Journal of Bioenergetics and Biomembranes, Vol. 14, Nos. 5/6, 1982

Isolation of a Highly Active H^+-ATPase from Beef Heart Mitochondria

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Received March 26, 1982

Abstract

The lysolecithin extraction procedure originally described by Sadler *et al.* (1974) has been modified to yield a H^+-ATPase with high levels of P_i–ATP exchange activity (400–600 nmol $\times$ min^{-1} $\times$ mg^{-1}). This activity is further enhanced (1400–1600 nmol $\times$ min^{-1} $\times$ mg^{-1}) following sucrose density gradient centrifugation in the presence of asolectin. This enhancement results in part from a lipid-dependent activation and in part from removal of inactive complexes. The H^+ translocating activity of the complex has been determined spectrophotometrically using binding of oxonol VI as an indicator of membrane potential. P_i-ATP exchange, ATP hydrolysis, and oxonol binding are sensitive to energy-transfer inhibitors (oligomycin, rutamycin) and/or uncouplers (DNP, FCCP).

KEY WORDS: H^+-ATPase; beef heart mitochondria; P_i–ATP exchange; lysolecithin.

Introduction

In general, the earlier preparations of beef heart mitochondrial ATPase which catalyzed P_i–ATP exchange demonstrated at least partial if not absolute dependence on exogenous phospholipid and coupling factors (Tzagoloff *et al.*, 1968a; Serrano *et al.*, 1976; Stiggall *et al.*, 1976; Ryrie, 1977; Berden and Vourn-Brouwev, 1978; Sanadi *et al.*, 1977). Even after reconstitution, the P_i–ATP exchange activity remained low (150–300 nmol $\times$ min^{-1} $\times$ mg^{-1}) and not much higher than that of the starting material, viz. submitochondrial particles. The ATPase activity was high (10–13 μmol $\times$ min^{-1} $\times$ mg^{-1}), indicating some degree of irreversible damage.

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0145-79X/82/1200-0287$03.00/0

The most promising preparation was described by Sadler *et al.* (1974), and while the P_i–ATP exchange activity was on the low side (180 nmol $\times$ min^{-1} $\times$ mg^{-1}), there was no requirement for phospholipid or coupling factors. Moreover, the low exchange activity may have resulted from the fact that extraction of ETPH with high lysolecithin (0.6–1.2 mg/mg protein) produced mostly small nonvesicular membrane fragments (Sadler *et al.*, 1974; Komai *et al.*, 1973). Therefore, in the present study the lysolecithin extraction conditions have been modified and the extract subjected to sucrose density gradient centrifugation to obtain a purified H^+-ATPase with the P_i–ATP exchange activity severalfold higher than that of previously reported preparations.

Methods

Beef heart mitochondria (Joshi and Sanadi, 1979), ETPH (Linnane and Ziegler, 1958), F_1 and F_B (Joshi *et al.*, 1979) were isolated by published procedures. ATPase (Tzagoloff *et al.*, 1968b), P_i–ATP exchange activity (Joshi *et al.*, 1979), NADH dehydrogenase (King and Howard, 1967), succinate dehydrogenase (Baginsky and Hatefi, 1969), and cytochromes (Williams, 1964) were determined as described. SDS-PAGE (Weber and Osborn, 1969) and protein determinations (Lowry *et al.*, 1951) were as described. For oxonol[3] binding, H^+-ATPase was suspended at 200 μg per 3.0 ml of 40 mM Tris-acetate (pH 7.4) containing 250 mM sucrose and 1 mM $MgCl_2$. A 5-μl aliquot of oxonol VI (1.5 mM in 95% ethanol) was added and allowed to equilibrate for 60 sec at 20°C. The reaction was initiated by addition of 2 μmol of ATP (with 0.2 μmol of ADP, pH 7.4). The shift in absorbance resulting from binding of oxonol VI was followed with an Aminco Chance dual-wavelength spectrophotometer using 603 and 630 nm, respectively, as the reference and measuring wavelengths (Smith and Chance, 1979). Oxonol VI was kindly provided by Drs. B. Chance and L. Bashford.

Results

The initial extraction of H^+-ATPase from ETPH essentially followed the procedure described by Sadler *et al.* (1974). ETPH (20 mg/ml) were suspended in cold Tris-HCl (pH 7.8) containing 250 mM sucrose. To the

[3] F_B, coupling factor B from beef heart mitochondria; Oxonol VI, bis-(3-propyl-5-oxoisoxazol-4-yl)pentamethineoxonol; DTT, dithiotreitol; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; DNP, dinitrophenol; DCCD, *N,N'*-dicyclohexylcarbodiimide; MES, 2-(*N*-morpholino)ethanesulfonic acid.

suspension was added an equal volume of the same buffer containing lysolecithin (4 mg/ml), and the suspension was stirred at 0–4°C. After exactly 30 min, 2 volumes of cold 100 mM MES-KOH (pH 6.0) were added and the suspension allowed to stand for 20 min at 0–4°C. After centrifugation at 105,000 × *g* for 15 min, the resulting supernate was recentrifuged at 140,000 × *g* for 150 min. The clear supernate was discarded and the tan pellet suspended at 20 mg/ml in 10 mM Tris-acetate (pH 7.5) containing 250 mM sucrose and 1 mM DTT. Under these conditions P_i–ATP exchange activity is stable for at least 2 hr at 20°C and for several months at −60°C. Increasing the lysolecithin concentration during extraction increased the yield of P_i–ATP exchange activity while decreasing the specific activity. Lower levels of lysolecithin decreased the yield of both exchange units and protein solubilized without affecting specific activity.

Aliquots of the lysolecithin-extracted material were thawed and diluted with an equal volume of 100 mM Tris-HCl (pH 7.5) containing 1 mM DTT, 15% sucrose, and 0.02% sonicated asolectin and incubated on ice for 15–30 min. Samples containing 5–7 mg of protein were transferred to 10-ml linear gradients containing 15–50% sucrose, 100 mM Tris-HCl (pH 7.5), 1 mM DTT, and 0.02% asolectin and centrifuged at 35,000 rpm (SW-41 rotor) for 15 hr.

Gradient centrifugation apparently resolves two distinct populations of complexes (pool I and pool II) which differ in P_i–ATP exchange activity but not in oligomycin-sensitive ATPase (Fig. 1). P_i–ATP exchange activity of the most active fractions was enhanced 3–4-fold with a yield of 40–60% of the applied protein (Table I). ATPase activity was increased 2–3-fold in pool 2. This amounts to a 1.5–2-fold increase in the yield of total units of P_i–ATP exchange activity during gradient purification. Consequently, the 3–4-fold increase in specific activity for P_i–ATP exchange is due in part (2-fold) to the removal of inactive proteins and possibly in part (1.5–2-fold) to the presence of asolectin in the gradient. A similar stimulatory effect of phospholipids can also be seen when pregradient H^+-ATPase is incubated with sonicated asolectin in a medium which approximates gradient conditions (100 mM Tris-HCl, pH 7.5, 0.25 M sucrose, 1 mM DTT). The maximum stimulation of P_i–ATP exchange achieved was 1.5–2-fold (Table I), with mitochondrial phospholipids being slightly more effective than asolectin. This level of stimulation was achievable using short incubations (30–60 min, 0.025% asolectin). The incubation of pregradient H^+-ATPase with additional lysolecithin (0.02–0.05%, 60 min at 0°C) did not result in a significant loss of P_i–ATP exchange activity.

The exchange activity of both pregradient material and pool II is 90% inhibited by energy-transfer inhibitors (0.2 μg oligomycin, 0.2 μg rutamycin) and uncouplers (10 μM DNP, 5 μM CCCP). Activity is 25% inhibited by 2 μg

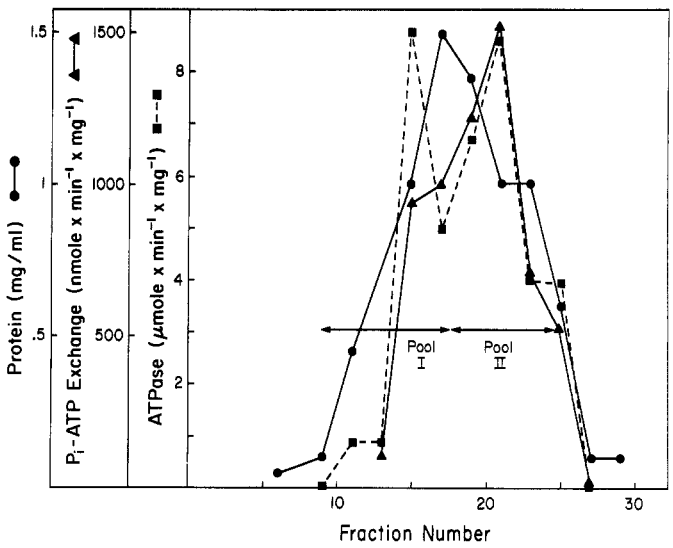

Fig. 1. Gradient purification of lysolecithin-extracted H^+-ATPase. Aliquots of the H^+-ATPase (20 mg/ml) were diluted to 10 mg/ml with 100 mM Tris-HCl (pH 7.5) containing 1 mM DTT, 0.44 M sucrose (15%), and 0.02% sonicated asolectin (Associated Concentrates, Woodside, New York) and incubated on ice for 15–30 min. Samples containing 5–7 mg of protein were transferred to 10-ml linear gradients containing 15–50% sucrose, 100 mM Tris-HCl (pH 7.5), 1 mM DTT, and 0.02% sonicated asolectin. After centrifugation for 15 hr at 35,000 rpm (SW-41), 0.3 ml fractions were collected and assayed as described.

Table I. Characterization of the Lysolecithin Extracted H^+-ATPase

	ETPH	Lysolecithin extract	Gradient pool II
Protein yield (μg/mg ETPH)	—	40–80	25–50
P_i–ATP exchange (nmol × min^{-1} × mg^{-1})	100–150	510	1400–1600
+0.05% lysolecithin	—	500	—
+0.25% asolectin	—	610	1540
+0.025% mitochondrial phospholipids	—	800	
Exchange units recovered (%)	100	20–30	50–70
ATPase (μmol × min^{-1} × mg^{-1})	0.5–1.0	1.5–3.0	5.0–7.0
Cytochromes (nmol × mg^{-1} protein)			
$a + a_3$	—	0.15–0.30	0.05–0.15
b	—	0.19–0.24	0.08–0.20
$c + c_1$	—	0.22–0.32	0–0.10
NADH dehydrogenase (μmol × min^{-1} × mg^{-1})		1.0–3.0	0.50–1.00
Succinate dehydrogenase (μmol × min^{-1} × mg^{-1})	—	0.05–0.15	0.05–0.1

of either nigericin or valinomycin plus K^+, but is completely inhibited by a combination of both ionophores in the presence of K^+. Exchange and ATPase activity are equally sensitive to DCCD (I_{50} = 3 nmol DCCD/mg protein, 20 min incubation at 0°C), which is in contrast to results obtained with Complex V (Stiggal *et al.*, 1976).

Exchange activity of the pregradient and pool II fractions are only minimally stimulated (<10%) following incubation with soluble F_1 and/or coupling factor B. Taken together with the high level of exchange activity and the low level of detectable contaminants (Table I), this would suggest that the lysolecithin extraction alters the structural integrity minimally, if at all. On the other hand, depletion of phospholipid with ionic detergents and reconstitution may be expected to lead to significant loss of coupling factors and scrambling of the arrangement of the protein subunits.

SDS-PAGE of the pregradient H^+-ATPase (not shown) indicates some similarity with complex V (Stiggall *et al.*, 1976) and the proton-translocating ATPase (Serrano *et al.*, 1976). Comparison of the pregradient material with material in fraction 22 (Fig. 1) suggests three obvious differences. First, three or four bands with molecular weight estimates of 60,000–100,000 are sometimes present in pregradient material but are reduced or absent in fraction 22. The staining intensity of these components varies from preparation to preparation, independently of exchange activity. The disappearance of these components following gradient centrifugation is consistent with the removal of respiratory-chain contaminants (Table I). Second, pregradient material contains, as a major component, a band with a slightly lower molecular weight estimate (29 kilodaltons) than band 3 (Fig. 2). This component is also prominent in pool I material and a H^+-ATPase made from pig heart mitochondria using our lysolecithin extraction procedure (Penin *et al.*, 1982), but not in pool II. The third difference is the relative contribution of the high- (bands 1 + 2) and low- (bands 8–10) molecular-weight components to total Coomassie Blue staining. In pregradient material, bands 1 + 2 and 8–10, respectively, account for 41 and 6% of the total staining. In pool II, the values are 45 and 15%. It is unclear whether the 29-kilodalton component seen in complexes with relatively low P_i–ATP exchange represents an aggregate of low molecular weight subunits or that pool II represents complexes which have not experienced aggregation and therefore remain active. However, aggregates of the DCCD-binding proteolipid with an approximate molecular weight of 29,000 have been reported to be resistant to SDS digestion (Graf and Sebald, 1978).

The subunits of F_1 (bands 1, 2, 3, 7, and 10) as well as OSCP (band 6) and Factor B and F_6 can be identified by co-migration with purified proteins. Furthermore, bands 1, 2, 3, 7, and 10 are substantially reduced or absent in F_0 prepared from pool II as described (Joshi, *et al.*, 1982), where absolute dependence upon added F_1 for reconstitution of P_i—ATP exchange was

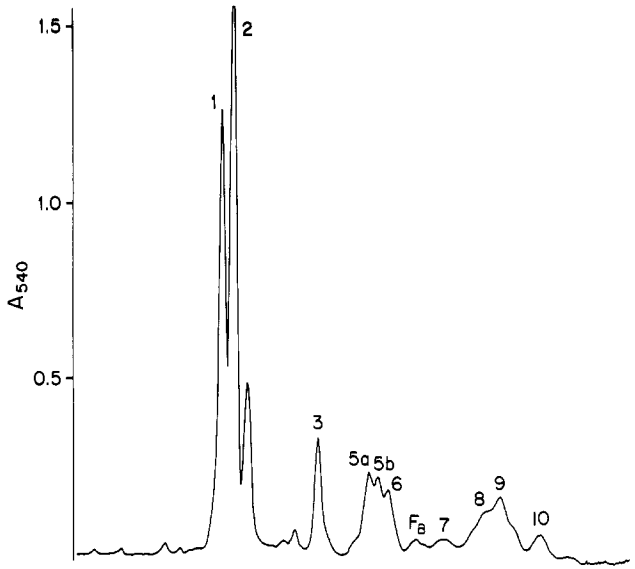

Fig. 2. SDS-gel electrophoresis of gradient fraction 22. Fraction 22 (50 μg/gel) was electrophoresed as described. Staining was for 15–18 hr in 0.5% Coomassie Blue in methanol/acetic acid/H_2O (40:10:50,v:v:v).

observed. Even when using 50 μg of H^+-ATPase per gel and extensive staining (0.5% Coomassie blue, 15 hr at 20°C), the F_B appears as a minor component of the complex (Fig. 2). This is consistent with the relatively poor staining properties of F_B (Joshi *et al.*, 1979).

The proton-translocating function of both the pregradient H^+-ATPase and pool II has been confirmed using the binding of the voltage-sensitive dye, oxonol VI. The shift in oxonol signal following addition of 2 μM ATP (+ 0.2 μM ADP) to aliquots of H^+-ATPase (Fig. 3) was similar to reports using ETPH (Smith and Chance, 1979). The voltage-sensitive binding of oxonol was reversed by addition of oligomycin (0.5 μg), or FCCP (2 μM) or valinomycin plus K^+ and completely abolished by the prior addition of oligomycin (data not shown) or FCCP.

Discussion

While the mechanism underlying the resolution by lysolecithin extraction is uncertain, available evidence suggests that the binding affinity of lysolecithin for some integral membrane proteins is higher than those of other phospholipids (Berger *et al.*, 1971). Consequently lysolecithin may displace

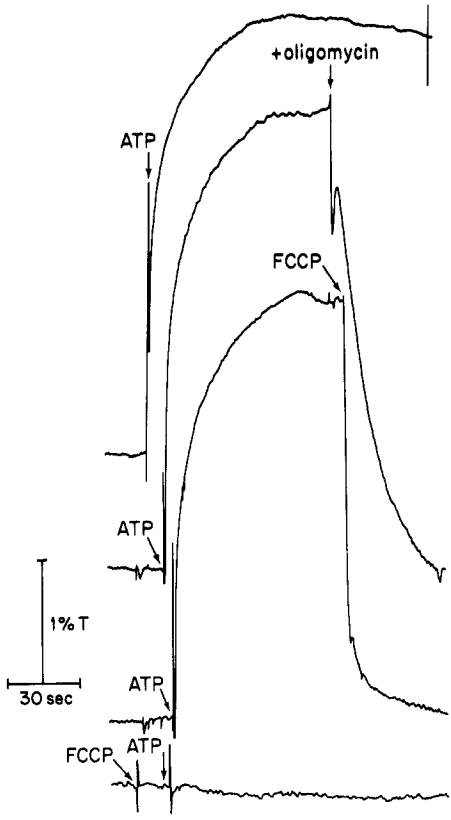

Fig. 3. Energy-Dependent binding of Oxonol VI. H^+-ATPase was suspended at 200 μg/3.0 ml of 40 mM tris-acetate (pH 7.4) containing 1 mM MgCl and 250 mM sucrose. After addition of 5 μl oxonol IV (1.5 mM in 95% ethanol), the reaction was initiated by addition of 2 μmol ATP (with 0.2 μmol ADP, pH 7.4). Other details as in the text. Additions: 2 μl oligomycin (100 μg/ml), 2 μl FCCP (100 μg/ml).

boundary lipids around integral proteins (Trauble and Overath, 1973; Weltzien, 1979) and render separation of different complexes easier. In contrast to other detergents, the lipid–protein interactions in the ATPase solubilized by lysolecithin are similar to the situation in the native membrane. The importance of such lipid–protein interactions to catalytic activity of the complex is indicated by studies relating lipid composition of the complex to P_i–ATP exchange activity (Kagawa *et al.*, 1972) and ATP hydrolysis (Parenti-Castelli *et al.*, 1979). In contrast, use of other detergents and

procedures which displace phospholipid introduces a need for reconstitution with exogenous phospholipid (e.g., Serrano *et al.*, 1976: Stiggal *et al.*, 1976; Berden and Voorn-Brouwer, 1978). This procedure could produce some scrambling of the subunits and alteration in polarity which in turn would lead to decreased P_i–ATP exchange activity.

One other reason for the high activity may be the retention of coupling factor B which has been shown to be required for P_i–ATP exchange activity (Joshi *et al.*, 1979). The lysolecithin-extracted complex contains F_1 and F_B in a 1:1 stoichiometry (Hughes *et al.*, 1979). While the stoichiometry of these two factors has not been reported for the other preparations of the H^+-ATPase, cholate treatment has been found to release F_B at least partially from membranes. The OSATPase made by the cholate procedure showed a doubling of the P_i–ATP exchange activity on addition of Factor B (Sanadi *et al.*, 1977). Consequently, the low exchange and high ATPase activity in cholate-extracted H^+–ATPase is consistent with loss of F_B during isolation.

Acknowledgments

This work was supported by grants from the National Institute of Health (GM 13641, GM 26764, and GM 26420). The authors acknowledge the expert technical assistance of Ms. Radha Narayana and Mr. Vincent Sy, and thank Ms. Angela Colombo for assistance in the preparation of this manuscript.

The anonymous reviews of this paper were arranged and its acceptability for publication determined by Dr. Peter Pedersen.

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