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# The assembly of yeast mitochondrial ATP synthase: subunit depletion in vivo suggests ordered assembly of the stalk subunits b, OSCP and d

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**Abstract**

The abundance in vivo of each of three subunits b, OSCP and d, components of the stalk region of the yeast mitochondrial ATP synthase complex, was manipulated by a controlled depletion strategy. Western blots of whole cell lysates were used to study the effect of depletion of each of these subunits on the cellular levels of other subunits of the enzyme complex. A hierarchy of subunit stability was determined and interpreted to indicate the order of assembly of these three subunits of the stalk region. Thus, subunit b is assembled first, followed by OSCP and then by subunit d. 

*Keywords:* Mitochondrion; ATP synthase; Stalk region; Assembly; Subunit depletion; (Yeast)

In higher organisms, the primary source of ATP is the mitochondrial ATP synthase (mtATPase). This multi-subunit complex harnesses the energy of the electrochemical proton gradient, produced by the active transfer of protons across the inner mitochondrial membrane resulting from the activity of the respiratory chain complexes, to catalyse the formation of ATP. The catalytic $F_1$ sector of the yeast mtATPase is located on the matrix side of the inner mitochondrial membrane. It is connected to the hydrophobic $F_O$ sector that forms the proton channel through the inner mitochondrial membrane by subunits that form a 'stalk' that is denoted the $F_A$ sector in yeast. This sector, which has been implicated in the coupling of proton passage across the membrane with ATP synthesis, is less well characterised than either the $F_O$ or $F_1$ sectors but is considered to include OSCP, subunit b and subunit d [1].

Investigations of mtATPase assembly have been pursued with the aim of understanding the overall structure of the complex at the level of subunit–subunit contacts and its mechanism of action. The assembly of mtATPase has been recently reviewed [1]. Briefly, the $F_1$ sector is considered as being assembled from newly imported subunits immediately following their import, with the availability of subunit $\beta$ possibly being a rate-limiting factor [2]. $F_1$ assembly can occur in the absence of an assembled $F_O$ sector [3]. The yeast $F_O$ sector subunits have been shown to assemble in the following order: 9, then 8, followed by 6 [4]. The assembly of subunit 6 also appears to

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require the assembly of the stalk sector. In yeast, mutants null for either the *ATP4* or *ATP7* genes (encoding subunits b and d, respectively) have been reported as failing to assemble subunit 6 [5,6]. The limited evidence available to date suggests that the assembly of the stalk components, subunits b, d and OSCP, requires a complete $F_O$ sector. Subunits b and d were not associated with immunoprecipitated mtATPase in a strain devoid of any of the $F_O$ subunits [4,7] (note OSCP, product of the *ATP5* gene, was not monitored in those experiments). In yeast cells lacking OSCP, subunits b, d and 6 all failed to be assembled or to be associated with assembled $F_1$ sectors, as detected by immunoprecipitation [8].

We have sought to extend our understanding of the assembly of the stalk sector of the yeast mtATPase complex. Our strategy has been to use controlled depletion of individual subunits and to measure by immunoblotting the abundance of other stalk subunits, specifically to monitor their retention or loss. It has been established that many subunits of mitochondrial inner membrane complexes are degraded quickly if they are not assembled into the complex, due to a lack of another subunit of that complex [9,10]. It is proposed, therefore, that the reduced stability of mtATPase subunits in depleted cells arises as a consequence of their failure to assemble into mtATPase complexes and their consequent degradation. Thus, the rationale behind the use of the controlled depletion strategy is that stalk subunits which are assembled will be stable and detectable by immunoblotting; conversely subunits which are not assembled will be degraded and not detected. Note that this approach to the analysis of mtATPase assembly does not rely on an ability to isolate complexes or subcomplexes. Such subcomplexes may be sufficiently unstable that they do not retain their structural integrity on isolation from the mitochondrial environment.

To achieve subunit depletion, we have used strains in which a disrupted $F_A$ subunit gene has been replaced by a plasmid-borne gene copy under the control of the GAL1 promoter [8,11]. Thus, strains A4NG, A5NG and A7NG (see legend to Fig. 1) express subunit b, OSCP or subunit d respectively when grown in medium containing galactose. By growing cells in the presence of galactose, then transferring them to galactose-free medium, it was possible to deplete the cells of the $F_A$ subunit under the control of the GAL1 promoter and thus to investigate the effects of depletion on the other $F_A$ subunits (for description of method, see legend to Fig. 1).

Growth curves of the cultures of strains A4NG, A5NG and A7NG propagated in medium lacking galactose (Fig. 1) indicate that the growth rate of cells became much less as the time period of propagation became longer (subsequent to their transfer to galactose-free medium). In the extreme, cultures with relatively small inocula, in which more than four doublings were expected, did not even reach the target $A_{650}$ of 3.0.

Cell cultures were sampled at the time of final harvesting to determine frequencies of petite cells and of galactose-independent cells, by plating on solid YEPE [11], YEPEGal and YEPDEGal (YEPEGal plus 0.1% (w/v) glucose) media. The proportions of each of these types of cells were found to be 7–8% of total cells. The low abundance of petite and galactose-independent cells is expected to have little effect on the presence or absence of a given subunit in the cell cultures sampled for analysis of subunit depletion.

Following harvesting of cells propagated in galactose-free medium for suitable periods, whole cell lysates were prepared. Polypeptides in samples containing equivalent amounts of protein were separated by SDS-PAGE before transfer to nitrocellulose membranes. Membranes were probed with primary antibodies raised against yeast subunits b, d, OSCP and $F_1$-$\alpha$, and binding detected as described in the legend to Fig. 2.

A single lane of each SDS-PAGE gel was loaded with a sample of a standard mtATPase preparation [16]. These lanes (Fig. 2A, B and C, lane 7) indicate that the signal arising from intact fully assembled mtATPase is directly comparable to that generated in lysates of cells not undergoing subunit depletion (Fig. 2A, B and C lane 1). The effect of depletion of each of the three stalk subunits can now be assessed in turn.

OSCP was considerably depleted in A5NG cells after just two doublings in medium lacking galactose (Fig. 2A, cf. lanes 1 and 3). After five doublings in the same medium, the level of OSCP had decreased to the limit of detection (Fig. 2A, lane 6). The depletion of OSCP was associated with a significant reduction in the level of subunit d, with similar

kinetics to those of OSCP, but had little effect on the level of subunit b (Fig. 2A, lanes 1–6). These results suggest that OSCP is required for maintaining the stability of subunit d, but not of subunit b.

The kinetics of depletion of subunit d in A7NG cells were similar to those for OSCP in A5NG cells (see Fig. 2B, lanes 1–6). However, depletion of subunit d did not lead to significant depletion of either subunit b or OSCP in A7NG cells, indicating that the presence of subunit d was not essential for maintaining the stability of these subunits.

The level of subunit b was reduced significantly as the growth of A4NG in medium lacking galactose progressed, but some subunit b was still detectable even in the A4NG cells grown for the longest period on medium lacking galactose (Fig. 2C, lanes 1–6). The growth curves of cells that were expected undergo three or more doublings in the absence of galactose approached a plateau prior to reaching the target $A_{650}$ for harvesting, indicating that cell growth ceased before the cells were completely depleted of subunit b (Fig. 1A). Strikingly, while some subunit b was still detectable in cells putatively depleted of subunit b for five doublings (Fig. 2C, lane 6), OSCP was detected at reduced levels and subunit d could not be detected in A4NG cells (Fig. 2C, lane 6). Note that the level of $F_1$-$\alpha$ was not decreased by the

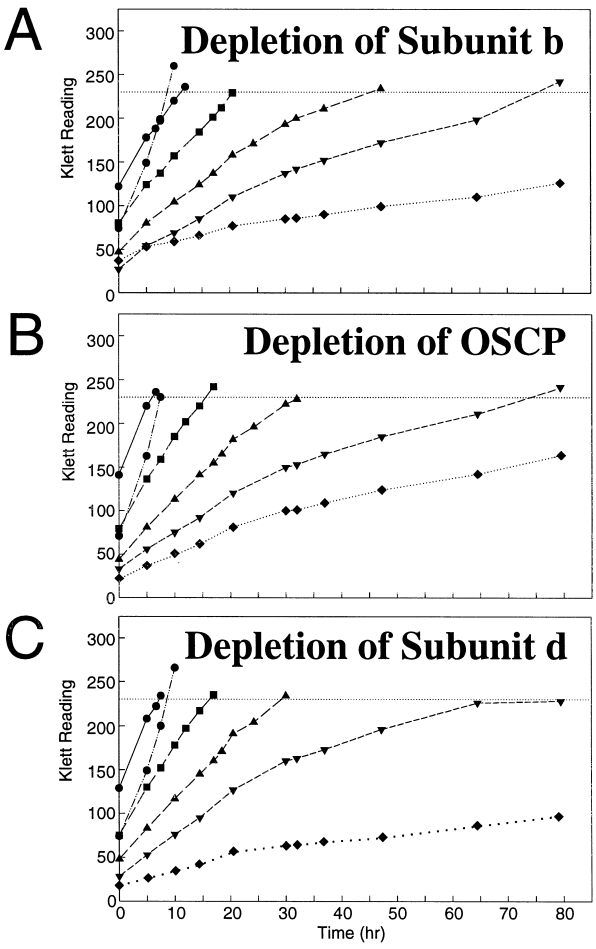


Fig. 1. Propagation of cells grown in medium lacking galactose. Cellular growth curves are represented for strains A4NG, A5NG and A7NG (panels A, B and C, respectively). Cultures were incubated at 28°C with shaking, and contained SaccE medium [11] without galactose unless specified otherwise. Flasks were inoculated at $A_{650}$ as indicated: ●, 0.75 (two doublings) in galactose-containing growth medium, SaccEGal [11]; ●, 1.5 (one doubling); ■, 0.75 (two doublings); ▲, 0.38 (three doublings); ▼, 0.19 (four doublings); ♦, 0.095 (five doublings). Strains: Strain A5NG [8] contains a plasmid-borne copy of the *ATP5* gene under GAL1 transcriptional control. Strains A4NG and A7NG were derived from the wild-type yeast strain YRD15 as follows. First, the chromosomal *ATP4* or *ATP7* gene was disrupted as described previously ([12]; except a 2.2 kb *Xho*I/*Sal*I LEU2 gene fragment [13] was used to disrupt the *ATP4* gene in place of a HIS3 gene fragment) to generate strains designated A4N and A7N, respectively. Second, the yeast expression vector pRJ21 [14] bearing a non-disrupted copy of the *ATP4* or *ATP7* gene was transformed [15] into these strains to form strains designated A4NG and A7NG, respectively. Method: Mid-log phase cells of each strain grown in YEPEGal medium [11] were inoculated into starter-culture flasks containing 100 ml of SaccEGal. Cultures were incubated at 28°C with shaking until an $A_{650}$ of 3.0 was reached. The cells were harvested by centrifugation (4000× *g*, 5 min, 4°C), washed twice in 200 ml of cold (4°C), sterile $dH_2O$ and resuspended in 10 ml of cold, sterile $dH_2O$. The $A_{650}$ of each suspension was then determined and five flasks, each containing 100 ml SaccE were inoculated so as to obtain $A_{650}$ as follows: 1.5, 0.75, 0.38, 0.19, and 0.095. The inocula were chosen such that the cultures would have to go through 1, 2, 3, 4 and 5 doublings respectively to reach a final $A_{650}$ of 3.0, immediately prior to harvesting. A sixth flask, containing SaccEGal medium was inoculated to a starting $A_{650}$ of 0.75; these cells should not be subject to subunit depletion. All cultures were incubated at 28°C with shaking. The $A_{650}$ of each culture was monitored approximately every 4 h using a Klett-Summerson Photoelectric Colorimeter. Upon the culture reaching the equivalent of an $A_{650}$ of 3.0, 5 mg (dry weight) of cells from the culture were snap frozen in a dry ice/ethanol bath for subsequent preparation of a whole cell lysate. For any culture that had not reached an $A_{650}$ of 3.0 after 80 h, the $A_{650}$ of the culture was determined at that time, and a sample harvested for the preparation of a whole cell lysate.

depletion of subunit b, indicating that subunit b is not required for the stabilisation of $F_1$-$\alpha$. Subunit b can be completely depleted by propagation of strain A4NG in chemostatic cultures containing low levels of glucose, sufficient to support cell growth, but below the levels at which carbon catabolite repression occurs. These experiments indicated that the loss of subunit b resulted in the complete loss of both OSCP and subunit d, but not $F_1$-$\alpha$ (data not shown).

On the basis of the observations made above, we conclude that subunit b is required for the continued stability and immuno-detection of both OSCP and subunit d, and that OSCP is required for the continued stability and immuno-detection of subunit d within the cell. These results indicate a hierarchy of stability for the $F_A$ subunits. If the stabilisation of each subunit is ascribed to its assembly into nascent mtATPase complexes or a sub-assembly (see above), then the interpretation is that the order of assembly of the $F_A$ subunits is as follows: subunit b followed by OSCP, and finally subunit d (as depicted in Fig. 3A).

In the series of experiments, that established the order of assembly of the $F_O$ subunits, Hadikusumo et al. [4] were able to isolate, from subunit 6 mutant strains, immunoprecipitates that contained a set of $F_1$ subunits together with the $F_O$ subunits 8 and 9, but which did not show the presence of subunit 6 and the $F_A$ subunits b and d. These immunoprecipitates were generated using an anti-$F_1$-subunit $\beta$ antibody able to recover aggregates of subunits of both $F_1$ and $F_O$ sectors. These results suggest that there are stable associations formed in vivo between the $F_1$ and $F_O$ sectors (as depicted in Fig. 3B) that do not require the $F_A$ subunits or subunit 6.

$F_O$ subunit 6 was not found in chloroform/methanol extracts of whole mitochondria from a strain null for expression of subunit b, but subunits 8 and 9 were recovered in those extracts [5]. Similarly, the absence of subunit 6 from a strain null for subunit d has been reported [6]. We have found subunit 6 to be absent, but subunits 9 and 8 present, in immunoprecipitates of mtATPase made using anti-

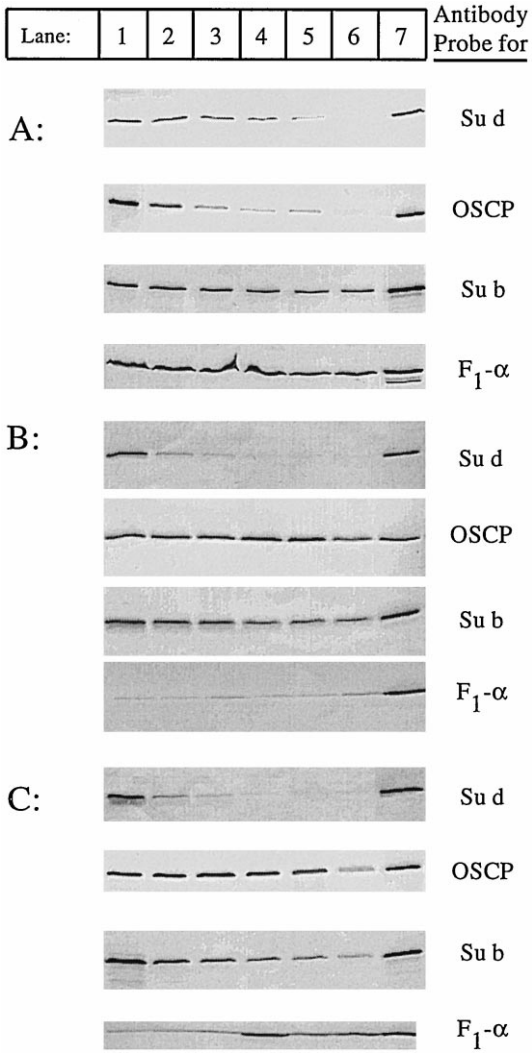


Fig. 2. Western blot analysis of depleted cells. Immuno-blot analyses are depicted of proteins transfered to nitrocellulose membranes from 15% polyacrylamide gels containing 0.1% SDS. The gels were loaded with 3.7 $\mu$g of total protein from the whole cell lysates prepared from cells depleted of the following subunits: OSCP (Panel A); subunit d (Panel B); subunit b (Panel C) for 0, 1, 2, 3, 4, or 5 doublings (lanes 1–6 in each panel, respectively). In each panel, lane 7 was loaded with mtATPase standard [16]. Membranes were probed with primary antibodies as indicated at the left of each strip. Su d: PF18-1 anti-subunit d rabbit polyclonal antibodies [17]; OSCP: PFOSCP-1 anti-OSCP rabbit polyclonal antibodies [17]; Su b: RH66 anti-subunit b mouse monoclonal antibody [5]; F1-$\alpha$: RH51 anti-$F_1$ $\alpha$ subunit mouse monoclonal antibody [5]. Methods: Whole cell lysates were prepared essentially as described by [18]. Proteins were subjected to SDS-PAGE and transfered to membranes using standard procedures as described previously [17]. Primary antibodies used are indicated above. Secondary antibodies were alkaline phosphatase conjugated anti-mouse or anti-rabbit IgG (Silenus, Hawthorn, Victoria, Australia). Signals were generated with bromochloro-indolyl/nitroblue tetrazolium.

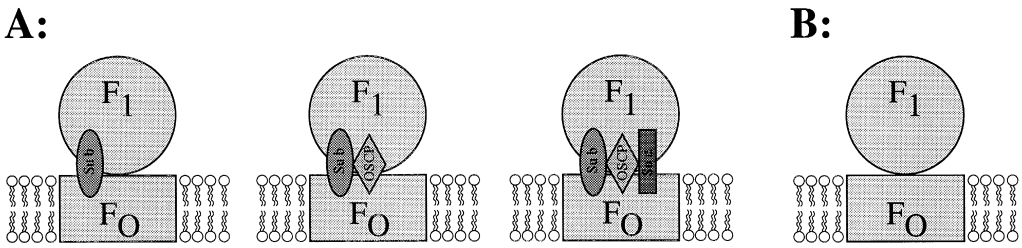


Fig. 3. Proposed assembly order of $F_A$ subunits. The proposed order of assembly of the $F_A$ subunits of the yeast mtATPase is depicted (panel A). Stable associations between the $F_1$ and $F_O$ sectors that do not require the $F_A$ subunits or $F_O$ subunit 6 may form in vivo (panel B; see text for discussion). The figure is not intended to represent the topology of the subunits or complex, nor does it depict any specific subunit–subunit interactions. The relative positions of subunits b, OSCP and d are drawn arbitrarily. Note that while the $F_1$ and $F_O$ sectors are represented as single units, the exact subunit composition of these sectors at various stages of the assembly of the $F_A$ subunits is not yet known. $F_1$: part or all of the mtATPase $F_1$ sector; $F_O$: part or all of mtATPase $F_O$ sector. Su b: subunit b; OSCP: oligomycin sensitivity conferring protein; Su d: subunit d.

$F_1$-$\alpha$ subunit antibodies from mitochondrial lysates of strain A5NG grown in the absence of galactose (and thus lacking OSCP) [8]. Thus, in cells that lack any one of the stalk subunits, b, OSCP or d, subunit 6 is not detected. Two conclusions can be reached. First, subunit 6 is required for the stabilisation (and thereby the continued detection) of these three $F_A$ subunits. Second, the assembly of each of subunits b, OSCP and d is required for the stabilisation of subunit 6. Note that this apparent co-dependence for assembly does not resolve the issue of whether subunit 6 is assembled into the nascent mtATPase before, during or after the assembly of the stalk subunits. Future studies using antibodies raised against subunit 6, at present unavailable, may identify whether this subunit is present in cells depleted of individual stalk subunits. Such experiments would, in turn, shed light on the integration of the $F_O$ sector and stalk pathways of assembly.

Collinson et al. [19] and Walker and Collinson [20] used an in vitro reconstitution approach to study the assembly with $F_1$ of stalk subunits of bovine mtATPase. Their data suggested that subunits b, $F_6$ and d interact with $F_1$ through OSCP, because these three subunits were unable to assemble with $F_1$ in the absence of OSCP. Subunits $F_6$ and d were assessed as interacting primarily with subunit b, rather than each other. Other components of the bovine stalk sector, subunits e, f and g, were not investigated in these studies. However, the work of Belogrudov et al. [21] suggests that each of these three small subunits has an inner mitochondrial membrane domain and therefore might be considered components of $F_O$ rather than the stalk.

Recently, putative yeast homologues of subunits e [22], f [23] and g [24] have been identified. In addition, a new subunit, designated subunit h, has been identified in yeast [25] but has not yet been reported to occur in the bovine mtATPase complex. The role of these subunits in the assembly of subunits b, d and OSCP, and of the complex as a whole, remains to be identified. Interestingly, subunit e has been reported to be identical to Tim11, a protein proposed to be involved in the sorting of proteins to the inner mitochondrial membrane [22]. A clear understanding of the subunit composition of the functional mtATPase complex will be essential for developing a conclusive understanding of its assembly.

The generally accepted view of the stalk as a group of subunits, including subunits b, OSCP and d, located at the inner interface of $F_1$ and $F_O$ has now been challenged. A recent radical structural model proposed by Engelbrecht and Junge [26] for the *Escherichia coli* ATP synthase complex, based on data from the bacterial, chloroplast and mitochondrial enzyme complexes, suggests there might be two structurally distinct segments of the stalk connecting $F_1$ and $F_O$. One of these is suggested to comprise subunits b and $\delta$ (OSCP homologue in *E. coli*) with subunit a (subunit 6) of $F_O$, at the outer surface of the complex. In this model, subunit $\delta$ is located near the top of the $\alpha_3$–$\beta_3$ hexagon of $F_1$ and the two subunits b extend from their membrane anchor to interact with the C-terminal domain of $\delta$. This view of the *E. coli*

complex is directly translatable to the mitochondrial complex (although it must be noted that the stoichiometry of subunit b differs in the two systems; two copies in bacteria compared with one in mitochondrial complexes [both bovine [27] and yeast (Bateson, M., Nagley, P., Prescott, M. and Devenish, R.J., unpublished data)]. Our results, which suggest an order of assembly for the b, OSCP and d subunits of yeast, are compatible with both the more traditional and the new radical view of the organisation of the stalk. Subunit b would play a central role in assembly, presumably on account of its membrane anchor and its interaction with $F_O$ subunit 6. Here, we have shown the presence of subunit b is required for the assembly and stability of subunits OSCP and d. As subunit d was lost when either b or OSCP was depleted it is possible that, even if subunit d is not directly associated with either or both of b and OSCP, the structure formed by their interaction is key to the assembly and stability of other stalk subunits including d. Indeed, it is conceivable that subunit d in association with $F_O$ subunit 8 (not present in bacterial ATP synthase) comprises the functional homologue of the second subunit b that is absent from mtATPase complexes.

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