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Mitochondrial F_0F_1 H^+-ATP synthase

Characterization of F_0 components involved in H^+ translocation

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The membrane F_0 sector of mitochondrial ATP synthase complex was rapidly isolated by direct extraction with CHAPS from F_1-depleted submitochondrial particles. The preparation thus obtained is stable and can be reconstituted in artificial phospholipid membranes to result in oligomycin-sensitive proton conduction, or recombined with purified F_1 to give the oligomycin-sensitive F_0F_1-ATPase complex. The F_0 preparation and constituent polypeptides were characterized by SDS-polyacrylamide gel electrophoresis and immunoblot analysis. The functional role of F_0 polypeptides was examined by means of trypsin digestion and reconstitution studies. It is shown that, in addition to the 8 kDa DCCD-binding protein, the nuclear encoded protein [(1987) J. Mol. Biol. 197, 89–100], characterized as an intrinsic component of F_0 (F_0I, PVP protein [(1967) J. Biol. Chem. 242, 2547–2551]) is involved in H^+ translocation and the sensitivity of this process to the F_0 inhibitors, DCCD and oligomycin.

Dicyclohexylcarbodiimide; F_0; F_1; H^+ transporting ATP synthase; Oligomycin; Proton translocation

1. INTRODUCTION

The F_0F_1 H^+-ATP synthase of mitochondria, bacteria and chloroplasts is a multi-subunit enzyme composed of two structurally and functionally distinct oligomeric moieties, F_1 and F_0, which are responsible for chemical catalysis and proton conduction, respectively (reviews [1–3]). The F_1 moiety can be readily released from the membrane as a soluble oligomer, consisting invariably of five non-identical protein subunits [2,4,5] with a stoichiometry of 3α, 3β, 1γ, 1δ, 1ϵ. The subunit composition of the F_0 sector, in contrast, varies from a minimum of 3 polypeptides in *E. coli* F_0 to 5–8 in F_0 of eukaryotic enzymes [2,3].

Bovine heart F_0 preparations thus far reported are generally obtained via elaborate procedures based on NaBr [6,7] or urea [8,9] treatment of the purified F_0F_1 complex.

Here, F_0 was purified directly after CHAPS extraction from F_1-depleted bovine heart submitochondrial particles (USMP). The F_0 thus obtained is stable and can be reconstituted in phospholipid membranes resulting in oligomycin-sensitive proton conduction.

The F_0 preparation contained 7 intrinsic components. The functional role of F_0 polypeptides was examined by extraction of F_0 from control and

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Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; ESMP, submitochondrial particles prepared in the presence of EDTA; USMP, submitochondrial particles devoid of F_1 (see section 2); SDS-PAGE, SDS-polyacrylamide gel electrophoresis; *Enzyme:* F_0F_1 ATP synthase (EC 3.6.1.34)

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trypsin-treated USMP and reconstitution in artificial phospholipid membranes.

It is shown that, in addition to the 8 kDa DCCD-binding protein (subunit c) [10,11], the nuclear encoded PVP protein [12] plays a critical role in transmembrane proton conduction by mitochondrial F_0.

2. MATERIALS AND METHODS

2.1. *Materials*

CHAPS, oligomycin, valinomycin and asolectin were purchased from Sigma (USA); SDS, goat anti-rabbit IgG labelled with peroxidase, horseradish peroxidase color development reagent and molecular mass standards from BioRad (USA); nitrocellulose membrane (0.45 μm pore size) from Schleicher and Schuell (FRG); and [^{14}C]DCCD (50 Ci/mol) from Sorin Biomedica (Italy).

2.2. *Preparation of membrane particles and ATP synthase components*

ESMP were prepared as in [13] by exposing heavy beef heart mitochondria to ultrasonic energy. F_1-depleted urea particles (USMP) were prepared from ESMP as described [14]. OSCP was isolated as in [15], F_6 according to [16] and PVP protein as described in [12,15]. For isolation of F_0 and F_1 see section 3.

2.3. *Electrophoretic analysis and immunoblotting procedure*

SDS electrophoresis was performed on slabs of a linear polyacrylamide gradient (14–20%) as in [15]. After electrophoresis the separated proteins were transferred to nitrocellulose and immunolabelled as in [12,15], using specific rabbit sera against F_1 (1:250) [15] or PVP protein (F_0I) (1:250) [12,15]. Densitometry was performed on a Camag TLC densitometer (Switzerland).

Table 1

Chloroform extraction of bovine heart mitochondrial F_1

Particles	Protein (mg)	Recovery (%)	ATPase activity (U/mg protein)
ESMP	156	100	2.27
ESMP incubated 3 h at 0°C	126	81	1.99
ESMP incubated 3 h at 37°C	112	72	7.55
F_1 from ESMP 3 h at 0°C	3.4	2.2	56.13
F_1 from ESMP 3 h at 37°C	2.8	1.8	99.60

For ESMP preparation and measurement of ATPase activity see section 2. For F_1 purification, particles incubated for 3 h at 0 or 37°C, in 0.25 M sucrose, 20 mM K^+-phosphate (pH 8.4), were centrifuged at 105000 × g for 20 min and suspended in 0.25 M sucrose, 10 mM Tris-sulfate, 1 mM EDTA (pH 7.6) to a final protein concentration of 3–5 mg/ml. Chloroform extraction was then performed as described in [4]

2.4. *ATPase assay and protein determination*

ATPase hydrolytic activity was measured in the presence of an ATP-regenerating system as described [17]. Proteins were assayed according to [15].

2.5. *Incorporation of F_0 into liposomes and measurement of proton conductivity*

F_0-containing liposomes were prepared by cholate-deoxycholate dialysis [18]: 3 mg F_0 were mixed with 30 mg acetone-washed sonicated asolectin in 1 ml of 0.1 M phosphate buffer (pH 7.2) containing 1.6% potassium cholate, 0.8% potassium deoxycholate and 0.2 mM EDTA. The mixture was dialyzed overnight vs 0.1 M potassium phosphate (pH 7.5), followed by 3 h dialysis against 10 mM Na^+-Tricine buffer (pH 7.5). Both dialysis media contained 0.25 mM EDTA and 2.5 mM $MgSO_4$.

H^+ translocation was evaluated by potentiometrically determining H^+ release induced by valinomycin-mediated K^+ influx [12,19]. H^+ translocation in USMP was analyzed by following anaerobic release of the respiratory proton gradient [17].

3. RESULTS

3.1. *Isolation and characterization procedures*

Direct extraction of F_1 from submitochondrial

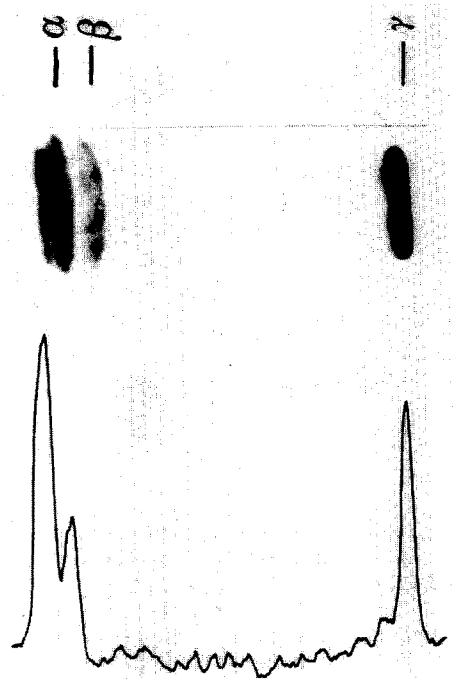

Fig.1. Immunoreactivity of the F_1 preparation. 10 μg protein of F_1 prepared as described in table 1 were subjected to SDS-PAGE and immunoblotting using rabbit antiserum against F_1 [15]. Nitrocellulose sheets were scanned at 590 nm.

particles with chloroform results in lower ATPase activity vs other F_1 preparations [4], probably due to the presence of the ATPase inhibitor protein (IF_1) [4] and contaminating proteins [4]. More active F_1 preparations were obtained by removing extrinsic proteins from the membrane before [20] or after [21] chloroform extraction, however the procedures described are rather lengthy.

In the method adopted here, effective treatment of ESMP to remove F_1 [22] was achieved by incubation for 3 h at 37°C and pH 8.4. In this way specific activity of ATPase increased 3–4-fold (table 1) and that of chloroform-extracted F_1 amounted to 100 μmol ATP hydrolyzed $\cdot$ $min^{-1} \cdot mg^{-1}$. SDS-PAGE of purified F_1 resolved the five subunits of F_1, of which α, β and γ reacted with antiserum raised against isolated F_1 (fig.1) (see also [15]).

Recently, a new zwitterionic detergent (CHAPS) has been used for purification of the F_0F_1 complex [23,24]. Using the same detergent, we succeeded in the direct purification of the F_0 sector from F_1-depleted bovine heart submitochondrial particles (USMP). The procedure consists of only two steps: Extraction of F_0 from USMP with CHAPS and purification of F_0 by centrifugation on 20% sucrose. This procedure, which thus avoids the time-consuming isolation of the F_0F_1 complex, yields purified F_0 which is in all respects comparable with F_0 preparations obtained from the purified F_0F_1 complex [6–9,25–27].

The results obtained from SDS-PAGE (fig.2)

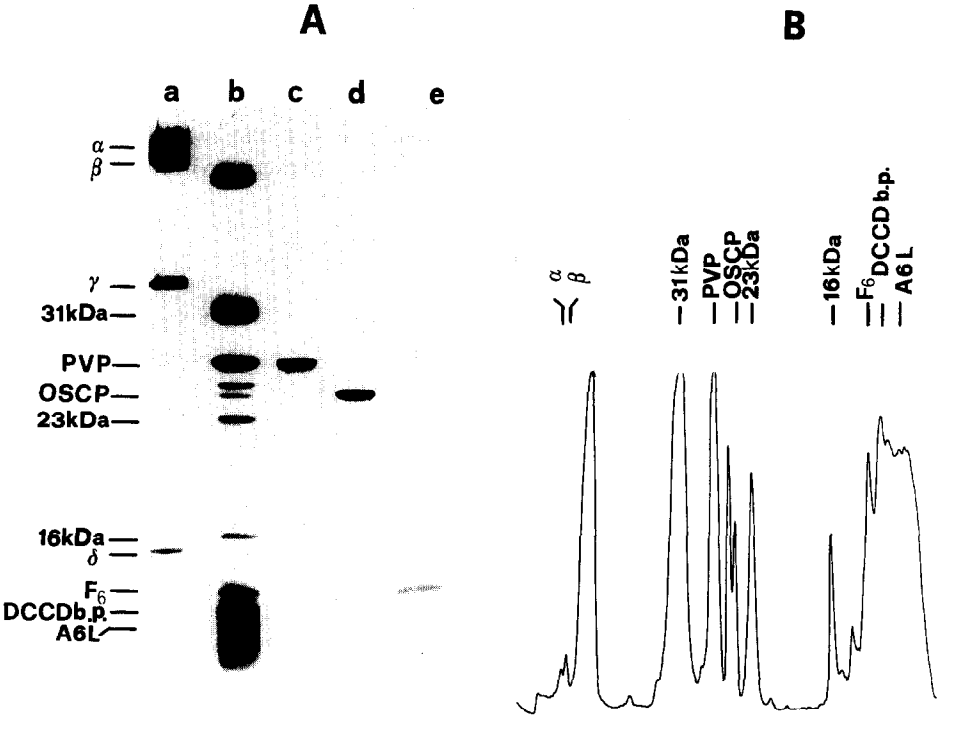

Fig.2. SDS-PAGE of isolated F_1, F_0, PVP protein, OSCP and F_6. For purification of F_0, USMP were suspended in 20 mM D-mannitol, 70 mM sucrose, 2 mM Hepes and 0.5 mg/ml of defatted bovine serum albumin (pH 7.4) at a concentration of 40–50 mg protein/ml and stored in liquid N_2. The stock USMP were thawed at room temperature and diluted to 2 mg protein/ml with 0.15 M K_2HPO_4, 1 mM ATP, 25 mM EDTA, 0.5 mM DTT and 5% ethylene glycol (pH 7.9). After centrifugation at 105000 × g for 45 min, the pellet was suspended and washed twice in the same buffer. The final pellet was suspended in TA buffer (50 mM Tricine, 1 mM ATP, 25 mM EDTA, 0.5 mM DTT and 5% ethylene glycol; pH 7.9) at a protein concentration of 4 mg/ml and incubated with 1% CHAPS for 10 min at 4°C. After 1 h centrifugation at 105000 × g the supernatant was layered on 20% sucrose in TA buffer (4 ml supernatant on 32 ml TA-sucrose) containing 0.2% CHAPS and centrifuged for 10 h at 27500 rpm at 2°C. After centrifugation the central 21 ml were concentrated (4–5 times) in an Amicon Diaflo apparatus with a PM-10 filter. The yield of purified F_0 sector was about 8–9 mg protein/100 mg protein of USMP. For F_1 preparation see table 1; other details, as given in section 2. (A) SDS-PAGE of: (a) 10 μg F_1, (b) 50 μg F_0, (c) 6 μg PVP, (d) 6 μg OSCP, (e) 6 μg F_6. (B) Densitometric analysis of F_0 sample (slot b of A) performed at 590 nm.

show that this F_0 preparation is devoid of F_1 polypeptides and contains the following characteristic components of the F_0 preparation: (i) a band of apparent molecular mass 27 kDa, identified as PVP-F_0I protein by immunoblot analysis (fig.3); (ii) a band of apparent molecular mass 25 kDa (consisting of a closely spaced doublet, the lower component being identified as OSCP [12,15]); (iii) a band of apparent molecular mass 23 kDa (F_0II in [26]) – this protein which was not labelled by thiol reagents [9,28] may represent the product of the ATPase 6 gene which has no codon for cysteine [29]. In addition, another four polypeptides with apparent values of 16, 10, 9–8 and 7 kDa (fig.2) were detected.

Among these bands, the 10 kDa species was identified as F_6 [16] and the 9–8 kDa band with the DCCD-binding protein (subunit c) by binding of [^{14}C]DCCD (fig.4). The 16 kDa band exhibited an even greater degree of [^{14}C]DCCD binding than that of the 9–8 kDa species (fig.4) and most likely represents the dimer of subunit c [11,30,31]. The 7 kDa band could represent the product of the A6L gene [29]. Additionally, a 31–33 kDa band was present which binds [^{14}C]DCCD (fig.4) but apparently does not belong to the F_0F_1 complex [30]. The 46 kDa band is not a component of F_0 and may represent a core protein of the *b*-c_1 complex [32].

3.2. *Reconstitution studies*

Purified F_0 per se exhibited no ATPase activity. When mixed with soluble F_1 in the presence of phospholipids, it produced marked inhibition of the hydrolytic activity which became oligomycin-sensitive (table 2). Thus, purified F_0 retained the capability of forming a functional complex with F_1.

F_0 reconstituted in liposomes exhibited oligomycin- and DCCD-sensitive proton conduction (fig.5). Proton release from F_0 liposomes, induced by a membrane potential difference generated by valinomycin-mediated K^+ efflux, amounted to about 900 ngion $H^+ \cdot min^{-1} \cdot mg\ protein^{-1}$.

F_0 extracted from trypsin-digested USMP [12,15] exhibited, when reconstituted in liposomes, lower proton conductivity which was practically insensitive to oligomycin (fig.5). It has been previously shown that the inhibition of passive proton conduction caused by trypsin treatment in

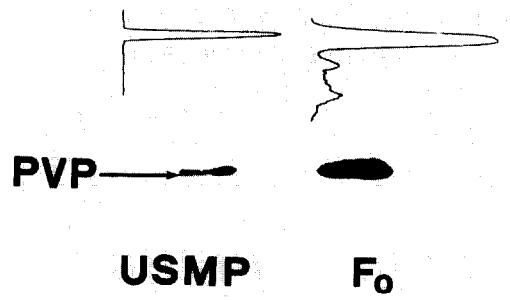

Fig.3. Comparison of PVP subunit content in USMP and F_0. 10 μg USMP and F_0 proteins were analysed by SDS-PAGE and immunoblotting, using antiserum against PVP subunit. Scans were performed at 590 nm.

USMP is directly correlated with digestion of the PVP (F_0I) protein [12,15]. Addition of purified PVP protein to liposomes inlaid with F_0 isolated from trypsinized USMP restored proton conductivity and its sensitivity to oligomycin (fig.5) (see also [2]).

In trypsin-treated USMP, passive proton con-

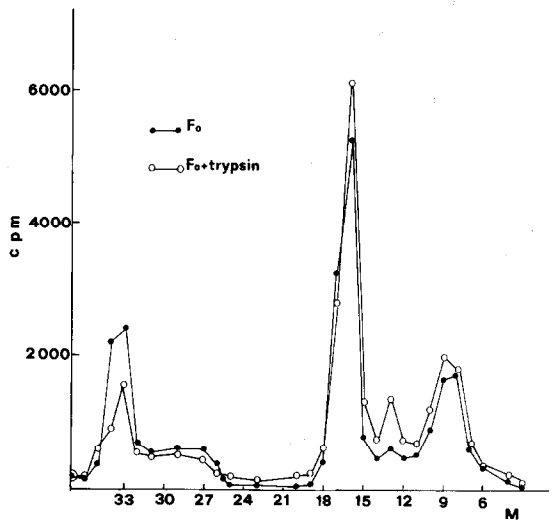

Fig.4. [^{14}C]DCCD binding to F_0 polypeptides. USMP or USMP treated for 20 min with trypsin (50 μg/mg protein) as in [12,15] were suspended, at a particle protein concentration of 3 mg/ml, in 0.25 M sucrose, 20 mM KCl and 30 mM K^+ succinate (pH 7.4). Particles were then incubated for 20 min with 30 μM [^{14}C]DCCD and centrifuged for 10 min at 105000 × *g*. F_0 was extracted as reported in the legend to fig.2, and subjected to SDS-PAGE (200 μg). Polypeptide bands were isolated as in [12,15] and used for determination of radioactivity [11]. The apparent molecular masses (kDa) of isolated proteins are reported on the abscissa.

Table 2

Reconstitution of F_0F_1 complex

	ATPase activity (μmol ATP hydrolyzed · min^{-1} · mg F_1^{-1})
F_1	99
F_0 + F_1	42
F_0 + F_1 + OSCP	37
F_0 + F_1 + F_6	33
F_1 + oligomycin	101
F_0 + F_1 + oligomycin	25
F_0 + F_1 + OSCP + oligomycin	23
F_0 + F_1 + F_6 + oligomycin	20

Purified F_0 (200 μg) was mixed with 85 μg liposomes, diluted to 200 μl with TA buffer (see legend to fig.2) and sonicated for 30 s. F_0 liposomes were incubated for 20 min at 25°C with 100 μg F_1 and 6 mM $MgCl_2$. An aliquot of this mixture was used for determination of ATPase activity. Where indicated 0.01 mg F_6 or 0.05 mg OSCP per mg F_0 were added to the F_1-F_0 complex and incubated for 20 min at 25°C. Addition of 20 μg oligomycin/ml is also indicated

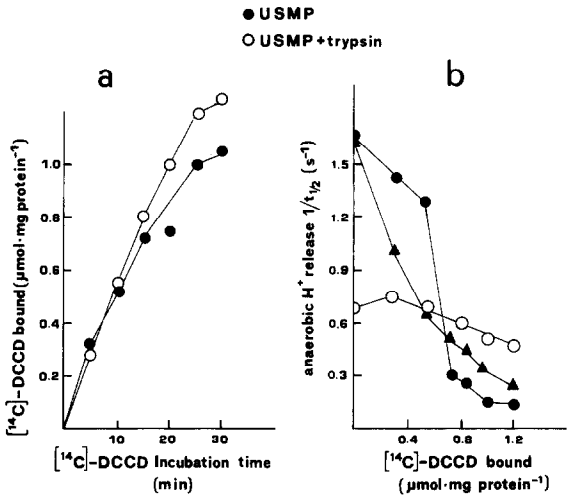

Fig.6. Effect of trypsin treatment of USMP on binding of [^{14}C]DCCD (a) and sensitivity of proton conduction to DCCD (b). Preparation of USMP and PVP protein and measurement of proton conduction were according to section 2. For treatment with [^{14}C]DCCD, see legend to fig.4 and [11]. (●—●) 3 mg/ml USMP; (○—○) 3 mg/ml trypsin-treated USMP [15], (▲—▲) trypsin-treated USMP (3 mg/ml) incubated for 15 min at 25°C with 0.5 μg PVP protein/ml.

ductivity was depressed by 50% with respect to untreated particles and was practically insensitive to the F_0 inhibitors oligomycin [12] and DCCD (fig.6b). However, no decrease in overall binding of [^{14}C]DCCD to F_0 was observed (fig.6a). Proton conduction and its sensitivity to DCCD were restored by addition of purified PVP protein to trypsinized USMP (fig.6b). Extraction of F_0 from [^{14}C]DCCD-labelled USMP showed that tryptic digestion producing 50% inhibition of proton conductivity did not affect [^{14}C]DCCD binding to the 8 and 16 kDa proteins (fig.4).

4. DISCUSSION

The 8 kDa DCCD-binding protein (subunit c) is an essential component of the H^+ channel of F_0 of H^+-ATP synthase from prokaryotes and eukary-

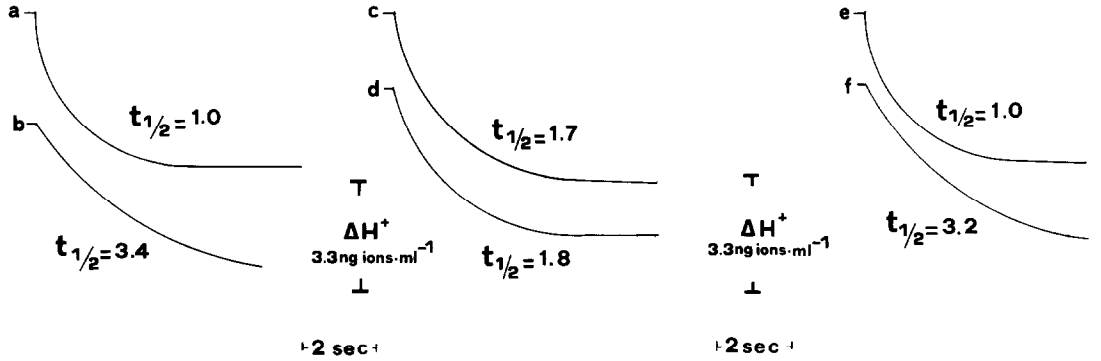

Fig.5. Restoration of H^+ conduction and oligomycin sensitivity by addition of purified PVP protein to liposomes reconstituted with F_0 extracted from trypsinized USMP. For preparation of F_0, see legend to fig.2; for PVP preparation and trypsin treatment of USMP see [12,16]. Details for F_0 liposomes and H^+ conduction are given in section 2. (a) F_0 liposomes (0.5 mg F_0/ml), (b) F_0 liposomes + 5 μg oligomycin/mg F_0 protein, (c) trypsinized F_0 liposomes, (d) trypsinized F_0 liposomes + 5 μg oligomycin/mg F_0 protein, (e) trypsinized F_0 liposomes + 6 μg PVP/mg F_0, (f) as (e) + 5 μg oligomycin/mg F_0.

otes [2,37], where it apparently functions in an oligomeric state [3,10,11].

Definite evidence is available showing that in the *E. coli* enzyme, in addition to subunit c, subunit a (uncB of the atp operon [2,33]) is also directly involved in proton conduction [34,35]. Subunit b (uncF of the atp operon) appears to be essential in *E. coli* for proper assembly of functional F_0 in the membrane [36,37].

Our studies show that the PVP (F_0I) protein of the mitochondrial enzyme plays a critical role in proton conduction by F_0. Previous results from our laboratories showed that this subunit, encoded by a nuclear gene, and consisting of 214 residues [38], extends out of the M side of the membrane where it is covered by F_1 [15].

Digestion by trypsin of the M region of the PVP protein (obtained after removal of F_1 [12,15]), resulting in the removal of a carboxyl-terminal tail of around 12 residues [28], causes partial (40–50%) inhibition of transmembrane proton conduction by F_0 in native membranes or, after isolation and reconstitution, in liposomes [12,28]. Residual proton conductivity of trypsinized F_0, which retained the remaining large N-terminal region of the PVP protein, was practically insensitive to inhibition by oligomycin (see also [12,28]) and DCCD.

Trypsin cleavage of the PVP protein, however, had no effect on DCCD binding to F_0 proteins, nor did oligomycin binding to F_0 on preventing digestion of the PVP protein (not shown).

Proton conduction by F_0 and its sensitivity to oligomycin and DCCD could be restored by adding back to trypsinized F_0 the native PVP protein, whilst addition of its N-terminal 18 kDa fragment or the 31 kDa protein, which was also digested by trypsin [15,28], was completely ineffective [28].

It is therefore apparent that the carboxyl-terminal region of the PVP protein exerts a critical influence on the oligomeric organization of the F_0 complex and its functional activity. The carboxyl-terminal region of PVP protein may promote proton conduction by favouring exchange of protons between the aqueous phase and the F_0 channel and/or by bringing the membrane-spanning protein segments of subunit c and other possible components of the channel into the proper active configuration.

These observations seem to support a model for proton conduction in F_0 based on dynamic interaction between the DCCD-reactive acidic residues of subunit c, the PVP protein (suggested to be analogous to subunit b of *E. coli*; see [37]) and possibly the product of the ATPase 6 gene, considered to be analogous to subunit a of *E. coli* [29].

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