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F_0F_1-ATP synthase of potato tuber mitochondria. Structural and functional characterization by resolution and reconstitution studies

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ATP hydrolase activity and oligomycin-sensitive proton conductivity have been measured in sonic potato submitochondrial particles with various degrees of resolution of the F_1-ATPase. In F_1-containing particles, the ATP hydrolase activity was oligomycin-sensitive both in particles prepared by ultrasonic exposure in the presence of EDTA or in the presence of Mg^{2+}. Removal of F_1 by urea treatment resulted in almost complete removal of the ATP hydrolase activity of the particles in both cases. Addition of purified F_1 to the urea-treated particles restored the membrane bound ATP hydrolase activity. The activity was oligomycin-sensitive when the reconstitution was carried out with urea-treated EDTA submitochondrial particles, but oligomycin insensitive when the reconstitution was carried out with urea-treated Mg^{2+} particles. Comparison of polyacrylamide gel electrophoresis patterns of Mg^{2+} particles and of the urea-treated Mg^{2+} particles showed, in the latter, that the decrease of the amount of the $F_1\alpha$ and $F_1\beta$ subunits was accompanied by the decrease of the amount of a protein of apparent molecular mass of 27 kDa. Addition of the purified 27 kDa protein upon reconstitution of the urea-treated Mg^{2+} particles with F_1, restored the oligomycn sensitivity of the membrane bound ATP hydrolase activity. In F_1-containing particles the oligomycin-sensitive proton conductivity was measured by kinetic analysis of anaerobic release of transmembrane proton gradient set up by respiration. Extrapolation of the initial slope of oligomycin titration of this process showed high affinity binding site at 0.4 μg oligomycin per mg particle protein, i.e., stoichiometry of 1 mol oligomycin per mol F_0F_1-ATP synthase. In F_1-depleted particles, the oligomycin sensitive proton conductivity was measured as proton release induced by a diffusion potential (positive inside) imposed by valinomycin mediated potassium influx. In these particles, also, the initial phase of oligomycin inhibition indicated a high-affinity binding site at 0.4 μg oligomycin per mg particle protein.

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Abbreviations: ESMP and MSMP, submitochondrial particles derived by sonication of mitochondria in the presence of EDTA or $MgCl_2$, respectively; UESMP and UMSMP, urea-treated submitochondrial particles; F_0 and F_1, proton-translocating and catalytic moieties of the mitochondrial ATP synthase, respectively; OSCP, oligomycin sensitivity conferring protein; F_6, subunit of F_0 of bovine heart mitochondrial ATP synthase; PVP protein, subunit of F_0 of bovine heart mitochondrial ATP synthase which corresponds to subunit F_0b of *E. coli* ATPase, PVP stands for proline, valine, proline at N-terminus of the protein; DCCD, *N,N'*-dicyclohexylcarbodiimide; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DTT, dithiothreitol; Mops, 3-(*N*-morpholino)propanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; Tricine, *N*-[2-hydroxy-1,1-bis (hydroxymethyl)methylglycine; PVDF, poly(vinylidine difluoride).

Introduction

The F_0F_1 ATP synthase of mitochondrial membrane is a multisubunit enzyme complex which utilizes the electrochemical proton gradient, generated by the respiratory chain, for synthesis of ATP from ADP and P_i [1,2]. This enzyme complex can be resolved into two moieties: the soluble F_1 sector bearing the catalytic site [3] and the integral F_0 sector which was shown to function as a transmembrane proton translocator in the native [4] and artificial phospholipid membranes [5–7]. In contrast to the prokaryotic F_0 system, which consists of three subunits, a, b and c [8], the F_0 sector of mitochondrial ATP synthase is more complex and was shown to consist, at least, of 7–9 subunits [9–11].

The plant mitochondrial F_0F_1-ATP synthase, recently isolated from spinach leaf mitochondria [12], was shown to consist of 12 polypeptides, of which seven were ascribed to the F_0 sector [13,14].

The role of the individual subunits in proton conduction is not known with certainty. Subunit c, which is found in all species examined, is thought to play a central role in proton conductivity [15]. Analysis of deletion mutants in *E. coli* [16] clearly shows that the presence of all F_0 subunits is required for formation of a complex active in proton translocation. In mammalian mitochondria, in addition to subunit c, subunit b (called PVP protein) was, also, shown to be involved in proton translocation by F_0 [17–19].

Tryptic digestion of the C-terminal region of PVP protein in the F_0 preparation or in F_1-depleted submitochondrial particles was shown to be accompanied by the loss of proton conductivity and sensitivity of the process to F_0 inhibitors [9,18,20]. Among the subunits of the mitochondrial F_0 so far examined, only b and c subunits were shown to be required for oligomycin- and DCCD-sensitive proton translocation. OSCP and F_6 [23] are not required for inhibitor-sensitive proton conductivity through F_0.

Little is known about the mechanism of proton translocation, its coupling to catalytic activities at F_1 level and the role of the individual subunits involved in these processes in F_0F_1-ATP synthase from plant mitochondria. This is mainly due to difficulties associated with preparation of a sufficient amount of mitochondria from plant sources.

In this paper we have studied the ATP hydrolase activity, proton conductivity and oligomycin sensitivity of the above listed processes in sonic submitochondrial particles derived from potato tubers with various degree of resolution of the F_1-ATPase.

Materials and Methods

Chemicals. Oligomycin and valinomycin were obtained from Sigma (St. Louis, MO, USA); phospho-*enol*pyruvate, pyruvate kinase, lactate dehydrogenase, reduced β-nicotinamide adenine dinucleotide (NADH), adenosine triphosphate and catalase from Boehringer (Mannheim, Germany); Percoll from Pharmacia (Uppsala, Sweden). All chemicals used were of high purity grade.

Isolation of mitochondria. Mitochondria from potato (*Solanum tuberosum* L.) tuber were purified on Percoll gradient according to the procedure described earlier [24] with several modifications. Sucrose was substituted for mannitol. Potato tubers were ground in a Moulinex juice centrifuge with continuous adjustment of the pH in the range of pH 7.0–7.5. A 25% instead of 32% (v/v) self-generating Percoll gradient was applied. The yield of mitochondria was high, 400–500 mg mitochondrial protein from 10 kg of starting material.

Preparation of submitochondrial particles. The isolated mitochondria, suspended in 0.25 M sucrose, 5 mM Mops (pH 7.5) at a protein concentration of about 10 mg/ml were washed in the same buffer containing 3% BSA. To obtain submitochondrial particles, the mitochondrial suspension was exposed to ultrasonic energy two times (setting 8) for 2 min in the presence of 4 mM EDTA or 30 mM $MgCl_2$, at pH 8.5, followed by centrifugation at $105\,000 \times g$ for 45 min [25]. Proteinase inhibitor, PMSF at 0.5 mM was present during sonication. Submitochondrial particles prepared in the presence of EDTA will be referred to as ESMP and in the presence of Mg^{2+} as MSMP.

For preparation of F_1-depleted submitochondrial particles, one volume of ESMP or MSMP (10 mg/ml) was incubated with one volume buffer containing 4 M urea, 0.1 M Tris-SO_4, 4 mM EDTA (pH 8.0) for 30 min. After the incubation, the suspension was centrifuged in 50.2 Ti rotor at $105\,000 \times g$ for 10 min at 4°C. The pellet was washed once in 0.25 M sucrose (pH 7.5). The resultant pellet, called UESMP or UMSMP, was resuspended in 0.25 M sucrose, 5 mM DTT (pH 7.5) at a protein concentration of 10 mg/ml.

Isolation of F_1 and of the 27 kDa protein. F_1 was isolated by the chloroform extraction method described previously [25]. Protein subunit of 27 kDa was isolated by preparative gel electrophoresis [18].

Reconstitution of oligomycin-sensitive ATP hydrolase activity. F_1-depleted ESMP (UESMP), 100 μg and F_1-depleted MSMP (UMSMP), 100 μg were incubated with purified F_1, 20 μg (at a ratio of membrane protein/F_1 protein = 5) for 30 min at room temperature in 100 μl medium containing 0.25 M sucrose, 10 mM Tris-SO_4 (pH 8.0), and 20 mM magnesium acetate (medium A). Bound F_1 was separated from the unbound F_1 by cenrifugation of the samples for 5 min in an Eppendorf centrifuge at 18000 rpm. The pellet containing reconstituted enzyme was washed in medium A, centrifuged and resuspended in the same medium. When the isolated 27 kDa protein was used in reconstitution experiments, the order of addition was as follows: membranes, F_1 and finally the isolated 27 kDa subunit. The amount of the isolated 27 kDa subunit used in these experiments was 3 μg subunit per 200 μg membrane protein.

Measurement of proton translocation. In ESMP, proton conduction by mitochondrial F_0F_1-ATP synthase was followed by kinetic analysis of the release of transmembrane proton gradient, set up by respiration, with anaerobiosis [26]. For this analysis ESMP (3 mg protein/ml) were incubated in a reaction mixture containing: 0.25 M sucrose, 30 mM KCl, 1.0 mg valinomycin/mg particle protein, 0.2 mg/ml purified catalase and 20 mM succinate (pH 7.5), under anaerobic conditions. Final volume was 1.5 ml. Respiration driven proton translocation was activated by repetitive addi-

tions of 1–3% H_2O_2 (5 μl/ml). The pH changes were monitored potentiometrically and converted into proton equivalents by titration with standard HCl. In F_1-depleted particles, UESMP proton conduction was measured potentiometrically in a reaction mixture containing 0.25 M sucrose and 30 mM KCl (pH 7.5), by kinetic analysis of proton release from the particles induced by diffusion potential (positive inside) imposed by valinomycin mediated potassium influx [2]. Valinomycin was added at a concentration of 0.5 mg/mg particle protein.

Activity measurements. The ATP hydrolase activity was determined spectrophotometrically by coupling the reaction of the pyruvate kinase and lactate dehydrogenase and following the oxidation of NADH at 340 mM as described earlier [7].

Electrophoresis. Polypeptide analysis was performed by SDS-PAGE in the buffer system of Laemmli [28], using a 12–22% linear acrylamide gradient.

Protein determination. Protein content was determined by modified Lowry-Folin assay [29].

Results

Both ESMP and MSMP show oligomycin-sensitive ATP hydrolase activity (Table I). In ESMP this activity was somewhat higher and less sensitive to oligomycin than that of MSMP. This observation together with that showing that ESMP from bovine heart [30] can not catalyze oxidative phosphorylation, while MSMP can do it both in bovine [31] and in plants [32] suggest a lower degree of coupling between F_0 and F_1 in ESMP.

TABLE I

Reconstitution of oligomycin-sensitive ATP hydrolase activity in F_1-depleted potato tuber submitochondrial particles

For preparation of submitochondrial particles, isolation of F_1, isolation of the 27 kDa protein and determination of ATPase activity see Materials and Methods. For reconstitution experiments, UESMP (100 μg), UMSMP (100 μg) and purified F_1 (20 μg) were incubated as described under Materials and Methods. The specific activity of purified F_1 was 12 μmol/min per mg protein. Where indicated, 3 μg of the purified 27 kDa protein were added as described in Materials and Methods. To determine inhibitor sensitivity, 5 nmol of oligomycin per mg particle protein were added. Standard error in these measurements does not exceed 10%.

Particles	Additions	ATPase activity (μmol/min per mg protein)	Oligomycin senstivity (%)
ESMP	–	0.38	68
UESMP	–	0.07	0
UESMP	F_1	0.43	65
MSMP	–	0.27	85
UMSMP	–	0.02	0
UMSMP	F_1	0.25	0
UMSMP	F_1 + 27 kDa protein	0.31	89

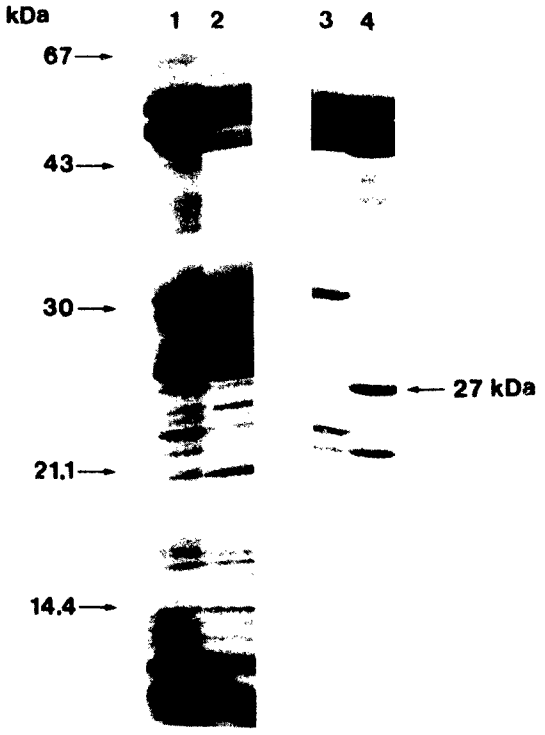

Fig. 1. SDS-PAGE profile of: Lane 1, 60 μg MSMP; lane 2, 40 μg of UMSMP; lane 3, 15 μg of purified F_1; lane 4, 15 μg of supernatant obtained after urea treatment of MSMP. The arrows on the left hand side indicate the molecular mass markers: bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, α-lactalbumin in order of decreasing molecular mass.

Urea treatment of both types of particle resulted in a membrane preparation which was almost depleted of F_1, as indicated by the almost complete removal of ATP hydrolase activity.

Reconstitution of F_0F_1 complex by addition of purified F_1 to urea-treated particles reconstituted membrane bound ATP hydrolase activity in both types of particles. However, whereas the reconstituted ATP hydrolase activity of UESMP plus F_1 was sensitive to oligomycin as that of the original ESMP, the ATP hydrolase activity of the reconstituted system, UMSMP plus F_1, was practically insensitive to oligomycin (Table I).

SDS polyacrylamide gel electrophoresis of UMSMP (Fig. 1) showed that in addition to the decrease of $F_1\alpha$ and $F_1\beta$ subunits there was also a decrease of the amount of one protein of apparent molecular mass of 27 kDa as compared to MSMP. This protein, together with the $F_1\alpha$ and $F_1\beta$ subunits was found in the supernatant obtained after urea treatment of MSMP (Fig. 1, line 4). Addition of the purified, electroeluted 27 kDa protein to the reconstituted system, UMSMP plus F_1, restored the oligomycin sensitivity of particles

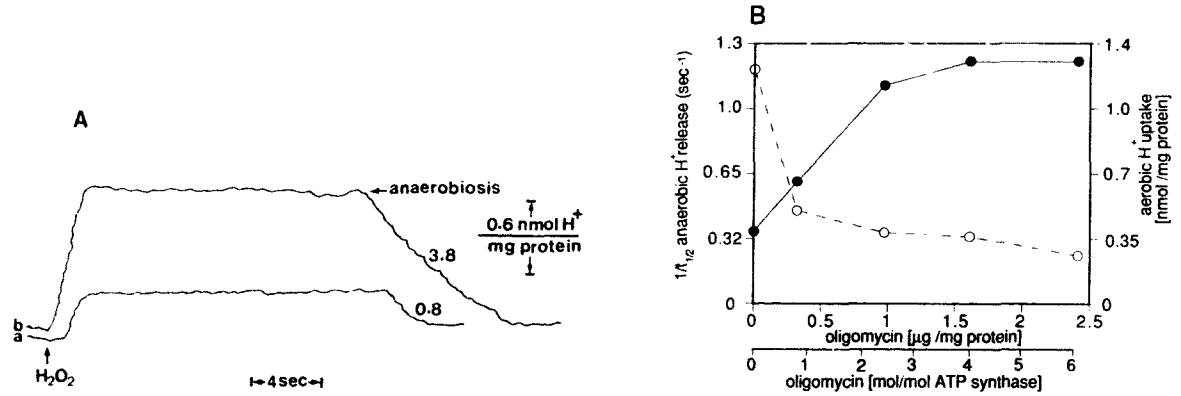

Fig. 2. Proton translocation induced by respiratory pulses in ESMP (A) and titrations of the inhibition by oligomycin (B) of anaerobic proton release (○); aerobic proton uptake (●) from ESMP. In (A) trace a, control; trace b, ESMP incubated in the reaction mixture for 5 min with oligomycin (2.41 µg /mg protein) before addition of H_2O_2. For experimental procedure, see Materials and Methods. The numbers written on the traces refers to $t_{1/2}$ of proton release under anaerobiosis.

(Table 1). The 27 kDa protein crossreacted with antibodies against the PVP protein of beef heart mitochondria (not shown).

Fig. 2A shows typical cycle of proton uptake and release induced by respiratory pulses of succinate supplemented ESMP. Initial rapid uptake of protons is followed by aerobic steady state [33]. When the particles become anaerobic the proton gradient decayed exponentially ($t_{1/2}$ = 0.8) (Fig. 2A). Oligomycin, a specific inhibitor of proton translocation by F_0 [33], enhanced the extent of aerobic proton uptake as consequence of inhibition of passive proton back-flow ($t_{1/2}$ = 3.8). Fig. 2B shows the oligomycin titration on the increase of the extent of the aerobic proton uptake and on the inhibition of the rate of anaerobic proton release (measured as reciprocal value of $t_{1/2}$). It can be observed that the extent of the proton uptake increased maximally 3-fold in the presence of oligomycin. Rapid increase of the extent is observed at the concentration of oligomycin below 1 µg oligomycin/mg particle protein. At this concentration 80% of the total increase of the extent is observed. At higher concentration of oligomycin, only a slow increase of the extent is seen. Addition of oligomycin does decrease the value of $1/t_{1/2}$ of the anaerobic backflow with a maximal effect of 4-fold at the concentration giving maximal increase of the proton uptake. Extrapolation of the initial slope of the plot of the inverted $t_{1/2}$ versus

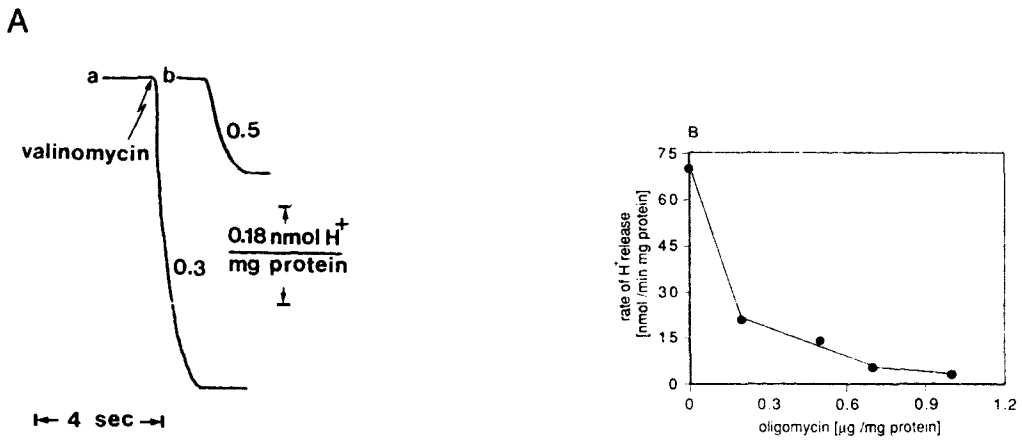

Fig. 3. Proton release from UESMP induced by valinomycin-mediated potassium influx (A). Titrations of the inhibitory effect by oligomycin on the rate of proton release (B). In (A) trace a, control; trace b, UESMP incubated in the reaction mixture for 5 min with oligomycin (0.5 µg /mg protein) before addition of valinomycin. For experimental procedure, see Materials and Methods. The numbers shown on the traces refers to $t_{1/2}$ of proton release.

oligomycin shows high affinity at about 0.4 μg oligomycin/mg particle protein (Fig. 2B).

The amount of F_1 in submitochondrial particles from potato tubers was estimated using Western blot technique with antibodies against the isolated potato F_1 (not shown). F_1 constitutes 14% of the total membrane protein. Assuming a molecular mass of F_1 to about 375 000, it was calculated [25] that the concentration of the ATP synthase in the membrane is 0.4 nmol/mg particle protein. Our results indicate thus that the proton conduction through F_0F_1 ATP synthase in ESMP can be almost completely inhibited by oligomycin at a molar stoichiometry of oligomycin per F_0F_1-ATP synthase of 1 : 1.

F_1-depleted submitochondrial particles were prepared by treatment of the EDTA submitochondrial particles with 2 M urea. This caused depletion of 80% of F_1 as estimated by measurement of the ATP hydrolase activity (see Table I). Measurement of proton conduction using repetitive pulses of oxygen (as described above for ESMP) was difficult to apply to UESMP. Therefore, proton conduction in UESMP was measured as proton release induced by valinomycin mediated K^+ influx. Typical traces of this experiment are shown in Fig. 3A (trace a). Both the extent and the velocity of H^+ release was gradually reduced by increasing concentration of oligomycin (Fig. 3A, trace b and Fig. 3B). Oligomycin, at a concentration of 0.2 μg/mg particle protein, reduced the rate of H^+ release to about 30%. Almost total inhibition of the process was obtained at a concentration of 0.7 μg/oligomycin per mg particle protein. Extrapolation of the initial slope if the titration curve of the oligomycin inhibition indicates high affinity binding site for oligomycin at a concentration of 0.3 μg oligomycin per mg particle protein, very similar to that obtained by extrapolation of the initial slope of the titration curve of oligomycin inhibition in ESMP (see above). These results show that oligomycin inhibits H^+ translocation through F_1-depleted particles with the same stoichiometry of 1 : 1 as in F_1-containing particles.

Discussion

In this paper the structure and function of F_0F_1-ATP synthase from potato tuber mitochondria has been studied by resolution and reconstitution of the enzyme in sonic submitochondrial particles. Particles derived by sonication of potato tuber mitochondria in the presence of EDTA exhibited higher ATP hydrolase activity and lower sensitivity to oligomycin of the hydrolase activity than the particles prepared in the presence of Mg^{2+}. These observations suggest that in ESMP, the catalytic ATPase activity at the F_1 level is not well coupled to H^+ translocation at the F_0 level and that there is better coupling between F_1 and F_0 in MSMP than in ESMP. It is feasible that positively charged Mg^{2+} ions present in the MSMP preparation improve binding of the negatively charged F_1 to the negatively charged membrane [34].

In ESMP, but not in MSMP (not shown), an oligomycin-sensitive proton conduction can be observed, confirming the above suggestion that F_1 is loosely coupled to F_0 in ESMP. Oligomycin inhibits this process with a molar stoichiometry of 1 : 1 oligomycin per F_0F_1 complex (cf. Fig. 2). Urea treatment of ESMP caused complete inhibition of ATP hydrolase activity, probably due to the removal of F_1 subunits as described for the urea-treated ESMP from beef heart mitochondria [4,7].

In UESMP, the oxygen induced proton cycles cannot be observed. This could be due either to higher passive proton conductivity of the membranes after urea treatment or to inhibition of electron flow of the respiratory chain. In any case, in UESMP, the passive proton conduction was induced by diffusion potential (positive inside) imposed by valinomycin mediated K^+ influx. This process was also inhibited by oligomycin with a molar ratio of 1:1 indicating that urea treatment does not alter the binding of oligomycin to F_0.

MSMP seem to be better coupled and, in fact, MSMP from pea cotyledon catalyse oxidative phosphorylation [31]. Treatment of these particles with 3 M urea resulted in a preparation of highly resolved particles which after reconstitution with F_1 showed low ATP hydrolase activity and no capacity of oxidative phosphorylation. The present study shows that urea treatment of MSMP from potato mitochondria removed, in addition to F_1 subunits, also a protein of apparent molecular mass of 27 kDa. Reconstitution of oligomycin-sensitive ATP hydrolase activity in UMSMP was possible only if the isolated 27 kDa protein was added together with the purified F_1. Thus, it is possible that in urea-treated MSMP from pea cotyledon [32] the 27 kDa protein, or its analogue, was removed. The fact that the 27 kDa protein is resolved from the MSMP in the presence of urea but not from ESMP indicates that this protein is intrinsically located in the F_0F_1-ATPase complex and that the presence of the positively charged ions facilitates dissociation of this protein from the complex. In trypsin-treated UESMP from beef heart mitochondria, the oligomycin-sensitive ATP hydrolase activity can be reconstituted only if, together with F_1, the 27 kDa protein and OSCP or F_6 are added [20]. This 27 kDa protein of beef heart mitochondria called also PVP protein has been identified, by sequence analysis, as corresponding to subunit b of F_0 of *E.coli* ATPase. The 27 kDa protein described in the present work is the component of F_0 and crossreacts with antibodies against the PVP protein (not shown). This protein corresponds also to the 28 kDa protein of spinach leaf mitochondria. We have recently shown

that the protein of 28 kDa of spinach leaf mitochondria crossreacts with antibodies against the PVP protein of beef heart mitochondria [13] and we have sequenced 32 amino acids at N-terminus. Sequence analysis reveals however no sequence similarity at N-terminus of the 28 kDa protein of spinach leaf mitochondria and the PVP protein of beef heart mitochondria. Lack of sequence similarity has also been observed between F_0b subunits from other sources [35].

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