Journal of Bioenergetics and Biomembranes, Vol. 32, No. 4, 2000

Partial Assembly of the Yeast Mitochondrial ATP Synthase[1]

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The mitochondrial ATP synthase is a molecular motor that drives the phosphorylation of ADP to ATP. The yeast mitochondrial ATP synthase is composed of at least 19 different peptides, which comprise the F_1 catalytic domain, the F_0 proton pore, and two stalks, one of which is thought to act as a stator to link and hold F_1 to F_0, and the other as a rotor. Genetic studies using yeast *Saccharomyces cerevisiae* have suggested the hypothesis that the yeast mitochondrial ATP synthase can be assembled in the absence of 1, and even 2, of the polypeptides that are thought to comprise the rotor. However, the enzyme complex assembled in the absence of the rotor is thought to be uncoupled, allowing protons to freely flow through F_0 into the mitochondrial matrix. Left uncontrolled, this is a lethal process and the cell must eliminate this leak if it is to survive. In yeast, the cell is thought to lose or delete its mitochondrial DNA (the petite mutation) thereby eliminating the genes encoding essential components of F_0. Recent biochemical studies in yeast, and prior studies in *E. coli*, have provided support for the assembly of a partial ATP synthase in which the ATP synthase is no longer coupled to proton translocation.

KEY WORDS: ATP synthase; F_1-ATPase; *Saccharomyces cerevisiae*; petite mutants; epistasis; mitochondrion; pet mutants.

BACKGROUND

The mitochondrial ATP synthase is molecular motor that couples the flow of protons down a chemical gradient to the phosphorylation of ADP. The ATP synthase is composed of two distinct domains: the F_1 that contains that catalytic site and F_0, which is thought to act like a proton turbine, funneling protons from the cytosol into the mitochondria during ATP synthesis. The F_1 has been shown to act as a molecular motor with the γ subunit rotating within the central core of three α/β pairs (Duncan *et al.*, 1995; Noji *et al.*, 1997; Sabbert *et al.*, 1996) to drive ATP synthesis by a scheme described by the binding site mechanism of Boyer (1989; Boyer *et al.*, 1973). Intrinsic features of this molecular motor include the rotor, which is minimally made of the γ subunit, and the stator which holds F_1, and prevents its rotation from the torque generated from the rotation of the rotor.

The basic structure and mechanism of the ATP synthase is conserved from *E. coli* to mammals. However, the biochemistry and the peptide compositions of the enzymes are not identical. Table I compares the known peptide composition of the bacterial, yeast, and bovine enzymes. The *E. coli* enzyme is a comparatively very simple enzyme containing only 8 different polypeptides (Senior, 1988), while the yeast mitochondrial enzyme contains at least 19 different peptides of which 14 are essential or critical. The nonessential subunits include the inhibitor peptides, Inh1p and Stf1p, that

[1] Key to abbreviations: DCCD, dicyclohexylcarbodiimide; IF_1, inhibitor of F_1; mt, mitochondrial; OSCP, oligomycin sensitivity-conferring protein; ρ^0/ρ^-, mtDNA either eliminated ρ^0 from the cell or containing a large deletion; ρ^-, UCP, uncoupler protein; YPD, 1% yeast extract, 2% peptone, 2% glucose; YPG, 1% yeast extract, 2% peptone, 3% glycerol.

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0145-479X/00/0800-0391$18.00/0

Table I. Subunit Composition of the Bovine, *E. coli*, and Yeast ATP Synthases[a]

Bovine	Alias	*E. Coli*	Yeast	Yeast gene	Genome	Importance
α	Sub. *1*	α	α	ATP1	Nuclear	Essential
β	Sub. *2*	β	β	ATP2	Nuclear	Essential
γ	Sub. *3*	γ	γ	ATP3	Nuclear	Essential
δ		ε	δ	ATP16, ATPδ	Nuclear	Essential
ε		None	ε	ATP15, ATPε	Nuclear	Important
OSCP	Sub. *5*	δ	OSCP	ATP5	Nuclear	Essential
Sub. *a*	Sub. *6*	Sub. *a*	Sub. *a*	ATP6	Mito	Essential
Sub. *b*	Sub. *4*	Sub. *b*	Sub. *b*	ATP4	Nuclear	Essential
Sub. *c*	Sub. *9*	Sub. *c*	Sub. *c*	ATP9	N (bovine) mt. (yeast)	Essential
Sub. *8*	A6L	None	Sub. *8*	ATP8	Mito	Essential
Sub. *d*	Sub. *7*	None	Sub. *d*	ATP7	Nuclear	Essential
Sub. *e*	TIM11	None	Sub. *e*	ATP21/TIM11	Nuclear	Dispensable
Sub. *f*		None	Sub. *f*	ATP17	Nuclear	Essential
Sub. *g*		None	Sub. *g*	ATP20	Nuclear	Dispensable
?		None	Sub. *h*	ATP14	Nuclear	Essential
?	Sub. *j*	None	Sub. *i*	ATP18	Nuclear	Essential
?		None	Sub. *k*	ATP19	Nuclear	Dispensable
F6		None	?	?	Nuclear	Essential
IF_1		None	Inh1p	INH1	Nuclear	Dispensable
?		None	Stf1p	STF1	Nuclear	Dispensable

[a] The known subunits of the ATP synthase for bovine, yeast, and *E. coli* are shown. "Alias" indicates other names that may be used to identify a peptide. "Genome" indicates where the gene is encoded. Refer to the text for relevant references.

prevent the hydrolysis, but not the synthesis of ATP (Hashimoto *et al.*, 1984; Hashimoto *et al.*, 1990; Ichikawa *et al.*, 1990), and three subunits, *e*, *g*, and *k*, that are involved in the dimerization of the ATP synthase (Arnold *et al.*, 1997, 1998). At present, there is not a yeast counterpart for the bovine F6 protein, yet it is likely to be one of the essential peptides of the yeast enzyme, e.g., subunit *h* or *i* (Arnold *et al.*, 1999; Paumard *et al.*, 2000; Roudeau *et al.*, 1999; Vaillier *et al.*, 1999) for which there is no bovine homolog. Conversely, the as-of-yet absence of bovine homologs for yeast subunits *h*, *i*, and *k* (Walker *et al.*, 1985, 1991) suggests either that the peptide composition of the yeast enzyme differs slightly from the mammalian enzyme or that the mammalian homologs have simply not been identified.

In addition to the differences in the structure of the bacterial and mitochondrial enzymes, their path of assembly appear to also differ. In yeast, at least two peptides are involved solely for the assembly or folding of F_1, Atp11p and Atp12p (Ackerman and Tzagoloff, 1990b; Wang and Ackerman, 1996; Wang and Ackerman, 2000), and at least one protein is involved solely for the assembly or folding of subunits in F_0, Atp10p (Ackerman and Tzagoloff, 1990a). These work with a number of general chaperones and proteases for the import, folding, and assembly of the F_1F_0-ATP synthase (Altamura *et al.*, 1996; Arlt *et al.*, 1996; Lemaire *et al.*, 2000; Neupert, 1997; Rep *et al.*, 1996; Tzagoloff *et al.*, 1994). Bacterial F_1, in contrast, can be assembled *in vitro* using the purified peptides. The increased complexity of the mitochondrial enzyme as compared to the bacterial enzyme begs the question, why? Typically, the increased complexity of the mammalian enzyme is a result of additional needs for the regulation of the enzyme. Indeed, the added complexity of the synthesis, assembly, and peptide composition, of the mitochondrial enzyme may be possible points of regulation in the cell.

Yeast is a very powerful system that can be used to study the structure/function/regulation relationship of the mitochondrial ATP synthase. The unique ability of yeast to perform a high rate of homologous recombination allows rapid, reliable, and thus simplifies sequential gene disruptions. Genetics also offers a tool that can be very sensitive, sometimes beyond the limit of standard biochemical methods. Mutations in genes can provide phenotypes even in genes whose products are at very low cellular concentrations. Once identified, however, routes can be devised for the biochemical

characterization of a protein that might otherwise go unnoticed. It is in this context that we have been able to provide evidence for the assembly of the mitochondrial ATP synthase that is missing essential subunits of F_1.

DISCUSSION

Phenotypes of Yeast Deletion Mutations in the Genes Encoding Subunits of F_1

The initial findings that led us onto this path of discovery were the differences in the phenotypes observed in yeast cells deleted of genes encoding subunits of F_1 (Lai-Zhang *et al.*, 1999). It was observed that a yeast strain that had a deletion in the gene encoding either the α or β subunit, had the expected phenotype of being unable to grow on a nonfermentable carbon source, such as glycerol (YPG). However, when the gene encoding the δ, γ, or ε subunit was deleted, then the cells obtained additional phenotypes including the loss of mtDNA and slow growth on rich medium containing glucose (YPD). For cells with a deletion in the γ and δ subunits, 100% of the cells became petite (ρ^0/ρ^-), meaning that they lost their mtDNA (or the DNA had large deletions). The petite phenotype is actually quite common in yeast and up to 10% of even wild-type cells can contain petite mutations. However, for 100% of the cells to become petite is quite unusual. An increased tendency to form petite mutations was reported for mutations in other genes encoding subunits of the ATP synthase, including OSCP (Uh *et al.*, 1990), subunits *d* (Norais *et al.*, 1991), *h* (Arselin *et al.*, 1996), and *f* (Spannagel *et al.*, 1997).

Still, the question remained, why did, for instance, a mutation in the gene encoding the γ subunit (or δ, or ε subunit) have phenotypes in addition to the phenotypes of the cells with mutations in the genes encoding the α or β subunit? There were two possible explanations for this phenomenon: either the γ subunit is involved in a second function, unrelated its role in the ATP synthase, or an enzyme complex is being made devoid of the γ subunit and this enzyme complex is responsible for the additional phenotypes. These two possibilities were easily resolved using genetic analysis.

Epistatic Interactions of the Deletion Mutations in the Genes Encoding Subunits of F_1

Epistasis means "to stand above" and is similar in meaning to "dominance." However, dominance refers to alleles of the same gene, while epistasis refers to mutations within different genes. Since the mutation in the genes encoding the α or β subunit gave a phenotype that differed from the phenotype observed in cells with a mutation in the δ, γ, or ε subunit, we were able to explore the epistatic relationship of these mutations. Thus, double mutations were constructed in which a mutation in the γ subunit, for instance, was paired with a mutation in the gene encoding the α or β subunit (Lai-Zhang *et al.*, 1999). The results clearly indicated that a mutation in the gene encoding the α and β subunit was epistatic to the mutation in the gene encoding either the γ, δ, or ε subunit. In other words, the phenotypes observed in the cells with the double mutations were that of the cell with a mutation in the gene encoding the α or β subunit. This result indicated that the first possibility discussed above could not be true: the cause of the additional phenotype in the cells containing the deletion mutation in the gene encoding the γ subunit was not due to an additional role of the γ subunit, something outside the ATP synthase. The same epistatic relationship was observed with mutations in the genes encoding the δ and ε subunits.

Of course, these experiments did not prove that a γ-less ATP synthase complex formed and was responsible for the secondary phenotypes. To provide further support for this hypothesis, double mutants were generated in cells with a null mutation in the gene encoding the γ subunit and coupled with a mutation in ATP11. Atp11p is not a subunit of the ATP synthase, but it is required for its assembly (Ackerman and Tzagoloff, 1990b; Wang and Ackerman, 2000). Cells with mutations in both ATP11 and the gene encoding the γ subunit had a phenotype like cells with the atp11 mutation alone. Thus, ATP11p is required in order to exhibit the secondary negative phenotypes of the mutations in the γ, δ, or ε subunit. Finally, by adding a missense mutation in the gene encoding the β subunit that inhibited enzymatic activity, but not the assembly of the enzyme, it was demonstrated that the secondary negative phenotypes required a functional active site. Overall, the genetic data indicated that for the cell to exhibit the secondary negative phenotypes associated with the null mutations in the γ, δ, or ε subunit, the cell required the α and β subunits, Atp11p, and a functional active site in the F_1-ATPase. What was not

needed, however, was the γ, δ, or ε subunit, as a yeast strain deleted in any one or two of these genes had a phenotype associated with the single deletion mutation, with the deletion in the gene encoding the γ subunit being epistatic to the other two mutations and the single deletion in the gene encoding the δ subunit being epistatic to the mutation in the gene encoding the ε subunit.

Implicit in these results is that the secondary negative phenotypes associated with the null mutation in the gene encoding the γ, δ or ε subunit was due to a gain-of-activity in the cell. This is concluded because the loss of the secondary phenotypes is observed after complete *deletion* of the gene encoding the α or β subunit or ATP11. This gain-of-activity is not to be confused with a gain-of-ATP synthetase activity, but it is likely a gain-of-activity that is not normally present, or present at a low level, that is dependent on the ATP synthase minus the deleted subunit.

A model to explain these results is shown in Fig. 1. The model suggests that with the deletion of the γ, δ, or ε subunit, an enzyme complex is formed that is missing the corresponding subunit. This partial enzyme complex is defective in coupling proton translocation to ATP synthesis, i.e., the enzyme is uncoupled. This uncoupling activity is proposed to be the "gain-of-activity," as discussed above. The proton pore in the F_0 is proposed to be intact, but the proton flow is proposed not to effectively couple the rotation of the γ subunit in F_1.

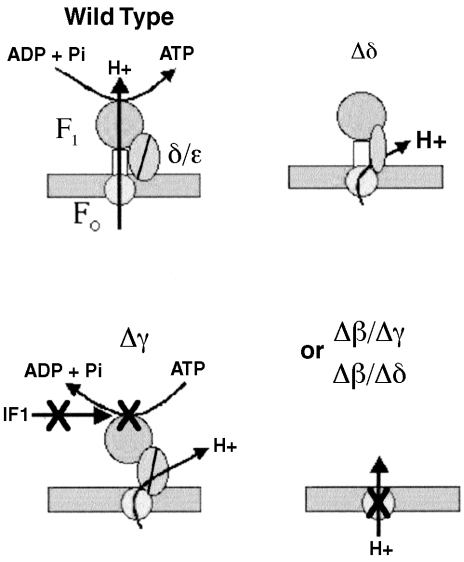


Fig. 1. Biochemical model to explain the results of the genetic data. The wild-type enzyme is shown as composed of the catalytic core, F_1, the proton pore, F_0, and ATP synthesis is driven by the flow of protons into the matrix of the mitochondria. When the gene encoding the γ or δ subunit is deleted, an enzyme complex is formed, devoid of the corresponding subunit, and passes protons into the matrix without the synthesis of ATP, i.e., proton flow uncoupled to ATP synthesis. When the γ subunit is missing, the γ-less enzyme hydrolyzes ATP and this hydrolysis is postulated to be insensitive to the inhibitor protein, IF_1. In the presence of a mutation in the α or β subunit, in F_0 does not form a proton permeable pore, for reasons that are not entirely clear.

The uncoupling defect is easy to understand in the enzyme missing the γ subunit, since the γ subunit is the rotor of the motor. Thus, removal of the rotor from a motor would result in a motor that ran effectively, but did not couple the energy from the proton gradient to produce ATP, i.e., uncoupled. For the δ and ε subunits, evidence from *E. coli* indicates that the ε subunit, which corresponds to the mitochondrial δ subunit, is also part of the rotor (Aggeler and Capaldi, 1996; Aggeler *et al.*, 1992, 1997; Bulygin *et al.*, 1998; Capaldi *et al.*, 1992; Kato-Yamada *et al.*, 1998; Tang and Capaldi, 1996; Wilkens and Capaldi, 1998). The mitochondrial ε subunit has been demonstrated to form a dimer with the δ subunit, thus implicating the mitochondrial δ/ε dimer as part of the rotor (Orriss *et al.*, 1996; Penin *et al.*, 1990). Thus, the available evidence on the structure/function of the γ, δ, and ε subunits is supportive of the predictive uncoupling effect of an ATP synthase complex that is missing either the γ, δ, or ε subunit.

The model in Fig. 1 suggests that the presence of the subunit-deficient enzyme complex will result in an uncoupling of the mitochondria. However, this is a lethal event, since the biogenesis of the mitochondrion requires a $\Delta\Psi$ across the mitochondrial membrane (for review, see Neupert, 1997). As such, in order for the yeast to survive, they must eliminate this proton leak. Since the proton leakage is via F_0 and since the mtDNA encodes three subunits of F_0, it is proposed that the loss of the mtDNA occurs in order to eliminate F_0 and thus the proton leak. This is proposed as the explanation as to why deletion of the genes encoding either the γ or δ subunit results in 100% petite formation. Furthermore, deletion of the gene encoding the ε subunit results in 60% of the cells becoming petite and very slow growth on YPG medium (Lai-Zhang *et al.*, 1999). The implication is that loss of the ε subunit causes a partial uncoupling of the ATP synthase and thus just 60% petite formation. This is reasonable since the ε subunit is not an essential component of the ATP synthase, having no apparent bacterial homolog, and deletion of the gene severely impairs, but does not eliminate, growth of yeast on medium containing glyc-

erol, a nonfermentable carbon source (YPG) (Lai-Zhang *et al.*, 1999).

Cells with a deletion mutation in the gene encoding the γ subunit grow significantly slower than those with a deletion mutation in any of the other genes encoding subunits of the ATP synthase that we have studied, including those encoding the α, β, and δ subunits, OSCP and ATP11. Figure 2, illustrates the difference in the growth of cells containing a deletion in the γ subunit as compared to cells with a deletion in the δ subunit. The experiment in Fig. 2 is a tetrad analysis of diploid cells with a heterozygous mutation

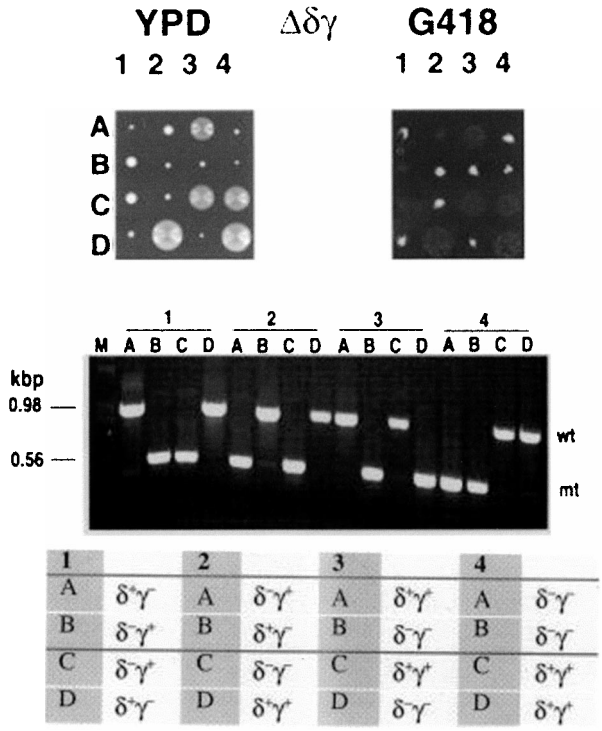


Fig. 2. Tetrad analysis demonstrating the epistatic relationship of mutations in the genes encoding the δ and γ subunits. Diploid cells containing a heterozygous mutations in the genes encoding the γ and δ subunits were sporulated and the four spores (A–D) dissected onto rich medium (YPD). The colonies were replica-plated to YPD plates containing 0.2 mg/ml G418 and also tested by whole-cell PCR analysis using primers that spanned the gene encoding the δ subunit. The G418 resistance module was used to disrupt the gene encoding the γ subunit and thus G418 resistance cells have a deletion in this gene. Determination of the genotype of the gene encoding the δ subunit was determined by whole-cell PCR analysis using primers that spanned the ATPδ. A PCR product of 942bp and 486bp is calculated for the reaction across the wild-type and deleleted gene encoding the δ subunit, respectively. The lower panel shows the genotype as determined from the growth on the G418 medium and the PCR analysis. This analysis is described in Lai-Zhang *et al.* (1999).

in the genes encoding the γ and δ subunits. The diploid cells are sporulated and the four spores are positioned onto a YPD plate and allowed to grow into colonies (labeled A–D). Since the genes encoding the γ and δ subunits are not linked genetically, their segregation is random and in a Mendelian manner. The genotype of these locus is easily identified by PCR analysis across the gene encoding the δ subunit and by growth on G418 medium, the marker for the deletion mutation in the γ subunit. There are only two combinations of the genes (ditype) represented in the first tetrad with cells in colonies A and D, having a mutation in the gene encoding the γ subunit and those in B and C having the mutation in the δ subunit. It is clear from the growth of these spores that the cells containing the deletion in the gene encoding the γ subunit grow slower than those with the deletion in the gene encoding the δ subunit. This indicates that the loss of the γ subunit is more deleterious to the cell than the loss of the δ-subunit. The model in Fig. 1 suggests an explanation for this differential effect. It is hypothesized that the enzyme missing the γ subunit can hydrolyze ATP, which reduces the intracellular level of ATP. Typically this backhydrolysis is controlled by IF_1, the inhibitor of the ATPase activity, but it is postulated here that IF_1 is not effective on the γ-less enzyme complex. This provides a molecular explanation of how IF_1 can inhibit the hydrolysis but not the synthesis of ATP. Possibly, IF_1 acts to block the rotation of the γ-subunit in the direction of ATP hydrolysis, but not ATP synthesis. Like a ratchet of a wrench, IF_1 could block the unidirectional rotation of the γ subunit. Of course, loss of the γ subunit of in the ATPase would result in a loss of the inhibition by IF_1, if this model is correct. The model in Fig. 1 thus predicts a large number features, many of which are testable.

The Proton Leak is Sensitive to Oligomycin

The model in Fig. 1 suggests that proton flow through F_0 in an uncontrolled fashion, thereby uncoupling the mitochondrion and causing the negative cellular phenotypes. If this is true, then blockage of proton movement through this pore would be predicted to correct the cellular phenotypes. Recent results in this laboratory indicate that a low concentration of oligomycin added to the medium corrects both the slow-growing phenotype and reduces the percentage of petites of the cells with the mutation in the gene encod-

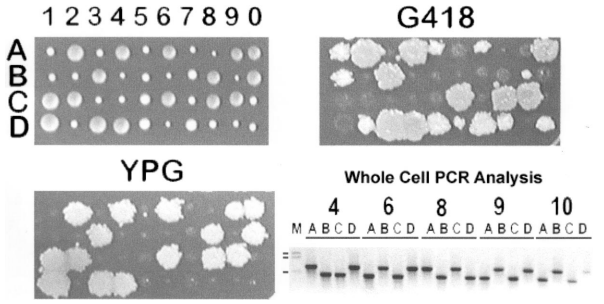
ing the γ subunit (unpublished results). Thus, oligomycin, a specific inhibitor of F_0, is able to prevent the negative cellular phenotypes. This effect is reminescent of the coupling effect of oligomycin on beef heart submitochondrial particles that have been partially stripped of the F_1 from the membrane (Lee and Ernster, 1968; Lee *et al.*, 1969). The implication is that oligomycin is blocking the leakage of protons through the F_0 component of the γ-less ATP synthase.

The γ-Less and δ-Less Enzyme Complexes Uncouple the Mitochondrion

A second prediction of this model is the assembly of the enzyme complex devoid of the γ or δ subunits results in an uncoupling of the mitochondrion. This prediction could not be tested directly in cells with a homozygous mutation in the either of these genes because it results in 100% petite formation, and thus loss of the F_0 portion of the enzyme. However, in a diploid cell, a heterozygous mutation was semidominant negative and provided a phenotype that was midway between the wild-type cell and the homozygous mutant phenotype (Xiao *et al.*, 2000). Based on a number of genetic studies, it was clear that this partial phenotype was due to a decrease in the gene dosage of either the γ or δ subunits relative to the level of the other genes of the ATP synthase. Thus, these cells provided an opportunity to test the hypothesis that the γ-less or δ-less enzyme complex uncouples the mitochondrion. Indeed, based on respiratory studies and $\Delta\Psi$ measurements, the mitochondria isolated from strains with a heterozygous mutation in either the genes encoding the γ or δ subunits resulted in an uncoupling of the mitochondria (Xiao *et al.*, 2000). Thus, it is suggested that the uncoupling of the mitochondrial membrane is responsible for the slow growth of the cells on rich medium. This is consistent with the effect of expression of human uncoupler protein 2, UCP2, in yeast, which partially uncouples the mitochondria and thereby inhibits cell growth (Fleury *et al.*, 1997).

Loss of IF_1 Inhibition in a Δδ Strain Does Not Augment the Effect of the Δδ Mutation

Another prediction made from the model in Fig. 1 is that the loss of IF_1 inhibition in the Δγ strain is the reason for the differences observed in the growth of the Δγ strain, as compared to the Δδ strain. This was tested by making a strain that was deleted in the genes encoding the δ subunit, IF_1 (INH1), and Stf1p (STF1), a second gene product that has IF_1-like activity (Fig. 3). The results were quite surprising since the growth of the strain with the deletion mutation in the genes encoding the δ subunit, IF_1 and Stf1p (e.g., 8D) did not differ as compared to the strain with a deletion mutation just in the gene encoding the δ subunit (e.g., 6B). This was a totally unexpected finding since it is logical that if the ATPase was uncoupled, that loss of IF_1 would allow uncontrolled hydrolysis of ATP, reducing the cellular level of ATP, which would be detrimental to the cell. However, even careful studies on the growth of those cells revealed no significant difference in their growth rates or in their final cell densities (by weight) after growth on minimal medium containing glucose as the carbon source. The same study was also performed with a strain with a deletion in the gene encoding the ε subunit and combined with



	3	4	6	8	9	10
A	δ⁻inh1⁻STF⁺	**δ⁺INH1⁺stf1⁻**	**δ⁺inh1⁻STF⁺**	**δ⁻INH1⁺STF⁺**	**δ⁺inh1⁻STF⁺**	**δ⁺inh1⁻stf1⁻**
B	**δ⁺INH1⁺stf1⁻**	δ⁻inh1⁻STF⁺	**δ⁻INH1⁺STF⁺**	**δ⁺inh1⁻STF⁺**	**δ⁻INH1⁺stf1⁻**	**δ⁺INH1⁺STF⁺**
C	δ⁻inh1⁻STF⁺	δ⁻inh1⁻STF⁺	**δ⁺inh1⁻stf1⁻**	**δ⁺INH1⁺stf1⁻**	**δ⁺inh1⁻stf1⁻**	δ⁻inh1⁻STF⁺
D	**δ⁺INH1⁺stf1⁻**	**δ⁺INH1⁺stf1⁻**	**δ⁻INH1⁺stf1⁻**	δ⁻inh1⁻stf1⁻	**δ⁻INH1⁺STF⁺**	**δ⁻INH1⁺stf1⁻**

Fig. 3. Effect of various combinations of deletion mutations in the genes encoding the δ subunit, IF1 (INH1) and STF1. Tetrad analysis was performed as described in Fig. 2 in a diploid cell containing a heterozygous deletion mutation in the genes encoding the δ subunit, IF_1 (INH1), and STF1. The colonies were replica-plated to rich medium containing glycerol as the carbon source (YPG) or YPD containing G418. The cells were also tested by whole-cell PCR using primers that spanned the INH1 gene. The cells that are unable to grow on YPG medium contain a deletion mutation in the gene encoding the δ subunit. Cells that are able to grow on G418 medium contain the deletion mutation in the gene encoding STF1. Cells that provide the smaller PCR product have a deletion in the INH1 gene. The lower panel shows the genotypes of the cells as indicated by the above tests. Note that all of the genes segregated 2:2 indicating a Mendelian segregation pattern consistent with a single heterozygous mutation in each of the three genes in the parent diploid cell.

mutations in IF_1 and STF1. Again, no differences were observed in this strain, as compared to the strain with a mutation in the gene encoding the ε subunit (unpublished results). There are at least two explanations for the lack of effect by the loss of IF_1 and Stf1p. First, it is possible that the δ-less or ε-less enzyme complexes are unstable and degraded to a level that is not deleterious to the cell, even in the absence of IF_1 and Stf1p. A second possibility is that there is a second mechanism for inhibiting ATP hydrolysis, which is independent of IF_1 or Stf1p, such as the inhibition by ADP. ADP has been shown to put the F_1 into an inhibited form (Minkov *et al.*, 1979). Possibly, the ADP levels rise in the cell, which then puts the δ-less or ε-deficient enzyme complex into the ADP inhibited form. Of course, if this was true, then this would suggest that the γ-less enzyme does not form the ADP inhibited form. There is some support for this since α/β core complexes made *in vitro* are insensitive to azide suggesting a role of the γ subunit in azide sensitivity (Gromet-Elhanan and Avital, 1992; Kagawa *et al.*, 1989; Miwa and Yoshida, 1989). Since azide is thought to inhibit ATPase activity by a mechanism that involves the binding of ADP and the formation of an ADP-inhibited complex (Vasilyeva *et al.*, 1982), this suggests that the γ-less enzyme does not form the ADP-inhibited form. Finally, we have been able to measure a low level of ATPase activity, which is sensitive to efrapeptin, but insensitive to azide, in mitochondria isolated from a Δγ yeast strain (unpublished data). This too supports the hypothesis that the γ-less enzyme does not form the ADP-inhibited form of the enzyme.

Thus, in general, it appears that many of the aspects of the model in Fig.1 are correct. The actual mechanism of IF_1 activity will require the atomic structure of IF_1 complexed with F_1 or the ATP synthase and this is certainly a logical goal.

Formation of the ATP Synthase Missing Two Subunits

If the prior interpretation of the data is correct, then the genetic evidence in Fig. 2 suggests that the ATP synthase can be formed devoid of the γ and δ subunits. In the tetrad analysis, the spores with a mutation in the genes encoding the γ and δ subunit (e.g., 2C) had a phenotype of a cell with only a mutation in the γ subunit (e.g., 2B). In other words, the mutation in the gene encoding the γ subunit was epistatic to the mutation in the δ subunit. This indicates that the putative complex responsible for the phenotype in the presence of the mutation in the gene encoding the γ subunit was also present with mutations in the genes encoding the γ and δ subunit. Other combinations of mutations indicate that the mutation in the gene encoding the γ subunit was also epistatic to the mutation in the gene encoding the ε subunit. Furthermore, the mutation in the gene encoding the δ subunit was epistatic to the mutation in the gene encoding the ε subunit. These results imply that the ATP synthase can be made in the absence of one or more of the γ, δ, or ε subunits.

The Role of the δ and ε Subunits

In *E. coli*, the subunit that corresponds to the δ subunit is the ε subunit, while the *E. coli* δ subunit corresponds to the mitochondrial oligomycin sensitivity-conferring protein. Studies from Capaldi's and other laboratories indicate that the *E. coli* ε subunit can be crosslinked to the γ subunit without altering activity (Bulygin *et al.*, 1998; Kato-Yamada *et al.*, 1998; Schulenberg *et al.*, 1997). The crystal structure of the yeast ATP synthase indicates that the mitochondrial δ subunit is closely associated with the γ subunit (Stock *et al.*, 1999). As such, it has been postulated that the mitochondrial δ subunit is part of the rotor and thus rotates with the γ subunit.

The mitochondrial ε subunit is not an essential component of the ATP synthase, but a yeast strain with a deletion in the gene is severely impaired or unable to grow on YPG medium (Arselin *et al.*, 1991; Lai-Zhang *et al.*, 1999). Studies also indicate that the δ subunit forms a heterodimer with the ε subunit (Orriss *et al.*, 1996; Penin *et al.*, 1990). Taken together with the results of the *E. coli* ε subunit, the conclusion can be made that the mitochondrial δ and ε subunits form a dimer that associates with the γ subunit. While the role of the δ/ε subunits might be simply to extend the rotor, it is also possible that the δ/ε pair acts as a molecular clutch. The clutch too must rotate with the rotor of the motor, but it differs in that provides the force that associates the rotor to the turbine, in this case F_0. Thus defining the δ/ε subunits as a clutch might more accurately describe their role in the motor. One possible misleading interpretation in defining δ/ε as a clutch would be to assume or suggest that the clutch can be released or engaged during the course of the lifetime of the ATP synthase. At this point, there is no evidence that the cell regulates the efficiency of

the ATP synthase by adding or subtracting the δ or ε subunits, although the possibility is there.

Is This Limited to Yeast?

There is some evidence from other species that the ATP synthase or F_1 ATPase can be assembled either *in vitro* or *in vivo* in the absence of the γ or δ subunit. In *E. coli*, deletion of the uncC gene, which encodes for the bacterial ε subunit, results in a unique and more severe phenotype growth than deletion of other genes, and it is referred to as the uncC phenotype (Klionsky *et al.*, 1984). A deletion mutation in the uncA gene, which encodes the α subunit, was epistatic to the deletion mutation in the uncC gene, identical to that observed in yeast. The lowered growth of the uncC mutant strains correlated well with the *in vivo* level of ATP, suggesting that the ATPase was hydrolyzing the cellular ATP. In addition, membranes from the uncC mutant strain were more leaky for protons than the wild strain, based on fluorescence quenching of 9-amino-6-chloro-2-methoxyacridine by the oxidation of NADH.

Similar results were obtained using thylakoid membranes stripped of CF1 and reconstituted with CF1 devoid of the ε (or δ) subunit (Patrie and McCarty, 1984). The subunit-deficient enzymes were shown to bind to stripped membranes as effectively as the wild-type enzyme, although they did not reconstitute photophosphorylation. Light-induced fluorescence quenching of quinacrine was also absent, but it could be restored by the addition of DCCD. The results suggest that the ε-less (corresponding to mitochondrial δ subunit) enzyme complex can form, but the F_0 freely passes protons, uncoupled to ATP synthesis.

In addition, a minimal active F_1-ATPase can be made with the subunit stoichiometry of $\alpha_3\beta_3$, $\alpha_3\beta_3\gamma$, and $\alpha_3\beta_3\delta$ in *E. coli*, chloroplast, and thermophilic bacteria, clearly indicating the lack of need for the mitochondrial δ or γ subunit for an active ATPase. (For review, see Gromet-Elhanan, 1992). There is less *in vivo* evidence for the presence of the γ-less enzyme complex in these systems likely due to the instability of the enzyme complex. However, it is clear that mutations in the γ subunit can provide a phenotype that is similar to the γ-less phenotype observed in yeast. The *E. coli* mutant NR70 was isolated as a mutant that was resistant to aminoglycoside antibiotics (Rosen, 1973). The mutation was later determined to be a 21-bp deletion at the 5′ end of the coding region for the γ subunit, uncG (Kanazawa *et al.*, 1985). The resistance was shown to be due to the increased permeability of protons through the membrane and this could be blocked by DCCD (Rosen, 1973). Similar results were also obtained with a α/γ fusion construct, which severely reduced the growth of the *E. coli* cells although it could be partially reversed by the addition of DCCD to the growth medium (Brusilow, 1987). Membranes isolated from these cells also had increased proton permeability. Thus, it is clear that it is possible to elicit a phenotype in *E. coli* that is similar to the phenotypes observed in yeast with a deletion mutation in the γ subunit. However, mutations that truncate a large portion of the N-terminus of the γ subunit, such as an amber mutation at position 15, do not increase the permeability of the membranes to protons (Miki *et al.*, 1986). Thus, it is likely that the γ-less enzyme complex is much less stable in *E. coli*, or at least more efficiently targeted for degradation, than that in the mitochondrion.

Does Partial Assembly of the ATP Synthase Occur in Mammalian Cells?

The mitochondrial ATP synthase is much more complex than the corresponding bacterial enzymes. However, the assembly of the enzyme still follows basic fundamental rules, such as the concentration dependence of the subunits on the assembly of the enzyme. Theoretically, unless there is a strict order in the assembly of the enzyme, a decrease in the level of a subunit will result in a increase in the level of the enzyme deficient in that subunit. By decreasing the concentration of, for example, the δ subunit, the level of the δ subunit-less enzyme complex must increase, although the level may be quite low. If the enzyme did form some δ-less enzyme complex, then the predicted effect would be to produce a partial uncoupling of the mitochondria. The mammalian cell, unlike the bacterial cell, has a number of mechanisms to produce a partial uncoupling of the mitochondrion, including the synthesis of UCP1, UCP2, and UCP3 (Boss *et al.*, 1997; Fleury *et al.*, 1997; Lin and Klingenberg, 1980). It is clear that there are cellular advantages to partially uncoupling the mitochondrion. In addition, the synthesis and assembly of the mitochondrial ATP synthase can be modified by a number of different mechanisms to provide a subunit-less enzyme that would be expected to partially uncouple the mitochondrion. As such, it is possible that these different means of regulation

could be used to alter the expression and synthesis of individual subunits of the ATP synthase, either in a normal or diseased state, thereby inducing the assembly of an ATP synthase complex devoid of the γ, δ, or ε subunit.

ACKNOWLEDGMENTS

Support for this work was provided by a grant from the NIH (GM44412). Thanks are given to Dr. John Keller for critically reading the manuscript.

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