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Modulation at a Distance of Proton Conductance through the *Saccharomyces cerevisiae* Mitochondrial F_1F_0-ATP Synthase by Variants of the Oligomycin Sensitivity-Conferring Protein Containing Substitutions near the C-Terminus

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We have sought to elucidate how the oligomycin sensitivity-conferring protein (OSCP) of the mitochondrial F_1F_0-ATP synthase (mtATPase) can influence proton channel function. Variants of OSCP, from the yeast *Saccharomyces cerevisiae*, having amino acid substitutions at a strictly conserved residue (Gly166) were expressed in place of normal OSCP. Cells expressing the OSCP variants were able to grow on nonfermentable substrates, albeit with some increase in generation time. Moreover, these strains exhibited increased sensitivity to oligomycin, suggestive of modification in functional interactions between the F_1 and F_0 sectors mediated by OSCP. Bioenergetic analysis of mitochondria from cells expressing OSCP variants indicated an increased respiratory rate under conditions of no net ATP synthesis. Using specific inhibitors of mtATPase, in conjunction with measurement of changes in mitochondrial transmembrane potential, it was revealed that this increased respiratory rate was a result of increased proton flux through the F_0 sector. This proton conductance, which is not coupled to phosphorylation, is exquisitely sensitive to inhibition by oligomycin. Nevertheless, the oxidative phosphorylation capacity of these mitochondria from cells expressing OSCP variants was no different to that of the control. These results suggest that the incorporation of OSCP variants into functional ATP synthase complexes can display effects in the control of proton flux through the F_0 sector, most likely mediated through altered protein–protein contacts within the enzyme complex. This conclusion is supported by data indicating impaired stability of solubilized mtATPase complexes that is not, however, reflected in the assembly of functional enzyme complexes *in vivo*. Given a location for OSCP atop the F_1-$\alpha_3\beta_3$ hexamer that is distant from the proton channel, then the modulation of proton flux by OSCP must occur "at a distance." We consider how subtle conformational changes in OSCP may be transmitted to F_0.

KEY WORDS: Yeast mitochondria; F_1F_0-ATPase; OSCP; proton conductance.

INTRODUCTION

F-type ATP synthases catalyze the formation of ATP from ADP and inorganic phosphate, driven by the proton-motive force (pmf) generated by respiratory or photosynthetic electron transport (Boyer, 1997; Weber and Senior, 1997). This enzyme complex has

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generally been considered as composed of two parts, F_1 and F_0. F_1 is the water-soluble portion that contains three high-affinity nucleotide binding sites used in ATP synthesis and consists of five different subunits in a defined stoichiometry ($\alpha_3\beta_3\gamma\delta\varepsilon$). F_0 encompasses the membrane-integral proton channel and has a less well-defined subunit composition and stoichiometry. F_0 consists of at least three subunits (ab_2c_{9-12}) in its relatively simple form as found in *Escherichia coli*, but includes many additional subunits in the mitochondrial enzyme of yeast and mammals (see Devenish *et al.*, 2000). Electron microscopy studies have shown that in *E. coli* the F_1 part is attached to the F_0 part by two stalks (Wilkens and Capaldi, 1998). One is a central stalk that includes the γ and ϵ subunits (Capaldi *et al.*, 1994), which rotate during ATP synthesis on the β subunits. The second is a peripheral stalk composed of the δ and *b* subunits and which acts as a stator to hold the $\alpha_3\beta_3$ hexamer during ATP synthesis (Rodgers *et al.*, 1997; Wilkens *et al.*, 1997; Rodgers and Capaldi, 1998). Indeed, cross-linking studies (Lill *et al.*, 1996; Ogilvie *et al.*, 1997) place the N-terminus of subunit δ near the top of F_1. This location is supported by more recent immunoelectron microscopy studies indicating that δ binds on top of the $\alpha_3\beta_3$ hexamer (Wilkens *et al.*, 2000). Subunit *b* is anchored in the membrane and extends into the matrix to contact subunit δ; the C-termini of the two subunits are in close contact (McLachlin *et al.*, 1998; Rodgers and Capaldi, 1998). On this basis, it is clear that bacterial subunit δ is certainly not a component of the membrane F_0 proton pore.

Evidence for a second peripheral stalk in mitochondrial complexes has recently been presented (Karrasch and Walker, 1999). By analogy with bacterial ATP synthase the peripheral stator stalk in mitochondrial complexes is minimally comprised of F_0 subunits *b* and OSCP (MacLennan and Tzagoloff, 1968; Tzagoloff, 1970). The latter subunit has long been considered the homolog of bacterial subunit δ (Ovchinnikov *et al.*, 1984a,b). Previous studies have established the importance of OSCP in making structural connections between F_1 and F_0 in mtATPase (Dupius *et al.*, 1983; Mukopadhyay *et al.*, 1992; Joshi *et al.*, 1996, 1997; Mao and Mueller, 1997; Golden and Petersen, 1998), as well as in the coupling of proton translocation to ATP synthesis (Pringle *et al.*, 1990; Mukhopadhyay *et al.*, 1992). This is emphasized by the demonstration in yeast that OSCP is essential for mtATPase assembly and function (Uh *et al.*, 1990). The available evidence suggests that OSCP in mtATPase makes physical contact with both the α and β subunits of F_1 (Joshi *et al.*, 1996; Golden and Pedersen, 1998), as well as with the C-terminus of subunit *b* (Velours *et al.*, 1998; Soubannier *et al.*, 1999). These observations support a location for OSCP in mtATPase equivalent to that of bacterial subunit δ on the outer upper surface of the F_1-$\alpha_3\beta_3$ hexamer. Thus, the following key questions arise. How does OSCP modulate proton flux through the F_0 sector? Is the modulation related to a role as an "inert" component of the stator stalk or can subtle changes in the structure of OSCP be communicated to the catalytic sites on $\alpha_3\beta_3$, or even the membrane F_0 subunit components of the rotor?

We sought to address these questions by expressing in yeast OSCP variants likely to perturb subunit–subunit interactions in mtATPase based on the observed phenotype conferred by the equivalent variants in bacteria. The variants carried either of two defined substitutions, Asp or Asn, at a highly conserved Gly residue (position 166 in mature OSCP and equivalent to Gly150 of *E. coli* subunit δ). In bacteria, these substitutions dramatically affect the coupling of proton transport through F_0 to ATP synthesis or hydrolysis (Hazard and Senior, 1994), such that Gly150 $\rightarrow$ Asp resulted in an assembly defect and Gly150 $\rightarrow$ Asn in an uncoupling of proton transport. Use of the yeast system allows a combined approach of molecular genetic manipulation to test variants in terms of overall function and mtATPase assembly, as well as more detailed examination of bioenergetic parameters, including the use of inhibitors, such as oligomycin, to probe proton channel function. This strategy permits the direct consequences of the substitutions in OSCP to be determined in whole cells, as well as in intact mitochondria.

The results reveal that the variants are both functionally assembled *in vivo*. Moreover, bioenergetic characterisation of mitochondria isolated from cells expressing the OSCP variants clearly demonstrate that OSCP is involved in the control of proton flow through the F_0 proton channel. We discuss how OSCP may participate in such control in view of its likely location in the mtATPase complex as a component of the stator stalk, distant from those F_0 subunits comprising the proton pore.

EXPERIMENTAL PROCEDURES

Materials

Rhodamine 123, oligomycin, *N,N′*-dicyclohexylcarbodiimide (DCCD), triethyltin bromide (TET-Br),

aurovertin B, citreoviridin, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), antimycin A, BSA (fatty acid free), trioctylamine, 1,1,2-trichlorotrifluroethane, β-octylpyranoside, ε-amino-*N*-caproic acid, *p*-aminobenzamide, and phenylmethylsulfonyl fluoride were purchased from Sigma. Firefly luciferase, firefly luciferin, ATP, and ADP and were purchased from Boehringer-Ingelheim (Sydney, Australia). Vistra Chemiluminesent Substrate Kit was purchased from Amersham Pharmacia Biotech (Sydney, Australia). Restriction enzymes and deoxynucleotides were purchased from Progen (Queensland, Australia).

Construction of Expression Vectors

The multicopy expression vector pAS1N containing a *Bgl*II/*Not*I cloning site was constructed by inserting into the *Bgl*II cloning site of pAS1 (Prescott *et al.*, 1995) a *Bgl*II/*Bam*HI fragment bearing an internal *Not*I site immediately adjacent to the *Bam*HI site. In this manner, the original *Bgl*II site 3′ to the insert was converted to an uncleavable *Bam*HI/*Bgl*II site fusion.

Mutagenesis of the *ATP5* Gene

For the studies reported here, use was made of a variant of OSCP containing two C-terminally substituted methionine residues, which is functionally equivalent to natural OSCP (Bateson *et al.*, 1996). A gene cassette (*ATP5-M*) encoding the precursor protein was retrieved by PCR using the oligonucleotide primers OSCPPCR2 (5′-GTCGACAGATCTACACAATGTTTAATAGAGTCTTTACCAGG-3′) and OSCPMNOT (5′-ATAGTTTAGCGGCCGCTTACATGCTGTCC-3′). Underlined nucleotides indicate the position of nucleotides specifying the unique *Bgl*II and *Not*I restriction sites, respectively. PCR products were cloned into the *Bgl*II/*Not*I expression site of pAS1N to form pAS1N:*ATP5-M*. Site-directed mutagenesis was performed using a megaprimer strategy involving two sequential PCR steps (Barik, 1993). The oligonucleotide primers used annealed to the 5′ end of the *ATP5-M* gene (OSCPPCR2), the region encoding G166 (YG166D/N, 5′-GATTAAGGGT**G**/**A**ATTTGATTGTAG-3′) of the pAS1N: *ATP5-M* template, and the *PGK1* transcriptional terminator (PGKTERM, 5′-CACCTGGCAATTCCTTACCTTCC-3′) respectively. Boldface nucleotides indicate the position of degeneracy within the primer YG166D/N that specified the two alternative codon changes [GGT (Gly) → AAT (Asp) or GGT (Gly) → GAT (Asn)] during a single PCR. The numbering of amino acids refers to that of the mature form of OSCP from yeast that lacks the 17 amino acids of the cleavable mitochondrial import signal peptide. A PCR product of 179 bp was amplified from the template pAS1N:*ATP5-M* using the oligonucleotides YG166D/N and PGKTERM. This PCR product was purified by agarose gel electrophoresis, and used as a megaprimer in a second hot start (5 min at 95°C) PCR containing, in addition to the standard reaction components, *Tth-Plus* polymerase (Amersham Pharmacia Biotech), pAS1N:*ATP5-M* as template (2 μg), OSCPPCR2 (50 pmol), megaprimer (800 ng), and 5% (v/v) DMSO in a final volume of 100 μl. The resulting product was digested with *Bgl*II and *Not*I, and the 651 bp fragment cloned into the expression site of pAS1N. Vectors (pAS1N:*ATP5-M,G166N* or pAS1N:*ATP5-M,G166D*) containing either of the two possible nucleotide substitutions were identified by sequencing of individual clones.

Expression of OSCP Variants

The strains of *S. cerevisiae* used in this study were derived from strain YRD15 (*MATa his3-11,3-15 leu2-3,2-112 ura3-251,3-373*). Gene cassettes encoding *ATP5-M* or variants in the multicopy expression vector pAS1N were expressed in the yeast strain, A5N, lacking endogenous OSCP (Prescott *et al.*, 1994). A dual-plasmid strategy (Law *et al.*, 1995) was used to generate strains bearing the relevant OSCP expression constructs. Introduction of yeast expression vectors into yeast cells was as described (Elble, 1992). The derivative strains used in this study are designated as follows; YG166D and YG166N denote strains expressing OSCP-M variants with the substitution Gly166 → Asp or Gly166 → Asn, respectively. The control strain, A5NPn, expresses OSCP-M.

Growth of Yeast Strains

Solid YEPE growth medium (Gray *et al.*, 1996) contained 1.5% (w/v) agar. For growth studies in liquid medium, two experimental regimes were used. In both regimes cells harvested from the midlogarithmic phase of growth were used to inoculate SaccE medium (Gray *et al.*, 1996), supplemented with 20 μg/ml each of

histidine, leucine, or uracil, as required. For growth studies in the presence of oligomycin, cells were inoculated at an optical density at 650 nm of 0.05 into the wells of a sterile 96-well microtiter plate (Nunc) containing 100 μl medium and incubated in a humidified atmosphere with shaking. Growth was followed with a plate reader (BioRad model 3550) using the 655-nm filter. In other growth studies, cells (50 μg dry weight) were used to inoculate 100 ml of fresh SaccE medium and incubated with shaking at 28 or 35°C. Experiments were performed in triplicate. Growth was followed using a colorimeter (Klett-Summerson).

Isolation of Mitochondria

Cells were cultured at 28°C in well-aerated SaccE medium, and harvested during early logarithmic phase of growth. Mitochondria were isolated as described (Boyle *et al.*, 1999). Mitochondria were used immediately for studies of bioenergetic parameters and F_1F_0-ATP synthase complex assembly. Protein estimation was made by Dye-Binding Protein Assay (BioRad).

Immunoprecipitation of F_1F_0-ATP Synthase from Isolated Mitochondria

Immunoprecipitation of F_1F_0-ATP synthase was performed as previously indicated (Bateson *et al.*, 1996), using an immobilized monoclonal antibody directed against the β subunit of the F_1 sector (Hadikusumo *et al.*, 1984).

ATPase Activity of Isolated Mitochondria

ATPase activity of isolated mitochondria was monitored spectrophotometrically by the oxidation of NADH in an enzyme-linked assay containing pyruvate dehydrogenase and lactate dehydrogenase, as previously described (Roberts *et al.*, 1979).

Mitochondrial Respiration

Respiration experiments were conducted as described (Boyle *et al.*, 1999). When required, F_0 inhibitors (oligomycin, DCCD, TET-Br) were added from an appropriate stock solution in ethanol or methanol. Respiratory substrate, ethanol, was added to a final concentration of 50 mM; this corresponded to state 4 respiration. State 3 respiration was initiated with the addition of between 0.25 to 1.0 mM ADP, pH 7.0. State 4b respiration was calculated after the phosphorylation of ADP (0.25 mM). Maximum respiration rates were measured after the addition of CCCP to a final concentration of 17 μM.

ATP Synthetic Activity of Isolated Mitochondria

ATP synthase activity was determined using a light-generating firefly luciferase/luciferin coupled enzyme assay as described (Boyle *et al.*, 1999). ATP production and oxygen consumption (see Mitochondrial Respiration Assay) were measured for the same assay.

Rhodamine 123 Fluorescence Assays of Membrane Potential in Isolated Mitochondria

Variations of mitochondrial transmembrane potential were followed by measurement of fluorescence quenching of rhodamine 123 (Emaus *et al.*, 1986; Guélin *et al.*, 1993). Mitochondria (300 μg) were resuspended in 3 ml of respiration buffer (see Boyle *et al.*, 1999) containing 0.09 μM rhodamine 123. Fluorescence due to rhodamine (Ex-485 nm; Em-533 nm) was followed using a Hitachi F-4000 fluorescence spectrophotometer. Mitochondria alone were added to the cuvette to give an initial quenched reading and then fluorescence recorded after each of the following additions: 50 mM ethanol, 50 μM ADP, 30 μg/mg oligomycin (9 μg per cuvette), 0.5 μM antimycin A, and 17 μM CCCP.

RESULTS

Growth of Cells Expressing OSCP Variants

Strains expressing OSCP variants, YG166D (OSCP Gly166 → Asp) and YG166N (OSCP Gly166 → Asn) were both capable of growth on solid medium containing the respiratory carbon source ethanol, similar to that of the control A5NPn expressing OSCP-M (data not shown). This primary test of function indicates that in each strain expressing an OSCP variant, mtATPase complexes were assembled and possessed

sufficient function to support respiratory growth. The growth properties of these strains were examined in more detail at 28°C in liquid medium. Generation times of strains expressing the OSCP variants (YG166D, 6.33 ± 0.28 h; YG166N, 8.35 ± 0.16 h) were significantly longer than those of A5NPn, the control strain (5.65 ± 0.28 h). The increase in generation time of the OSCP variant strains was also observed when strains were grown at 35°C (data not shown).

To test for alterations in structure and function of the F_0 sector of the complex, cellular growth in the presence of a specific inhibitor of mtATPase, oligomycin, was monitored. Oligomycin binding occurs at a region that includes F_0 subunits 9 and 6 (see Nagley, 1988; Cox *et al.*, 1992) and inhibits proton translocation through the proton channel (Matsuno-Yagi and Hatefi, 1993). The growth of control strain A5NPn at 28°C was substantially inhibited in liquid medium containing oligomycin at a concentration of 200 ng/ml and was completely inhibited at 400 ng/ml (Fig. 1). Strain YG166D showed a lowered threshold of sensitivity to inhibition by oligomycin: substantial inhibition of growth was observed at 100 ng/ml, with complete inhibition of growth at 200 ng/ml. Strain YG166N was far more sensitive to oligomycin: inhibition of growth was apparent at the lowest concentration of inhibitor tested, 20 ng/ml, with complete inhibition

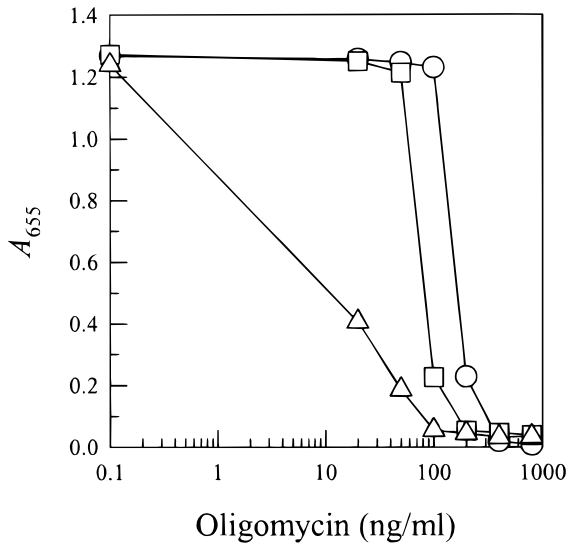

Fig. 1. Growth of strains expressing OSCP variants in the presence of oligomycin. Cells were inoculated into sterile 96-well microtiter plates and growth was followed as described in Experimental Procedures. Each data point represents the mean of triplicate experiments, with all readings being within 2% after 96-h growth. Strains: ○, A5NPn; □, YG166D; Δ, YG166N.

of growth at 100 ng/ml. The presence of the OSCP variants Gly166 → Asp or Gly166 → Asn in the mtATPase complex of intact cells thus confers increased sensitivity to oligomycin. This increased sensitivity was not due to an alteration of levels of mtATPase in the OSCP variant cells, as Western blot analysis indicated equivalent levels of OSCP and other subunits when compared to the control strain A5NPn (data not shown). This finding suggests that the functional interaction of F_1 with F_0 mediated by OSCP is intact, but modulated in some way. This change may be due to a structural alteration at the inhibitor-binding sites in F_0 (more efficient binding of oligomycin) or modulation of the transmission of the inhibitory effects of oligomycin through the complex to F_1.

Isolated Mitochondria from OSCP Variant Strains Have Elevated State 4 Respiration

The function of mtATPase in these OSCP variant strains was examined by investigation of respiration properties of mitochondria isolated from the control strain and both of the variant strains (Table I). For each mitochondrial preparation, the addition of 0.25 mM ADP was able to induce a transition from state 4a to state 3 respiration. Furthermore, the state 3 respiration rate for each mitochondrial preparation (variants and control) was similar, suggesting no apparent defects in these OSCP variant strains. These results also show that in isolated mitochondria the F_1 catalytic sector in each mtATPase complex containing an OSCP variant is functionally linked to the membrane F_0 sector, at least during state 3 respiration.

On the other hand, state 4 respiration rates in mitochondria isolated from OSCP variant strains were similar to each other but were significantly higher than those observed for the control strain A5NPn (Table I). This increase was observed for the respiration rates in both state 4a (before the addition of ADP) and state 4b (after complete phosphorylation of added ADP) respiration. The increased state 4 respiration rates were not due to a decrease in the level of OSCP or other mtATPase subunits, as Western blot analysis indicated similar levels of proteins in OSCP variants and control mitochondria (data not shown). This result suggested that during state 4 respiration in OSCP variant mitochondria, there is increased proton translocation across the inner membrane. Note that the arithmetic difference between state 4a and 4b respiration rates in variant mitochondria was similar to that in control mitochon-

Table I. Respiration and ATP Synthetic Rates in Isolated Mitochondria from OSCP Variant Strains[a]

Strain	Respiration rate (nmol O/min/mg protein)[b]				ATP synthetic rate	
	State 4a	State 3	State 4b	Maximal rate	nmol ATP/min/mg protein[c]	ATP/O ratio[d]
A5NPn	81.3 ± 3.3	238.6 ± 5.3	40.4 ± 6.9	259.2 ± 6.3	516.8 ± 43.0	2.20
YG166D	138.2 ± 11.1	238.1 ± 12.0	85.1 ± 7.3	257.2 ± 4.8	517.6 ± 104.5	2.18
YG166N	148.6 ± 10.0	243.0 ± 17.9	96.2 ± 15.1	257.2 ± 5.7	527.3 ± 60.8	2.14

[a] Results are presented as mean ± SD from at least triplicate assays of three independent mitochondrial preparations.
[b] Respiration rates were measured using ethanol (50 mM) as substrate. State 4a respiration was measured before the addition of ADP; state 3 respiration was measured after addition of ADP (0.25 mM); state 4b respiration corresponded to the rate after ADP was consumed; the maximal respiration rate was measured after addition of 17 μM CCCP.
[c] ATP synthetic rates shown represent triplicate assays from a single preparation of mitochondria.
[d] Similar ATP/O ratios were observed in three independent mitochondrial preparations.

dria. Thus, in variant mitochondria it is unlikely that the elevated respiratory activity during state 4 (a and b) is related to the mechanism responsible for differences between state 4a and 4b respiration rates for control mitochondria. Respiration rates in the presence of the protonophore CCCP were similar, indicating that mitochondria from all strains had the same maximal respiratory capacity with no defects elsewhere within the respiratory chain (Table I).

State 4 Respiration of OSCP Variant Mitochondria Is Hypersensitive to F_0 Inhibitors

It was important to identify the route of the enhanced proton translocation in mitochondria isolated from OSCP variant cells. Experiments were, therefore, carried out to distinguish between a nonspecific permeabilization of the mitochondrial inner membrane to protons or a more specific leak through the mtATPase itself. The respiratory activities of isolated mitochondria were titrated with the F_0 inhibitors oligomycin, TET-Br, and DCCD (Fig. 2; Table II). The abnormally high respiration rates of variant mitochondria were reduced in the presence of relatively small quantities of oligomycin (0.5 μg/mg protein) to the basal state 4 respiration rates determined for control A5NPn mitochondria in the absence of oligomycin both for states 4a (Fig. 2A) and 4b (Fig. 2B). No further reduction in state 4 respiration rates was observed at higher concentrations of oligomycin up to 10 μg/mg protein. State 4 respiration rates for control A5NPn mitochondria were essentially insensitive to oligomycin at concentrations up to 10 μg/mg protein. DCCD (10 μg/mg protein) or TET-Br (10 μM) were effective in a similar manner to oligomycin in reducing state 4 respiration rates for mitochondria from YG166N to the normal basal rate exhibited by the A5NPn control mitochondria (Table II). These inhibitors had equivalent inhibitory effects on state 3 respiration in both variant and control mitochondria (Table II). Similar results were obtained for mitochondria isolated from YG166D (data not shown). These results further suggest that the abnormally high respiration rates during state 4 are a result of increased proton translocation through the F_0 part of mtATPase, rather than a nonspecific permeability of the membrane to protons.

The effects of oligomycin on state 3 respiration of mitochondria were also determined (Fig. 2C). State 3 respiration in OSCP variant strains YG166D and YG166N is more sensitive to oligomycin than that in the control. The concentration of oligomycin giving half maximal inhibition of state 3 respiration in mitochondria from the strain A5NPn was approximately 8.1 μg/mg protein, compared to 3.1 and 2.2 μg/mg protein for mitochondria isolated from the variants YG166D and YG166N, respectively. This observation was consistent with the inhibition of cellular growth by oligomycin, which showed that strains YG166D and YG166N were approximately two- and fourfold more sensitive to oligomycin, respectively, than the control A5NPn (Fig. 1). It seems likely, therefore, that the increased effect of oligomycin on the growth of OSCP variant strains is due to a disruption of state 3 respiration in mitochondria.

Membrane Potential Reflects Increased Proton Translocation Capacity in State 4 Mitochondria from OSCP Variant Strains

Although results presented above suggested that the increased respiration rate during state 4 of OSCP

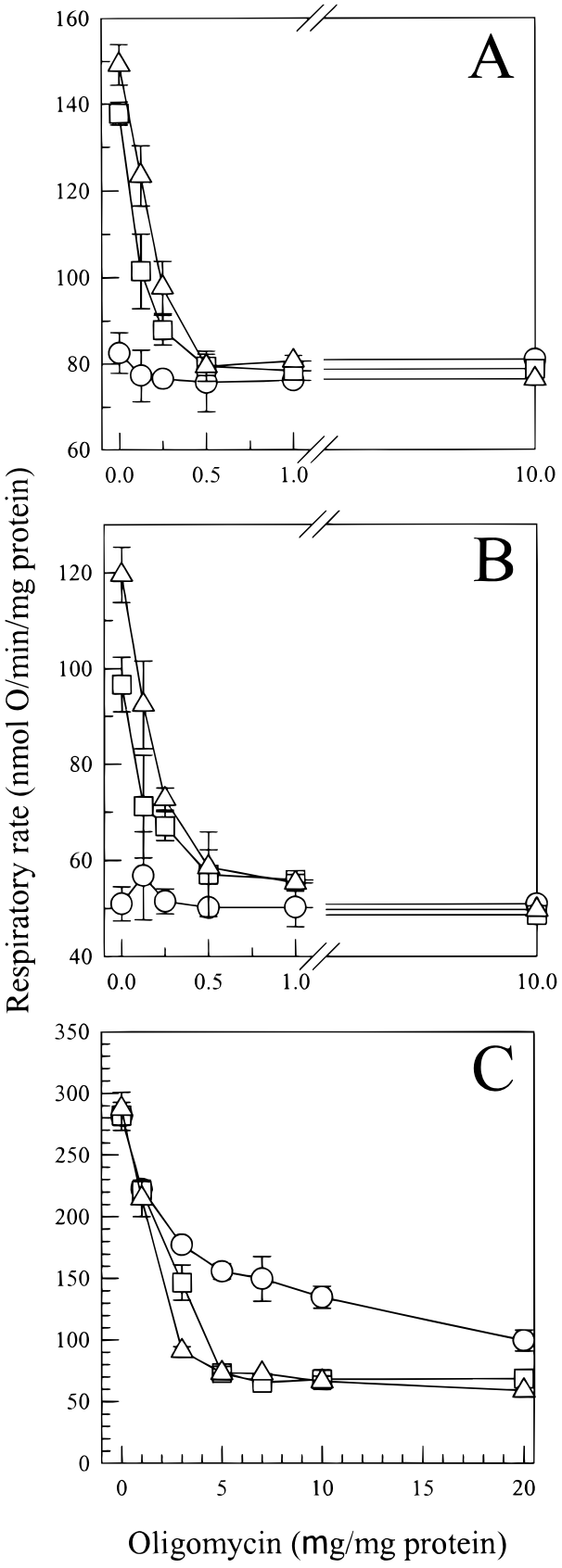

Table II. Inhibition by F_0 Inhibitors of Elevated State 4 Respiration in Isolated Mitochondria[a]

Strain	Inhibitor[c]	Respiration rate (nmol O/min/mg protein)[b] State 4a	State 3	State 4b
A5NPn	None	92.0 ± 1.6	298.3 ± 1.7	52.5 ± 2.7
	DCCD[d]	91.3 ± 1.8	146.8 ± 5.4	52.0 ± 4.5
	TET-Br[e]	93.1 ± 3.9	129.7 ± 0.1	53.4 ± 2.3
YG166N	None	150.9 ± 5.0	296.9 ± 2.4	109.3 ± 6.0
	DCCD[d]	89.7 ± 0.6	138.9 ± 3.2	53.9 ± 3.6
	TET-Br[e]	88.6 ± 1.3	93.6 ± 5.4	56.0 ± 3.3

[a] Mitochondria were prepared from cells grown at 28°C with ethanol as the carbon source.
[b] Results presented are mean ± SD from triplicate assays from a single mitochondrial preparation.
[c] The inhibitors were added after an initial state 4a respiration rate had been established, during state 3 respiration after addition of ADP (0.25 mM) or after the consumption of ADP, to observe the effect on state 4b respiration.
[d] DCCD was added to a final concentration of 10 μg/mg of mitochondrial protein.
[e] TET-Br was added to a final concentration of 10 μM.

variant mitochondria represented an increased proton translocation through the F_0 sector, we sought further evidence in support of this presumption. Verification that the increased state 4 respiration in mitochondria from OSCP variant strains was due to an increased proton translocation through the F_0 sector was obtained by use of the fluorescent dye rhodamine 123, whose uptake into mitochondria depends on the membrane potential (Fig. 3). In the case of mitochondria isolated either from A5NPn (Fig. 3A) or from YG166N (Fig. 3B), the addition of ADP to energized mitochondria (ethanol as respiratory substrate) resulted in a reduced membrane potential (detected as an increase in fluorescence) as the mitochondria entered state 3 respiration. The membrane potential returned to a steady state level when ADP was depleted and the mitochondria entered state 4b respiration. When oligomycin was subse-

Fig. 2. Oligomycin titration of respiration in mitochondria isolated from OSCP variant strains. (A) State 4a respiration; (B) state 4b respiration; (C) state 3 respiration. State 3 respiration was initiated by the addition of 0.25 mM ADP. Oligomycin was added to the concentrations indicated, either before the addition of ADP to find the effect on state 4a and b respiration, or alternatively, after the utilization of the ADP to find the effect on state 4b respiration alone. State 4a and b respiration rates were consistent using either of these methods. Note the different scale used in (C). Strains: ○, A5NPn; □, YG166D; Δ, YG166N.

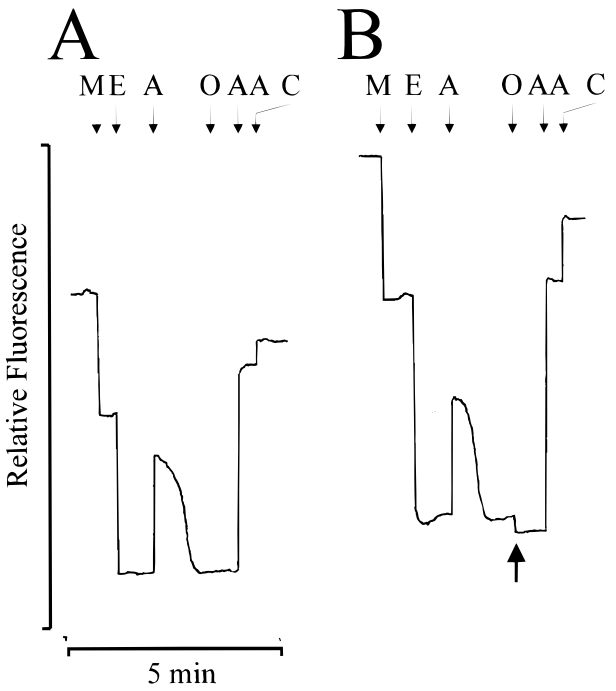

Fig. 3. Mitochondrial membrane potential monitored by fluorescent quenching of rhodamine 123. (A) A5NPn mitochondria; (B) YG166N mitochondria. Additions were 300 μg of mitochondrial protein (M), 50 mM ethanol (E), 50 μM ADP (A), 9 μg oligomycin (O), 0.5 μM antimycin A (AA), and 17 μM CCCP (C). The arrow under (B) indicates the decrease of fluorescence quenching on addition of oligomycin to YG166N mitochondria in state 4b respiration [not observed in (A) with A5NPn mitochondria].

quently added to YG166N mitochondria, a further increase in membrane potential resulted, as evident from the quenching of rhodamine 123 fluorescence (arrow in Fig. 3B). By contrast, an equivalent increase in transmembrane potential was not observed when oligomycin was added to A5NPn control mitochondria. This result shows that the F_0 inhibitor-sensitive respiration rate in the absence of ADP for YG166N mitochondria represents a decrease in mitochondrial transmembrane potential, indicative of an increase in proton translocation across the mitochondrial inner membrane. This was quite distinct from the situation in control A5NPn mitochondria.

Proton Translocation through F_0 during State 4 Respiration Is Not Coupled to Phosphorylation

To address the issue of whether such proton translocation in state 4 was coupled to phosphorylation, we investigated the effects of the F_1 inhibitors aurovertin B or citreoviridin on the respiration of mitochondria from YG166N and A5NPn. These inhibitors have been shown to inhibit F_1-ATPase from yeast by binding to the β subunit (Gause *et al.*, 1981). Both inhibitors were without effect upon state 4a respiration at a concentration of 5 μM. Under the same conditions, state 3 respiration for both mitochondrial preparations was significantly inhibited (48–60%) in the presence of aurovertin B or citreoviridin (data not shown). These results suggest that the increased proton translocation through the F_0 sector of F_1F_0-ATP synthase during state 4 respiration in OSCP variant mitochondria is not coupled to the synthesis of ATP. Moreover, these results also exclude the activity of any other ATPase in these mitochondrial preparations from YG166D or YG166N that would lead to the presence of ADP within the mitochondria for phosphorylation by mtATPase.

Efficiency of Oxidative Phosphorylation in OSCP Variant Mitochondria Is Normal

The rate of ATP synthesis during state 3 respiration for the mitochondria isolated from strains A5NPn, YG166D, and YG166N was examined to determine if the mitochondria from OSCP variant strains were functionally equivalent to those from A5NPn. In each case, the rate of ATP synthesis was similar (Table I). Similar ATP/O ratios in each case indicate that the efficiency of ATP synthesis is the same for mitochondria prepared from both variants and the control strain (Table I). There are two alternative explanations for these results. First, it is possible that the proton leak identified through F_0 during state 4 respiration does not persist during ATP synthesis, as one would expect the efficiency of phosphorylation to be reduced. Second, the proton leak may persist during ATP synthesis, but must contribute to phosphorylation. At present, a distinction between these explanations can not be made.

mtATPase Complexes from OSCP Variant Strains Are Structurally Unstable *in vitro*

ATPase (ATP hydrolase) activity was assayed in mitochondria prepared from A5NPn and the two vari-

ants YG166D and YG166N (Table III). Total ATPase rates were similar across all strains in this study, showing that the mitochondrial preparations contain the same amount of assembled F_1. The ATPase activity of A5NPn mitochondria was inhibited by approximately 77% in the presence of an excess of oligomycin (100 μg/mg protein). This value is consistent with values obtained from mitochondria routinely prepared from the control yeast cells. Oligomycin-sensitive ATP hydrolysis by mitochondria isolated from cells expressing OSCP variants was significantly reduced. Mitochondria prepared from YG166N exhibited the most severe reduction in oligomycin-sensitive ATPase activity, showing approximately 11% of ATPase activity sensitive to this inhibitor. Mitochondria from YG166D showed a less severe reduction with approximately 27% of ATPase activity being oligomycin sensitive. These data imply there is some disruption of functional links between F_1 and F_0 in OSCP variant mtATPase once mitochondria are lysed osmotically.

Physical Integrity of mtATPase Isolated from OSCP Variant Strains

Further evidence in support of instability of mtATPase complexes, following their solubilization from mitochondrial membranes with mild detergent, was sought 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. Immunoprecipitates of mtATPase were subjected to SDS–PAGE and polypeptides visualized by silver staining (Fig. 4A) or immunoblotting with antibodies directed against different subunits of the mtATPase (Fig. 4B). It was apparent that the amount of intact complex recovered

Table III. Oligomycin-Sensitive ATPase Activity in Isolated Mitochondria[a]

Strain	ATPase activity (nmol ATP/min/mg protein)[b]		Inhibition (%)
	−Oligomycin	+Oligomycin[c]	
A5NPn	978.8 ± 154.1	221.0 ± 7.2	77.4
YG166D	969.6 ± 154.5	705.2 ± 77.1	27.3
YG166N	965.7 ± 127.7	860.8 ± 78.5	10.9

[a] Mitochondria were isolated from cells grown at 28°C with ethanol as the carbon source.
[b] Results shown are presented as the mean ± SD from at least triplicate assays of three independent mitochondrial preparations.
[c] Oligomycin was added to 100 μg/mg mitochondrial protein.

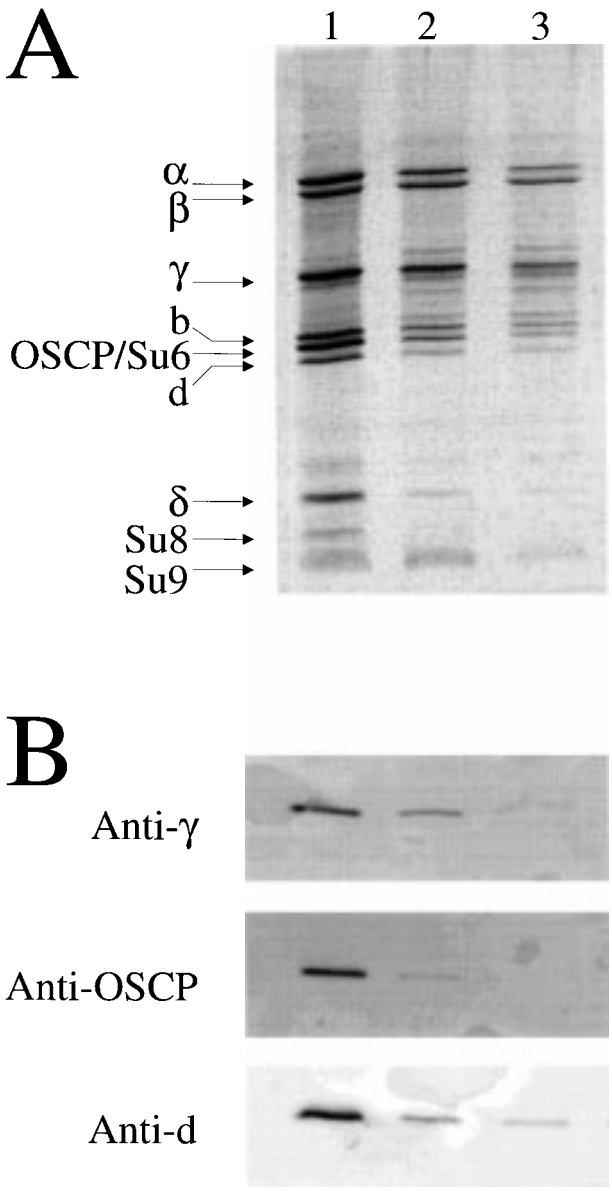

Fig. 4. Immunoprecipitation of mitochondrial ATP synthase containing OSCP variants. Samples of mitochondrial lysates were analyzed by electrophoresis on 15% SDS–polyacrylamide gels, pH 6.8 at 200 V. Subunits of mtATPase were detected by staining with silver (A) or by immunodetection with polyclonal antibodies directed against γ, OSCP, or subunit *d* (B) using Vistra chemiluminescent substrate with detection using the blue fluorescence mode on the Storm 860 Fluorescence Scanning System (Molecular Dynamics). Lane 1, A5NPn; lane 2, YG166D; lane 3, YG166N.

from mitochondria prepared from strains YG166D (Fig. 4A and B, lane 2) and YG166N (Fig. 4A and B, lane 3) was reduced when compared to that recovered from A5NPn mitochondria. Mitochondria prepared from strain YG166N showed a more severe depletion of subunit levels than YG166D mitochondria. However, quantitative immunoblotting of the proteins solubilized in SDS from whole cells or isolated mitochondria indicated that the levels of OSCP and other subunits of mtATPase were similar (data not shown). Furthermore, since the OSCP variant strains and A5NPn control strain have similar efficiencies of oxidative phosphorylation (Table I) and ATPase activities (Table III), the levels of F_1 subunits would be expected to be equivalent. A possible explanation for the disparity is that only the oligomycin-sensitive portion of the ATPase activity is the portion that is visualized in the immunoprecipitation experiment, the remaining mtATPase complexes having been structurally disaggregated as indicated by loss of oligomycin-sensitive ATPase (see above). The results do support the contention that incorporation of the OSCP variants Gly166 → Asp or Gly166 → Asn has resulted in the instability of the F_1 sector of the complex upon solubilization from mitochondrial membranes.

DISCUSSION

Properties of Cells Expressing OSCP Variants and Effects on mtATPase

Cells expressing OSCP variants, Gly166 → Asp and Gly166 → Asn, are able to assemble functional mtATPase complexes and grow by oxidative phosphorylation. Isolated mitochondria from these strains show the same level of ATPase activity, ATP synthetic rate, and ATP/O ratio as control (A5NPn) mitochondria. Furthermore, the data obtained in this study indicate that within intact and energized mitochondria the presence of normal OSCP is required for proper control of proton translocation through the F_0 sector of the complex, at least under conditions where no net ATP synthesis is occurring. In cells completely lacking OSCP mtATPase complexes are not assembled properly; the partially assembled F_1 sector is no longer bound to F_0 subunits (Uh *et al.*, 1990; Prescott *et al.*, 1994).

The altered rate of proton translocation in mtATPase complexes containing the two OSCP variants studied here is a specific feature of state 4a and state 4b respiration. Normal rates of ATP synthesis and state 3 respiration rates (and, in consequence, ATP/O values) indicate that proton conductance through the F_0 sector is normal under conditions where mtATPase is actively catalyzing ATP formation. The most likely explanation of the data is the existence of a conductance pathway common to both respiration states 3 and 4 in the mutant mtATPase complexes. Three F_0 inhibitors having different modes of action (oligomycin, DCCD, TET-Br), at low concentrations, each inhibit this unusual state 4 proton conductance. Previous observations by Razaka-Jolly *et al.* (1994) of differential inhibition of respiration by these F_0 inhibitors led to the identification of alternative proton dissipating pathways in the yeast mtATPase complex containing a variant of subunit *b*. Likewise, a proton flux in state 4, highly sensitive to very low concentrations of oligomycin, was found in mitochondria prepared from cells lacking the F_1-ε subunit, although, in this case, these mitochondria did not possess a functional F_1 sector (Guélin *et al.*, 1993). We cannot, however, eliminate the possibility that there is a novel and separate proton dissipation route in mtATPase complexes containing OSCP variants, which closes down upon transition from state 4 to 3 respiration.

Although oxidative phosphorylation in intact mitochondria isolated from OSCP variant strains was found to be normal, strains YG166D and YG166N show a generation time that was significantly increased over that of cells expressing normal OSCP. One possible explanation for the impaired growth in ethanol of YG166D and YG166N is that mitochondria *in vivo* are not always in an active state of net ATP synthesis (defined by state 3 respiration). It has previously been suggested that the energized state in mitochondria within actively growing cells lies, on average, between state 3 and 4 as defined in isolated mitochondria (Law *et al.*, 1995). If this were so, there being periods where mitochondria *in vivo* are effectively in state 4 (or close to that state), then the OSCP variant strains characterized here would pay a price in terms of reduced efficiency of respiratory growth because of partial dissipation of the electrochemical proton gradient *in vivo*.

Interestingly, the yeast OSCP variants confer on mtATPase a less debilitating effect than does the expression of the equivalent variants of subunit δ in bacteria. This may reflect differences in the molecular architecture and subunit composition of the mtATPase compared with bacterial ATP synthase (see Devenish *et al.*, 2000). It is clear from the results presented in

this study that subtle changes in the structure of OSCP, resulting from single amino acid substitutions, can influence inhibition of mtATPase activity by oligomycin. However, even a "variant" with several amino acid changes is compatible with OSCP function in yeast. Thus, the expression in yeast of rat OSCP (Prescott *et al.*, 1995), which has 58% homology with yeast OSCP (in terms of conserved amino acids) was able to satisfy both the spatial orientation requirements and subunit–subunit contact interactions required of OSCP within assembled mtATPase. It is noteworthy that, like the complexes incorporating either of the OSCP variants studied here, the chimeric complexes are more sensitive to inhibition by oligomycin and are structurally less stable upon isolation from mitochondrial membranes. Significantly, when equivalent substitutions of Gly166 → ASP or Gly166 → Asn are made in rat OSCP, such variants are nonfunctional when expressed in yeast (G.M. Boyle, P. Nagley, R.J. Devenish, and M. Prescott, unpublished data 1998). These observations strongly support the suggestion that residue 166 is important for the conformation of OSCP, such that, upon substitution, key contacts are altered (yeast OSCP) or lost (rat OSCP).

Molecular Communication between F_1 and F_0

The present evidence clearly suggests a proton modulation function can be ascribed to OSCP. It then remains to be determined how such regulation of mtATPase activity is mediated; presumably this occurs through molecular communication between the F_1 and F_0 sectors. In support of such a transmission of conformational changes through mtATPase are the data of Matsuno-Yagi *et al.* (1985) indicating that the binding of oligomycin to F_0 of bovine mtATPase induces changes in the properties of F_1. These changes include binding of ADP or the binding of the fluorescent inhibitor, aurovertin, effects ascribed to conformational changes between the F_1 and F_0 sectors. The protein–protein interactions that form the route for the transmission from F_0 to F_1 of the effects of such initiating events, however, were not defined.

While the experiments reported here do not directly address the nature of the contacts of OSCP with F_1 or with F_0, they do imply that the normal contacts must be altered in a manner such that asssembly of mtATPase is not significantly perturbed. At this stage it is unclear whether the F_1 to F_0 communication occurs by transmission through contacts made by OSCP with the α and β subunits, or whether it is 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 or other components of the rotor. In the latter case, the structural deformities are transmitted to the membrane sector of F_0 via the stator stalk.

In terms of transmission of long-range conformational changes to components of the rotor consideration of recent new structural evidence concerning interactions between rotor subunits helps to delineate possible transmission "pathways." Determination of the X-ray crystal structure of yeast mitochondrial F_1, in association with the subunit 9 ring, has revealed that subunits γ and δ of mtATPase together form a footlike structure at the bottom of F_1. This structure sits on the surface of the subunit 9 ring and contacts the loop region of 6 to 7 monomer subunit 9 units (Stock *et al.*, 1999). In view of the recent demonstration that the bacterial homolog of subunit 9 (subunit *c*) rotates during ATP hydrolysis (Sambongi *et al.*, 1999) taken together these observations provide the evidence for the structural connection between a subunit 9 ring and the γ subunit such that they can rotate in concert. These connections suggest that OSCP could exert an influence on rotor function and hence proton translocation via transmission of conformational changes through subunits α and β and their interaction with the γ subunit.

With regard to the possibility of transmission through the stator stalk to the rotor, it is well established that there is an interaction between the C-termini of subunit *b* and OSCP (Velours *et al.*, 1998; Soubannier *et al.*, 1999). Furthermore, deletions at the C-terminus of OSCP affect mtATPase assembly and function (Joshi *et al.*, 1992; Mao and Mueller, 1997). Interestingly, amino acid substitutions in subunit *b* can also lead to reduced sensitivity to inhibition by oligomycin (Razaka-Jolly *et al.*, 1994). Conformational changes in OSCP could presumably be transmitted from subunit *b* to subunit 6 via contacts made in the inner mitochondrial membrane. In the bacterial complex evidence in support of a linkage between the equivalent subunits, *b* and *a*, has recently been reported (Altendorf *et al.*, 2000; McLachlin *et al.*, 2000). The N-terminal third of subunit *b* in yeast is embedded in the membrane via two transmembrane stems in close proximity to subunit 6 (Velours *et al.*, 1998), a membrane integral protein with several transmembrane stems (see Nagley, 1988). Two transmembrane stems of subunit 6 are implicated in proton transfer since amino acid substitu-

tions have been identified that result in ATP synthase complexes resistant to inhibition by oligomycin (see Nagley, 1988). What remains to be delineated in this context for mtATPase are the structural connections between subunit *b* and subunit 6 in membrane relative to each other and also to subunit 9. Such information may shed light on how these subunits could participate in interactions with subunit 9 leading to proton tranfer. Accepting the likely existence of such structural connections at face value, it is then possible that long-range transmission of conformational changes in OSCP could be transmitted through subunit *b* to subunit 6 and finally to the subunit 9 ring, thereby influencing proton transfer.

Returning to the proposition that OSCP may regulate proton translocation in relation to F_1 catalytic activity, one may particularly consider those bioenergetic phases when mtATPase is intended to be inactive. It is expected that significant and dynamic modulation of particular protein–protein contacts and alterations in individual subunits conformations would occur within the ATP synthase complex in transition from state 3 to 4 respiration as well as during individual cycles of ATP synthesis or hydrolysis. Indeed, it has been recently shown that subunit δ in chloroplast ATP synthase 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). An "idling" phenomenon involving conformational changes to subunit γ had earlier been suggested to take place in the chloroplast complex that was suppressed in the presence of added ADP. The molecular components of the "clutch" mechanism enabling the resumption of coupling between proton translocation and ATP synthesis were not identified, but were proposed to be in the F_1 sector (Frische and Junge, 1996). In these terms, OSCP may function as part of such a clutch mechanism in the yeast mtATPase complex.

In conclusion, improved knowledge of the contacts that OSCP and other F_0 subunits make in mtATPase, together with an understanding of the modulation of these contacts during different bioenergetic phases of enzyme function, will further elucidate the role of OSCP in structure and function of mtATPase.

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