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Role of the carboxyl-terminal region of the PVP protein (F_0I subunit) in the H^+ conduction of F_0F_1 H^+-ATP synthase of bovine heart mitochondria

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By means of protein sequencing, labelling with thiol reagents and reconstitution studies it is shown that the carboxyl-terminal region of the PVP protein (F_0I subunit, nuclear-encoded protein of M_r 25000) of mitochondrial F_0 promotes transmembrane proton conduction by F_0 and the sensitivity of this process to oligomycin.

H^+-ATP synthase; F_0F_1 complex; Oligomycin; Proton conduction

1. INTRODUCTION

Recent work from our laboratories [1-3] has provided evidence showing that a protein of M_r 25 000 (apparent M_r from gels 24 000-27 000) is a genuine functional component of the membrane sector (F_0) of the ATP synthase (EC 3.6.1.34) of bovine heart mitochondria (cf. [4-6]). The protein revealed an amino acid sequence at the N-terminus consisting of PVPPLPEHGGKVRF which is exactly that determined for the protein of apparent M_r 24 000 isolated by Walker et al. [6] from bovine heart enzyme and corresponds to the nucleotide sequence of the cognate cDNA which they prepared. The protein, denoted PVP from the single-letter notation of the first three residues at the N-terminus [2,3], could be digested by trypsin in everted vesicles of the inner mitochondrial membrane only after removal with urea of the peripheral moiety (F_1) of the ATP synthase [1-3]. Digestion produced a fragment of apparent M_r 18 000 which remained attached to the membrane, could be co-purified with F_0 and reacted with an antibody prepared against the isolated intact PVP protein [1-3]. Proteolytic cleavage of the protein resulted in partial inhibition of passive proton conduction in submitochondrial particles and F_0-reconstituted vesicles and loss of the sensitivity of this process to oligomycin [7].

Both proton conductivity and its sensitivity to oligomycin were restored upon reconstitution of digested particles, or liposomes containing F_0 isolated from trypsinized particles, with the isolated intact PVP protein [2]. Thus, the PVP protein (F_0I according to the nomenclature in [1,5]) is the second subunit of mitochondrial F_0, in addition to that of 8 kDa (DCCD-binding protein) [8] which

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Abbreviations: USMP, submitochondrial particles devoid of F_1; PAGE, polyacrylamide gel electrophoresis; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DCCD, *N,N'*-dicyclohexylcarbodiimide; DACM, *N*-(7-dimethylamino-4-methyl-3-coumarinyl)maleimide; NEM, *N*-ethylmaleimide

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appears to be involved in the proton conducting activity in mitochondrial F_0F_1 ATP synthase.

It is shown here, by protein sequencing and labeling with thiol reagents, that the proteolytic product of apparent M_r 18 000 is produced by removal from the PVP protein of a carboxyl-terminal tail. At variance with reconstitution with intact PVP protein, its 18 kDa fragment was ineffective in restoring proton conductivity and oligomycin sensitivity of this process when added to digested F_0 in native or artificial phospholipid membranes. Similarly, addition of 31 kDa protein, present in F_0 preparations, which was also digested by trypsin, was without effect.

2. MATERIALS AND METHODS

CHAPS, oligomycin, valinomycin and asolectin were obtained from Sigma; SDS, goat anti-rabbit IgG labeled with peroxidase, horseradish peroxidase color development reagent and molecular mass standards from Bio-Rad; nitrocellulose membranes (0.45 μm pore size) from Schleicher and Schuell; PVDF membranes (immobilon transfer; 0.45 μm pore size) from Millipore and sequencing-grade reagents from Applied Biosystems. DACM was obtained from Serva.

2.1. *Enzyme preparations*

Heavy bovine heart mitochondria were prepared as described in [9] and EDTA submitochondrial particles (ESMP) as in [10]. F_1-depleted urea particles (USMP) were obtained from ESMP according to [11]. F_0 was isolated by CHAPS solubilization from USMP [12]. F_0 subunits were isolated by preparative gel electrophoresis and F_0 vesicles were obtained by the dialysis method as in [13].

2.2. *Trypsin digestion*

USMP (1 mg/ml) suspended in 0.25 M sucrose, 10 mM Tris-acetate, 1 mM EDTA, 6 mM $MgCl_2$ (pH 7.5) were incubated with 50 μg tyrypsin/mg particle protein at 25°C. After 20 min, digestion was stopped by using soybean trypsin inhibitor (Boehringer) in 5-fold excess over trypsin, and cooling to 0°C. The particle suspension was then centrifuged at 105 000 × g and the pellet suspended in 0.25 M sucrose.

2.3. *Electrophoresis and immunoblotting procedures*

SDS-PAGE was performed on slabs of linear gradient polyacrylamide (14–20%) gel as in [14] and immunoblot analysis as in [1,2]. Individual proteins of F_0 were isolated as in [1]. The isolated PVP protein was used for immunization of rabbits as described [1].

2.4. *Treatment of USMP with thiol reagents*

USMP (30 mg/ml) were incubated for 20 min at 25°C with 30 μM DACM or [^{14}C]NEM in 0.1 M phosphate buffer (pH 7.2). The suspension was then diluted 50-fold with 0.25 M sucrose and centrifuged at 105 000 × g for 10 min.

2.5. *Amino acid sequence analysis*

Electrophoretically homogeneous protein bands were transferred to PVDF membranes (immobilon transfer) as in [15] and stained with Coomassie blue. The cut membrane pieces were centered on a TFA-treated glass-fiber filter coated with polybrene and placed in the cartridge block of the sequencer. Proteins were sequenced using an Applied Biosystems sequencer (model 477A) equipped with an on-line PTH analyzer.

2.6. *Assays*

Proton translocation in submitochondrial particles was analyzed potentiometrically, following anaerobic release of the respiratory proton gradient [1,13]. Proton conduction in F_0 liposomes was determined potentiometrically, by monitoring the H^+ release induced by a diffusion potential imposed by valinomycin-mediated K^+ influx [2].

3. RESULTS AND DISCUSSION

Fig.1 shows the SDS-PAGE pattern of the F_0 moiety of bovine heart ATP synthase isolated from everted vesicles of the inner mitochondrial membrane from which F_1 had been removed (USMP) [1,2,13].

Coomassie blue staining revealed the characteristic bands of these preparations [1–6] with apparent M_r values around 31 000, 27 000 (PVP protein, see [1–3]), 25 000 (this band consisted in fact of a closely spaced doublet) and 23 000. Additional bands of M_r 16 000 and 10 000–8000 were also detected. Treatment with the fluorescent thiol reagent DACM resulted in labeling of all bands except that of apparent M_r 23 000 (see also [13]). This may represent the product of the mitochondrial ATPase 6 gene which has no codon for cysteine [16].

The 27 kDa protein and that of 18 kDa produced by tryptic digestion of USMP [1,2] were sequenced after electrotransfer onto PVDF membranes [15] directly from the gel slab of isolated F_0 or after electroelution in glycerol (the eluted proteins were electrophoretically homogeneous [13]). Both the 27 and 18 kDa proteins produced by digestion had the same N-terminal sequence: PVP-PLPEHGGKVRF. Furthermore, the 18 kDa product reacted with a specific antiserum raised against the purified PVP protein [1], in both submitochondrial particles and F_0 extracted from trypsinized particles (fig.2).

The 18 kDa fragment of the PVP protein retained DACM labeling as indicated by fluorescence of this band (fig.1E). When USMP were labeled with [^{14}C]NEM prior to and after incubation with tryp-

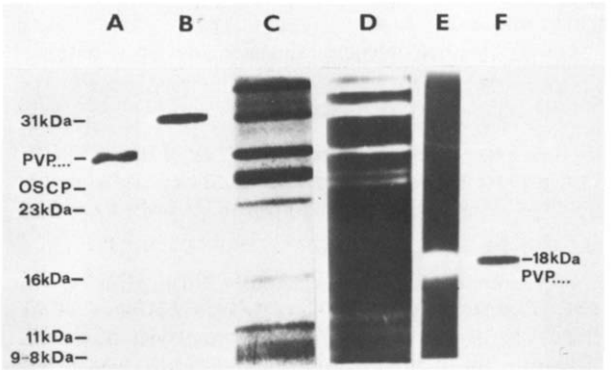

Fig.1. SDS-PAGE of F_0 and isolated F_0 polypeptides; effect of trypsin digestion. F_0 was isolated, as reported in section 2, from USMP (C) and DACM-treated USMP (D). For trypsin digestion, DACM treatment, gel electrophoresis and isolation of PVP (A), 31 kDa (B) and 18 kDa proteins (E,F) see text. Proteins were detected by Coomassie blue (A,B,C,F) or by fluorescence under ultraviolet light (D,E). For SDS-PAGE 3 μg protein of each single polypeptide or 50 μg protein of F_0 were used.

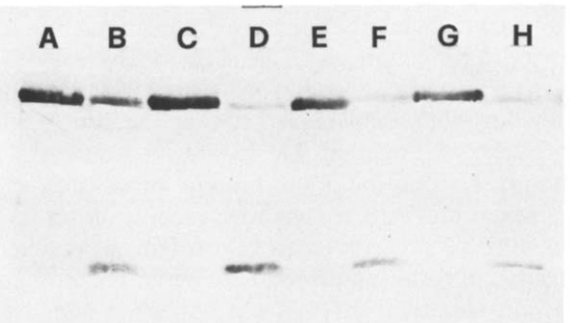

Fig.2. Immunoblot of PVP protein in USMP (A,D) and purified F_0 (E–H) before and after trypsin digestion. USMP (50 μg) treated with DACM (C,D) or untreated (A,B) were subjected to SDS-PAGE before (A,C) or after trypsin digestion (B,D). From these particles F_0 was purified and 10 μg F_0 protein was subjected to SDS-PAGE (E, F_0; F, trypsinized F_0; G, F_0 from DACM-treated USMP; H, trypsinized F_0 from DACM-treated USMP). PVP protein and its 18 kDa fragment were detected by immunoblotting with a specific antiserum raised against isolated PVP protein [2].

sin, it was found (fig.3) that in trypsinized samples the radioactivity of both the 31 kDa protein and of the PVP protein decreased while the cleavage product of 18 kDa was labeled by [^{14}C]NEM. On the other hand, trypsinization did not affect labeling by [^{14}C]NEM of the 8 kDa protein (DCCD-binding, c subunit [17,18]). Thus, the 18 kDa protein is produced by tryptic digestion of the PVP protein at its carboxyl-terminal region. The nuclear cDNA coding for the PVP protein has only one triplet coding for cysteine in position 197 (PVPPL-PEHGGKVRF.....EKETIAK$_{190}$C$_{197}$IA-DLKLLSKKAQAQPVM$_{214}$) [6]. The finding that this cysteine is retained in the large fragment restricts the site of cleavage of the PVP protein by trypsin (which acts specifically on the carboxyl side of lysine residues) to Lys 202 (or Lys 206, 205). The first site is the most probable in view of the substantial decrease in apparent M_r of the protein, although it is clear from the present observations that proteolytic digestion alters the electrophoretic mobility of the protein via effects other than only the decrease in M_r. Interestingly, an anomalous mobility of the F_0I subunit was noted before [1].

Tryptic digestion of USMP resulted in partial inhibition of passive proton conduction and loss of its sensitivity to inhibition by oligomycin, in both USMP and F_0 extracted from trypsinized USMP and reconstituted in phospholipid vesicles [2,3] (table 1). Proton conduction and sensitivity to oligomycin could be restored by adding to trypsinized USMP the intact PVP protein (table 1). In contrast, additions of the purified protein of 31 kDa or of the purified 18 kDa tryptic product were completely ineffective in restoring proton conduc-

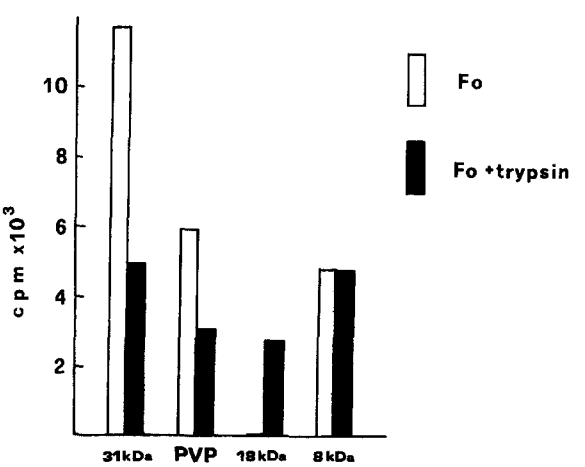

Fig.3. Binding of [^{14}C]NEM to F_0 proteins. F_0 was isolated from [^{14}C]NEM-treated control or trypsinized USMP as reported in section 2. 50 μg protein of F_0 were subjected to SDS-PAGE, the polypeptides isolated as reported in the text and used for determination of radioactivity [13]. On the abscissa the apparent molecular masses of isolated proteins are reported.

Table 1

Restoration of H^+ conduction and oligomycin sensitivity by addition of purified F_0 proteins to USMP treated with trypsin and to F_0 liposomes obtained by reconstitution with F_0 extracted from trypsinized USMP

	$1/t_{1/2}$ H^+ release (s^{-1})	
	Control	+ oligomycin (2 μg/mg protein)
USMP	1.82	0.25
Tryp.-USMP	0.91	0.80
Tryp.-USMP + PVP	2.00	0.57
Tryp.-USMP + 18 kDa protein	1.00	0.90
Tryp.-USMP + Mr 31 kDa protein	1.00	0.91
Tryp.-USMP + PVP-DACM	2.00	0.59
F_0 liposomes	0.93	0.30
Tryp.-F_0 liposomes	0.50	0.60
Tryp.-F_0 liposomes + PVP protein	1.10	0.35
Tryp.-F_0 liposomes + 18 kDa protein	0.52	–

Tryp.-F_0, F_0 purified from trypsinized USMP. Where indicated, Tryp.-USMP or Tryp.-F_0 liposomes were preincubated for 15 min at 25°C with purified PVP, 31 or 18 kDa proteins (6 μg/mg particle protein) before measurement of passive proton conduction. For other details see section 2. PVP-DACM, PVP protein purified from DACM-treated USMP

tion and its sensitivity to oligomycin. It can also be noted that thiol modification of the single cysteine residue in the PVP protein did not affect its ability to restore proton conductivity. This is consistent with previous observations showing that modification by [^{14}C]NEM in F_0 of the protein of apparent M_r 27 000 was not responsible for the inhibition that this thiol reagent caused on passive proton conduction in F_0 liposomes [13]. The lack of any effect of the 31 kDa band is consistent with previous observations demonstrating that it does not belong to F_0 [19].

In conclusion, the present data show that the proteolytic removal from F_o of the peripheral carboxyl-terminal tail (not longer than 14 residues) of the PVP protein, protruding from the M surface of the membrane and covered by F_1 [1], is responsible for the partial inhibition of proton conduction in the proton channel of F_0 and the loss of sensitivity to oligomycin. Because of its peripheral location at the M surface of the membrane this carboxyl-terminal tail cannot participate directly in transmembrane proton conduction. The present results show, however, that this segment of the PVP protein promotes proton conduction in F_0 possibly by favouring exchange of protons between the aqueous phase and the channel and/or by bringing the membrane-spanning protein segment [17,20] of the channel into the proper active configuration. This conclusion is also supported by the fact that removal of the carboxyl-terminal tail of the PVP protein induces loss of sensitivity of the F_0 proton channel to oligomycin. This inhibitor, which is a bulky hydrophobic molecule, probably blocks proton conduction in F_0 by interacting with a number of residues in subunit c of F_0 (8 kDa, DCCD-binding protein) exposed towards the lipid core of membrane phospholipids [21].

Since the PVP protein is in intimate contact with F_1 [1] its cleavable residues could also take part in directing protons from the transmembrane channel in F_0 to subunits of F_1.

It seems appropriate to recall here a recent paper by Takayama et al. [22] showing that chromosomal unc F mutants of *E. coli* defective in the carboxyl-terminal region of the b subunit of F_0 resulted in impairment of the normal assembly of functional F_0F_1 ATP synthase in the membrane. Walker et al. [6] consider the nuclear-encoded PVP protein of bovine ATP synthase to be analogous to the b subunit of *E. coli* F_0 (for a more extensive discussion of the latter aspect, however, see [1,3].

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