Mitochondrial Genome Integrity Mutations Uncouple the YeastSaccharomyces cerevisiae ATP Synthase*

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The mitochondrial ATP synthase is a molecular motor, which couples the flow of protons with phosphorylation of ADP. Rotation of the central stalk within the core of ATP synthase effects conformational changes in the active sites driving the synthesis of ATP. Mitochondrial genome integrity (mgi) mutations have been previously identified in the α-, β-, and γ-subunits of ATP synthase in yeast Kluyveromyces lactis and trypanosome Trypanosoma brucei. These mutations reverse the lethality of the loss of mitochondrial DNA in petite negative strains. Introduction of the homologous mutations in Saccharomyces cerevisiae results in yeast strains that lose mitochondrial DNA at a high rate and accompanied decreases in the coupling of the ATP synthase. The structure of yeast F1-ATPase reveals that the mgi residues cluster around the γ-subunit and selectively around the collar region of F1. These results indicate that residues within the mgi complementation group are necessary for efficient coupling of ATP synthase, possibly acting as a support to fix the axis of rotation of the central stalk.

The mitochondrial ATP synthase couples energy of the proton gradient across the mitochondrial membrane, derived by respiration, to the phosphorylation of ADP to ATP. Mutations or drugs that interfere with this process can reduce the efficiency of ATP synthesis resulting in wasting of the energy derived from the respiratory chain. On a cellular level, uncoupling the mitochondrion can result in a loss of the membrane potential inducing apoptosis (1).

The ATP synthase is composed of a membrane-bound F0 portion and a water soluble F1. F0 acts as a proton turbine, which drives the rotation of the central stalk in F1 effecting the phosphorylation of ADP. F1 has 3 catalytic sites formed by three pairs of α/β subunits, which are arranged as a sphere forming the core of the enzyme (2). The central stalk, composed of the γδε, is in the center of the core of F1 and is physically coupled to F0. F0 is minimally composed of abc10 with the c-subunits arranged as a cylinder in the membrane. A peripheral stalk formed by subunits b, d, h, and subunit 5, links the a-subunit to the periphery of the α/β core and acts as a stator, preventing the core from following the rotation of the central stalk (3–5).

The F1-ATPase is a molecular motor, which can drive the rotation of the central stalk with hydrolysis of ATP. This process is estimated to be nearly 100% efficient generating a torque of 90 pN/nm (6). Rotation of the stalk is central to the mechanism of the ATP synthase where the active sites alternate between a state having a high affinity, with that having a low affinity, for nucleotides (7). The eccentric γ-subunit rotates within the core of the F1-ATPase, sequentially converting the biochemistry of the catalytic sites through the catalytic cycle. Disruption of the interactions of the central stalk with the core of the enzyme is therefore predicted to alter the efficiency of the enzyme, i.e. uncouple the enzyme.

Yeast Saccharomyces cerevisiae is a facultative anaerobe and thus is viable in the absence of oxidative phosphorylation. The mtDNA2 encodes genes needed to express the genome and encodes subunits of the electron transport chain and the ATP synthase. Not surprisingly, yeast S. cerevisiae is able to lose mtDNA. Loss, or a major deletion of mtDNA, is defined as the cytoplasmic “petite” mutation. S. cerevisiae is referred to as “petite positive” because it is able to survive with the loss or deletion of mtDNA.

Yeast Kluyveromyces lactis is also a facultative anaerobe but unlike S. cerevisiae, the cytoplasmic petite mutation is lethal and thus K. lactis is petite negative. A family of nuclear mutations, mitochondrial genome integrity (mgi), has been identified in K. lactis, which convert it from petite negative to petite positive. Without exception, these mutations map to the α-, β-, or γ-subunits of the ATP synthase (8).

Nuclear mutations have long been known to exist in S. cerevisiae, which convert it to petite negative. These mutations have been identified in a number of genes including the genes encoding the ATP/ADP translocase (9), the PGS1 gene whose product is involved in cardiolipin biosynthesis (10, 11), and the YME1 gene whose product is involved in the migration of mtDNA from the mitochondrion to the nucleus (12). Mutations in S. cerevisiae containing the yme1 mutation have also been isolated, which convert it from petite negative to petite positive. These mutations were mapped to the gene encoding the α- and γ-subunit of the ATP synthase and are homologous to the mgi mutations isolated from K. lactis (12, 13).

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2 The abbreviations used are: mtDNA, mitochondrial DNA; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; RCR, respiratory control ratio.

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The bloodstream form of *Trypanosoma brucei* normally requires expression of the mitochondrial genome despite the absence of oxidative phosphorylation in this stage of the life cycle. However, there is a naturally occurring bloodstream form of trypanosome, dyskinetoplastic, that lacks mtDNA. This form has been shown to have sequence variation in the gene encoding the γ-subunit, which allows this bloodstream form of trypanosome to survive without mitochondrial gene expression (14). Introduction of the corresponding mutation in yeast *K. lactis* converts it to a petite positive strain and thus, this mutation may be defined as an mgi mutation.

There have been a number of hypotheses to explain the biochemical effect of mgi mutations on the ability of the cell to lose mitochondrial gene expression, as in the petite mutation in yeast. The mgi mutations have been postulated to decrease the proton permeable channel made by F₀ (15, 16), to increase ATPase activity by decreasing the inhibitory effects of natural inhibitory factors (17), to increase the intrinsic ATPase activity of F₁ in the absence of F₀ (8), or by decreasing the Kᵅᵤ for ATP of the F₁·ATPase (18). Whereas there is ambiguity as to the effect of the mgi mutations on the biochemistry of the F₁-ATPase, there seems to be consensus that the petite negative phenotype is related to mitochondrial biogenesis, which if blocked, results in death of the cell (14, 18–22).

The corresponding mgi mutations have been made and analyzed in yeast *S. cerevisiae*. Genetic analysis and vital staining of the yeast containing the corresponding mutations and biochemical analysis of the mitochondria derived from the corresponding strains indicate that the mutations uncouple the ATP synthase. Analysis of the crystal structure of the wild type yeast F₁-ATPase suggests a structural role of the corresponding residues where these residues secure the axis of rotation of the central stalk. Changes in the axis of rotation of the central stalk are predicted to alter the interactions of the γ-subunit with the core of the F₁-ATPase and reduce the coupling of the ATP synthase. These results provide information on the structure/function relationship for the residues classified as mgi.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains and Genetics**—The yeast *S. cerevisiae* strain, W303-1A (a, ade2-1, his3-1, 15, leu2-3, 112, trpl-1, ura3-1) (obtained from A. Tzagoloff, Columbia University, New York) was used throughout this study as the parent of the mutant strains. The percentage of cytoplasmic petites (loss or deletion of mtDNA) was determined after growing a single colony in YPD medium (3 ml) for 3 days, plating cells on YPD plates (about 100 colonies/plate) and incubating the plates at 30 °C for 3–4 days. The percentage of petites was measured by determining the percentage of white colonies (the petite strain does not turn red with the ade2- mutation (21, 23)). The percentage of petite cells was confirmed genetically by mating the colonies to a tester strain (21), followed by testing for growth on YPG medium.

The null deletion yeast mutants were made by homologous recombination of PCR products using the KanMX resistance module (24). Yeast transformation was performed by the LiAc method (25). Mutations were introduced into the α, β, and γ genes of yeast ATPase. The αN67I, αA295V, αF405S, βV279F, βR408G, βR408I, γT264A, and γL270T mutants were prepared in plasmids (pRS305, pRS304, and pRS306 (26) for the α, β, and γ genes, respectively) using the QuikChange method (Stratagene) (supplemental materials Table S1).

Yeast media are standard recipes as described (27): YPD, 2% glucose, 1% yeast extract, 2% peptone; YPG, 2% glucose, 1% yeast extract, 2% peptone, and 20 mg/liter adenine sulfate. Minimal medium (SD) with 2% glucose was supplemented at 20 mg/liter with amino acids, adenine, and uracil.

**Isolation of Wild Type and Mutant Mitochondria**—Cells were grown at 30 °C in a complete liquid medium containing 2% galactose and 0.05% glucose (28). Cells were harvested during logarithmic growth phase. Mitochondria were prepared as described (29). Protein concentration was determined using the BCA assay (30).

**Measurement of ATPase Activity**—ATPase activity was determined by a coupled enzyme assay (31) at 30 °C. Oligomycin-sensitive ATPase activity assay was performed after incubation (5 min) in the presence and absence of oligomycin (5 μg/ml). For determination of the Kᵅᵤ and Vₚₘₓ values, mitochondria were first diluted with an equal volume of isolation buffer containing 0.75% Triton X-100 (w/v). The rate of ATP hydrolysis by mitochondrial protein (2 μg) was measured at concentrations of ATP·MgCl₂ ranging from 2 μM to 2 mM with 1 mM free MgCl₂.

**Measurement of Respiration Rates**—Respiration was measured as described (28, 32) at 30 °C, using 0.6 mg of mitochondrial protein in buffer (0.65 M mannitol, 0.3 mM EGTA, 3 mM Tris phosphate, 10 mM Tris maleate, pH 6.75) (33) with NADH (8 mM) and carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) (4 μg) as indicated.

**Membrane Potential Analysis**—For vital staining of the mitochondria, the yeast cells were grown to the mid-log phase, yeast cells (10⁶) were incubated with Rhodamine 123 (2 μM) in phosphate-buffered saline plus 20 mM HEPES, 1% glucose with shaking at 30 °C for 20 min, and the cells were washed with phosphate-buffered saline 3–4 times. Stained cells (2 × 10⁵) were analyzed by fluorescence-based flow cytometry (FACS caliber Flow Cytometer, BD Biosciences) with excitation at 488 nm and emission at 525 nm. Stained cells were visualized and photographed using a Leica DMRB microscope.

**RESULTS**

The mutations identified in *K. lactis, S. cerevisiae*, and *T. brucei* that act with the mgi phenotype are summarized in Table 1. The mutations cluster in both regions and on specific residues in the α-, β-, and γ-subunits. Assuming that the frequency for a given mutation follows a Poisson distribution, this clustering suggests that there is a very limited number of residues that when mutated, give an mgi phenotype. To gain a better understanding on the effect of these mutations on the biochemistry of the ATP synthase, we have made a subset of the corresponding mutations in yeast *S. cerevisiae* and tested the resulting genetic, growth, and biochemical phenotypes.

The mutant genes were introduced into yeast strains containing a deletion mutation in the gene corresponding to that with the mgi mutation. The yeast strains also had a deletion
mutation in the gene encoding the \( \alpha \)-subunit (ATP2) of the ATP synthase. The \( \alpha \) mutation served two purposes. First, we re-introduced the wild type ATP2 gene as a chimeric construct with a His\(_6\) affinity tag on the amino end of the \( \alpha \)-subunit (34). The His\(_6\) tag allows nickel affinity purification of the F\(_1\)-ATPase. Second, a deletion in the gene encoding the \( \gamma \)-subunit (ATP3) has been shown to cause the cells to completely lose their mtDNA, i.e. become petite (21). However, in the presence of a null mutation in ATP2, the cells do not become petite (21), making analysis of the \( mgi \) mutations in ATP3 possible.

The yeast, when grown on rich medium containing glucose as the carbon source, showed a strong sectoring effect (Fig. 1). The sectoring is due to the rapid formation of petite mutations in the presence of the ade2 mutation. Cells that are respiratory competent and that contain the ade2 mutation turn red after adenine is depleted. However, yeast with the ade2 mutation that have lost mtDNA (petite mutation), remain white. The result is that cells that form petites at a high frequency exhibit a red/white color sectoring. Whereas not always definitive, the color of the yeast can be used here to determine whether the yeast is petite. This rapid sectoring indicated that the \( mgi \) mutations induced a large percentage of petite mutations in the cell. This propensity to become petite is known to occur in strains with mutations that uncouple the ATP synthase (19, 21, 35).

Table 2 shows the percentage of the cells that are petite after growth in YPD medium. The petite mutation was confirmed by genetic analysis by mating a wild type strain containing a cytoplasmic petite mutation with the test colonies. In each case, the resulting diploids from the white colonies were unable to grow on YPG medium indicating that the cells in the white colonies were petite. All of the mutations caused an increased rate of petite formation ranging from 15 to 44% of the colonies. This is a conservative analysis because the sectored colonies were classified as grande (nonpetite).

The effect of the mutations on the ability of the yeast to grow on complete medium with glycerol as the sole carbon source (YPG) medium is shown in Fig. 2. Cells that are defective in oxidative phosphorylation are unable to grow on medium that contains a nonfermentable carbon source, such as glycerol, but are able to grow on medium that contain a fermentable carbon source, such as glucose. There was a large variation of the effect that the mutations had on the ability of the yeast to grow on YPG medium. Some strains displayed near normal growth on YPG medium, such as that containing ATP1–75, whereas others were completely defective, such as that containing ATP3–5.

The haploid strains were mated to a wild type haploid strain and the resulting diploids were tested for growth on YPG medium. This assay indicated that the mutations, which imparted a negative growth phenotype on YPG, were semi-dominant negative.

| \( \alpha \)-subunit | \( \beta \)-subunit |
|---------------------|---------------------|
| \( \beta \)-subunit | \( \gamma \)-subunit |

**Primary sequence comparison of regions of the \( \alpha \)-, \( \beta \)-, and \( \gamma \)-subunits with the amino acid residues that have been identified in the \( mgi \) complementation group**

The mutations in the genes encoding the \( \alpha \)-, \( \beta \)-, and \( \gamma \)-subunits (ATP1, ATP2, and ATP3) are shown corresponding to the \( mgi \) complementation group identified from \( K. \) lactis, \( S. \) cerevisiae, and \( T. \) brucei (8, 12–14). The altered residues are indicated in bold and underlined. The mutations ATP1–75, ATP3–1, and ATP3–5 were identified from \( S. \) cerevisiae and classified in those studies as dominant mutations. The abbreviation used for \( T. \) brucei is T.B.
Mutations Uncoupling the ATP Synthase

The semi-dominant negative behavior was also displayed in the percentage of petite mutation formed by the heterozygous diploid strain (Table 2). A mutation can exhibit a dominant phenotype if the mutant protein is assembled into a complex that imparts a new activity that gives a negative phenotype. For mutations that uncouple the ATP synthase, the new activity is the free flow of protons into the mitochondrion and a negative phenotype is, for example, the slow growth on glycerol medium. The mutations are not entirely dominant negative, presumably because of the added capacity provided by the wild type enzyme to create a gradient via ATP hydrolysis and diminished overall level of the mutant enzyme complex.

The prior phenotypes are indicative of mutations that uncouple the ATP synthase. To test this hypothesis further, the cells were stained with Rhodamine 123. Rhodamine 123 accumulates in the mitochondrion in response to a negative potential across the mitochondrial membrane. Partial uncoupling of the ATP synthase results in protons entering the mitochondrion causing a decrease in the efficiency of ATP synthesis. Uncoupling of the ATP synthase would be predicted to decrease the potential across the mitochondrial membrane. Fig. 3 shows the staining with Rhodamine 123 with the wild type (WT), mutant (αN67I), and petite cells. The staining was moderately decreased in the mutant cells and severely decreased in the petite strain. A fluorescent-activated cell sorter was used to count and quantify the level of fluorescence in each cell (Table 2, fourth column). This analysis indicated that the cells from all of the mutant strains had a decreased staining and thus lower potential across the mitochondrial membrane. The decreased staining by Rhodamine 123 in cells containing the mutations is consistent with the mgi mutations altering the coupling of the ATP synthase.

Biochemical analysis was performed on mitochondria isolated from the wild type and mutant strains to specifically address the coupling capacity of mitochondria isolated from the strains. Respiratory control was measured in the absence of ADP (State 4) and stimulated with the uncoupler, FCCP. The ratio of the uncoupled rate divided by the State 4 rate is referred to as the respiratory control ratio (RCR). The higher the RCR, the more tightly coupled respiration is with ATP synthesis. An RCR of 1 would indicate that the mitochondria are completely uncoupled. In all cases the RCR dropped, from 3.1 for the wild type to as low as 1.3 for the mitochondrial from the mutant strains (Table 2, seventh column). Frequently, State 4 rate is higher for mitochondria isolated from the mutant strains indicating that respiration is stimulated in the absence of FCCP (e.g. βV279F, Table 2, fifth column). In some cases, State 4 rate is not any higher than that of wild type mitochondria, but there is a corresponding decrease in the rate of respiration in the presence of FCCP (e.g. βR408I, Table 2, sixth column). This decrease in respiration is likely due to the formation of petite mutations during cellular growth. Because the mtDNA encodes subunits of the respiratory chain, petite mutations decrease the overall rate of respiration in the mitochondrial preparation.

The oligomycin-sensitive ATPase activity can also be useful as an indicator of the coupling of the ATP synthase. Oligomycin binds to Fo and inhibits ATP hydrolysis by the ATP synthase, but does not inhibit ATP hydrolysis by the F1-ATPase when it is not associated with Fo. Thus, an oligomycin-sensitive ATPase can indicate that F1 is bound to Fo forming an intact and presumably, coupled enzyme. A decrease in the sensitivity to oligomycin suggests that the ATP synthase is less coupled. Both the level of the oligomycin-sensitive ATPase and the percent inhibition by oligomycin decreased for mitochondria isolated from the mutant cells (Table 2, columns 8–10). The specific ATPase activity decreased from 2.6 for the wild type to a low value of 0.7 for mitochondria isolated from the mutant cells. The oligomycin sensitivity was 96% for the wild type and ranged...
from 86 to 26% for the ATPase for mitochondria isolated from the mutant strains. Thus, the activity of the ATPase and the oligomycin sensitivity were decreased in each of the mutant strains as compared with that of the wild type strain.

The isolated mitochondria were also tested for the extent of the membrane potential generated through the respiratory chain (by the oxidation of ethanol forming NADH) or by the hydrolysis of ATP. In the former case, the membrane potential is partly dependent on the amount of the respiratory chain in the mitochondria and the leakage of protons back into the mitochondria, in this case presumably via the ATP synthase. In the latter case, the membrane potential is partly dependent on the level of the ATP synthase and on the coupling potential of the hydrolysis of ATP with the pumping of protons out of the mitochondria. In both assays, the membrane potential was decreased for mitochondria isolated from the yeast with the mgi mutations relative to mitochondria isolated from the wild type strain. The relative decrease in the potential was much greater, however, when the mitochondrial potential was generated with hydrolysis of ATP rather than with oxidation of ethanol (Table 2, columns 11 and 12).

**DISCUSSION**

The initial high resolution structure of the bovine F$_1$-ATPase defined the three catalytic sites based upon their occupancy: TP, DP, and E for bound adenine nucleotide triphosphate, adenine nucleotide diphosphate, and empty, respectively (2). Whereas the yeast enzyme does not have the same nucleotide content, the structures are similar and comparable (accession number 2HLD) (36). Thus, for the point of discussion, we will refer to the active sites of the yeast enzyme with the names corresponding to the equivalent sites in the bovine enzyme: TP, DP, and E. When discussing a single subunit, the nomenclature will be used based on the active site formed by that subunit, e.g. $\alpha$TP and $\beta$DP.

The positions of the mgi within the structure of the yeast F$_1$-ATPase are shown in Fig. 4 (supplemental materials Movie 1). All of the residues lie in a region that is either directly or indirectly linked to interactions with the $\gamma$-subunit. The sites of interaction cluster into 2 regions: the collar region and the bottom of the $\alpha$- and $\beta$-subunits (cf. Fig. 4A). The collar region, defined in the structure of the bovine F$_1$-ATPase, is formed by a loop in the $\alpha$- and $\beta$-subunits (2). Because of the eccentric rotation of the $\gamma$-subunit, the plane of the collar is not perpendicular to the axis of the $\gamma$-subunit, but tilted at an angle of about 45°.

The collar is hydrophobic and seems to form a structural support holding the axis of rotation of the $\gamma$-subunit. The tilting of the collar is a consequence of the asymmetric features of the structure.
enzyme. Whereas there are three pairs of the α/β-subunits arranged in a sphere-like structure, the conformations differ and this is reflected in three active sites with distinct biochemical properties. The position of the central stalk is expected to be critical for the asymmetric interactions of the γ-subunit with the core of F₁, and the active sites.

There is a cluster of residues in this region, which when mutated give the mgi phenotype, including αAla-295, βVal-279, γThr-264, and γyle-270 (cf. Fig. 4). Interestingly, αAla-295 is adjacent to γThr-264 and γyle-270, depending on the conformation of the subunit, i.e. TP or E. αAla-295 is within 4.1 Å of γyle-270 in αₜ and within 3.9 Å of γThr-264 in the αₜp conformation. βₜ Val-279 interacts with γThr-264 further illustrating the importance of γThr-264.

αAsn-67 is at the top of the β-barrel domain of the α-subunit far from the collar region and the γ-subunit. Whereas αAsn-67 does not make direct contact with the γ-subunit, it forms two H-bonds with αArg-289 in both αₜ and αₜp. αArg-289 is adjacent to, and in the same loop as αAla-295, and thus may be important for the position of this loop keeping the tilt of the collar.

γLeu-254 is located in the beginning of the coiled-coil region of the γ-subunit (cf. Fig. 4B, C and Table 1) and γL254P likely alters the structure of the central stalk. The Pro residue may put a kink in the α-helix of the γ-subunit thereby altering the axis of rotation, at least for the region below that point in the γ-subunit, which would alter the functional interactions with the α- and β-subunits.

The mutations in the α-helix bundle of the α- and β-subunits occur in residues αPhe-405 and βArg-408. αₜPhe-405 is in close contact with γThr-22 (3.9 Å), βArg-408 is in one of two helices in a finger-like structure that forms the second catch region in the β-subunit (residues 377–419), originally defined in the bovine enzyme (2). βₜPArg-408 form H-bonds with βGlu-454, βArg-412, and βTyr-381. βTyr-381 is in the second α-helix that forms the finger-like structure and this H-bond may be important for the structure of the second catch region.

The consensus is that these residues are involved in a structural role in the enzyme. The distribution among the α-, β-, and γ-subunits eliminates the possibility that one of the subunits acts in a yet undefined and secondary role, for instance, somehow related to DNA replication, which is responsible for the mgi phenotype. The residues clearly are not part of the catalytic site and are far removed from the site. Instead, clustering of the mutations around the γ-subunit and within the γ-subunit, indicate that the mgi phenotype is somehow related to the γ-subunit.

Introduction of the mgi mutations in yeast S. cerevisiae enzyme uncouples the enzyme. This conclusion is based on the genetic and biochemical data and there is a structural basis to support this conclusion. The genetic evidence is based on the formation of cytoplasmic petite mutations and the semi-dominant nature of the mutations. Prior studies have shown that mutations that uncouple the ATP synthase result in an increased rate of cytoplasmic petite formation (19, 21, 35). The rationale for the increased petite formation is that the import of
proteins into the mitochondrion is dependent on the $\Delta \Psi$ across the membrane. Loss in the potential across the mitochondrial membrane inhibits biogenesis of the mitochondrion and this is lethal. The cell survives by eliminating the source of the proton leak, in this case $F_0$ or the ATP synthase, and this is most readily done by eliminating the mitochondrial genome that encodes key subunits of $F_0$. The dominant or semi-dominant nature of the mutation is also predicted for mutations that uncouple the ATP synthase. These mutations give an additional activity: the free flow of protons into the mitochondrion without the synthesis of ATP. This activity should be present in either a homozygous or heterozygous background and hence the genetic dominance of the mutation. There is still the question as to the extent the mutant enzymes will assemble in relationship to the wild type enzyme in a heterozygous background and thus the degree of the negative effect is uncertain.

The biochemical evidence is based on the decreased mitochondrial staining in vivo with the membrane potential probe, Rhodamine 123, the decreased RCR, the decrease in the oligomycin sensitivity of the ATPase, and the decreased potential generated with either ethanol or ATP, by mitochondria isolated from the mutant strains. All of these studies gave consistent decreases in the coupling of the ATP synthase.

There are two issues that might confuse the interpretation. First, the various $mgi$ mutations do not affect the coupling, or the growth phenotype, to the same extent. However, this is expected because the $mgi$ mutations are not equally efficient at producing the $mgi$ phenotype (8). Second, the results of the different assays used to measure coupling did not always apparently agree on the level of uncoupling caused by any one mutation (Table 2). However, the assays were measuring different aspects of coupling. In some cases, coupling was measured in the direction of ATP synthesis, whereas in other cases ATP hydrolysis. ATP synthesis may not simply be the reverse of ATP hydrolysis. There are a number of examples that demonstrate that the structural requirements for ATP hydrolysis are not the same as those for ATP synthesis. For instance, the natural inhibitor protein IF1, like the $\epsilon$-subunit of the Escherichia coli enzyme, inhibits hydrolysis but not the synthesis of ATP (37–43). The $E. coli$ $\epsilon$-subunit is required for ATP synthesis, but is not needed for torque generated by ATP hydrolysis (44). Mutations that were thought to alter the coupling of the $E. coli$ enzyme had no effect on the torque generated by ATP hydrolysis (45). These are just three of the growing number of examples where ATP hydrolysis does not appear to be simply the reverse of ATP synthesis. Thus, it is possible that a mutation...
could uncouple ATP hydrolysis from pumping of protons, but not affect coupling the flow of protons into the mitochondrion with the phosphorylation of ADP or vice versa. If a mutation altered the coupling for ATP hydrolysis and not synthesis, then the results in Table 2 should show no change in the potential generated by NADH, but a severe defect when the potential is generated by ATP hydrolysis. However, this extreme situation is not likely to be observed because uncoupling mutations cause the cell to become petite and thus decrease NADH oxidation with an accompanied loss in membrane potential generated by oxidation by NADH. Furthermore, few mutations can be expected to affect only ATP hydrolysis and not synthesis. With these considerations taken into account, some, but not all of the mutations, may have a differential effect on ATP synthesis versus ATP hydrolysis.

The results from this study suggest that mgi mutations alter the coupling of the ATP synthase. The structural basis for this uncoupling is postulated to be a displacement in the axis of rotation of the γ-subunit and the central stalk. Changes in the position of the axis of rotations are predicted, and expected, to alter the interactions between the γ-subunit and the α- and β-subunits and these interactions help define the conformation of the active site. Any changes in these interactions are expected to alter the coupling efficiency of the enzyme.

There have been a number of hypotheses to explain the biochemical effect of the mgi mutation on the ATP synthase including, lowering the Km for nucleotides and an increase in the level of the ATPase activity of the free F1-ATPase when the mtDNA, and thus F0, is lost. In the former case, the increase in the level of the ATPase is thought to be due to the decrease in the Km and thus a corresponding increase in the rate of hydrolysis at low ATP concentrations. In the latter case, the increase in the rate of hydrolysis is thought to be due to the loss of inhibition by the natural inhibitor protein, IF1. In both cases, the increased rate of ATP hydrolysis is necessary for the formation of ADP3−, which is then exchanged for ATP4− by the ATP translocase establishing a ΔΨ across the mitochondrial membrane. However, there are difficulties with both of these hypotheses. First, deletion of the gene coding for IF1 gives just a weak mgi phenotype (13). Second, whereas a decrease in the Km for Mg:ATP was observed for the mitochondrial ATPase, there was also a parallel reduction in the total mitochondrial ATPase activity in the mgi strains (based on mitochondrial protein). This reduction offsets any increase in ATPase activity (at low ATP concentrations) that might be expected due to the decrease in Km (18) (supplemental materials Table S2). Third, there is no structural basis for the proposed effects on the Km, ATPase activity, or the loss of inhibition by IF1. These hypotheses do not explain the clustering of the mgi mutations in the collar region or around the γ-subunit. Fourth, the premise that hydrolysis of ATP coupled with the exchange of matrix ADP for cytosolic ATP is sufficient for the generation of the potential is suspect. At pH 7.5, the hydrolysis of ATP releases 0.69 H+ and the exchange of ADP for ATP has a charge difference of −1. Thus, this process has a charge difference of only −0.31/ATP and it establishes a pH gradient in the wrong direction. The pH gradient might be dissipated by the electroneutral exchange of H3PO4− for OH−, depending on the concentration gradient of phosphate, but this still leaves just a minor effect for hydrolysis of ATP in generating the ΔΨ across the membrane. At pH 7.0, the numbers are better, with a calculated charge difference of −0.57/ATP, but these numbers still raise doubt as to this being the only process to establish the potential. Even if this is the mechanism to establish the ΔΨ, it does not establish that mgi mutations act by increasing the rate of ATP hydrolysis in the mitochondrion. Finally, it is difficult to connect an increase in the uncoupling of the ATP synthase with an increase in ATP hydrolysis because the ATP synthase is not coupled in the petite cell. Thus mutations that uncouple the ATP synthase should not stimulate ATP hydrolysis in the petite cell.

The conclusion that mgi mutations decrease the coupling of the ATP synthase does not, by itself, provide a mechanism of how this gives the mgi phenotype. Whereas the data in this paper does not directly address this issue, the results from others studies do. One possible explanation is that mgi mutations condition cells allowing them to adapt and survive the loss of mtDNA and the components encoded therein. A partial uncoupling of the ATP synthase mimics some of the effects of the petite mutation, including a decrease in the ΔΨ and a decrease in the concentration of intracellular ATP. These partial defects may cause the cell to alter gene expression to compensate for these changes so that when mtDNA is lost, the cell is able to survive. This is similar to preconditioning myocardium where brief episodes of ischemia (first identified in the canine but later observed with other mammals) reduced lethal myocardial injury due to a subsequent larger ischemic episode (46). The adaptive hypothesis suggests that differences in gene expression explain why some yeast strains are petite negative, whereas others are petite positive. The adaptive hypothesis explains why mutations in some genes, such as YME1, convert a cell to petite negative and why expression of some proteins can convert a cell from petite negative to petite positive (17, 22). As such, a decrease in expression or function of a gene or gene product is able to cause a petite negative phenotype, and conversely, induction of a single gene product or multiple gene products can convert a petite negative cell to petite positive. Consistent with this hypothesis, overexpression of any number of proteins involved in import of proteins into the mitochondrion convert petite negative cells to petite positive (17, 22). Thus, the fault in the petite negative cell can be overcome without directly altering the ΔΨ. However, we know of no studies that indicate that the mgi mutations in the ATP synthase have an effect on gene expression, as predicted by the adaptive hypothesis.

Ultimately, it is clear that a ΔΨ must be present across the mitochondrial membrane for the biogenesis of the mitochondrion and viability. It is not as clear as to the relative contributions of the various processes that establish this potential, how large a potential is required, and if a decrease in protein import, due to a decrease in ΔΨ, can be compensated by changes in gene expression.

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3 This calculation assumes pertinent pKs values of 6.95, 6.88, and 7.2 for ATP, ADP, and Pi, respectively.
Mutations Uncoupling the ATP Synthase

The results in this study give insights into the physiological basis of the ability of the cell to survive without oxidative phosphorylation. This is critical in understanding human diseases associated with loss of mtDNA and in survival after ischemic episodes. The results also give unique insights into the structure/function of the ATP synthase. Current crystallographic studies are ongoing to establish the effect of mgi mutations on the structure of the ATP synthase.

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