Identification of nucleus-encoded F_0I protein of bovine heart mitochondrial H^+-ATPase as a functional part of the F_0 moiety

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The F_0I protein of apparent M_r 27000, previously characterized [(1988) Eur. J. Biochem. 173, 1–8] as a genuine component of bovine heart F_0, has been sequenced and shown to be identical with the nucleus encoded 24668 Da protein characterized earlier [(1987) J. Mol. Biol. 197, 89–100]. It is directly shown by proteolytic cleavage and reconstitution experiments that this protein, denoted here as PVP from the single-letter codes of the last three residues of the N-terminus, is involved in proton conduction by F_0 and in its sensitivity to oligomycin.

H^+-ATPase; F_0 complex; Oligomycin; H^+ conduction

1. INTRODUCTION

Definite progress is being made in the elucidation of the genetics, structure and function of the protein subunits of the F_0F_1 H^+-ATPase (EC 3.6.1.34) of coupling membranes [1–3]. The catalytic sector, F_1, consists invariantly of 3 α, 3 β, 1 γ, 1 δ and 1 ϵ protein subunits, whose genes, structure and interspecies homologies have been described [1–5]. On the other hand, the polypep-

tide composition of the F_0 sector, responsible for transmembrane proton translocation, varies among species [1–3]. In *E. coli* F_0 consists of three subunits, a–c, encoded by genes of the unc or atp operon [3,6]. Their structures have been described and they have been shown to be essential for the proper assembly and functioning of F_0 in the ATP synthase [7–9]. The subunit composition of F_0 in eukaryotic ATP synthase is more complex [1–4] and even preparations from bovine heart mitochondria exhibit, besides the five F_1 subunits and the ATPase inhibitor protein [10], 7–9 proteins which may belong to F_0 [11–14].

In a previous paper we have characterized a protein of apparent M_r 27000 as being a genuine component of bovine heart F_0 (F_0I) ([14]; see also [11–13,15,16]). Walker et al. [15] have isolated two membrane-associated proteins of apparent M_r 24000 and 19000 from bovine heart ATP synthase, cloned their corresponding nuclear cDNA and determined the DNA and protein sequences. These authors propose [15] that the protein of apparent M_r 24000 (M_r value from sequence: 24668) is analogous to subunit b of *E. coli* F_0 and to similar proteins found in ATP synthases from other

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Abbreviations: F_1, catalytic part of mitochondrial H^+-ATPase; F_0, membranous sector of mitochondrial H^+-ATPase; F_6, coupling factor 6 involved in the binding of F_1 to F_0; OSCP, oligomycin-sensitivity-conferring protein; ESMP, submitochondrial particles prepared in the presence of EDTA; USMP, submitochondrial particles devoid of F_1 (see section 2); PAGE, polyacrylamide gel electrophoresis; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate

sources, having in common a hydrophobic segment near the N-terminus [15], and is involved, like the b subunit of $E.\ coli$ [15], in the binding of F_1 to F_0.

This paper provides direct evidence that the F_0I protein characterized by us corresponds to the nucleus-encoded protein of M_r 24 668 of bovine heart ATP synthase preparations [15], and is a functional component of the F_0 sector. This subunit is involved in proton conduction by F_0 and in its sensitivity to oligomycin. We propose to term this protein PVP from the single-letter notation of the first three residues at the N-terminus, pending its analogy to the b subunit of $E.\ coli$.

2. MATERIALS AND METHODS

CHAPS, oligomycin, valinomycin and asolectin were obtained from Sigma; nucleotides and enzymes from Boehringer; SDS, goat anti-rabbit IgG labeled with peroxidase, horseradish peroxidase color development reagent and molecular mass standards from Bio-Rad; nitro cellulose membrane (0.45 μm pore size) from Schleicher and Schüll; PVDF membranes (immobilon transfer); 0.45 μm pore size) from Millipore and sequencing-grade reagents from Applied Biosystems.

2.1. Enzyme preparations
Heavy beef heart mitochondria were prepared as described [17] and EDTA submitochondrial particles (ESMP) as in [18]. F_1-depleted urea particles (USMP) were obtained from ESMP according to [19]. F_0F_1 complex [20,21] and F_0 [21] were isolated by CHAPS solubilization from ESMP and USMP, respectively. F_0 subunits were isolated by preparative gel electrophoresis as described in [13,14]. F_0 vesicles were obtained by the dialysis method in [13].

2.2. Trypsin digestion
USMP (1 mg) were suspended in 1 ml of 0.25 M sucrose, 10 mM Tris-acetate, 1 mM EDTA, 6 mM $MgCl_2$ (pH 7.5) and incubated with various concentrations of trypsin at 25°C. After 20 min digestion was stopped by adding trypsin inhibitor 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. Trypsin-digested samples of USMP, characterized by electrophoresis, were tested for proton translocation and used for F_0 extraction.

2.3. Electrophoresis and immunoblotting procedures
SDS-PAGE was performed on slabs of linear gradient polyacrylamide (14–20%) gel as in [22]. SDS gels with separated proteins were subjected to immunoblot analysis as in [14]. Immunodecorated blots were photographed and/or subjected to densitometry using a Camag TLC scanner at 590 nm. The quantity of antigen detected was evaluated from the computed peak areas. Individual proteins of F_0 were isolated as in [13,14]. The isolated 27 kDa protein was used for immunization of rabbits as in [14].

2.4. Amino acid sequence analysis
About 200 pmol homogeneous protein bands were transferred to PVDF membranes (immobilon transfer) as in [23]. Proteins electroblotted onto PVDF membranes were stained with Coomassie blue and the bands cut. The membrane pieces were centered on a teflon seal 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 analyser.

2.5. Assays
ATPase hydrolytic activity was measured in the presence of an ATP-regenerating system as described [24]. Proton translocation in submitochondrial particles was analyzed potentiometrically, following anaerobic release of the respiratory proton gradient [13,24]. Proton conduction in F_0 liposomes was analyzed potentiometrically, by following the H^+ release induced by a diffusion potential imposed by valinomycin-mediated K^+ influx [13]. Proteins were estimated by a modified Lowry-Folin assay [25].

3. RESULTS

Fig.1. illustrates the polypeptide composition of the bovine heart ATP synthase preparation from ESMP '[21]. SDS-polyacrylamide gradient (14–20%) electrophoresis gave rase to good separation of proteins in the range 33–20 kDa, in which the components under investigation are located. Four protein bands below the F_1 γ-subunit (33 kDa), with apparent molecular masses of 31, 27, 25 and 23 kDa could be identified. The 25 kDa band, in fact, consisted of a closely spaced doublet (see also [14]). The protein of 31 kDa is not consistently found in bovine heart ATP synthase preparations [21,26], and its removal, when present, does not affect activities of the complex [26,27]. Its attribution to the ATP synthase is, therefore, questionable.

The F_0 membrane sector was also directly purified from submitochondrial particles after extensive removal of F_1 subunits with urea [21]. This F_0 preparation, enriched in the five subunits described above, was applied to large gel slots. After electrophoretic separation the bands of apparent molecular masses 27, 26, 25 and 23 kDa were cut and electroeluted separately in glycerol-H_2O [13]. The homogeneity of the proteins was checked electrophoretically (see fig.1) and samples were used for production of polyclonal antibodies [14] or subjected to automated Edman degradation for determination of the amino acid sequence at the N-terminus [23]. Sequencing of the protein of ap-
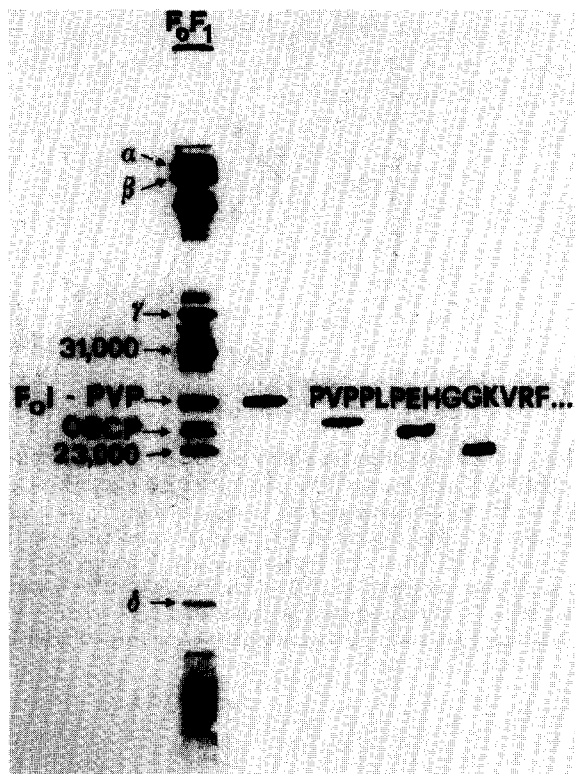
Fig.1. Gel electrophoresis of purified F_0F_1 complex and of isolated 27 kDa F_0 protein. For preparation of F_0F_1, isolation of F_0 proteins and gel electrophoresis see section 2. Where indicated, 20 μg F_0F_1 complex and 2 μg individual F_0 proteins were applied to the gel.

parent M_r 27 000 revealed an amino acid sequence at the N-terminus consisting of PVPPLPEHG-GKVRF which is exactly that determined on the protein of apparent M_r 24 000 isolated by Walker et al. [15] from bovine heart ATP synthase and corresponds to the nucleotide sequence of cognate cDNA [15]. It may be noted that this cDNA has one codon for cysteine and the protein isolated and sequenced here reacts with thiol reagents [13,14]. Thus the protein of apparent M_r 27 000 that we isolated from F_0 is the same as that described by Walker et al. [15] and designated subunit b. The difference in apparent M_r obtained from the electrophoretic mobility is clearly due to the different conditions for SDS-PAGE. The F_0I protein can be thus called PVP from its first three residues at the N-terminus.

The band of apparent M_r 25 000 (lower compo-

nent of the doublet) is the OSCP protein, as revealed by direct comparison with the gel for OSCP isolated in other laboratories [14].

In a previous study on the accessibility to exogenous protease of ATP synthase subunits in submitochondrial particles from bovine heart [14], we found that the F_0I protein, identified here as the PVP subunit of F_0, could be cleaved by trypsin to a fragment of M_r 18 000 (reacting with polyclonal antibodies raised against the intact protein) only when F_1 subunits were removed from the particles with urea. The 18 kDa fragment remained associated to the membrane. Thus, it appears that the PVP subunit is an F_0 subunit with hydrophilic extension(s) covered by F_1 and possibly involved in the F_1-F_0 junction [14].

Fig.2 shows that the PVP subunit, detected by an antibody specific for this protein, was cleaved during trypsin digestion of USMP to an immunoreactive fragment of 18 kDa which remained associated to the particles and was co-purified, as well as the intact PVP protein, with the other components of F_0.

The pH traces presented in fig.3 show that trypsin digestion of USMP caused inhibition of proton conduction in both submitochondrial particles (see also [14]) and liposomes reconstituted with F_0 from trypsinized USMP as compared to those reconstituted with F_0 from untreated particles. The plot in fig.3 shows that increasing the trypsin concen-
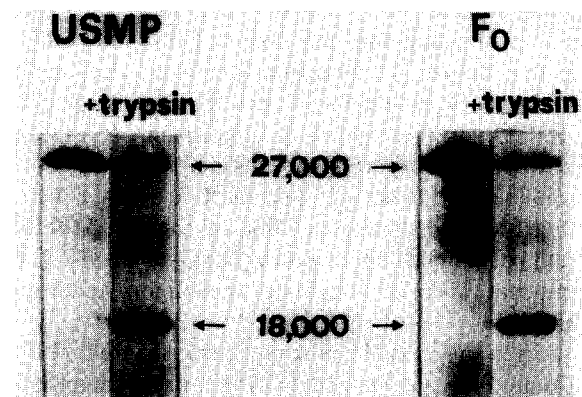

Fig.2. Comparison of the content of PVP protein in USMP and purified F_0. Effect of trypsin digestion. F_0 was isolated from USMP and USMP treated with 50 μg trypsin/mg protein and subjected to SDS-PAGE. The content of PVP protein was analyzed by immunoblotting with specific antiserum against this protein (see section 2 and [14]).
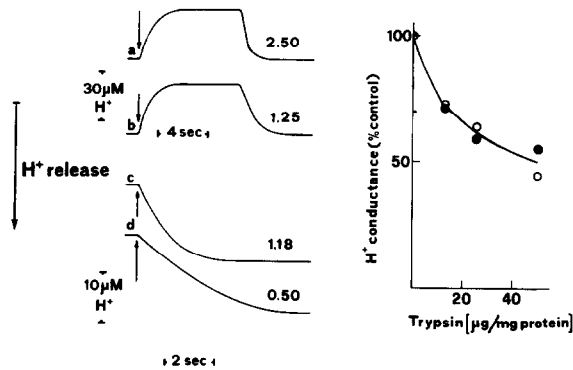
Fig.3. Effect of trypsin on H^+ conduction in USMP and in liposomes reconstituted with F_0 isolated from control and trypsinized USMP. For preparation of USMP and F_0, trypsin treatment and measurement of H^+ conduction, see section 2. Traces: (a) USMP (3 mg protein/ml); (b) USMP (3 mg protein/ml) treated with trypsin (50 μg/mg particle protein); (c) liposomes (0.5 μg F_0 protein/ml) obtained using F_0 isolated from control USMP; (d) liposomes (0.5 μg F_0 proteins/ml) obtained using F_0 isolated from USMP treated with trypsin (50 μg/mg particle protein). (●——●) USMP, (○——○) liposomes reconstituted with F_0 isolated from USMP before or after treatment with trypsin at the concentrations indicated. The numbers beside traces give the apparent rates of H^+ release ($1/t_{1/2}$). Arrows indicate the addition of valinomycin (c,d) or H_2O_2 (a,b).
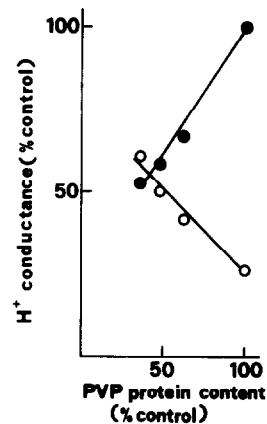

Fig.4. Relationship between observed passive H^+ conduction in USMP and the content of PVP protein. PVP content was determined by densitometric analysis of immunoblots, as described in section 2. The content of PVP and H^+ conduction (as the reciprocal value of $t_{1/2}$ for anaerobic release of respiratory proton gradient; see legend to fig.2) of untreated USMP were taken as 100%. H^+ conduction was measured in the presence (○——○) or absence (●——●) of 0.5 μg oligomycin/mg particle protein.

tration used to digest USMP caused the same progressive inhibition of proton conduction in USMP and liposomes reconstituted with F_0 from treated USMP.

Fig.4 shows that progressive digestion of the PVP subunit, affected by incubation of USMP with increasing concentrations of trypsin, resulted in the progressive inhibition of proton conduction in the particles and loss of sensitivity of this process to the inhibitory action of oligomycin. It appears, however, that there also remained significant proton conducting activity (about 50% of the control) in USMP when almost 70% of the PVP subunit had been digested by trypsin (figs 2–4; see also [14]) but that this residual activity was oligomycin insensitive.

Fig.5 shows that liposomes reconstituted with F_0 isolated from trypsinized USMP, in which approx. 70% of the PVP protein had been cleaved, exhibited a rate of proton conduction which amounted to only 60% of that exhibited by liposomes with untreated F_0 and was completely insensitive to oligomycin. Proton conduction in the untreated F_0 liposomes was markedly inhibited by
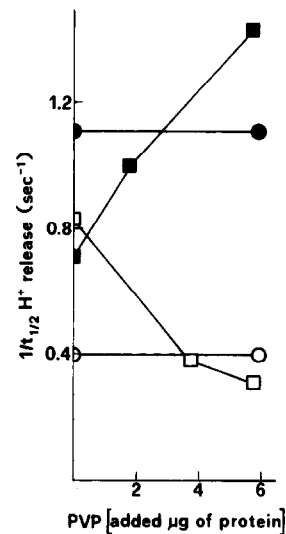

Fig.5. Reconstitution of H^+ conduction and oligomycin sensitivity by addition of purified PVP proteins to liposomes with F_0 isolated from trypsin-treated USMP. USMP were treated with 50 μg trypsin/mg particle proteins. Where indicated 1 ml of F_0 liposomes (0.5 mg protein/ml) was mixed for 10 min at 25°C with PVP protein, at the concentrations shown. F_0 liposomes reconstituted with F_0 extracted from untreated USMP (●——●) or trypsin-treated USMP (■——■). Effects of oligomycin on H^+ conduction by liposomes reconstituted with F_0 extracted from untreated USMP (○——○) or from trypsin-treated USMP (□——□).

oligomycin. Reconstitution of liposomes containing F_0 from trypsinized USMP with increasing amounts of isolated PVP protein resulted in the progressive and full recovery of proton conduction by F_0 which became concomitantly oligomycin-sensitive. At the higher concentrations of added PVP protein, the extent of proton conduction of reconstituted vesicles with trypsinized F_0 became even greater than that exhibited by vesicles reconstituted with untreated F_0, however, this extra proton conduction was still suppressed by oligomycin.

It thus appears that the isolated intact PVP protein is able to replace its inactive fragment in F_0 and thus restore normal proton conductivity.

4. CONCLUSIONS

The present data show that the protein of apparent M_r 27 000 of bovine heart H^+-ATPase [13,14], previously characterized by us as a genuine subunit of the F_0 sector and termed F_0I [14], is the same as that isolated by Walker et al. [15] and described as subunit b. This protein, which is encoded by a nuclear gene (from cDNA sequence: M_r 24 668), has an N-terminal sequence consisting of PVPPLPEHGGKVRF as obtained from the gene sequence [15] and direct protein analysis in [15] and in this work.

The present paper provides clear-cut evidence that this protein, that we propose calling the PVP subunit, is a functional component of F_0, essential for proton conductivity in the ATP synthase and for the inhibition by oligomycin of proton conduction in mitochondrial F_0. Thus, the PVP protein (F_0I) is the second subunit of mitochondrial F_0, in addition to the 8 kDa, DCCD-binding protein [28], which is shown to contribute to the proton-conducting activity of the mitochondrial ATP synthase.

We have previously reported [14] that the PVP subunit (F_0I) is, like the b subunit of *E. coli* ATP synthase [29,30], protected by the presence of F_1 against cleavage by trypsin into smaller membrane-associated fragments. However, trypsin digestion of the *E. coli* b subunit in F_1-depleted everted membranes abolished binding of F_1 to F_0 but left proton translocation unaffected [29,30]. It might also be noted that oligomycin, whose inhibitory action requires the PVP protein, is specific for the mito-

chondrial ATP synthase but has no effect on the *E. coli* enzyme. These observations, taken together with the absence of extensive sequence homology [15] and lack of immunological cross-reactivity between mitochondrial PVP subunit and *E. coli* b subunit [14], would indicate that the two proteins are not directly related (but see [15]). It may, however, be possible that in the course of evolution additional segments and functions have been acquired in mitochondria by a bacterial protein ancestor.

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