fig. 2. Mutant OM1G had four mutations, all within the leader sequence of OSCP. These mutations hit the initiating methionine (−17), and codons coding for residues −10, −7, −5, and −3. The initiating methionine codon was mutated from ATG to ATA (Ile). The mutation at −10 was a silent mutation and only changed the codon from AGG to AGA. The other mutations caused amino acid replacements within the leader sequence.

The mutant OM2A3 had just one mutation in the ATP5 gene and that was also at the initiating methionine residue. The mutation, ATG to AAG, changed the codon from coding for Met to coding for Lys. These mutations in the initiating Met codon were very curious since there is only one methionine residue in the whole molecule. Since protein translation typically starts with Met, the immediate question was how much OSCP is in the cells?

Fig. 3 shows a Western blot analysis of total yeast proteins after growth of wild type OM1G cells at 18°C, 30°C and 37°C. The cells were grown under conditions in which expression of the ATP5 gene is derepressed (see section 2). Under these conditions, the level of gene expression is similar to that as cells grown in glycerol medium. Antibody directed against OSCP was used to determine the relative amount of OSCP in the mutant strain as compared to the wild type strain. Densitometric analysis indicated that the level of OSCP decreased about 80% independent of the temperature that the cells were grown. These results indicated that OSCP was synthesized, despite the mutation in the initiating methionine. Furthermore, the cold sensitivity is not due to a decrease in synthesis of OSCP at 18°C versus 30°C or 37°C.

Fig. 3 also indicates that OSCP from OM1G was processed into the mature size OSCP. This is despite the four mutations that are present in the leader sequence that directs import of OSCP to the mitochondrion. In addi-

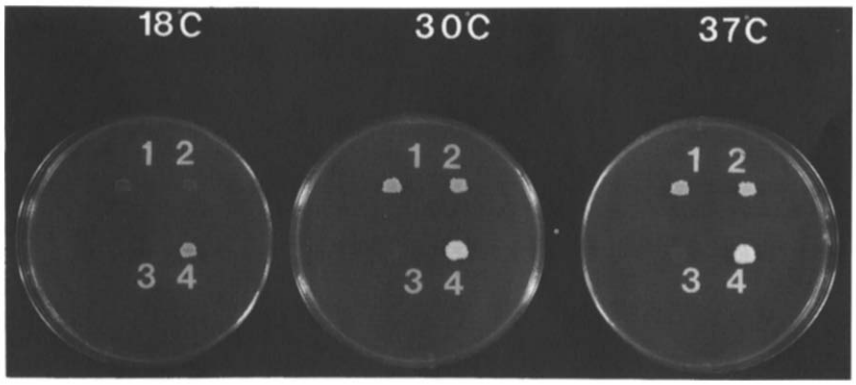

Fig. 1. Growth phenotypes of the mutants. Mutants, OM1G (1) and OM2A3 (2), were grown at 18°C, 30°C, and 37°C, on complete medium containing glycerol as the sole carbon source (YPG). Yeast strain, MLY007, transformed with the vector pRS314 (3), and with the vector containing the wild type ATP5 gene (4) were used as negative and positive controls, respectively.

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wt     Met Phe Asn Arg Val Phe Thr Arg Ser Phe Ala Ser Ser Leu Arg
       ATG TTT AAT AGA GTC TTT ACC AGG TCA TTT GCA TCA AGC TTA AGA

OM1G   Ile                      Arg         Thr     Ile     Ile
       ATA                      AGA         ACA     ATC     ATA

OM2A3  Lys
       AAG
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Fig. 2. Mutations identified in the cold sensitive mutants. The nucleotide sequence and the predicted amino acids of the first 15 of 17 residues that constitute the leader sequence of OSCP are shown. The mutations are indicated by underlines and the resulting amino acid changes due to this mutation are shown in bold.

tion, there is little to none of the precursor to OSCP as this peptide migrates just above the mature OSCP in this gel system. These result are not surprising, however, since the primary sequence of the leader peptide is not conserved. Instead, the leader sequences tend to simply have basic and hydroxyl containing amino acids that determine its function [20].

The decrease in the level of OSCP should be reflected in a corresponding decrease in the level of the oligomycin-sensitive ATPase or ATP synthase. Table 1 shows the level of the oligomycin-sensitive ATPase from mitochondria isolated from wild type and OM1G strains after growth at 30°C under derepressive growth conditions (see section 2). The level of ATPase that was sensitive to oligomycin in OM1G was decreased from a specific activity of 1.18 to 0.18, or by 85%, relative to the wild type strain. This indicates that the decrease in OSCP resulted in a corresponding decrease in the level of the oligomycin sensitive ATPase. The strong correlation between the decrease in the level of OSCP and the decrease in the specific activity of the oligomycin sensitive ATPase suggests that the level of free OSCP in the cell that is not complexed with the ATPase is very low.

4. Discussion

This study reports on an upper limit (15%) of the level of oligomycin-sensitive ATPase necessary for yeast to grow on a nonfermentable carbon source at 30°C. This study is unique since the decrease in the level of oxidative phosphorylation is due to a decrease in the expression of a subunit, OSCP, which is essential for the formation of the ATP synthase complex. While a plethora of mutations have been isolated in various subunits of the ATP synthase, correlations made between in vitro studies and in vivo studies are difficult. In the case of a mutant enzyme, it is difficult to know if a decrease in the activity assayed in vitro is reflective on the activity in vivo. This is further confounded because it is a formidable task to determine if the level of the mutant enzyme is the same as the level of the wild type enzyme in their respective strains. In this study, OSCP and the ATP synthase are normal. The defect in the ATP synthesis is not due to formation of a defective enzyme complex, but rather due to insufficient amount of complex being synthesized. Since the level of ATP synthase is limiting the amount of ATP via oxidative phosphorylation, this level reflects the level oxidative phosphorylation necessary for cell to grow on a nonfermentable carbon source. This value is very useful, since mutagenesis studies typically identify *pet* mutants by their growth phenotype. This study indicates that the mutation must impair oxidative phosphorylation by at more than 85% to identify a *pet* mutant at 30°C.

The 80% decrease in the level of OSCP is presumably due to the decrease in the translation of OSCP. Initiation of translation generally starts with a Met, though there are examples of other codons serving this role [21,22]. The cold sensitivity is not due to a further decrease in the level of OSCP, because this level is rather constant from cells grown at 18°C, 30°C, and 37°C (Fig. 3). The cold sensitivity must be reflecting the differences in the biochemistry or physiology of the cells at 18°C vs. 30°C. The likely reason why the cells are cold sensitive is due to the biochemistry of the ATP synthase. The ATP synthase is a membrane bound enzyme with an essential hydrophobic portion, F_0. Membrane bound enzymes typically show a break in Arrehenius plots which is thought to be due to a transition in the state of the membrane [23]. The mitochondrial oligomycin sensitive ATPase from yeast shows a break at 20°C in Arrehenius plots [24]. This transition reflects an increase in the energy of activation from 53 kJ/mol above 20°C to 150 kJ/mol below 20°C. As such, the cold sensitivity of the yeast mutant strains OM1G and OM2A3 is probably due to a large decrease in activity of the ATP synthase going from 30°C to 18°C which is added onto the 85% decrease in activity due to the decrease expression of

Table 1
ATPase activity of the wild type and mutant enzymes

Strain	ATPase (μmol/min/mg)		Inhibition	Og-sensitive ATPase activity (μmol/min/mg)	Reduction in Og-sensitive ATPase activity
	−Og	+Og			
wt	1.9 ± 0.4	0.72 ± 0.06	62%	1.18	
OM1G	1.1 ± 0.2	0.92 ± 0.16	15%	0.18	85%

Mitochondrial ATPase activity was determined from yeast strains in the presence (+) and absence (−) of oligomycin (Og) at 30°C as described in section 2. Oligomycin sensitive ATPase activity was calculated by subtracting the ATPase activity in presence of oligomycin (+Og) from that in absence of oligomycin (−Og). The results are a mean of three different experiments.

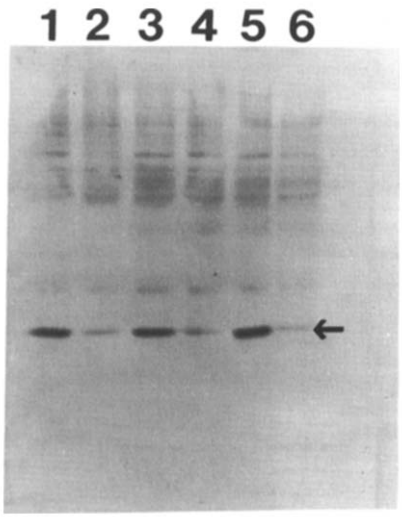

Fig. 3. Western blot analysis of wild type and mutant yeast proteins for OSCP levels. Total protein was extracted from wild type and the mutant, OM1G, yeast strains, after growth at 18°C (lanes 1 and 2), 30°C (lanes 3 and 4) and 37°C (lanes 5 and 6) as described in section 2. Lanes 1, 3 and 5 contain wild type protein samples, whereas lanes 2, 4 and 6 contain the mutant proteins. The relative levels of OSCP as determined by densitometric scanning of the film were 0.17, 0.24 and 0.21 for lanes 2, 4 and 6 relative to lanes 1, 3 and 5, respectively. The arrow indicates the position of mature OSCP.

OSCP. The resulting activity at 18°C must go below a threshold value of ATP synthase activity required for growth of yeast on glycerol media. This hypothesis is very similar to the threshold hypothesis for mitochondrial diseases which suggests that mitochondrial mutations need to accumulate to a threshold limit before a phenotype is observed in human tissues [25].

This same phenomena is probably true in *E. coli*. The *unc* mutations in *E. coli* typically have dramatic effects on the activity of the ATPase. For example, a mutation in subunit a, a-P230L, decreases the dicyclohexylcarbodiimide (DCCD) sensitivity by 80% with only a corresponding 50% decrease in the growth yield on medium containing succinate [26]. As discussed earlier, however, conclusions drawn from mutant enzymes are difficult. In same report, another mutation in subunit a, a-R210Q, reduces the growth yield to less than 3% of the wild type strain and yet there was 2-fold more DCCD sensitive ATPase activity in this mutant strain as compared to the strain with a-P230L. This discrepancy is probably related to differences between in vitro vs. in vivo effects of the mutation on the activity of the enzyme.

The binding change hypothesis for ATP synthesis proposed by Boyer [27] suggests a rotational model for ATP synthesis. There is some evidence that rotation through all catalytic sites is obligatory [28–31]. That is, inactivation of one of the three active sites inactivates the enzyme. Despite this, there has not been any dominant negative mutations isolated in the ATPase, even in the β-subunit. The reason for this may now be clear. If a mutant β-subunit is present in equal concentration as the wild type subunit, and if the mutant and wild type subunit form homo and hetero type hybrid molecules, then the population of homo type enzymes with only the wild type subunit will be 12.5%. This level may be sufficient for yeast cells to grow at 30°C on a nonfermentable carbon source and thus would not be observed as dominant negative.

There are at least two ways to identify biochemically dominant negative mutations in the β-subunit of the ATPase. One way is to look for the defective growth phenotype at 18°C. Since there is a higher demand for the level of ATP synthase at the colder temperature, this may be sufficient to identify the dominant negative mutations. The second way would be to decrease the level of the ATP synthase by decreasing the expression of an essential subunit, as OSCP. If the level of the ATP synthase is decreased, then a further decrease caused by the dominant negative mutation could be observed as a growth defect even at 30°C. Both of these methods are being now used to identify dominant negative mutations in the β-subunit.

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