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ISOLATION OF AN ATPase FROM THE MEMBRANE COMPLEX OF THE HEN'S EGG

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SUMMARY

The ATPase (ATP phosphohydrolase, EC 3.6.1.3) activity of the vitelline–plasma membrane complex markedly increases at the time of ovulation. A fraction, high in ATPase activity, that remains in the supernatant after centrifugation at 100000 × *g* for 60 min, can be isolated from this membrane complex. The method for solubilizing this fraction utilizes repeated extraction in buffered KCl and differential centrifugation, a procedure analogous to that used to solubilize muscle ATPase. The solubilized fraction provides three bands on gel electrophoresis and demonstrates a 30-fold increase in activity over the bound fraction. It is stable during storage at 4° for many days. The fraction can be isolated from the unfertilized egg and appears to differ from the cleavage-membrane ATPase of the fertilized egg.

INTRODUCTION

ATPase (ATP phosphohydrolase, EC 3.6.1.3) has been found in plasma membrane preparations from a variety of animal and bacterial sources[1–5]. In mammalian organisms, a membrane-bound ATPase is thought to play a role in inorganic ion transport[1,6,7], in oxidative phosphorylation[8], and in mitochondrial[9] and plasma membrane contraction[10,11]. In bacteria a membrane-bound ATPase is thought to regulate the permeability of the external membrane to extracellular solutes such as oligosaccharides[4]. The ubiquity of distribution and variety of function attributed to ATPase (whether a single multifunctional enzyme or a family of enzymes) suggest its basic importance in membrane function and the need for its detailed characterization.

The function of an enzyme in a membrane system can be clarified by study of the enzyme *in situ* and in the solubilized form. Recent work in our laboratory[12] has demonstrated a marked increase in the activity of the ATPase of the vitelline–plasma membrane complex of the hen's oocyte, associated with ovulation. Furthermore, changes in the functional characteristics of this membrane-bound enzyme, coincident to maturation, have been identified in intact membrane specimens. This paper reports the isolation and partial purification of a cation-activated ATPase from this vitelline–plasma membrane complex of the hen's egg using techniques similar to those used in the isolation of muscle ATPase[13]. The subsequent paper of this series[14] details the

characterization and comparison of this soluble ATPase fraction to that in the bound or intact membrane form.

The term "soluble" has been problematic for workers in the cell membrane field, and different definitions exist. Our "soluble" ATPase exists in the supernatant after centrifugation at a mean 100000 $\times g$ for 60 min. Recent work by RAPAPORT[15] casts some doubt on the validity of this and similar definitions, since he demonstrated the presence of membranous vesicles by subjecting such "soluble" fractions to 200000 $\times g$ centrifugation followed by electron microscopy of the pellets. Using phosphotungstic acid for negative staining, we were unable to demonstrate the presence of vesicles in the soluble fraction following 100000 $\times g$ for 60 min. Following centrifugation of 230000 $\times g$, however, 500–1000 Å membrane vesicles can be seen in the pellet using electron microscopy. It was not demonstrated, however, that the ATPase activity could be attributed exclusively to these vesicles.

MATERIALS AND METHODS

Unfertilized eggs of the chicken (Gallus domesticus) were obtained from the Poultry Extension, Department of Animal Science, University of Minnesota, and were processed within 24 h of laying. All solutions were prepared using doubly distilled, deionized water. Disodium adenosine triphosphate (Na_2ATP) was purchased from Boehringer Mannheim Corp., New York, N.Y. All reagents were analytic grade. Centrifugation was performed in either a Sorvall or Spinco Model L centrifuge, with swinging bucket rotors. Sonication was performed with a Sonifier cell disruptor (Heat Systems Co., Melville, N.Y.) with a 20000 cycles/sec probe with a maximum output of 150 W. Dialysis was performed in 1-inch-diameter cellophane tubing, pretreated by the method of ROBERTS *et al.*[16]. All routine extractions, centrifugations and dialyses were performed at 4°. Disc electrophoresis was performed by the method of ORNSTEIN[17]. Calibrated gel filtration was performed with Sephadex G-200 and BioGel, A-0.5, mesh 100–200, using a mixed buffer of 0.2 M Tris and histidine with 0.5 M KCl as eluant. The columns were packed at 4° and were allowed to stabilize for 48 h prior to use. The Sephadex and BioGel columns were calibrated using Blue Dextran 2000, cytochrome *c*, γ-globulin and apoferritin. Eluent fractions were collected on a Buchler automatic fraction collector. Vacuum dialysis for concentration of proteins was done with a collodion bag apparatus, obtained from Carl Schleicher and Scheell Co., Keene, N.H.

ATPase assay

Routine assays for ATPase activity were performed by incubating either a solubilized enzyme sample or a membrane piece at 37° for 30 min in a final reaction volume of 0.85 ml containing Tris–histidine or imidazole at varied pH's, 5 mM Na_2ATP, and 7.35 mM $MgCl_2$; for quantitative studies during the extraction procedures the reaction mixture also contains an added 90 mM NaCl and 20 mM KCl. The reaction is stopped by the addition of cold 30% (w/v) trichloroacetic acid. At this point the intact membrane samples are removed for protein assay. P_i in the supernatant is determined by the method of LOWRY AND LOPEZ[18]. Specific activity is defined as the μmoles of P_i released per h per mg of protein whereas the assayed activity is expressed as the μmoles of P_i released per h per ml.

Protein assay

Protein content of the fractions and the membrane pieces was determined by the method of LOWRY *et al.*[19], using crystalline bovine serum albumin as a standard. Intact membrane pieces are picked from the enzyme assay reaction mixture following the prescribed incubation period and are then dissolved in 1 M NaOH for the Lowry protein determination. To assure that the ATPase reaction does not interfere with the Lowry protein determination, membrane pieces of known surface area have been assayed separately for ATPase activity and protein count. No interference is detectable

Isolation procedures

Eggs are processed in batches of 24, and all extraction steps, concentrations and volumes of reagents refer to this total number of eggs or resulting fractions. Crude separation is accomplished by separating the intact egg yolks from the egg whites; the yolks are placed in a shallow dish containing 500 ml distilled water and the vitelline–plasma membrane complex (hereafter called the "membranes") is cut with a scissors. Gentle agitation of the container frees the membranes of the bulk of the yolk substance. The membranes are then transferred to another container of distilled water and swirled free of yolk. This is repeated until no yolk residues remain. Homogenization is performed with a conical, loose-fitting (200–300 μm) clearance) Teflon pestle and a glass mortar until the membranes are wispy shreds. Sonication is done with the processing vessel in an ice bath, using a power output of 100 W for 5–10 sec, energy sufficient to result in a finely dispersed, particulate suspension.

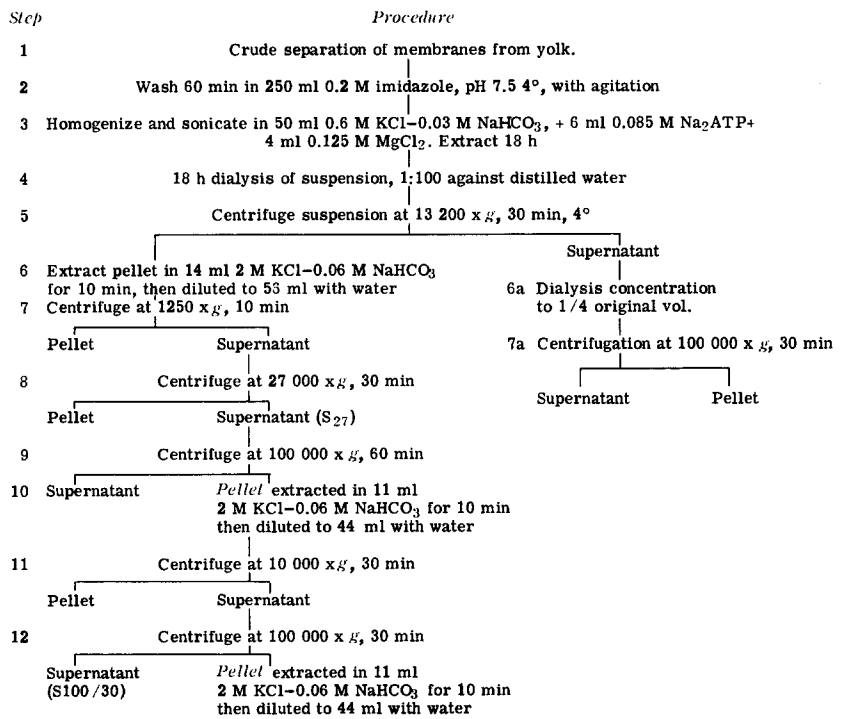

Fig. 1. Flow diagram for the isolation of ATPase.

RESULTS

Isolation steps

A step-wise flow diagram for the isolation of ATPase is presented in Fig. 1. The approach used parallels in some respects that used by MOMMAERTS[13] for the isolation of muscle ATPase.

(*1*) *Preliminary treatment.* Washing the membrane for 1 h in imidazole buffer, pH 7.5 (Step 2, Fig. 1) results in a 60% loss of inactive protein from the membrane mass with no measurable ATPase released. An attempt was made to isolate a soluble fraction by treatment with urea using a factorial treatment of washed membranes with 1 M, 3 M, 5 M, and 8 M urea for 5, 15, 30 and 60 min and 24 h, in the presence of 14 mM mercaptoethanol, at a pH of 6.35, 7.65, 7.9 and 8.40, respectively, for each of the urea concentrations. Treatment was followed by centrifugation at 48000 $\times g$ for 20 min and resulted in no significant release of ATPase activity into the supernatant solution following any exposure time. Just detectable enzyme activity in the supernatant was noted following treatment with urea: 8 mM, 30 min; 1 mM, 60 min; and 3 mM, 60 min (Table I). Specific activity could not be ascertained since the protein present was not detectable. Intact membrane activity persisted, however, with slightly decreasing magnitude following exposure to all urea concentrations. The entire membrane went into solution after 24 h in 8 M urea. ATPase was assayed in the supernatant and pellet after each time and concentration level following dialysis against distilled water (100:1) for 4 h.

(*2*) *Homogenization and sonication.* Homogenization and sonication (Step 3, Fig. 1) markedly enhances the extraction of ATPase activity from the membranes. This mechanical disruption treatment can be replaced in the isolation technique at step 3 by a prolonged extraction (148 h or longer) of the membranes in 0.5 or 0.6 M

TABLE I

ENZYME ACTIVITY AND SPECIFIC ACTIVITY (IN PARENTHESES) FOLLOWING UREA TREATMENT.

All values are the mean of two observations. Membrane pieces were soaked in the urea concentration specified for the times indicated. Supernatant aliquots and the piece (or pellet) remaining were dialyzed before assaying. The initial membranes treated were not necessarily equal in size. (See text for additional information.)

Fraction assayed	*Treatment time*	*Enzyme activity** (*specific activity***)			
		1 M urea	*3 M urea*	*5 M urea*	*8 M urea*
Supernatant	5 min	0 (0)	0 (0)	0 (0)	0 (0)
	15 min	0 (0)	0 (0)	0 (0)	0 (0)
	30 min	0 (0)	0 (0)	0 (0)	0
	60 min	0	0	0 (0)	0 (0)
Piece	60 min	(3.8)	(3.3)	(2.1)	(1.7)
Supernatant	24 h	0	0	0	0
Piece		(2.0)	(2.0)	(4.1)	
Pellet		(1.1)	(0.1)	(0.1)	(1.0)

* μmoles P_i per h per ml.
** μmoles P_i per h per mg protein

KCl at 4°. This effect is not simply due to ionic strength since prolonged extraction in 0.5 M NaCl will not release ATPase activity from the membrane pieces to the supernatant. This treatment in NaCl, however, causes an increase in the specific activity of the membrane pieces, since there is loss of inactive protein from the membranes.

Sonication of the homogenized membrane fraction (Step 3, Fig. 1) must be regulated, since variations in intensity and duration of sonication alter the yield of ATPase, especially in samples "unprotected" by added Na_2ATP (8.5 mM) and $MgCl_2$ (8.3 mM). If relatively intense sonication is used without the addition of ATP *plus* Mg^{2+} (2–5 sec bursts, 100 W output of a 20000 cycles/sec. probe) to the point of heavy frothing of the sample, there will be a 60% reduction of ATPase activity of the sonified suspension, measured at both pH 6 and pH 9, as compared to low intensity sonication (30 sec bursts, 50–75 W output, 20000 cycles/sec probe) with avoidance of frothing. Similarly the derived S27 crude sample has no ATPase activity at pH 6 when the relatively high intensity sonication is used in the absence of Na_2ATP and $MgCl_2$. The pH 6 activity is preserved when this intensity of sonication of the homogenized sample is performed in the presence of ATP and Mg^{2+}. The S27 crude fraction has ATPase activity at pH 9 following sonication regardless of the presence or absence of the ATP and Mg^{2+}. Low intensity sonication of the homogenized sample, in the absence of the ATP and Mg^{2+} reduces the pH 6 activity and enhances the pH 9 activity of the S27 crude fraction as compared to low intensity sonication performed in the presence of these species.

The homogenized-sonicated specimen with ATP and Mg^{2+} can be stored at −20° overnight with no loss of ATPase activity, but freezing in the absence of the substrate and divalent salt destroys all ATPase activity.

(3) *Post-dialysis fractions.* The pellet resulting from precipitation that occurs during dialysis has a 2 M KCl-soluble component (Step 6, Fig. 1) which immediately dissolves into a visibly clear supernatant upon adding KCl. This high concentration of KCl, however, is detrimental if prolonged. The ATPase activity in the supernatant rapidly declines after 10 min of exposure to the concentrated KCl. Upon dilution of the solution to 0.5 M KCl, there is a significant (approx. 10-fold) increase in the activity assayed in the supernatant as compared to the activity just prior to dilution. This increase in enzyme activity in response to a change in the ionic environment suggests a conformational change which confounds attempts to quantitate the degree of purification for the resulting fractions based on increases in specific activity.

When the diluted (0.5 M KCl) extract of the pellet (Step 6, Fig. 1) is treated with saturated ammonium sulfate and dialyzed, a precipitate forms. Though all the enzyme activity is retained the specific activity decreases 4-fold, revealing that this treatment poorly separates the active enzyme from inactive protein.

Extraction of the pellet by a 1% digitonin solution instead of the 2 M KCl solution results in a comparable release of ATPase activity but the digitonin interferes with the protein determination. Alternating centrifugation with KCl extraction, as shown in Fig. 1, proved to be an effective isolation procedure, though resulting in a low yield in the first cycle.

Nearly complete (98%) recovery of ATPase activity can be obtained in the pellet at Step 5 (Fig. 1) by centrifugation of 48000 × *g* for 30 min. The extraction of ATPase activity with 2 M KCl is incomplete, since this extraction (Step 6, Fig. 1)

and subsequent centrifugations (Steps 7 and 8) may be repeated up to 5 times without exhausting the yield of ATPase activity to the S27 crude fraction. This recycling may be done over a 3-month period if the pellet is stored in 0.5 or 0.6 M KCl at pH 7.4 and can improve the total yield per batch of eggs as much as 10-fold.

If the supernatant of Step 5, Fig. 1 is diluted 10-fold with distilled water or concentrated with Lyphogel (Gelman Instrument Co.) a crystalline birefringent, precipitate occurs; this precipitate is stable for several months, has an ATPase specific activity greater than 17 times that of the intact membrane, and is insoluble in 0.5 M and 2.0 M KCl, and in distilled water.

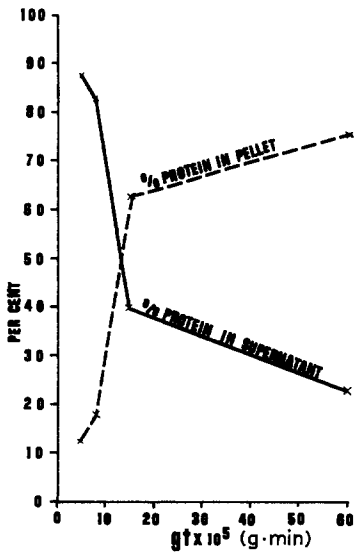

Fig. 2. Total protein (per cent) in pellet and supernatant as functions of centrifugal force.

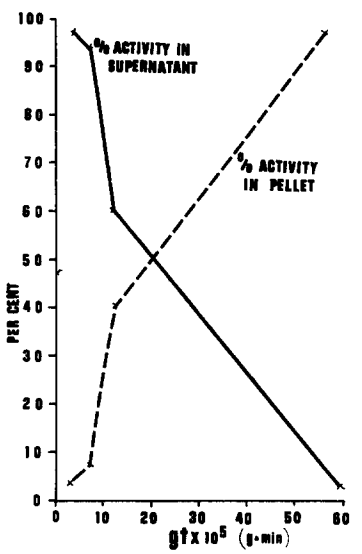

Fig. 3. Total ATPase enzyme activity (per cent) in pellet and supernatant as functions of centrifugal force.

(*4*) *The S27 crude fraction.* The crude S27 fraction was reproducible over many replications of the extraction procedure with a mean protein of 734 mg/ml in 6 different preparations. This fraction can be stored at 4° for at least a week without significant loss of specific activity, which on the average is twice that of the intact membrane complex. Gel filtration of the S27 crude fraction on Sephadex G-200 columns, results in the appearance of ATPase activity at numerous elution volumes (13 peaks). This may be due to a disassociation phenomenon, or due to variable binding of an ATPase–RNA complex to the Sephadex. The RNA content of the S27 crude fraction is 4%, by weight. Based on electron microscope observations at 200000 × *g*, however, it is likely that membrane vesicles are responsible for the numerous peaks with no relationship to molecular weight. Centrifugation data (Figs. 2 and 3) relating total protein and assayed enzyme activity show a continuous distribution of active enzyme by weight. Total protein and enzyme assays of the Sephadex filtered S27 fraction corroborates this.

(*5*) *Characteristics of the soluble fraction* (*S100/30*). The supernatant of Step 12,

Fig. 1 contains the soluble ATPase fraction and is designated S100/30. When assayed at pH 9, the soluble fraction has a mean specific activity of 650 μmoles P_i per h per mg protein, as compared with a specific activity of 40 for the intact membrane complex. This is a nominal 16-fold purification. The mean protein content is 11 μg/ml. This concentration of protein is near the lower limit of resolution of the Lowry technique in our hands and the per cent error is large. Calculation of specific activity, then can vary significantly because of the small amounts of protein and the error in the measurements at this level.

Dialysis of the S100/30 fractions results in a 40% reduction in enzyme activity in the supernatant with approximately a 50% reduction in protein (the limit of our resolution using the Lowry technique). The maximum specific activity of the dialyzed supernatant in the presence of Na^+, K^+ and Mg^{2+} is about 1220 μmoles P_i per h per mg protein whereas in the presence of only Na^+ and Mg^{2+} the specific activity is about 840. Under the conditions of this assay this is 30-fold purification with respect to the intact membrane. Concentration of the S100/30 fraction by vacuum dialysis results in loss of activity. After such concentration the S100/30 fraction demonstrates three narrow bands following disc electrophoresis whereas disc electrophoresis of the S100/30 pellet (Step 12, Fig. 1) demonstrates four bands, three of which are in the same relative locations as those in the S100/30 fraction.

Biogel filtration of the S100/30 fraction resulted in 8 elution peaks having enzyme activity in both pH 6 and 9 assays. The continued elution of active samples beyond the point at which cytochrome *c* is eluted led us to conclude that the enzyme fractions collected were not being separated on the basis of molecular weight. The addition of Na_2ATP and $MgCl_2$ to the eluant, followed by dialysis prior to assaying does not alter the gel filtration pattern. The separation achieved by gel filtration is stable, however, since a given fraction that is rerun through the column reappears at the same elution volume.

DISCUSSION

The method for solubilizing an ATPase, reported in this study, utilizes repeated extraction in buffered KCl and subsequent differential centrifugation. Of the several similarities between the methods for the isolation of muscle ATPase and this egg membrane ATPase, the most striking is the notable increase in both activity and specific activity upon dilution of the 2 M KCl-soluble protein fraction to 0.5 M KCl. Not only does dilution of the 2 M KCl extracted sample to 0.5 M KCl markedly increase its activity, but it confers a stability that allows storage for many days at 4°. Another similarity is the precipitation of an ATPase active substance with dilution of the solution. Experiments to define the nature of the semi-crystalline precipitate are in progress.

The appearance of relatively few bands following disc electrophoresis is not consistent with the number of elution peaks following BioGel column separation. Future experiments are planned to elucidate this discrepancy.

The definition of the role of this ATPase in development is highly desirable. The striking variation of this enzyme activity with development which we have previously shown[12] prompted us to pursue the isolation described in this paper. In our previous paper[12] we assayed the enzyme's function in single and multiple cell membranes

during development. As we have shown, an active ATPase can be isolated from the laid egg using procedures analogous to those for muscle ATPase. Based on these studies the hypothesis can be advanced that the ATPase (or ATPase family) functions in both transport and contractility during development. The sensitivity of early cleavage membrane ATPase to ouabain and the specific activity variation between the animal and vegetal pole has not been demonstrated, however, for the unfertilized laid egg. In the development of the hen's egg we have shown a persisting increase in the ATPase specific activity of the fertilized egg following ovulation. Considering the increasing number of cleavages and concomitant cell number increase we would anticipate higher assays of both transport and contractile ATPase in the fertilized egg system assayed by our procedures. This increase in total ATPase activity during development of the fertilized egg was demonstrated previously[12]. If fertilization does not occur, however, there is little increase in specific ATPase activity of the egg membrane during the period from 1 h after ovulation to the freshly laid egg stage. The enzyme system we have isolated as described in this paper is derived from the freshly laid unfertilized egg and, as such, its function as assayed can be expected to be altered from that assayed during the cleavage stages of the fertilized egg.

Subsequent papers will attempt to identify the function of membrane bound and isolated ATPase during development. In the next paper of this series[14] we characterize the soluble fraction S100/30 and compare the ATPase activity of this soluble fraction to that of the membrane complex-bound ATPase activity of the intact membranes.

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