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VESICULAR PREPARATION OF A HIGHLY COUPLED ATPase-ATP SYNTHASE COMPLEX FROM PIG HEART MITOCHONDRIA

F. PENIN, C. GODINOT *, J. COMTE and D.C. GAUTHERON

Laboratoire de Biologie et Technologie des Membranes du Centre National de la Recherche Scientifique, Université Claude Bernard, 43 Bd. du 11 Novembre 1918, 69622 Villeurbanne (France)

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1. A method is described to prepare an ATPase-ATP synthase complex from pig heart mitochondria exhibiting a very high ATP-$^{32}P_i$ exchange activity (1.6 μmol/min per mag protein in optimal conditions). 2. The preparation is virtually devoid of nucleoside diphosphokinase and adenylate kinase activities. 3. Freeze-fracture studies show that the ATPase-ATP synthase complex is integrated in lipid vesicles of 400–600 Å in diameter. 4. It contains the endogenous natural proteic inhibitor which seems to behave as a coupling factor. 5. The rate of ATP hydrolysis catalyzed by the ATPase-ATP synthase complex is competitively inhibited by ADP, while the presence of ADP increases the initial rate of $^{32}P_i$ incorporation into ATP. 6. The $^{32}P_i$ incorporation into ATP can occur at a rate almost equal to that of nucleoside triphosphate (NTP) hydrolysis provided that the rate of NTP hydrolysis is kept low and that the ADP concentration is high enough. In these conditions, a very high coupling between NTP hydrolysis and ATP synthesis can be demonstrated.

Introduction

According to the chemiosmotic hypothesis proposed in 1961 by Peter Mitchell [1], the energy provided during substrates oxidation in mitochondria permits the formation of a proton gradient between the two faces of the inner mitochondrial membrane. The ATPase-ATP synthase complex present in this membrane phosphorylates ADP to form ATP by a reverse flow of protons. This reaction is reversible which means that the complex can couple the formation of an electrochemical gradient of protons with the hydrolysis of ATP.

Many attempts have been made to purify the ATPase-ATP synthase complex in an active form or to reconstitute vesicles capable of energy transduction from purified components [2–8]. However, in most cases, the main concern of the investigators was to obtain a preparation containing a minimum number of polypeptides while keeping the enzyme capable of catalyzing inhibitor-sensitive $^{32}P_i$-ATP exchange and ATP-dependent proton uptake. In the present study, by using an appropriate purification procedure we have obtained a vesicular preparation of the ATPase-ATP synthase complex which also kept its inhibitor-sensitivity but exhibited a much higher specific activity for ATP synthesis than the preparations obtained by previous procedures. This specific activity is 5-fold that measured in submitochondrial particles. With this preparation, which is virtually devoid of adenylate kinase and nucleoside diphos-

* To whom correspondence should be addressed.

Abbreviations: F_1, coupling factor F_1 (solubilized ATPase); Mes, 2-(*N*-morpholino)ethanesulfonic acid; Ap_5A, P_1,P_5-di-(adenosine-5'-)-pentaphosphate; NTP, nucleoside triphosphate; CCCP, carbonylcyanide *m*-chlrophenylhydrazone.

phokinase activities, it can be demonstrated that the energy provided by nucleoside triphosphate (NTP) hydrolysis can be almost completely recovered to make ATP from ADP and P_i. It is also shown that the concentration of the nucleotides present in the assay and the activity of the natural protein inhibitor [9] are important factors in the apparent efficiency of coupling between NTP hydrolysis and ATP synthesis.

Materials and Methods

Previously described procedures were used to prepare pig heart mitochondria [10], submitochondrial particles* [11], F_1 [12] and complex V [13]. Liposomes were made from pig heart mitochondrial lipids [14]. Mitoplasts were obtained from freshly prepared mitochondria. The mitochondrial pellets were suspended in 20 mM potassium phosphate at pH 7.4 at a protein concentration of 10 mg per ml. After 30 min at 0°C, the suspension was centrifuged at 105 000 × *g* for 1 h. The supernatant liquid contains soluble enzymes of the intermembrane space and in particular adenylate kinase and creatine kinase [15]. The pellets are suspended in 0.25 M sucrose/10 mM Tris-HCl (pH 7.4) and centrifuged at 11 500 × *g* for 15 min. Only mitoplasts were sedimented while outer membranes remained in the supernatants [16]. The mitoplasts were suspended in 0.25 M sucrose/10 mM Tris-H_2SO_4/1 mM ATP/15 mM $MgSO_4$ (pH 7.5). The suspension maintained below 6°C was sonicated for 3 min in a Branson Sonifier B12 used at a power of 60 W and centrifuged at 22 500 × *g* for 10 min at 4°C. The supernatant was centrifuged again at 105 000 × *g* for 75 min. The resulting pellet (submitochondrial particles) was suspended in 0.25 M sucrose/10 mM Tris-H_2SO_4/10 mM $MgSO_4$ (pH 7.5) and centrifuged again under the same conditions. When it was convenient, these submitochondrial particles could be kept at −80°C and thawed later on.

The extraction of the ATPase-ATP synthase complex was performed by treating the submitochondrial particles with α-lysophosphatidylcholine according to Sadler et al. [4] as modified by Joshi et al. [17] and as follows: The submitochondrial particles were suspended in 0.25 M sucrose/10 mM Tris-H_2SO_4/10 mM $MgSO_4$ (pH 7.5) at a protein concentration of 10 mg per ml. After centrifugation at 105 000 × *g* for 75 min, the pellets were suspended at a protein concentration of 10 mg per ml in 0.25 M sucrose/10 mM Tris-H_2SO_4/10 mM $MgSO_4$/2.6 mM $CaCl_2$ (pH 7.5) at 0°C. A 10% solution of α-lysophosphatidylcholine in the same buffer was added to give a final concentration of 0.1%. 30 min later, an equal volume of 100 mM Mes-KOH buffer (pH 6.5) was added. After a further 20 min incubation at 0°C the suspension was centrifuged at 105 000 × *g* for 15 min. The pellets were discarded and the supernatants were centrifuged for 2 h at 155 000 × *g*. The pellets containing the ATPase-ATP synthase complex were homogenized with a Potter glass homgenizer in a minimum volume of 0.25 M sucrose/10 mM Tris-H_2SO_4/10 mM $MgSO_4$ (pH 7.5). Aliquots were kept in liquid nitrogen until used. All operations starting from the collection of pig hearts at the slaughter-house were performed the same day. Protein concentration was determined by the biuret method [18] in the presence of 1% deoxycholate or by the method of Lowry et al. [19].

The rate of hydrolysis of ATP or GTP (free from any ATP) was estimated by measuring the amount of $^{32}P_i$ liberated after hydrolysis of [γ-^{32}P]ATP or [γ-^{32}P]GTP. The enzyme was rapildy thawed and preincubated for 15 min at 37°C in 0.25 M sucrose/25 mM Tris-H_2SO_4/15 mM $MgSO_4$ (pH 7.5) at a protein concentration of 1–5 mg per ml. The (γ-^{32}P)-labeled nucleotides were prepared as described by Penefsky [20] and salts were eliminated by charcoal treatment as described by Gautheron and Morélis [21]. Their radiochemical purity was monitored on a radiochromatogram Scanner (Packard) after thin-layer chromatography on PEI-cellulose F (Merck) using 1 M LiCl as a developing solvent. Unless otherwise indicated, the incubation mixture (0.95 ml) contained 25 mM Tris-acetate/0.3 M sucrose/15 mM $MgSO_4$/10 mM [γ-^{32}P]ATP or [γ-^{32}P]GTP (10^4 cpm/μmol)/5 mM ADP/20 mM potassium phosphate (pH 7.5) 37°C. To inhibit adenylate kinase 0.2 mM Ap_5A was routinely added [22]. The reaction was initiated by adding 0.05 ml of the enzyme suspension and stopped 5 min later with 0.1 ml 35% perchloric acid at 0°C. The assays were centrifuged at 14 000 × *g* for 5 min and the $^{32}P_i$ liberated was measured by introducing as quickly as possible 0.5 ml aliquots of

* In this paper the term submitochondrial particles is applied to those submitochondrial particles prepared by sonication of mitoplasts followed by differential centrifugation.

the supernatant solutions in 4 ml 1.25 M $HClO_4$/2.5 ml benzene/5.5 ml isobutanol/1 ml 4% ammonium molybdate. When using the purified complex, the $HClO_4$ treatment could be avoided. After shaking the mixture vigorously for 20 s, the upper organic phase (8 ml) containing more than 99.9% of the free phosphate was removed and counted for radioactivity. It was checked that in all conditions used the rate of NTP hydrolysis remained constant during the incubation time. Alternatively, the ATPase activity was measured spectrophotometrically at 340 nm with an ATP regenerating system: the reaction mixture contained 3.7 mM phospho*enol*pyruvate/0.32 mM NADH/50 μg pyruvate kinase/10 μg lactate dehydrogenase/10 mM ATP/15 mM $MgSO_4$/25 mM Tris-acetate/0.3 M sucrose/20 mM potassium phosphate (pH 7.5), 37°C.

ATP-P_i and GTP-P_i exchange activity was measured in a parallel assay in exactly the same incubation conditions as those used for the determination of the rate of ATP or GTP hydrolysis by the radioactive procedure except that the nucleotides were unlabeled at the beginning of the experiment and that $^{32}P_i$ was included ($(1-2) \cdot 10^6$ cpm/ml). The ^{32}P-labeled ATP or GTP was measured as described [13]. Correction was made for ATP hydrolysis during the exchange [13], but could not be made for GTP hydrolysis in our experimental conditions.

The incorporation of $^{32}P_i$ into individual nucleotides was detected by counting the radioactivity after separation of the different nucleotides. The exchange was stopped by addition of 10% trichloroacetic acid (final). After centrifugation at 14 000 × *g* for 5 min, the nucleotides were purified from the supernatants using charcoal as described [21]. After elution with pyridine and evaporation of pyridine, the nucleotides were separated by two-dimensional thin-layer chromatography on PEI-cellulose; the chromatograms were developed in the first dimension with 1 M LiCl, the plates were washed 10 min in distilled water, and the second dimension was run with 0.9 M potassium phosphate, pH 3.4. The nucleotides were located with an ultraviolet lamp at 254 nm. The individual spots were scraped off and eluted three times with 0.2 ml 1 M LiCl. The LiCl extracts were pooled, diluted with 10 ml distilled water and counted for radioactivity. This technique permitted the recovery of at least 93% of the radioactivity.

Adenylate kinase and nucleoside diphosphokinase activities were estimated by measuring the initial rate of ATP formation using the firefly luciferase assay as described [12]. The enzyme was incubated in the same mixture as that used for nucleoside triphosphate hydrolysis or synthesis studies. To measure the adenylate kinase activity, Ap_5A was omitted and 25 μg/ml olygomycin was added to prevent ATP formation through the ATP-P_i exchange system. 5 mM ADP was present and ATP was absent. To measure the nucleoside diphosphokinase activity 10 mM GTP, 5 mM ADP, 0.2 mM Ap_5A and 25 μg/ml oligomycin were present.

Phospholipids were extracted by the method of Folch et al. [23], separated and estimated by testing the amount of lipid phosphorus according to Portoukalian et al. [24]. Gel electrophoresis in the presence of SDS was carried out according to the procedure of Laemmli [25] using 12% acrylamide in the separating gel and 3% acrylamide in the stacking gel. Gels were stained overnight with 0.02% Coomassie blue R (Sigma) in 25% isopropanol, 10% acetic acid and destained with 10% acetic acid. The gel plates were dried under vacuum between two sheets of cellophane. Densitometric traces were obtained with a Vernon PHI 3 gel scanner.

Freeze-fracture electron microscopy was performed as previously described [14].

^{32}Pi was obtained from the Centre d'Etudes Atomiques, France. Nucleotides and carboxyatractyloside were purchased from Boehringer, mersalyl and α-lysophosphatidylcholine from Sigma Chemical Co. Bongkrekic acid was a generous gift of Prof. P.V. Vignais, University of Grenoble and Nigericin from Dr. Y. Briand, University of Clermont-Ferrand, France.

Results

Purification and characterization of the ATPase-ATP synthase preparation

The specific approach of the procedure was to prepare the ATPase-ATP synthase complex from inner membrane after elimination of as many loosely bound proteins as possible without affecting the activities of the complex. Table I summarizes the ATPase and ATP-$^{32}P_i$ exchange activities of the main fractions in a typical isolation run of purification.

TABLE I

PURIFICATION OF THE ATPase–ATP SYNTHASE COMPLEX FROM PIG HEART MITOCHONDRIA

The preparation of the different fractions are described in Material and Methods.

Step	Total protein (mg)	Phospholipids / protein (mg/mg)	ATPase activity (μmol/min per mg protein)	$^{32}P_i$-ATP exchange (μmol/min per mg protein)	Purification ATPase	Purification Exchange
1 Mitochondria	4 032	0.34	0.24	0.07	(1)	(1)
2 Mitoplasts	3 010	0.32	0.24	0.034	1	0.5
3 Submitochondrial particles	624	0.59	1.2	0.36	5	5
4 ATPase-ATP synthase	36	1.14	5.36	1.65	22	24

It is striking that ATPase and ATP-P_i exchange activities increased in parallel along the purification steps; especially, they increased 4–5-fold between the submitochondrial particles step and the final step of purified ATPase-ATP synthase. The complex was stable when kept as small aliquots in liquid nitrogen. However it was sensitive to freezing-thawing cycles which were each accompanied by a loss of 10–20% of exchange activity while the ATPase activity increased. Additionally, heating should be carefully avoided during the sonication step leading to the preparation of the submitochondrial particles. Increasing the ratio lysophosphatidylcholine/protein decreased the rate of ATP-P_i exchange. The procedure has been repeated at least seven times. The ATP-$^{32}P_i$ eschange was always remarkably high. The ATPase activity measured in the presence of an ATP regenerating system (see Material and Methods) was not significantly different from that measured in the absence of ADP. In the experiments described in Table I, the ATPase activity was measured in the presence of 5 mM ADP (necessary to obtain linear rates of ATP-P_i exchange; see below Fig. 3). This ADP concentration decreased the rate of ATP hydrolysis by about 10%. The ATPase activity can be further increased by 25% in the presence of uncouplers (see Table III).

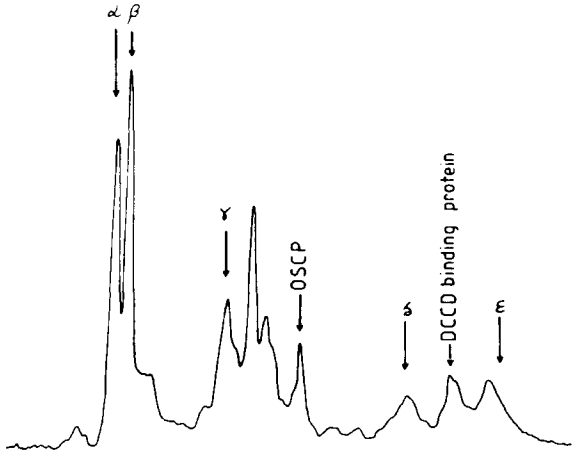

Fig. 1. Densitometric trace of ATPase-ATP synthase complex (30 μg) electrophoresed on sodium dodecyl sulfate 12% acrylamide gel. Proteins were stained with Coomassie blue. The arrows indicate the position of the bands of either an F_1 sample run in parallel (α, β, γ, δ and ε) or a sample of OSCP (oligomycin-sensitivity conferring protein) or a sample of DCCD binding protein.

The polypeptide composition of the ATPase-ATP synthase preparation as determined by SDS-polyacrylamide gel electrophoresis is shown in Fig. 1. The most striking feature of this densitometric trace is the presence of a band of high intensity between γ and OSCP at a calculated M_r value of approx. 28 000–30 000, which was absent or very weak in the preparation of Serrano et al. [6] or in complex V [7]. Further studies are needed to tell if the presence of this band is of any importance for the ATP-$^{32}P_i$ exchange activity which is at least 4–5-fold higher than for complex V or for the preparation of Serrano et al. [6].

Freeze fracture studies of the ATPase- ATP synthase preparation (Fig. 2) shows that most proteins are included in lipid vesicles of 400–600 Å in diam-

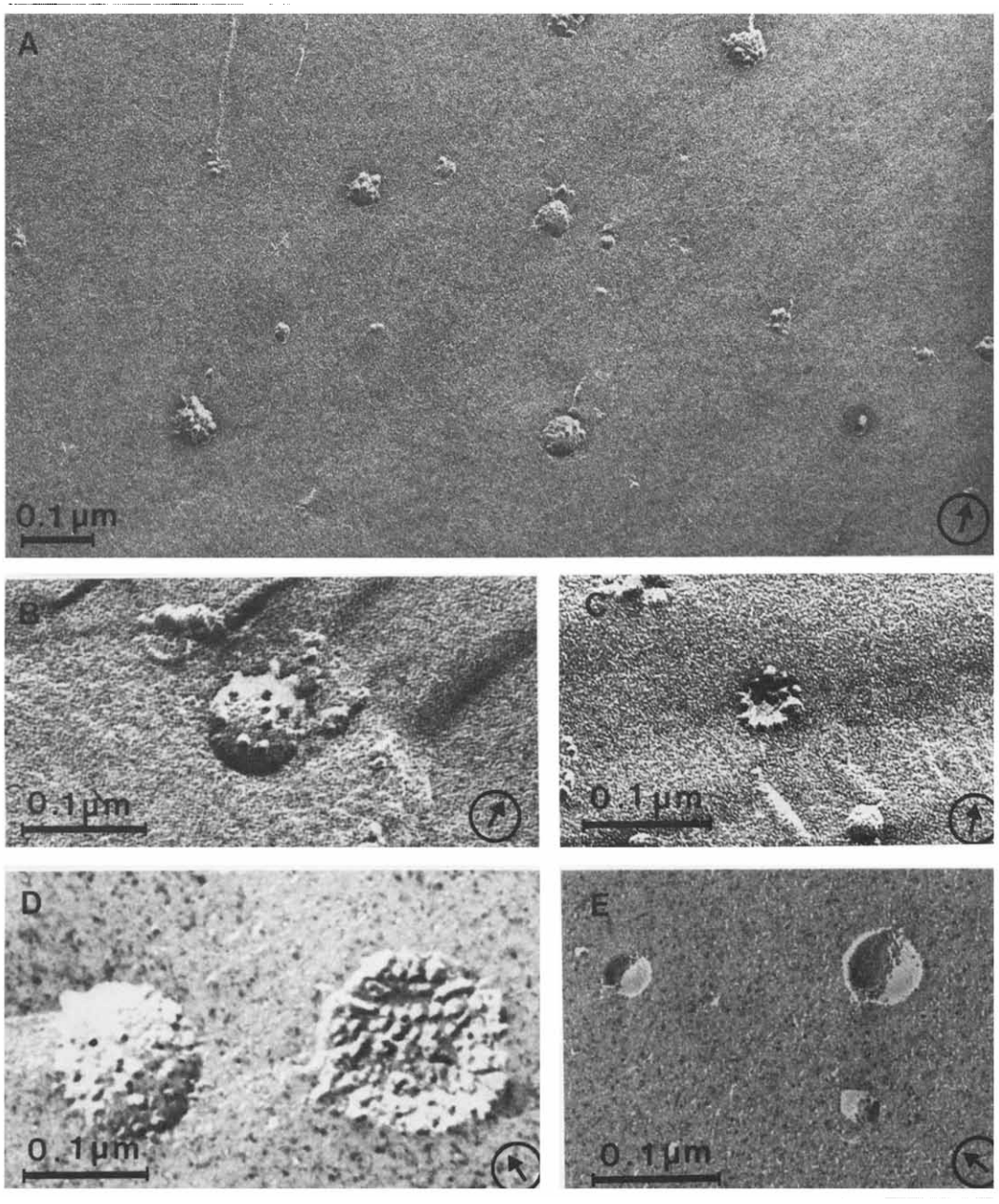

Fig. 2. Freeze-fracture studies of the ATPase-ATP synthase complex as compared to submitochondrial particles and liposomes. (A) low magnification view of the ATPase-ATP synthase complex; ×100 000. (B and C) higher magnification of this vesicular preparation, showing a convex fracture of EF face (B) and a concave fracture of PF face (C); ×180 000. (D) submitochondrial particles; ×180 000. (E) liposomes prepared from pig heart mitochondrial lipids; ×180 000.

eter (Fig. 2A), which corresponds approximately to the size of liposomes (Fig. 2E) prepared with phospholipids extracted from pig heart mitochondria [14]. Less than 1–2% protein aggregates were observed in the freeze-fracture replicas. The amount of particles representing the proteins in each vesicle is low when compared to that present in submitochondrial particles prepared from the same mitochondria (Fig. 2D). A comparison between convex (Fig. 2B) and concave (Fig. 2C) fracture faces shows an even distribution of particles between the two lipid layers of the membrane. On the contrary, an asymmetrical distribution of submitochondrial particles was observed [14]. The proteins seem to be more diluted in the lipid phase than in the original membranes. This conclusion is in agreement with the results shown in Table I: the ratio between phospholipids and proteins was 3-fold higher in the ATPase-ATP synthase preparation than in the original mitochondria or mitoplasts. Analysis of the different phospholipids present shows a much higher level of lysophosphatidylcholine (22%) as compared to that present in mitoplasts (0.6%). Therefore, some of the lysophosphatidylcholine added during the preparation was incorporated into the membrane. If the lysophosphatidylcholine present was deduced from the total lipid by calculation, the distribution of other phospholipids was not significantly different from that of the starting mitochondrial membranes (not shown). Therefore there was no removal of any specific phospholipid during the preparation.

When compared to the rate of oligomycin-sensitive incorporation of $^{32}P_i$ into ATP supported by the NTP hydrolysis, the rate of ATP formation through adenylate kinase or nucleoside diphosphokinase activities was very low (Table II). The adenylate kinase activity could be completely inhibited by the addition of 0.2 mM Ap_5A while this inhibitor did not modify the rate of $^{32}P_i$ incorporation into ATP. Ap_5A was therefore routinely used in all assays. It will be seen below that, in the conditions used here, no detectable incorporation into GTP occurred.

The effects of various inhibitors on the ATP-P_i exchange and the ATPase activities of the ATPase-ATP synthase preparation are shown in Table III. Oligomycin fully inhibits both ATPase and ATP-P_i exchange activities. Uncouplers inhibit the ATP-P_i exchange and increase the ATPase activity. Compounds known to inhibit the adenylate kinase activity (Ap_5A) or the nucleotide translocation (carboxyatractyloside or bongkrekic acid) have no significant effect. Bovine serum albumin and dithiothreitol do not increase the exchange. Mersalyl inhibits the ATPase less than the ATP-P_i exchange activity.

When the complex was preincubated at pH 6.7 in the presence of 2 mM ATP and 2 mM Mg^{2+} as described by Galante et al. [27], the ATPase activity was decreased to a larger extent (56% of the control)

TABLE II

COMPARED ACTIVITIES OF $^{32}P_i$ INCORPORATION INTO ATP, OF ADENYLATE KINASE AND OF NUCLEOSIDE DIPHOSPHOKINASE

When present, the inhibitors were used at the following concentrations: oligomycin: 25 μg/mg protein, Ap_5A : 0.2 mM. The nucleotide concentrations were 10 mM for ATP and GTP and 5 mM for ADP.

Enzyme	Inhibitor present	Nucleotides	Activity (μmol ATP or [γ-^{32}P]ATP formed/min per mg protein)
$^{32}P_i$ incorporation into ATP	0	ATP + ADP	1.7
	0	GTP + ADP	0.22
	Oligomycin	ATP + ADP	<0.008
	Ap_5A	ATP + ADP	1.65
Adenylate kinase	Oligomycin	ADP	0.020
	Oligomycin + Ap_5A	ADP	<0.006
Nucleoside diphosphokinase	Oligomycin + Ap_5A	GTP + ADP	<0.006

TABLE III

INFLUENCE OF VARIOUS INHIBITORS ON ATP-P_i EXCHANGE AND ATPase ACTIVITIES OF THE ATPase-ATP SYNTHASE PREPARATION

All inhibitors were present in the assays at the indicated concentrations. Assays with valinomycin and nigericin were made in the presence of 0.1 M KCl.

Inhibitor	Concentration	ATP-P_i exchange (% of control)	ATPase (% of control)
Oligomycin	5 μg/mg protein	<0.5	2
CCCP	1 μM	12	128
2,4-DNP	100 μM	42	122
Ap_5A	200 μM	97	103
Carboxyatractyloside	100 μM	99	100
Bongkrekic acid	20 μM	98	100
Valinomycin ($+K^+$)	2 μg/ml	68	95
Nigericin ($+K^+$)	2 μg/ml	18	156
Valinomycin + Nigericin ($+K^+$)	2 μg/ml 2 μg/ml	0.6	23
Mersalyl	30 μM	24	79
Dithiothreitol	5 μM	95	102
Bovine serum albumine	2.5 mg/ml	96	133
Endogenous protein inhibitor	(a)	70	56

[a] The endogenous protein inhibitor is activated as described by Galante et al. [27].

than the ATP-P_i exchange activity (70% of the control), It was shown [27] that this treatment permitted the expression of the activity of the natural protein inhibitor. This indicates that the natural protein inhibitor is present in the ATPase-ATP synthase preparation and that it increases the apparent coupling between ATP hydrolysis and $^{32}P_i$ incorporation into ATP (see below).

Kinetic properties of the ATPase-ATP synthase complex: correlation between the rate of nucleoside triphosphate hydrolysis and the rate of $^{32}P_i$ incorporation into ATP

The rates of [γ-^{32}P]ATP hydrolysis and ATP-$^{32}P_i$ exchange have been studied simultaneously in parallel assays with the ATPase-ATP synthase complex. Fig. 3 shows that the rate of ATP hydrolysis remains constant during 10 min of incubation. The presence of 5 mM ADP induces a 15% inhibition of this rate when ATP was present at an initial concentration of 10 mM. The rate of $^{32}P_i$ incorporation into ATP increases progressively with time until a constant rate is reached after about 6 min of incubation. When 5 mM ADP is present in the medium before the addition of the enzyme which initiates the reaction, the rate of $^{32}P_i$ incorporation into ATP is independent of the time of reaction and is equal to that obtained after 6 min of incubation in the absence of added ADP. This suggests that the amount of ADP available was a limiting factor for $^{32}P_i$ incorporation. When the initial ADP concentration increased from zero to 10 mM (Fig. 4A), the rate of ATP hydrolysis decreased while the rate of $^{32}P_i$ incorporation into ATP slightly increased at low ADP concentration and slightly diminished after 5 mM ADP. This diminution may be a reflection of the decreased ATPase activity although the ATP-$^{32}P_i$ exchange activity was corrected for hydrolysis of ATP during the exchange as described [13]. Qualitatively, similar effects are observed with complex V (Fig. 4B) and submitochondrial particles (Fig. 4C). In the experiments described here, the energy necessary for the incorporation of inorganic phosphate into the terminal phosphoryl group of ATP must come from the ATP hydrolysis. The ratio between the rate of ATP hydrolysis and the rate of $^{32}P_i$ incorporation into ATP appears related to the recovery of the energy of ATP hydrolysis and thus indicates the degree of coupling between the two

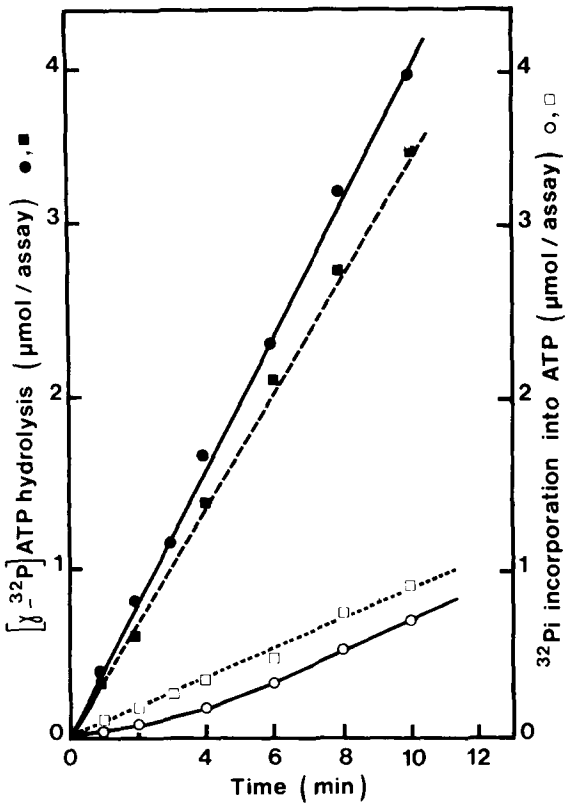

Fig. 3. Rate changes of the ATP hydrolysis or of the ATP-P_i exchange reaction as a function of time and initial presence of ADP. The initial ATP concentration was 10 mM in all cases. Circles: no ADP added, squares 5 mM ADP added before the initiation of the reaction.

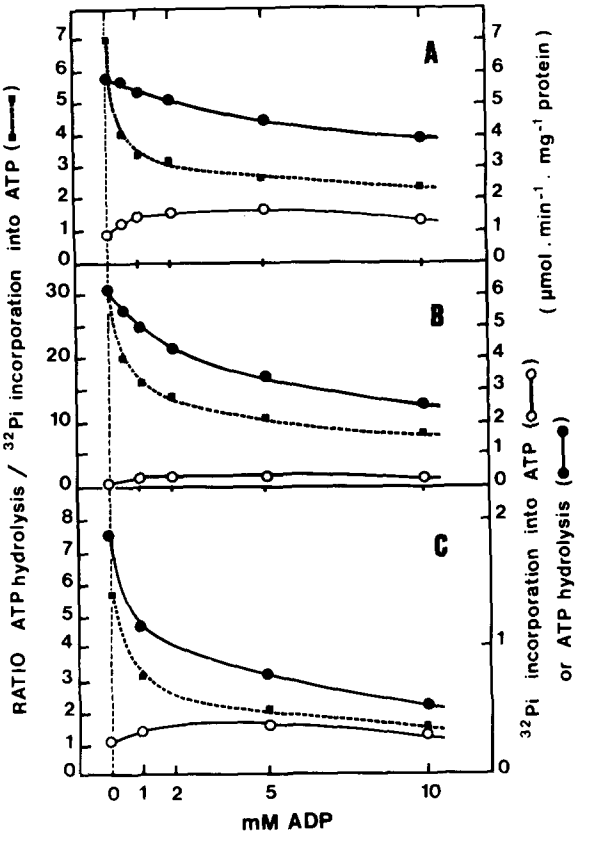

Fig. 4. Effect of initial ADP concentration on the ATP hydrolysis, the $^{32}P_i$ incorporation into ATP and the ratio ATP hydrolysis over $^{32}P_i$ incorporation into ATP catalyzed by three different enzyme preparations: A: ATPase-ATP synthase complex; B: complex V; C: submitochondrial particles.

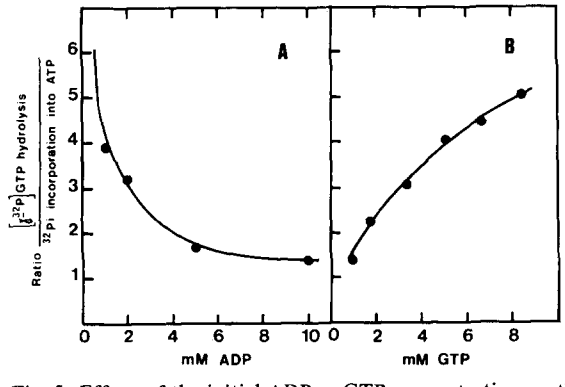

Fig. 5. Effect of the initial ADP or GTP concentration on the ratio between the rate of [γ-^{32}P]GTP hydrolysis and the rate of $^{32}P_i$ incorporation into NTP. A. Initial GTP concentration, 5 mM; ADP as indicated. B. Initial ADP concentration, 5 mM; GTP, as indicated.

reactions, the highest ratios corresponding to the lowest coupling. This ratio evolves in the conditions used here from 7 to 2.5 for the ATPase-ATP synthase complex (Fig. 4A), from 31 to 8.5 for the complex V (Fig. 4B) and from 5.7 to 1.5 for the submitochondrial particles (Fig. 4C). This result shows that the coupling properties of the ATPase-ATP synthase complex are very close to those of the original particles.

The ATPase-ATP synthase complex is known to be able to hydrolyze several nucleoside triphosphates with variable affinities [26]. This was observed previously with submitochondrial particles or with other oligomycin-sensitive ATPase preparations [26,7]. It is shown in Table IV that 10 mM GTP is hydrolyzed at a lower rate than 10 mM ATP. GDP inhibited the

TABLE IV

COMPARED SPECIFICITY OF THE ATPase-ATP SYNTHASE COMPLEX TOWARDS NUCLEOSIDE TRIPHOSPHATE HYDROLYSIS OR $^{32}P_i$ INCORPORATION INTO NUCLEOSIDE TRIPHOSPHATE

The assays made in the absence of added nucleoside triphosphate were done exactly as other assays except that $^{32}P_i$ was added at a higher specific activity: $37 \cdot 10^6$ cpm per assay instead of about $2 \cdot 10^6$ cpm per assay. Ap_5A was present in all assays at a concentration of 0.2 mM.

Added nucleotides		NTP hydrolysis (A) (μmol/min per mg protein)	$^{32}P_i$ incorporation (B) (μmol/min per mg protein)	Ratio A/B
Nucleoside triphosphate (10 mM)	Nucleoside diphosphate (5 mM)			
ATP	–	6.9	1.1	6.3
ATP	ADP	6.1	1.7	3.6
ATP	GDP	6.3	0.96	6.6
–	ADP		0.0013	
GTP	–	2.4	0.03	80
GTP	ADP	0.4	0.22	1.9
GTP	GDP	1.5	0.04	40

rate of ATP or GTP hydrolysis less efficiently than ADP. ADP behaves as a competitive inhibitor of ATP or GTP (not shown). Therefore there is a higher affinity for nucleotides containing adenine than for those containing guanine. GDP does not significantly improve the rate of $^{32}P_i$ incorporation into nucleoside triphosphate, while ADP improves the $^{32}P_i$ incorporation into nucleoside triphosphates both when ATP or GTP were hydrolyzed. The ratio between nucleoside triphosphate hydrolysis and $^{32}P_i$ incorporation into ATP was the lowest when GTP and ADP were present together (1.9) (Table IV). This ratio could be further diminished to a minimum of about 1.2 by decreasing the concentration of GTP or increasing that of ADP (Fig. 5). In this case the system appears to reach a steady-state equilibrium between NTP hydrolysis and $^{32}P_i$ incorporation into ATP and seems to be strictly coupled. It was checked that $^{32}P_i$ was incorporated into ATP after separation of the nucleotides by two-dimensional thin-layer chromatography. When the

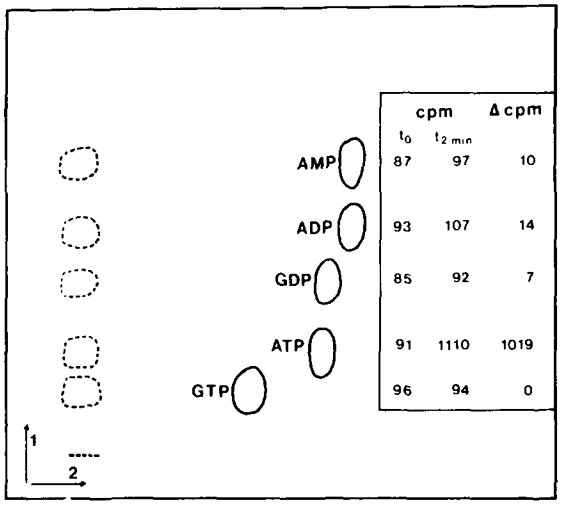

Fig. 6. Chromatography on PEI-cellulose of nucleotides labeled with $^{32}P_i$ by the ATPase-ATP synthase complex. The samples were spotted together with a mixture of unlabeled GTP, GDP, ATP, ADP, AMP (5 nmol of each) used as carriers. 1: first dimension : M LiCl. 2: seond dimension : 0.9 M potassium phosphate, pH 3.4. Dotted spots: location of the nucleotides after the development of the chromatogram in the first dimension; dotted line : origin. The radioactivity present in each spot was counted (see Material and Methods). The background for a spot without nucleotide was around 90. Δcpm ($t_{2\,min} - t_0$) represents the difference between the cpm found in each nucleotide at $t_{2\,min}$ and that found at zero time when $^{32}P_i$ was incorporated into NTP for 2 min ($t_{2\,min}$) in the presence of 10 mM GTP/5 mM ADP/0.2 mM Ap_5A/20 mM P_i (43 μCi/μmol)/25 mM Tris-acetate/0.3 M sucrose/15 mM $MgSO_4$/0.8 mg protein of ATPase-ATP synthase complex (pH 7.5), 37°C (final volume 0.2 ml). The cpm counted at t_0 and $t_{2\,min}$ for AMP, ADP, GDP, GTP are within the range of variations in the background.

incubation was performed in the presence of GTP and ADP all the $^{32}P_i$ incorporated was found in ATP. No significant radioactivity was ever detected in GTP (Fig. 6). GDP, ADP and AMP were not labeled either: the variations observed between no and 2 min incubation are within the range of the variations of the background.

Discussion

The ATPase-ATP synthase complex purified from pig heart by the method described in this paper has a specific activity for ATP-$^{32}P_i$ exchange (1.6 μmol/min per mg protein) very much higher than that reported for preparations obtained from beef heart mitochondria. Indeed, in the literature this activity varied from 0.025 [28] to 0.2–0.5 for complex V [7] or for the proton-translocating ATPase complex of Serrano et al. [6]; Joshi et al. [17] reported that they could obtain an exchange activity of 0.6 to 0.9 by using the procedure of Sadler et al. [4]. Our purification procedure yields an ATPase-ATP synthase complex, the specific activity of which is about 5-fold that of the starting submitochondrial particles; this has never been reported before. The close relationship between ATPase activity and ATP-P_i exchange along the purification also indicates that the whole physiological ATPase-ATP synthase complex has been purified. The very low nucleoside diphosphokinase and adenylate kinase activities (less than 4% of that present in the original mitochondrial fraction [15] and the fact that the remaining adenylate kinase activity can be completely inhibited by Ap_5A without significant modification of the ATP-P_i exchange activity are other interesting features of this preparation. The complete purification can be achieved within 1 day starting from the collection of pig hearts at the slaughter house. The ATPase-ATP synthase complex does not need the addition of any cofactor or phospholipid to be highly active. As in submitochondrial particles, it is sensitive to oligomycin, uncouplers, mercurials or valinomycin plus nigericin. All these characteristics prove the excellent behavior of this preparation on a functional point of view.

Although most of the protein in this preparation corresponds to F_1, the ATPase activity is about 20-fold lower that that obtained with F_1 purified from the same source [12]. This can be explained by the fact that the endogenous protein inhibitor [9] is present in the ATPase-ATP synthase complex (Table III). The low specific ATPase activity may also come from the close association between F_1 and its membrane sector F_0. It is quite possible that this association maintains the enzyme in a conformation showing a low specific ATPase activity as in intact mitochondria and that the high specific activity of the purified F_1-type preparations would be somewhat artefactual!

The polypetide composition of this preparation is similar to that reported by others except for the presence in relatively high amount of a polypeptide in the 28 000 to 30 000 M_r region. Carboxyatractylate and bongkrekic acid each alone or together have no effect on the ATP-P_i exchange activity. In the same way, Serrano et al. [6] have shown that the removal of a 28 000 to 30 000 M_r polypeptide, responsible for a high adenine nucleotide translactor activity from their preparation did not diminish the exchange activity. It is therefore unlikely that the high exchange activity of our preparation can be directly related to the presence of the adenine nucleotide translocator. Berden and Henneke [28] have shown that a 30 000 M_r polypeptide, identified as the uncoupler-binding protein [29], can be removed from the oligomycin-sensitive ATPase complex and is not essential for a functional complex. However, in their preparation the maximal ATP-P_i exchange activity was 0.030 as compared to 1.6 μmol/min per mg protein in our experiments. Galante and Hatefi [30] also reported the non-essential role of the uncoupler-binding protein for the proton pumping activity of the ATPase complex, using a preparation with an exchange activity of 0.2–0.3 μmol/min per mg protein. Several possibilities remain open. Either the 28 000 to 30 000 M_r polypeptide abundant in our preparation is a contaminant unrelated to the high specific activity, or it is an important component still unidentified. It could also be the adenine nucleotide translactor the uncoupler-binding protein or even a polypeptide of the phosphate transporter which also has a similar M_r [31,32]. These components alone or together could maintain the complex in such a conformation that the activity would be higher.

The contamination of our preparation by cytochromes (not shown) is low and of the same order of magnitude as that reported for complex V [7] or for the proton-translocating ATPase complex [6].

Any attempt to further purify the complex has resulted so far in an irreversible loss in ATP-P_i exchange activity. It has possible in the case of yeast [33] and of thermophilic bacterium [34] to obtain an ATP-P_i exchange activity in the absence of electron transport components. Other studies are needed to conclude whehter it is possible or not to obtain a complex completely free of identifiable respiratory components as described by Galante et al. [8] but still capable of high physiological level of ATP-P_i exchange activity.

The vesicular structure of this preparation, well demonstrated by freeze fracture studies, may be an important factor in the high specific activity for ATP-P_i exchange. The sensitivity of the complex to valinomycin plus nigericin indicates the involvement of a proton gradient in the activity and it is very likely that this proton gradient is established between the inner and the outer faces of the vesicle membrane. When the amount of protons provided by nucleoside triphosphate hydrolysis is limited by using GTP (which has a low affinity) at a low concentration and by adding ADP which inhibits the rate of GTP hydrolysis, the ratio between GTP hydrolysis and ATP synthesis rates decreases. In other words the coupling between the proton production and the ATP synthesis appears very good. This coupling may be limited by the capacity of the vesicles to maintain a proton gradient; one cannot exclude some leakage of protons, perhaps due to the high content of lysophosphatidylcholine in the vesicles, especially when the rate of NTP hydrolysis becomes too high. On the contrary, when the rate of hydrolysis is limited, the protons are used up for ATP synthesis as soon as they are produced and the ratio between the two processes approaches 1. After activation by incubation of the complex with MgATP as described by Galante et al. [27], the natural protein inhibitor diminishes less the rate of ATP synthesis than its rate of hydrolysis. Therefore, it decreases the ratio between the two rates and behaves as a coupling factor.

The experiments presented here demonstrate that the energy produced by nucleoside triphosphate hydrolysis can be almost completely recovered and used for ATP synthesis. The fact that the energy produced by GTP hydrolysis can be used to produce ATP synthesis in the absence of significant kinase activity and without any labeling of GTP, GDP, AMP or ADP can be explained if the reactions can take place together at two sites or two different pathways. Either, on each ATPase-ATP synthase complex, there is one site specialized for hydrolysis and one site specialized for synthesis or else there are at least two different complexes present in the same vesicle. One of them hydrolyses GTP and produces a ΔpH between the inner and outer faces of the vesicle, while another complex uses up the energy provided by this proton gradient to make [γ-^{32}P]ATP from the ADP and $^{32}P_i$ present in the assay. The experiments presented here do not permit a choice between these two possibilities, but the experiments reported by Kohbrenner and Boyer [35] favor rather the second possibility. The alternating site mechanism proposed by Boyer [36] would implicate the uncoupler-sensitive energized state being induced through the membrane, maybe by the proton gradient, and that different complexes are involved. At a given time, some complexes would hydrolyze NTP while others would be used for ATP synthesis. The same complex could serve alternatively for either function. Statistically the number of complexes used for hydrolysis or synthesis would depend on the respective affinity of the complex for the nucleotides, NTP or NDP present, or on the efficiency of the inhibitor protein.

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