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The Oligomycin Axis of Mitochondrial ATP Synthase: OSCP and the Proton Channel

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Oligomycin has long been known as an inhibitor of mitochondrial ATP synthase, putatively binding the F_o subunits 9 and 6 that contribute to proton channel function of the complex. As its name implies, OSCP is the oligomycin sensitivity-conferring protein necessary for the intact enzyme complex to display sensitivity to oligomycin. Recent advances concerning the structure and mechanism of mitochondrial ATP synthase have led to OSCP now being considered a component of the peripheral stator stalk rather than a central stalk component. How OSCP confers oligomycin sensitivity on the enzyme is unknown, but probably reflects important protein–protein interactions made within the assembled complex and transmitted down the stator stalk, thereby influencing proton channel function. We review here our studies directed toward establishing the stoichiometry, assembly, and function of OSCP in the context of knowledge of the organization of the stator stalk and the proton channel.

KEY WORDS: Yeast mitochondrial ATP synthase; oligomycin sensitivity-conferring protein (OSCP); oligomycin; F_o subunit organization; proton channel; stator stalk.

INTRODUCTION

Over the last decade our view of the molecular structure of F_1F_o-ATP synthase has undergone considerable refinement (see Boyer, 1999; Fillingame, 1999). F_1 is the soluble sector with catalytic sites for ATP synthesis from ADP and phosphate. The proton channel through the membrane is within the F_o sector. Further, subunits classified as belonging to F_o are peripheral to the membrane and make structural and functional links to F_1. Earlier theoretical predictions of a rotatory mechanism for ATP synthesis (see Cox *et al*., 1984; Boyer, 1993) have been supported by the elegant demonstrations of the rotation of bacterial F_1 subunit γ or chloroplast F_1 subunit ε (mitochondrial homologs subunits γ and δ, respectively), with respect to the $\alpha_3\beta_3$ hexamer during ATP hydrolysis (Noji *et al*., 1997; Kato-Yamada *et al*., 1998). The primary advances in detailed knowledge of ATP synthase structure include solution of crystal structures of bovine $F_1\alpha_3\beta_3\gamma$, and of yeast mitochondrial F_1 in association with the subunit 9 ring, solved by Walker and colleagues (Abrahams *et al*., 1994; Stock *et al*., 1999). The latter structure revealed that subunits γ and δ of mitochondrial ATP synthase (mtATPase) together form a footlike structure at the bottom of F_1, sitting on the surface of the membrane-embedded subunit 9 ring and contacting the loop region of 6 to 7 monomer subunit 9 units (Stock *et al*., 1999). In conjunction with the recent demonstration that the bacterial homolog of subunit 9 (subunit *c*) rotates during ATP hydrolysis (Sambongi *et al*., 1999), the available evidence supports the existence of a structural connection between the subunit 9 ring and γ subunit such that they can rotate in concert. The coupling process is now thought to involve a ring of subunit 9 monomers rotating relative to the membrane integral F_o subunit 6 (homolog of bacterial subunit *a*). The passage of protons across the membrane depends on the concerted interactions of a series of charged amino acid side chains in one transmembrane helix of each of the multiple members of the subunit 9/*c* ring and two transmembrane helices of subunit 6/*a* (see Dimroth, 2000; Fillingame *et al*., 2000; Groth, 2000).

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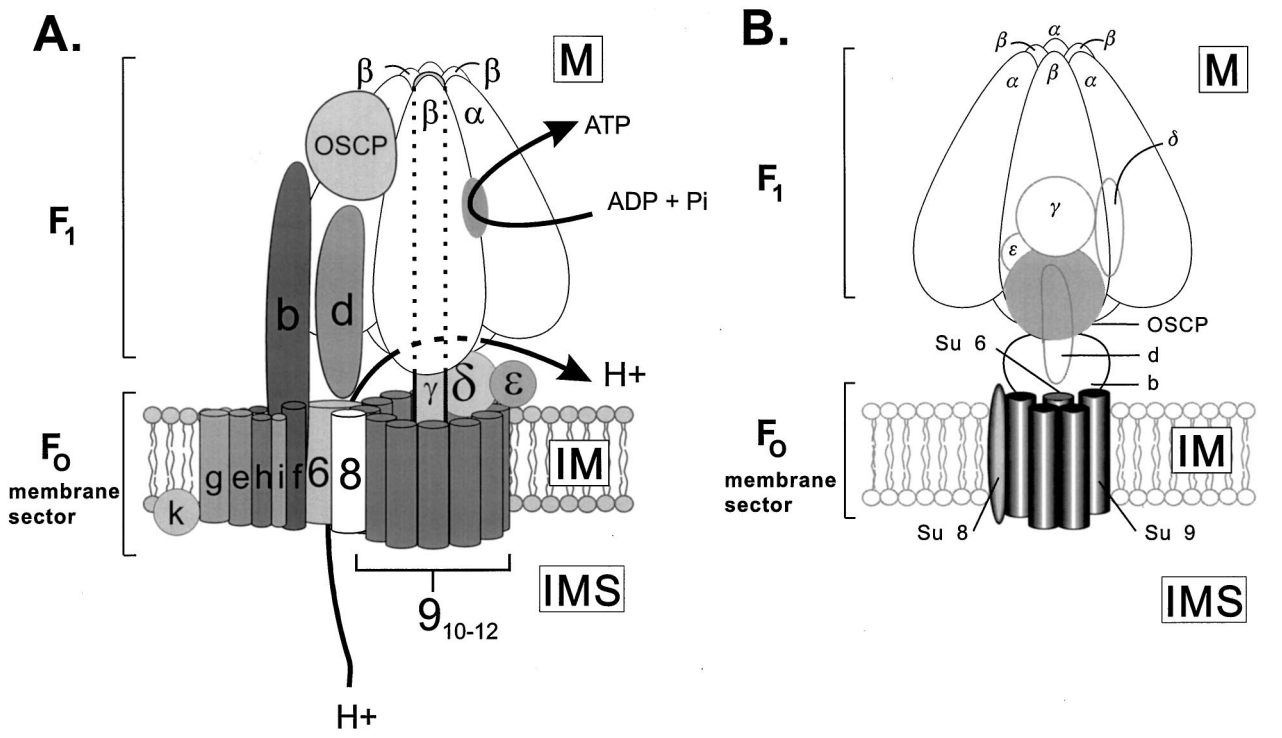

Fig. 1. Schematic representation of yeast mitochondrial ATP synthase. (A) A schematic representation of yeast mtATPase including the more recently identified subunits is shown (see Devenish *et al*., 2000 for details of subunit composition). Two stalks connecting F_1 with F_o are depicted; the central 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* with possible contributions by subunits 8 and *f*. Subunits are depicted in positions indicated by the results of cross-linking 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 Devenish *et al*., 2000). 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 localized on the inner membrane space surface of the inner membrane in close proximity to subunits *e* and *g*. Protons are depicted as passing through the inner mitochondrial membrane (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 Boyer, 1997). (B) Schematic representation of yeast mtATPase prior to the concept of the stator stalk and the enumeration of the additional subunits in yeast *e, f, g, h, i*, and *k* (modified from Prescott *et al*., 1996). The full complement of subunit 9 monomers is not shown. Note the location of OSCP (shaded in gray) then thought of as a component of the central stalk, directly linking F_1, and the membrane-associated F_o.

In parallel to the development of the rotational mechanism for ATP synthase, the single stalk "mushroom" view of the enzyme complex embodying the catalytic F_1 sector connected by a central stalk to the membrane F_o sector (see Fig. 1) has been supplanted by a two-stalk model. This model incorporates the previous basic structural arrangement including a central stalk, but introduces a second peripherally located stator stalk (Wilkens and Capaldi, 1998; Bottscher *et al*., 1998; Karrasch and Walker, 1999). The stator stalk was proposed (Engelbrecht and Junge, 1997) to prevent the futile rotation of the $\alpha_3\beta_3$ hexamer relative to the remainder of the complex (see Fig. 1). In *E. coli*, this stalk is comprised of subunits *b* and δ (Ogilvie *et al*., 1997; Rodgers and Capaldi, 1998). Subunit δ is the bacterial homolog of OSCP (Ovchinnikov *et al*., 1984a,b). Subunit *b*, present in two copies per bacterial ATP synthase complex, extends from the membrane up the side of the $\alpha_3\beta_3$ hexamer to contact bacterial subunit δ (see McLachlin *et al*., 2000), which interacts with the top of the $\alpha_3\beta_3$ hexamer (Wilkens *et al*., 2000). Eukaryotic subunit *b* (homolog of the bacterial subunit *b*) and OSCP are the prime candidates for components of a stator stalk in mtATPase. However, the composition of the stator in mtATPase is evidently more complex than that in bacteria because mtATPase contains additional subunits that do not have any bacterial homolog (see Devenish *et al*., 2000). Possible candidate subunits include *d* and 8 of the F_o sector. Subunit *b* is present in only one copy in mtATPase (Hekman *et al*., 1991; Collinson *et al*., 1996; Bateson *et al*., 1999).

The yeast, *Saccharomyces cerevisiae*, has provided an attractive system for the analysis of OSCP and other

mtATPase subunits. The highly developed system of molecular genetic manipulation available in this unicellular eukaryote permits the genes for mtATPase subunits to be manipulated and questions of stoichiometry, structure, and function to be addressed. This review focuses on understanding the oligomycin axis within mtATPase, in particular, how OSCP influences proton channel function and acts to confer upon the complex sensitivity to oligomycin. Our studies commenced in the early 1980s with mitochondrial molecular genetic analysis to define the mitochondrial oligomycin-resistance loci and have more recently focused on the analysis of products of nuclear genes in yeast.

THE BINDING SITE OF OLIGOMYCIN IN mtATPase

Oligomycin is an inhibitor of proton translocation in mtATPase (see Slater, 1967). Sensitivity to oligomycin can be defined in two contexts. The first relates to the "coupling" of the enzyme *in vitro* and assayed in terms of oligomycin-sensitive ATP hydrolytic (ATPase) activity. It is a measure of the structural integrity of the enzyme such that detachment of F_1 from F_o leads to a decrease in oligomycin-sensitive ATPase activity (Tzagoloff, 1970). ATP hydrolysis by F_1 takes place, but without the coupling of ATP hydrolysis to extrusion of protons through F_o, the sensitivity to oligomycin is no longer evident. The second relates to the ability of the assembled complex *in vivo* to bind oligomycin and is dependent on the mitochondrially encoded amino acid sequence of individual mtATPase subunits. Thus, in yeast, aside from plasma membrane permeability mutants, the oligomycin resistance phenotype is conferred by mutations in one of two mitochondrially encoded genes (*oli1* and *oli2*), those encoding the F_o subunits 6 (Y6) and 9 (Y9), respectively. These membrane-integral proteolipid subunits also form the proton channel of mtATPase (see Nagley, 1988; Cox *et al*., 1992).

Y6 is predicted to span the inner mitochondrial membrane at least five times (Nagley, 1988), mirroring the topology demonstrated for the bacterial homolog of Y6, subunit *a* (see Devenish *et al*., 2000). The two helices most proximal to the C-terminus of Y6, designated h4 and h5 (see Fig. 2), are well conserved across species and contain those residues considered to be involved in proton translocation, Arg186, His195, and Glu233 (homologous to residues Arg210, Glu219, and His245 of bacterial subunit *a* respectively; for reviews see Nagley, 1988; Weber and Senior, 1997; Fillingame, 1997). Moreover, h5 of Y6 also contains two residues, Leu232 and Val242, that when substituted, confer an oligomycin-resistant phenotype (Macino and Tzagoloff, 1980; Slott *et al*., 1983; John and Nagley, 1986). Three other residues have been recognized that can be substituted to confer this phenotype and lie within the loop connecting h3 and h4 of subunit 6 that protrudes into the mitochondrial matrix (Fig. 2); these are Ile171 and Ser175 in yeast (Macino and Tzagoloff, 1980; John and Nagley, 1986) and Glu172 in the hamster (Breen *et al*., 1986).

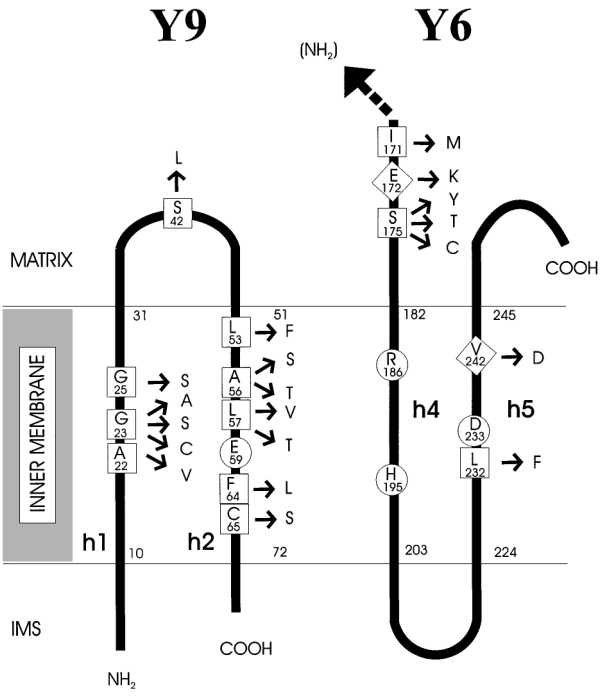

Fig. 2. Schematic representation of subunits 6 (Y6) and 9 (Y9) of yeast mtATPase highlighting residues determining oligomycin resistance. Proteins are represented as thick solid lines; the Y9 protein is drawn in its hairpin configuration and, for Y6 only, the C-terminal portion of the protein is drawn. The inferred boundaries of the transmembrane stems are shown as thin lines and are based on hydropathy plots and consideration of homology with the corresponding bacterial subunits (see Fillingame *et al*., 2000; Vik *et al*., 2000). Charged residues, thought to participate in proton translocation across the membrane are shown circled. Amino acid substitutions that confer oligomycin resistance are shown boxed for yeast and within a diamond for mammalian cells. Orientation of proteins with respect to the matrix and intermembrane space (IMS) is indicated. F_1 is in the matrix compartment.

Y9 also has a primary sequence that is well-conserved across species and includes a number of invariant residues (see Nagley, 1988). This subunit has long been predicted to have a hairpin topology, recently confirmed by NMR spectroscopic analysis (Girvan *et al*., 1998), with two the transmembrane domains, denoted h1 and h2, separated by a small loop containing polar residues that protrudes into the mitochondrial matrix (see Nagley, 1988; Cox *et al*., 1992). The single charged residue in the C-terminal transmembrane helix of subunit 9, Glu59

(corresponding to Asp61 in subunit *c*), is a key player in proton channel function.

A number of amino acid substitutions in Y9 conferring resistance to oligomycin (as well as venturicidin and ossamycin) were determined in studies examining yeast mutants isolated in our laboratory at Monash University (Ooi *et al*., 1985; Nagley *et al*., 1986; Nagley and Linnane, 1987; Willson and Nagley, 1987; Galanis *et al*., 1989), as well as in other laboratories (for example, Sebald *et al*., 1979; Macino and Tzagoloff, 1980). Together this extensive collection of mutants and the definition of the substitutions they contain in Y9 led to the definition of an oligomycin-binding domain. The oligomycin-resistance domain is centered on Gly23 in h1 and Glu59 in h2, and thus encompasses both the N- and C-terminal domains of Y9. It is particularly noteworthy that the bacterial equivalent of Glu59 has been identified as the proton-binding site in F_o and is considered to undergo protonation–deprotonation as each proton is transported (for review see Fillingame *et al*., 2000). The venturicidin-resistance domain overlaps the oligomycin-resistance domain (see Galanis *et al*., 1989 for details). The ossamycin-resistance domain encompasses only residues 53 to 57 of h2. These residues also lie close to the boundaries of the oligomycin- and venturicidin-resistance domains, suggesting that this region of Y9 interacts in some way with all three inhibitors (see Galanis *et al*., 1989). It is noteworthy that one residue, Ser42, of the loop region of Y9 is found to be substituted in oligomycin resistant revertants of an *oli1 mit*$^-$ mutant (Sebald *et al*., 1979).

The identification of domains in both Y6 and Y9 that influence oligomycin resistance is consistent with the view that proton translocation through F_o requires the concerted action of both subunits. Indeed, it may be postulated that there is a functional interaction between the C-terminal region of Y6 (h4 and h5) and the two transmembrane domains of Y9, such that the oligomycin-binding site encompasses both subunits 9 and 6. Note that the binding site is likely to involve the membrane-spanning regions of both subunits, as well as the Y9 loop and the loop connecting h3 and h4 of Y6, since all regions contain residues that when substituted lead to an oligomycin-resistant phenotype. It remains to be determined whether binding of oligomycin to F_o prevents proton translocation across the membrane, or impedes the rotation of the subunit 9 ring relative to subunit 6.

OSCP INTERACTION WITH PROTON CHANNEL SUBUNITS

It has long been established that OSCP makes important structural connections between F_1 and F_o within mtATPase (Dupius *et al*., 1983; Mukopadhyay *et al*., 1992; Joshi *et al*., 1996,1997; Mao and Mueller, 1997; Golden and Pedersen, 1998), as well as in the coupling of proton translocation to ATP synthesis (Pringle *et al*., 1990; Mukopadhyay *et al*., 1992). Although the physical location of OSCP in mtATPase remains to be definitively established, evidence has been presented in support of physical contact being made by OSCP with both the α and β subunits of F_1 (Joshi *et al*., 1996; Golden and Petersen, 1998), as well as the C-terminus of subunit *b* (Velours *et al*., 1998; Soubannier *et al*., 1999). This evidence supports a location for OSCP equivalent to that of bacterial subunit δ, that is, on the outer upper surface of the $\alpha_3\beta_3$ hexamer of F_1. Such a location for OSCP raises the question of how OSCP modulates proton flux through the distant membrane-integral F_o sector subunits that constitute the proton channel. It would seem that such modulation would require subtle changes in the structure of OSCP to be communicated elsewhere within the enzyme complex by subunit–subunit interactions.

We sought to address this issue by expressing in yeast OSCP variants having either of two defined substitutions, Asp or Asn, of the Gly residue at position 166 (numbered within mature OSCP). Gly at this position is highly conserved in OSCP homologs and is equivalent to Gly150 of *E.coli* subunit δ. The equivalent substitutions in the bacterial subunit δ have previously been shown to abolish coupling of proton transport through F_o to ATP synthesis or hydrolysis (Hazard and Senior, 1994), such that Gly150 $\rightarrow$ Asp (G166D) resulted in an assembly defect and Gly150 $\rightarrow$ Asn (G166N) in an uncoupling of proton transport.

Our results provide evidence suggesting specific functional links between OSCP and the proton channel. Expression of OSCP variant G166D resulted 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 (Boyle *et al*., 2000). We have interpreted these results to mean that OSCP has a role in modulating proton conductance through the complex. We have not established whether the phenotype was due merely to futile rotation of F_1 or to some direct involvement of conformational and functional changes in OSCP or other F_o subunits. A number of additional observations provide evidence in support of the latter possibility.

First, the presence of the OSCP variants G166D or G166N in the mtATPase complex of intact cells confers increased sensitivity to oligomycin when measured in intact cells (Boyle *et al*., 2000). This increased sensitivity

was not due to an alteration of levels of mtATPase in the OSCP variant cells, as immunoblot analysis indicated equivalent levels of OSCP and other subunits when compared to control cells. This finding suggested that the functional interaction of F_1 with F_o mediated by OSCP was intact, but modulated in some way. This change may be due to an induced structural alteration at the inhibitor-binding sites in F_o (more efficient binding of oligomycin) or modulation of the transmission of the inhibitory effects of oligomycin through the complex to F_1.

Second, mtATPase complexes from the same OSCP variant strains are structurally unstable *in vitro* (following solubilization of mitochondrial membranes) as judged by measurement of ATPase activity. Total ATPase rates were similar for control and variant strains indicating that the mitochondrial preparations assayed contained the same amount of assembled F_1. However, while the ATPase activity of control mitochondria was inhibited by 77% in the presence of oligomycin (consistent with the levels of activity routinely obtained with mitochondria prepared from wild-type yeast cells), the oligomycin-sensitive ATPase activity by mitochondria isolated from cells expressing OSCP variants was significantly reduced, to 11% for G166D and 27% for G166N. Confirmatory evidence in support of instability of mtATPase complexes was obtained by determining levels of assembled mtATPase using immunoprecipitation with a monoclonal antibody directed against the β subunit (Hadikusumo *et al*., 1984) coupled to Sepharose beads. It was apparent that the amount of intact complex recovered from mitochondria prepared from the OSCP variant strains was reduced when compared to that recovered from control mitochondria (Boyle *et al*., 2000).

Finally, there is the recent demonstration in the chloroplast complex that the OSCP homolog, 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 (Svergun *et al*., 1998).

The importance of contacts made by OSCP with other subunits of the complex is emphasized by the results observed upon the expression of the cDNA for rat OSCP in yeast cells lacking endogenous OSCP. Rat OSCP is able to restore mtATPase function in such cells (Prescott *et al*., 1995); however, these cells express a number of phenotypes that are not observed in cells expressing native yeast OSCP. Cells expressing rat OSCP showed a late onset of logarithmic growth at 35°C in liquid growth medium and an increased sensitivity to oligomycin (similar to that of cells expressing yeast OSCP variants discussed above). Further, upon isolation of mtATPase (by immunoprecipitation from mitochondrial lysates), there is found to be an instability of complexes containing rat OSCP compared to those containing yeast OSCP. These phenotypes are ascribed to changes in protein–protein contacts normally made by OSCP. Thus, rat OSCP, which has 58% overall homology with the yeast protein, is able to maintain the essential contacts normally made by yeast OSCP, but they are subtly altered to produce the observed phenotypes. That fact that there are, nevertheless, changes in the contacts made by rat OSCP is shown by the demonstration that variants having either of the substitutions G166N or G166D were no longer able to functionally replace yeast OSCP (M. Prescott, G. M. Boyle, P. Nagley, and R. J. Devenish, unpublished data 1998).

In conclusion to these studies, it is evident that substitution at G166 markedly decreased the stability of OSCP. Thus, G166 is important for a stable interaction of OSCP with mtATPase and without the ability to form a stable interaction the unassembled subunits are removed, presumably by proteolysis. To further understand the contacts made by G166 with other subunits in the complex will require the three-dimensional structure of OSCP to be determined and to precisely locate OSCP (and G166) with respect to F_1 subunits.

The growth and oligomycin-sensitivity phenotypes associated with expression of rat OSCP in yeast cells offered the possibility of mapping regions of OSCP responsible for conferring these phenotypic differences. We expressed hybrid yeast/rat OSCP proteins (see Fig. 3) with the aim of defining such "functional" domains. Strain OCA1 in which the C-terminal 40 residues of rat OSCP replaces the equivalent yeast residues showed a twofold increase in oligomycin sensitivity relative to that shown by a strain expressing yeast OSCP. Strain OCA2, in which the C-terminal 69 residue segment of rat OSCP replaced the corresponding region in yeast, showed a more marked increase in sensitivity; the sensitivity was comparable to that of the strain expressing rat OSCP. This can be compared to data obtained with strains OCA4, 5, and 6 that expressed hybrid proteins in which the rat C-terminal 40, 69, or 151 residues were replaced with the corresponding regions of yeast. These strains showed significant growth at higher concentrations of oligomycin, such that for OCA5 and OCA6 growth was comparable to that of the control strain expressing yeast OSCP (see Fig. 3). These results suggest that in yeast/rat OSCP hybrids, the source of the C-terminal 40 residues is important for determining the level of oligomycin sensitivity shown by whole yeast cells. This correlates with the importance of the region containing Gly166.

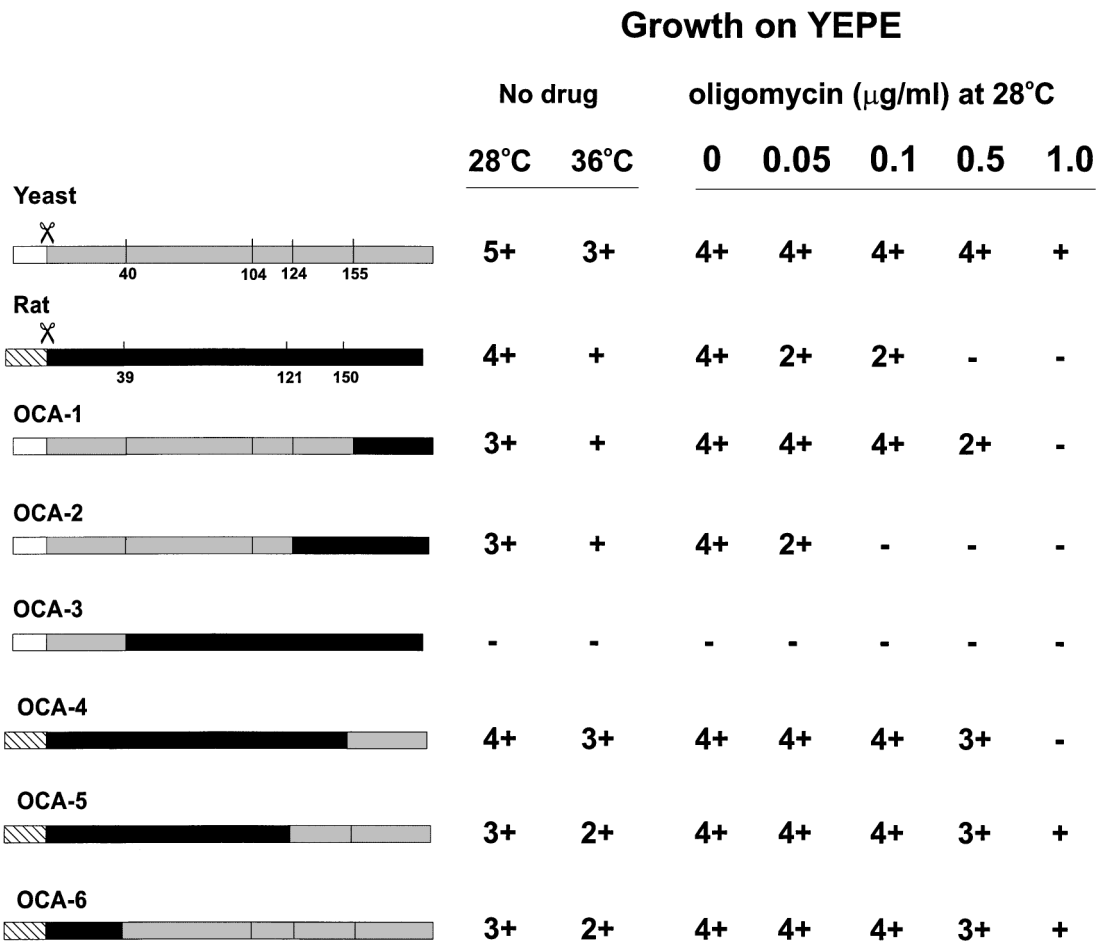

Fig. 3. Growth of strains expressing yeast/rat hybrid OSCP. The yeast and rat OSCP plus yeast/rat OSCP hybrid (designated OCA1–6) coding regions are represented by shaded bars. Gray shading represents yeast segments and black bars represent rat segments. The mitochondrial targeting sequence is represented by a white bar for yeast and a hatched bar for the rat. The scissor symbol indicates the site of cleavage of the precursor protein by mitochondrial matrix protease. The positions of the mutations introduced to specify restriction sites, which allow segment swapping, are indicated by vertical solid lines. The numbers below these lines indicate the residue numbers in the mature protein, which are the fusion points between the OSCP proteins. To assess growth, cells expressing these OSCP variants were replica-plated onto either solid YEPE medium alone or the same medium containing indicated concentrations of oligomycin. Plates were then incubated at 28°C for 4 days, or at 36°C for 5 days, before scoring the level of growth. Growth was related to a parental control strain YRD15 (not shown; which was scored as 5+ on YEPE at 28°C): 4+, strong growth, slightly less than YRD15; 3+, normal growth; 2+, less than normal growth; +, weak growth; −, no growth.

The yeast OSCP variants conferred on mtATPase a less debilitating effect than did the expression of the equivalent variants of subunit δ in bacteria (Hazard and Senior, 1994). This may reflect differences in the molecular architecture and subunit composition of mtATPase compared with bacterial ATP synthase (see Devenish *et al.*, 2000). In this context it is noteworthy that the bacterial complex is only weakly inhibited by oligomycin, but strongly inhibited by venturicidin (Perlin *et al.*, 1985). One possible explanation is that the additional subunit components of F_o in the mitochondrial complex facilitate binding of oligomycin, or mediate, at least in part, the effect of oligomycin on proton flow through the proton channel.

STOICHIOMETRY AND ASSEMBLY OF OSCP

In the context of establishing a detailed structure for F_o, several investigators have determined the stoichiometry of OSCP together with other subunits. Thus, a stoichiometry for subunits *b*:OSCP:*d*:F_6 in the bovine system was first determined as 2:1:1:2 (Hekman *et al.*, 1991) and

later as 1:1:1:1 (Collinson *et al*., 1996). The stoichiometry of 1 for OSCP from these reports contradicts the earlier report of a stoichiometry of 2 for OSCP in the porcine enzyme (Penin *et al*., 1985). To address the question of the stoichiometry of OSCP in yeast, we developed an approach that utilized Ni–NTA affinity chromatography, such that intact mtATPase complexes incorporating a hexahistidine (h6)-tagged subunit could be isolated (Bateson *et al*., 1996). Strains were constructed in which h6-tagged versions of each of three subunits, OSCP, *b*, and *d*, were coexpressed 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 the 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 (Bateson *et al*., 1999).

Relatively little data are available concerning the assembly of OSCP, whether considered either as a component of the stator stalk or of the overall F_o sector. The results of *in vitro* reconstitution experiments performed by Collinson *et al*. (1994) indicated the primary interactions of OSCP to be with F_1 or subunit *b* and were interpreted in the context of the then extant single-stalk models of mtATPase. Little definitive information was available for yeast OSCP and thus we sought to examine the assembly of OSCP (in conjunction with subunits *d* and *b*) using a strategy based on controlled depletion of individual subunits (Prescott *et al*., 1994; Law *et al*., 1995) and the measurement by immunoblotting of the abundance of other subunits, specifically to monitor their retention or loss. Our observations using this approach (see Straffon *et al*., 1998) led us to conclude that subunit *b* was required for the continued stability and immunodetection of both OSCP and subunit *d* and that OSCP was required for the continued stability and immunodetection of subunit *d* within the cell. Thus, the results indicated a hierarchy of stability for the three subunits studied. On the basis that the stabilization of each subunit could be ascribed to its assembly into nascent mtATPase complexes or a subassembly, we interpreted the order of assembly to be as follows: subunit *b* followed by OSCP and then subunit *d* (Straffon *et al*., 1998). It is possible that this assembly of F_o subunits takes place on already assembled F_1 complexes.

Our still somewhat limited knowledge of OSCP assembly, and indeed the overall assembly of F_o, especially in light of the now recognized subunit complexity of F_o within mtATPase, deserves a more detailed investigation. Any new information will have to be integrated with the ordered assembly of the three mitochondrially encoded F_o subunits 9, 8, and 6 previously recognized in studies carried out by the Monash laboratory (see Cox *et al*., 1992; Prescott *et al*., 1996). Examination of immunoprecipitates of mtATPase (made using anti-F_1-α subunit antibodies) from mitochondrial lysates, prepared from cells exhaustively depleted of OSCP, revealed that of the three mitochondrially encoded subunits only subunit 6 was found to be absent (Prescott *et al*., 1994). Moreover, strains lacking one of several other nuclear-encoded F_o subunits also lack subunit 6, suggesting an interdependence of a number of subunits, including OSCP, with subunit 6 in F_o assembly (see Devenish *et al*., 2000). Taken together, the data concerning yeast F_o assembly can be interpreted as being dependent on ordered assembly of the mitochondrially encoded subunits 9, 8, and 6, such that coincident with assembly of subunit 6 other membrane integral subunits, such as *b* and *f*, assemble with the complex. Since the bulk of subunit *d* protrudes from the membrane into the matrix, then this provides a scaffold for assembly of OSCP and subunit *d*, as suggested by subunit-depletion studies (see above). The assembly of F_1 is likely to precede, or be coincident with, that of the stator stalk.

The stoichiometry of subunit *b*, in the bovine and yeast systems, is one (Hekman *et al*., 1991; Collinson *et al*., 1994; Spannagel *et al*., 1998; Bateson *et al*., 1999). This raises the question, when comparison is made with the bacterial complex containing two *b* subunits, of what takes the place of the missing subunit *b* in mtATPase. Considering the additional F_o subunits found in mtATPase, it seems most probable that one or more of these F_o subunits takes the place of the "missing" subunit *b*. Elsewhere, we have presented evidence suggesting that subunit *d* together with subunit 8 may be components of the stator stalk in yeast (Devenish *et al*., 2000; Stephens *et al*., 2000). Another candidate subunit for fulfilling the role of the second *b* subunit in the stator stalk of mtATPase is subunit F_6. Clearly this cannot pertain to the yeast complex because, although F_6 is considered an F_o component of the mammalian complex, no homolog has yet been identified in searches of the yeast genome database. To investigate any possible role for F_6 in the yeast complex, we have expressed the rat cDNA for F_6 in yeast cells. Overexpression of F_6 did not confer any obvious growth phenotype on cells and no association of F_6 with mtATPase could be demonstrated, although the protein was correctly localised to yeast mitochondria as determined by tagging F_6 with green fluorescent protein (M. Prescott, A. Lourbakos, P. Nagley, and R. J. Devenish, unpublished data 1997).

FUTURE DIRECTIONS

In terms of transmission of long-range conformational changes, recent, structural evidence concerning the enzyme complex (see above) helps to delineate possible transmission "pathways." Two possible suggestions concerning F_1 to F_o communication are that it may occur by transmission through contacts made by OSCP with the α and β subunits, or alternatively through the contact OSCP makes with subunit *b*. In the former case, influence could be exerted via the nucleotide binding/catalytic sites in F_1, or even transmitted further to the γ subunit and then to other components of the rotor, such as Y9. Alternatively transmission could occur through the stator stalk to the rotor. Here conformational changes in OSCP could presumably be transmitted from subunit *b* to Y6 via contacts made between hydrophobic membrane domains of these two subunits located in the inner mitochondrial membrane and, finally, to the Y9 ring, thereby influencing proton transfer. Close contacts between Y6 and Y9 are established, but not well defined, on the basis of their involvement in proton channel function and oligomycin resistance. The normal structural connections between subunit *b* and Y6/Y9 in the membrane are beginning to be elucidated through cross-linking studies (Velours *et al*., 2000). Details of the structural connections between the equivalent subunits in the bacterial complex (see Deckers-Hebestreit *et al*., 2000; Fillingame *et al*., 2000; Vik *et al*., 2000) should also be informative when extrapolated to mtATPase, although it is likely that the contacts seen may be modified by the presence of additional F_o subunits not present in the bacterial complex.

The role of subunit 8 (Y8) in proton channel or stalk function also warrants further examination as substitutions in Y8 can influence the physical coupling between F_1 and F_o. Cells expressing Y8 variants containing adjacent negative charges, such as Leu23 → Asp and Leu24 → Asp showed functional defects in mtATPase function. Further, the enzyme showed a 30% reduction in ATPase activity; the remaining activity was almost insensitive to the F_o inhibitor oligomycin (Roucou *et al*., 1999). Here the structural alterations resulting in the insensitivity ATPase to oligomycin may not result from a direct effect of the double substitution within Y8, but may be mediated by one or more other subunits proposed to be involved in the physical linking of F_o to F_1. Primary candidates for subunits involved in destabilization include subunits *d* and *f*, since in yeast mitochondria Y8 can be cross linked to these subunits (Stephens *et al*., 2000). Significantly, the efficiency of cross linking of Y8 to subunit *f* is quite different when the mtATPase is actively hydrolyzing ATP, compared to when it is resting with no nucleotide conversions (A. N. Stephens, R. J. Devenish, and P. Nagley, unpublished data 2000). This suggests the proximity of Y8 to subunit *f* varies according to the activity state of the enzyme complex. One reason for this may be that cycles of contraction and expansion of the stator stalk and central stalk take place during active ATP synthesis, as shown for the bacterial enzyme (Capaldi *et al*., 2000). These "vertical movements" within the ATP synthase presumably reflect cyclic storage and release of energy generated by proton pumping, transduced to the stator movements and linked to adenine nucleotide phosphorylation on the active sites of F_1-β subunits.

CONCLUDING REMARKS

Clearly, oligomycin has been a powerful tool to dissect structure and function of mtATPase. Improved knowledge of the contacts that OSCP makes in mtATPase will further elucidate the role of OSCP in structure and function of mtATPase. Particularly important will be to gain an understanding of the modulation of these contacts during different bioenergetic phases of enzyme function. The concerted interactions and shifts in protein structure that take place in the ATP synthase complex are an important aspect of the function of this enzyme in energy transduction. The study of protein variants relating to the oligomycin axis should continue to play an important role in understanding the workings of this "splendid molecular machine" (Boyer, 1997).

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