PREPARATION OF OLIGOMYCIN-SENSITIVE ATPase ENRICHED SUBMITOCHONDRIAL FRACTION FROM BEEF HEART MITOCHONDRIA

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Received October 30th, 1978

A simple method for purification of oligomycin-sensitive ATPase from beef heart mitochondria is described. The isolation procedure is based on short term solubilization of mitochondrial membrane in deoxycholate and 1M-KCl followed by sequentional precipitation of hydrophobic proteins and isopycnic centrifugation of crude particulate enzyme on sucrose density gradient. The oligomycin-sensitive ATPase preparation has a specific activity 15—20 μmol P/min/mg protein and contains 5% of the total mitochondrial protein which can be separated by SDS-polyacrylamide gel electrophoresis into 13 protein components of relative molecular weight from 6000—65000 daltons, respectively.

The oligomycin-sensitive ATPase (OS-ATPase) plays a fundamental role in oxidative phosphorylation and other energy dependent reactions[1-3]. It has been demonstrated that the ATPase complex is composed of three functionally distinct entities: the oligomycin-insensitive F_1-ATPase, the "OSCP protein" and the membrane sector[1,2]. Problems of isolating OS-ATPase arise from the fact that its membrane sector is strongly bound in the hydrophobic interior of the inner mitochondrial membrane and drastic experimental conditions have to be used for solubilization. High ionic strength and the presence of detergents required for solubilization results in the inactivation of OS-ATPase and are responsible, above all, for a very low yield of the isolated enzyme.

The optimum isolation procedure should resolve the above mentioned methodological dilemma, preserve the functional integrity of the OS-ATPase complex and solubilize the enzyme without inactivation. This demand was successfully met in the case of yeast and thermophilic bacteria ATPase only, when high purity and specific activities as high as 30—40 U/mg were reached[4-6]. On the other hand, specific activities of the most commonly used beef heart OS-ATPase preparations[7,8] do not exceed 8 U/mg protein and most activity present in the starting material is lost during isolation. The addition of exogenous phospholipids is necessary for the recovery of ATPase activity and ATP-P exchange reaction in the two most recent[9,10] preparations of the enzyme from the heart muscle.

The aim of this communication was to describe a rapid and simple method for the isolation of particulate OS-ATPase preparation from beef heart mitochondria. The

method offers a highly active enzyme preparation which does not require reactivation by exogenous phospholipids.

EXPERIMENTAL

Beef heart mitochondria were prepared according to Smith[11], suspended in 0·25M sucrose with 10 mM-Tris-HCl and 1 mM-EDTA (pH 7·4) at protein concentration 25—35 mg/ml, and stored at —20°C. ATPase activity was determined as a release of inorganic phosphate[12] during a 3 min incubation period at 30°C in 2 ml of medium containing 0·05M-KCl, 0·02M-Tris-sulfate, 3 mM-MgCl and 5 mM-ATP (pH 8·2). Specific activity was expressed as U/mg and 1 unit represents 1 μmol of inorganic phosphate released per 1 min. Protein was determined by the method of Lowry and coworkers[13]. Analyses of cytochromes were performed as described by Williams[14]. Polyacrylamide gel electrophoresis was performed according to Weber and Osborn[15]. Total phospholipids were extracted from OS-ATPase preparations according to Folch and coworkers[16] and phospholipid composition was tested by thin-layer chromatography on Silikagel Merck G. Inorganic phosphorus was determined according to Fiske and Subbarow[17].

RESULTS

Solubilization of Oligomycin-Sensitive ATPase

OS-ATPase can be solubilized when mitochondria are treated with deoxycholate (0·3 mg per 1 mg protein) and 1M-KCl. After high speed centrifugation 50% of mitochondrial proteins including OS-ATPase remain in the supernatant[7,10]. This commonly used arrangement, however, has a disadvantage. The treatment of the solubilized enzyme by deoxycholate during the period of high speed centrifugation inactivates the enzyme complex and more than 50% of the original enzyme activity is lost[7,8].

The inactivation of ATPase during solubilization can be avoided if the concentration of deoxycholate is decreased by dilution before high speed centrifugation (Table I). In this case a higher concentration of deoxycholate can be used for a short period of time which results in a complete enzyme solubilization (Table I) and 156% of original enzyme activity is found in the soluble fraction.

Based on these results the following solubilization procedure was used. The suspension of frozen-thawed mitochondria (25 mg/ml) was diluted with an equal volume of water and freshly prepared 10% deoxycholate was added to a final concentration 0·9% (0·8 mg deoxycholate per mg protein). Then solid potassium chloride was added to 1M concentration. The solution was stirred for 5 min at 0°C, diluted with 4 volumes of 0·25M sucrose with 10 mM-Tris-HCl (pH 7·4) and 1 mM-EDTA (sucrose medium), and centrifuged (0°C, 60 min, 100 000*g*). A red-green pellet (P_1) was discarded and the supernatant (S_1) was used for further purification. A five-fold dilution of the deoxycholate-solubilized mitochondria decreases deoxycholate and potassium chloride concentrations to the level which is without any inhibitory effect on the ATPase activity for at least 2 h.

The specific activity of the enzyme in the soluble fraction increased to 2—3 U/mg protein (Table II). Total enzyme activity increased to 156% which can be explained by the removal of an endogenous ATPase inhibitor[19,20].

Precipitation of Particulate Oligomycin-Sensitive ATPase

Further dilution of the supernatant containing solubilized OS-ATPase (S_1) with 2 volumes of cold sucrose medium decreases deoxycholate and potassium chloride concentrations to 0·06% and 0·07M respectively. A slight turbidity appears. After 90 min of centrifugation at 100000*g* the particulate OS-ATPase was obtained in the pellet (P_2). In this fraction as much as 230% of the original enzyme activity was recovered and the specific activity of crude OS-ATPase preparation increased up to 8–10 U/mg (Table II). This enzyme activity corresponds to values described

TABLE I

Solubilization of OS-ATPase from Beef Heart Mitochondria

Mitochondria were added to the sucrose-medium containing 0·9% deoxycholate and 1M-KCl. After 5 min at 0°C the suspension was diluted 5times and centrifuged (60 min, 100000*g*, 0°C). The results are expressed as per cent of the total protein content and enzyme activity present in the starting mitochondria.

Deoxycholate mg/mg protein	OS-ATPase, %		Protein, %	
	particulate	soluble	particulate	soluble
0·2	57	38	71	26
0·3	52	81	59	37
0·4	47	98	57	41
0·5	17	134	52	44
0·8	20	156	49	45

TABLE II

Purification of OS-ATPase from Beef Heart Mitochondria

The incubation mixture (2 ml) contained 0·2—0·5 mg of mitochondria or solubilized proteins, 0·08 mg of crude or 0·01—0·2 mg of purified OS-ATPase.

Fraction	Protein %	Total activity %	Specific activity U/mg
Mitochondria	100	100	0·7
Solubilized proteins (S_1)	45	156	2·43
Crude OS-ATPase (P_2)	18	234	9·1
Purification by isopycnic centrifugation	5	110	15·4

earlier for purified OS-ATPase preparations[7,8]; however, the SDS-gel electrophoresis revealed several contaminating polypeptides of an approximate molecular weight of 73000, 65000, 49000, 47000 and 30000 daltons. Furthermore, residual cytochromes $a + a_3$ (0·42 nmol/mg) and cytochromes b and $c + c_1$ (1·60 and 0·31 nmol/mg, respectively) were detected. These values are significantly higher than those of other OS-ATPase preparations[7-10]. Therefore the crude ATPase preparation was further purified by isopycnic centrifugation.

Purification of Particulate Oligomycin-Sensitive ATPase

Particulate OS-ATPase was suspended in sucrose medium to a protein concentration of 5 mg/ml and 10% deoxycholate was added to 0·3% (0·6 mg per mg protein). The suspension (1 ml) was layered on the top of continuous linear sucrose density gradient (1·05 – 1·2 g/cm^3, total volume 9 ml) containing 4 mM-EDTA. After centrifugation (90 min, 80000*g*, 0°C, MSE Superspeed – 50, swing-out rotor 3 . 10 ml) the sharp band in the middle of the tube appeated (1·13M1·15 g/cm^3). The fraction containing OS-ATPase was diluted with a sucrose medium and washed twice with the same medium by centrifugation (30 min, 1000*g*). The resulting sediment was suspended in a sucrose medium (1 mg protein/ml) and stored at −20°C. The isopycnic centrifugation increased the specific activity of OS-ATPase up to 15 U/mg (Table II) and and 110% of the original enzyme activity was recovered.

Properties of Particulate Oligomycin-Sensitive ATPase

The activity of OS-ATPase isolated by the described procedure can be further increased up to 18·3 U/mg when the measurement is carried out at pH 9·0 (Table III). The specific activities varied in the range 16 to 23 U/mg in 6 independent experiments. While the enzyme activity assayed at pH 7·4 and 8·2 can be increased by adding bicarbonate no activating effect of this ion was found at pH 9·0 (Table III). The purified OS-ATPase can be stored for at least one week at −20°C without any loss of enzyme activity; storage at 0°C for 24 h results in 30% inactivation.

The OS-ATPase is inhibited by oligomycin as well as dicyclohexylcarbodiimide. The concentrations needed for 50% inhibition (at pH 7·4) were 0·2 μg of oligomycin and 0·6 μg of dicyclohexylcarbodiimide per 1 mg protein which is in agreement with the earlier observation[7,21]. The purified enzyme contains 30 – 40 μg of lipid phosphorus per 1 mg protein. This amount is sufficient for the maintenance of maximum enzyme activity and the addition of exogeneous phospholipids is not required. The phospholipid composition of purified OS-ATPase is similar to that found in the original heart mitochondria: 43% phosphatidylcholine, 37% phosphatidylethanolamine, 15% cardiolipine and 5% minority phospholipids. The purified enzyme has a higher content of cytochrome b (0·8 nmol/mg) than other OS-ATPase prepara-

tions[7,10] and also cytochromes c + c_1 can be detected (0·1 nmol/mg) whereas cytochromes a + a_3 were completely removed.

A subunit composition of OS-ATPase evaluated by polyacrylamide gel electrophoresis[15] indicates the presence of 13 distinct protein bands with apparent molecular weights 65000 (1), 55000 (2), 52000 (3), 49000 (4), 47000 (5), 32000 (6), 30000 (7), 23000 (8), 21000 (9), 19000 (10), 13000 (11), 8000 (12), and 6000 (13) daltons. Subunits 2, 3 and 6 can be identified as A, B and C subunits of F_1-ATPase[1,2]. Subunits 1, 4 and 5 are evidently contaminants of the respiratory chain components, most probably[20] succinate dehydrogenase and cytochrome b. Subunits 7–13 have molecular weights similar to that described[1,2,21] for the membrane sector subunits, oligomycin sensitivity conferring protein, and for D and E subunits of F_1-ATPase. A high content of subunit 7 with a molecular weight of about 30000 daltons appears to be a characteristic feature of particulate enzyme preparations[22].

DISCUSSION

Procedures developed so far for the isolation of OS-ATPase from beef heart mitochondria result in enzyme preparations with low specific activity[7,8] or require phospholipids for recovery of the functional ATPase activity[9,10]. Therefore modifications were introduced to preserve the functional integrity of this complex lipoprotein molecule.

It was found that sequentional precipitation of proteins solubilized by deoxycholate and potassium chloride by dilution of the detergent and saline followed by density gradient centrifugation results in OS-ATPase preparation with a relatively

TABLE III

Specific Activity of Purified OS-ATPase Measured under Different Experimental Conditions
Specific activity of ATPase (U/mg protein) was measured as described in Experimental.

Conditions	Oligomycin	
	0	$2{\cdot}5 \cdot 10^{-7}$ M
pH 7·4	12·2	1·3
pH 7·4, 30 mM-$KHCO_3$	17·2	2·3
pH 8·2	15·4	3·2
pH 8·2, 30 mM-$KHCO_3$	18·0	4·2
pH 9·0	18·3	5·4
pH 9·0, 30 mM-$KHCO_3$	18·2	5·1
pH 9·0, 24 g at 0°C	12·8	0·9
pH 9·0, 7 days at —20°C	17·6	5·3

high specific activity and a good yield. As much as 110% of the original enzyme activity can be recovered in the final enzyme preparation (Table II). However, this represents only the relative evaluation of the yield of the enzyme because correction for the removal of an endogeneous ATPase inhibitor[19] is not included during the isolation procedure.

The enzyme preparation is rich in phospholipids (30–40 μg lipid phosphorus per 1 mg protein), the composition of which is almost identical with that of the original mitochondrial membrane. Therefore, no reconstitution of artificial membrane structure is necessary to measure enzyme activity and oligomycin resp. dicyclohexylcarbodiimide sensitivity as was described for other OS-ATPase preparations[9,10]. The used procedure results in the particulate form of the enzyme rich in phospholipids and preserves the functional integrity of the enzyme complex.

Further investigation, will be required to clarify the problem of additional constituent contaminants in OS-ATPase preparation (polypeptides 1, 4, 5 and 7 in Fig. 1). The main contaminating band is the polypeptide with a molecular weight of 30000 daltons. This polypeptide was identified as a translocator of adenine nucleotiees[9,23,24]. This result may be regarded as persistent contamination but on the other hand it may be indicative of a close physical relationship between the mitochondrial ATPase and adenine nucleotides carrier, as was suggested by Vignais and coworkers[25].

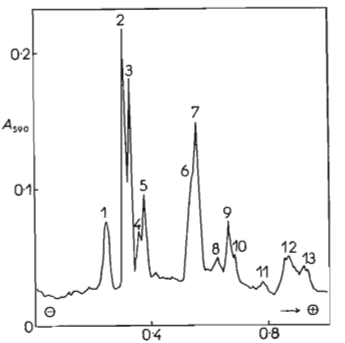

FIG. 1

SDS-Polyacrylamide Gel Electrophoresis of Purified Particulate OS-ATPase

Samples of OS-ATPase were dissociated by 3 min incubation at 100°C in the presence of 10% SDS and 2% mercaptoethanol and 25 μg of protein was applied per tube. Electrophoresis[15] in polyacrylamide gel (10%) in the presence of 1% SDS was carried out for 6 g at 3 mA per tube. Proteins were stained with Coomassie Brilliant Blue R-250 and densitometric traces were made using a Beckman Acta III spectrophotometer equipped with gel-scanner attachment.

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