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RAT TESTIS MITOCHONDRIAL ADENYLATE CYCLASE

PARTIAL PURIFICATION AND CHARACTERIZATION

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Summary

1. Adenylate cyclase (EC 4.6.1.1) from rat testis mitochondria has been solubilized by treatment with the non-ionic detergent Lubrol PX. The soluble enzyme was further purified by DEAE-cellulose chromatography.

2. The specific activity of the adenylate cyclase eluted from the DEAE-cellulose column was found to be four times higher than that of an intact mitochondrial preparation. At this step the enzyme shows a sedimentation coefficient of 4.2 S and a diffusion coefficient (D) of $3.12 \cdot 10^{-7}$ cm^2/sec.

3. Solubilization of the adenylate cyclase resulted in loss of responsiveness to gonadotrophic hormones. Addition of phosphatidylserine to the soluble preparation partially restored the activation of adenylate cyclase by human chorionic gonadotrophin.

4. The results of this study suggest that the activity of the adenylate cyclase may be dependent on the membrane-bound phospholipids and that the enzyme attached to the mitochondrial membranes has some properties which are similar to the adenylate cyclase found to be associated with other membrane systems of the cell.

Introduction

In mammalian tissues adenylate cyclase (ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1) has been reported to be attached mainly to the plasma membranes [1]. In some tissues examined this enzyme was also found to be associated with other membrane systems of the cell, such as: the T system of

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cardiac and skeletal muscle [2,3], synaptic membranes of the nerves [4], liver nuclei [5], mitochondria and particulate fractions of adrenal cortex [6], mitochondria [7,8] and submitochondrial membranes [9] isolated from testicular tissue. Purification of the membrane-bound adenylate cyclase has been hampered by its resistance to solubilization. Early works employed Triton, a non-ionic detergent, for partial solubilization of adenylate cyclase from brain, heart and skeletal muscle [2]. More recently, Levey [10] described a one-step method for solubilizing the adenylate cyclase from heart homogenates using a non-ionic detergent, Lubrol PX, an ethylene oxide condensate of dodecanol. This latter method was also applied for the solubilization of the adenylate cyclase associated with different membranous structures in aminal cells [8,11] and bacteria [12].

Recent studies have shown that Lubrol PX solubilized 75—80% of adenylate cyclase from rat testis mitochondria [8]. The present report is concerned with the purification of rat testis mitochondrial adenylate cyclase and the determination of some of its properties. Portions of these studies have appeared in abstract form [13].

Materials and Methods

Source of materials

[^{3}H]adenosine 3′:5′-cyclic monophosphate (cyclic AMP) (spec. act. 27.5 Ci/mmole) was obtained from the Radiochemical Centre (Amersham, England). Human chorionic gonadotrophin 3800 IU/mg was purchased from Ikapharm (Israel). The non-ionic detergent Lubrol PX was from I.C.I. (England) and phosphatidylserine was from Applied Science Laboratries (U.S.A.). DEAE-Cellulose, anion exchange was obtained from Sigma (U.S.A.) and Sepharose 2B from Pharmacia (Uppsala, Sweden). Other chemicals were of analytical reagent grade.

Preparation of mitochondria

Testicular tissue was obtained from mature male rats (Charles River Colony) 2—3 months old weighing 250—300 g. The rats were killed by a sharp blow in the head and the testes were removed, decapsulated and homogenized in ice-cold 0.25 M sucrose. Preparation and purification of mitochondria was performed as previously described [14].

Evaluation of mitochondrial contamination with plasma membrane

The contamination of mitochondria with plasma membrane was evaluated by centrifugation on a discontinuous sucrose density gradient [15]. The washed mitochondrial pellets (9000 × *g*) [14] and a crude membrane preparation isolated from the same tissue (3000 × *g*) [15] were separately suspended in sucrose solution to a final concentration of 50%. The samples (15 ml) were distributed into Spinco (SW 27) rotor centrifuge tubes. Over this suspension were layered 10 ml of 35.5% and 10 ml of 25.5% sucrose. The tubes were finally, filled with 19% sucrose. The gradients were centrifuged at 54 000 × *g* for 3 h. The fraction between 25.5 and 35.5% sucrose contained the plasma membrane whereas the band between 35.5 and 50% sucrose contained the

mitochondria [15]. These bands were collected with Pasteur pipettes and assayed for ($Na^+ + K^+$) activated ATPase [16]. The inorganic phosphate released from ATP during the incubation was measured using the technique described by Gomori [17].

Solubilization of mitochondrial adenylate cyclase

The washed mitochondrial preparation was suspended in 0.05 M Tris · HCl buffer, pH 7.8. This mixture was incubated with Lubrol PX at a concentration of 0.3 mg/mg mitochondrial protein [8]. Incubations were carried out at 4°C for 30 min, followed by centrifugation at 165 000 × *g* for 90 min. After this step approximately 80% of the adenylate cyclase activity was recovered in the supernatant fluid [8]. Lubrol PX used at this concentration extracted 60% of the mitochondrial protein.

Ion-exchange chromatography

DEAE-cellulose was packed into a column (1.3 cm × 15 cm) in the presence of 50 mM Tris · HCl buffer, pH 7.4. Elution of the enzyme was performed with a linear gradient of KCl (0—0.75 M) prepared in the same buffer [12]. Fractions of 2.5 ml were collected at a flow rate of 1 ml per min. The volume of the elution system was 75 ml.

Gel filtration on Sepharose 2B

Sepharose 2B was allowed to swell for 8 days in 0.1 M Tris · HCl buffer, pH 7.4. A glass column with an internal diameter of 4 cm was filled with Sepharose 2B and was allowed to run overnight with Tris · HCl buffer, pH 7.4, to pack the Sepharose. The height of the gel column was 38 cm. Fractions of 3 ml were collected at a flow rate of 1 ml per min. The volume of the elution system was 270—280 ml.

Adenylate cyclase activity

Adenylate cyclase activity was determined with a 10 min reaction period at 37°C, following incubation of the mitochondria with Mg^{++}, theophylline and ATP as substrate [8,9]. The mitochondrial pellets were first suspended in 0.05 M Tris · HCl by hand homogenization. An ATP-regenerating system was employed to maintain the concentration of ATP for maximal rates of activity. The findings that adenine nucleotides are carried across the mitochondrial membrane [18,19] and that Mg^{++} is accumulated by mitochondria [20] made it possible to add these substances to an intact mitochondrial preparation. The reaction was terminated by addition of trichloracetic acid to give a final concentration of 5%. The cyclic AMP was isolated and purified as previously reported [8,9] and measured by the technique described by Gilman [21] modified by using charcoal plus 2% bovine serum albumin [22] to separate the bound from the free nucleotide. Adenylate cyclase activity is expressed as pmol of cyclic AMP formed per mg of protein in 10 min. Protein concentration was measured by the method of Lowry et al.]23] and crystalline bovine serum albumin was used as standard.

Results

Contamination of the mitochondria with plasma membrane

Electron microscopic examination of a purified mitochondrial preparation showed no contamination with plasma membrane [14]. Density gradient centrifugation was further used to evaluate if the mitochondria, were contaminated with plasma membrane. Centrifugation of a purified mitochondrial preparation (9000 × *g*) resulted in the appearance in the gradients of a single band located between 35.5 and 50% sucrose. On the other hand, density gradient centrifugation of the membrane fractions which sedimented at 3000 × *g* resulted in the appearance of three distinct zones [15]. The first zone was located between 19 and 25.5% sucrose. The second zone migrated between 25.5 and 35.5% sucrose and appeared as two bands, one floating slightly above the interface. This fraction, as described by Henn et al. [15] contains plasma membranes. The third zone migrated between 35.5 and 50% sucrose and contains mitochondria [15]. The bands which migrated between 25.5—35.5% and 35.5—50% sucrose were collected and assayed for $(Na^+ + K^+)$-ATPase. The results illustrated in Table I indicate that the specific activity of this enzyme was 5 times higher in the fractions containing the plasma membrane (25.5—35.5% sucrose) than in those containing the mitochondria (35.5—50% sucrose).

Purification of soluble mitochondrial adenylate cyclase

Fig. 1 shows an elution profile of Lubrol-treated mitochondrial adenylate cyclase from a DEAE-cellulose column. Under these conditions the detergent was excluded from the column [12]. The first peak containing the bulk of Lubrol extractable mitochondrial proteins was devoid of adenylate cyclase activity. The adenylate cyclase activity was eluted at a KCl concentration of about 0.28 M and approximately 30% of the mitochondrial protein applied to the column were recovered in this fraction. The enzyme eluted from DEAE-cellulose column, has a maximum specific activity of about 28 pmol/10 min per mg protein approximately four times higher than that of an intact mitochondrial preparation before treatment with Lubrol PX (Table II). The sedimenta-

TABLE I

SPECIFIC ACTIVITY OF $(Na^+ + K^+)$-ATPase OF SOME FRACTIONS ISOLATED FOLLOWING CENTRIFUGATION IN A DISCONTINUOUS SUCROSE DENSITY GRADIENT OF A 3000 × *g* MEMBRANE FRAGMENTS AND A MITOCHONDRIAL FRACTION OF RAT TESTIS

Assay mixture: 0.2—2.0 mg protein in 0.05 M Tris · HCl buffer pH 7.8 (220 nmol) containing 0.3 nmol NaCl, 60 μmol KCl, 18 μmol ATP, 17.5 μmol $MgCl_2$. The total volume was 3 ml and the reaction was carried out at 37°C for 15 min. The data represent the mean of three determinations each from separate preparations. The 3000 × *g* fraction was prepared according to the procedure of Henn et al. [15]. Mitochondria (9000 × *g*) were isolated and purified as previously described [14].

Fraction	$(Na^+ + K^+)$-ATPase (nmol P_i/min per mg protein) Bands migrated on sucrose tubes	
	25.5—35.5%	35.5—50%
3000 × *g*	970(3)	210(3)
Mitochondria	—	190(3)

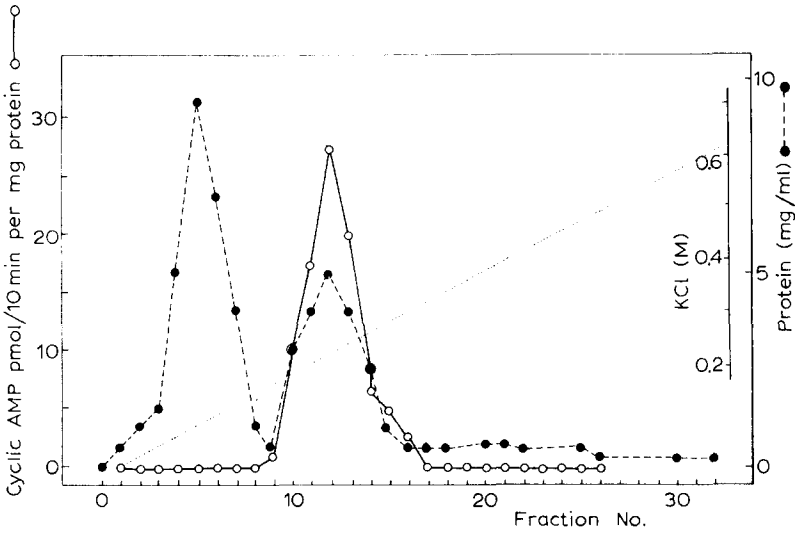

Fig. 1. DEAE-cellulose column chromatography of the solubilized form of adenylate cyclase from rat testis mitochondria. - - - - - -, protein; ———, adenylate cyclase activity. A 10 ml sample (14 mg protein/ml) of 165 000 × *g* supernatant of a Lubrol treated mitochondria was applied to a DEAE-cellulose column. The conditions of preparation of the column and the elution of the enzyme, were as described under the section on methods.

tion coefficient $S^{\circ}_{20,w}$ of adenylate cyclase eluted from DEAE-cellulose column was 4.2 S, whereas the diffusion coefficient D was found to be $3.12 \cdot 10^{-7} cm^2/s$. Both coefficients were determinated in an analytical ultracentrifuge Model E.

After the DEAE-cellulose step the adenylate cyclase was found to be very unstable. Storage at 0°C for 3—5 days led to an 80% loss of enzymic activity. The fractions obtained from the DEAE-cellulose column having the adenylate cyclase activity were pooled and dialyzed for 18 h against 50 mM Tris · HCl buffer, pH 7.4. After that, this fraction (approximately 15 ml; 45 mg protein) was loaded on a Sepharose 2B column prepared as described in the section on methods. The column was eluted with 0.1 M Tris · HCl buffer, pH 7.4. A typical elution pattern is illustrated in Fig. 2. The fractions of the peaks I and

TABLE II

ADENYLATE CYCLASE ACTIVITY IN RAT TESTIS MITOCHONDRIA AT DIFFERENT STAGES OF PURIFICATION

The preparation of different fractions was carried out as described in the Methods section. Incubation mixture: 1.5 mg protein in 0.05 M Tris · HCl buffer (29.5 pmol) (pH 7.8) containing 1.2 μmol $MgSO_4$, 4 μmol theophylline, 2 μmol NaF, 0.4 μmol ATP, 2 μmol phosphoenolpyruvate and 60 μg/ml pyruvate kinase to a total volume of 0.4 ml. Results are given as mean ±S.E. of the mean followed by the number of observations in parenthesis.

Fraction	Adenylate cyclase activity (pmol cyclic AMP/10 min per mg protein)
Intact mitochondria	6.70 ± 0.10 (4)
165 000 × *g* Lubrol supernate	15.00 ± 0.14 (5)
DEAE-cellulose	27.80 ± 0.20 (4)

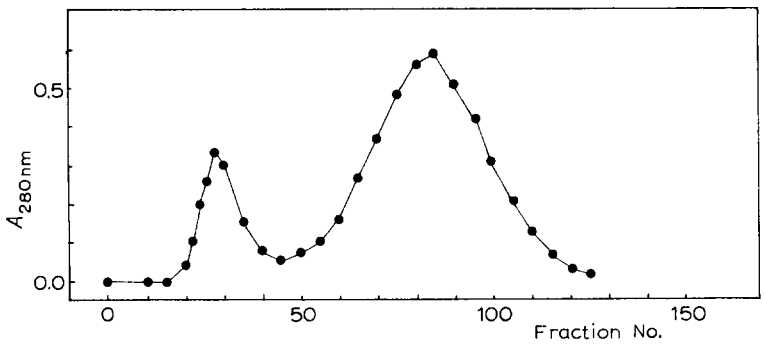

Fig. 2. Resolution of the DEAE-cellulose fraction containing adenylate cyclase activity by gel-filtration on Sepharose 2B. The eluate fractions numbered 10—15 (Fig. 1) from the DEAE-cellulose column (15 ml, 3 mg protein/ml) were added to a Sepharose 2B column. The proteins were eluted from the column by means of 0.1 Tris · HCl buffer ph 7.4. The preparation of the column and the conditions of the elution were as described under the section on methods.

II were placed in dialysis sacks and concentrated with polyvinylpyrolidone to 2—3 ml, and then were dialyzed for 5 h against 50 mM Tris · HCl buffer, pH 7.4. The fractions were then assayed for adenylate cyclase. No enzymic activity could be detected, indicating that fractionation on Sepharose resulted in loss of adenylate cyclase activity.

Effect of phosphatidylserine on the human chorionic gonadotrophin-mediated activation of the solubilized mitochondrial adenylate cyclase

Gonadotrophic hormones stimulate the adenylate cyclase attached to an intact mitochondria preparation [8]. After chromatography in DEAE-cellulose, the solubilized enzyme is not activated by these hormones. Addition of phosphatidylserine to the incubation mixture partially restored the responsiveness of the adenylate cyclase to human chorionic gonadotrophin (Table III). No

TABLE III

THE EFFECT OF PHOSPHATIDYLSERINE ON RESTORING THE RESPONSIVENESS OF A PARTIAL PURIFIED MITOCHONDRIAL ADENYLATE CYCLASE TO HUMAN CHORIONIC GONADOTROPHIN

The incubation mixture was the same as described in Table II, with the exception of NaF which was omitted from these incubations. The concentration of human chorionic gonadotrophin was 10 IU/incubation and of phosphatidylserine 20 μg/incubation (50 μg/ml). Results are given as mean ±S.E. of the mean followed by the number of observations in parenthesis. The preparation of the DEAE-cellulose column and the elution of the enzyme from the column was the same as described under Methods. Phosphatidylserine was prepared by sonication in cold 0.1 M Tris · HCl buffer pH 7.7 (1.0 mg/ml) and added to the enzyme immediately before incubation [29].

Fraction	Addition	Cyclic AMP - (pmol/10 min mg protein)	% Stimulation
Intact mitochondria	None	3.10 ± 0.05 (3)	
Intact mitochondria	Human chorionic gonadotrophin	5.80 ± 0.10 (3)	87%
DEAE-cellulose	None	13.00 ± 0.15 (4)	
DEAE-cellulose	Human chorionic gonadotrophin	13.30 ± 0.15 (4)	—
DEAE-cellulose	Human chorionic gonadotrophin + phosphatidylserine	19.30 ± 0.20 (4)	48.4%

activation was obtained when phosphatidylserine was used at concentrations lower than 20 μg/incubation and when it was added to the enzyme in the presence of Lubrol PX (165 000 × *g* supernatant). It should be noticed that NaF was not added to these incubations and the specific activity of the adenylate cyclase was 4 times higher than in an intact mitochondrial preparation.

Discussion

In the present study, contamination of mitochondria by plasma membrane was assessed by comparing the behaviour of purified mitochondria and a fraction rich in plasma membrane by density gradient centrifugation. At the end of the run, mitochondria appeared as one single band in the gradient tubes, whereas the enriched plasma membrane fraction (3000 × *g*) had three distinct zones. The $(Na^+ + K^+)$-ATPase was selected as a marker enzyme for plasma membrane and its specific activity was found to be 5 times higher in the zone containing plasma membrane than in those containing mitochondria (Table I). Thus the present results, as well as previously described morphological evidence [14], indicate that the mitochondria preparation used as the enzyme source for adenylate cyclase was freed of plasma membrane.

Several investigators have reported the use of the non-ionic detergent Lubrol PX for the solubilization of the membrane bound adenylate cyclase [8,10—12]. This detergent was previously shown to solubilize the $(Na^+ + K^+)$-ATPase from brain microsomes [24,25]. In the present investigation the solubilized adenylate cyclase from rat testis mitochondria was further purified by DEAE-cellulose column chromatography. The adenylate cyclase eluted from the DEAE-cellulose column exhibits a 4-fold increase in specific activity over that found for the same enzyme in an intact mitochondrial preparation (Table II). These results show that ion-exchange chromatography increased the specific activity of the soluble mitochondrial adenylate cyclase of testis in contrast to the inactivation of the enzyme reported for the solubilized adenylate cyclase from brain [11] and heart [26] tissues.

A general feature of the adenylate cyclase, when solubilization and purification was attempted, was the instability of this enzyme. In contrast to reports for soluble brain adenylate cyclase [11], addition of NaF and Mg^{2+} to the soluble mitochondrial adenylate cyclase did not protect the enzymic activity. The loss in adenylate cyclase activity after chromatography on Sepharose 2B observed in the present experiments, may also be attributed to the lability of this enzyme during storage and processing.

The analytical ultracentrifuge patterns of the fractions containing the adenylate cyclase activity showed one single band. Although, in the present experiments we were unable to establish the homogeneity of this fraction, the molecular weight of the material sedimented in this fraction was 114 000 as calculated from the sedimentation and diffusion coefficients [27,28]. This value was of the same order of magnitude as that reported for the purified adenylate cyclase from cat myocardium [10] and *Neurospora crassa* membranes [12].

Human chorionic gonadotrophin (10 IU/incubation) increased the adenylate cyclase activity in an intact mitochondrial preparation approximately 80%.

The stimulatory effect of this hormone and of other gonadotrophic hormones on the adenylate cyclase was abolished by solubilization [8]. The present data demonstrate that the responsiveness of the soluble mitochondrial adenylate cyclase to human chorionic gonadotrophin (10 IU/incubation) was partially restored (50%) upon addition of phosphatidylserine to the enzyme (Table III). These results are in good agreement with those obtained with the soluble myocardial adenylate cyclase, where the responsiveness of the solubilized enzyme to glucagon [29] and norepinephrine [30] was restored by addition of phosphatidylserine and phosphatidylinositol, respectively. The distribution pattern of phospholipids in the testis showed that phosphatidylserine is present in smaller amounts than phosphatidylcholine or phosphatidylinositol [31]. The capability of the latter phospholipids to restore the response of the soluble mitochondrial adenylate cyclase to human chorionic gonadotrophin was not tested. It was suggested that the adenylate cyclase may be bound to membrane phospholipids [2] and the activity of this enzyme in vivo may be dependent on the integrity of the lipid-protein complex [32]. The results of the present investigation support this suggestion and indicate that this enzyme attached to mitochondrial membranes might have some similarities with the adenylate cyclase found to be associated with other membrane systems of the cell.

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