

# Mitochondrial $F_0F_1$ $H^+$ -ATP synthase

## Characterization of $F_0$ components involved in $H^+$ translocation

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Received 23 March 1989

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  $3\alpha$ ,  $3\beta$ ,  $1\gamma$ ,  $1\delta$ ,  $1\epsilon$ . 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)

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 aroclorin 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  $\mu$ m 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],  $F_0$  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 ( $F_0$ ) (1:250)

Table 1

Chloroform extraction of bovine heart mitochondrial  $F_1$

| Particles                   | Protein Recovery (mg) | ATPase activity (%) | 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  $105000 \times g$  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]

[12,15]. Densitometry was performed on a Camag TLC densitometer (Switzerland).

### 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 aroclorin 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

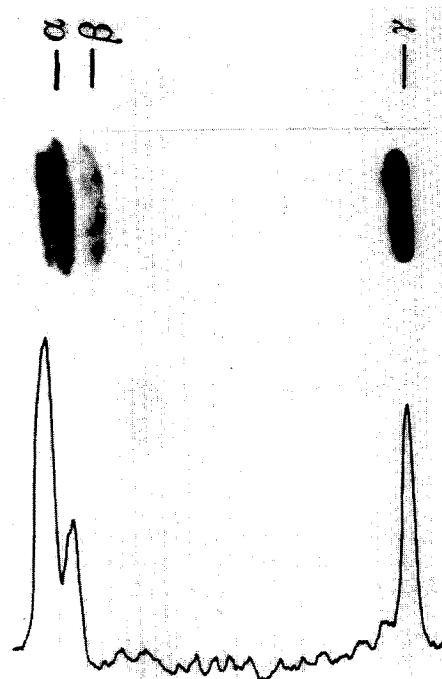


Fig.1. Immunoreactivity of the  $F_1$  preparation. 10  $\mu$ g protein of  $F_1$  prepared as described in table 1 were subjected to SDS-PAGE and immunoblotting using rabbit antiserum against  $F_1$  [15]. Nitrocellulose sheets were scanned at 590 nm.

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<sub>1</sub>) [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  $\mu\text{mol}$  ATP hydrolyzed  $\cdot\text{min}^{-1} \cdot \text{mg}^{-1}$ . SDS-PAGE of purified  $F_1$  resolved the five subunits of  $F_1$ , of which  $\alpha$ ,  $\beta$  and  $\gamma$  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)

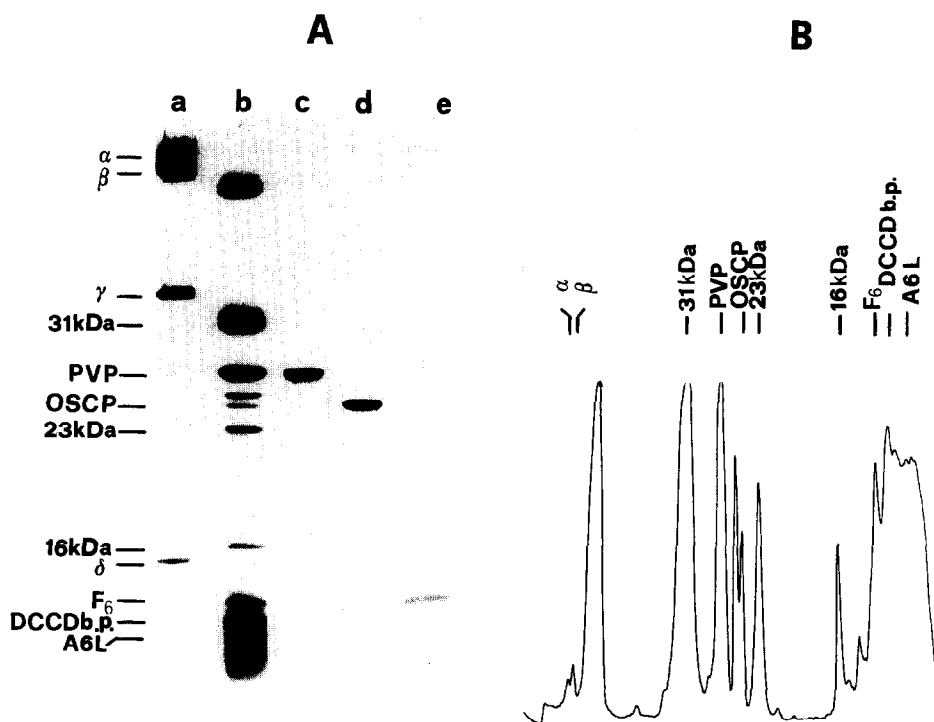


Fig.2. SDS-PAGE of isolated  $F_1$ ,  $F_0$ , PVP protein, OSCP and  $F_6$ . For purification of  $F_0$ , USMP were suspended in 20 mM D-mannitol, 70 mM sucrose, 2 mM Hepes and 0.5 mg/ml of defatted bovine serum albumin (pH 7.4) at a concentration of 40–50 mg protein/ml and stored in liquid  $N_2$ . 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  $105\,000 \times g$  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  $105\,000 \times g$  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 27 500 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  $\mu\text{g}$   $F_1$ , (b) 50  $\mu\text{g}$   $F_0$ , (c) 6  $\mu\text{g}$  PVP, (d) 6  $\mu\text{g}$  OSCP, (e) 6  $\mu\text{g}$   $F_6$ . (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- $F_0$ I 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 ( $F_0$ II 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  $H^+ \cdot \min^{-1} \cdot \text{mg protein}^{-1}$ .

$F_0$  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

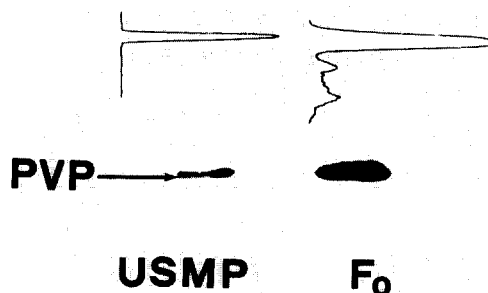


Fig.3. Comparison of PVP subunit content in USMP and  $F_0$ . 10  $\mu$ g USMP and  $F_0$  proteins were analysed by SDS-PAGE and immunoblotting, using antiserum against PVP subunit. Scans were performed at 590 nm.

USMP is directly correlated with digestion of the PVP ( $F_0$ I) 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-

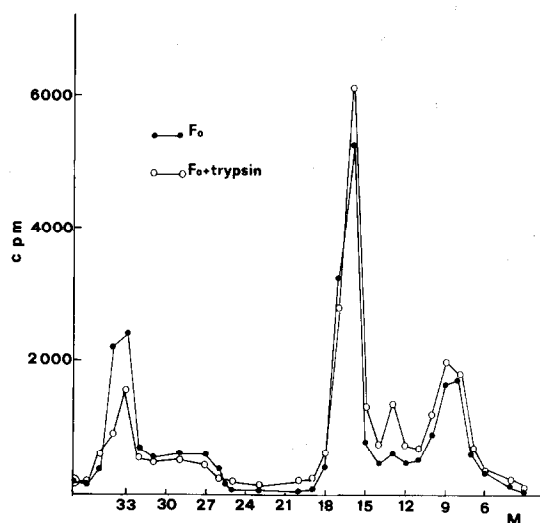


Fig.4. [ $^{14}$ C]DCCD binding to  $F_0$  polypeptides. USMP or USMP treated for 20 min with trypsin (50  $\mu$ g/mg protein) as in [12,15] were suspended, at a particle protein concentration of 3 mg/ml, in 0.25 M sucrose, 20 mM KCl and 30 mM  $K^+$  succinate (pH 7.4). Particles were then incubated for 20 min with 30  $\mu$ M [ $^{14}$ C]DCCD and centrifuged for 10 min at  $105000 \times g$ .  $F_0$  was extracted as reported in the legend to fig.2, and subjected to SDS-PAGE (200  $\mu$ g). 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 ( $\mu\text{mol}$<br>ATP hydrolyzed $\cdot \text{min}^{-1} \cdot$<br>$\text{mg F}_1^{-1}$ ) |
|---|---|
| $F_1$   | 99  |
| $F_0 + F_1$                                   | 42  |
| $F_0 + F_1 + \text{OSCP}$                     | 37  |
| $F_0 + F_1 + F_6$                             | 33  |
| $F_1 + \text{oligomycin}$                     | 101   |
| $F_0 + F_1 + \text{oligomycin}$               | 25  |
| $F_0 + F_1 + \text{OSCP} + \text{oligomycin}$ | 23  |
| $F_0 + F_1 + F_6 + \text{oligomycin}$         | 20  |

Purified  $F_0$  (200  $\mu\text{g}$ ) was mixed with 85  $\mu\text{g}$  liposomes, diluted to 200  $\mu\text{l}$  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  $\mu\text{g}$   $F_1$  and 6 mM  $\text{MgCl}_2$ . 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  $\mu\text{g}$  oligomycin/ml is also indicated

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 [ $^{14}\text{C}$ ]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 [ $^{14}\text{C}$ ]DCCD-labelled USMP showed that tryptic digestion producing 50% inhibition of proton con-

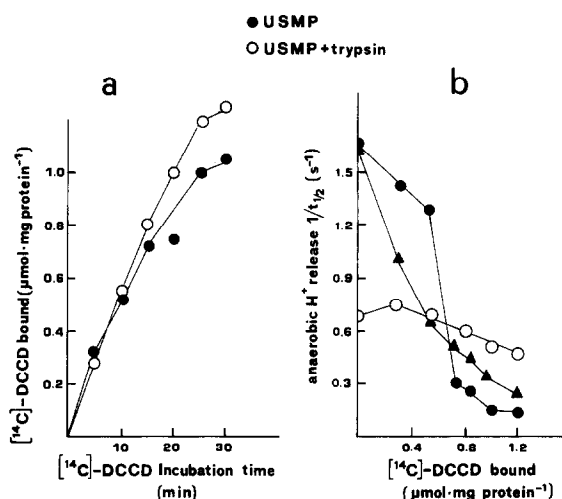


Fig.6. Effect of trypsin treatment of USMP on binding of [ $^{14}\text{C}$ ]DCCD (a) and sensitivity of proton conduction to DCCD (b). Preparation of USMP and PVP protein and measurement of proton conduction were according to section 2. For treatment with [ $^{14}\text{C}$ ]DCCD, see legend to fig.4 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  $\mu\text{g}$  PVP protein/ml.

ductivity did not affect [ $^{14}\text{C}$ ]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  $\text{H}^+$  channel of  $F_0$  of  $\text{H}^+$ -ATP synthase from prokaryotes and eukary-

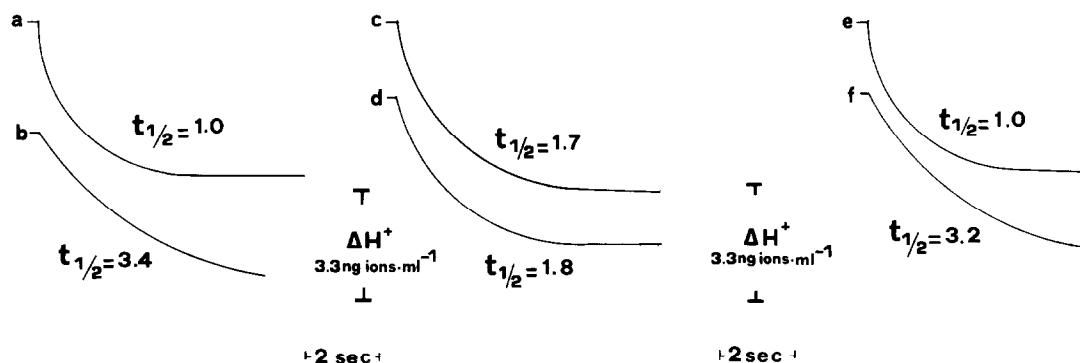


Fig.5. Restoration of  $\text{H}^+$  conduction and oligomycin sensitivity by addition of purified PVP protein to liposomes reconstituted with  $F_0$  extracted from trypsinized USMP. For preparation of  $F_0$ , see legend to fig.2; for PVP preparation and trypsin treatment of USMP see [12,16]. Details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