Subunit γ-Green Fluorescent Protein Fusions Are Functionally Incorporated into Mitochondrial F₁F₀-ATP Synthase, Arguing Against a Rigid Cap Structure at the Top of F₁

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We have investigated the question of the presence of a cap structure located at the top of the F₁ αβ₃ hexamer of the yeast mitochondrial F₁F₀-ATP synthase complex. Specifically, we sought to determine whether the putative cap has a rigid structure and occludes the central shaft space formed by the αβ₃ hexamer or alternatively whether the cap is more flexible permitting access to the central shaft space under certain conditions. Thus, we sought to establish whether subunit γ, an essential component of the F₁ central stalk housed within the central shaft space and whose N and C termini would both lie beneath a putative cap, could be fused at its C terminus to green fluorescent protein (GFP) without loss of enzyme function. The GFP moiety serves to report on the integrity and location of fusion proteins containing different length polypeptide linkers between GFP and subunit γ, as well as being a potential occluding structure in itself. Functional incorporation of subunit γ-GFP fusions into ATP synthase of yeast cells lacking native subunit γ was demonstrated by the ability of intact complexes to hydrolyze ATP and retain sensitivity to oligomycin. Our conclusion is that the putative cap structure cannot be an inflexible structure, but must be of a more flexible nature consistent with the accommodation of subunit γ-GFP fusions within functional ATP synthase complexes.

F₁F₀-ATP synthase uses energy produced from the electrochemical gradient to produce ATP from ADP and Pᵢ. The enzyme complex is described as having two major structural domains, F₁ and F₀. The globular F₁ catalytic sector contains three α and three β subunits arranged alternately in a hexamer thereby forming a central shaft space that houses part of the γ subunit. Each β subunit contains a catalytic site for the synthesis of ATP. These sites are repetitively and sequentially driven through defined conformational states by the rotation of the γ subunit, which is in turn linked to the translocation of protons through the membrane bound F₀ sector (1). Models of the F₁ sector derived from x-ray crystallographic data indicate the presence of an apparent “dimple” in the top of the F₁ sector ~15 Å deep that is contiguous with the central shaft space housing the C- and N-terminal portions of subunit γ (2).

Despite the considerable advances in determining much of the structure of F₁ (2–4), there are many questions that remain unanswered concerning the structure/function relationships of F₁F₀-ATP synthase components. One important question relates to the structure of F₁ in the vicinity of the dimple at the very top of F₁. A “cap” at the top of F₁ has been visualized in electron microscopic images of ATP synthase isolated from Escherichia coli (5, 6) and bovine mitochondria (7). This cap structure is not seen in models of ATP synthase derived from x-ray crystallographic data of F₁. The identity of the proteins that contribute to the cap structure is not clear but may represent, in part, the N-terminal 20–30 amino acids of subunits α and β that were disordered and therefore not represented in models derived from the x-ray crystal data, or alternatively, F₀ proteins lost upon crystallization of the complex. Using an immuno-electron microscopy approach, bacterial subunit δ, in particular its C-terminal portion, has been localized to the dimple region (8). In earlier studies, proteolysis experiments had indicated that the eukaryotic homologue of subunit δ, OSCP, binds to the N-terminal end of the α subunit (9). Subsequent studies have positioned OSCP in association with F₁, but sometimes at the top and sometimes at the side (10). Recent evidence obtained using a protein chemistry approach for ATP synthase isolated from rat mitochondria indicates that specific regions at the top of F₁ are shielded by F₀ components (11). It was suggested that this shielding represents an extension of the stator stalk and that the cap, composed in part by F₀α (whose functional homologue in yeast is subunit h (12)) and possibly subunit d, completely covers the N termini of all α and β subunits.

It is not clear whether the cap represents a solid structure closing off the surface area at the top of F₁, as represented by the mouth of the dimple seen in F₁ crystal structures. Under the experimental conditions used by Böttcher and colleagues (6) the cap of the E. coli ATP synthase (EF₃F₀) was shown to cover much of the area represented by the dimple. Thus, upon binding of the nucleotide analogue AMP-PNP to EF₃F₀ a cap structure could be detected concomitant with significant shrinkage in the diameter of F₁ (6). It was suggested that some of the differences observed on binding AMP-PNP were a result of a significant rearrangement in the N-terminal domains of the α and β subunits. When a three-dimensional reconstruction of EF₃F₀ was viewed from the top looking down the axis of the

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References

1 The abbreviations used are: OSCP, oligomycin sensitivity-confering protein; AMP-PNP, adenosine 5′-(β,γ-imino)triphosphate; mt, mitochondrial; GFP, green fluorescent protein.
central stalk a triangular shaped cap appeared to cover the central shaft space. A flexible cap that shifts its position throughout the catalytic synthesis of ATP on F1 would be consistent with the idea that the conformationally flexible N termini of the α and β subunits contribute to its structure. The appearance of the cap would alter depending on the state in which the complex was “captured,” sometimes completely covering the top of the F1, and sometimes not.

In this study we have investigated the question of the presence of a flexible cap structure in the yeast mitochondrial F1-F0-ATP synthase (mtATPase) complex. Although direct evidence for a cap structure has yet to be reported for yeast mtATPase, the high degree of conservation documented between eukaryotic mtATPase complexes would favor the existence of a cap. Thus we have sought to determine whether the putative cap precludes the assembly into mtATPase complexes of subunit γ, an essential component of the central stalk, fused to a reporter protein, green fluorescent protein (GFP). The N and C termini of subunit γ would both lie beneath the cap within the central shaft space formed by the F1 αβ hexamer, with the C-terminal glycine residue about 15 Å below the position of the N-terminal end of subunits α and β (2).

Our strategy has been to express in yeast cells subunit γ fused at its C terminus to GFP via a polypeptide linker. Correctly folded GFP forms a very rigid and stable 11-stranded β-barrel structure 24 Å in diameter and 48 Å in length threaded by an α-helix running up the axis of the barrel (13) that is remarkably stable to the action of a range of denaturing agents and proteases (14). The fluorescence properties of GFP have been utilized widely as a convenient tag to report the presence of a protein in various subcellular locations. We have used these properties of GFP to report on the integrity and location of fusion proteins containing different length polypeptide linkers between GFP and mtATPase subunit γ. Functional incorporation of subunit γ-GFP fusions into mtATPase would suggest that the putative cap structure in vivo is not at any time a “solid” inflexible structure that completely occludes the dimple in the top of the F1, but must be of a more flexible nature consistent with the incorporation of subunit γ-GFP fusions within functional ATP synthase complexes. In such complexes it is presumed that polyepitope linker connecting subunit γ with GFP would not be obstructed in exiting the central shaft space at the top of F1.

EXPERIMENTAL PROCEDURES

Materials—Dodecyl β-maltoside and complete protease inhibitors were purchased from Roche Molecular Biochemicals (Sydney, Australia). Vistra ECF substrate was purchased from Amersham Biosciences (Arlington Heights, IL). ATPase separated on clear native gels was determined by digestion at the unique XbaI site of the multicopy yeast ATP3DNA fragment encoding subunit γ. The PCR product was generated with the primer pair ATP3POUP/ATP3TDO using pRS306:ATP3-YEGFP3L4 as a template and Dynamez EXT thermostable polymerase (Finnzymes, Espoo, Finland).

Assays of mitochondrial lysates were performed as described previously (22). ATPase activity of mtATPase separated on clear native gels was determined in situ by incubating the gel slices in a solution of 5 mM ATP, pH 8.6, in 50 mM glycine/NaOH, pH 9.5, containing 0.05% lead acetate, 1 mM magnesium acetate with gentle agitation at room temperature (23). A white precipitate in the gel was indicative of ATPase activity. Images of gel sections made for detection of ATPase activity were recorded against a black background using a Wallac multi-wavelength imager in “edge-illumination mode” equipped with filters for excitation (480 ± 25 nm) and detection at 520 ± 25 nm.

Construction of Yeast Strains—prRS306:ATP3-YEGFP3L27 was linearized by digestion at the unique XbaI site 363 bp upstream of the initiation codon for subunit γ and used to transform YRD15 cells by the method of Schiestl and Gietz (19).

A PCR product was generated with the primer pair ATP3POUP/ATP3TDO using pRS306:ATP3-YEGFP3L4 as a template and Dynamez EXT thermostable polymerase (Finnzymes, Espoo, Finland). The product was digested with XbaI and used to transform YRD15 cells. Transformants (ura+) and then ATP3 gene replacement events were selected as described above. Strain MPγ-4 expresses γ-4-GFP. A PCR product encoding YEGFP3 linked, without any intervening amino acids, to the C terminus of subunit γ (γ-0-GFP), the yeast ADH1 terminator, and the Kluyveromyces lactis URA3-selecting region of homology sufficient for recombination with the ATP3 gene was prepared using a template to be described elsewhere. The PCR product was used to transform YRD15 cells. Following selection of ura+ transformants and then ATP3 gene replacement events (as described above), one isolate was designated strain MPγ-0 (expresses γ-0-GFP).

Expression vector pAS1NB:YEGFP3L was introduced into strain YRD15 to produce the strain MPγ-27 and expresses γ-27-GFP. A PCR product was cloned into the BamHI restriction site of pRS306:ATP3-YEGFP3L27 by removal of a 69-bp fragment flanked by restriction sites (Fig. 1). Following selection of ura+ transformants and then ATP3 gene replacement events (as described above), one isolate was designated strain MPγ-0 (expresses γ-0-GFP).

For expression of GFP tagged with a polypeptide linker of 27 amino acids (pRS306::ATP3-YEGFP3L27) encoding subunit γ fused to YEGFP3 with a polypeptide linker of 4 amino acids (γ-4-GFP) was derived from pRS306:ATP3-YEGFP3L27 by removal of a 69-bp fragment flanked by BamHI sites (Fig. 1).
20 nm) and emission (535 ± 20 nm). After imaging, gels were separated from gel plates and sliced into sections for further individual analysis. Protein was stained with Coomassie Brilliant Blue G-250 (24). Proteins extracted from cells (25) were subjected to SDS-PAGE on 4–20% gradient acrylamide gels according to standard procedures (26) and using a Bio-Rad mini-gel apparatus.

**Western Blotting**—Proteins were transferred to polyvinylidene difluoride membranes (Gelman Laboratory, Pall Corporation) after SDS-PAGE by standard procedures. Membranes were probed with rabbit polyclonal antisera against subunit γ (diluted 1:1000). Secondary antibodies were alkaline phosphate-conjugated anti-rabbit. Signals were generated using chemifluorescent Vistra substrate and incubating for 10 mins at room temperature. Chemiluminescence was detected using an Amersham Biosciences Storm PhosphorImager (27). Image data were analyzed using ImageQuant software (Amersham Biosciences). Integrated volumes for each of the relevant bands were determined and expressed as a percent of the relevant intact fusion protein after background correction.

**Modeling of Fusion Proteins**—The x-ray crystal structures of bovine mitochondrial F$_{1}$-ATPase (Ref. 2; Protein Data Bank identifier 1BMF) and GFP (Ref. 28; Protein Data Bank identifier 1EMB) were obtained from the Protein Data Bank (www.rcsb.org; Ref. 29). Using the tools generated using chemifluorescent Vistra substrate and incubating for 10 mins at room temperature. Chemiluminescence was detected using an Amersham Biosciences Storm PhosphorImager (27). Image data were analyzed using ImageQuant software (Amersham Biosciences). Integrated volumes for each of the relevant bands were determined and expressed as a percent of the relevant intact fusion protein after background correction.

To build the model for the zero length linker, the GFP structure was positioned “end on” with its N terminus of GFP proximal to the C terminus of subunit γ. Manual positioning of the GFP structure was carried out so that steric clashes were minimized (using the contacts package within Quanta to highlight close contacts) in the merging of the two termini such that the GFP moiety became a C-terminal extension of subunit γ. The entire F$_{1}$-derived portion of the model was then constrained (i.e. so that only the GFP portion of the model was allowed to move) and the model subjected to CHARMM minimization. Further minimization was performed using dihedral constraints applied to residues in the N-terminal region of GFP. Minimization was performed to convergence, and upon completion of the modeling procedure all residues in the GFP molecule were in allowed conformations. We predict that in order for the GFP molecule to correctly link to subunit γ, the N-terminal helix must partially unwind. To create the full model of F$_{1}$ containing subunit γ fused to GFP, the coordinates of the fusion were copied back into 1BMF (since the F$_{1}$-derived portion of the template was constrained during minimization the subunit γ chain of 1BMF could be simply replaced with that of the template). Models containing longer linkers were created similarly, the linkers being added to the C terminus of subunit γ using the “edit protein” facility available within Quanta. Linkers were modeled as extended regions so as to create the maximum possible distance between the C terminus of subunit γ and the GFP moiety.

**RESULTS**

In YRD15 yeast cells lacking endogenous subunit γ, we expressed individually the fusions γ-27-GFP, γ-4-GFP, and γ-0-GFP (Fig. 1) and tested their ability to act as a functional replacement for the native subunit. Yeast cells lacking expression of subunit γ are unable to grow on non-fermentable substrates because of the absence of a functional ATP synthase (30). Thus, strains MPγ-27, MPγ-4, and MPγ-0 were assessed for growth in liquid SacC medium containing ethanol as carbon source (31). All three strains grew on ethanol-containing medium, enabling comparison of their growth rates at 28 °C with that of the parental strain YRD15 (Table I). There was no significant difference (p > 0.05) in generation time for yeast MPγ-27 (10.9 h) and the control strain YRD15 (10.4 h). The generation times for MPγ-4 (14.7 h) and MPγ-0 (23.9 h), however, were significantly longer than that for the control strain (p > 0.05). Collectively these results indicate that functional mtATPase complexes are assembled in each of the strains MPγ-27, MPγ-4, and MPγ-0 at levels sufficient to support growth on a non-fermentable substrate.

Individual cells of strains MPγ-27, MPγ-4, and MPγ-0 were observed by fluorescence microscopy. For cells of each strain fluorescence due to GFP was distributed in a filamentous manner characteristic of a mitochondrial location (data not shown). This result indicated that GFP had been correctly targeted to the mitochondrion and that the correct folding of the protein and maturation of the chromophore had occurred.

The integrity of the γ-GFP fusion proteins was further investigated as follows. Proteins were extracted from cells of yeast strains MPγ-27, MPγ-4, MPγ-0, and YRD15 and subjected to SDS-PAGE. Proteins were then transferred to polyvinylidene difluoride membrane. Polypeptides of M$_{r}$ ~63,800 (Fig. 2, lane 2) and M$_{r}$ ~61,600 (Fig. 2, lanes 3 and 4) were detected when a blot was probed with polyclonal antibodies against subunit γ. The polypeptides migrated with mobility corresponding to a size slightly larger than that predicted for the corresponding fusion proteins (M$_{r}$ 59,997, 57,891, and 57,495 respectively, for γ-27-GFP, γ-4-GFP, and γ-0-GFP). Native subunit γ (not fused to GFP) from YRD15 mitochondria (Fig. 2, lane 1) was also found to migrate more slowly, M$_{r}$ 32,400, than its predicted size (M$_{r}$ 30,661). Probing an equivalent blot with antibodies against GFP confirmed that each of these polypeptides contained GFP (data not shown). An additional band (M$_{r}$ ~38,000) not present in YRD15 and representing only minor amounts (1.9%, MPγ-27; 0.47%, MPγ-4; and 2.8%, MPγ-0) of intact fusion protein was observed when blots were

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2 Significance levels of difference between generation times were determined using Student’s t test, comparing control strain YRD15 and cells expressing subunit γ-GFP fusions.
Proteins extracted from cells of strains YRD15, MPγ-27, MPγ-4, and MPγ-0 were subjected to reducing SDS-PAGE. Blots were probed with polyclonal antiserum against subunit γ. Blots were developed with Vistra ECF substrate and bands visualized by scanning for chemiluminescence using a Wallac phosphorimager. The mobilities of protein size standards (Rainbow® Markers, Amersham Biosciences) are indicated at the left.

One gel slice was imaged for fluorescence due to GFP (excitation 480 ± 20 nm, emission 535 ± 20 nm). A single fluorescent species with mobility similar to that observed for mtATPase stained with Coomassie Blue (Fig. 3A, lanes 1–4) was observed for mitochondria lysates isolated from the three strains expressing a subunit γ-GFP fusion protein (Fig. 3B, lanes 2–4). As expected, fluorescence was not detectable for the mitochondrial lysate isolated from YRD15 cells (Fig. 3B, lane 1). A cytosolic extract prepared from cells of strain MPGFPc (Fig. 3B, lane 5) was used to indicate the mobility of GFP not fused to another protein, wtGFPL.

In other experiments regions of clear native gels corresponding to mtATPase complexes isolated from mitochondrial isolates of MPγ-27, MPγ-4, and MPγ-0 cells were excised and subjected to a second dimension of reducing SDS-PAGE. Proteins were transferred to polyvinylidene difluoride membrane and probed with antibodies against subunit γ. Polypeptides with mobilities corresponding to each of the fusion proteins were detected (data not shown). These findings provide additional confirmation that complete fusion proteins are present in assembled and functional mtATPase complexes.

The ATP hydrolytic activity of mtATPase complexes containing γ-GFP fusions was investigated for osmotically lysed mitochondria using a spectrophotometric assay. ATPase activity probing with antisera against subunit γ (Fig. 2). If it is assumed this polypeptide was assembled into functional mtATPase complexes, the amounts of this polypeptide relative to the intact fusion protein cannot explain the levels of oligomycin sensitive ATPase assayed in isolated MPγ-27, MPγ-4, and MPγ-0 mitochondria (see Table I and below). Thus, compared with YRD15 reductions in ATPase activity of greater than 97% would be expected instead of the 40% observed for MP-0 (Table I). These findings confirm the identity of the fusion proteins and indicate that only complete fusion proteins are present within the vast majority of ATP synthase complexes. In other experiments in which a hexahistidine tag was added to the C terminus of the fluorescent protein moiety, it was possible using nickel-nitrilotriacetic acid chromatography, to recover assembled mtATPase complexes that contain intact γ-fusion proteins. Under conditions where mtATPase complexes from YRD15 mitochondria did not bind to nickel-nitrilotriacetic acid resin, complexes containing γ-4-GFP or γ-0-GFP were recovered from lysates of mitochondria that when assayed for ATPase were found to be oligomycin sensitive (data not shown). Thus, it can be concluded that γ-27-GFP, γ-4-GFP, and γ-0-GFP are able to functionally replace native subunit γ in ATP synthase complexes. This conclusion is consistent with the results of other studies from our laboratory that show different subunits of the yeast mtATPase complex can be functionally replaced by the cognate GFP fusion protein (16, 32, 33).

The integrity and function of isolated ATP synthase complexes containing γ-GFP fusion proteins were next investigated. Lysates of mitochondria isolated from cells of yeast strains MPγ-27, MPγ-4, MPγ-0, and YRD15 were subjected to clear native gel electrophoresis. This gradient gel electrophoresis technique (23) is capable of separating proteins and protein complexes over a wide size range. After electrophoresis, gels were sliced longitudinally into several sections. Each section was subjected to one of the following: staining for protein, Coomassie Blue staining and fluorescence profiles for monomeric mtATPase complexes from MPγ-0 cells shown in Fig. 3A and B were of reduced intensity and presumably result from a decreased extractability of complexes. However, similar amounts of each of the γ-GFP fusion proteins were present in whole cell lysates (Fig. 2). ATP synthase complexes in a dimer form can be isolated if digitonin is used to extract the ATP synthase (35). In a separate experiment fluorescent bands of lower mobility corresponding to ATP synthase dimers were observed on clear native gels when samples of MPγ-27, MPγ-4, and MPγ-0 mitochondria were extracted using digitonin (data not shown).

The ATP synthase complexes in a dimer form can be isolated if digitonin is used to extract the ATP synthase (35). In a separate experiment fluorescent bands of lower mobility corresponding to ATP synthase dimers were observed on clear native gels when samples of MPγ-27, MPγ-4, and MPγ-0 mitochondria were extracted using digitonin (data not shown).
was measured in the absence and presence of oligomycin, an inhibitor of the F$_1$ proton channel. Such an assay gives an indication of the degree of functional coupling between the F$_1$ and F$_0$ sectors of the complex, since ATPase activity is only sensitive to inhibition by oligomycin if the two sectors are functionally coupled. Inhibition by oligomycin (Table I) was found to be in the range of 64–83% within the range observed for ATPase isolated from wild-type yeast cells. These findings indicate that the fusion of GFP to the C terminus of subunit γ does not compromise the ability of the γ subunit to assemble into active mtATPase complexes.

In a separate experiment the ATP hydrolytic activity of mtATPase complexes containing γ-GFP fusions and separated on clear native gel electrophoresis was confirmed using an in situ gel ATPase assay. In the presence of lead acetate, free phosphate liberated by hydrolysis of ATP forms a white precipitate indicating the site of ATPase action within the gel (23). Enzyme activity (as indicated by the presence of a white precipitate) was observed for mitochondrial lysates (Fig. 3C) isolated from the control strain YRD15 and the three strains expressing a subunit γ-GFP fusion at a position in the gel corresponding to the Coomassie-stained or fluorescent species detected in the corresponding lanes of A and B. This result confirms that mtATPase complexes migrating with the expected mobility of monomers and containing GFP retain ATPase activity.

**DISCUSSION**

In this study we have shown that fusion proteins in which GFP is linked to the C terminus of mtATPase subunit γ with a polypeptide linker of 0, 4, or 27 amino acids are able to assemble into complexes that are functional in vivo. Furthermore, ATP synthase complexes isolated from these yeast cells expressing each of these fusion proteins are correctly assembled, fluorescent, and functionally coupled.

There are little structural data available concerning the nature of the cap structure. The results in this study indicate that any putative cap structure in yeast mtATPase complexes containing the γ-GFP fusion proteins cannot entirely cover the dimple located at the top of F$_1$. There must be a route passing through, or around, the cap structure sufficient to accommodate the polypeptide chain linking the C terminus of subunit γ to GFP. Furthermore, such a route accommodating the linker must be present throughout all states of the catalytic cycle of the mtATPase. It would be expected that ATP synthase complexes prevented from undergoing the full range of co-operative subunit interactions required for multisite catalysis would be severely compromised in their ability to synthesize ATP. Such complexes would not be capable of providing sufficient ATP synthetic capacity to support growth on respiratory substrates such as ethanol. Cells in which subunit γ was replaced by each one of the fusion proteins were capable of growth on ethanol and exhibited specific activities for oligomycin-sensitive ATPase similar to that of the control. Unless the linker of the γ-GFP fusion causes some particular displacement of the cap compatible with continued mtATPase function, it can be concluded that complexes containing native subunit γ would also possess potential polypeptide “threading” routes through the cap structure. A significant displacement of the cap might be expected to result in some uncoupling of the F$_1$ and F$_0$ sectors of the complex because of an adverse influence on interactions between OSCP and αβ pairs. OSCP is known to support important structural interactions between F$_1$ and F$_0$ and also modulates proton channel function at a distance (36, 37). However, instability of mtATPase arising from uncoupled F$_1$–F$_0$ was not apparent according to the results presented here.

The generation time for yeast expressing γ-27-GFP is not significantly different from that of the cells expressing γ subunit not fused to GFP. The x-ray crystal structure coordinates for F$_1$ and GFP were used to model the position of GFP in relation to the top of the F$_1$ αβ$_3$ hexamer. The models show a side view of F$_1$ and the position of the bottom end face of the β-barrel of GFP relative to the N termini of the F$_1$ α and β subunits in mtATPase complexes containing γ-4-GFP (left of panel) and γ-0-GFP (right of panel).

**Fig. 4.** Modeling of the F$_1$ αβ$_3$ hexamer with the γ-GFP fusion proteins. The x-ray crystal structure coordinates for F$_1$ and GFP were used to model the position of GFP in relation to the top of the F$_1$ αβ$_3$ hexamer. The models show a side view of F$_1$ and the position of the bottom end face of the β-barrel of GFP relative to the N termini of the F$_1$ α and β subunits in mtATPase complexes containing γ-4-GFP (left of panel) and γ-0-GFP (right of panel).
dimple formed by several β sheets at the N termini of α and β and that contact regions cannot extend downwards into the central shaft space housing subunit γ. It is noteworthy that the exact location of the OSCP (or subunit δ) component of the cap remains uncertain and variously has been positioned at the top of F₁ or alternatively on the outer face of F₁ extending from the nucleotide binding sites toward the top of F₁ (7, 39).

Does GFP rotate during catalysis? It is now accepted that ATP synthase is a rotatory motor and that subunit γ together with subunit ε (δ in mitochondria) and the subunit c (nine in mitochondria) ring forms the rotor of the motor (40–42). Rotation of the γ subunit appears to be an absolute requirement for the synthesis or hydrolysis of ATP. However, ATP synthase is capable of synthesizing ATP through uni-site catalysis where the subunit γ is not required to rotate (43). In this case ATP synthesis occurs at a greatly reduced rate compared with complexes in which the subunit γ is allowed to rotate without interference. No evidence is yet available concerning rotation of GFP in the strains investigated here, but rotation of subunit γ is assumed on the basis of restoration of mtATPase function in host cells lacking native subunit and the results of in situ ATPase assays for purified mtATPase complexes. However, it is conceivable that sufficient freedom exists in the polypeptide linker, joining the C-terminal region of subunit γ to GFP, to allow an uncoupling of rotation between subunit γ and the relatively bulky GFP. Evidence suggesting such a possibility is feasible is provided by the observation of forced full rotation of subunit γ in EF₁F₀ complexes when the penultimate residue of subunit γ was cross-linked to subunit α (44). Further experiments are now under way in an attempt to obtain evidence for the rotation of GFP in these complexes. As we have already shown that other subunits of yeast ATP synthase can be functionally replaced with GFP fusions proteins (16, 32, 33),⁵ complexes containing binary combinations of appropriate fusion proteins may provide the means for monitoring catalytic activity by ATP synthase in live cells.

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