Structure/Function of the \( \beta \)-Barrel Domain of \( F_1 \)-ATPase in the Yeast *Saccharomyces cerevisiae* (*

The first 90 amino acids of the \( \alpha \) - and \( \beta \)-subunits of mitochondrial \( F_1 \)-ATPase are folded into \( \beta \)-barrel domains and were postulated to be important for stabilizing the enzyme (Abrahams, J. P., Leslie, A. G., Lutter, R., and Walker, J. E. (1994) *Nature* 370, 621–626). The role of the domains was studied by making chimeric enzymes, replacing the domains from the yeast *Saccharomyces cerevisiae* enzyme with the corresponding domains from the enzyme of the thermophilic bacterium *Bacillus* PS3. The enzymes containing the chimeric \( \alpha \) - , \( \beta \) - , or \( \alpha \) - and \( \beta \)-subunits were not functional. However, gain-of-function mutations were obtained from the strain containing the enzyme with the chimeric PS3/yeast \( \beta \)-subunit. The gain-of-function mutations were all in codons encoding the \( \beta \)-barrel domain of the \( \beta \)-subunit, and the residues appear to map out a region of subunit-subunit interactions. Gain-of-function mutations were also obtained that provided functional expression of the chimeric PS3/yeast \( \alpha \) - and \( \beta \)-subunits together. Biochemical analysis of this active chimeric enzyme indicated that it was not significantly more thermostable or labile than the wild type. The results of this study indicate that the \( \beta \)-barrel domains form critical contacts (distinct from those between the \( \alpha \) - and \( \beta \)-subunits) that are important for the assembly of the ATP synthase.

The mitochondrial ATP synthase is a multimeric enzyme composed of the water-soluble \( F_1 \) and the membrane-bound \( F_0 \) complexes. \( F_1 \)-ATPase is composed of five unique subunits in the stoichiometry of \( \alpha_3 \beta_3 \gamma_6 \). The \( \alpha/\beta \) - interfaces form six nucleotide-binding sites, three of which are catalytic and three noncatalytic. \( F_1 \) is bound to \( F_0 \) by one or two stalks that probably comprise the rotor and the stator of the enzyme (1–9). Possible subunits that provide the stalks include the oligomycin sensitivity-conferring protein (OSCP)\(^1\) and subunits a and b of \( F_0 \). There are also various other peptides in the yeast and mammalian enzymes whose specific role is not certain, including subunits d–i, and \( F_0 \) (10–14). Thus, many subunits must associate to form a complete and functional ATP synthase with interactions spanning from \( F_0 \) to \( F_1 \).

The crystal structures of bovine (15), rat liver (16), and *Bacillus* PS3 (17) \( F_1 \)-ATPases indicate that the \( \alpha \)- and \( \beta \)-subunits of the ATPase are divided into three domains. The top domain is composed of a 90-amino acid \( \beta \)-barrel structure that forms a crown on top of \( F_1 \). This crown is 50 Å from the nucleotide-binding site and does not appear to be critical in the formation of the active site. The role of the \( \beta \)-barrel domains is not certain, but they were postulated to be important for stabilizing \( F_1 \)-ATPase (6). This hypothesis is tested here by making chimeric enzymes that replace the \( \beta \)-barrel domains from yeast *Saccharomyces cerevisiae* ATPase with those from the thermophilic bacterium *Bacillus* PS3 enzyme. It was predicted that if the \( \beta \)-barrel structures were critical in stabilizing the enzyme, then the chimeric enzyme would be more thermostable than the wild-type yeast enzyme. No support for this hypothesis was obtained, but instead the results indicate that the crown made by the \( \beta \)-barrel domains forms critical interactions necessary for the assembly of the ATP synthase. These interactions appear to map on the face of the \( \beta \)-barrel crown at the noncatalytic interface between the \( \alpha \) - and \( \beta \)-subunits.

**EXPERIMENTAL PROCEDURES**

*Yeast Strains and Media—* A list of the parent strains used in this study is shown in Table I. The yeast *S. cerevisiae* strain BY101 was made by making a null mutation in the *ATP1* gene in the host strain W303-1A. The strain BY6A is a meiotic progeny of the diploid formed from mating BY101 with DMY301.

The yeast media are standard recipes as described (20): YPD, 1% yeast extract, 2% peptone, and 2% glucose; YPG, 1% yeast extract, 2% peptone, and 3% glycerol; and YPAD, 1% yeast extract, 2% peptone, 20 mg/liter adenine sulfate, and 2% glucose. Synthetic minimal medium contained 2% glucose (SD) and was supplemented with adenine, histidine, arginine, methionine, tyrosine, lysine, leucine, isoleucine, and tryptophan or uracil at 20 mg/ml.

**Gene Disruptions—** The genes encoding the \( \alpha \)-subunit (*ATP1*) and \( \beta \)-subunit (*ATP2*) of the ATPase were disrupted following a single-step procedure using linear plasmid DNA (21). The plasmid for the null mutation in *ATP2* was described earlier (18). The knockout plasmid for the *ATP1* gene was made by digesting *ATP1* with BglII and replacing the 210-base pair fragment with the 1767-base pair *BamHI* fragment containing the *HIS3* gene. The resulting *ATP1/HIS3* construct was released from the plasmid after digestion with *BamHI* and EcoRI and then used to transform (22) yeast W303-1B.

**Construction of the Chimeric Genes—** The chimeric subunits containing the \( \beta \)-barrel domain from PS3 and the remaining portion from *S. cerevisiae* were made by taking advantage of the ability of yeast to perform gap repair (23). A schematic diagram for the production of the chimeric genes for the *α*-subunit (*ATP1*) and *β*-subunit (*ATP2*) is shown in Fig. 1. The method requires that the gene of interest (the \( \alpha \) - and \( \beta \)-subunits) is cloned into a yeast centromere vector, in this case, pRS316 and pRS314 (19), respectively. The method also requires the presence of a unique restriction enzyme site in the region that is to be exchanged. There was not a unique restriction site available in the DNA encoding the \( \beta \)-barrel domains of the \( \alpha \) - or \( \beta \)-subunits. Thus, a unique restriction site was added to the appropriate region by inserting the gene encoding either the chloramphenicol (Cm) or kanamycin (Kan) resistance into the region of the portion of the gene encoding the \( \beta \)-barrel domain of the \( \alpha \) - and \( \beta \)-subunits, resulting in plasmids pY16ATP1/Cm and pRS344ATP2SB/Kan, respectively. The Cm and Kan genes were obtained by PCR using the primers shown in Table I.

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\(^{1}\) The abbreviations are: OSCP, oligomycin sensitivity-conferring protein; Cm, chloramphenicol; Kan, kanamycin; PCR, polymerase chain reaction.

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the PS3 replacement sequence was amplified by PCR using primers (Table I) containing the genes encoding the ATP1α units of the ATPase from PS3, pUC118 (kindly provided by Dr. Masasuke Yoshida). The PCR products consisted of the CDS that contained the sequence of the desired target for homologous recombination and the DNA that encodes the N-terminal sequence analysis of the purified α- and β-subunits of PS3 (Table I). The numbering system used in Fig. 2 assigns position 0 to the yeast and PS3 α- and β-barrel domains in the structure and function of F1-ATPase. The initial hypothesis was that the β-barrel domains from the α- and β-subunits of PS3 replaced the β-barrel domains of the corresponding subunits from yeast.

The primary sequence comparison of the β-barrel domains of the α- and β-subunits from yeast and PS3 is shown in Fig. 2A. The yeast and PS3 α- and β-subunits are 45 and 57% identical in this region as compared with an overall identity of 59 and 71%, respectively. Thus, this region is less well conserved than the other portions of the molecule. Since only the circular plasmid DNA was sequenced, the entire region that was amplified by PCR and the DNA that encodes the upstream and downstream borders of the ligation sites were sequenced to ensure that accurate homologous recombination had occurred and to ensure the absence of other mutations generated by PCR.

**Phenotypic Testing**—The function of the chimeric subunits was tested by determining the growth phenotypes of cells containing each of the chimeric constructs. The plasmids containing the chimeric constructs were introduced into the yeast strains BY101 (α), DMY111 (β), and BY6A (αβ); selected on minimal glucose medium; and tested on YPG medium at 18, 30, and 37 °C. Growth on medium containing glycerol as the sole carbon source indicates a functional ATP synthase.

**Gain-of-Function Mutant Isolation**—To obtain strains able to grow on YPG medium, the colonies obtained after transformation of DMY111 with the PCR product and the single-cut pRS344ATP2SB/Kan were replica-plated onto YPG medium and incubated at 30 °C. Colonies that grew were selected and restested on YPG medium to confirm the phenotype. Plasmid DNA was isolated from the yeast, transformed into E. coli XL1-Blue, and sequenced as described above. The plasmid was also retransformed into DMY111 and tested for its ability to rescue the YPG− phenotype of DMY111. For the isolation of strains 14.1–14.20, BY6A [αATP1]βATP2-4 (the brackets indicate nonchromosomal plasmids) was grown on SD-Trp-Ura medium (3 ml); the cells were washed once with sterile water; and ~106 cells were plated onto YPG medium and incubated at 30 °C. The plasmids were isolated from each of the 20 strains, amplified in E. coli, and retransformed into BY6A. The plasmids did not provide BY6A the ability to grow on YPG medium, indicating that the YPG− phenotype was not due to a mutation on either cATP1 or cATP2-4.

**Biochemical Studies**—N-terminal sequence analysis of the purified α- and β-subunits of the ATPase was performed as described (24). Mitochondria were isolated from yeast after growth on minimal medium containing the auxotrophic requirements (25). The extraction of the mitochondria with chloroform, Western blot analysis, and ATPase measurements were done as described (25). Protein concentration was determined by the BCA method (26) for samples that contained membrane proteins and by a modified Bradford method for soluble proteins (26). All biochemical experiments were repeated at least twice starting from the growth of the cells, and all assays were done in at least duplicates for each preparation.

**RESULTS**

The goal of this study was to determine the role of the β-barrel domain in the structure and function of F1-ATPase. The initial hypothesis was that the β-barrel domains are important for stabilizing F1-ATPase (15). To test this hypothesis, chimeric subunits were made in which the β-barrel domains from the α- and β-subunits of PS3 replaced the β-barrel domains of the corresponding subunits from yeast.

FIG. 1. Method used to make the chimeric constructs. Homologous recombination effected in yeast between a PCR product and the target gene was used to make chimeric constructs. The yeast vectors containing the genes encoding the α-subunit (ATP1) and the β-subunit (ATP2) are shown in A and B, respectively. A unique NcoI site was added in the ATP1 gene by inserting the Cm resistance gene into the BseRI site, whereas a unique NruI site was introduced into the ATP2 gene by inserting the Kan resistance gene into the KpnI site. The PS3 replacement sequence was amplified by PCR using primers (Table I) that primed DNA synthesis at the beginning of the region that encoded the PS3 α- or β-subunit β-barrel domain with cloned DNA containing the gene encoding the α- or β-subunit. The 5’ end on the PCR primer contained the sequence of the desired target for homologous recombination. Upon transformation of yeast with the PCR product and the linearized plasmid, recombination closed the gap and allowed replication of the plasmid. The transformants were identified by complementation of the auxotrophic markers ura3-52 (for py16ATP1/Cm) and trp1 (for pRS344ATP2SB/Kan). CDS, coding sequence.

The Kan primers contained a KpnI site at their 5’-end to simplify the ligation of the gene into the KpnI site of ATP2. The Cm gene was blunt end-ligated into the BseRI site of ATP1 after the BseRI site was made blunt with the Klenow fragment of DNA polymerase. The ligation mixture was transformed into Escherichia coli XL1-Blue, and the transformants were selected on LB medium containing either Cm or Kan. Selected clones were checked by restriction digestion to ensure the proper construct as well as to determine the orientation of the Cm or Kan resistance gene. These constructs provided unique NcoI and NruI sites in the plasmids that were used to make the chimeric constructs.

The DNA encoding the PS3 β-barrel domain sequence and the junction point of the PS3/yeast sequence was amplified by PCR. PCR was performed with forward and reverse primers that contained ~30 bases at the 5’-end that corresponded to the desired site of recombination. The last 15 bases served to prime the PS3 sequence and corresponded to the border encoding the β-barrel domain from PS3 (Table I). PCRs were performed using plasmids containing the genes for the α- and β-subunits of the ATPase from PS3, pUC118α, and pUC118β (kindly provided by Dr. Masasuke Yoshida). The PCR products consisted of the β-barrel region of either the α- or β-subunit of PS3 flanked by 30 base pairs of DNA, which corresponded to the desired sites of recombination in either the yeast ATP1 (encodes the α-subunit) or ATP2 (encodes the β-subunit) gene. The PCR products were used to transform yeast strain BY101 (α) or DMY111 (β) with the NcoI- or NruI-cut plasmid DNA, respectively. (Note that this method does not require that the yeast strain contain a null mutation in the target gene. Use of these strains, however, allowed us to directly test the ability of the chimeric constructs to complement the respective null mutation.) Upon transformation in yeast, homologous recombination occurs between the PCR product and the corresponding linear plasmid, thereby replacing the DNA encoding the yeast β-barrel domain with the PS3 DNA and circulating the plasmid DNA. Since only the circular plasmid DNA is replicated in yeast, nearly all the resulting transformants result from the correct integration event. The transformed yeast cells were selected on minimal medium lacking the appropriate auxotrophic requirement. Colonies were taken and grown on liquid minimal medium; the plasmid DNA was isolated from the yeast; and the DNA was transformed into E. coli XL1-Blue. The plasmid DNA was isolated from E. coli, and the DNA was sequenced by cycle sequencing (Amersham Pharmacia Biotech) using [3P]-dideoxynucleotides and dITP in place of dGTP. The entire region that was amplified by PCR and the DNA that encodes the upstream and downstream borders of the ligation sites were sequenced to ensure that accurate homologous recombination had occurred and to ensure the absence of other mutations generated by PCR.
peptides, whereas the prior published determinations were obtained either by homology (27) or by an indirect method (28). The amino acids in lowercase letters correspond to the sequence derived from PS3, whereas the remaining sequence is that from yeast. For the α-subunit (Atp1p), the resulting chimera (cAtp1p) had the same number of residues as the yeast α-subunit. However, the sequence QHKARNE (cf. Fig. 2A) is unique to the β-subunit of PS3 and thus to cAtp2p. These 7 residues (residues 39–45) make the chimera 7 amino acids longer (485 residues) than the wild-type (478 residues) yeast β-subunit (Atp2p). This difference results in the slower migration of cAtp2p as compared with yeast Atp2p on SDS-polyacrylamide gel (cf. Fig. 5).

The chimeric constructs were tested individually and together to see if they were able to complement the corresponding null mutations. The tests were performed in strains DMY111 (atp2Δ) and BY6A (atp1Δ). These strains had null mutations in the gene encoding the β-subunit (ATP2) or the α-subunit (ATP1) and β-subunit of the ATPase and thus are unable to grow on medium containing glycerol as the sole carbon source (e.g., YPG). In addition, the ATP1 and ATP2 genes (both the wild type and PS3/yeast chimeras) were carried on yeast vectors that also contained the URA3 and TRP1 genes, respectively, which allowed selection of cells containing the plasmids.

The functional complementation of cAtp1p and cAtp2p, as assessed by the cells’ ability to grow on YPG medium, is shown in Fig. 3. The wild-type yeast ATP1 and ATP2 genes complement the respective mutations as seen in positions 1 and 5. However, cAtp2p did not complement the mutations either alone (position 3) or together with cAtp1p (position 7). Thus, despite forming a clear distinct domain structure separate from the rest of the α- or β-subunits, the β-barrel domains must have some species-specific structural requirements beyond just the interactions between themselves.

A further understanding of the secondary requirements for function was obtained by isolating gain-of-function mutations in cAtp2p. Cells were isolated that were able to grow on YPG medium from DMY111 (atp2Δ) containing the chimeric β-subunit gene. One example of these cells is shown in Fig. 3 (position 4, cAtp2-4). The plasmids in these cells were isolated and amplified in E. coli, and the sequence of cAtp2-4 was determined. In all but one case (cAtp2-4), a single missense mutation in a codon in the β-barrel domain of the β-subunit was identified as summarized in Fig. 2A. The clone (cAtp2-4) had a deletion of the codon for G4 in addition to the missense muta-
SD-TRP

| Key |
|-----|
| 1   |
| 2   |
| 3   |
| 4   |
| 5   |
| 6   |
| 7   |
| 8   |
| 9   |

SD-TRP-URA

YPG 18°C
YPG 30°C

FIG. 3. Complementation analysis of the chimeric PS3/yeast genes in yeast. Cells were grown on minimal glucose medium devoid of tryptophan or tryptophan and uracil (SD-TRP and SD-TRP-URA) at 30 °C or on complete medium containing glycerol (YPG) at 18 and 30 °C. The host strain was DMY111 (atp2Δ) in spots 1–4 and BY6A (atp1Δ-atp2Δ) in spots 5–9. The strains contained the following plasmids genes: [ATP2] (where the brackets indicate nonchromosomal spot 1); [pRS314] (vector alone) (spot 2); [cATP2] (spot 3); [cATP2-4] (spot 4) (see Fig. 1); [ATP1][ATP2] (spot 5); [pRS314][pRS316] (spot 6); [cATP1][cATP2] (spot 7); [cATP1][cATP2-4] (spot 8); and gain-of-function strain 14.1 (spot 9), derived from the strain shown in spot 8.

tation, T2K. Note that all of the mutations identified changed residues that are conserved between PS3 and yeast, rather than mutating nonconserved residues. Thus, single-base mutations in the β-barrel domain were able to provide a functional ATP synthase containing the chimeric PS3/yeast β-subunit.

The β-barrel domain structures of the PS3 α- and β-subunits are nearly identical to those of the bovine subunits (15, 17). The main difference is the presence of the 7 additional residues in the PS3 β-subunit, which extends the second β-sheet, forming a finger-like projection from the domain. Otherwise, the overall conformation and the relative positions of the individual residues are comparable between the bovine and PS3 proteins. Inspection of the crystal structure of PS3 F1-ATPase indicates that all of the gain-of-function mutations face the interface of the α- and β-subunits that forms the noncatalytic nucleotide-binding site (Fig. 4). None of the gain-of-function mutations lie at the interface between the α- and β-subunits, where residues from the α- and β-subunits make contact. Instead, the residues (especially βT2, βP24, and βE49) appear to map out a region on the surface of the β-barrel domain located on the front face on the surface of the interface that forms the noncatalytic nucleotide-binding site.

The identification of the gain-of-function mutation in cATP2-4 followed by retransformation of BY6A with the plasmids. In all cases, the cells were unable to grow on YPG medium, indicating that the gain-of-function mutation did not lie in either cATP1 or cATP2-4. Furthermore, retransformation of the gain-of-function strain, e.g. 14.1, which had been cured of the plasmids with cATP1 and cATP2-4, with cATP1 and cATP2-4 resulted in strains that were able to grow on YPG medium. Thus, the gain-of-function mutation(s) allowing the functional expression of cATP1 with cATP2-4 were not in either cATP1 or cATP2-4, but must be in either the chromosomal or mtDNA.

For reasons that will be more evident under “Discussion,” the initial hypothesis was that the gain-of-function mutations, which allowed the functional expression of cATP1 and cATP2-4, were in the gene encoding OSCP (ATP5 gene). To test this, ATP5 was amplified by PCR from five different strains that contained cATP1 and cATP2-4 and that were able to grow on YPG medium, and the DNA was directly sequenced. No mutations were identified in the coding region of ATP5, which indicates that the gain-of-function mutations must lie in another gene. Thus, at this point, the identity of the second gain-of-function mutations is unknown.

The ability of the cells to grow on YPG medium does not provide quantitative information on the effectiveness of the ATP synthase to make ATP. This is because only 15% of the wild-type activity of the ATP synthase is necessary for the cell growth on YPG medium (29). Therefore, the functional complementation of the chimeric genes was tested by measuring the oligomycin-sensitive ATPase from isolated mitochondria. The results of this study are shown in Table II. In general, the amount of oligomycin-sensitive ATPase in the mitochondria...
correlated well with the ability of the cells to grow on YPG medium. The gain-of-function mutations allowed the expression of the oligomycin-sensitive ATPase at nearly the same level as the wild-type enzyme. In a second set of experiments, F1-ATPase was released from the membrane by chloroform, and the efrapeptin-sensitive ATPase activity was measured. The level of the ATPase activity in the chloroform extract correlated well with that in the mitochondria.

The activity of the chimeric enzymes may be due to either the lack of assembly or an assembled but inactive enzyme. To test between these two possibilities, Western blot analysis using antibodies against yeast F1 was performed on the mitochondrial and chloroform-extracted preparations, as tested in Table II (Fig. 5). Chloroform has been used to selectively release assembled F1 from the membrane (30) and thus can be used as a test for assembly of F1-ATPase. This is illustrated in lane 1, where the α-subunit is present in the mitochondria isolated from cells with a null mutation in ATP2, but not in the chloroform extract of the mitochondrial membranes from the same cells. The analysis shows that cAtp2p in the presence of the wild-type α-subunit resulted in the degradation of wild-type Atp1p and cAtp2p (lane 2). This is unusual since the α- and β-subunits are normally stable when expressed in yeast strains with the null mutations ATP1 and ATP2, respectively (31), as is seen in lane 1 for the α-subunit. The gain-of-function mutation in cAtp2p restored the stable expression of the α- and β-subunits of F1 to near wild-type levels (lane 3). Note also that the migration of cAtp2p-4p (e.g. lane 3) was slightly slower than that of wild-type Atp2p (e.g. lane 4). This difference is due to the 7 added residues present in cAtp2p (cf. Fig. 2A).

The chimeric PS3/yeast α- and β-subunits were not stable when they were expressed together in yeast (Fig. 5, lane 7). Upon adding the gain-of-function mutation in the chimeric β-subunit (cAtp2p-4), the β-subunit, but not cAtp1p, was present together with a degradation product of either the α- or β-subunit (lane 8). Finally, strain 14.1, the strain containing cAtp2p-4 with cAtp1p and an additional undetermined mutation that allowed the functional expression of the PS3/yeast chimeras, had a stable, assembled, and functional F1-ATPase (lane 9). Thus, the gain-of-function mutations stabilized F1-ATPase assembled from the chimeric PS3/yeast α- and β-subunits.

The stable expression of a functional chimeric ATPase that contained the β-barrel domains for both the α- and β-subunits of the thermophilic enzyme PS3 allowed the biochemical analysis of the effect of these replacements on the thermostability of the enzyme. The thermostability of the membrane-bound enzyme was assessed using isolated mitochondria, whereas the thermostability of F1-ATPase was determined using the chloroform extract of the mitochondrial membrane. Thermodenaturation studies at 55 and 60 °C failed to show any differences between the thermostability of the wild-type and chimeric enzymes (data not shown). Although the chimeric enzymes were not any more thermostable than the wild-type enzyme, it is important to note that the enzymes were not any less thermostable, which suggests that the conformation of the chimeric enzymes was not grossly altered.

**DISCUSSION**

This work used a novel method to make fusion protein constructs, although it is based on a prior method used to make new plasmids (23). The method (see Fig. 1 and “Experimental Procedures”) takes advantage of the high rate of homologous recombination in yeast and the ability to perform gap repair of plasmids. The method is efficient, quick, and not restricted to any sequence or gene. In addition to the constructs presented in this study, this laboratory has used this method for making numerous other fusion constructs with 100% success. Also, the number of incorrect clones obtained or identified was only ~10%, making this method very efficient. Thus, this method can be used as a general tool for making protein or gene fusion constructs.

The initial hypothesis that was being tested in this study was that the β-barrel domains of the α- and β-subunits function to increase the stability of F1-ATPase. This hypothesis was originally proposed based on the crystal structure of bovine F1-ATPase (15), which showed that these domains were far from the catalytic site (50 Å) and clearly formed a domain structure in the form of a crown. The α- and β-subunits interact with each other along a single face that forms the catalytic and noncatalytic nucleotide-binding sites. As such, the β-barrel domain structures of the α- and β-subunits have large faces of interactions, but the β-barrel domains are far from the nucleotide-binding sites and do not contribute directly to their formation.

Initially, it was predicted that the chimeric PS3/yeast α- and β-subunits would not be able to function without each other. This prediction was based on a prior study that indicated that the top domain of the E. coli α-subunit could not be replaced with the corresponding region from Bacillus megaterium whereas the catalytic domain could be exchanged (32). Furthermore, the E. coli uncD strain could not be complemented by expression of the chloroplast β-subunit, although the chloroplast chimeras containing the E. coli β-barrel domain was able to restore oxidative phosphorylation (33). These results suggested that there was a greater requirement for species-specific

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**TABLE II**

*Specific ATPase activity of isolated mitochondria and the chloroform-extracted preparation*

|          | Mitochondria | Chloroform extract |
|----------|--------------|--------------------|
|          | Specific activity + oligomycin | Specific activity + Efrapeptin |
| DMY11 [pRS314] | 0.11 0.11 | 0.06 0.05 |
| DMY11 [cATP2] | 0.07 0.05 | 0.03 0.02 |
| DMY11 [cATP2-4] | 0.43 0.18 | 1.36 0.13 |
| DMY11 [ATP2] | 0.92 0.16 | 3.5 0.13 |
| BY6A [ATP1][pRS314] | 0.07 0.05 | 0.02 0.02 |
| BY6A [ATP1][cATP2] | 0.43 0.05 | 4.7 0.11 |
| BY6A [cATP1][cATP2] | 0.1 0.03 | 0.1 0.1 |
| BY6A [cATP1][cATP2-4] | 0.1 0.03 | 0.16 0.07 |
| 14.1 | 1.0 0.08 | 3.9 0.9 |

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**Fig. 5. Western blot analysis of mitochondrial and chloroform-extracted proteins.** The mitochondria were purified, and chloroform extraction was done as described under "Experimental Procedures." A total of 2 μg of protein was separated in each lane by SDS gel electrophoresis, and the protein was transferred to nitrocellulose and probed with antiserum directed against the yeast α- and β-subunits of the ATPase. Lanes 1–4 and lanes 5–9 correspond to protein isolated from strains DMY111 (atp2) and BY6A (atp1 atp2), respectively, containing the following plasmids (genes): [pRS314] (where the brackets indicate nonchromosomal); lane 1; [cATP2] (lane 2); [cATP2-4] (lane 3); [ATP2] (lane 4); [ATP1][pRS314] (lane 5); [ATP1][ATP2] (lane 6); [ATP1][cATP2] (lane 7); [cATP1][cATP2-4] (lane 8); and gain-of-function mutant strain 14.1 (lane 9), derived from the strain shown in lane 8.
residues in the β-barrel domains as compared with those in the remaining portions of the β-subunit.

The species-specific primary structural requirements in the β-barrel domain of the β-subunit might be due to the interactions with the corresponding domain of the α-subunit, interactions with another subunit(s) of the ATP synthase, or a combination of both types of interactions. However, the structural requirement does not appear to be due to the interactions with the corresponding β-barrel domain of the α-subunit for two reasons. First, expression of the chimeric PS3/yeast α- and β-subunits together does not provide a functional enzyme. This indicates that despite restoring the putative interactions between the domains, the enzyme is still unable to function. Second, a number of gain-of-function mutations in the β-barrel domain of cAtp2p allowed the functional expression of the ATP synthase. These mutations were all in residues that were conserved between PS3 and yeast, suggesting that the mutations were not simply restoring specific interactions that were lost in cAtp2p (Fig. 2A). These mutations (especially those at T2, P24, and E49) map to the groove that lies between the α- and β-subunits in the interface that forms the noncatalytic nucleotide-binding site.

The gain-of-function mutation T2K, ΔG4 may be very telling because it is at the very end of the N terminus of the β-subunit. The T2 side chain points directly into the groove formed at the interface of the α- and β-subunits, but does not interact with residues in either the α- or β-subunit. The simplest explanation for the mechanism of this gain of function is that the mutation affects the interactions with another subunit(s) of the ATPase and that this interaction occurs within this groove. This is also the simplest explanation for the other gain-of-function mutations, although some of the mutant residues must act indirectly with the putative subunit as the side chains are not positioned at the surface of the domain. It is important to remember that although the gain-of-function mutations are different, they must all act by the same mechanism, i.e. restoring interactions that were lost by replacing the β-barrel domain of the yeast subunit with that of the PS3 subunits. As such, the simplest explanation for the mechanism of gain of function also seems to be the most likely mechanism.

Other studies have also indicated that the β-barrel domain is important for the assembly of the ATP synthase, and in E. coli, for specific interactions with the E. coli δ-subunit. First, mutations in the β-barrel domains of the E. coli α- and β-subunits have been shown to impair the assembly of the ATP synthase by inhibiting binding of F1 to F0 (34–36). Second, limited tryptic digestion of bovine F1 removed 15 and 7 amino acids from the N terminus of the α- and β-subunits, respectively, resulting in the decoupling of ATP hydrolysis from proton transport (37). Similarly, proteolytic removal of the first 50 amino acids of the E. coli α-subunit impaired binding of F1 to F0 (38). Finally, cross-linking studies have indicated that the E. coli δ-subunit interacts with the α- and β-subunits and likely with the β-barrel domain of the β-subunit (6, 7). Thus, it appears that a probable reason why cAtp1p and cAtp2p are not functional either alone or together in yeast is because of structural requirements in the β-barrel domain for interactions with other subunits of the ATP synthase. Since OSCP is homologous to the E. coli δ-subunit, it seemed likely that the interaction with OSCP was disrupted in the ATPase formed with cAtp1p and cAtp2p.

The isolation of gain-of-function mutations in BY6A (atp1-1 atp2-2) with the expression of cAtp1p and cAtp2-4p (e.g. strain 14.1) provided a possible source for genetic evidence to support the presence of an interaction of OSCP with the β-barrel domains. The gain-of-function mutations in these strains were not in either the α- or β-subunits based on a number of observations. First, after curing the plasmids from the strain, reintroduction of the strain with cATP1 and cATP2-4 was sufficient to restore cell growth on YPG medium. Second, purification of the plasmids from the strains that were able to grow on YPG medium and the subsequent transformation of the purified plasmids into BY6A did not provide a cell able to grow on YPG medium. Therefore, the gain-of-function mutation must be in a gene(s) other than cATP1 or cATP2-4. The first postulate was that the mutation was in the gene encoding OSCP (ATP5). However, we sequenced ATP5 from five different gain-of-function mutants, including 14.1, and were unable to identify any mutations in the coding region. Although some of the other isolates may indeed have mutations in ATP5, for at least the five studied, a mutation in another gene must be responsible for the gain of function. Possible candidate genes include those that encode subunit b of F0 or possibly genes encoding other ancillary proteins, such as subunits d–i.

The initial hypothesis that was tested in this study was that the β-barrel domains are important for stabilizing the ATPase (15). The prediction that followed from this hypothesis was that an increase in the thermostability of this domain structure would result in an increase in the overall thermostability based on the ATPase activity of the enzyme. However, the chimeric enzyme containing the β-barrel domains from PS3 showed no significant difference in the thermostability of the enzyme. Although this negative result did not disprove the initial hypothesis, the data do not support it either. With the wealth of data that show the importance of this region in the assembly of the ATP synthase, it is likely that a major role of this domain in the enzyme is its interactions with other subunits helping in forming the ATP synthase. Indeed, as it appears that the subunits that interact with the domain form the stator of the enzyme (6–9, 37–41), the easiest way to hold F1 in place during rotation of the γ-subunit would be by holding it at the top of the molecule. Furthermore, subunit-barrel interactions occurring in the groove formed at the noncatalytic nucleotide interface would be expected to be the least disruptive during the catalytic cycle.

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