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Structural and functional characterization of subunits of the F_o sector of the mitochondrial F_oF_1-ATP synthase

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Proteolytic digestion of F_1-depleted submitochondrial particles (USMP), reconstitution with isolated subunits and titration with inhibitors show that the nuclear-encoded PVP protein, previously identified as an intrinsic component of bovine heart F_o (F_o1) (Zanotti, F. et al. (1988) FEBS Lett. 237, 9–14), is critically involved in maintaining the proper H^+ translocating configuration of this sector and its correct binding to the F_1 catalytic moiety. Trypsin digestion of USMP, under conditions leading to cleavage of the carboxyl region of the PVP protein and partial inhibition of transmembrane H^+ translocation, results in general loss of sensitivity of this process to F_o inhibitors. This is restored by addition of the isolated PVP protein. Trypsin digestion of USMP causes also loss of oligomycin sensitivity of the catalytic activity of membrane reconstituted soluble F_1, which can be restored by the combined addition of PVP and OSCP, or PVP and F_6. Amino acid sequence analysis shows that, in USMP, modification by [^{14}C]*N,N'*-dicyclohexylcarbodiimide of subunit *c* of F_o induces the formation of a dimer of this protein, which retains the ^{14}C-labelled group. Chemical modification of cysteine-64 of subunit *c* results in inhibition of H^+ conduction by F_o. The results indicate that proton conduction in mitochondrial F_o depends on interaction of subunit *c* with the PVP protein.

Introduction

The H^+-translocating membrane sector (F_o) of the F_oF_1-ATP synthase of coupling membranes has a different polypeptide composition in various species [2]. Bacterial F_o (*Escherichia coli*) is composed of three subunits *a,b,c* [2] which are essential for the proper assembly and function of F_o [3–6] and are present, in a stoichiometry of 1:2:10–12, respectively [7,8]. Subunit *c* is generally considered to represent the essential element of proton conduction system in F_o [2,6]. Models for proton conduction in F_o, based on interaction of subunits *c* and *a*, have been proposed [9,10].

The eukaryotic F_o is more complex, consisting of 7–8 proteins (see Ref. 11 for review). Among these is the M_r 8000 protein, which binds *N,N'*-dicyclohexylcarbodiimide (DCCD), shows high homology to bacterial subunit *c* [12], is apparently present in multiple copies and is essential for proton conduction [12,13]. Recent work from our laboratories has shown [1,14,15] that a nuclear encoded [16] protein of M_r 25 000 (PVP protein, F_o1) is a genuine functional component of the membrane sector F_o in bovine heart mitochondria. The protein is digested by trypsin only after removal of F_1 from inside-out submitochondrial particles (USMP). Tryptic digestion of the PVP protein removed a tail extending from the carboxyl terminal M-214 to residues

Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DCCD, *N,N'*-dicyclohexylcarbodiimide; DPT, 2,2'-dithiobispyridine; NEM, *N*-ethylmaleimide; DACM, *N*-(7-dimethylamino-4-methyl-3-coumarinyl)maleinimide; ESMP, submitochondrial particles prepared in the presence of EDTA; F_o, membranous sector of mitochondrial F_oF_1-ATP synthase; F_1, catalytic part of mitochondrial F_oF_1-ATP synthase; F_6 coupling factor 6 involved in binding of F_1 to F_o; OSCP, oligomycin-sensitivity-conferring protein; PVP (F_o1) protein, subunit of membranous sector of F_oF_1-ATP synthase; SDS, sodium dodecyl sulfate; TID, 3-(trifluoromethyl-3-phenyl)diazerine; USMP, submitochondrial particles devoid of F_1 (see Materials and Methods); TPT, triphenyltin; PAGE, polyacrylamide gel electrophoresis.

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K-202 or K-206, K-205 [15]. This resulted in depression of proton conduction which became insensitive to inhibition by oligomycin [1] and DCCD [17].

The present results show that the PVP protein modulates the general sensitivity to inhibitors and the activity of the transmembrane proton channel in F_o and is essential, together with OSCP [18,19] and/or F_6 [18], for the correct binding of F_1 to F_o. DCCD modification of subunit *c* results in the formation of a dimer. Direct modification of cysteine-64 in subunit *c* results in inhibition of H^+ conduction.

Materials and Methods

3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), *N*-ethylmaleimide (NEM), oligomycin and valinomycin were obtained from Sigma; *N*-(7-dimethylamino-4-methyl-3-coumarinyl)maleinimide (DACM) from Serva; sodium dodecyl sulfate (SDS), goat anti-rabbit IgG labeled with horseradish peroxidase, horseradish peroxidase color development reagent and molecular weight standards from Bio-Rad; [^{14}C]DCCD (50 Ci/mol) was purchased from Sorin Biomedica; nitrocellulose membrane (0.45 μm pore size) from Schleicher and Schuell; PVDF membrane (immobilon transfer, 0.45 μm pore size) from Millipore and sequencing grade reagents from Applied Biosystems. Soyabean trypsin inhibitor was from Boehringer. All other chemicals were of high purity grade.

Enzyme preparations

F_1-depleted urea particles (USMP) were prepared by sonication according to Racker and Horstmann [20]. F_o was isolated by CHAPS solubilization from USMP [17]. F_o subunits were isolated by preparative gel electrophoresis as described in Zanotti et al. [21].

Preparation of F_o vesicles

For reconstitution experiments F_o vesicles were prepared by dialysis method [17]: 3 mg of F_o were mixed with 30 mg of 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 against 0.1 M potassium phosphate buffer (pH 7.5), followed by 3 h dialysis against 10 mM sodium tricine buffer (pH 7.5). Both dialysis media contained 0.25 mM EDTA and 2.5 mM $MgSO_4$.

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 at 25°C, with trypsin. 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 gel electrophoresis, were tested for proton translocation and used for F_o extraction.

Electrophoresis, immunoblotting procedures and amino acid sequence analysis

SDS-PAGE was performed on slab gels with a linear gradient of polyacrylamide (14–20%) [21]. SDS gels were subjected to immunoblot analysis [14]. The isolated PVP protein, exhibiting in the present PAGE an apparent M_r of 27 000 (see also Refs. 1, 14 and 15), was used for immunization of rabbits [14]. For amino acid sequence analysis, electrophoretically homogeneous protein bands were transferred to immobilon (PVDF membranes) and proteins were sequenced using an Applied Biosystems sequencer (model 477 A) as in Zanotti et al. [1].

Assays

Proton translocation in submitochondrial particles was analyzed potentiometrically, following anaerobic release of the respiratory proton gradient [22]. Proton translocation in F_o reconstituted liposomes was analyzed following potentiometrically H^+ release induced by a diffusional potential (positive inside) imposed by valinomycin-mediated potassium influx [22]. ATPase hydrolytic activity was measured in the presence of an ATP-regenerating system [22].

Measurement of binding [^{14}C]DCCD to submitochondrial particles and to individual F_o proteins

USMP (3 mg protein/ml) were incubated at 22°C with 30 μM [^{14}C]DCCD, (added as ethanolic solution) under anaerobic conditions. Then, immediately after the oxygen pulses, 15 μl of particles suspension were taken and the estimation of binding was carried out as reported in Kopecky et al. [13]. 5 mg of [^{14}C]DCCD treated USMP were used for isolation of F_o, then SDS-PAGE of isolated F_o was carried out and, after staining and densitometric analysis using a Camag TL Scanner (Switzerland) at 590 nm, the bands of the gel were sliced, treated with 1.0 ml of Beckman Tissue Solubilizer at 60°C for 12 h and radioactivity was determined by liquid scintillation counting.

Spectrophotometric analysis of thiol residues in DACM treated purified F_o

Labelling of F_o with DACM was carried out as follows: F_o (5 mg) was incubated with the reagent (at the concentrations reported in the legend to the figures) for 10 min in 0.1 M phosphate buffer (pH 7.2) at 22°C. After incubation, the reaction was stopped by addition of 2-mercaptoethanol (100-fold molar excess over DACM). The labelled protein was dialyzed for 12 h, at 4°C, against 1000 volumes of 0.1 M phosphate

TABLE I

Effect of trypsin treatment on anaerobic release of respiratory proton gradient

For details on preparation of USMP, treatment with trypsin (50 μg/mg protein) and measurement of proton translocation, see Materials and Methods. Where indicated DCCD 30 μM (incubation time: 20 min), triphenyltin (TPT) 600 μM (incubation time: 10 min) or PVP protein 2 μg (incubation time: 10 min.) were added before activation of respiration with the H_2O_2 pulse. Figures represent means ($n = 5$) ±S.E.

	Anaerobic H^+ release $1/t_{1/2}$ (s^{-1})					
	–			+PVP		
	–	+DCCD	+TPT	–	+DCCD	+TPT
Normal USMP	1.70±0.15	0.12±0.03	0.27±0.05			
Trypsin-USMP	0.77±0.06	0.59±0.06	0.70±0.05	1.85±0.20	0.33±0.03	0.45±0.04
Trypsin-USMP *		0.36±0.03	0.70±0.05			

* Indicates that trypsin treatment occurred after DCCD or TPT treatment of USMP.

buffer (with 1 change after 3 h), than reconstitution was carried out as described above. Samples of acetone washed DACM-treated purified F_o were suspended in 50 μl of a solution containing 2% SDS and the amount of reacted DACM was determined by measurement of absorbance at 380 nm ($\Delta\epsilon_{mM} = 19.8$) [23].

Results

Trypsin digestion of USMP

It has been reported that trypsin digestion of USMP results in partial inhibition of passive proton conduction [1,14]. The residual proton translocation was insensitive to oligomycin [1] and DCCD [17]. The experiment illustrated in Fig. 1 shows that the residual proton conduction measured in trypsin-digested USMP becomes also insensitive to triphenyltin (TPT). The inhibition of proton conduction by F_o, caused by trypsin digestion, was correlated to digestion of the PVP protein to a membrane bound immunoreactive fragment of apparent M_r 18000 [1,14]. Pretreatment of USMP with oligomycin or DCCD does not, however, affect this pattern of trypsin digestion of the PVP protein (results not shown).

Addition of purified PVP protein to trypsin treated USMP restored proton conductivity, which becomes again sensitive to F_o inhibitors (Table I). The addition of the M_r 18000 fragment of PVP protein or of the M_r 31000 protein, which was also digested by trypsin, were ineffective in this respect (see Ref. 15). DCCD and TPT inhibited the residual proton conduction, measured in trypsin digested USMP, only by 23% and 9%, respectively. After addition of PVP the restored proton conductivity was inhibited by 82% and 76% by DCCD and TPT, respectively. Trypsin treatment of USMP,

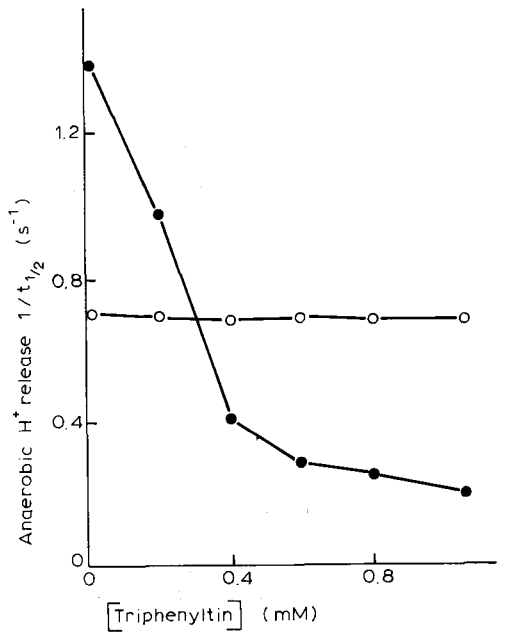

Fig. 1. Titration of the inhibitory effect of triphenyltin on anaerobic proton release in USMP and trypsin treated-USMP. For details of trypsin digestion (50 μg/mg protein) and measurement of proton conduction, see under Materials and Methods. Symbols: (●———●) USMP (3 mg protein/ml); (○———○) trypsinized USMP (3 mg protein/ml).

TABLE II

Effect of F_1 and trypsin treatment on anaerobic release of respiratory proton gradient in USMP

USMP particles were treated with trypsin (40 μg/mg particle protein) for 20 min. Chloroform extraction of F_1 was performed as described in Ref. 17. USMP or trypsinized USMP (0.5 mg particles protein/ml) were incubated in 0.25 M sucrose, 10 mM Tris-acetate, 1 mM EDTA, 6 mM $MgCl_2$ (pH 7.5), with purified F_1 at a F_1 protein/USMP protein ratio of 0.2. After 30 min at 25°C, incubation was stopped by centrifugation at 105000×g at 0°C. Analysis of the anaerobic release of respiratory proton gradient was carried out on the sedimented particles (3 mg protein/ml) as described under Materials and Methods. Figures represent the means ($n = 5$) ±S.E.

	Anaerobic H^+ release $1/t_{1/2}$ (s^{-1})	
	–	+F_1
Normal USMP	1.82±0.12	1.05±0.05
Trypsin-USMP	1.17±0.08	1.24±0.10

preincubated with TPT or DCCD, partially removed their inhibitory effect (Table I).

Binding of F_1 to USMP depressed the rate of H^+ release (Table II; see also Refs. 13,24). Trypsin digestion of USMP suppressed this effect of F_1 on proton conductivity in USMP (Table II). Thus, trypsin digestion of USMP caused alterations in the interaction of F_1 with F_o.

USMP do not exhibit significant ATPase activity (Table III). However, an oligomycin sensitive ATPase activity can be reconstituted by addition of purified F_1 to the particles. After treatment of USMP with trypsin, the particles were still capable of binding F_1 but the sensitivity of the reconstituted ATPase activity to oligomycin was lost (Table III). Addition of purified PVP protein, OSCP or F_6 caused per se some inhibition of the ATPase activity of reconstituted F_1; F_6 was the most effective in this respect. Control experiments showed that bovine serum albumin, added even in excess (50 μg/mg particle protein) had no effect on the hydrolase activity (results not shown). The PVP protein, OSCP and F_6 added individually did not induce significant oligomycin sensitivity. However, combined addition of PVP protein and OSCP and, even better, of PVP protein and F_6 were very effective in restoring oligomycin sensitivity (Table III).

Chemical modification of subunit c

The experiments of Fig. 2 show the effect of the hydrophobic reagent *N*-(7-dimethylamino-4-methyl-3-coumarinyl)maleinimide (DACM), which forms a stable, strongly fluorescent adduct with -SH groups [23,25],

TABLE III

Reconstitution of ATPase activity in USMP by addition of purified F_1. Effect of trypsin

For particle preparations, trypsin treatment (50 μg/mg particle protein) and determination of ATPase activity see Materials and Methods. F_1 was isolated by chloroform extraction of ESMP previously activated by exposure at 37°C for 3 h at pH 8.2 [17]. For reconstitution, USMP were incubated with F_1 at a protein ratio 0.2 mg F_1/mg particle protein, for 20 min at 25°C [14] and then separated from the supernatant after centrifugation at 105000 × *g* for 20 min. Where indicated particles were preincubated with oligomycin (1 μg/mg particle protein) after reconstitution with F_1. F_6, OSCP, PVP were added to trypsin treated USMP before reconstitution with purified F_1 at a concentration of 4 μg/mg particle protein. Figures represent ATP hydrolase activity expressed as μmol ATP hydrolyzed per min per mg protein.

	−		+PVP	
	Control	+oligo	Control	+oligo
Normal USMP	0.04			
Normal USMP + F_1	3.51	0.75	3.04	0.75
Trypsin-USMP + F_1	3.22	2.50	2.57	2.11
Trypsin-USMP + F_1 + F_6	2.06	1.70	2.14	0.20
Trypsin-USMP + F_1 + OSCP	2.90	2.05	2.10	1.15

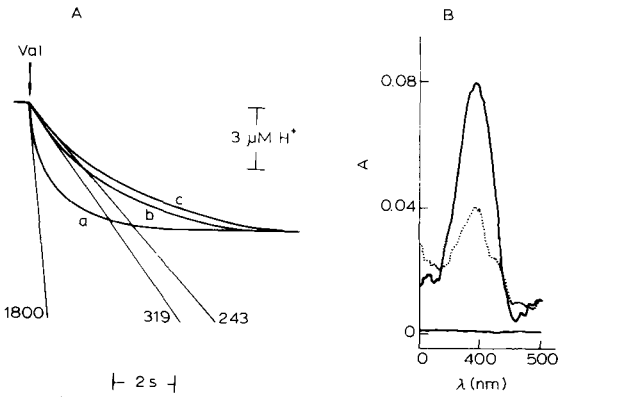

Fig. 2. Inhibition by DACM of proton conduction by F_o-liposomes. analysis of DACM bound by differential spectra. For F_o preparation, DACM treatment, reconstitution in artificial phospholipid vesicles see under Materials and Methods. (A) Valinomycin + K^+ induced H^+ release; (a) none; (b) + DACM (40 nmol/mg F_o protein); (c) + oligomycin (1 μg/mg protein). The figures presented on the traces represent the initial rates of valinomycin induced H^+ release expressed as ng ions H^+ min^{-1} mg F_o^{-1}. (B) Differential spectra. 130 μg of F_o treated with DACM (40 nmol/mg F_o) were suspended in 1 ml phosphate buffer (0.1 M) (pH 7.2) and the spectrum was run. Dotted line indicated F_o preincubated with 0.15 mM NEM, for 30 min, before DACM treatment.

on oligomycin sensitive proton conduction by purified F_o reconstituted in liposomes. H^+ release from reconstituted F_o phospholipid vesicles was 80% inhibited either by oligomycin or by DACM (Fig. 2A). Spectral analysis of DACM treated F_o showed the specific peak of the thiol adduct at 380 nm (Fig. 2B). Controls (not presented) showed that the binding of DACM to F_o was completed in 10 min incubation. Preincubation of F_o with another thiol reagent NEM, decreased the binding of DACM (Fig. 2B). Addition of DACM to liposomes inlaied with F_o pre-treated with a concentration of NEM giving per se about 50% inhibition of proton conduction [21], caused further inhibition, practically additive with that exerted by NEM (Fig. 3).

Fluorescent analysis of PAGE of DACM treated F_o showed binding of DACM to the PVP protein (exhibiting in the present PAGE an apparent M_r of 27000 (see also Refs. 1, 14 and 15), an M_r 25000 band (possibly OSCP) [15], an M_r 11000 band and the DCCD binding protein (Fig. 4A). Fluorescence was also observed in the residual γ subunit of F_1 and in an M_r 31000 band which does not, however, belong to F_o preparations [26]. When F_o was preincubated with NEM, no DACM binding could be detected in the F_o subunits with the exception of subunit *c* (DCCD binding protein) (Fig. 4B).

F_o, isolated from USMP pretreated with DCCD, showed a decrease of DACM induced fluorescence on the M_r 11000 band and subunit *c* (Fig. 4D). Fluorescence of the M_r 31000 band, PVP protein (exhibiting in the present PAGE an apparent M_r of 27000) and

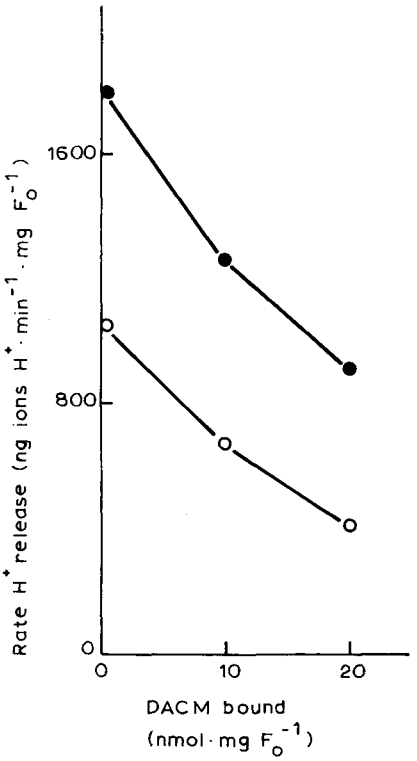

Fig. 3. Additivity of inhibitory effect by thiol reagents on H^+ conduction in F_o liposomes. For F_o purification, treatment with thiol reagents, reconstitution in liposomes and measurement of H^+ release see under Materials and Methods. Symbols: (●———●) F_o; (○———○) F_o preincubated 30 min with 0.15 mM NEM.

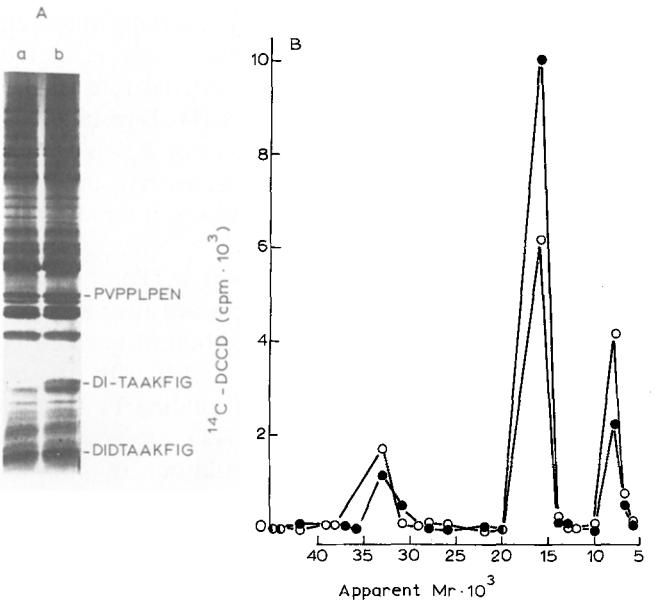

Fig. 5. SDS-PAGE of control and DCCD treated USMP (A). Effect of DACM on binding of ^{14}C-DCCD on F_o polypeptides (B). For USMP preparation, SDS-PAGE, isolation of F_o and determination of binding of ^{14}C-DCCD on F_o polypeptides see under Materials and Methods. (A) SDS-PAGE of USMP; (a) 30 μg USMP; (b) 30 μg of USMP incubated 20 min with 30 μM [^{14}C]DCCD. The protein bands were detected by silver staining. (B) 200 μg of F_o extracted from USMP treated with [^{14}C]DCCD (●———●) or from USMP pretreated with DACM (40 nmol/mg protein) before treatment with DCCD (○———○) were used for SDS-PAGE and then the [^{14}C]DCCD bound to F_o polypeptides was determined.

the M_r 25000 band was practically unaffected by DCCD (Fig. 4).

SDS gel electrophoresis of [^{14}C]DCCD treated USMP revealed, when compared to the control, the appearance of a second band, in the M_r region of 16000 (Fig. 5a). This new band was labelled together with the M_r 8000 band by [^{14}C]DCCD (Fig. 5). Amino acid sequence analysis showed that the M_r 8000 band

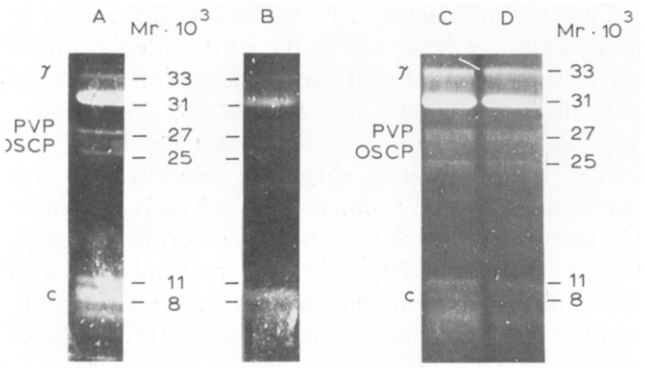

Fig. 4. Fluorescent analysis of binding of DACM on F_o polypeptides. effect of NEM and DCCD. For F_o preparation, treatment with DACM (40 nmol/mg F_o) and SDS-PAGE see under Materials and Methods. (A) and (C) DACM treated F_o. (B) F_o pretreated with 0.15 mM NEM for 30 min before DACM treatment. (D) F_o pretreated with [^{14}C]DCCD 30 μM for 20 min before DACM addition.

is the DCCD-binding subunit *c* [12]. Analysis of the M_r 16000 band appearing after [^{14}C]DCCD treatment, revealed the sequence DI-TAAKFIG... of the DCCD-binding subunit *c* [27]. Thus, the new M_r 16000 band is a dimer formed from subunit *c* after reaction with DCCD. The fact that the dimer retained the radioactive ^{14}C group of the reagent shows that dimerization of the two copies of subunit *c* did not involve reaction of the modified glutamic residue. Such reaction would, in fact, result in the loss of radioactive ^{14}C from the addition product [28]. Pre-treatment of USMP with DACM decreased the appearance of [^{14}C]DCCD in the M_r 16000 region, while it enhanced the radioactivity in the M_r 8000 region (Fig. 5B).

Discussion

The present work shows that proteolytic digestion of F_1-depleted submitochondrial particles, under conditions leading to cleavage of the carboxyl-terminal region of the PVP protein (F_o1) [15] and partial inhibition of passive proton conduction [1], results in a general loss of the sensitivity of this process to different F_o inhibitors like oligomycin [1], DCCD [17] and triphenyltin.

Tryptic digestion of USMP also results in the loss of

oligomycin-sensitivity of the ATPase activity of soluble F_1 reconstituted with the particles. Trypsin digestion does not, however, affect binding of DCCD to F_o [17] neither do the inhibitors affect the pattern of proteolytic digestion of PVP protein by trypsin. H^+ translocation and sensitivity of this process to F_o inhibitors can be specifically restored by the addition of the purified PVP protein (see also Ref. 15). Restoration of oligomycin sensitivity of the ATPase activity of soluble F_1 added to digested particles required, on the other hand, addition of OSCP, or even better of F_6, together with the PVP protein.

It seems possible to conclude that the PVP protein is critically involved in maintaining a normal H^+ translocating configuration of F_o and in assuring, together with OSCP and F_6, a correct functional binding of F_1 to F_o. It should be recalled that the two latter proteins are not necessary for H^+ conduction by F_o [29,30]. Thus, PVP protein appears to represent an essential functional component of the F_oF_1 complex of mitochondria. The carboxyl region of the PVP protein may play a critical role in the gating and coupling function of the F_oF_1-ATP synthase.

Studies with monothiol [15] and dithiol [31] reagents have shown that the single cysteine-197 of the PVP protein is not involved in transmembrane proton translocation [15]. However, modification of purified F_o with the thiol reagent DACM is found to inhibit proton conduction in F_o reconstituted liposomes. Thiol groups in F_o proteins are very few [32]. In addition to the single cysteine present in the PVP protein, one cysteine each is present in OSCP, subunit *d* and subunit *c* [32]. The present results show that DACM inhibition of H^+ conduction by F_o was additive with that exerted by non saturating amounts of NEM. After NEM treatment the only F_o protein that was still able to bind DACM was subunit *c*. It is, therefore, conceivable that modification of the single cysteine of subunit *c* in position 64, four residues apart from the essential glutamic residue, is responsible for DACM inhibition of H^+ conduction by mitochondrial F_o. This is confirmed by the observation that preincubation of USMP with DCCD decreased labelling of subunit *c* by DACM.

Treatment of USMP with [^{14}C]DCCD results in the appearance of a new band with bound [^{14}C]DCCD. This new band is shown, by direct amino acid sequencing, to be a dimer of the M_r 8000 protein. It is conceivable that DCCD binding, at the critical glutamic residue, induces a change in the structure of subunits *c*, which results in the formation of dimers. This might occur through cross-linking of the cysteine-64 as suggested by the observation that preincubation with DACM decreased specifically the radioactivity of [^{14}C]DCCD in the M_r 16000 region whilst increasing that in the M_r 8000 region.

Subunit *c* appears to exist in the membrane in a highly dynamic state. It possibly rotates on its main axis so as to get critical residues exposed alternatively to lipophilic reagents like DCCD and TID in the lipid phase [33]. Pertubation of structure and rotation of subunit *c*, caused by modification of glutamic-58 by DCCD or of cysteine-64 by thiol reagents, may be involved in the inhibition of proton conduction by these reagents.

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