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Subunit composition and cold stability of the pea cotyledon mitochondrial F_1-ATPase *

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The F_1-ATPase isolated from pea cotyledon submitochondrial particles contained six types of subunit with molecular weights of 57000 (α), 55000 (β), 36500 (γ), 26500 (δ), 22500 (δ′) and 8000 (ε). The same polypeptide composition was observed even when the purification was carried out at 4°C in the presence of proteolytic inhibitors, suggesting that the sixth subunit was not a proteolytic product formed during the isolation procedure. The six-subunit F_1-ATPase exhibited considerable cold stability: it retained 65% of its activity after 24 h of incubation at 0°C and was more than 90% active after 48 h of incubation at 4°C. The 26.5 kDa protein could be dissociated from the remaining F_1-ATPase by centrifugation in a linear sucrose gradient containing $(NH_4)_2SO_4$ and deoxycholate. The resulting five-subunit F_1-ATPase was considerably less stable at 0°C than the six-subunit enzyme. Several features suggest the possibility of a functional and structural relationship between the 26.5 kDa protein of the pea cotyledon mitochondria and the mammalian oligomycin-sensitivity-conferring protein.

Introduction

Recently, we have described the purification to homogeneity of the pea cotyledon mitochondrial F_1-ATPase [1]. We have shown that the purified enzyme contained an unusual complement of six subunits when electrophoresed in the presence of sodium dodecyl sulfate [1].

The hydrophilic, peripheral component of the coupling factor complex from chloroplasts, bacteria, yeast and mammalian mitochondria contains five different polypeptides. During recent years purification of the F_1 portion of proton-translocating ATPases from higher plant mitochondria has been achieved in several laboratories. The enzymes purified from maize [2,3], oat root [4] and cuckoo-pint [5] mitochondria were reported to contain the usual complement of five subunits (α, β, γ, δ, ε), while in the purified mitochondrial adenosine triphosphatases from sweet potatoes [6,7] and from pea cotyledons [1] six kinds of subunits (α, β, γ, δ, δ′, ε) were present. The possibility that the sixth polypeptide observed in the pea cotyledon F_1-ATPase represented a contamination with a protein not related to the coupling factor complex has been excluded in the previous report [1]. In this paper we present evidence that the sixth polypeptide is not a proteolytic product formed from one of the larger subunits during the F_1 isolation. Moreover, we report that the six-subunit enzyme exhibits cold stability

* This paper is dedicated to Dr. Saul Zalik on the occasion of his 65th birthday.

Abbreviations: F_1, mitochondrial coupling factor; PMSF, phenylmethylsulfonylfluoride; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; P_i, inorganic phosphate.

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which is considerably decreased upon dissociation of the 26.5 kDa protein from the ATPase.

Materials and Methods

Preparation of pea cotyledon mitochondrial F_1-ATPase. The pea cotyledon F_1-ATPase has been isolated by three different procedures.

(1) The first isolation procedure has been carried out at room temperature. The enzyme was released from submitochondrial particles by a washing procedure using a low-ionic strength sucrose solution and purified by DEAE-cellulose chromatography followed by sucrose density gradient centrifugation exactly as described before [1].

(2) To minimize the possibility of proteolysis during the isolation, the second procedure has been carried out at 4° C with the inclusion of proteolytic inhibitors; 1 mM phenylmethylsulfonylfluoride (PMSF; Sigma) as well as 1 mM *p*-aminobenzamidinehydrochloride (Sigma) were included during the mitochondrial disruption [2], 1 mM PMSF was present during chloroform extraction of the mitochondrial membranes [2] and during sucrose density gradient centrifugation. Mitochondria isolated by differential centrifugation [1,8] were suspended at 10 mg protein/ml in 10 mM Tricine-KOH (pH 7.2), 1 mM EGTA, 0.4 M mannitol, and repelleted by centrifugation at 15000 × *g* for 15 min. The final pellets were frozen in dry ice and stored at −80° C. Mitochondrial membranes were prepared by 'osmotic shocking' of frozen and thawed mitochondria [2] and the ATPase was extracted from these membranes by chloroform as described by Hack and Leaver [2] for maize mitochondrial adenosine triphosphatase. Chloroform extraction was the only step in this purification procedure that was carried out at 20° C. All subsequent steps were performed at 4° C and 10%–12.5% (v/v) methanol was included in the buffers in order to minimize the possible cold-induced inactivation of the F_1-ATPase [2]. The aqueous phase obtained after removal of chloroform was supplemented with 1/9 volume of methanol, chilled to 4° C and loaded on a 1.5 × 17 cm DEAE-cellulose (Whatman DE-52) column [1] equilibrated with 'buffer A' containing 300 mM sucrose, 2 mM EDTA, 2 mM ATP, 20 mM Tris-H_2SO_4 (pH 7.4) and 10% (v/v) methanol. After washing with 50 ml of this buffer, the proteins were eluted from the column at 4° C with 150 ml of a linear 0–0.2 M K_2SO_4 gradient in 'buffer A'. The fractions with highest ATPase activity were pooled, concentrated on an Amicon YM-10 membrane and loaded on a 35 ml linear 12.5%–35% (w/v) sucrose gradient [1] containing 1 mM EDTA, 1 mM ATP, 1 mM PMSF, 20 mM Tris-H_2SO_4 (pH 7.6) and 12.5% (v/v) methanol [2]. After centrifugation at 44000 × *g* for 41 h at 4° C, the fractions with highest ATPase activity were pooled, dialysed against 'buffer A' and stored at −80° C.

(3) The third procedure for isolation of the F_1-ATPase was the same as procedure number one, except that the DEAE-cellulose chromatography and sucrose density gradient centrifugation were carried out at 4° C instead of room temperature. No methanol or proteolytic inhibitors were included during this purification.

Sucrose density gradient centrifugation in the presence of $(NH_4)_2SO_4$ and deoxycholate. Purified F_1-ATPase (400 μg, specific activity 25 μmol P_i/min per mg protein) that was stored in 'buffer A' at −80° C, was thawed, dialyzed against 'buffer A' without methanol at room temperature and concentrated using Centricon-30 Microconcentrators (Amicon) to 200 μl. $(NH_4)_2SO_4$ was added to the sample to a final concentration of 0.25 M, followed by addition of 40 μg sodium deoxycholate (100 μg/mg protein) and allowed to stand for 15 min at room temperature [9]. Subsequently, the sample was layered on a 5 ml linear 12.5%–35% (w/v) sucrose gradient containing 20 mM Tricine (pH 7.1), 2 mM EDTA, 1 mM ATP, 0.25 M $(NH_4)_2SO_4$, 0.1% sodium deoxycholate, and centrifuged at 175000 × *g* for 19 h at 20° C. After the centrifugation, fractions with the highest ATPase activity were pooled, dialyzed against 'buffer A' without methanol, concentrated, and used for SDS-polyacrylamide gel electrophoresis and cold stability studies.

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis was carried out in slab gels containing a linear 7.5–15% acrylamide concentration gradient under conditions described by Chua [10]. The molecular weight standards (Sigma) included

bovine serum albumin (66 000), ovalbumin (45 000), glyceraldehyde 3-phosphate dehydrogenase (36 000), carbonic anhydrase (29 000), trypsinogen (24000), soybean trypsin inhibitor (20 100), α-lactalbumin (14200) and aprotinin (6500). The gels were stained for protein with Coomassie Brilliant Blue R-250. The stained gels were scanned with a model 750 densitometer (Corning Instruments).

ATPase activity of F_1 and protein determination. The ATPase activity of the mitochondrial F_1 was assayed as described previously [1]. Protein was measured by the method of Bradford [11].

Results and Discussion

Subunit composition of the F_1-ATPase from pea cotyledon mitochondria

Results in Fig. 1 show that the F_1-ATPase isolated from pea cotyledon mitochondria contained six types of polypeptide regardless of whether it was extracted from the mitochondrial membranes by a low-ionic strength sucrose solution and further purified by DEAE-cellulose chromatography and sucrose density gradient centrifugation at room temperature (Fig. 1, lane b), or whether it was released from the mitochondrial membranes by chloroform extraction and purified by DEAE-cellulose chromatography and sucrose density gradient centrifugation at 4 °C in the presence of proteolytic inhibitors (Fig. 1, lane a). The subunits with apparent molecular weights of 57 000 (α), 55 000 (β), 36 500 (γ), 26 500 (δ), 22 500 (δ′) and 8000 (ε), were present in approximately the same quantity in both F_1 preparations. The α and β subunits of F_1 migrated close to each other and did not separate clearly in Fig. 1. However, they separated completely when small amounts of protein (1–2 μg) were used for electrophoresis. The results strongly indicate that the additional δ subunit present in our preparation is not a proteolytic product of one of the larger subunits formed during the isolation procedure. The presence of the δ and δ′ subunits in pea mitochondrial F_1-ATPase is in agreement with the observations of Iwasaki and Asahi [6,7] who found two δ subunits in the preparation of sweet potato mitochondrial F_1-ATPase. The tryptic peptide maps prepared from purified δ and δ′ subunits differed completely from each other, indicating that the subunits were different molecular species [7].

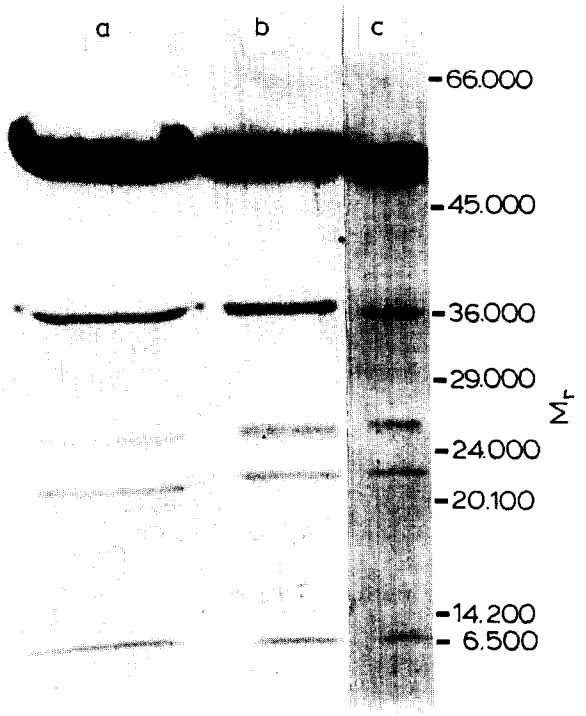

Fig. 1. SDS-polyacrylamide gel electrophoresis of purified pea cotyledon mitochondrial F_1-ATPase. The enzyme was isolated and electrophoresis carried out as described in Materials and Methods. The numbers along the ordinate indicate the molecular weights of the molecular-weight standards (bovine serum albumin, ovalbumin, glyceraldehyde-3-phosphate dehydrogenase, carbonic anhydrase, trypsinogen, soybean trypsin inhibitor, α-lactalbumin, aprotinin). Lane (a), 12 μg F_1 prepared by chloroform extraction and purified at 4 °C in presence of methanol, PMSF, and *p*-aminobenzamidinehydrochloride (procedure no. 2 in Materials and Methods); lane (b), 12 μg F_1 prepared by low-ionic strength extraction and purified at 20 °C in absence of proteolytic inhibitors (procedure no. 1 in Materials and Methods); lane (c), 10 μg F_1 prepared by low-ionic strength extraction and purified at 4 °C in absence of proteolytic inhibitors or methanol (procedure no. 3 in Materials and Methods).

Moreover, both subunits were synthesized under the direction of sweet potato poly(A)$^+$RNA in the wheat germ system [7]. Other investigators have also detected two δ subunits in preparations of F_1-ATPases from plant sources. Randall et al. [4] occasionally observed two δ subunits in their preparations of oat root mitochondrial F_1-ATPase. While Fava bean mitochondrial F_1-ATPase was

reported to contain only five types of subunit [12], the molecular weight of Fava bean ε subunit (22900) was closer to that of the δ′ subunit (22500) in our preparation than to the usual molecular weight of ε subunits from a variety of organisms (6000–16000).

It is interesting to note that our pea cotyledon mitochondrial δ and δ′ subunits seem to exhibit higher molecular weights than the rat liver mitochondrial δ (molecular weight: 12000, Ref. 13) or bovine heart mitochondrial δ subunit (molecular weight: 15000, Ref. 14). Similarly, Dunn et al. [5] noted the discrepancy in molecular weights between δ subunits of cuckoo-pint and rat liver F_1-ATPase, with the plant enzyme being comparable in size to the δ subunit of *Escherichia coli*. *E. coli* and bovine heart δ subunits are not equivalent; rather *E. coli* δ shows sequence and function homologies with bovine heart oligomycin-sensitivity-conferring protein [15] and bovine heart mitochondrial δ is related to the bacterial ε subunit [14]. It is possible that the plant mitochondrial δ subunit may be more closely related to its bacterial counterpart than is the δ subunit of the animal F_1-ATPase.

Cold stability of the F_1-ATPase

With the exception of thermophilic bacteria [16,17], the isolated F_1-ATPases from a variety of energy-transducing membranes including mammalian mitochondria [18,19], yeast mitochondria [20] and mesophilic bacteria [21] exhibit pronounced cold lability. Partially purified pea cotyledon mitochondrial ATPase has been rapidly inactivated by exposure to cold and most of the ATPase activity was lost after 20 min incubation at 0°C [22]. Corn mitochondrial F_1-ATPase lost practically all activity after 1 h at 0–2°C [3]. Purified sweet potato mitochondrial ATPase exhibited some cold lability, though the decline in the activity was considerably slower than for the majority of other F_1-ATPase preparations [6]. About 40% of activity was still remaining after 15 h of incubation at 2°C [6].

Methanol has been found to provide protection against cold inactivation to a variety of F_1-ATPase preparations, including the mitochondrial ATPases from bovine heart [19], yeast [20], pea cotyledons [22] and maize [2]. We have, therefore, initially included methanol in buffers during the purification of the pea cotyledon F_1-ATPase when it was performed at 4°C (second purification procedure described in Materials and Methods).

However, subsequently we have observed, that the F_1-ATPase which was extracted from pea cotyledon mitochondria by a low-ionic strength sucrose solution and further purified at room temperature as described in Materials and Methods (procedure 1), did not loose any activity when it was stored overnight in the refrigerator, even in the absence of methanol. When the purification of the ATPase was carried out at 4°C in the absence of methanol (procedure 3 in Materials and Methods), the specific activity of the purified enzyme was 27 μmol P_i/min per mg protein. This activity was comparable to the specific activity of 23–30 μmol P_i/min per mg protein obtained after purification at room temperature (procedure 1 in Materials and Methods). This result suggested considerable cold stability of the pea cotyledon F_1-ATPase. The enzyme purified at 4°C contained the same six subunits (Fig. 1, lane c) as the enzyme purified at room temperature (Fig. 1, lane b), or the enzyme which was released from the membranes by chloroform extraction and purified at 4°C in the presence of methanol and proteolytic inhibitors (Fig. 1, lane a).

Fig. 2 shows the effect of temperature on the ATPase activity of the purified enzyme when it was stored over an extended period of time at 0°C or 4°C. When stored at 4°C in a buffer containing 2 mM ATP, the enzyme retained more than 90% of its activity after 48 h and still had 75% of its activity even after 72 h of incubation (Fig. 2). The same stability of the enzyme was observed at 4°C in the absence of ATP. When stored at 0°C, the ATPase activity decreased faster than at 4°C, however, the inactivation was still slow. In the presence of 2 mM ATP, the enzyme lost 35% of its activity after 24 h at 0°C. In the absence of ATP, the activity decreased by 50% in the same time period (Fig. 2). Thus, the inclusion of ATP provided some protection to the enzyme at 0°C. This is in agreement with the results reported for the purified yeast oligomycin-sensitive ATPase [23], but in contrast to the lack of an ATP effect on cold inactivation of the purified bovine heart [18], yeast [20], or partially purified

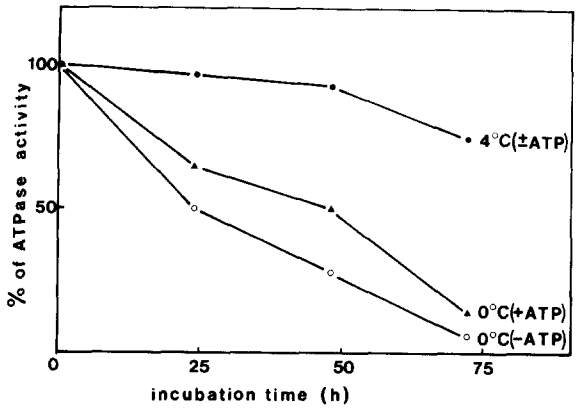

Fig. 2. Effect of temperature on the activity of the purified pea cotyledon mitochondrial F_1-ATPase. Purified F_1-ATPase prepared by the procedure no. 3 in Materials and Methods was incubated at a concentration of 170 μg of ATPase protein/ml in a buffer containing 300 mM sucrose, 2 mM EDTA, 20 mM Tris-H_2SO_4 (pH 7.4) and 0 or 2 mM ATP either at 4 °C or 0 °C. At the times indicated, a 10 μl aliquot of the sample was withdrawn and its ATPase activity assayed as described previously [1].

pea cotyledon [22] mitochondrial F_1-ATPase.

We considered it probable that one of the two δ subunits observed in our preparation of the pea cotyledon F_1-ATPase was responsible for the high degree of cold stability of the isolated enzyme. The pronounced cold lability of the partially purified pea cotyledon ATPase [22] could have been caused by removal of the sixth polypeptide during the harsh sonication used for disruption of mitochondria and for release of the ATPase [24]. As mentioned above, purified sweet potato mitochondrial F_1-ATPase exhibiting a six-subunit composition [6] was inactivated by exposure to cold much more slowly than the five-subunit corn mitochondrial F_1-ATPase [3].

Bovine heart mitochondrial F_1-ATPase is composed of five subunits [25] and is rapidly inactivated at low temperature [19]. Hundal et al. [26] observed that when the purified F_1 was complexed with the bovine heart oligomycin-sensitivity-conferring protein (OSCP), the resulting complex was more resistant to cold inactivation than the F_1-ATPase alone. Both free F_1 and the F_1-OSCP complex were shown to release upon cold exposure a part (probably 1 out of 3) of their β subunits [26]. As the OSCP itself was found to interact with the α and β subunits [27], it is possible that binding of this protein to the F_1-ATPase interferes with the cold-induced dissociation of the subunits. Fisher et al. [28] isolated from rat liver mitochondria a complex of a five subunit F_1-ATPase with a 26.5 kDa protein. This protein was functionally and structurally related to the bovine heart OSCP [29] and its presence stabilized the ATPase to cold inactivation [28].

Another protein shown to confer increased cold stability upon the soluble F_1-ATPase was the mitochondrial ATPase inhibitor discovered by Pullman and Monroy [30]. However, it does not seem to be very probable that one of the δ components in our preparation of the pea mitochondrial F_1-ATPase is related to the inhibitor protein. The molecular weights of our δ and δ′ components are substantially higher than the molecular weight of 9578 reported for the inhibitor protein [31] and, more significantly, all six subunits remained associated even after prolonged sucrose density gradient centrifugation carried out at room temperature in the presence of 1 mM ATP and 2 mM EDTA. Under these conditions, the inhibitor protein should be dissociated from the remaining F_1 complex [30,32].

Similar to its mammalian counterpart, the plant mitochondrial membrane-bound ATPase has been shown to be completely inhibited by oligomycin [33], indicating the presence of an oligomycin-binding site and, possibly, of the oligomycin-sensitivity-conferring protein. In 1968, Vallejos et al. [34] isolated from bovine heart mitochondria a complex of F_1 with a protein that was later identified as oligomycin-sensitivity-conferring protein [35,9]. This protein could be separated from F_1 in a linear sucrose gradient in the presence of $(NH_4)_2SO_4$ and deoxycholate [9]. We have employed the conditions similar to those of Van de Stadt et al. [9] in order to attempt the separation of the extra δ subunit from the pea mitochondrial F_1-ATPase complex. Fig. 3 shows the gel-electrophoretic profiles of the pea mitochondrial F_1-ATPase before and after centrifugation in a linear sucrose gradient in the presence of 0.25 M $(NH_4)_2SO_4$ and 0.1% deoxycholate. The two δ components are clearly visible in the sample before centrifugation (a), while the 26.5 kDa δ subunit is missing in the F_1-ATPase obtained after the sucrose gradient centrifugation step (b). Thus, the δ subunit present in our preparation of the pea

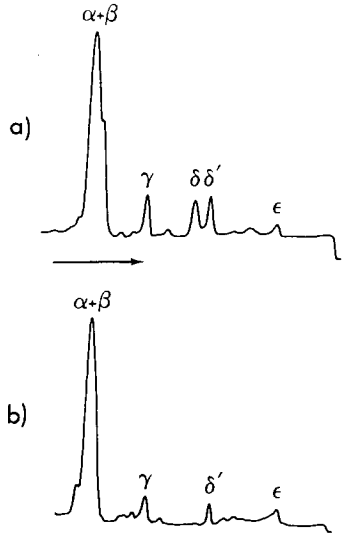

Fig. 3. Gel electrophoretic profiles of the purified pea cotyledon mitochondrial F_1-ATPase. Purified F_1-ATPase was centrifuged in a linear sucrose gradient containing 0.25 M $(NH_4)_2SO_4$ and 0.1% deoxycholate as described in Materials and Methods; 15 μg of the ATPase before the centrifugation (a), as well as 15 μg of the enzyme after the centrifugation (b) were electrophoresed on SDS-polyacrylamide slab gels. After the electrophoresis the gels were stained and scanned as described in Materials and Methods. The arrow indicates the direction of the run.

cotyledon mitochondrial F_1-ATPase dissociated from the remaining complex under the same conditions that allowed complete separation of the oligomycin-sensitivity-conferring protein from the bovine heart mitochondrial F_1 [9].

The six-subunit as well as the five-subunit enzymes were incubated at 0 °C under the same conditions as described in the legend to Fig. 2 (with the inclusion of 2 mM ATP). After 24 h at 0 °C the six-subunit enzyme lost 38% of its ATPase activity, while the activity of the five-subunit enzyme declined by 76%. Incubation for 48 h at 0 °C resulted in 83% activity loss in case of the five subunit ATPase, while the six-subunit enzyme retained 50% of its activity after the same period of time. The results confirmed our original hypothesis, that the presence of the sixth subunit increases the cold stability of the purified pea mitochondrial F_1. The molecular weight of this subunit (26 500) corresponds to the molecular weight of the rat liver mitochondrial oligomycin-sensitivity-conferring protein [29] and is close to the molecular mass of 20 967 reported for bovine heart mitochondrial oligomycin-sensitivity-conferring protein [36]. Thus, several features including the cold stability of our six-subunit F_1, dissociation of the 26.5 kDa protein from the F_1 complex in the presence of $(NH_4)_2SO_4$ and deoxycholate as well as the molecular weight of this subunit suggest the possibility of a functional and structural relationship between the mammalian oligomycin-sensitivity-conferring protein and the 26.5 kDa protein of the pea cotyledon mitochondrial F_1-ATPase.

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