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Yeast F_1F_0-ATP Synthase Complex Interactions *In Vivo* Can Occur in the Absence of the Dimer Specific Subunit e

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Evidence suggests membrane bound F_1F_0-ATPase complexes form stable associations such that dimers can be retrieved from detergent lysates of mitochondria isolated from a range of sources including algae, higher plants, yeast and bovine heart, and plant chloroplasts. The physiological relevance of these interactions is not clear but may be connected with the formation and structure of mitochondrial cristae. We sought to demonstrate, in vivo, the association of F_1F_0-ATPases in yeast cells co-expressing two b subunits each fused at its C-terminus to a GFP variant appropriate for fluorescence resonance energy transfer (FRET; BFP as the donor and GFP as the acceptor fluorophore). Both subunit b-GFP and b-BFP fusions were assembled into functional complexes. FRET was observed from enzyme complexes in molecular proximity in respiring cells providing the first demonstration of the association, in vivo, of F_1F_0-ATPase complexes. Moreover, FRET was observed within cells lacking the dimer specific subunit e, indicating structured associations can occur within the inner membrane in the absence of subunit e.

KEY WORDS: Yeast; ATP synthase; GFP; FRET.

INTRODUCTION

F_1F_0-ATP synthases catalyse the formation of ATP from ADP and inorganic phosphate (Boyer, 1997). They reside in the inner mitochondrial membrane of eukaryotes, the thylakoid membrane of chloroplasts and the plasma membrane of bacteria. It has long been thought from the results of studies on isolated membranes and reconstituted membranes using electron microscopy, that mitochondrial F_1F_0-ATP synthase (mtATPase) complexes may be closely spaced in the membrane (Fernàndez-Moràn, 1962). More recently, evidence has been presented in vitro that mtATPase complexes of yeast and bovine mitochondria may associate into dimers (Arnold *et al*., 1998; Schägger and Pfeiffer, 2000).

Arnold *et al*. (1998) provided the first evidence for the association of mtATPase complexes in yeast by demonstrating that mtATPase dimers could be recovered from detergent extracts of mitochondria subjected to blue native polyacrylamide gel electrophoresis (BN-PAGE). Dimeric mtATPase could not be isolated from mitochondria lacking either of the peripheral mtATPase subunits e or g, suggesting their involvement in promoting mtATPase dimer formation and/or stabilisation (Arnold *et al*., 1998). Subsequent work has shown that small amounts of dimeric mtATPase can be recovered from digitonin extracts of mitochondria from yeast cells lacking subunit g (Brunner *et al*., 2002; Paumard *et al*., 2002b). Dimeric mtATPase has also been recovered from detergent extracts of mitochondria isolated from bovine heart (Pfeiffer *et al*., 2003; Schägger and Pfeiffer, 2000), algae (van Lis *et al*., 2003) and higher plants (Eubel *et al*., 2004). Dimers of H^+ATP-synthase have been isolated from the chloroplast membranes of *Chlamydomonas reinhardtii* (Rexroth *et al*., 2004). Most recently oligomeric arrangements for

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Key to abbreviations: BN-PAGE, blue native-polyacrylamide gel electrophoresis; CN-PAGE, clear native-polyacrylamide gel electrophoresis.

mtATPases have been proposed following the recovery of higher order mtATPase structures containing more than two enzyme complexes from mitochondrial lysates using BN-PAGE (Paumard *et al*., 2002b).

Independent evidence for dimeric mtATPase complexes in isolated mitochondrial membranes has been provided by the generation of disulfide bridges between two b subunits (Spannagel *et al*., 1998). Subunit b of yeast mtATPase is a 209 amino acid protein with two membrane spanning domains (Soubannier *et al*., 2002; Velours *et al*., 1989) and is a component of the stator stalk that holds the catalytic F_1 stationary relative to F_0 during enzyme activity (Gavin *et al*., 2003; Razaka-Jolly *et al*., 1994; Straffon *et al*., 1998). As the yeast enzyme contains only one copy of subunit b (Bateson *et al*., 1999), disulfide formation between two b subunits was interpreted as resulting from the proximity of mtATPase complexes within the membrane (Spannagel *et al*., 1998). Notably, disulfide bridges were generated between b subunits in mitochondria lacking either of the dimer specific subunits e or g (Paumard *et al*., 2002b). Chemical cross-links have subsequently been demonstrated between the i subunit of separate mtATPase complexes within intact mitochondria (Paumard *et al*., 2002a).

The physiological relevance of mtATPase dimerisation remains unclear. Dimerisation has been postulated to confer stability to the enzyme (Arnold *et al*., 1998; Schägger, 2001), or promote efficient regulation of ATPase activity by the inhibitor protein (IF_1/Inh1p) that simultaneously binds two F_1 sectors to prevent ATP hydrolysis (Cabezón *et al*., 2000; Dienhart *et al*., 2002). Rexroth *et al*. (2004) have proposed that dimerisation of the chloroplast ATP synthase provides an enzymatic advantage by reducing futile rotational movement of the stator relative to the rotor.

Recent work has reported altered morphology of the inner mitochondrial membrane in yeast in the absence of subunits e or g. A role for mtATPase dimerisation, or even oligomerisation, in cristae formation was proposed (Paumard *et al*., 2002b) consistent with an earlier hypothesis proposed by Allen and colleagues (Allen, 1995; Allen *et al*., 1989). We have recently demonstrated the ability to trap mtATPase complexes in an oligomeric arrangement in vivo, by fusing mtATPase subunit b or γ to DsRed; the tetrameric nature of the fluorescent protein anchors multiple complexes together (Gavin *et al*., 2002, 2004). Cells containing trapped mtATPase oligomers displayed altered mitochondrial morphology and an absence of cristae, consistent with a role for the correct arrangement of mtATPase in cristae formation (Gavin *et al*., 2004). However, it was unclear whether the DsRed fusion protein trapped endogenous oligomeric mtATPase associations, or its tetramerisation promoted the oligomerisation of mtATPase complexes. Evidence supporting the existence of endogenous dimeric mtATPase complexes in vivo, has therefore, yet to be presented, and the possibility exists that mtATPase dimers recovered in the studies to date are inadvertently created by the very methods used to recover them. Arguments justifying the techniques used and for the organisation of the enzyme in vivo are still being presented (Arselin *et al*., 2003; Dienhart *et al*., 2002; Paumard *et al*., 2002b; Schägger, 2002) seven years after the initial observation of mtATPase dimers (Arnold *et al*., 1998). Thus, we sought to demonstrate the association of mtATPase complexes within intact cells sampled during respiratory growth.

Fluorescence resonance energy transfer (FRET) is the non-radiative transfer of energy from an excited donor fluorophore to an acceptor fluorophore in molecular proximity, and has been used to non-invasively monitor the interactions of a variety of proteins tagged with GFPs in vivo (see Zhang *et al*., 2002 for review). We have previously shown that dynamic changes in orientation between two subunits (subunit b and OSCP) assembled within yeast mtATPase complexes can be monitored by tagging the subunits with GFP variants appropriate for FRET (Gavin *et al*., 2003). In the present study we have used the same technology with the aim of establishing the association of mtATPase complexes in intact cells sampled during respiratory growth. To this end we have co-expressed two b subunits in cells, each fused at its C-terminus with a GFP variant appropriate for FRET (GFP or BFP), that can be assembled into functional mtATPase complexes. FRET could be detected between the fluorescent protein moieties of b-GFP and b-BFP fusions assembled in neighbouring mtATPase complexes in whole cells. The association of mtATPase complexes was also detected in vivo by the same manner, in cells lacking subunit e, previously reported to be required for dimer recovery.

EXPERIMENTAL

Materials

Dodecyl β-maltoside, digitonin, BSA (fatty acid free), antimycin A, ε-amino-*N*-caproic acid and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma. Anti-(mouse IgG) and anti-(rabbit IgG) were purchased from Amrad-Pharmacia (Melbourne, Australia). Antibodies to subunit b and GFP/BFP were as described previously (Bateson *et al*., 1999; Gavin *et al*., 2003; Prescott *et al*., 1997). Antibodies to subunits e and g were a generous gift from Dr. Jean Velours (Institut de Biochimie et Génétique Cellulaires du CNRS, Université Victor Segalen, Bordeaux, France). Vistra ECF substrate was purchased from Amersham Biosciences (Sydney, Australia).

Yeast Protocols

S. cerevisiae strain YRD15 (Table I) was the parental strain used in this study. Growth media used were as described (Boyle *et al*., 1997) supplemented with uracil, histidine and leucine as required. Cells were grown at 28°C in liquid medium (SaccE) with agitation and followed using a colorimeter (Klett-Summerson). All studies utilised cells in the mid-logarithmic phase of growth. Plasmid shuffling was performed as in Boyle *et al*. (1997), while integrative transformations followed the protocol of Agatep *et al*. (1998).

Construction of Expression Cassettes Encoding Subunit b-GFP and b-BFP Fusion Proteins

Construction of the cassette encoding the b-GFP fusion protein has been described in detail (Gavin *et al*., 2003). To generate the subunit b-BFP expression vector, a variant of pRS306: ATP4POT-YEGFP3 (Gavin *et al*., 2003) denoted pRS306:ATP4PO7T-YEGFP3 (transcription terminator fragment from *ATP4* (ATP4T) replaced with a terminator fragment from the gene encoding subunit d, *ATP7*), was digested with *Pvu*II enzyme at sites flanking the ATP4PO7T-YEGFP3 cassette. This fragment was cloned into the *Pvu*II site of episomal expression vector pRS416 (Sikorski and Hieter, 1989) to generate pRS416:ATP4PO7T-YEGFP3. The YEGFP3 gene was released using *Bam*HI and *Not*I restriction enzymes and a *Bam*HI-*Not*I fragment encoding BFP11h6 (Gavin *et al*., 2003) inserted to create pRS416:ATP4PO7T-BFP11h6. This construct is expressed under the transcriptional control of the native *ATP4* promoter and expresses BFP fused to the C-terminus of subunit b via a 27 amino acid linker, in identical context to the b-GFP fusion.

Construction of Yeast Strains Co-Expressing Subunit b-GFP Fusion Proteins

Yeast strains used within this study are detailed in Table I. Subunit b-GFP and b-BFP fusion proteins were co-expressed in the absence of native subunit b. The chromosomal *ATP4* gene in strain YRD15 was replaced with the ATP4-YEGFP3 cassette as described

Table I. Yeast Strains Used in This Study

Yeast strain	Description	Replaced chromosomal gene	Disrupted chromosomal mtATPase gene	Expression vector
YRD15	Parental strain (*MATα, his3, ura3, leu2, rho*$^+$)	—	—	—
YRD:b-GFP	Expresses the chromosomally integrated subunit b-YEGFP3 fusion in place of endogenous subunit b	ATP4 with ATP4POT-YEGFP3	—	—
YRD:b-BFP	Expresses the episomal subunit b-BFP11h6 fusion in place of endogenous subunit b	—	ATP4	pRS416:ATP4PO7T-BFP11h6
YRD:b-GFP/ b-BFP	Co-expresses the integrated subunit b-YEGFP3 fusion with the episomal subunit b-BFP11h6 fusion in place of endogenous subunit b	ATP4 with ATP4POT-YEGFP3	—	pRS416:ATP4PO7T-BFP11h6
Δ*E*	Null for subunit e	—	ATP21	—
Δ*E*:b-GFP/ b-BFP	Co-expresses the integrated subunit b-YEGFP3 fusion with the episomal subunit b-BFP11h6 fusion in a null e background	ATP4 with ATP4POT-YEGFP3	ATP21	pRS416:ATP4PO7T-BFP11h6
YRD:mitGFP	Expresses YEGFP3 targeted to the mitochondrial matrix	—	—	pAS1N:mitGFP
Δ*E*:mitGFP	Expresses YEGFP3 targeted to the mitochondrial matrix in a null e background	—	ATP21	pAS1N:mitGFP
OSCP-BFP/ b-GFP	Expresses two fusion proteins used for FRET studies, b-YEGFP3 and OSCP-BFP11h6, in the absence of endogenous subunits	ATP4 with ATP4POT-YEGFP3	ATP5	pRS416:ATP5POT-BFP11

previously (Gavin *et al*., 2003). Cells exhibiting mitochondrial fluorescence and able to survive on the non-fermentable carbon source ethanol were examined for the presence of the fusion protein using SDS-PAGE and western analysis. Positive cells were denoted YRD:b-GFP. This strain was then transformed with pRS416:ATP4PO7T-BFP11h6. Transformants selected on the basis of exhibiting BFP fluorescence and the appropriate auxotrophic markers were named YRD:b-GFP/b-BFP. Plasmid pRS416:ATP4PO7T-BFP11h6 was transformed into a strain null for expression of subunit b to create YRD:b-BFP.

A PCR-based integration protocol was used to disrupt the gene encoding subunit e (*TIM11*/*ATP21*) in strains YRD15 and YRD:b-GFP/b-BFP to create strains Δ*E* and Δ*E*:b-GFP/b-BFP.

Isolation of Mitochondria and Assay of ATPase Activity

Cells were grown in well-aerated SaccE medium at 28°C, and harvested during mid-logarithmic growth phase. Mitochondria were isolated (Boyle *et al*., 1999), flash-frozen and stored at −80°C. Thawed mitochondria were used for analysis of ATPase activity, mtATPase subunit assembly and fluorescence. Protein estimation was made using the Dye-Binding Protein Assay (Biorad, Australia). ATPase activity was followed by the oxidation of NADH in an enzyme-linked assay (Roberts *et al*., 1979).

Isolation of Monomeric/Dimeric mtATPase

Clear native polyacrylamide gel electrophoresis (CN-PAGE) (Gavin *et al*., 2002; Schägger *et al*., 1994) was used to separate mtATPase complexes in monomeric, dimeric and oligomeric states. Mitochondria (0.5–1 mg) were pelleted (100,000 g, 10 min) at 4°C and resuspended in extraction buffer (50 mM NaCl, 2 mM aminohexanoic acid, I mM EDTA, 5 mM PMSF, 50 mM imidazole, pH 7). To recover mtATPase monomers, dimers or oligomers, mitochondria were solubilised on ice for 20 min after addition of lauryl maltoside (4 g/g protein) or digitonin (4 g/g protein or 1 g/g protein), respectively. Lysates were centrifuged (100,000 g, 20 min) and supernatants loaded onto Clear Native polyacrylamide gels (gradient 3–13%). Electrophoresis was performed at 4°C for 4 h at 15 mA. Separated mtATPase complexes were visualised by GFP fluorescence using a Pro-Express Multi-Wavelength Fluoroimager (Perkin-Elmer).

Analysis of Fluorescence

Yeast strains were sampled during mid-logarithmic growth in SaccE for examination by fluorescence microscopy and examined using an Olympus BX60 fluorescence microscope equipped with a 1.35 NA oil immersion lens (Olympus 100X; UPlanapo). Images were captured using a DAGE CCD-72 ETX camera (Berthold, Australia) and MCID acquisition software (Imaging Research Inc., USA).

Fluorescence spectra were recorded for intact mitochondria (200 μg) suspended in 750 μl 0.6 M mannitol, 2 mM EGTA, 5 mM phosphate, 10 mM Tris/maleate pH 6.8 using a Cary Eclipse spectrofluorometer (Varian, Melbourne, Australia). Scans were performed at 120 nm/min with 5 nm slit widths. Spectra remain uncorrected.

Fluorescence spectra were recorded for yeast cells sampled during logarithmic growth in SaccE medium, immediately after washing in distilled water and resuspension in 50 mM Tris/HCl pH 8 to an OD of 2 at 650 nm. 10 nm emission slit widths were used. Emission spectra recorded for YRD15 control cells were used to correct spectra for autofluorescence.

RESULTS

The proximity of two b subunits from separate mtATPase complexes has been demonstrated in the inner membrane of isolated mitochondria (Paumard *et al*., 2002b; Spannagel *et al*., 1998). If mtATPase complexes adopt a similar arrangement in vivo, we predicted this proximity should be detectable using FRET. Subunit b-GFP and b-BFP fusion proteins were co-expressed in the absence of the endogenous b subunit under the transcriptional control of the native *ATP4* promoter. As subunit b of mtATPase has a stoichiometry of 1 (Bateson *et al*., 1999), each complex is labelled with a single fluorescent protein (GFP or BFP). Only mtATPase associations that bring the GFP and BFP variants together will result in energy transfer. Cells were also examined in the absence of subunit e, to examine the effect on the association of mtATPase complexes in vivo.

Cells Expressing b-GFP Fusions Have Functional mtATPase

Yeast cells lacking expression of endogenous subunit b are unable to grow on non-fermentable substrates owing to the absence of functional ATP synthase complexes (Paul *et al*., 1989). Strains YRD:b-GFP/b-BFP and Δ*E*:b-GFP/b-BFP expressing both subunit b-GFP fusions,

Table II. Generation Times of Strains and Oligomycin-Sensitive ATPase Activities of Isolated Mitochondria

Strain	Generation time *h*	ATP hydrolysis ($\mu mol\ ATP\ min^{-1}\ mg\ protein^{-1}$)		Inhibition (%)
		No addition	+Oligomycin	
YRD15	8.67 ± 0.14	3.16 ± 0.33	1.35 ± 0.08	57.3
YRD:b-GFP/b-BFP	8.28 ± 0.73	3.08 ± 0.08	1.32 ± 0.05	57.1
Δ*E*:b-GFP/b-BFP	11.45 ± 0.49	2.58 ± 0.14	1.00 ± 0.04	61.2

Note. Mitochondria were isolated from cells grown at 28°C with 2% ethanol as the carbon source. Assays were performed in the presence of 100 μg of oligomycin/mg of protein, where indicated. Numbers represent the mean ± standard deviation.

were able to grow using ethanol as carbon source, demonstrating the ability to generate ATP via oxidative phosphorylation in the absence of the endogenous b subunit. Comparison of the generation time for cells (Table II) showed that YRD:b-GFP/b-BFP (8.28 h) was similar to YRD15 (8.67 h), while Δ*E*:b-GFP/b-BFP (11.45 h) displayed a relative increase in generation time from the wild-type comparable to published reports (Arselin *et al*., 2003; Paumard *et al*., 2002b).

Functional coupling of the F_1 and F_0 sectors was also found to be equivalent for all strains (Table II). ATPase activity was moderately sensitive to oligomycin in YRD15 (57.3% inhibition), YRD:b-GFP/b-BFP (57.1% inhibition) and Δ*E*:b-GFP/b-BFP (61.2% inhibition) mitochondria. Uninhibited ATPase rates were similar for both YDR15 and YRD:b-GFP/b-BFP (Table II), and slightly decreased in Δ*E*:b-GFP/b-BFP mitochondria.

Collectively, the above results demonstrate that the addition of a GFP moiety to the C-terminus of subunit b does not significantly alter the function of mtATPase. Such observations are in agreement with our previous findings (Gavin *et al*., 2003).

Subunit b-GFP and b-BFP Fusion Proteins Are Present in Cells

Proteins were extracted from yeast cells and subjected to SDS-PAGE and transferred to PVDF membrane. Blots were probed with antisera directed against subunit b and GFP (Fig. 1(a) and (b)). Antisera directed against subunit b (panel A) detected a polypeptide of ∼25 kDa in YRD15 (lane 1), corresponding to endogenous subunit b. Subunit b antisera highlighted a band of decreased mobility (∼55 kDa) in strains YRD:b-GFP/b-BFP (lane 2) and Δ*E*:b-GFP/b-BFP (lane 3) close to the predicted size (52.1 kDa) for a subunit b-GFP fusion. The GFP antisera used (detects both GFP and BFP), confirmed the identity of the subunit b-GFP fusions (panel B, lanes 2–3). Given the similar sizes of the b-GFP and b-BFP fusions and the lack of independent antisera for GFP and BFP, these blots cannot establish the presence of both fusion proteins within cells. However, both fluorescence microscopy (data not shown) and spectrofluorimetry (see below) demonstrate the presence of both GFP and BFP within whole cells and isolated mitochondria. As expected, no endogenous subunit b is seen in lysates from YRD:b-GFP/b-BFP (lane 2) or Δ*E*:b-GFP/b-BFP (lane 3), evidence that intact subunit b-GFP fusion proteins are assembled in functional enzymes. Only minor degradation of the fusion protein is evident in lanes 2 and 3 (panel A), leading us to conclude that the majority of mtATPase complexes contain an intact fusion protein. Furthermore, analysis of mitochondrial lysates using clear native gel analysis indicated

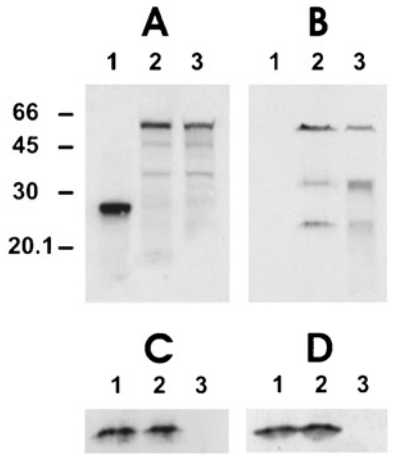

Fig. 1. *Immunological detection of fusion proteins in whole cell lysates.* Whole cell lysates prepared from strains YRD15 (lane 1), YRD:b-GFP/b-BFP (lane 2) and Δ*E*:b-GFP/b-BFP (lane 3) were subjected to SDS-PAGE and proteins transferred to PVDF membrane (panels A–D). Blots were probed with antisera directed against subunit b (panel A), GFP (panel B), subunit e (panel C) and subunit g (panel D). The position of molecular size markers is shown to the left.

that the vast majority of GFP fluorescence was associated with intact complexes (see below; Gavin *et al*., 2003; and data not shown). Some free GFP ($M_r \sim 35$ kDa) is seen in whole cell lysates from these same strains (panel B), however small amounts of GFP free within the mitochondrial matrix does not contribute to FRET (data not shown) and cannot therefore produce misleading instances of FRET in the experiments reported here.

Mitochondria isolated from cells of yeast strains YRD15, YRD:b-GFP/b-BFP and Δ*E*:b-GFP/b-BFP were lysed, subjected to SDS-PAGE and the proteins transferred to PVDF membranes. Blots were probed with antisera directed against subunits e (panel C) and g (panel D), to confirm the presence or absence of the dimer specific subunits in mitochondrial membranes. Subunit e was present in mitochondria isolated from YRD15 (panel C, lane 1) and YRD:b-GFP/b-BFP (lane 2), and absent in mitochondria from Δ*E*:b-GFP/b-BFP (lane 3). Subunit g was also present in all mitochondria except those isolated from Δ*E*:b-GFP/b-BFP (panel D, lane 3), consistent with previously published results (Arnold *et al*., 1998; Brunner *et al*., 2002).

These results confirm the presence of the subunit b fusion proteins and the absence of endogenous subunit b. The mtATPase complexes therefore contain an associated fluorescent protein, allowing the proximity of the enzymes within the membrane to be investigated using FRET.

Δ*E*:b-GFP/b-BFP Cells Have Abnormal Mitochondria

Subunit b of the yeast mtATPase has recently been reported to influence mitochondrial morphology (Paumard *et al*., 2002b; Soubannier *et al*., 2002). For this study it was important to show substitution of endogenous subunit b with a subunit b-FP fusion, in cells null for expression of subunit e, retain the mitochondrial morphology typical of these cells. Yeast cells sampled from cultures (grown on the non-fermentable carbon source ethanol) during mid-logarithmic growth phase were examined both by fluorescence microscopy, and in more detail by electron microscopy (data not shown). The fluorescence pattern observed for Δ*E*:b-GFP/b-BFP cells indicates mitochondria exist as punctate balls and larger ring or "donut-like" structures distributed throughout the periphery of the cell, with no continuous mitochondrial network evident. YRD:b-GFP/b-BFP cells showed a tubular network of mitochondria typical of wildtype cells undergoing respiratory growth (Egner *et al*., 2002; Hermann and Shaw, 1998). A closer examination of the mitochondrial ultrastructure using electron microscopy revealed a striking lack of cristae and multiple layers of unfolded inner membranes in Δ*E*:b-GFP/b-BFP cell sections, as reported recently for cells lacking subunits e or g (Paumard *et al*., 2002b). These results indicate that the incorporation of subunit b-GFP fusion proteins into functional mtATPase does not alter the morphology of the mitochondrial network.

Recovery of mtATPase Oligomers from Mitochondria Containing Subunit b-GFP Fusions

The association of mtATPase was studied using clear native polyacrylamide gel electrophoresis (CN-PAGE) to ensure complexes incorporating subunit b-GFP fusion proteins could form associations of types previously reported to be recovered from mitochondrial lysates (Arnold *et al*., 1998; Paumard *et al*., 2002b). Lysates were prepared from YRD:b-GFP/b-BFP and Δ*E*:b-GFP/b-BFP mitochondria using lauryl maltoside (4 g/g detergent/protein) or digitonin (4 g/g or 1 g/g detergent/protein). Lysates were subjected to CN-PAGE and gels then imaged for GFP fluorescence (Fig. 2). An intense fluorescent band corresponding to the monomer form of mtATPase was observed in the YRD:b-GFP/b-BFP mitochondrial lysate (Fig. 2, lane 1) prepared using lauryl maltoside. Digitonin lysates (4 g digitonin/g protein) of YRD:b-GFP/b-BFP mitochondria contain fluorescent mtATPase in a predominately dimeric state (Fig. 2, lane 2). *In situ* analysis showed these fluorescent bands display oligomycin ATPase activity confirming they contain functionally coupled mtATPase complexes (data not shown). Decreasing the digitonin/protein ratio (1 g digitonin/g protein) led to the recovery of three oligomeric forms of mtATPase in addition to the dimer (Fig. 2, lane 5). Similar relative recovery of monomer, dimer higher oligomer forms were observed for mitochondrial lysates containing native subunit b (data not shown; Gavin *et al*., 2003). These results indicate the presence of GFP/BFP fused to subunit b does not interfere with the ability to recover dimers or higher order associations of mtATPase from mitochondria.

A single fluorescent band corresponding to monomeric mtATPase was detected in mitochondrial lysates of Δ*E*:b-GFP/b-BFP mitochondria prepared using lauryl maltoside (Fig. 2, lane 3). However, mtATPase was recovered only as a single monomeric species from digitonin (4 g/g) extracts of these mitochondria, with no evidence of mtATPase dimers (Fig. 2, lane 4). Oligomeric and dimeric forms of the enzyme were absent from Δ*E*:b-GFP/b-BFP mitochondria solubilised using digitonin/protein ratios of 1 g/g, with monomeric mtATPase being the only form recovered (data not shown).

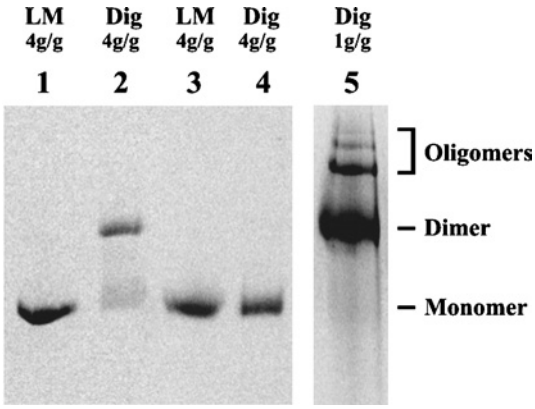

Fig. 2. *Recovery of mtATPase on CN-PAGE*. Mitochondria were lysed with lauryl maltoside (LM; 4 g/g detergent to protein ratio, lanes 1 and 3) or digitonin (Dig; 200 μg mitochondria at 4 g/g detergent/protein, lanes 2 and 4; 1 mg mitochondria at 1 g/g detergent/protein, lane 5) and run on a 3–13% Clear Native polyacrylamide gel to separate mtATPase forms. Lanes 1, 2 and 5, YRD:b-GFP/b-BFP; Lanes 3 and 4, ΔE:b-GFP/b-BFP. Intact mtATPase was visualised by YEGFP3 fluorescence using a Pro-Express Multi-Wavelength Fluoroimager.

FRET Occurs Between mtATPase Complexes *In Vivo*

We next sought evidence for the close association of mtATPase in vivo using FRET. Cells were sampled during respiratory growth and emission spectra recorded from control strains YRD:b-BFP (Fig. 3(a)) and YRD:b-GFP (Fig. 3(b)). Upon excitation with wavelengths of light specific for the excitation of BFP (360 nm) or GFP (480 nm), emission maxima of 455 and 513 nm were observed for suspensions of YRD:b-BFP (Fig. 3(a)) and YRD:b-GFP cells, respectively. Importantly, little emission at 513 nm was seen from YRD:b-GFP cells upon excitation with 360 nm light (Fig. 3(b)).

Peak emission of 513 nm was also recorded from YRD:b-GFP/b-BFP cells when illuminated with 480 nm light (Fig. 3(c)), although this was slightly reduced relative to YRD:b-GFP cells (Fig. 3(b)). Significantly, a second emission peak at 513 nm corresponding to GFP was observed in YRD:b-GFP/b-BFP cells upon specific excitation (360 nm) of the BFP (Fig. 3(c)). The fluorescence intensity ratio of emission $R_{513/455\,nm}$ (for excitation at 360 nm) from YRD:b-GFP/b-BFP cells was 1.56. GFP emission is relatively minor in YRD:b-GFP cells following excitation at 360 nm, despite the increased levels of GFP fluorescence (Fig. 3(b)). Emission spectra recorded from a 1:1 mixture of YRD:b-GFP and YRD:b-BFP cells (data not shown), reveal the absence of significant GFP emission ($R_{513/455\,nm} = 0.57$) following excitation at 360 nm as seen in YRD:b-GFP/b-BFP. The emission maximum at 513 nm recorded from YRD:b-GFP/b-BFP cells must therefore result from energy transfer between the BFP and GFP moieties of the subunit b fusion proteins in close proximity within the membrane. We conclude the close association of mtATPase complexes observed using FRET in vivo most likely represent mtATPase in a dimeric arrangement.

We next addressed whether the association of mtATPase complexes could be detected using FRET in cells lacking subunit e. Like YRD:b-GFP/b-BFP cells, ΔE:b-GFP/b-BFP cells displayed two emission peaks following excitation at 360 nm. The first emission maximum of 455 nm corresponds to BFP, while the second peak at 513 nm corresponds to GFP emission produced by FRET (Fig. 3(d)). FRET observed in ΔE:b-GFP/b-BFP cells is of lower efficiency ($R_{513/455\,nm} = 0.96$) compared to that of YRD:b-GFP/b-BFP, possibly reflecting a subtle alteration to the conformation of mtATPase complexes within the mitochondrial inner membrane produced by the absence of subunit e.

Collectively, these results support the hypothesis that mtATPase complexes interact in vivo, most likely as dimers. Moreover the detection of FRET in ΔE:b-GFP/b-BFP cells provides evidence that these interactions can still occur in vivo in the absence of the dimer specific subunit e.

Solubilised mtATPase Complexes Do Not Show FRET

Emission spectra were recorded from suspensions of intact mitochondria (Fig. 4). As with intact cells, exposure to wavelengths of light specific for the excitation of BFP (360 nm) led to emission maxima of 455 and 513 nm for intact mitochondria isolated from YRD:b-GFP/b-BFP (panel A) and ΔE:b-GFP/b-BFP (panel B). The ratio of fluorescence intensity $R_{513/455\,nm}$ was 0.88 and 0.86 for YRD:b-GFP/b-BFP mitochondria and ΔE:b-GFP/b-BFP mitochondria, respectively. As expected, BFP does not exhibit significant emission at 513 nm in YRD:b-BFP mitochondria (panel C, $R_{513/455\,nm} = 0.67$), however some emission at of 513 nm was seen from YRD:b-GFP mitochondria following excitation at 360 nm (panel D). A blue/green emission intensity ratio is not presented for YRD:b-GFP mitochondria, as the lack of a BFP would exaggerate the relative level of GFP emission relative to other mitochondria. Some fraction of this emission at 513 nm is attributable to emission of fluorescent species within the mitochondria because, in contrast to the data for whole cells, no correction has been made to account for autofluorescence which is more prevalent in whole cells than isolated mitochondria. This is evident in the

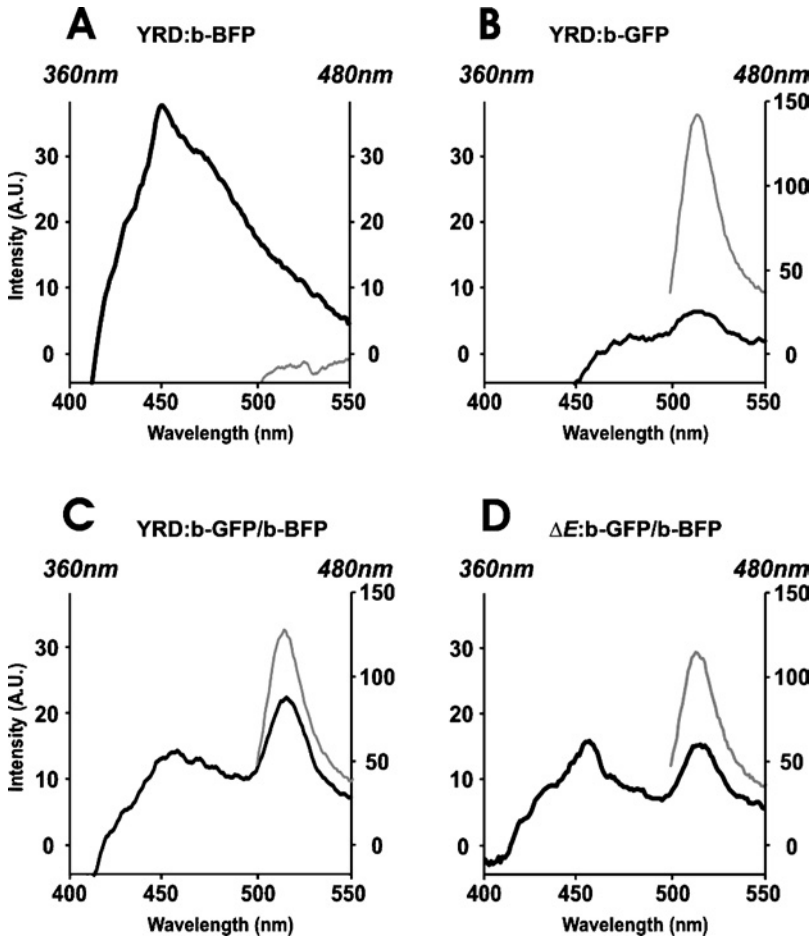

Fig. 3. *Emission spectra of whole cells expressing subunit b-GFP fusions*. Yeast cells were sampled from mid-logarithmic cultures, washed and resuspended to an OD of 2 (at 650 nm) in cuvettes within the Cary Eclipse spectrofluorometer (Varian). Emission spectra were recorded upon 360 nm (black lines) or 480 nm (grey lines) excitation. Emission spectra recorded from YRD15 were subtracted from strains expressing subunit b-GFP fusions in order to correct for cellular autofluorescence. Emission intensity following 360 or 480 nm excitation is presented on the left and right *Y*-axis, respectively. Yeast strains were YRD:b-BFP, (A); YRD:b-GFP, (B); YRD:b-GFP/b:BFP, (C) and Δ*E*:b-GFP/b:BFP, (D).

relative increase in emission at 513 nm following excitation at 360 nm in YRD:b-BFP mitochondria (panel C) compared to that seen in whole cells (Fig. 3(a)).

In order to positively attribute GFP emission following excitation at 360 nm to FRET in YRD:b-GFP/b-BFP and Δ*E*:b-GFP/b-BFP mitochondria, we sought to disrupt putative mtATPase associations using detergents, altering the proximity of the fluorescent protein tags and thereby abolishing any FRET signal present. Mitochondria isolated from a separate strain, OSCP-BFP/b-GFP (Table I), were used as a control in these experiments. These cells express both subunit b-GFP and OSCP-BFP fusion proteins assembled within the same mtATPase complex (Gavin *et al*., 2003). In this instance the fluorophores have a defined 1:1 stoichiometry within each mtATPase complex and an orientation that promotes efficient FRET ($R_{513/455\,nm} = 1.46$; Fig. 4, panel E) within the monomeric complex (Gavin *et al*., 2003).

Lauryl maltoside was added to mitochondria at a detergent/protein ratio of 4 g/g, which yields essentially monomeric mtATPase on Clear Native gels (Fig. 2). Mitochondrial lysates from YRD:b-GFP/b-BFP (Fig. 4, panel A), and Δ*E*:b-GFP/b-BFP (panel B) treated in this manner maintained strong GFP emission at 513 nm upon excitation at 480 nm. BFP emission at 455 nm was increased following lysis and excitation at 360 nm, however the

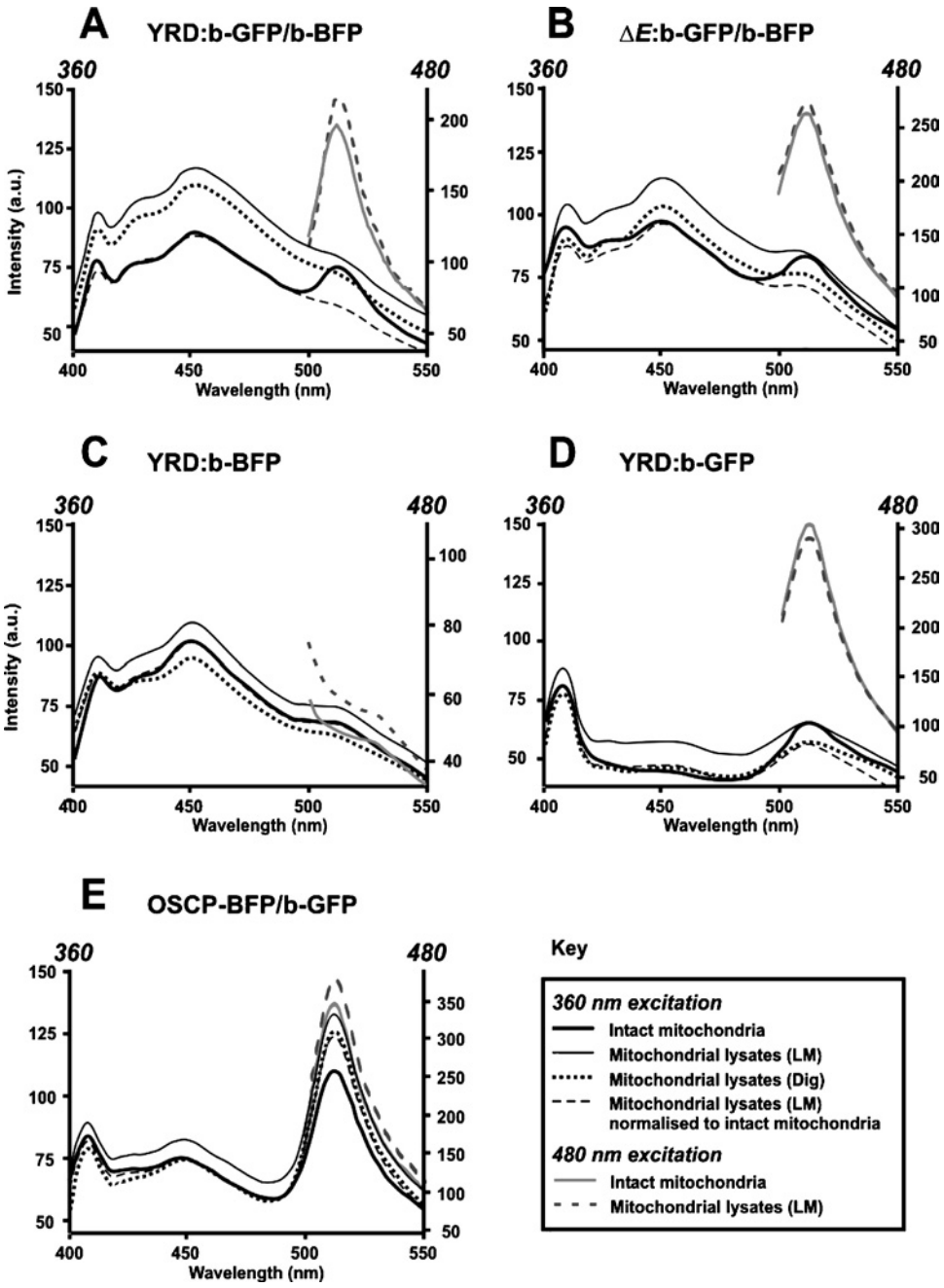

Fig. 4. *Emission spectra of mitochondrial suspensions*. Emission spectra with excitation at 360 nm (left ordinate axis, solid black line) or 480 nm (right ordinate axis, solid grey line) were recorded for suspensions of mitochondria isolated from strains YRD:b-GFP/b-BFP (A); Δ*E*:b-GFP/b-BFP (B), YRD:b-BFP (C); YRD:b-GFP (D) and OSCP-BFP/b-GFP (E). In addition emission spectra were recorded for mitochondria solubilised with lauryl maltoside using 360 nm excitation (solid thin black line) and 480 excitation (dotted grey line), and for mitochondria solubilised with digitonin using 360 nm excitation (dotted black line). Emission spectra from mitochondria solubilised with lauryl maltoside following 360 nm excitation (solid thin black line) were normalised with spectra from intact mitochondria (dashed black line) to show the relative decrease in 513 nm emission.

relative GFP emission at 513 nm seen with intact mitochondria was now reduced. $R_{513/455\,nm}$ for YRD:b-GFP/b-BFP mitochondria fell from 0.88 in intact mitochondria, to 0.68 after lauryl maltoside lysis, equivalent to levels observed in YRD:b-BFP mitochondria ($R_{513/455\,nm} = 0.67$) containing BFP alone. No reduction in FRET was observed upon lysis of OSCP-BFP/b-GFP mitochondria in which both BFP and GFP are attached to subunits contained within each monomeric mtATPase (panel E). Lysis of YRD:b-GFP mitochondria also led to a small reduction of emission at 513 nm upon excitation at 360 nm, demonstrating a specific effect of the detergent lysis and/or change in the nearby molecular environment upon GFP fluorescence. However the magnitude of the decrease was 50% less than that for YRD:b-GFP/b-BFP mitochondria, despite the increased levels (~33%) of GFP fluorescence in YRD:b-GFP mitochondria. Thus, while a component of the GFP fluorescence in YRD:b-GFP/b-BFP mitochondria following illumination at 360 nm may result from the non-specific excitation of the subunit b-GFP fusion, the majority of 513 nm fluorescence can be attributable to FRET between complexes in close association.

A smaller reduction was seen in ΔE:b-GFP/b-BFP mitochondria ($R_{513/455\,nm}$ from 0.86 to 0.74) upon detergent lysis (panel B). The decrease in emission at 513 nm following excitation at 360 nm is greater than that observed in YRD:b-GFP mitochondria suggestive of FRET produced by dimeric mtATPase, however the degree of non-specific GFP excitation makes it difficult to establish the degree of FRET between mtATPase dimers in isolated mitochondria lacking subunit e.

Analysis of mitochondrial lysates prepared using digitonin at detergent/protein ratios (4 g/g) by CN-PAGE led to recovery of mtATPase dimers (Fig. 2). Following solubilisation of mitochondria with digitonin at the same detergent/protein ratio, FRET was again reduced in both YRD:b-GFP/b-BFP and ΔE:b-GFP/b-BFP mitochondria (Fig. 4, panels A and B) to levels similar to those established following lysis with lauryl maltoside (YRD:b-GFP/b-BFP $R_{513/455\,nm} = 0.68$; ΔE:b-GFP/b-BFP $R_{455/513\,nm} = 0.74$).

DISCUSSION

To date, the evidence suggesting yeast mtATPase complexes associate closely within mitochondria has been derived from results of two independent experimental approaches conducted in vitro. Native PAGE performed on detergent lysates of mitochondria (Arnold *et al*., 2000; Schägger and Pfeiffer, 2000), and cross-linking in mitochondrial membranes (Paumard *et al*., 2002a,b; Spannagel *et al*., 1998). Since mitochondria undergo dramatic rearrangement in morphology and structure after isolation from cells we sought to determine whether mtATPase associates within *intact* cells sampled during respiratory growth by monitoring FRET between fusions with fluorescent proteins assembled into functional complexes.

FRET in intact cells was detected between subunit b-GFP fusion proteins assembled into separate mtATPase complexes. While it is not possible to estimate with precision the distance between subunit b of different complexes, taking into account length of the linker and properties of the fluorescent proteins we calculate the maximum possible separation of subunit C-termini to be ~180 Å. It is likely the actual separation would be significantly less when other considerations, such as steric hinderance around the complex and unfavourable orientation of the fluorophores are taken into account. These estimated distances are consistent with the molecular dimensions of the yeast mtATPase monomer. Single molecule electron microscopy studies (Rubinstein *et al*., 2005; Stock *et al*., 1999) indicate F_1 to be ~110 Å in diameter and stand ~90 Å from the F_0 sector. These results imply the close proximity of mtATPase reported by FRET.

What is the nature of the molecular associations between mtATPase complexes in these cells reported by FRET? Paumard *et al*. (2002b) proposed a speculative model for the stable association of multiple mtATPase complexes into oligomers, mediated through two distinct interfaces (Fig. 5, panel A). The first involves peripheral F_0 subunits e and g, while the second is mediated by subunit b of the stator stalk. In this model the precise position of these two interfaces is not defined. FRET between differently labelled b subunits may take place between complexes in *trans* across the b/b interface as depicted in Fig. 5, panel A, or in *cis* spanning the e/g interface, or a combination of both. Our data show that FRET was eliminated in mitochondria solubilised with digitonin, a treatment shown to preserve the e/g dimeric interface (Arnold *et al*., 2000; Paumard *et al*., 2002b; Schägger and Pfeiffer, 2000). The data upon which the model proposed by Paumard is based, predicts this detergent treatment would disrupt the b/b interface. We conclude that FRET most likely occurs in these cells between subunit b-fusions across the *trans* interface and indicate that in vivo mtATPase can be arranged into supramolecular structures consisting of at least two mtATPase complexes. These results do not indicate whether the entire mtATPase population, or only a subpopulation of mtATPase complexes are involved in these dimeric structures, or whether higher order associations of mtATPase (e.g. tetramers) might be present in mitochondria.

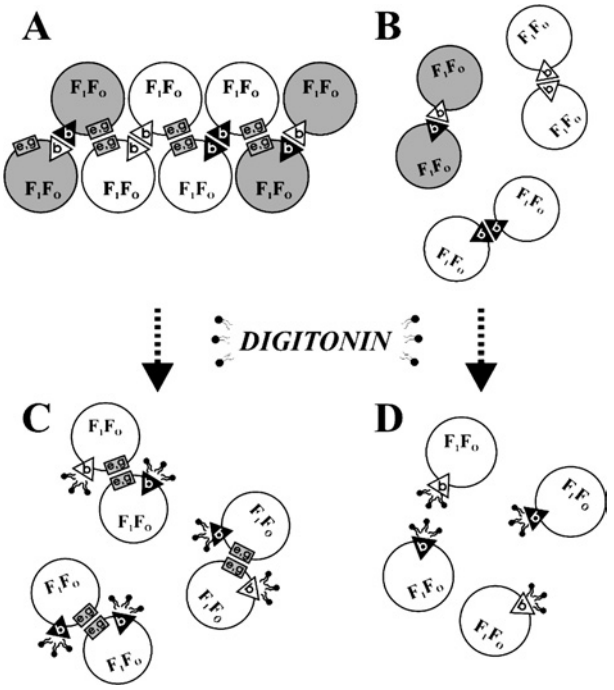

Fig. 5. *Oligomeric associations of mtATPase as monitored by FRET.* (A) Schematic model of mtATPase association based on that proposed by Paumard *et al.* (2002b). The transmembrane domains of subunit b represented by triangles are coloured black or white to represent the subunit b-GFP and b-BFP fusion proteins respectively. Two separate dimeric interfaces are present. One is mediated by subunits e and g and the other by subunit b. FRET is considered to occur in *trans* between immediate subunit b neighbours. Oligomerisation of mtATPase results from the association of multiple dimers. The combination of subunit b fusion proteins are shown within each b/b dimer, with only 50% of complexes theoretically containing the appropriate fusion proteins necessary for FRET (coloured grey). (B) In the absence of subunits e and/or g, mtATPase is unable to oligomerise, however the b/b interface still allows FRET between mtATPase dimers incorporating the correct combination of fusion proteins. (C) Digitonin preserves the e/g interface in solubilised mitochondria while presumably breaking the putative b–b interface and reducing FRET across this *trans* interface. (D) Digitonin disrupts the b/b interface in mitochondria lacking subunits e and/or g, reducing mtATPase to a monomeric state and abolishing FRET. Note that lauryl maltoside disrupts both the e/g and b/b interfaces, reducing mtATPase to a monomeric state in solution.

It can be predicted using the model (Fig. 5; Paumard *et al.*, 2002b) that FRET should occur in isolated oligomers of ATP synthase in which the b/b interface remains intact. Presumably these oligomers would be represented in solubilised complexes comprising three or more monomer mtATPases. Small amounts of such complexes have been observed in gels (Fig. 2, lane 5), (Paumard *et al.*, 2002b). However, analysis of the fluorescence spectra of these oligomers in situ did not provide convincing evidence for FRET (data not shown). Only small alterations in the relative positioning of mtATPase complexes would be required to eliminate FRET, as the phenomenon is highly sensitive to the relative proximity and orientation of the acceptor and donor fluorophores. It is probable the b/b interface is somewhat more labile than the e/g interface as oligomers higher than dimers are recovered in much reduced amounts and only from lysates containing low amounts of digitonin (Fig. 2, lane 5). In a recent study, dimers of mtATPase isolated using digitonin were observed to behave as a doublet on BN-PAGE, suggesting the individual complexes may assume alternative configurations upon isolation (Arselin *et al.*, 2004). Furthermore, our results show FRET efficiency is slightly reduced in cells lacking subunit e (see discussion below). Other proteins present in intact mitochondrial membranes may play a role in 'tuning' mtATPase interactions in vivo. A recent study reports the isolation from mammalian mitochondrial membranes and three-dimensional characterisation by electron microscopy of the 'ATP synthasome,' mtATPase complexed with the adenine nucleotide carrier and phosphate transporter (Chen *et al.*, 2004). Alternatively, the oligomeric species migrating with mobility less than that of dimers on native gels may constitute only a minor proportion of the total mtATPase population in vivo or illegitimate interactions produced upon isolation. These observations reinforce the importance of making studies of mitochondria in vivo, as they suggest the relative orientation of mtATPase complexes isolated within a dimeric/oligomeric arrangement in vitro, differ from the endogenous conformation of complexes within the mitochondrial membrane in vivo.

Our data suggest that close interactions between mtATPase complexes persist in ΔE:b-GFP/b-BFP cells. Since mtATPase dimers cannot be isolated from such cells in the absence of subunit e it might be concluded that the interactions observed to persist in these cells result from a different set of interactions represented perhaps, by those across the b/b interface (Fig. 5). Importantly the nature of the interactions between mtATPase complexes that persist in ΔE:b-GFP/b-BFP cells is not sufficient to support normal cristae structure. Further investigations in yeast using genetic approaches will help to define the nature of the interfaces between the complexes.

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