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Oxidative phosphorylation in a hybrid system containing bovine heart membranes and pea mitochondrial F_1-ATPase

Helena Horak, Mary Packer and Arnost Horak

Department of Plant Science, University of Alberta, Edmonton, Alberta (Canada)

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Purified pea mitochondrial F_1-ATPase reconstituted oxidative phosphorylation in both partially and completely F_1-depleted bovine heart mitochondrial membranes. The isolated plant enzyme exhibited high rates of ATP synthesis when combined with bovine heart membranes, suggesting great evolutionary conservation of the ATP synthase complex in mitochondria.

Proton-translocating ATP synthases are proteins responsible for coupling the formation of ATP to electron transport in membranes of bacteria, chloroplasts and mitochondria. The enzymes from all these sources possess remarkably similar overall structure. They are composed of two sectors: the F_1 sector on which ATP synthesis and hydrolysis occur and the membrane sector (F_0) which provides energy for ATP synthesis in the form of an electrochemical proton gradient [1]. The F_1 portion can be easily dissociated from the membrane. It has been purified from many sources and shown to be composed of five subunits. The main criterion for the functional integrity of the isolated enzyme is its ability to restore ATP synthesis in membranes which are depleted of their own F_1. The exogenously added F_1 fulfills two functions [2]: a structural one in sealing the proton channels created by previous removal of endogenous F_1 (thereby preventing dissipation of the electrochemical proton gradient) and a functional one in catalyzing the synthesis of ATP. In membrane preparations completely depleted of their own F_1, all ATP synthesis observed on addition of the exogenous F_1 can be attributed to the catalytic function of the added enzyme. Recently [3], we described purification of F_1-ATPase from pea cotyledon mitochondria. The isolated enzyme was composed of six subunits [3,4]. It was capable of reconstituting photophosphorylation in pea chloroplast membranes [3] as well as of restoration of ATP synthesis in F_1-deficient pea mitochondrial membranes [5]. So far, we have not been able to prepare pea mitochondrial membranes completely devoid of their own F_1 without affecting their capacity for reconstitution [5]. We have, therefore, tried to use F_1-depleted bovine heart mitochondrial membranes, the preparation of which has been well described [2,6,7–9]. We report here that the purified pea mitochondrial F_1-ATPase is capable of reconstituting oxidative phosphorylation in both partially and completely F_1-depleted bovine heart mitochondrial membranes. Moreover, the isolated

Abbreviations: F_1, mitochondrial coupling factor; ASU-particles, bovine heart submitochondrial particles treated with ammonia, Sephadex and urea; OSCP, oligomycin sensitivity-conferring protein.

Correspondence: A. Horak, (temporary address:) Department of Biochemistry, University of Aberdeen, Marischal College, Aberdeen, AB9 1AS, U.K.

plant enzyme exhibits higher coupling activity than the F_1-containing extract of bovine heart mitochondria.

The first series of experiments was done with bovine EDTA-submitochondrial particles [6,7]. Being only partially depleted in their own F_1, these particles still possessed some residual oxidative phosphorylation, which was (in accordance with a previous report – Ref. 8) stimulated by addition of low levels of oligomycin (Table I). Using various oligomycin concentrations (not shown), we have obtained the optimal stimulation at 0.1 μg oligomycin per mg of EDTA-particles.

Oligomycin binds to the F_0 portion of the enzyme complex and by sealing the proton channels [10,11] it restores the electrochemical proton gradient. This gradient is utilized by the residual F_1 for increased ATP synthesis. The F_1-containing extract of bovine heart mitochondria stimulated ATP synthesis to a similar degree as did oligomycin alone (Table I). In agreement with previous observations [8], oligomycin substantially increased the level of oxidative phosphorylation when added in combination with the F_1-containing extract of bovine mitochondria. Rather unexpectedly, purified pea cotyledon mitochondrial F_1-ATPase stimulated the ATP formation in bovine heart EDTA-particles to almost the same degree as did the bovine heart F_1 in the presence of oligomycin (Table I).

This suggested that the pea mitochondrial enzyme could fulfill not only its structural, but also its catalytic function, when it was bound to bovine-heart mitochondrial membranes.

Encouraged by these results, we wanted to see whether our purified pea cotyledon enzyme would be capable of stimulating oxidative phosphorylation in bovine heart mitochondrial membranes that are virtually devoid of their own F_1. The membranes were prepared by successive treatment of submitochondrial particles with ammonia, Sephadex and urea (ASU-particles) according to methods published previously [2,8,9]. The resulting membranes exhibited ATPase activity of 0.12 μmol ATP hydrolyzed per min per mg protein, which represented 0.6% of the original activated ATPase value obtained after Sephadex treatment. There was no detectable residual oxidative phosphorylation associated with these particles, not even upon addition of low levels of oligomycin (Table II). Thus the lack of ATP synthase activity was indeed due to the F_1 deficiency rather than

TABLE I

RECONSTITUTION OF OXIDATIVE PHOSPHORYLATION IN BOVINE HEART EDTA-PARTICLES

Heavy bovine heart mitochondria were isolated as described by Azzone et al. [17] and stored at −80 °C at 56 mg/ml. To prepare the EDTA-particles [6,7] 56 mg of mitochondria were suspended in 40 ml of 250 mM sucrose, 2 mM EDTA (pH 7.4) and sonicated at 0 °C in a 100 ml beaker with four 1-min bursts at 90% of full power on the Artek 300 sonicator using the full size tip. The suspension was centrifuged at 10000 × *g* for 10 min at 4 °C to remove the unbroken mitochondria and the EDTA-particles were sedimented by centrifuging the supernatant at 100000 × *g* for 50 min at 4 °C. The pellet was rinsed and suspended in 250 mM sucrose. The F_1-containing extract of beef heart mitochondria [18] was prepared by suspending 56 mg of mitochondria in 40 ml of a solution containing 250 mM sucrose, 2 mM EDTA, 4 mM ATP (pH 7.4) and sonicating at room temperature by using two 4-min bursts at 90% full power. The temperature during sonication was not allowed to rise above 45 °C. The suspension was centrifuged at 160000 × *g* for 60 min at 20 °C and the resulting supernatant concentrated to 1 ml using an Amicon-stirred cell with a YM-10 membrane. The ATPase activity of the concentrated supernatant was 7.4 units (μmol ATP hydrolyzed per min) per mg protein. Purified pea cotyledon mitochondrial F_1-ATPase (specific activity of 20 units per mg protein) was stored at −80 °C in a buffer containing 300 mM sucrose, 2 mM EDTA, 2 mM ATP, 20 mM Tris-H_2SO_4 (pH 7.4) and 10% methanol. Before use, the enzyme was concentrated using Centricon-30 microconcentrators (Amicon) and passed through a Sephadex G-50 centrifuge column [19] equilibrated with 250 mM sucrose, 2 mM EDTA, 4 mM ATP (pH 7.4). The reconstitution was done using basically the conditions of Penefsky et al. [20] by incubating 600 μg of EDTA-particles in a solution containing 250 mM sucrose, 13 mM $MgCl_2$, 2 mM ATP, 10 mM Tris-HCl (pH 7.4) in a total volume 0.12 ml. Where indicated, 60 ng of oligomycin, 400 μg of crude bovine heart F_1 or 130 μg of purified pea mitochondrial F_1 were included in the incubation. After 15 min at 25 °C, 40 μl of the mixture were removed and oxidative phosphorylation measured with NADH as substrate as described before [5]. Protein was determined by the method of Lowry et al. [21].

Additions to EDTA-particles	ATP formed (nmol per min per mg protein)
None	13
Oligomycin	57
Bovine F_1 (crude extract)	57
Bovine F_1 (crude extract) + oligomycin	92
Purified pea mitochondrial F_1	83

TABLE II

RECONSTITUTION OF OXIDATIVE PHOSPHORYLATION IN BOVINE HEART ASU-PARTICLES

For the preparation of A-particles [8,9] 170 mg of heavy bovine heart mitochondria were suspended in 40 ml of a solution containing 50 mM sucrose, 0.6 mM EDTA, 2 mM Tris-HCl (pH 7.8). After raising the pH to 9.2 with 0.5 M NH_4OH, the mitochondria were sonicated and the resulting suspension centrifuged as described for preparation of the EDTA-particles in Table I. The final pellet (40 mg of A-particles) was suspended in 1 ml of a buffer containing 75 mM sucrose, 2 mM EDTA, 250 mM KCl, 30 mM Tris-H_2SO_4 (pH 8.0), loaded on a 1×40 cm Sephadex G-50 (coarse) column and the procedure of Racker and Horstman [2] for the preparation of ASU-particles was followed precisely. The particles were finally suspended in 250 mM sucrose and stored in 400 μg/25 μl aliquots in liquid N_2. The F_1-containing extract of bovine heart mitochondria was prepared as described in Table I. It had an ATPase activity of 7.2 units per mg protein. Purified pea mitochondrial F_1-ATPase (specific activity of 20 units per mg protein) was processed as described in Table I. The reconstitution was done by incubating 400 μg of ASU-particles in a solution containing 250 mM sucrose, 13 mM $MgCl_2$, 2 mM ATP, 10 mM Tris-HCl (pH 7.4) in a total volume of 0.1 ml. Where indicated, 80 ng of oligomycin, 890 μg of crude bovine heart mitochondrial F_1 or 150 μg of purified pea mitochondrial F_1 were included in the incubation. After 15 min at 25 °C, 40 μl of the mixture were removed for the oxidative phosphorylation assay.

Additions to ASU-particles	ATP formed (nmol per min per mg protein)
None	0
Oligomycin	0
Bovine F_1 (crude extract)	0
Bovine F_1 (crude extract) + oligomycin	66
Purified pea mitochondrial F_1	61
Purified pea mitochondrial F_1 + Oligomycin	70

due to oligomycin-repairable uncoupling as seen in particles partially depleted in F_1. Crude bovine heart mitochondrial F_1 was unable to stimulate ATP synthesis in the ASU-membranes to any significant extent unless oligomycin was also added (Table II). Optimal stimulation was obtained at 0.2 μg of oligomycin per mg ASU-particles. The purified pea cotyledon mitochondrial F_1-ATPase stimulated the ATP formation in bovine heart ASU-particles to almost the same degree as did the crude bovine heart F_1 in the presence of oligomycin (Table II). Contrary to the profound effect on oxidative phosphorylation in the presence of bovine heart F_1, the oligomycin only slightly increased the level of ATP synthesis when added in combination with the purified pea enzyme (Table II). It can be concluded that the pea mitochondrial F_1-ATPase is capable of catalyzing the synthesis of ATP when combined with bovine heart mitochondrial membranes and that the level of ATP synthesis observed is comparable to the level obtained in the presence of crude bovine heart F_1 and oligomycin.

To the best of our knowledge, a similar finding has not been reported previously. The results were surprising to a certain degree, especially in view of the studies done with bovine heart membranes and purified yeast mitochondrial F_1-ATPase [12]. The yeast enzyme was found to exert its effect on the membrane structure only, without participating in phosphate-transfer reactions in bovine heart membranes [12].

We have previously reported [3] that the purified pea mitochondrial F_1 was able to substitute functionally for the chloroplast coupling factor in reconstituting photophosphorylation in pea chloroplast membranes. However, the reverse has not been found to be true, i.e., purified pea chloroplast coupling factor was unable to stimulate ATP synthesis in pea mitochondrial membranes [5]. The reconstitution efficiency of our pea mitochondrial F_1-ATPase may be related to the presence of the sixth subunit in our enzyme preparation. We have observed [4] that the pea enzyme contains two δ subunits (with molecular weights of 26 500 and 22 500) and suggested the possibility of a functional relationship between the $M_r = 26\,500$ protein and mammalian oligomycin sensitivity-conferring protein (OSCP). The OSCP is a part of the mitochondrial complex and controls its sensitivity to oligomycin [13]. Our present finding of the high coupling activity of the isolated pea F_1, which was not substantially increased by oligomycin, supports the possibility of an OSCP-like protein being part of the pea enzyme complex. When Vallejos et al. [14] isolated a six-subunit F_1-ATPase from bovine heart mitochondria (F_1-X complex), they found that it had much higher coupling activity than a five-subunit F_1. Moreover, contrary to the F_1, the ATP synthesis observed on addition of F_1-X to the bovine heart membranes

was not further stimulated by oligomycin [14]. The X component was later identified as OSCP [15]. Higher coupling activity of the F_1-OSCP complex could be explained by recent findings of Dupuis and Vignais [16]. The authors reported that the association between F_1 and bovine heart particles was readily reversible, whereas the ternary complex formed by the particles, F_1 and OSCP was much more difficult to dissociate, as if the OSCP would secure a 'lock' between the F_1 and the membranes.

The present finding that the isolated plant mitochondrial F_1 is capable of association with the bovine heart membranes in a way that enables the added plant F_1 to synthesize ATP, shows the great evolutionary conservation of the ATP synthase complex in mitochondria.

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