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SHORT COMMUNICATION

Bioenergetic Consequences of FLAG Tag Addition to the C-Terminus of Subunit 8 of Yeast *Saccharomyces cerevisiae* Mitochondrial ATP Synthase

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The yeast mitochondrial F_1F_0-ATP synthase is a multisubunit complex that contains at least 17 different subunits. Subunit 8 of yeast mitochondrial ATP synthase is a hydrophobic protein of 48 amino acids encoded by the mitochondrial *ATP8* gene. Subunit 8 has three distinct domains; an N-terminal domain, a central hydrophobic domain and a C-terminal domain. FLAG tag addition to subunit 8 protein potentially facilitate elucidation of its topology, structure, and function. It has been shown that following incorporation of FLAG tag to its C-terminus, subunit 8 still assemble into functional ATP synthase complex. In order to analyze bioenergetic consequences of the FLAG tag addition, a yeast strain expressing FLAG tagged-subunit 8 was subjected to cellular respiration assays. Results obtained showed that addition of FLAG tag to the C-terminus of subunit 8 does not impair its proper functioning. The FLAG tag system, therefore, can be employed to study subunit 8's detailed structure, topology, and function.

Key words: bioenergetic, ATP synthase, mitochondria, yeast

INTRODUCTION

ATP synthase, also known as F_0F_1-ATPase, is a multisubunit, membrane-associated protein complex that catalyzes the phosphorylation of ADP to ATP at the expense of a proton motive force generated by an electron transport chain in energy transducing membrane. In some organisms, it also works in the reverse direction by hydrolyzing ATP and generating an electrochemical proton gradient across a membrane to support locomotion or nutrient uptake (Hong & Pedersen 2008). ATP synthase is an exceptionally complicated protein complex which in yeast is composed of at least 17 subunits (Stephens *et al*. 2003).

The subunit 8 of yeast mitochondrial ATP synthase is a small hydrophobic polypeptide of 48 amino acids encoded by the *ATP8* gene (Macreadie *et al*. 1983). Analysis of its primary structure has led to identification of three distinct domains; an N-terminal domain, a central hydrophobic domain (CHD) and a C-terminal domain (Devenish *et al*. 1992). The topology of subunit 8, which is determined by unique introduced cysteine residues, indicates that its N-terminus is located in the intermembrane space of mitochondria whereas the C-terminus is located within the mitochondrial matrix (Stephens *et al*. 2000). Further analysis employing cysteine-scanning mutagenesis showed that the first 14 and the last 13 amino acids were extrinsic to the lipid bilayer, indicating the existence of a 21 amino acid transmembrane spanning region (Stephens *et al*. 2003).

As a mitochondrially encoded protein, subunit 8 is transcribed and translated entirely within the organelle. Subunit 8 is not present in prokaryotes, but is an additional subunit present in the mitochondrial ATP synthase of eukaryotes. This means that bacterial ATP synthase can naturally function without the presence of subunit 8 (Artika 2007). The immediate question therefore is to resolve the detailed structure and roles of this subunit in the enzyme complex. Several lines of evidence suggested that subunit 8 is part of the stator stalk in the yeast mitochondrial ATP synthase. No amino acid of subunit 8 directly participates in either ATP synthesis/hydrolysis or proton pumping, suggesting that subunit 8 is a structural component of the mitochondrial ATP synthase complex (Stephens *et al*. 2003).

In order to elucidate its detailed structure, function, and membrane topology, an allotropic expression system for subunit 8 has been developed. Allotopic expression is the deliberate relocation of mitochondrial genes to the nucleus and delivery of the gene products from the cytoplasm to the mitochondria. For allotropic expression of subunit 8, a nuclear version of subunit 8 gene to be expressed in the nucleocytosolic system, has been designed. To ensure that the cytoplasmically synthesized

subunit 8 was imported into mitochondria, sequences encoding a mitochondrial signal peptide were fused to the N-terminus of the gene (Gearing *et al*. 1985). The allotopic expression system has been applied to study various aspects of subunit 8 molecular biology. This system has also been successfully used to express FLAG tagged-subunit 8 (Artika 2006). In these experiments, the FLAG epitope tag was incorporated into subunit 8 by using a PCR-based mutagenesis technique. The primers used were designed to incorporate additional nucleotide sequences encoding hexapeptide (DYKDDD) representing the FLAG epitope tag at the C-terminus of subunit 8 gene with two serine residues functioning as a bridge between the subunit 8 and the FLAG tag. The allotopically expressed FLAG tagged-subunit 8 protein was imported into mitochondria and assembled into a functional ATP-synthase-complex. The main purpose of the FLAG tag addition to subunit 8 protein was to enable detection of subunit 8 protein by means of an anti-FLAG tag monoclonal antibody. In the present study, strain expressing FLAG tagged-subunit 8 was subjected to cellular respiration assays to analyze bioenergetic consequences of the FLAG tag addition.

MATERIALS AND METHODS

Materials. *Saccharomyces cerevisiae* strain M31 [atp8, mit$^-$, *his*6, *ade*1] a collection strain of the Department of Biochemistry and Molecular Biology, Monash University, has previously been described (Nagley *et al.* 1988). Strain YM2 is strain M31 expressing non-tagged-subunit 8 gene fused with a mitochondrial signal peptide (Roucou *et al*. 1999). Strain FTC2 is strain M31 expressing FLAG-tagged-subunit 8 gene fused with a mitochondrial signal peptide (Artika 2007).

Determination of Generation Time. Generation time of each strain was determined as described by Gray *et al*. (1996). Generation times represent mean ± SD of triplicate assays.

Cellular Respiration Assay. Cellular bioenergetic analysis was carried out by measuring the whole cell respiration as described by Law *et al*. (1995). Yeast cells were grown at 23 °C on *Saccharomyces* salt medium [1% (w/v) yeast extract, 0.12% (w/v) $(NH_4)_2SO_4$, 0.1% (w/v) KH_2SO_4, 0.01% (w/v) $CaCl_2$, 0.0005% (w/v) $FeCl_3$, 0.07% (w/v) $MgCl_2$, 0.05% (w/v) NaCl] plus 2% (v/v) ethanol, until they reached mid-logarithmic growth phase. The cells were then harvested by centrifugation, washed twice in distilled water and then resuspended at OD_{550} = 30. The cellular respiration rate was determined by measuring the oxygen consumption rate using the Clarke electrode (Gilson). The oxygraph was calibrated so as the condition of oxygen saturated distilled water in the chamber represented 100% oxygen and in the present of sodium dithionate in the chamber represented 0% oxygen concentration. To the chamber was then added 300 µl respiration buffer (*Saccharomyces* salt medium without ethanol). Following addition of 1 µl of absolute ethanol, 10 µl of cell suspension was added to the chamber. The rate of oxygen consumption, J_0 (nmol/O/min/mg dry mass cell) was measured. The oxygen consumption rate measured at this state represents the physiological function of the cells. To measure the respiration rate in the absence of oxidative phosphorylation, J_0^{TET} (nmol/O/min/mg dry mass cell), triethyltin (TET) was added to the chamber to a final concentration of 80 µM. From these measurements, the respiratory control ratio (RCR), J_0/J_0^{TET}, was calculated. To determine the maximal (uncoupled) respiratory rate, carbonylcyanide *m*-chlorophenylhydrazone (CCCP) was added to the oxygraph chamber to a final concentration of 5 mM. The maximal respiration rate, J_0^{CCCP}, was then measured. From these measurements, the maximum respiratory capacity (MRC), J_0^{CCCP}/J_0^{TET}, was calculated. Values reported (mean ± SD) are from two independent experiments and assays were performed in triplicate.

RESULTS

Basic Growth Characteristics of Strains Expressing Non-Tagged and FLAG Tagged-Subunit 8. The growth properties of strains expressing non-tagged and FLAG tagged-subunit 8 were examined by determining their generation times for growth on liquid-ethanol-medium. Generation time is the time needed for the population to double. The generation time (Table 1) was calculated from the growth curve of each strain.

Cellular Respiration Parameters of Strains Expressing Wild-type and FLAG Tagged-subunit 8. The cellular respiration parameters of strains YM2 and FTC2 are shown in Table 2.

DISCUSSION

The FLAG tag system has been widely used in epitope tagging of various proteins. Epitope tagging is a process of fusing a set of amino acid residues, serving as antigenic determinant, to a protein of interest in order to allow the surveillance of the tagged-protein with a specific monoclonal antibody. This approach has been found to

Table 1. Generation time of strains YM2 and FTC2

Strain	Generation time (h)
YM2	6.3 ± 0.4
FTC2	6.9 ± 0.4

Table 2. Cellular respiration parameters of strains YM2 and FTC2

Strain	Cellular respiration parameters				
	J_0 (nmol/O/min/mg cell)	J_0^{TET}(nmol/O/min/mg cell)	J_0^{CCCP}(nmol/O/min/mg cell)	J_0/J_0^{TET}	J_0^{CCCP}/J_0^{TET}
YM2	14.3 ± 0.6	6.4 ± 0.9	25.3 ± 4.8	2.2 ± 0.2	4.0 ± 0.2
FTC2	17.5 ± 1.2	6.3 ± 1.8	27.3 ± 4.6	2.7 ± 0.8	4.3 ± 0.5

be useful in the studies of abundance, cellular location, posttranslational modifications, protein-protein interactions, and purification of particular tagged-protein (Kolodziej & Young 1991). The practicability of the FLAG tag system relies on the specificity of the binding of the anti-FLAG antibody to the FLAG fusion proteins with no or very little cross reactivity to other cellular proteins (Schafer & Braun 1995). The small size of the FLAG epitope is proposed as having a minimal effect on protein conformation thereby reducing the possibility of disrupting the function of any tagged-protein.

The present study attempted to analyze the functional performance of subunit 8 following FLAG tag addition to its C-terminus by determining its basic growth characteristics and bioenergetic parameters. The generation time of the strain FTC2 was found to be similar to that of YM2 indicating that FLAG tag addition to subunit 8 does not impair its function. The generation time reflects the performance of subunit 8 in the enzyme complex.

Bioenergetic analysis of the functional performance of mitochondrial ATP synthase in yeast can be carried out both at the cellular and the mitochondrial level. In the present study measurement of respiration parameters was conducted at cellular level. Measurement of cellular respiration parameters is a convenient means to obtain information about the bioenergetic capacity of mitochondria. Cellular respiration analysis can also be used as an alternative means to analyze mitochondrial ATP synthase function when it is proved to be difficult to isolate mitochondria from certain strains.

To determine if addition of the FLAG epitope tag on the wild-type subunit 8 causes functional impairment, strains YM2 and FTC2 were also subjected to bioenergetic analysis under the same conditions. Based on the cellular respiration data, generally the functional performances of the two strains were similar, except the respiration rate of the FTC2 was found to be higher. However, the RCR of the two strains was not different. Other bioenergetic parameters (J_0^{TET}, J_0^{CCCP}, and MRC) were found to be similar. These results indicated that addition of FLAG epitope tag does not affect the function of the native subunit 8.

The respiration rate measured in the presence of respiratory substrate, ethanol, and absence of ATP synthase inhibitors (J_0), corresponds to the physiological function of the cells and is therefore intermediate between absence of phosphorylation (state 4) and maximal phosphorylation rate (state 3). Ethanol is conventionally used as the respiratory substrate. Although it was reported that a further addition of glucose increases the respiration rate, this is not recommended because the mechanism of this stimulation involves a modified response of the respiratory chain to the ATP/ADP ratio (Law *et al.* 1995).

Much of current knowledge of mitochondrial function results from the study of toxic compounds. Specific inhibitors can be used to distinguish the electron transport system from the phosphorylation system. The respiration rate measured in the presence of trietyltin (J_0^{TET}) corresponds to absence of phosphorylation (state 4). Triethyltin is a potent inhibitor of oxidative phosphorylation (Cain & Griffits 1977). Triethyltin belongs to organotin compounds known to inhibit both ATP hydrolysis and ATP synthesis catalyzed by the membrane-bound and isolated F_0F_1 complex. Triethyltin reacts noncovalently with the ATP synthase and its inhibitory effect is reversed by mono- and dithiols such as dithiothreitol and mercaptoethanol. The site of triethyltin action is located in the ion channel within the F_0 sector. Here it is believed to inhibit ATP synthase by competing with H^+ for the same binding site (Hong & Pedersen 2008).

The respiration rate measured in the presence of carbonyl cyanide *m*-chlorophenylhydrazone, (J_0^{CCCP}), corresponds to a state in which the respiratory chain is not kinetically controlled by the electrochemical proton gradient and therefore is dependent only on the activity of the respiratory chain. CCCP is a protonophoric uncoupler that abolishes the obligatory linkage between the respiratory chain and the phosphorylation system (Law *et al*. 1995). CCCP is a small amphipathic molecule which dissolves in phospholipid bilayers and enormously increases their ionic permeability. CCCP shields the electric charge as the ion passes through the membrane, providing a polar environment for the ion and a hydrophobic face to the membranous region. CCCP specifically increases the proton permeability, and disconnect the electron transport chain from the formation of ATP.

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