A novel fluorescent marker for assembled mitochondria ATP synthase of yeast

OSCP subunit fused to green fluorescent protein is assembled into the complex in vivo

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Abstract **We have shown that OSCP, a subunit of yeast mitochondrial ATP synthase, can be incorporated into the intact enzyme as a fusion protein representing OSCP fused at its C-terminus to the green fluorescent protein (GFP) of *Aequorea victoria*. The relevant fusion OSCP-GFP-h6 additionally contains a hexahistidine tag at the C-terminus. Expression of OSCP-GFP-h6 in yeast cells lacking endogenous OSCP led to the efficient restoration of growth of cells on the non-fermentable substrate, ethanol. Confocal laser scanning microscopy revealed fluorescence due to GFP in mitochondria of cells expressing OSCP-GFP-h6. Use of immobilised metal ion affinity chromatography enabled the recovery of assembled ATP synthase complexes which contained OSCP-GFP-h6 identified by its mobility on SDS-PAGE and immunoreactivity to anti-OSCP and anti-GFP antibodies. The successful isolation of the assembled multisubunit ATP synthase containing GFP fused to one of the essential subunits of the complex widely expands the potential applications of GFP. In principle, these include the spatial and temporal monitoring of ATP synthase complexes in vivo, and the exploration of interactions involving ATP synthase subunits by fluorescence resonance energy transfer (FRET).**

Key words: Green fluorescent protein; Hexahistidine; ATP synthase; OSCP; (Saccharomyces cerevisiae)

1. Introduction

The study of multisubunit complexes such as mitochondrial ATP synthase (mtATPase), comprised of at least 12 non-identical subunits, can take place at two levels. First, the complex can be viewed holistically, in the context of its structural and functional organisation in the fully assembled state. Second, protein-protein interactions involving individual subunits that comprise the complex are of importance in the elucidation of enzyme assembly, function and regulation (often through interaction with yet other proteins).

A novel means of investigating mtATPase at both levels could be achieved if it were possible to incorporate into the complex individual subunits fused to the green fluorescent protein (GFP) of *Aequorea victoria*. Use of this tool would enable, for example, localisation of intact enzyme complexes by microscopic visualisation in cellular and subcellular preparations [1] to be made. Further, by fluorescence resonance energy transfer (FRET) techniques, the interactions of individual subunits with other closely apposed proteins (themselves tagged with appropriate GFP variants) could be analysed [2,3].

As a first step to these ends, we have tested the proposition that a particular subunit of yeast mtATPase can be incorporated in vivo into fully assembled complexes, when fused to GFP at the C-terminus of that subunit. We report here that this can be achieved using the OSCP subunit of the so-called stalk sector that connects F_0 (membrane sector, encompassing the proton channel) and F_1 (soluble 'knob' on which ATP synthesis/hydrolysis occurs). Specifically, we have expressed, in host cells of *Saccharomyces cerevisiae* lacking the endogenous natural subunit, OSCP fused to GFP itself containing a C-terminal hexahistidine tag (Fig. 1A). Sets of polypeptides that correspond to correctly assembled mtATPase complexes can be recovered by chromatography of mitochondrial lysates on immobilised Ni^{2+} (Ni-NTA resin). The stable integration of GFP into correctly assembled mtATPase complexes opens the way for new dimensions of investigation of the enzyme complex both holistically and in terms of individual pairs of protein-protein interactions.

2. Materials and methods

2.1. Construction of expression vectors

PCR was used simultaneously to retrieve a gene cassette encoding wild-type GFP together with the multiple cloning site of pGFPN3 (Clontech laboratories Inc, Palo Alto, CA, USA) and to modify GFP by the C-terminal addition of hexahistidine. The DNA segment so produced contained a *Bgl*II site proximal to the 5′ end of the modified GFP coding region and a *Not*I site distal to the 3′ end of the coding region. This GFP expression cassette was cloned into the *Bgl*II/*Not*I expression site of the yeast multicopy expression vector pASlN, a derivative of pAS1 [4] containing a *Not*I site just downstream of the original *Bgl*II site, to form pAS1N-GFP-h6. GFP (denoted L1-GFP to indicate the presence of the linker) expressed from this vector contains a 22-amino acid N-terminal extension and a C-terminal hexahistidine tag (Fig. 1C). For fusions involving OSCP, a variant of OSCP containing two C-terminally substituted methionines, functionally equivalent to natural OSCP, was employed [5]. A gene cassette encoding such modified OSCP was retrieved by PCR and cloned into the *Bgl*II site embedded within the multiple cloning site of pAS1N-GFP-h6, to form pAS1N-OSCP-GFP-h6. The novel fusion protein (OSCP-GFP-h6) now encoded by this vector (Fig. 1A) contains OSCP linked at its C-terminus to GFP-h6 via a 28-amino acid linker (Fig. 1B).

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2.2. Construction of yeast strains

Using methods and strains previously described [6] the vector pAS1N-OSCP-GFP-h6 was introduced into the yeast strain A5N, null for the expression of the corresponding chromosomal OSCP gene, to form strain AL1. The vector pAS1N-GFP-h6 was introduced into the wild-type strain YRD15 [6], to form strain AL2.

2.3. Confocal laser scanning microscopy

Yeast cells were grown in liquid medium containing ethanol as respiratory substrate [6] and harvested in mid-log-phase of growth. Cells were washed in distilled water and air-dried onto the underside of poly-L-lysine coated coverslips. Fluorescence due to GFP was visualised using the Leica TCS NT system (Leica Instruments, Australia) fitted with an FITC emission filter and scanning with the 488 nm laser line of a Krypton-Argon ion laser source.

2.4. Isolation of mtATPase from mitochondrial lysates

Procedures for the isolation of mtATPase complexes from mitochondrial lysates by metal ion chelate chromatography (where one subunit is tagged with hexahistidine), or immunoprecipitation using an anti-F_1-β monoclonal antibody, have been described [5].

2.5. SDS-PAGE

As previously described [5] proteins were separated by SDS-PAGE and stained with silver or transferred to nitrocellulose and probed with rabbit polyclonal antisera against OSCP diluted 1/1000. GFP was similarly detected by probing with mouse monoclonal antiserum against GFP diluted 1/1000 (kindly provided by Dr. John Gill, Boehringer-Mannheim Biochemicals, Indianapolis, IN). For immunoblotting, the secondary antibody was alkaline phosphatase-conjugated anti-rabbit IgG; signals were generated using the chemifluorescent substrate (ECF substrate, Amersham International, UK). Fluorescence was detected after excitation at 450 nm using the Storm 860 scanner system (Molecular Dynamics, Australia).

3. Results

3.1. Strategy for the expression of OSCP as a fusion with GFP

Salient features of the OSCP-GFP-h6 fusion depicted in Fig. 1A are as follows. The N-terminal cleavable presequence of 17 amino acids functions as a mitochondrial targeting sequence [7,8]. GFP was fused at the C-terminus of OSCP in anticipation of minimal interference with mitochondrial targeting and accurate proteolytic maturation of the OSCP moiety in mitochondria. We noted that OSCP can tolerate substantial additions at its C-terminus ([5]; M. Prescott, unpublished observations). In addition, the gene cassette for GFP was modified to encode a C-terminal addition of hexahistidine. This modification was made to allow assembly of the fusion protein into mtATPase complexes to be readily assessed. The entire mtATPase complex containing a single subunit tagged at the C-terminus with hexahistidine can be rapidly and conveniently isolated under relatively mild conditions using metal ion chelate chromatography [5].

3.2. Expression of the OSCP-GFP-h6 fusion in yeast supports growth on ethanol

OSCP is an essential component of mtATPase. Cells of yeast strain A5N that are null for the expression of OSCP are unable to grow on carbon sources that rely on mitochondrial respiratory capacity for utilisation (e.g. ethanol) and fail to assemble functioning mtATPase complexes [6]. Strain AL1 (A5N expressing OSCP-GFP-h6) was tested for growth on ethanol-containing medium and exhibited (not shown) a vigorous growth pattern barely distinguishable from that of the

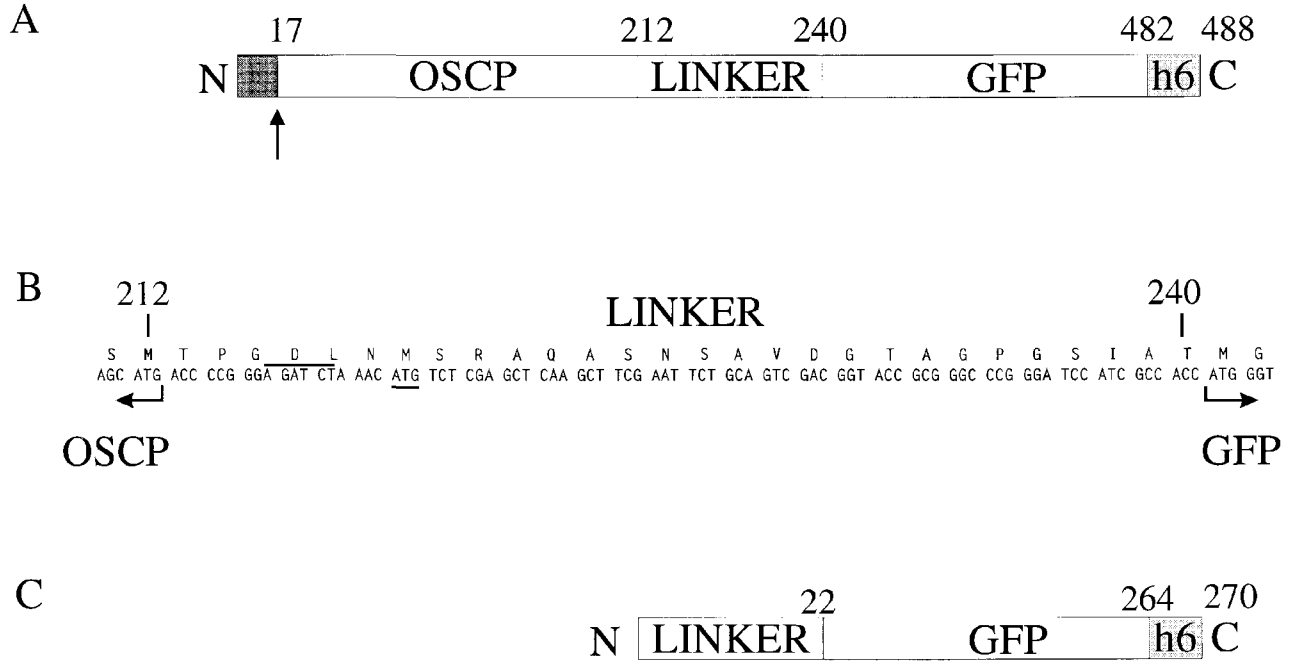

Fig. 1. The structure of OSCP-GFP-h6 fusion protein. (A) Schematic (not to scale) indicating relevant elements of the fusion protein. Numbers are the cumulative amino acid residues indicated immediately to the left of the relevant junction (numbering starts at the N-terminal methionine residue 1). The position of the protease cleavage site for removal of the mitochondrial targeting sequence (shaded region) is indicated by a vertical arrow. The hexahistidine tag is denoted as h6. (B) Sequence detail of the linker region between OSCP and GFP. The position of the *Bgl*II site embedded in the polylinker, into which was cloned a DNA expression cassette encoding the OSCP import precursor, is shown overlined. The initiation codon for expression of the short fusion (when OSCP is not inserted) is shown underlined. (C) The structural elements of the short fusion protein, L1-GFP-h6 (indications as for (A)).

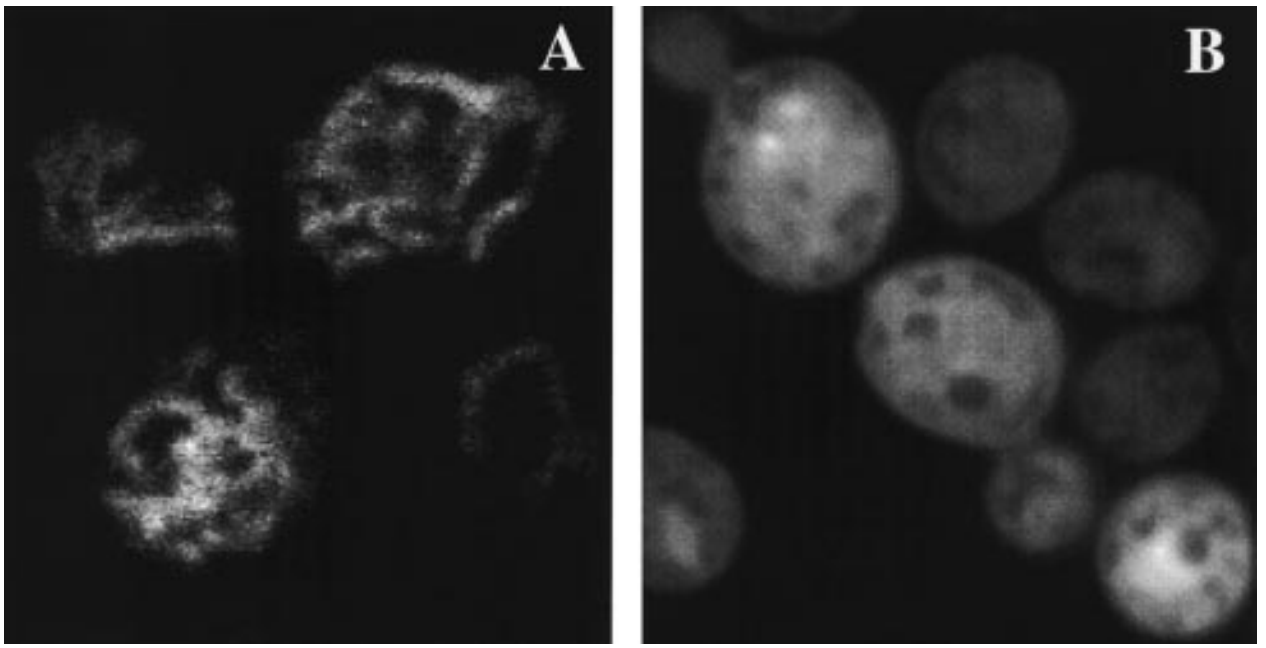

Fig. 2. Cells expressing fluorescent GFP in the mitochondrion or cytoplasm. Confocal laser scanning microscope images were generated using FITC filter sets and illumination with a 488 nm laser line. (A) Cells expressing GFP targeted to the mitochondrion as the fusion protein OSCP-GFP-h6. (B) Cells expressing the cytoplasmic form of GFP (L1-GFP-h6).

parent YRD15 cells at 18 and 28°C. However, there was a notable temperature sensitivity of strain AL1 at 36°C. The growth properties (at least at 18 and 28°C) strongly suggest that the OSCP-GFP-h6 fusion was successfully imported into the mitochondrion, correctly processed and that functional mtATPase complexes were assembled.

3.3. *Visualisation of fluorescence in vivo due to GFP*

AL1 cells were cultured in ethanol-containing medium and fluorescence due to GFP was visualised using confocal laser scanning microscopy. The stimulated fluorescence resulting from excitation with 488 nm laser light was monitored in cells using a filter combination optimal for FITC (Fig. 2A). The strong fluorescence was observed within AL1 cells was distributed in a punctate manner and exhibited a filamentous array, which is consistent with a location within the mitochondria. In other studies, similar structures and distribution in *S. cerevisiae* mitochondria have been observed when using membrane potential-sensing dyes such as $DiOC_6$ [9]. These results (Fig. 2A) indicate that GFP, when directed to the mitochondrion as a fusion with OSCP, was successfully imported into that organelle. Further, maturation of the GFP chromophore clearly occurred in yeast mitochondria to produce strong fluorescence characteristic of GFP [10].

As a control yeast strain AL2 was used, in which a cytoplasmic form of GFP (L1-GFP-h6) is expressed. Note that the linker in L1-GFP-h6 upstream of GFP has no particular targeting function. Fluorescence in AL2 cells was distributed throughout the cell (Fig. 2B), indicative of cytosolic retention, in contrast to the putative mitochondrial location of GFP in AL1 cells.

3.4. *The OSCP-GFP-h6 fusion protein is assembled into mtATPase*

It was critical to establish that the OSCP-GFP-h6 fusion protein in mitochondria (presumably cleaved by the matrix processing protease to remove the N-terminal targeting sequence) was present in assembled mtATPase complexes. Use was made of the Ni-NTA chromatography technique to purify assembled mtATPase complexes [5] through selective adsorption of the hexahistidine tag attached to the fusion protein. Mitochondrial lysates of AL1 cells and cells of the control strain YRD15 were prepared and subjected to chromatography on Ni-NTA resin. Eluates from the resin were subjected to SDS-PAGE and the gels stained for protein with silver (Fig. 3A). The polypeptide profile of fully assembled mtATPase (lane 1) was generated using a preparation of the purified complex [11] obtained from cells of the wild-type strain YRD15. Polypeptides corresponding to each of the subunits of mtATPase can be identified in eluates from the Ni-NTA resin loaded with lysates of mitochondria from AL1 cells containing OSCP-GFP-h6 (lane 2). This result clearly indicates the adsorption to Ni-NTA of assembled mtATPase from this strain. A novel polypeptide not present in lane 1, with a mobility expected for the OSCP-GFP-h6, was observed to migrate at a position above the F_1-α subunit (arrow adjacent to lane 2). By contrast, polypeptides corresponding to mtATPase were not retrieved in eluates from Ni-NTA loaded with lysates of mitochondria isolated from cells of the control strain YRD15 (lane 3). A few mitochondrial polypeptides non-specifically absorbed to and eluted from Ni-NTA resin have been previously observed [5].

To confirm the presence of OSCP in the novel protein represented within assembled complexes isolated from AL1 cells (see Fig. 3A, lane 2), the contents of that lane were transferred to a nitrocellulose membrane and probed with antibodies specific for OSCP (Fig. 3B, lane 1). A strongly reactive band at precisely the same position as the novel silver-stained protein was revealed. Moreover, a parallel blot probed with antiserum specific for GFP revealed a strong band in this same position (see Fig. 3C, lane 1). These results demonstrate that the novel band, as predicted, contains both OSCP and GFP. On the

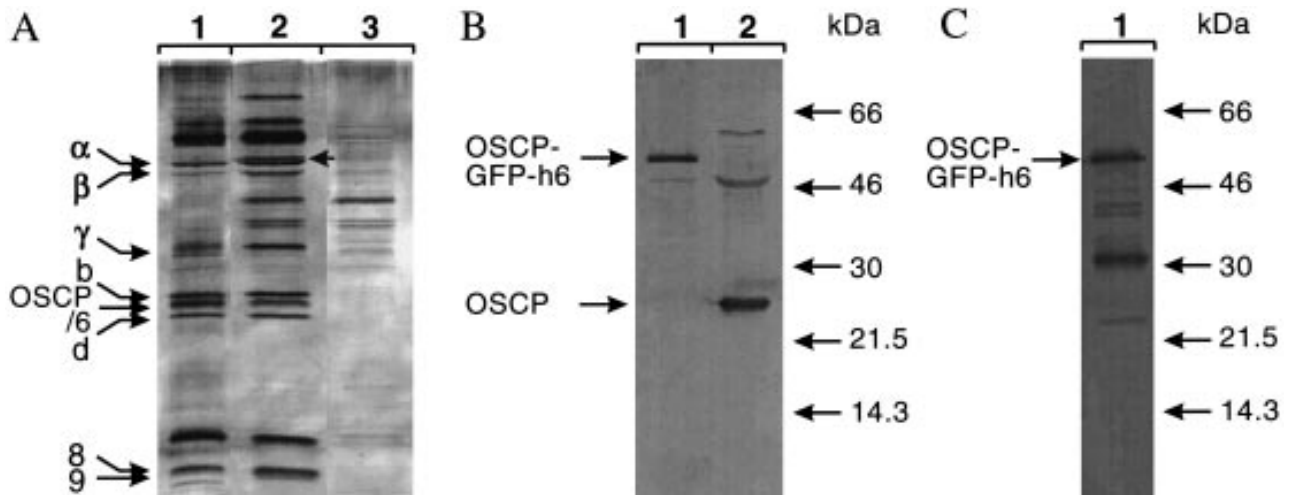

Fig. 3. Isolation of assembled ATP synthase complexes containing OSCP-GFP-h6. Protein preparations include lysates of mitochondria subjected to adsorption/elution on Ni-NTA resin to retrieve sets of polypeptides associated with a subunit (Ni-NTA eluates), or purified mtATPase complexes as subunit mobility references. The preparations were separated by SDS-PAGE and silver stained (A) or subjected to immunoblotting using either anti-OSCP antibodies (B) or an anti-GFP monoclonal antibody (C). Contents of lanes are as follows. (A) Lane 1: intact mtATPase from YRD15 prepared according to Rott and Nelson [11]; lane 2: Ni-NTA eluate from strain AL1; lane 3: Ni-NTA eluate from strain YRD15. The positions of prominent subunits of normal mtATPase are indicated at left. The arrow (at right of lane 2) indicates the position of OSCP-GFP-h6. (B) Lane 1: Ni-NTA eluate from strain AL1; lane 2: intact mtATPase from YRD15 purified by immunoprecipitation [5] using an anti-F_1-β monoclonal antibody. The positions of OSCP-GFP-h6 and natural OSCP are indicated at left. Positions of reference proteins as size markers are indicated at right. (C) Lane 1: Ni-NTA elute from strain AL1. The position of OSCP-GFP-h6 is indicated at left. Position of reference proteins as size markers are indicated at right. The size-mobility relationships of proteins in (A), (B) and (C) are essentially the same.

basis of its mobility relative to standard markers (Fig. 3B) an apparent size of 51.4 kDa is suggested for the novel band. This is in close agreement with the calculated M_r of the OSCP-GFP-h6 fusion (after N-terminal proteolytic maturation), namely 51 589. This novel polypeptide contains the complete GFP moiety since its presence in the profile, along with other associated polypeptides, is dependent on an intact C-terminal hexahistidine tag. Furthermore, OSCP of natural size was absent from complexes isolated from AL1 cells (Fig. 3B, lane 1). The normal OSCP is clearly visible as the strongly staining band in control mtATPase from strain YRD-15 (Fig. 3B, lane 2). Collectively these results indicate that OSCP-GFP-h6 is assembled into intact mtATPase complexes, in place of normal OSCP.

4. Discussion

4.1. Assembly of OSCP-GFP-h6 into mtATPase in yeast cells

We have shown that when expressed in yeast cells GFP can be targeted to, and imported into, yeast mitochondria fused to the C-terminus of OSCP, an essential subunit of mtATPase. Strong fluorescence within these cells indicated that the chromophore in GFP had matured correctly within mitochondria. Previous studies on targeting GFP to mitochondria have been achieved by fusion to GFP of an N-terminal signal and expression in mammalian cells [10]. In recent studies (data not shown), we have shown that GFP can be efficiently targeted to yeast mitochondria in vivo using as leader that of the nuclearly encoded precursor to subunit 9 of mtATPase from *Neurospora crassa*. The significance of the present work is that we have achieved specific targeting of GFP to mitochondria in the form of a fusion protein containing a full length protein assembled into a multisubunit enzyme complex. The results suggest that GFP, when fused to OSCP as described here, can be used to indicate not only a mitochondrial location for this protein but also the temporal and spatial distribution in vivo of assembled mtATPase complexes within the mitochondrial array under a variety of physiological regimes.

It might be inferred that the mtATPase complexes containing OSCP-GFP-h6 are functional. Immunoblotting of whole cell lysates with an antibody specific for OSCP (results not shown) failed to detect natural OSCP within these cells, under conditions in which abundant quantities of the fusion protein were visualised. In any event, the growth of these cells using ethanol as carbon source must have depended on a functional OSCP moiety which could only have originated from expression of an intact OSCP-GFP-h6 fusion protein. We have yet to formally eliminate the possibility that the restoration of function occurs from a sub-population of mtATPase complexes in which some proteolytic cleavage of the OSCP-GFP-h6 fusion has occurred. We regard this, however, as unlikely. If our supposition holds, GFP fused to assembled OSCP does not compromise its function in mtATPase.

4.2. General applications of GFP-tagged subunits in multisubunit complexes

The paradigm we adopted here for furnishing the GFP fusion protein with a C-terminal hexahistidine tag has general application. Not only can the hexahistidine tag be used to recover readily the GFP-fusion protein under investigation, but also the opportunity is now available to recover (as described here for mtATPase) a collection of other proteins with which the fusion protein of interest is associated. This adds a very advantageous molecular dimension to the type of information obtainable, in a cell biological context, from visualising assembled GFP as a monitor of the subcellular localisation of a particular complex of interest.

The present results have important implications for the fu-

ture analysis of interactions between individual proteins using FRET. We are now in a position to study such interactions involving one particular protein properly assembled to its host enzyme complex (in this case, OSCP in mtATPase). FRET has been demonstrated between suitable pairs of GFP variants [3,12]. In principle it would be possible to monitor interactions between different mtATPase subunits as they assembled into the enzyme complexes, using GFP and the FRET phenomenon, if each subunit were tagged with appropriate GFP variants. Alternatively, the interactions could be analysed of individual subunits of mtATPase with other proteins in mitochondria, either proteins transiently associated with the mtATPase complex [13] or those of other enzyme complexes in mitochondria. FRET in these cases could be monitored by confocal laser scanning microscopy in vivo, or by spectrofluorimetry of isolated mitochondria or even of the purified complexes.

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