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Review

Insights into ATP synthase assembly and function through the molecular genetic manipulation of subunits of the yeast mitochondrial enzyme complex

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Abstract

Development of an increasingly detailed understanding of the eucaryotic mitochondrial ATP synthase requires a detailed knowledge of the stoichiometry, structure and function of F_0 sector subunits in the contexts of the proton channel and the stator stalk. Still to be resolved are the precise locations and roles of other supernumerary subunits present in mitochondrial ATP synthase complexes, but not found in the bacterial or chloroplast enzymes. The highly developed system of molecular genetic manipulation available in the yeast *Saccharomyces cerevisiae*, a unicellular eucaryote, permits testing for gene function based on the effects of gene disruption or deletion. In addition, the genes encoding ATP synthase subunits can be manipulated to introduce specific amino acids at desired positions within a subunit, or to add epitope or affinity tags at the C-terminus, enabling questions of stoichiometry, structure and function to be addressed. Newly emerging technologies, such as fusions of subunits with GFP are being applied to probe the dynamic interactions within mitochondrial ATP synthase, between ATP synthase complexes, and between ATP synthase and other mitochondrial enzyme complexes.

Keywords: Yeast mitochondrial ATP synthase; F_0 subunit organization; Proton channel; Stator stalk; Stoichiometry; Green fluorescent protein fusion

1. Introduction

Recent advances in our understanding of the F_1F_0-ATP synthase complex have led to its being described as a 'remarkable molecular machine' [1]. Important progress has been made concerning the molecular structure of F_1 and the rotatory mechanism of ATP synthesis. A largely complete crystal structure of bovine F_1 $\alpha_3\beta_3\gamma$ has been solved by Abrahams et al. [2]. Moreover, the rotation of bacterial subunit γ and chloroplast subunit ε, each of which has a mitochondrial homologue (subunit γ and δ, respectively), inside the $\alpha_3\beta_3$ hexamer during catalysis has been unequivocally demonstrated [3,4]. Notwithstanding these advances, much remains to be discovered about the molecular structure and contribution to the mechanism of the remainder of the complex. The 'classical' view of ATP synthase has traditionally been of the catalytic F_1 sector connected by a central stalk to the membrane F_0 sector. Although this basic structural arrangement is main-

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tained for bacterial, chloroplast and mitochondrial complexes, it is now recognised that there exists a second 'stator' stalk that links F_1 with the membrane [5–7]. Furthermore, an increasingly large number of subunits supernumerary to those found in bacteria and chloroplasts (five F_1 subunits and three F_0 subunits; see below) are being identified in mitochondrial ATP synthase (mtATPase) complexes. Thus, the next phase of understanding ATP synthase requires a detailed knowledge of the stoichiometry, structure and function of F_0 sector subunits in the following contexts: the proton channel, the stator stalk, and the precise location and role of the supernumerary subunits present in mtATPase complexes.

The yeast, *Saccharomyces cerevisiae*, provides an attractive system for the analysis of mtATPase. The highly developed system of molecular genetic manipulation available in this unicellular eucaryote permits the genes for subunits of ATP synthase to be manipulated and questions of stoichiometry, structure and function to be addressed. Indeed, it is in the yeast system that the largest number of supernumerary subunits has been identified to date. This review focuses on recent advances on understanding F_0 gained by study of yeast mtATPase.

2. Classification of F_0 subunits in mtATPase

F_1 has long been defined as consisting of the five subunits denoted α, β, γ, δ and ε in bacteria, chloroplasts and mitochondria. Although Nagley [8] introduced the classification F_A for yeast mtATPase, to encompass non-F_1 subunits that were not integral membrane proteins, it is now more generally considered that the non-F_1 subunits in mtATPase are all classified as belonging to the F_0 sector irrespective of the extent of their association with the membrane. As indicated above, the F_0 sector is more complex in eucaryotic mtATPase than in bacteria. In yeast, the F_0 sector is composed of at least nine polypeptides which are nucleus-encoded: b, OSCP, d, e, f, g, h, i/j (the nomenclature i/j is used to indicate that the same subunit has been denoted i by Vaillier et al. [9] and j by Arnold et al. [10]) and k, together with three subunits, 6, 8 and 9, which are mitochondrion-encoded (Table 1). Of this large F_0 subunit complement, only three, subunits b, 6 and 9, have homologues in the bacterial enzyme (denoted b, a and c, respectively; Table 1). Homologues of some, but not all, the yeast proteins have been described for bovine mtATPase including subunits 8 (denoted A6L), b, OSCP, d, e, f and g. The bovine enzyme also includes subunit F_6 for which no homologue has been found in yeast, bacteria or chloroplasts (Table 1).

In the bacterial complex, the three F_0 subunits a, b and c are all are membrane-associated. In yeast mtATPase, a membrane location has been proposed for all of the numerous F_0 subunits that have been characterised, except for OSCP, d and k. It is more informative, however, to consider these subunits, where possible, in terms of function (either established or postulated). Thus, in Table 1, subunits are grouped as follows.

(a) *Proton channel*. This group comprises the membrane integral proteolipids subunits 6 and 9. Subunits 6 and 9 (homologues of bacterial subunits a and c, respectively) have long been considered to be components of the proton channel [8] and the evidence will be only briefly reviewed here.

(b) *Unassigned requirement for function*. This group includes seven subunits and these are discussed as follows:

(1) Subunit 8, like subunits 6 and 9, is a proteolipid, integral membrane protein. The application of allotopic expression [29] has permitted a unique study of this mitochondrion-encoded subunit aimed at elucidating its functional role, and its topology in the inner mitochondrial membrane.

(2) Subunits b, OSCP and d. These subunits are proposed as components of the stator stalk in yeast [30] based on analogies drawn with the bacterial system together with consideration of differences in F_0 subunit stoichiometry and complexity between bacteria and yeast. Note that this classification would suggest that subunit δ of bacteria and chloroplasts, now shown to be a component of the stator stalk (see Section 4.3) raises the question as to whether this subunit ought to be continued to be classified as belonging to the catalytic F_1 sector in these organisms.

(3) Subunits f [17] and h [18] have been demonstrated by gene disruption to be essential for assembly and function of the complex. The situation is not clear for subunit i/j with two conflicting reports presented to date (see below). At present, there is insuf-

Table 1
F_0 subunit composition, genetic specification and stoichiometry of yeast mitochondrial ATP synthase

	Yeast	Yeast gene[a]	*E. coli* homologue	Bovine homologue	Stoichiometry[b]	Subunit size (aa residues)[c]	Apparent mass (kDa)[d]	Actual mass (kDa)[d]
Proton channel	6	$ATP6^M$ (*oli2*)	Subunit a	Subunit 6	1	249	20	27.9
	9	$ATP9^M$ (*oli1*)	Subunit c	Subunit 9	12	76	3.5	7.8
Unassigned requirement for function								
Subunit 8	8	$ATP8^M$ (*aap1*)	–	A6L	1	48	5.8	5.9
Stator stalk	b (4)	*ATP4*	Subunit b	Subunit b	1	209	25	23.3
	OSCP (5)	*ATP5*	δ[e]	OSCP	1	195	20	20.9
	d (7)	*ATP7*	–	Subunit d	1	173	18	19.7
Subunits f, h, i/j	f	*ATP17*	–	Subunit f	1	95	12	10.6
	h	*ATP14*	–	?	?	92	15	9.5
	i/j	*ATP18*	–	?	?	59	10	6.7
Inter-complex	e	*ATP21/TIM11*	–	Subunit e	2 per dimer	96	12	10.9
Interactions	g	*ATP20*	–	Subunit g	2 per dimer	115	11	12.8
	k	*ATP19*	–	?	?	68	?	7.5
Inhibitor complex	Inh1p	*INH1*	–	INH	1	63	10	7.4
	9 kDa	*STF1*	–	–	1	63	9	7.3
	15 kDa	*STF2*	–	–	1	84	15	9.5
(Mammalian)	–	–	–	F_6	2	77	9	8.9

Subunits are groups according to current understanding of function. Homologues present in *E. coli* or the bovine complexes are indicated; the single subunit present in the bovine complex without a homologue in yeast or bacteria is also listed.

[a]Genes located in the mitochondrial genome are designated by '$_M$'. References for the individual genes are: *ATP6* (*oli2*), [11]; *ATP8* (*aap1*), [12]; *ATP9* (*oli1*), [13]; *ATP4*, [14]; *ATP5*, [15]; *ATP7*, [16]; *ATP17*, [17]; *ATP14*, [18]; *ATP18*, [9,10]; *ATP21/TIM11*, [19,20]; *ATP20*, [21–23]; *ATP19*, [22]; *INH1*, [24]; *STF1*, [25]; *STF2*, [26]; F_6, [27].

[b]After Cox et al. [28] plus references cited here.

[c]The size of the mature yeast subunit is given (in amino acid residues). Nearly all nucleus-encoded subunits are synthesised in the cytosol as precursors that include an N-terminal targeting sequence, that is removed by proteolytic cleavage after import into the mitochondria. Exceptions here are subunits d, i/j and the 15-kDa protein, which are believed to contain internal, non-cleavable targeting sequences. However, subunit d and the 15-kDa protein are processed to remove the N-terminal methionine residue.

[d]Subunit masses shown indicate apparent mass as determined by SDS-PAGE analysis, as well as the actual size as determined from nucleic acid or amino acid sequences.

[e]Subunit δ has historically been classified as a component of the catalytic F_1 sector (see note on this point in the text under b(2) in Section 2

ficient information to suggest the function of any of these subunits in mtATPase.

(c) *Inter-complex interactions*. This group represents a new class of subunits including subunits e, g and k. Studies by Stuart and colleagues [22] suggest all three subunits are involved in formation and stability of mtATPase dimers. It is noteworthy that subunits in this group do not satisfy the traditional definition of a mtATPase subunit since upon disruption of the relevant gene, cells do not have the 'classical' phenotype of respiratory deficiency on non-fermentable growth substrates. Moreover, our studies implicate subunit g in interactions between mtATPase and cytochrome *c* oxidase [23].

(d) *Inhibitor complex*. This group includes three proteins, Inh1p, Stf1p (9-kDa stabilising factor) and Stf2p (15-kDa stabilising factor). These proteins have been reviewed elsewhere [31] and are not considered further here.

3. The proton channel subunits 6 and 9

3.1. Subunit 6

Yeast subunit 6 (Y6) is the largest of the three mitochondrially encoded and synthesised proteolipids. The primary translation product of 259 amino acid residues is post-translationally cleaved after residue Thr-10 [32]. Analysis of hydropathy plots and structural predictions of Y6 have suggested the presence of at least five hydrophobic α-helices believed to be transmembrane spanning regions [8]. Similar predictions were made for the bacterial homologue of Y6, subunit a [28], and subsequently explicitly demonstrated [33,34], although other studies have presented evidence for four to eight transmembrane stems [35–37]. For the remainder of this discussion, Y6 will be considered as a protein having five transmembrane domains. In this case, the topology for Y6 would be such that the N-terminus protrudes into the inner membrane space and the C-terminus into the matrix. Experimental support for this topology is limited, but is consistent with the substitution by Lys of the C-terminal residue Thr-248 (in a naturally occurring mutant) having been shown to interfere with the coupling of mtATPase proton translocation to ATP synthesis [38]. Presumably an interaction with one or more residues from F_1 proteins is perturbed. More recently, crosslinking experiments have shown a disulfide bridge can be formed between a cysteine residue introduced at residue 54 of subunit b and Cys-33 of Y6 [39].

Two helices of the five transmembrane helices of Y6, designated h4 and h5, are well conserved across species and contain those residues considered to be involved in proton translocation as shown by detailed studies on bacterial subunit a (for reviews see [8,36,40]). Thus, residue Arg-186 of Y6 (R210 in subunit a) is likely to play a role in H^+ translocation. Other residues predicted to be involved in the process of H^+ translocation include His-195 (Glu-219 in subunit a) and Glu-233 (His-245 in subunit a) which are in close proximity being located on h4 and h5, respectively. Amino acid substitutions occurring in these regions of Y6 led to cells having an oligomycin-resistant phenotype. This phenotype of the Y6 mutants suggests a functional interaction between the C-terminal region (helices 4 and 5) of Y6 and the two transmembrane domains of Y9 (see [8]), such that the oligomycin binding site encompasses both subunits 9 and 6.

Mutant yeast strains specifically lacking expression of Y6 fail to assemble subunits b and d [38], in terms of achieving stable association with a fully assembled F_1. There is a need to re-assess the effect of the specific loss of Y6 on other F_0 subunits in the light of the recent proliferation of reports about additional subunits. It seems clear that Y6 occupies a sensitive position in relation to the assembly of other subunits within the F_0 sector. In a mutant yeast strain expressing truncated Y6 (40 native residues plus 17 generated by reading frame shift downstream of a base insertion in the mitochondrial ATP6 gene), subunit b is assembled, suggesting that at least the transmembrane domain helix 1 of Y6 may be required as a nucleation site for assembly of F_0 subunits [41]. Interestingly, mtATPase prepared from strains lacking one of several subunits (OSCP, b, d, f, h and j) also lack Y6 [9,14,16–18,42,43] supporting an interdependence of the interactions of Y6 with several other subunits in F_0 assembly.

3.2. Subunit 9

Subunit 9, also known as the DCCD-binding pro-

teolipid, has a primary sequence that is well conserved across species and includes a number of invariant residues (see [8]). This subunit has long been recognised to have a hairpin topology, with the transmembrane domains (residues 11–29 (h1), and 53–71 (h2)) separated by a small loop of polar residues that protrudes into the mitochondrial matrix [8,28,44,45]. Structural analysis by NMR spectroscopy supports the hairpin model [46]. Recent work strongly supports a stoichiometry of 12 for subunit 9, with the subunit monomers arranged in a ring [47,48]. Rotation of this ring with respect to subunit 6 is proposed as a key element of models of proton channel function proposed by several research groups in the field. In the earliest model of rotational catalysis subunit a (6) was envisaged to rotate within the oligomeric ring of subunit c (9) [49,50], but subunit a (6) is now envisaged as located outside of the subunit c (9) ring [51–55].

Functional domains of Y9 were investigated through analysis of a series of strains carrying naturally occurring mutations in the mitochondrial gene for this subunit. For example, substitution at Gly-18 led to failure of Y9 to assemble, while substitution at Gly-23 resulted in defective function of mtATPase that was otherwise assembled properly (see [8,56]). Mutant strains having defective assembly of Y9 also showed a failure of both subunits 6 and 8 to assemble [38] and suggest an important role of Y9 in assembly of the F_0 sector. These same strains also showed pleiotropic effects in the assembly of cytochrome *c* oxidase, implying the assembly of F_0 sector may also be important for other mitochondrial respiratory complexes. Study of a strain having the substitution Arg-39→Met and two revertants showed the absolute requirement for a positive charge in the polar loop between the two transmembrane domains, h1 and h2 [8]. There is evidence in support of interaction between the loop of subunit c in bacteria and chloroplasts with F_1 (see [57]).

The analysis of yeast strains that are resistant to inhibitors that block proton flow through the enzyme complex (such as oligomycin, venturicidin and TET-Br) due to mutations in mtDNA, enabled identification of regions in subunit 9 implicated in proton translocation. Thus, Gly-23 in h1, a cluster of amino acids in h2 (Leu-53, Ala-56, Leu-57, Phe-64, Leu-65) and a single residue in the polar loop between the two transmembrane domains (Ser-42) were, on this basis, suggested to be involved in binding of oligomycin and/or venturicidin (see [8,58]). The bacterial equivalent of Glu-59 has been identified as the H^+-binding site in F_0 and is considered to undergo protonation–deprotonation as each H^+ is transported [59,60]. The identification of domains in Y6 that influence oligomycin resistance (see above) is consistent with the view that proton translocation through F_0 requires the concerted action of Y9 (subunit c) and Y6 (subunit a).

4. Subunits having unassigned requirement for function

4.1. Subunit 8

Subunit 8 (Y8), the third of the mitochondrially encoded subunits of the yeast F_0 sector, is unique to mtATPase of fungi and mammals (denoted A6L in the latter). It was suggested by the study of naturally occurring mutants that this subunit has an essential structural role in mtATPase, being required for the assembly of a functional F_0 sector [61]. However, evidence for a specific role in function of the complex has proved harder to establish. One approach to probing function was taken by Grandier-Vazeille et al. [62] who studied the effects of binding of polyclonal antibodies specific for Y8 on mtATPase activity in isolated mitochondria. Anti-subunit 8 antibodies enhanced respiratory control and stimulated the ATP synthesis rate resulting in an increase in the ATP/O ratio without modification of proton permeability of the membrane. Antibody binding was considered to have modified the conformation of the F_0 sector and, in turn, that of the F_1 sector. On this basis, it was proposed that, in addition to its role in assembly of mtATPase, Y8 may have a negative regulatory role in oxidative phosphorylation [62].

We introduced the technique of allotopic expression [29] to study the structure/function of Y8, whereby an artificial gene encoding Y8 is expressed in the nucleus of cells lacking a functional version of the corresponding mitochondrial gene, to generate assembly of a functional mtATPase complex. Such cells expressing an essentially unmodified version of

Y8 have ATP synthesis and hydrolysis rates, as well as efficiency of oxidative phosphorylation, very similar to those of the parental wild-type strain in which Y8 is naturally expressed in mitochondria [63]. Allotopic expression has provided the opportunity to generate mutants of this subunit by site-directed mutagenesis of the artificial nuclear gene. The functional consequences of expression of variants of Y8 can thus be assessed in order to probe the relation between structure and function, and other aspects of Y8 topology and subunit interactions.

To this end, many variants have been allotopically expressed in cells lacking endogenous Y8. From such studies, the positively charged C-terminal region has been shown to be required for assembly of Y8 into the F_0 sector [64–66]. The central hydrophobic domain (CHD) of Y8, residues 14–32, considered to represent a transmembrane domain, is generally able to accommodate charged residues without grossly impairing the assembly and function of Y8 in mtATPase [63,67–69]. The functional expression in yeast of subunit 8 from *Aspergillus nidulans* [70], which has only about 50% sequence homology, but shares the presence of CHD and C-terminal charged residues with Y8, confirmed that the conserved structural motifs of subunit 8 are important for function rather than particular amino acid residues.

As part of these studies, we investigated the requirement for the CHD of Y8 to retain its hydrophobic character by introducing adjacent negative charges at different positions [68]. One such variant, Leu-23→Asp, Leu-24→Asp, did give rise to cells which displayed structural and functional alterations in mtATPase [63]. Such cells were only able to grow very slowly by oxidative phosphorylation. Measurement of bioenergetic parameters showed major defects in these cells relative to control cells that allotopically expressed unmodified Y8. The overall activity of the complex was significantly decreased and ATP synthesis and state 3 of respiration were reduced by about 30–40%. Moreover, the physical coupling between the two sectors of the enzyme, as well as the stability of the F_1 sector itself, were affected as shown by decreased recovery of F_0 (8, 9, b, OSCP, d, h and f) and F_1 (α, β, γ) sector subunits in immunoprecipitates that collected mtATPase from mitochondrial lysates. Support for a physical coupling defect was provided by the reduction by about 30% of ATP hydrolysis which was almost insensitive to the F_0 inhibitor oligomycin [63]. The defects we observed are not specific for negative charges at positions 23 and 24 of Y8. Indeed, cells expressing a Y8 variant having substitution of both residues Leu-23 and Leu-24 by arginines display similar bioenergetic defects at the whole cell level (I.M. Artika, X. Roucou, P. Nagley, R.J. Devenish, unpublished data). The structural alterations of mtATPase may not result from a direct effect of the double aspartate substitution within Y8, but may be mediated by one or more other subunits proposed to be involved in the physical linking of F_0 to F_1. One possibility is subunit d since in mammalian mitochondria, A6L (bovine homologue of Y8) can be crosslinked to subunit d [71].

Our conclusion is that Y8 not only performs an important role in the structure and assembly of mtATPase, but also in determining its activity (as suggested above in relation to the findings of Grandier-Vazeille et al. [62]). In particular, the hydrophobic character of amino acids 23 and 24 in the middle of the putative transmembrane domain of Y8 is essential for coupling proton transport through F_0 to ATP synthesis on F_1. Thus, Y8 may take part in conformational changes that occur between the F_0 and the F_1 sectors of the enzyme during catalysis. The potential for Y8 to undergo dynamic movements (possibly in concert with other F_0 subunits) during proton translocation coupled to ATP synthesis warrants further investigation. Such movement may have influence on the rotation of the subunit 9 ring and on the stator stalk (see below).

4.2. Topology of Y8

Our findings have indicated that Y8 mediates key molecular interactions within the complex that are essential not only for the structure, but also for the activity of the mitochondrial complex. To understand this in more detail, one needs to know the topology of Y8 in the enzyme complex. Based on hydropathy plots, the suggestion has been made that the CHD of Y8 represents a transmembrane domain [8,72]. The C-terminus of Y8 was localised to the matrix by labelling with an affinity reagent which specifically bound primary amines of hydrophilic protein domains of integral membrane proteins

exposed in a preparation of inside-out inner membrane vesicles. The reagent labelled only the ε-amino group of Lys-47, this residue having the only free amino group in Y8 (since the N-terminal Met residue is blocked by *N*-formylation [73]). Antibody binding experiments with mammalian mtATPase [74,75] provided evidence in support of this location for the C-terminus of subunit 8, but did not enable definitive identification of the location of the N-terminus.

In order to resolve the uncertainty about the topology of Y8, we have inserted cysteine residues by PCR site-directed mutagenesis at the N- and C-termini of Y8 (A. Stephens, X. Roucou, P. Nagley, R.J. Devenish, unpublished results). Using the fluorescent sulfhydryl reagent F5M (fluorescein 5-maleimide, non-permeant to lipid bilayer membranes) the N-terminal cysteine of modified Y8 assembled into mtATPase could be labelled in preparations of intact mitochondria and, therefore, is accessible from the aqueous solvent. This indicated that the N-terminus of Y8 is located in the intermembrane space. Conversely, the C-terminal cysteine could be labelled by F5M only in lysed mitochondria, but in intact mitochondria could be labelled with fluorescent CPM (7-diethylamino-3-(4′-maleimidylphenyl)-4-methylcoumarin, permeant to the lipid bilayer). Moreover, in intact mitochondria, mersalyl (highly non-permeant to the bilayer) efficiently suppressed F5M labelling of the N-terminus of Y8 assembled into mtATPase. In lysed mitochondria, mersalyl suppressed labelling by both reagents at both the N- and C-termini of Y8. These data clearly indicate Y8 to have a transmembrane domain, with the C-terminus accessible from the matrix. Penetrating questions can now be posed in the context of a possible dynamic role for Y8 in F_0, given its transmembrane topology in the native state and its ability to tolerate charged residues within the transmembrane domain. For example, when expressed as a variant containing charged residues in the transmembrane domain, must the protein obligatorily maintain a transmembrane topology or can it adopt another functional topology with respect to the inner membrane?

4.3. The putative stator stalk subunits

Recent studies have demonstrated that one element of the mechanism driving ATP synthesis is the rotation of the γ and ε (mitochondrial homologue δ) subunits in the F_1 sector of the complex [3,4]. The coupling process is now thought to involve a ring of subunit 9 (c) monomers rotating relative to subunit 6 (a). Thus, a stator stalk is proposed [76] to prevent the futile rotation of the $\alpha_3\beta_3$ hexamer relative to the remainder of the complex (Fig. 1). A second stalk has been visualised in *Escherichia coli* [5] and evidence has been presented for a stator stalk of comprised of subunits b and δ [77,78]. Conservation of the general mechanism of ATP synthesis between the F_1F_0-ATPases would suggest that a stator of similar structure exists in mitochondrial enzymes. Indeed, second stalks visualised by electron microscopy have been reported for the chloroplast and bovine mitochondrial enzymes [6,7]. In eucaryotes, subunits b and OSCP, which are homologues of the bacterial subunits b and δ respectively, would be prime candidates for components of a stator stalk in mtATPase. The composition of the stator in higher organisms may be more complex than that in bacteria because mtATPase contains additional subunits that do not have any bacterial homologue.

In order to have an understanding of the structure and function of this stator stalk in the eucaryotic mtATPase, it is important to know the identity and number of each subunit within the enzyme complex. However, the stoichiometry and composition of several subunits in mtATPase enzymes remains ill-defined. It is generally agreed that bacterial ATP synthase contains two identical copies of subunit b per complex, and the corresponding chloroplast enzyme contains one copy each of two non-identical but homologous subunits, b and b′ (I and II). By contrast, in the mammalian system, the stoichiometry of subunit b and other possible stator stalk components such as OSCP, d and F_6 varies according to the report. Collinson et al. [79] determined the molar ratio of b:OSCP:d:F_6 in the bovine system to be 1:1:1:1, while Hekman et al. [75] proposed a stoichiometry of 2:1:1:2 for the same group of subunits. Some support for a stoichiometry of 2 for subunit b comes from the earlier studies of Lippe et al. [80] in the bovine enzyme. A stoichiometry of 1 for OSCP reported by Collinson et al. and Hekman et al. contradicts the earlier report of Penin et al. [81] who proposed a stoichiometry of 2 for OSCP in the porcine enzyme.

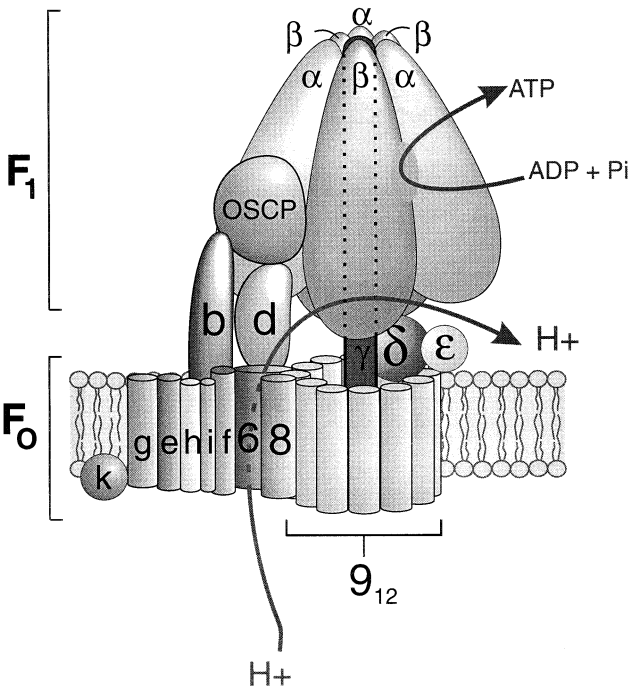

Fig. 1. Schematic representation of yeast mitochondrial ATP synthase. A schematic representation of yeast mtATPase including the more recently identified subunits is shown (see Table 1 for details of subunit composition). Two stalks connecting F_1 with F_0 are depicted; the central stalk is comprised of subunits γ, δ and ε, while the stator stalk (depicted at the left) is proposed to comprise OSCP together with subunit b and d. Subunits b, d, OSCP, e, f, h and i/j are depicted in positions indicated by the results of crosslinking experiments (both in the yeast and bovine systems), as well as on the basis of effects on other subunits, especially subunit 6, observed following disruption of individual subunit genes (see text). Subunit k, one of three subunits proposed to be involved in formation of the ATP synthase dimer (the others being subunits e and g) is depicted as a membrane peripheral protein localised on the inner membrane space surface of the inner membrane in close proximity to subunits e and g. The components of the inhibitor complex, subunits Inh1p, 9 kDa and 15 kDa, have been excluded from the figure for clarity. Protons are depicted as passing through the inner mitochondrial membrane (narrow arrow; see text for details of the proton channel) to reach the F_1 sector. ATP synthesis is indicated as occurring at the interface of the α and β subunits (see [1]).

We have recently resolved the question of the stoichiometry of yeast subunits b, OSCP and d using Ni-NTA affinity chromatography by which intact mtATPase complexes incorporating a hexahistidine (h6)-tagged subunit can be isolated [82]. Strains were constructed in which h6-tagged versions of each of the three subunits were co-expressed with the corresponding wild-type subunit resulting in a mixed population of mtATPase complexes containing untagged wild-type and h6-tagged subunits. The stoichiometry of each subunit was then assessed by determining whether or not untagged wild-type subunit could be recovered from Ni-NTA purifications as an integral component of those complexes absorbed by virtue of the h6-tagged subunit. As only the h6-tagged subunit was recovered from such purifications, it was concluded that the stoichiometry of subunits d, OSCP and b, in yeast, is one in each case [30]. Independently, by means of radiochemical labelling of cysteinyl groups, the stoichiometry of subunit b in yeast was shown to be 1 [83].

In *E. coli*, the stoichiometry of subunit b and the δ subunit (OSCP homologue) is well established as $b_2\delta$ [84,85]. The N-terminal portion of subunit b is anchored in the membrane; however, the remainder of the protein is considered to form an α-helical segment which extends away from the membrane and interacts with the F_1 sector [86]. Chemical crosslinking experiments suggest that the C-terminal portion of subunit b interacts with the δ subunit, which in turn interacts with subunits of the F_1 sector [77,87]. An equivalent interaction between subunits b and OSCP in yeast has been demonstrated using two hybrid analysis [39].

The stoichiometry of one for subunit b in yeast contrasts with the two copies of this subunit in the bacterial enzyme. This difference in stoichiometry means that in mtATPase either another subunit fulfills the role of the second copy of b or that the stator stalk has a different composition to that found in the bacterial enzyme. It should be noted that considerable (approximately 30%) homology has been reported between bovine OSCP and the hydrophilic C-terminal region of the bacterial δ subunit [88]. Thus, OSCP in the eucaryotic complex may fulfill the role of both the second b subunit and δ.

OSCP is known to have important structural links to the proton channel portion of F_0. On the other hand, specific functional links between OSCP and the proton channel have been suggested, but not unequivocally demonstrated. For example, expression in yeast of an OSCP variant having the substitution Gly-166→Asp results in an open proton channel, or proton leak through F_0 under state 4 respiration conditions (that is exquisitely sensitive to very low

concentrations of oligomycin), but normal proton channel function under state 3 respiration conditions (G. Boyle, M. Prescott, P. Nagley, R.J. Devenish, unpublished data). These results are interpreted to mean that OSCP has a role in modulating proton conductance through the complex, in support of several earlier observations in bacteria and chloroplast systems concerning the role of the δ subunit [89,90]. Further work is needed to establish whether such phenomena are due merely to futile rotation of F_1 or to some direct involvement of conformational and functional changes in OSCP or other F_0 subunits. Recently, it has been shown that subunit δ can alter the catalytic reaction occurring on F_1, and that this subunit undergoes significant conformational alterations depending on the catalytic state of the enzyme [91]. Clearly, a better understanding of the function of OSCP (δ) is required. In relation to this goal, it will be important to gain more structural information for OSCP and, in particular, to identify the precise location of the subunit in relation to other subunits and to investigate possible conformational changes in the subunit during catalysis as suggested by the observations of Svergun et al. [91].

Alternatively, the role of the second copy of subunit b may be fulfilled by a subunit for which there is no homologue in bacteria. We have suggested [30] that in yeast, subunit d in combination with Y8 may take the place of a second subunit b. The function of the small membrane integral and hydrophobic Y8 would be to replace, in functional terms, the membrane-anchored N-terminal region of subunit b. Evidence in support of the requisite proximity of the two subunits comes from the observation that bovine subunit d could be crosslinked to subunit A6L (bovine homologue of Y8 [71]). As stated above, the C-terminal portion of Y8 includes three positively charged residues and extends into the matrix; Y8 would thus be available to make contact with subunit d. A closer examination of the relationship of subunits 8 and d in yeast is warranted not only in terms of stator stalk structure in yeast, but also in terms of possible dynamic movements of Y8 in mtATPase regulation (see above).

4.4. Subunits f, h and i/j

4.4.1. Subunit f

Subunit f was originally identified as a component of highly purified bovine mtATPase [92] and subsequently shown to be present in functionally active complexes [93]. The N-terminal region was shown to be exposed on the matrix side, and the C-terminus on the cytosolic side, of the mitochondrial inner membrane, suggesting subunit f spans the membrane once only. Furthermore, crosslinking studies showed that subunit f is in close proximity to A6L (homologous to subunit 8) and subunit g [93]. In parallel studies, a novel protein showing significant homology to bovine subunit f was identified in highly purified yeast mtATPase and named subunit f [17]. The yeast protein contains a short hydrophobic stretch near its C-terminus and remained associated with other F_0 subunits upon stripping of the F_1 sector. Deletion of the gene encoding subunit f resulted in cells that were unable to grow by oxidative phosphorylation, and showed no oligomycin-sensitive ATPase activity, although the F_1 sector showed high levels of activity. Immunoprecipitation of mtATPase from the null mutant revealed that complexes lacked subunit f as well as subunits 6 and 8, and contained decreased amounts of other F_0 subunits [17]. Crosslinking studies have indicated a close proximity between subunit f and subunit b [83].

4.4.2. Subunit h

Subunit h is a component of yeast mtATPase [18] unique to yeast; to date, no subunit h homologue has been identified in mammalian complexes or any other species. Deletion of the gene encoding subunit h resulted in cells unable to grow by oxidative phosphorylation and containing a high proportion of petite cells. Mitochondria prepared from these cells showed no oligomycin-sensitive ATPase activity, although the mitochondria contained normal F_1 sector activity [18]. The levels of subunits d and OSCP were reduced and subunit 6 was absent from mitochondria of this deletion strain [18] leading to a non-assembled F_0 sector. Later studies showed that subunit f was also absent in these mitochondria [17].

4.4.3. Subunit i/j

A small protein of 59 amino acids has been inde-

pendently characterised in two laboratories as the product of the ATP18 gene. This subunit is denoted i by Velours and colleagues [9] and j by Stuart and colleagues [10]. The protein has the characteristics of an integral membrane protein with the hydrophobic N-terminal part presumably representing a single transmembrane domain. In addition to the confusion over nomenclature of this subunit there also exists a disparity in the phenotype of cells lacking its expression as reported by the two above-mentioned laboratories. This may reflect different genetic backgrounds of the strains used to generate null cells and the precise nature of the gene constructs. Thus, total gene deletion reported by Vaillier et al. [9] resulted in cells showing low growth on non-fermentable carbon sources. Mutant mitochondria were reported to have a low ADP/O ratio and a decrease with time in proton pumping after ATP addition. On the basis of a two-fold reduction in specific ATPase activity, these authors hypothesised that the observed decrease in turnover of the mutant enzyme may be linked to a proton translocation defect through F_0. By contrast, partial deletion and disruption of the gene in a different strain led to cells completely deficient in oligomycin-sensitive ATPase activity and unable to grow on non-fermentable carbon sources [10]. Moreover, in this case, the presence of subunit j was found to be required for the stable expression of subunits 6 and f.

All three of the subunits, f, h and i/j, could conceivably be involved in mediating proton channel and/or stator stalk function. It is notable that loss of each subunit can show effects on subunit 6 as does loss of putative yeast stator stalk subunits d, OSCP and d. Further information on the assembly and possible interaction of all these subunits is necessary and will provide an area for potentially fruitful investigation contributing much to our understanding of F_0 structure and assembly.

5. Inter-complex interactions (definition of a new class of subunit)

5.1. Subunit e

Subunit e was first identified by Walker and colleagues [27] as a component of bovine mtATPase complex. Its presence in a highly purified preparation of mtATPase together with previously characterised subunits provided the initial evidence for its classification as a bona fide subunit of the bovine complex. Studies by Hatefi and colleagues [93] verified that subunit e was present in preparations of bovine mtATPase, having fully oligomycin-sensitive ATPase and uncoupler-sensitive ATP-$^{32}P_i$ exchange activities, and that it could be immunoprecipitated with an antibody directed toward OSCP. Further analysis using antibodies directed against the C-terminus of subunit e showed that this region of the polypeptide was exposed on the cytosolic side of the inner mitochondrial membrane. Subunit e was found to be in close proximity to subunit g upon crosslinking and appeared to form e–e dimers [93].

A yeast homologue to mammalian subunit e was identified by searching of the yeast (*S. cerevisiae*) genome sequence database [20]. The protein identified in this manner had previously been characterised as Tim11p, a component of the TIM (translocase of inner membrane) complex of the mitochondrial inner membrane, and as such was considered to be involved in the process of sorting proteins to the inter-membrane space [19]. Further examination by Arnold et al. showed that subunit e was present in complexes much larger than the TIM complex, and could be co-immunoprecipitated with the α subunit of mtATPase. Furthermore, stable expression of subunit e was shown to require the presence of the mitochondrially encoded F_0 sector subunits. It was therefore concluded that subunit e was a component of yeast mtATPase [20]. It is was previously held that in order to classify a yeast protein as an integral component of mtATPase, the disruption of its gene should necessarily lead to cells defective in oxidative phosphorylation (see [94]). Interestingly, disruption of subunit e resulted in a 50% reduction in both respiratory activity and cytochrome *c* oxidase activity [19]. Recently, subunit e has been proposed as being essential for the formation of a newly identified, dimeric form of yeast mtATPase. The protein appears to play a central role in the dimerisation process [22].

5.2. Subunit g

Bovine subunit g was originally characterised as a

mtATPase subunit by Walker and colleagues [27]. A yeast homologue was identified by means of a yeast genome database search [21,23]. The yeast protein is characterised as an integral membrane protein based on its extractability with sodium carbonate [23] in agreement with the earlier finding for the bovine protein [93]. These authors also demonstrated a transmembrane topology for the bovine protein, such that the N- and C-termini are on the matrix and cytosolic sides of the inner mitochondrial membrane, respectively.

Cells in which the gene encoding subunit g was insertionally disrupted and therefore lacked expression of this subunit, retained the ability to grow by oxidative phosphorylation although having a slower growth rate compared to control cells. This result indicates that such cells retain ATP synthetic capacity sufficient for growth using respiratory substrates, and that subunit g, therefore, does not play an essential role in the function of yeast mtATPase. Moreover, cells apparently contained correctly assembled complexes at normal levels as oligomycin-sensitive ATP hydrolase activity in mitochondria was normal [23].

Stuart and colleagues have presented evidence that subunit g is essential for formation of mtATPase dimers [22]. These studies used conditions for solubilisation of mitochondrial enzyme complexes based on low detergent:protein ratios. The monomer form of the complex generated using higher detergent:protein ratios apparently does not contain subunit g. On this basis, Arnold et al. [22] proposed subunit g to be a component only of the dimeric form of mtATPase.

5.3. Pleiotropic effects on respiratory chain complexes

Interestingly, in the study by Boyle et al. [23] the absence of subunit g reduced the activity of cytochrome *c* oxidase by some 26%, and altered the kinetic control of this complex as demonstrated by experiments titrating ATP synthetic activity with cyanide. This result indicated that subunit g is required for maximal levels of cytochrome *c* oxidase activity in yeast.

Pleiotropic effects upon respiratory chain complexes, particularly cytochrome *c* oxidase, have previously been observed in mutant strains either lacking a particular yeast mtATPase subunit, or expressing a mutant variant of that subunit. In particular, the altered assembly of cytochrome *c* oxidase has been observed, as revealed by reduced levels of optically detectable cytochromes $a+a_3$ in cells having mutations in subunits 6, 8 and 9 [61,95,96]. However, in cells lacking subunit g, no reduction in the level of cytochromes $a+a_3$, or $c+c_1$ and b was observed [23]. Furthermore, it can be assumed that the mitochondrially encoded subunits of cytochrome *c* oxidase were present in similar amounts in each strain, as the proportion of respiratory deficient petite cells from cells lacking subunit g was similar to that determined for control cultures. These observations suggest that gross assembly defects in cytochrome *c* oxidase are not responsible for the observed alterations in the activity of this complex in cells lacking subunit g.

How might subunit g mediate its effect upon cytochrome *c* oxidase? One possibility is that the role of subunit g could be mediated through a physical interaction with a component of cytochrome *c* oxidase. In support of this notion a specific association of the bovine mtATPase and cytochrome *c* oxidase has been observed in reconstitution studies involving these two complexes [97]. Indeed, these authors suggested that it was the membrane-bound F_0 sector of mtATPase that interacted with cytochrome *c* oxidase. It may be through its postulated role in stabilising the dimeric form of mtATPase that subunit g can influence interactions with other components of the mitochondrial inner membrane. Thus, further investigation is warranted on the question of how subunit g may mediate a physical association of mtATPase with cytochrome *c* oxidase, and how kinetic control of oxidative phosphorylation is exerted by cytochrome *c* oxidase.

5.4. Subunit k

Subunit k is a newly recognised component of mtATPase also characterised as a small (68 amino acid) dimer-specific subunit [22]. Disruption of the gene for subunit k shows it to be, like subunits e and g, non-essential for enzymatically active mtATPase. The role of subunit k in the dimerisation process is unclear. Neither is its presence required for stable expression of subunit e or g, nor did it appear to be essential for dimer formation. Subunit k ap-

pears to be peripherally associated with the mitochondrial inner membrane, facing the intermembrane space.

6. Inhibitor complex

It is now amply evident that mtATPase has considerable complexity relative to the bacterial enzyme. The subtleties of F_0 subunit composition, structure and function are only now being uncovered. With the description of subunits whose ablation genetically does not grossly diminish the activity of the complex, there needs to be a re-evaluation of criteria for the assignment of subunits as integral components or ancillary components of this enzyme. Therefore, it may be appropriate to re-appraise the classification of those proteins that form the inhibitor complex of F_1. This complex comprises three proteins: the inhibitor protein, Inh1p, as well two stabilising factors Stf1p (9-kDa protein) and Stf2p (15-kDa protein). Cells lacking expression of these proteins show no gross growth defects on non-fermentable substrates [24–26]. Traditionally, they have not been classified as subunits of mtATPase on this basis, not being required for enzymatic function and only associated with the complex under certain conditions. In this sense, they can be classified with the dimer-specific subunits e, g and k. A more appropriate classification of all these subunits might be as regulatory subunits of mtATPase.

7. Use Of GFP fusions to probe F_0 subunit function and assembly

The green fluorescent protein (GFP) of *Aequorea victoria* has proven very useful in studying events within cells. Potentially GFP can be used to investigate and visualise in situ the spatial and temporal distribution of mtATPase, particularly the recently identified dimeric form of the complex, as well as to study interactions between mtATPase and other mitochondrial enzyme complexes.

The potential versatility of mtATPase subunit-GFP fusions in providing novel insights into the formation, function and topology of mtATPase has been demonstrated in yeast. Three subunits, OSCP, b and d fused at the C-terminal to GFP are able to replace, in vivo, the function of the respective native protein ([98]; M. Prescott, P. Gavin, P. Nagley, R.J. Devenish, unpublished data). Using the technique of native gel electrophoresis, the ATP synthase solubilised from isolated mitochondria and containing these fusion proteins was shown to be fluorescent, fully assembled and enzymatically active as an oligomycin-sensitive ATP hydrolase (Fig. 2). The availability of such functional subunit-GFP fusions facilitates the development of new procedures based on fluorescence resonance energy transfer (FRET) for the study of subunit–subunit interactions. To this

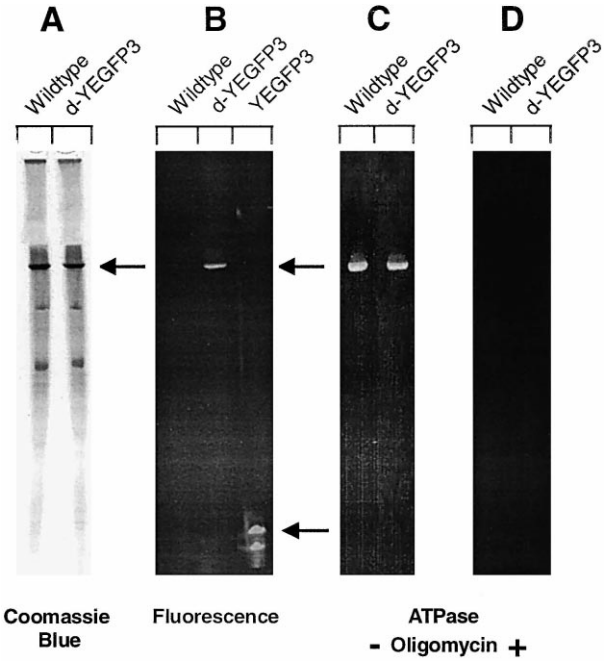

Fig. 2. ATP synthase containing a subunit d–GFP fusion is functional and fluorescent. Lysates of mitochondria isolated from cells expressing a subunit d–GFP fusion, or from wild-type cells were subjected to native gel electrophoresis. Equivalent sections of gel were subjected to a number of tests. (A) Visualisation of protein complexes following staining with Coomassie blue. The assembled ATP synthase is indicated by the arrow at right. (B) Fluorescence due to GFP observed after excitation with laser light at 450 nm. The upper arrow at right of this panel indicates mitochondrial fluoresence due to GFP which is associated with mtATPase as intact d–GFP fusion. The lower arrow at right of this panel indicates the position of GFP expressed in cells not fused to another protein. (C) In situ assay for ATP hydrolase activity (the presence of activity is indicated by a white precipitate). (D) Sensitivity of ATP hydrolase activity to oligomycin.

end, we have expressed a pair of subunits (d and OSCP) each fused to an appropriate variant of GFP suitable for FRET (a blue fluorescent protein or a red shifted GFP) in yeast cells lacking the endogenous proteins. One of the GFP variants contained a C-terminal hexahistidine tag. Using Ni-NTA chromatography assembled complexes containing both subunit d-GFP and OSCP-BFP fusion proteins could be isolated. Furthermore, isolated mitochondria could be shown to have the fluorescent properties of both GFP variants. Thus, it was shown that mtATPase can be assembled into active complexes containing not only one, but two different subunits fused to variants of GFP suitable for FRET.

These newly emerging techniques can be applied to probe the dynamic interactions between subunits, between mtATPase complexes (dimer formation), as well as between mtATPase and other mitochondrial enzyme complexes, such as cytochrome *c* oxidase.

8. Concluding remarks

While the study of the bacterial ATP synthase has provided us with an archetype on which to develop models for mitochondrial complexes, its different subunit stoichiometry and more simple composition mean that some features, such as the composition of the stator stalk, cannot be simply be directly applied to mtATPase. The advantage of the yeast system is that it allows a wide range of molecular biological approaches to be used for the study of the complex. At the same time, yeast mtATPase provides a more suitable model on which to develop our detailed understanding of the structure and function of the eucaryotic mtATPase. It is perhaps not surprising that these benefits have led to new developments in terms of our ideas concerning subunit composition and structure of the complex. With a better understanding of subunit composition, it will be appropriate to investigate in further detail the assembly of the F_0 sector for which there is a relative paucity of data [28,99]. A useful approach in this respect may be that of Straffon et al. [100] who studied the effects of depletion of each of subunits b, OSCP and d on the cellular levels of other subunits of the complex to determine a hierarchy of subunit stability that was interpreted to indicate an order of assembly. The influence of nuclear genes (see [28,99]) on subunit assembly, both in general, and for specific subunits also warrants further evaluation.

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References

[1] P.D. Boyer, Annu. Rev. Biochem. 66 (1997) 717–749.
[2] J.P. Abrahams, A.G.W. Leslie, R. Lutter, J.E. Walker, Nature 370 (1994) 621–628.
[3] H. Noji, R. Yasuda, M. Yoshida, K. Kinosita Jr., Nature 386 (1997) 299–302.
[4] Y. Kato-Yamada, H. Noji, R. Yasuda, K. Kinosita Jr., M. Yoshida, J. Biol. Chem. 273 (1998) 19375–19377.
[5] S. Wilkens, R.A. Capaldi, Nature 393 (1998) 29.
[6] B. Bottcher, L. Schwartz, P. Graber, J. Mol. Biol. 281 (1998) 757–762.
[7] S. Karrasch, J.E. Walker, Biochim. Biophys. Acta EBEC Short Reports 10 (1998) 49.
[8] P. Nagley, Trends Genet. 4 (1988) 46–52.
[9] J. Vaillier, G. Arselin, P.-V. Graves, N. Camougrand, J. Velours, J. Biol. Chem. 274 (1999) 543–548.
[10] I. Arnold, K. Pfeiffer, W. Neupert, R.A. Stuart, H. Schägger, J. Biol. Chem. 274 (1999) 36–40.
[11] G. Macino, A. Tzagoloff, Cell 20 (1980) 507–517.
[12] I.G. Macreadie, C.E. Novitski, R.J. Maxwell, U.P. John, B.G. Ooi, G.L. McMullen, A.W. Linnane, P. Nagley, Nucleic Acids Res. 11 (1983) 4435–4451.
[13] W. Sebald, J. Hoppe, Curr. Top. Bioenerg. 12 (1981) 1–64.
[14] J. Velours, P. Durrens, M. Aigle, B. Guerin, Eur. J. Biochem. 170 (1988) 637–642.
[15] M. Uh, D. Jones, D.M. Mueller, J. Biol. Chem. 265 (1990) 19047–19052.
[16] N. Norais, D. Prome, J. Velours, J. Biol. Chem. 266 (1991) 16541–16549.
[17] C. Spannagel, J. Vaillier, G. Arselin, P.-V. Graves, J. Velours, Eur. J. Biochem. 247 (1997) 1111–1117.
[18] G. Arselin, J. Vaillier, P.-V. Graves, J. Velours, J. Biol. Chem. 271 (1996) 20284–20290.
[19] K. Tokatlidis, T. Junne, S. Moes, G. Schatz, B.S. Glick, N. Kronidou, Nature 384 (1996) 585–588.
[20] I. Arnold, M.F. Bauer, M. Brunner, W. Neupert, R.A. Stuart, FEBS Lett. 411 (1997) 195–200.

[21] M. Prescott, G. Boyle, A. Lourbakos, P. Nagley, R.J. Devenish, Yeast S13 (1997) 137.
[22] I. Arnold, K. Pfeiffer, W. Neupert, R.A. Stuart, H. Schägger, EMBO J. 17 (1998) 7170–7178.
[23] G. Boyle, X. Roucou, P. Nagley, R.J. Devenish, M. Prescott, Eur. J. Biochem. 262 (1999) 315–323.
[24] N. Ichikawa, Y. Yoshida, T. Hashimoto, N. Ogasawara, H. Yoshikawa, F. Imamoto, K. Tagawa, J. Biol. Chem. 265 (1990) 6274–6278.
[25] N. Ichikawa, Y. Yoshida, T. Hashimoto, N. Ogasawara, H. Yoshikawa, F. Imamoto, K. Tagawa, J. Biol. Chem. 265 (1990) 6274–6278.
[26] Y. Yoshida, T. Sato, T. Hashimoto, N. Ichikawa, H. Yoshikawa, F. Imamoto, K. Tagawa, Eur. J. Biochem. 192 (1990) 49–53.
[27] J.E. Walker, R. Lutter, A. Dupuis, M.J. Runswick, Biochemistry 30 (1991) 5369–5378.
[28] G.B. Cox, R.J. Devenish, F. Gibson, S.M. Howitt, P. Nagley, in: L. Ernster (Ed.), Molecular Mechanisms in Bioenergetics, Elsevier, Amsterdam, 1992, pp. 283–315.
[29] P. Nagley, R.J. Devenish, Trends Biochem. Sci. 14 (1989) 31–35.
[30] M. Bateson, R.J. Devenish, P. Nagley, M. Prescott, J. Biol. Chem. 274 (1999) 7462–7466.
[31] T. Hashimoto, Y. Yoshida, K. Tagawa, J. Bioenerg. Biomembr. 22 (1990) 27–38.
[32] T. Michon, M. Galante, J. Velours, Eur. J. Biochem. 172 (1988) 621–625.
[33] J.C. Long, S. Wang, S.B. Vik, J. Biol. Chem. 273 (1998) 16235–16240.
[34] F.I. Valiyaveetil, R.H. Fillingame, J. Biol. Chem. 273 (1998) 16241–16247.
[35] H. Yamada, Y. Moriyama, M. Maeda, M. Futai, FEBS Lett. 390 (1996) 34–38.
[36] J. Weber, A.E. Senior, Biochim. Biophys. Acta 1319 (1997) 19–58.
[37] H. Jager, R. Birkenhagen, W.-D. Stalz, K. Altendorf, G. Deckers-Hebestriet, Eur. J. Biochem. 251 (1998) 122–132.
[38] R.G. Hadikusumo, S. Meltzer, W.M. Choo, M.J. Jean-Francois, A.W. Linnane, S. Marzuki, Biochim. Biophys. Acta 933 (1988) 212–222.
[39] J. Velours, C. Spannagel, S. Chaignepain, J. Vaillier, G. Arselin, P.-V. Graves, G. Velours, N. Camougrand, Biochimie 80 (1998) 793–801.
[40] R.H. Fillingame, J. Exp. Biol. 2000 (1997) 217–224.
[41] A.L.A. Andrews, PhD Thesis, Monash University 1995.
[42] M.-F. Paul, J. Velours, G. Arselin de Chateaubodeau, M. Aigle, B. Guérin, Eur. J. Biochem. 185 (1989) 163–171.
[43] M. Prescott, N. Bush, P. Nagley, R.J. Devenish, Biochem. Mol. Biol. Int. 34 (1994) 789–799.
[44] M. Futai, T. Noumi, M. Maeda, Annu. Rev. Biochem. 58 (1989) 111–136.
[45] R.H. Fillingame, M.E. Girvan, D. Fraga, Y. Zhang, Ann. N.Y. Acad. Sci. 671 (1992) 323–334.
[46] M.E. Girvan, V.K. Rastogi, F. Abildgaard, J.L. Markley, R.H. Fillingame, Biochemistry 37 (1998) 8817–8824.
[47] G. Groth, J.E. Walker, FEBS Lett. 410 (1997) 117–123.
[48] P.C. Jones, W. Jiang, R.H. Fillingame, J. Biol. Chem. 273 (1998) 17178–17185.
[49] G.B. Cox, D.A. Jans, A.L. Fimmel, F. Gibson, L. Hatch, Biochim. Biophys. Acta 768 (1984) 3896–3903.
[50] G.B. Cox, A.L. Fimmel, F. Gibson, L. Hatch, Biochim. Biophys. Acta 849 (1986) 62–69.
[51] S.B. Vik, B.J. Antonio, J. Biol. Chem. 269 (1994) 30364–30369.
[52] T.M. Duncan, V.V. Bulygin, Y. Zhou, M.L. Hutcheon, R.L. Cross, Proc. Natl Acad. Sci. USA 92 (1995) 10964–10968.
[53] W. Junge, H. Lill, S. Engelbrecht, Trends Biochem. Sci. 22 (1997) 420–423.
[54] P. Dimroth, G. Kaim, U. Matthey, Biochim. Biophys. Acta 1365 (1998) 87–92.
[55] T. Elston, H. Wang, G. Oster, Nature 391 (1998) 510–513.
[56] P. Nagley, A.W. Linnane, in: T. Ozawa, S. Papa, (Eds.), Bioenergetics: Structure and Function of Energy Transducing Systems, Japan Sci. Soc. Press, Tokyo/Springer, Berlin, 1987, pp. 191–204.
[57] T. Lichter, E. Kellner, H. Lill, FEBS Lett. 431 (1998) 419–422.
[58] M. Galanis, J.R. Mattoon, P. Nagley, FEBS Lett. 249 (1989) 333–336.
[59] R.H. Fillingame, Bacteria 12 (1990) 345–391.
[60] P. Dimroth, Biochem. Soc. Trans. 23 (1995) 770–775.
[61] S. Marzuki, L.C. Watkins, W.M. Choo, Biochim. Biophys. Acta 975 (1989) 222–230.
[62] X. Grandier-Vazeille, R. Ouhabi, M. Guerin, Eur. J. Biochem. 223 (1994) 521–528.
[63] X. Roucou, I.M. Artika, R.J. Devenish, P. Nagley, Eur. J. Biochem. 261 (1999) 444–451.
[64] D. Nero, S.M. Ekkel, L.-F. Wang, D.G. Grasso, P. Nagley, FEBS Lett. 270 (1990) 62–66.
[65] D.G. Grasso, D. Nero, R.H.P. Law, R.J. Devenish, P. Nagley, Eur. J. Biochem. 199 (1991) 203–209.
[66] T. Papakonstantinou, M. Galanis, P. Nagley, R.J. Devenish, Biochim. Biophys. Acta 1144 (1993) 22–32.
[67] T. Papakonstantinou, R.H.P. Law, S. Manon, R.J. Devenish, P. Nagley, Eur. J. Biochem. 227 (1995) 745–752.
[68] T. Papakonstantinou, R.H.P. Law, W.S. Nesbitt, P. Nagley, R.J. Devenish, Curr. Genet. 30 (1996) 12–18.
[69] W.S. Nesbitt, PhD Thesis, Monash University 1998.
[70] A.F.L. Straffon, P. Nagley, R.J. Devenish, Biochem. Biophys. Res. Commun. 203 (1994) 1567–1573.
[71] G.I. Belogrudov, J.M. Tomich, Y. Hatefi, J. Biol. Chem. 270 (1995) 2053–2060.
[72] J. Velours, M. Esparza, J. Hoppe, W. Sebald, B. Guerin, EMBO J. 3 (1984) 207–212.
[73] J. Velours, B. Guerin, Biochem. Biophys. Res. Commun. 138 (1986) 78–86.
[74] T. Oda, S. Futaki, K. Kitagawa, Y. Yoshihara, I. Tani, T. Higuti, Biochem. Biophys. Res. Commun. 165 (1989) 449–456.
[75] C. Hekman, J.M. Tomich, Y. Hatefi, J. Biol. Chem. 266 (1991) 13564–13571.

[76] S. Engelbrecht, W. Junge, FEBS Lett. 414 (1997) 485–491.
[77] I. Ogilvie, R. Aggeler, R.A. Capaldi, J. Biol. Chem. 272 (1997) 16652–16656.
[78] A.J.W. Rodgers, R.A. Capaldi, J. Biol. Chem. 273 (1998) 29406–29410.
[79] I.R. Collinson, J.M. Skehel, I.M. Fearnley, M.J. Runswick, J.E. Walker, Biochemistry 35 (1996) 12640–12646.
[80] G. Lippe, F.D. Sala, M.C. Sorgato, J. Biol. Chem. 263 (1988) 18627–18634.
[81] F. Penin, P. Archinard, M. Moradi-Ameli, C. Godinot, Biochim. Biophys. Acta 810 (1985) 346–353.
[82] M. Bateson, R.J. Devenish, P. Nagley, M. Prescott, Anal. Biochem. 238 (1996) 14–18.
[83] C. Spannagel, J. Vaillier, S. Chaignepain, J. Velours, Biochemistry 37 (1998) 615–621.
[84] D.L. Foster, R.H. Fillingame, J. Biol. Chem. 257 (1982) 2009–2015.
[85] S.D. Dunn, J. Chandler, J. Biol. Chem. 273 (1998) 8646–8651.
[86] S.D. Dunn, J. Biol. Chem. 267 (1992) 7630–7636.
[87] D.T. McLachlin, J.A. Bestard, S.D. Dunn, J. Biol. Chem. 273 (1998) 15162–15168.
[88] Y.A. Ovchinnikov, N.N. Modyanov, V.A. Grinkevich, N.A. Aldanova, P.V. Kostetsky, O.E. Trubetskaya, T. Hundal, L. Ernster, FEBS Lett. 175 (1984) 109–112.
[89] R.A. Monticello, W.S.A. Brusilow, J. Bact. 176 (1994) 1383–1389.
[90] S. Engelbrecht, W. Junge, Eur. J. Biochem. 172 (1988) 213–218.
[91] D.I. Svergun, I. Aldag, T. Sieck, K. Altendorf, M.H.J. Koch, D.J. Kane, M.B. Kozin, G. Gruber, Biophys. J. 75 (1998) 2212–2219.
[92] I.R. Collinson, M.J. Runswick, S.K. Buchanan, I.M. Fearnley, J.M. Skehel, M.J. van Raaij, D.E. Griffiths, J.E. Walker, Biochemistry 33 (1994) 7971–7978.
[93] G.I. Belogrudov, J.M. Tomich, Y. Hatefi, J. Biol. Chem. 271 (1996) 20340–20345.
[94] A. Tzagoloff, C.L. Dieckman, Microbiol. Rev. 54 (1990) 211–225.
[95] W.M. Choo, R.G. Hadikusumo, S. Marzuki, Biochim. Biophys. Acta 806 (1985) 290–304.
[96] M.J.B. Jean-Francois, R.G. Hadikusumo, L.C. Watkins, H.B. Lukins, A.W. Linnane, S. Marzuki, Biochim. Biophys. Acta 852 (1986) 133–143.
[97] Z.-H. Qiu, L. Yu, C.-A. Yu, Biochemistry 31 (1992) 3297–3302.
[98] M. Prescott, A. Lourbakos, M. Bateson, G. Boyle, P. Nagley, R.J. Devenish, FEBS Lett. 411 (1997) 97–101.
[99] M. Prescott, R.J. Devenish, P. Nagley, in:F.-U. Hartl (Ed.), Protein Targeting to Mitochondria, (Advances in Molecular and Cell Biology, Vol. 17), JAI Press, Greenwich, CT, 1996, pp. 299–339.
[100] A.F.L. Straffon, M. Prescott, P. Nagley, R.J. Devenish, Biochim. Biophys. Acta 1371 (1998) 157–162.