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Restoration of ATP synthesis in urea-treated membranes prepared from pea cotyledon mitochondria

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Submitochondrial particles were prepared from pea cotyledon mitochondria by sonication in a medium containing 5 mM $MgCl_2$. The resulting particles (Mg^{2+}-submitochondrial particles) catalyzed oxidative phosphorylation at the rate of 100–200 nmol ATP formed/min per mg protein. Treatment of Mg^{2+}-submitochondrial particles with 3.0 M urea resulted in a preparation of highly resolved particles with low ATPase activity and no capacity for oxidative phosphorylation. However, the resulting membranes were not capable of reconstitution of oxidative posphorylation with the purified mitochondrial F_1-ATPase. Urea particles capable of reconstitution of oxidative phosphorylation could be prepared by extracting Mg^{2+}-submitochondrial particles with concentrations of urea ranging from 1.7 to 2.0 M. We have used 1.9 M urea for large-scale preparation of urea particles that could be stored in liquid nitrogen without any loss of reconstitution capacity. The residual oxidative phosphorylation rate of these particles was 6–8 nmol ATP/min per mg protein and this rate could increase to 60–70 nmol ATP/min per mg protein on incubation with saturating amounts of purified mitochondrial F_1-ATPase. In contrast to the mitochondrial F_1, purified activated pea chloroplast CF_1 was unable to stimulate ATP synthesis in 1.9 M urea particles.

Introduction

Proton-translocating ATP synthetases are essential components of both mitochondrial oxidative phosphorylation as well as of chloroplastic photophosphorylation. Higher plants possess both of these systems. The chloroplast coupling factor has been studied in great detail, while relatively little information is available on the plant mitochondrial enzyme. This is, in large part, due to the difficulties involved in isolating large quantities of plant mitochondria. The proton-translocating ATPase of higher plant mitochondria resembles generally that of mammalian mitochondria, chloroplasts and bacteria. The enzyme consists of two sectors, an F_1 sector on which ATP synthesis and hydrolysis occur and a membrane sector (F_0) which binds F_1 to the membrane and transports protons across the membrane. Similar to mammalian mitochondria, the F_0 sector contains a binding site for oligomycin [1] and *N,N'*-dicyclohexylcarbodiimide [2]. Partial purification of the F_1 sector from pea cotyledon mitochondria has been reported by Malhotra and Spencer [3] and the enzyme was further purified by Grubmeyer [4]. More recently,

Abbreviations: F_1, mitochondrial coupling factor; CF_1, chloroplast coupling factor; DCCD, *N,N'*-dicyclohexylcarbodiimide; Tes, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino} ethanesulphonic acid; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

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purification and the subunit composition of the F_1-ATPase from sweet potato [5], maize [6,7], Fava bean [8], oat root [9] and pea cotyledon mitochondria [10] has been described. The enzyme has been reported to contain the usual complement of five subunits in maize mitochondria [6,7], while reports on sweet potato [5] and pea cotyledon [10] F_1-ATPases suggest a possibility of six different types of polypeptides in these F_1 preparations.

Recently, we have described the purification of pea cotyledon F_1-ATPase [10] and showed that the preparation possessed coupling factor activity. It stimulated ATP formation in CF_1-depleted pea chloroplast membranes [10]. Thus, the coupling factor activity of the mitochondrial enzyme has been demonstrated in a heterologous system. In order to demonstrate the activity in a homologous system, it is necessary to obtain reconstitutively active F_1-depleted mitochondrial membranes. No such membrane preparation has been described for plant mitochondria, while in mammalian mitochondria, reconstitution of oxidative phosphorylation has been achieved many years ago. Some of the early reports on preparation of F_1-depleted submitochondrial particles from bovine heart involved disruption of mitochondria in the presence of EDTA [11], passage of submitochondrial particles through Sephadex followed by urea treatment [12] or urea-ATP treatment of submitochondrial particles [13]. More recently, successful preparation of F_1-depleted rat liver mitochondrial membranes has been reported [14] using urea extraction under controlled conditions. The particles obtained had very low residual ATP-synthetic and ATPase activities, yet they were reconstitutively active.

We have adapted the method of Pedersen and Hullihen [14] to prepare urea-extracted membranes from pea cotyledon submitochondrial particles. In this paper we describe the preparation, some properties, and the reconstitutive response of these membranes to the addition of purified pea mitochondrial F_1-ATPase. We have also examined the effect of the addition of purified chloroplast CF_1 to these membranes in view of the fact that both CF_1 as well as F_1 can reconstitute photophosphorylation in CF_1-depleted thylakoid membranes [10].

Materials and Methods

Purification of mitochondrial coupling factor (F_1). Preparation of pea cotyledon mitochondria, submitochondrial particles and purification of F_1 were performed exactly as described in our previous paper [10]. The purified F_1 was dialyzed at room temperature against buffer containing 300 mM sucrose/2 mM EDTA/2 mM ATP/20 mM Tris-H_2SO_4 (pH 7.4)/10% methanol and stored frozen at −80°C. Before use in reconstitution experiments the enzyme was dialyzed at room temperature against the 'reconstitution buffer' containing 100 mM sucrose/100 mM K_2HPO_4/2 mM EDTA/1 mM Tes (pH 7.5) and concentrated using Centricon-30 Microconcentrators (Amicon).

Purification and activation of chloroplast coupling factor (CF_1). Preparation of chloroplasts from pea leaves and purification of CF_1 was performed as described before [10]. As in the case of mitochondrial F_1, the purified chloroplast CF_1 was dialyzed against 300 mM sucrose/2 mM EDTA/2 mM ATP/20 mM Tris-H_2SO_4 (pH 7.4)/10% methanol and stored frozen at −80°C. Before use in reconstitution experiments, the enzyme was activated by dialysis at room temperature against 20 mM Tes (pH 8.0) containing 50 mM dithiothreitol. After dialysis, the sample was concentrated on Centricon-30 Microconcentrators and finally passed through a Sephadex G-50 centrifuge column [15] equilibrated with the 'reconstitution buffer' containing 0.1 mM dithiothreitol.

Preparation of F_1-depleted mitochondrial membranes. 'Mg^{2+}-submitochondrial particles' were prepared by sonication of mitochondria in the presence of 5 mM $MgCl_2$ in addition to the 250 mM sucrose/50 mM Tes (pH 7.0) used to prepare submitochondrial particles for F_1 purification. The final suspension of these particles was made in 250 mM sucrose. They were stored in 0.5-ml aliquots at 14 mg protein/0.5 ml at −80°C.

Urea extraction was done by a modification of the procedure described by Pedersen and Hullihen [14] for rat liver mitochondria. To 0.5 ml of Mg^{2+}-submitochondrial particles in 250 mM sucrose, 0.1 ml of 12 mM Tes/0.6% fatty acid poor bovine serum albumin (pH 7.4) was added, followed by addition of 0.6 ml of urea in water to obtain a final urea concentration between 1.0 M

and 4.0 M. The suspension was incubated on ice for 5 min and the extraction stopped by addition of 20 ml of cold 250 mM sucrose/2 mM Tes/0.1% fatty acid poor bovine serum albumin (pH 7.4). Unless mentioned otherwise, the final suspension of the urea particles was made at 10 mg protein/ml in 'reconstitution buffer' containing 1% fatty acid poor bovine serum albumin. For storage in liquid nitrogen, the urea particles were distributed in 40 μl (400 μg protein) aliquots that were used directly for the reconstitution experiments.

Reconstitution of oxidative phosphorylation in urea extracted submitochondrial particles. Reconstitutions were done according to Pedersen and Hullihen [14] with some modifications. Urea particles (400 μg) were incubated with various amounts of purified F_1, or purified activated CF_1, or DCCD (added in 5 μl ethanol) in a final volume of 0.1 ml. The incubations were carried out in the presence of 1% fatty acid poor bovine serum albumin in the 'reconstitution buffer' consisting of 100 mM sucrose/100 mM K_2HPO_4/2 mM EDTA/1 mM Tes (pH 7.5). After 1 h on ice, 40-μl aliquots were taken for the oxidative phosphorylation assay. Unextracted submitochondrial particles or urea-extracted membranes alone (for measurements of residual oxidative phosphorylation) were subjected to the same reconstitution procedure prior to the oxidative phosphorylation assay.

Oxidative phosphorylation assay. Oxidative phosphorylation was measured in a final volume of 1 ml with 0.88 mM NADH as substrate. Conditions described by Grubmeyer et al. [16] were followed, except that 0.2% fatty acid poor bovine serum albumin was also included in the assay medium. To stop the reaction, 0.1 ml of 60% trichloroacetic acid was added and after centrifugation to remove precipitated protein, the $^{32}P_i$ incorporation into glucose-6-phosphate was measured using the procedure of Nielsen and Lehninger [17].

ATPase activity determinations. The ATPase activity of submitochondrial particles or of urea particles was measured in the same way as described before for the ATPase activity of F_1 [10]. When oligomycin was included, a final concentration of 1 μg/ml of incubation mixture was used.

Protein determinations. Protein was determined by the method of Lowry et al. [18].

Results and Discussion

Preparation of F_1-depleted mitochondrial membranes

Starting material for preparation of F_1-depleted mitochondrial membranes were 'Mg^{2+}-submitochondrial particles' prepared by sonciation of mitochondria in 250 mM sucrose/50 mM Tes/5 mM $MgCl_2$ (pH 7.0). The preparation exhibited about two times higher rates of oxidative phosphorylation than the particles obtained by sonication in the absence of $MgCl_2$. Similar results have been obtained with bovine heart sumitochondrial particles in the presence of $MgCl_2$ and ATP. These particles had higher phosphorylation capacity than particles prepared in the absence of $MgCl_2$ and ATP [19,20]. In our preparations obtained by sonication in the pesence of $MgCl_2$, the oxidative phosphorylation rates ranged from 100–200 nmol ATP formed/min per mg protein and the inclusion of 1 mM ATP into the sonication medium did not increase the rates any further. These values are lower than the oxidative phosphorylation capacity of bovine heart submitochondrial particles [21], but comparable to the phosphorylation rates of the inner membrane vesicles prepared from rat liver mitochondria [14]. We have stored the Mg^{2+}-submitochondrial particles for several months at $-80°C$ without appreciable loss in phosphorylation rates and have used these particles for preparation of F_1-depleted membranes.

In our initial attempts to prepare F_1-depleted membranes from pea cotyledon mitochondria we have tried extracting the Mg^{2+}-submitochondrial particles with 300 mM sucrose/2 mM Tricine/1 mM EDTA (pH 7.4). This low ionic strength EDTA extraction procedure resulted in a 90% decrease in oxidative phosphorylation rates, but virtually no decrease in oligomycin-sensitive ATPase. Similar results, i.e., loss of oxidative phosphorylation and, at the same time, retention of ATPase activity, have been reported for EDTA extracted bovine heart submitochondrial particles [22], indicating only a partial extraction of F_1 moieties by the EDTA treatment.

In order to prepare particles more resolved with respect to the ATPase activity, we have extracted the Mg^{2+}-submitochondrial particles with urea following the procedure of Pedersen and Hullihen developed for rat liver mitochondria [14]. Fig. 1 shows the levels of oxidative phosphorylation and of oligomycin sensitive ATPase found in Mg^{2+}-submitochondrial particles extracted with various urea concentrations. It can be seen that at a urea concentration higher than 1.0 M, ATP synthesis rapidly decreased, while the decline in ATPase activity was more gradual. As found for rat-liver mitochondrial membranes [14], 3.0 M urea treatment completely abolished oxidative phosphorylation and decreased the ATPase activity to a low level.

The reasons for the increases in ATP synthetic as well as ATPase activities in Mg^{2+}-submitochondrial particles extracted with 1.0 M urea are not clear at the moment. One possibility is that at lower concentrations urea causes preferential extraction of proteins other than the mitochondrial coupling factor, thereby increasing the specific activities of the extracted membranes.

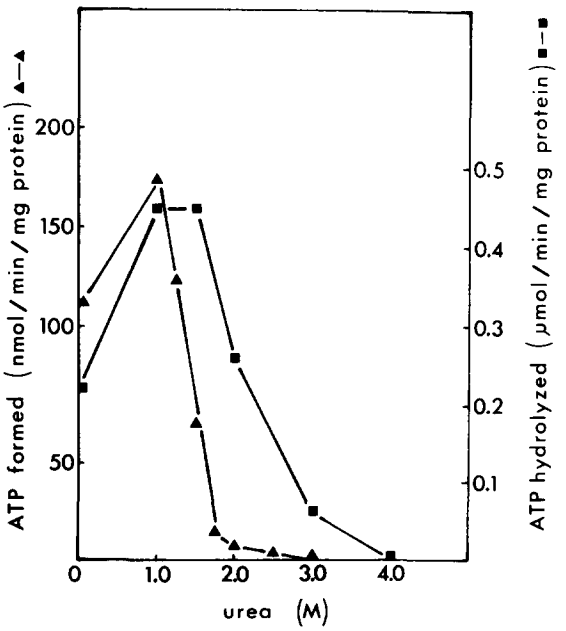

Fig. 1. Oxidative phosphorylation and oligomycin sensitive ATPase in urea-extracted Mg^{2+}-submitochondrial particles. Mg^{2+}-submitochondrial particles were extracted using various urea concentrations as described in Materials and Methods. Final suspension of urea extracted membranes was made in 250 mM sucrose at 1 mg/ml. Freshly prepared particles were used for ATPase (30–60 μg protein) or oxidative phosphorylation (100–200 μg protein) assays as described in Materials and Methods.

Since the Mg^{2+}-submitochondrial particles used in our experiments are prepared directly by sonication of whole mitochondria, they might contain outer membrane fragments in addition to the vesicles derived from the inner mitochondrial membrane. It is possible that the elimination of outer membrane fragments from the membrane preparation used for subsequent urea extraction could increase the rates of oxidative phosphorylation and ATP hydrolysis to the levels observed in 1.0 M urea particles.

Pedersen and Hullihen [14] used inner membrane vesicles as a starting material for urea extraction and they did not observe any increase in ATP synthetic or ATP hydrolytic activities in 1.0 M urea particles. We have, therefore, tried to remove the outer mitochondrial membrane by digitonin treatment [23,24] prior to sonication. Treatment of pea cotyledon mitochondria with a suspension of crystallized digitonin as described in Chan et al. [23], followed by sonication in 250 mM sucrose/50 mM Tes/5 mM $MgCl_2$ (pH 7.0), resulted in particles which had substantially lower oxidative phosphorylation and ATPase activities than the Mg^{2+}-submitochondrial particles which were prepared by sonication of whole mitochondria (not shown). Moreover, when the digitonin-treated particles were kept overnight at room temperature, complete dissociation of the ATPase activity from the membranes (no oligomycin inhibition) was observed (not shown). In the Mg^{2+}-submitochondrial particles which were prepared from whole mitochondria, the ATPase activity remained oligomycin sensitive after 20 h at room temperature. Thus, digitonin treatment used to remove the outer mitochondrial membrane from rat-liver mitochondria without damaging the inner membrane is detrimental to the inner membrane of pea cotyledon mitochondria.

Another possible explanation for the increase in ATPase activity in 1.0 M urea particles, is removal (or partial removal) of the ATPase inhibitor protein [25] by urea treatment. Bovine heart submitochondrial particles prepared in the presence of Mg-ATP have been reported to contain a

high amount of the mitochondrial inhibitor protein [26]. However, the removal of the inhibitor protein would not explain the observed increase in oxidative phosphorylation; the inhibitor has been reported to interfere with initial stages of phosphorylation only, while having no effect on phosphorylation when measured over a 3–15 min period [27].

Activation of ATPase in Mg^{2+}-submitochondrial particles and in urea-extracted membranes

We did try to remove the inhibitor protein from Mg^{2+}-submitochondrial particles in an attempt to obtain a maximal value of the 'unmasked' ATPase activity in the starting material, in order to express the percentage of ATPase activity remaining in the membranes after urea extraction. Table I demonstrates the results. Activation of ATPase by 'aging' of Mg^{2+}-submitochondrial particles for 5 h at room temperature (experiment 1) increased the activity two times, suggesting partial dissociation of the inhibitor [25,28]. A substantial activation was achieved by washing the Mg^{2+}-submitochondrial particles with a solution containing 150 mM KCl (experiment 2), a treatment that was also successful in activating the ATPase of bovine heart submitochondrial particles [29]. It has been reported that further activation of the enzyme was obtained by alkaline treatment of the KCl-washed bovine heart membranes [30]; however, such an effect was not observed with pea cotyledon membranes (experiment 3). Washing five times with a phosphate buffer containing 250 mM K_2HPO_4/5 mM EDTA, a procedure used to activate maxi-

TABLE I

EFFECT OF ATPASE ACTIVATING CONDITIONS ON ATPASE ACTIVITY IN Mg^{2+}-SUBMITOCHONDRIAL PARTICLES AND IN UREA PARTICLES

Mg^{2+}-submitochondrial particles were prepared as described in Materials and Methods and, in experiment 1, activated by allowing them to sit at room temperature for 5 h before ATPase activity was determined as described under Materials and Methods. In experiment 2, untreated urea particles from liquid nitrogen were diluted 10 times in 250 mM sucrose before the ATPase assay in order to decrease the high phosphate concentration of the reconstitution buffer used for storage of urea particles. KCl wash was done according to Penefsky [29]: Mg^{2+}-submitochondrial particles or urea particles were suspended at 1 mg/ml in 250 mM sucrose/150 mM KCl/2 mM EDTA/10 mM Tris-sulfate (pH 8.0), suspension was incubated at 30°C for 20 min, centrifuged at 160000 × *g* for 40 min at room temperature, pellet suspended in 250 mM sucrose/2 mM EDTA/10 mM Na_2HPO_4/50 mM Tris-sulfate (pH 8.0), centrifuged again and finally suspended in 250 mM sucrose. In experiment 3, the alkaline treatment was done by suspending the KCl washed membranes in 250 mM sucrose/2 mM EDTA/10 mM ATP/0.1% fatty acid poor bovine serum albumin (pH 8.0), increasing the pH to 10 by addition of 1 : 10 dilution of concentrated NH_4OH and incubating for 30 min at room temperature [30]. The pH was returned to 8 before assay of ATPase activity by addition of 1 M HCl. In experiment 4, K_2HPO_4 wash was done by suspending the Mg^{2+}-submitochondrial particles at 1 mg/ml in 250 mM K_2HPO_4/5 mM EDTA (pH 7.5) at 0–4°C, centrifuging at 160000 × *g* for 40 min at 4°C and washing four times in the same buffer [14,31]. Final suspension was made in 250 mM sucrose.

Exp.	Starting material	Initial oligomycin-sensitive ATPase (μmol/min per mg protein)	Treatment	Subsequent oligomycin-sensitive ATPase (μmol/min per mg protein)
1	fresh Mg^{2+}-submitochondrial particles	0.22	aging 5 h, 20°C	0.41
2	frozen and thawed Mg^{2+}-submitochondrial particles	0.64	KCl wash	2.64
	1.9 M urea particles from liquid N_2	1.84	KCl wash	4.78
3	frozen and thawed Mg^{2+}-submitochondrial particles	0.66	KCl wash	1.50
			KCl wash + alkaline activation	1.50
4	frozen and thawed Mg^{2+}-submitochondrial particles	0.57	K_2HPO_4 wash	0.78

mally the ATPase of inner membrane vesicles from rat liver mitochondria [31,14], resulted in only marginal activation of the pea cotyledon enzyme (experiment 4). With bovine heart submitochondrial particles, maximal activation of the ATPase was achieved by passing the particles through a Sephadex G-50 column [12]; however, this treatment was reported to result in complete inactivation of pea cotyledon ATPase [28]. Thus, so far, maximal ATPase activation has only been achieved by the KCl wash. However, exposure of 1.9 M urea particles to the same treatment resulted in an even higher specific activity of the enzyme (experiment 2) making it doubtful that the value obtained for KCl washed Mg^{2+}-submitochondrial particles represents the optimum 'unmasked' ATPase activity of the starting material.

Reconstitution of oxidative phosphorylation in urea-treated membranes

In initial experiments we tried to reconstitute the oxidative phosphorylation with purified mitochondrial F_1 in urea particles which had low amounts of residual ATPase activity (3.0 M urea particles, Table II). However, in contrast to the liver membranes, no reconstitution occurred with these particles. Lowering the concentration of urea used for extraction of the membranes resulted in a gradual increase in ATP synthesis. In the experiment shown in Table II, with 1.7 M urea particles, ATP synthesis after reconstitution was approximately the same as the value obtained for nonextracted Mg^{2+}-submitochondrial particles.

TABLE II

RECONSTITUTION OF OXIDATIVE PHOSPHORYLATION IN UREA PARTICLES BY PURIFIED F_1

Urea particles were prepared as described under Materials and Methods. For reconstitution, freshly prepared urea particles (400 μg protein) were incubated with 100 μg of purified F_1 as described in Materials and Methods

Membrane preparation	ATP formed (nmol/min per mg protein)
Mg^{2+}-submitochondrial particles	120
1.7 M urea particles	35
1.7 M urea particles + F_1	133
2.0 M urea particles	11
2.0 M urea particles + F_1	54
2.3 M urea particles	2
2.3 M urea particles + F_1	17
3.0 M urea particles	0
3.0 M urea particles + F_1	0

The percentage increase in ATP synthesis after reconstitution was the same regardless of whether it was carried out at room temperature or on ice, but the absolute values of oxidative phosphorylation were substantially higher for samples kept on ice (not shown). Consequently, we carried out the reconstitution experiments on ice. The inclusion of 4 mM ATP in the reconstitution system, which enhanced the capacity of the reconstituted membranes to catalyze ATP synthesis in liver membranes [14], did not have any effect on freshly prepared urea particles from pea cotyledon mitochondria (not shown).

The concentration of urea used for extraction seems to be very critical. Particles that were extracted too much either did not respond or responded very feebly to the addition of purified F_1. As we had difficulties in obtaining reproducible extractions from one experiment to another, we tried to prepare a larger amount of extracted particles and store them in liquid nitrogen. Membranes extracted with 1.9 M urea and stored in liquid N_2 were used in subsequent experiments. Neither their residual ATP synthetic activity (6–8 nmol ATP formed/min per mg protein), nor their capacity for reconstitution (9–10-fold increase in oxidative phosphorylation on incubation with saturating amounts of F_1) were affected by storage. The results obtained with these particles were highly reproducible.

As shown in Fig. 2, purified mitochondrial F_1 could readily reconstitute oxidative phosphorylation in 1.9 M urea particles, reaching maximal levels when 100 μg of F_1 were incubated with 400 μg of urea particles. Contrary to the purified mitochondrial enzyme, chloroplast CF_1, isolated from pea leaves as described in our previous paper [10], was not capable of increasing ATP synthesis in urea extracted membranes from pea cotyledon mitochondria (Fig. 2). The CF_1 used in this experiment has been activated by dithiothreitol treatment prior to use in the reconstitution experiment. It was reported by Andreo et al. [32] that activation of spinach CF_1-ATPase by dithiothreitol

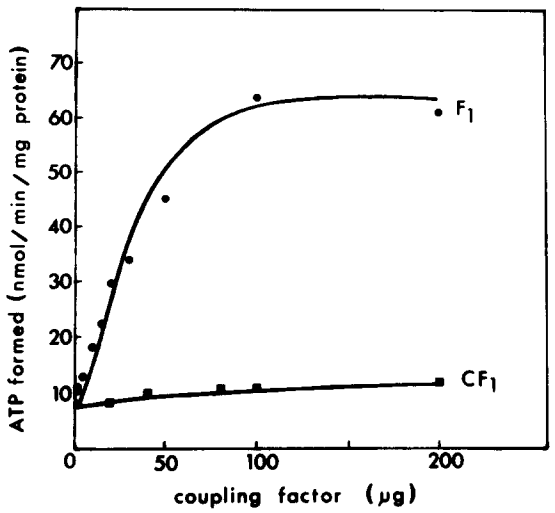

Fig. 2. ATP-synthetic capacity of 1.9 M urea particles reconstituted with various concentrations of purified F_1 or CF_1. Mg^{2+}-submitochondrial particles were extracted with 1.9 M urea and stored in liquid nitrogen as described in Materials and Methods. For reconstitution, 400 μg of urea particles were incubated with the indicated amount of purified mitochondrial F_1 or purified activated chloroplast CF_1 and subsequently 40 μl aliquots were used in the oxidative phosphorylation assay. The conditions for activation of chloroplast CF_1, for reconstitution, and for oxidative phosphorylation assays are given in Materials and Methods.

increased its coupling factor activity in thylakoid membranes. In our experiments, neither non-activated nor activated CF_1 exhibited any effect on ATP synthesis in urea particles. This result is interesting in view of the fact that both chloroplast CF_1 as well as mitochondrial F_1 could reconstitute the photophosphorylation in CF_1-depleted pea chloroplast membranes [10].

We have measured the binding of CF_1 to urea-extracted mitochondrial membranes in order to determine whether its failure to stimulate ATP synthesis in urea particles was due to its inability to bind to these membranes, or due to its failure to bind to them correctly to restore ATP synthesis. Binding of CF_1 and F_1 to urea particles was measured by incubating 25 μg of chloroplast CF_1 or 25 μg of mitochondrial F_1 with various amounts of 1.9 M urea particles under conditions described for the reconstitution experiments in Materials and Methods. After 1 h on ice, reconstituted particles were centrifuged down. The amount of CF_1 and F_1 remaining in the supernatant fractions was determined by measuring their ATPase activity (after the supernatants were dialysed against 250 mM sucrose/2 mM Tes (pH 7.4) to remove the phosphate present in the 'reconstitution buffer'). The binding experiments have shown that more than 60% of F_1 was bound to 1.9 M urea particles when 400 μg of particles were incubated with 25 μg of F_1. In contrast, no binding of CF_1 to urea particles was observed, all CF_1 remained in the supernatant fractions.

The urea particles used in our reconstition experiments were partially deficient in, but not devoid of, endogenous ATPase activity. As mentioned above, using urea treatment, we were not able to prepare more resolved particles that were also reconstitutively active. It seems that plant mitochondrial membranes are more fragile than mitochondrial membranes prepared from animal sources. For example, the plant membranes are damaged by digitonin treatment (see Results in this paper), passage through a Sephadex G-50 column [28], as well as by tratment with high urea concentrations (Table II).

In the experiment shown in Table III, we tried to distinguish between whether the added F_1 was

TABLE III

STIMULATION OF OXIDATIVE PHOSPHORYLATION IN 1.9 M UREA PARTICLES BY DCCD AND BY PURIFIED F_1

Mg^{2+}-submitochondrial particles were extracted with 1.9 M urea and stored in liquid nitrogen as described in Materials and Methods. For reconstitution, 400 μg urea particles were incubated with the indicated amount of DCCD or with purified mitochondrial F_1 under conditions described in Materials and Methods.

Addition to 1.9 M urea particles	ATP formed (nmol/min per mg protein)
A. DCCD (nmol)	
0	6
0.1	8
0.5	14
1	25
2	44
3	48
5	27
10	3
B. F_1 200 μg	61

fulfilling its structural role only (by filling the proton pores created by previous removal of F_1 and thus enabling the residual F_1 on the membrane to form more ATP by utilizing the increased proton gradient) or whether it was catalytically active as well [33]. The proton leak resulting upon removal of F_1 can be blocked by low concentrations of DCCD which binds to the proton conducting part of the mitochondrial proteolipid [2]. We preincubated the 1.9 M urea particles with low concentrations of DCCD in an experiment analogous to that of McCarty and Racker [34]. As shown in Table III, the phosphorylation rates measured after preincubation of 1.9 M urea particles with F_1-ATPase were aout 25% higher than the highest rates observed after preincubation of 1.9 M urea particles with DCCD (to block the proton channels). This result suggests that the added F_1-ATPase, besides fulfilling its structural role, was also catalytically active in ATP synthesis. However, more resolved mitochondrial membranes containing lower amounts of residual F_1-ATPase will be required for unequivocal proof of catalytic activity of the added enzyme.

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