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Solubilization, Purification, and Characterization of a Nucleoside Triphosphatase from Avian Myeloblastosis Virus*

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1. A nucleoside triphosphatase (EC 3.6.1.3) was solubilized from avian myeloblastosis virus after treatment with ethanol and sonication at an alkaline pH. The enzyme was purified by ammonium sulfate precipitation, chromatography on Bio-Gel A-0.5m, and sucrose density gradient centrifugation.

2. In sodium dodecyl sulfate-acrylamide gels, the protein dissociated into five subunits with molecular weights of 62,000, 60,000, 28,000, 24,000, and 18,000. Assuming a subunit structure of $\alpha_2\beta_2\gamma\ \delta\ \epsilon$ (based on stain intensities), a molecular weight of about 314,000 was calculated. In sucrose density sedimentation, an $s_{20,w}$ value of 19 S was observed, corresponding to a molecular weight of 650,000 suggesting formation of a dimer.

3. The enzyme required Ca^{2+} or Mg^{2+} for activity and hydrolyzed ATP, GTP, CTP, UTP, and ITP at a similar rate. ADP was hydrolyzed at one-tenth the rate of ATP and no significant hydrolysis of AMP or PP_i was detected.

4. The enzyme activity was resistant to a variety of inhibitors of mitochondrial and $(Na^+\text{-}K^+)$-ATPase but was sensitive to mercurials and *N,N'*-dicyclohexylcarbodiimide (DCCD). Treatment of the enzyme with phospholipase A stimulated ATPase activity and reduced its sensitivity to DCCD. The enzyme was extremely sensitive to most of the ionic and nonionic detergents such as cholate, deoxycholate, Triton X-100, Tween 80, NP-40, Lubrol, or lysolecithin, which explains the failure of previous attempts to solubilize the enzyme. Trypsin inactivated the ATPase activity which was protected in the presence of ATP or EDTA.

5. A rabbit antiserum against the enzyme inhibited the activity by 40 to 50%. The protein was, however, quantitatively precipitated and the antiserum-insensitive activity was recovered in the precipitate.

It has been known for a long time that avian myeloblastosis virus contains a very active ATPase (1-6). Available evidence indicates that the enzyme is incorporated into the virus during the cytoplasmic budding from its host cells, the myeloblasts (7, 8). Previous attempts to solubilize the enzyme were unsuccessful (9). In the present paper we describe a method for the solubilization and purification of the enzyme as well as its biochemical and immunological properties.

EXPERIMENTAL PROCEDURES

Materials

Absolute ethanol was a product of Commercial Solvents Incorporation. Bio-Gel A-0.5m (100 to 200 mesh), TEMED,[1] acrylamide, bisacrylamide, sodium dodecyl sulfate, and agarose were obtained from Bio-Rad Laboratories; sucrose, DCCD, and ammonium sulfate from Schwarz/Mann; nucleotides, *N*-ethylmaleimide, crystalline bovine serum albumin, 5,5'-dithiobis(2-nitrobenzoic acid), and ammonium persulfate from Sigma; trypsin and soybean trypsin inhibitor from Worthington Biochemical Corp.; phospholipase A (B grade) from Calbiochem and sodium mersalyl from K & K Laboratories. [^{32}P]ATP was prepared as described previously (10) and purified by passage through Dowex 1-X8-Cl (20 to 50 mesh). All other chemicals were of reagent grade. Arylazidoaminopropionyl-ATP (azido-ATP) was a gift of Dr. R. T. Guillory, University of Hawaii. Mitochondrial inhibitor was prepared as described (11).

Virus

Blood was collected from chickens infected with avian myeloblastosis virus and the plasma was generously provided by Dr. J. W. Beard and G. E. Houts authorized by the Office of Resources and Logistics of the Virus Oncology Program. The plasma (100 ml) was centrifuged for 10 min at 2,000 × *g* to remove residual cells and the supernatant was collected. The sedimented cells were washed once with 12 ml of 10 mM Tris/Cl (pH 7.2) and the combined supernatants were centrifuged for 90 min at 65,000 × *g*. The virus pellet was collected, suspended in 16 ml of Tris/Cl (pH 7.2), and recentrifuged for 30 min at 70,000 × *g*. The virus pellet was then suspended in 10 ml of 10 mM Tris/Cl (pH 7.2) at a concentration of 5 to 10 mg/ml. The virus ATPase was stable for months at 4°.

Assay of ATPase

The samples were diluted with 0.2 ml of 0.25 M sucrose, 10 mM Tris/Cl, 0.5 mM EDTA (pH 8.0), and the reaction was started by the addition of 0.5 ml of a solution containing 50 mM Tris/Cl and 5 mM

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[1] The abbreviations used are: TEMED, *N,N,N',N'*-tetramethylethylenediamine; Tris, tris(hydroxymethyl)aminomethane; EGTA, ethylene glycol bis(β-aminoethyl ether)*N,N'*-tetraacetate; DCCD, *N,N'*-dicyclohexylcarbodiimide; F_1, coupling factor 1 from bovine heart mitochondria; CF_1, coupling factor 1 from chloroplast; azido-ATP, arylazidoaminopropionyl-ATP (arylazidoaminopropionyl-ATP, (3'-*O*-{3-[*N*-(4-azido-2-nitrophenyl)amino] propionyl}adenosine 5'-triphosphate).

Ca-ATP or 7 mM Mg-ATP (pH 8.0) which were kept at 37°. After incubation for 5 min at 37°, the reaction was terminated by addition of 0.1 ml of 50% trichloroacetic acid. The amount of phosphate liberated was assayed according to Taussky and Shorr (12). For radioactive assay, [^{32}P]ATP (3.8×10^4 cpm/μmol) was used under the same conditions and the $^{32}P_i$ released was assayed after extraction (13). Occasionally, enzyme activity was measured in a coupled assay with phosphoenolpyruvate, NADH, pyruvate kinase, and lactate dehydrogenase (14). One unit of activity was defined as the amount of enzyme liberating 1 μmole of P_i per min and specific activity was expressed as units/mg of protein.

Electrophoresis of Active Enzyme

Electrophoresis of the native enzyme was carried out at 4° with cold water flowing through the outer jacket of the lower chamber using 4% acrylamide gel, Tris/Cl (pH 8.9) as gel buffer, and Tris/glycine (pH 8.5) as electrode buffer as described by Davis (15). The gels were first run for 15 min with 2 mA per tube and, following addition of the sample, were run for another 10 to 15 min. Then the current strength was increased to 2.5 to 3 mA per tube for about 2½ h. The gels were stained with 0.25% Coomassie brilliant blue in 7% acetic acid and destained in 7% acetic acid. For location of the enzyme activity, the gels were incubated at 37° for 30 min in a reaction mixture containing 50 mM Tris/Cl, 5 mM ATP, and 50 mM $CaCl_2$ (pH 8.0) and then were washed in distilled water until the background was clear. The position of the ATPase showed up as a white band.

Electrophoresis in Sodium Dodecyl Sulfate

This was performed with 10% acrylamide gel according to Weber and Osborn (16). Samples were prepared by heating at 100° for 3 min in the presence of 10 mM sodium phosphate (pH 7.0), 1% sodium dodecyl sulfate, 5% (v/v) mercaptoethanol, 0.5 M sucrose, and 0.01% bromphenol blue in a final volume of 0.1 ml. Electrophoresis was carried out at 7 mA per tube. The gels were fixed in a solution containing 7.5% trichloroacetic acid and 50% methanol, stained with 0.25% Coomassie brilliant blue, destained with 7.5% acetic acid and 5% methanol solution, and finally scanned at 550 nm in a Gilford spectrophotometer equipped with a linear scanner. Standards used were β-galactosidase (M_r = 130,000), bovine serum albumin (M_r = 68,000), ovalbumin (M_r = 43,000), yeast alcohol dehydrogenase (M_r = 37,000), chymotrypsinogen (M_r = 25,700), and myoglobin (M_r = 17,200).

Other Analytical Methods

Sedimentation coefficient and molecular weight were determined by density gradient centrifugation as described by Martin and Ames (17) with β-galactosidase as a standard. Galactosidase activity was assayed according to Wallenfels (18). Determinations of phospholipids (19), —SH groups (20), and carbohydrates (21) were performed as described in the references. Immunodiffusion was carried out according to Ouchterlony (22). An antiserum was prepared by injecting rabbits subcutaneously with 0.8 mg of the purified enzyme suspended in Freund's complete adjuvant followed by a booster injection of 0.3 mg after 3 weeks. Blood was collected 1 week after the last injection. Protein was determined according to Lowry *et al.* (23).

Purification of ATPase

Preparation A

Step 1: Ethanol Treatment – Virus was suspended at 13 mg/ml in 0.25 M sucrose, 10 mM Tris/Cl, 0.5 mM EDTA, and 1 mM ATP (pH 7.2). Forty volumes of ice cold absolute ethanol were added and the mixture was stirred for 30 min at 0°. After centrifugation for 20 min at 44,000 × *g*, the pellet was suspended at pH 10.0 in 60 ml of 25 mM Tris/Cl, 0.5 mM EDTA, and 1 mM ATP at a protein concentration of 1 to 2 mg per ml.

Step 2: Sonication – The enzyme (4-ml batches) was placed in a test tube and sonicated in a bath-type sonicator (model T-80-80-1-RS by Laboratory Supplies Corp.) at 4° for 4 min. The preparation was centrifuged at 160,000 × *g* for 60 min and the supernatant was collected. About 50% of the activity that was recovered after ethanol precipitation was now in the soluble fraction.

Step 3: Concentration by Ammonium Sulfate – Solid ammonium sulfate, 390 mg/ml, was added to the supernatant at 0° and the mixture was stirred magnetically for 10 min. After centrifugation for 20 min at 44,000 × *g*, the precipitate was dissolved in 5 ml of 25 mM Tris/Cl, 0.5 mM EDTA, and 1 mM ATP (pH 8.0).

Step 4: Chromatography on Bio-Gel A-0.5m – The preparation was placed on a Bio-Gel A-0.5m column (1.35 × 55 cm) previously equilibrated with Tris/EDTA/ATP solution (pH 8.0). Fractions of 1.2 ml were collected at a flow rate of 12 ml per h. ATPase activity appeared as a symmetrical peak shortly after the void volume.

Step 5: Sucrose Density Gradient Centrifugation – The pooled active fractions were concentrated at 4° by dialysis against 2.5 M sucrose for 2 to 3 h to ¼ the original volume and dialyzed for 3 h against two changes of 250 ml of Tris/EDTA/ATP buffer. The enzyme was subjected to density gradient centrifugation in a linear gradient of 0.8 to 2 M sucrose containing the same buffer. About 0.5 ml of the concentrated enzyme (containing not more than 5 mg of protein per ml) was layered on top of 11.5 ml of the buffered sucrose. After centrifugation for 16 h in a SW 41 rotor at 150,000 × *g*, 0.5-ml fractions were collected by piercing the bottom of the tube. One tube without enzyme was run at the same time and the fractions served as controls for protein determinations. The enzyme appeared in a somewhat asymmetrical peak. The rising phase of the curve had the highest specific activity (tube 8–14). A pool of the most active fractions had a specific activity of about 60.

Preparation B

More enzyme activity was recovered by suspending the pellet obtained after sonication in a buffer at pH 10 and repeating the sonication for 10 min. Centrifugation and precipitation with ammonium sulfate were performed as with the first extract. If turbid, the preparation was clarified by centrifugation for 10 min at 44,000 × *g*. The supernatant was then either passed through the Bio-Gel column as with the first sample or dialyzed and subjected to sucrose density gradient centrifugation. The active pooled fractions had a specific activity of 80.

RESULTS

Purification of ATPase – Avian myeloblastosis virus has a remarkably high ATPase activity of about 30 μmoles of ATP cleaved per min per mg of protein. After treatment of the virus with 98% ethanol, about 75% of the activity was lost, either because of enzyme inactivation or removal of an activating component. Attempts to restore activity by addition of the crude ethanol extract, which contained phosphatidylcholine, phosphatidylethanolamine, and sphingomyelin, were unsuccessful. Of the remaining 25% activity in the pellet, about 50% was recovered after sonication in the soluble supernatant. Re-extraction and purification of the enzyme from the pellet obtained after the first exposure to sonication yielded preparations with a specific activity of about 80 (Table I). The major loss in activity and relative small increase in specific activity after purification suggests that in the virus the enzyme is in an activated state which is lost when the enzyme is solubilized.

Stability – The enzyme obtained after sucrose density gradient centrifugation was stable at −15° for several months. Repeated freezing and thawing did not cause significant loss of activity, but dialysis against Tris/EDTA/ATP buffer for 3 h at 4° (pH 8.0) resulted in 20 to 30% loss of activity. The enzyme was not cold-labile.

Purity – Enzyme preparations with a specific activity of 60 were 80 to 85% pure as judged by polyacrylamide gel electrophoresis which revealed one major and three minor contaminating proteins. Activity stain for ATPase activity showed that the major band was in fact the enzyme but some activity was noted at the top of the gel, presumably due to aggregated enzyme. The preparations with a specific activity of 80 showed a single protein band in gel electrophoresis (Fig. 1).

Subunit Structure – For determination of the subunit structure, the enzyme was first electrophoresed in the absence of sodium dodecyl sulfate and the band corresponding to the activity stain was cut out and used for gel electrophoresis in

TABLE I

Purification of ATPase from avian myeloblastosis virus

	Total units	Protein	Specific activty	Yield of enzyme
		mg	*units/mg*	%
Preparation A				
Virus	4224	128	33	100
Virus pellet after ethanol extraction	1392	123	11.3	32.9
Supernatant after sonication	840	117	7.1	20
Ammonium sulfate precipitate	619	84.6	7.3	14.6
Bio-Gel eluate	708	24.0	30.7	16.7
Sucrose gradient fraction	390	6.75	57.7	9.2
Preparation B				
Pellet obtained after sonication	480	22.5	21.3	11.3
Supernatant	234	11.75	19.9	5.5
Ammonium sulfate precipitate	154	5.1	30.2	3.6
Sucrose gradient fraction	80	1.0	80	1.9

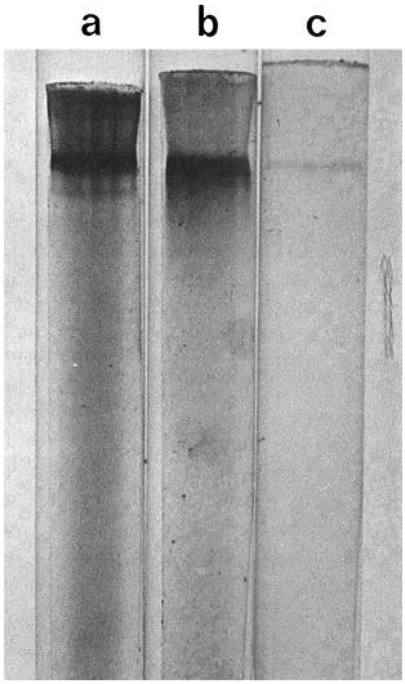

FIG. 1. Polyacrylamide gel electrophoresis of the purified enzyme. Gel electrophoresis of the native enzyme was carried out as described under "Experimental Procedures." *a*, enzyme with a specific activity of 60; *b*, enzyme with a specific activity of 80; and *c*, same as *b* but stained for Ca^{2+}-ATPase activity.

the presence of sodium dodecyl sulfate. The purified enzyme revealed five subunits with molecular weights estimated as 62,000, 60,000, 28,000, 24,000, and 18,000 (figure deleted on request of the editors). From estimation of stain intensity, a tentative formula of $\alpha_2\beta_2\gamma\ \delta\ \epsilon$ may be suggested. The resulting molecular weight corresponds to about 314,000. The $s_{20,w}$ value determined by sucrose density gradient centrifugation with β-galactosidase as a standard as shown in Fig. 2 was 19 S suggesting a dimer of molecular weight of about 650,000. Removal of sucrose and ATP by dialysis resulted in aggregation and some loss of activity.

Chemical Analysis of the Enzyme—The enzyme contained

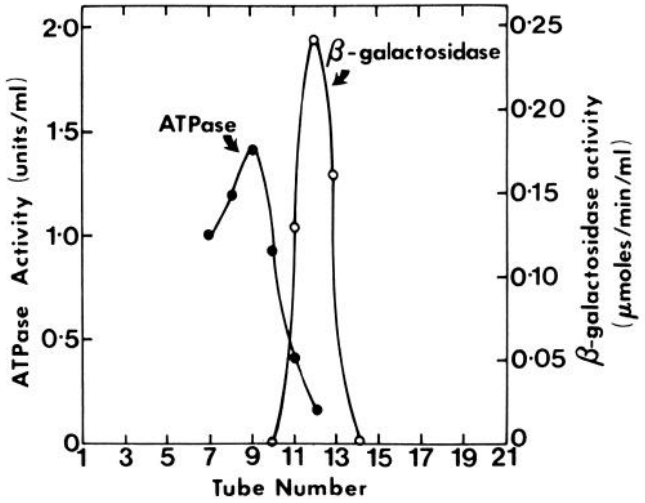

FIG. 2. Determination of $s_{20,w}$ of ATPase by sucrose density gradient centrifugation. About 200 μg each of purified ATPase and β-galactosidase were layered on the top of a linear gradient made with 5% and 20% sucrose containing Tris/EDTA/ATP buffer. After centrifugation at 150,000 × *g* for 12 h in a SW 41 rotor, 0.5 ml fractions were collected. ATPase activity was assayed by P_i determination and β-galactosidase activity was measured according to Wallenfels (18).

about 40 nmoles of phospholipid per mg of protein. Titration of —SH groups in the native and in the sodium dodecyl sulfate-treated enzyme with 5,5′-dithiobis(2-nitrobenzoic acid), revealed the presence of 13 and 20 moles of —SH per mole of enzyme, respectively.

Kinetic Properties and Substrate Specificity—In all these studies, properties of the soluble enzyme were compared with those of the virus ATPase. The hydrolysis of ATP was linear up to 5 min. Optimal activity was at pH 8.0 with either Mg^{2+} or Ca^{2+} as activator. As shown in Table II, higher rates of hydrolysis were observed with 5 mM Ca^{2+} than with 7 mM Mg^{2+} which were the optimum ion concentrations. Higher concentration inhibited particularly in the case of virus ATPase. The apparent K_m for Mg-ATP and Ca-ATP was 1 mM and 0.74 mM, respectively, for the virus ATPase and slightly lower, 0.64 and 0.58 mM, respectively, for the purified enzyme. The V_{max} values were 25 and 33 μmoles/min/mg for virus ATPase and 60 and 80 μmoles/min/mg for the purified enzyme (Fig. 3).

The enzyme hydrolyzed ATP, GTP, CTP, ITP, and UTP at similar rates (Table III). The rate of hydrolysis of ADP was about one-tenth. AMP and inorganic pyrophosphate were not significantly hydrolyzed. As shown in Table IV, the hydrolysis of [^{32}P]ATP was inhibited in the presence of GTP, CTP, UTP, and ITP, suggesting a common active site of hydrolysis.

The formation of ADP during hydrolysis of ATP was measured in the coupled assay with phosphoenolpyruvate, NADH, pyruvate kinase, and lactate dehydrogenase. The specific activity of the purified enzyme by this method was 68 compared to 60 obtained by the standard assay.

The effect of various divalent cations on the hydrolysis of ATP is shown in Table II. Fe^{2+}, Mn^{2+}, and Sn^{2+} were considerably less effective than Ca^{2+} or Mg^{2+} at 5 mM concentration. Cu^{2+} was inactive.

Effect of Inhibitors—It is known that energy transfer inhibitors like oligomycin or rutamycin, trialkyltin, and DCCD inhibit the ATPase activity of energy-transducing membranes. The effect of DCCD on the virus and purified ATPase

TABLE II

Metal ion specificity of the ATPase

Samples (1.5 to 2.5 μg) of the virus or of the purified enzyme were incubated at 37° in 0.2 ml of a solution containing 0.25 M sucrose, 10 mM Tris/Cl, and 0.5 mM EDTA (pH 8.0) followed by addition of 0.5 ml of the reaction mixture containing 5 mM metal ion, 5 mM ATP, and 50 mM Tris/Cl (pH 8.0).

Divalent cations	Specific activity	
	Virus	Purified enzyme
Ca^{2+}	33.1	56.4
Mg^{2+}	26.3	45.5
Fe^{2+}	9.2	19.2
Mn^{2+}	9.1	15.4
Sn^{2+}	6.8	15.3
Cu^{2+}	0	2.5

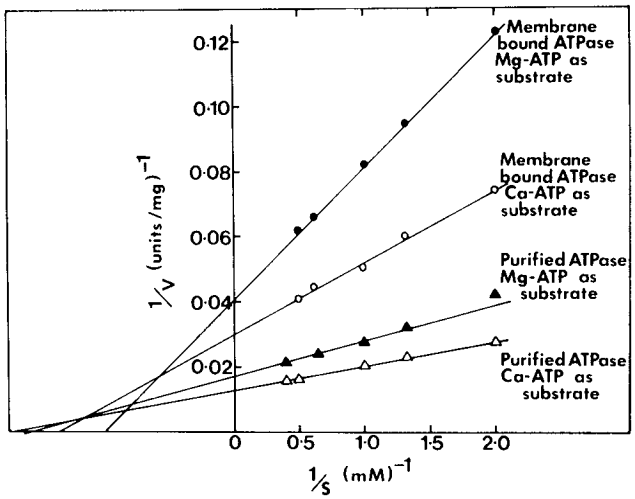

FIG. 3. Lineweaver-Burk double reciprocal plot of activities of virus ATPase and purified enzyme. ATPase activity was assayed as described earlier at varying concentrations of Mg-ATP or Ca-ATP. ●——●, membrane-bound ATPase with Mg-ATP as substrate; ○——○, membrane-bound ATPase with Ca-ATP as substrate; ▲——▲, purified ATPase with Mg-ATP as substrate; and △——△, purified enzyme with Ca-ATP as substrate.

TABLE III

Substrate specificity of the ATPase

The experimental procedure was as described in Table II except that 0.5 ml of the reaction mixture containing a 5 mM concentration of the various substrates, 5 mM Mg^{2+} or Ca^{2+} and 50 mM Tris/Cl (pH 8.0) were used.

Additions	Specific activity			
	Virus		Purified enzyme	
	Mg^{2+}	Ca^{2+}	Mg^{2+}	Ca^{2+}
ATP	26.6	34.2	45.0	64.1
GTP	25.0	29.7	45.0	56.1
CTP	27.0	34.3	45.0	73.0
UTP	30.0	27.4	48.4	64.1
ITP	30.0	29.7	38.8	59.0
ADP	4.0	5.7	6.0	6.4
AMP	0	0	0	0
Pyrophosphate	0	0	0	0

is shown in Fig. 4. DCCD at 125 μM inhibited both the Mg^{2+}- and Ca^{2+}-ATPase activity by 70 to 90%. Rutamycin had little or no effect with less than 30% inhibition observed at concentrations as high as 100 μg/ml.

As shown in Table V, the enzyme was insensitive to ouabain, sodium azide, or mitochondrial inhibitor. Quercetin at 20 μg/ml inhibited the Mg^{2+}- and Ca^{2+}-ATPase of the virus by 30 to 40% but had no effect on the purified enzyme. The enzyme was not stimulated by dinitrophenol or by Na^+ or K^+. The enzyme was sensitive to mersalyl or *p*-hydroxymercuribenzoate, but not to *N*-ethylmaleimide.

Azido-ATP inhibited the viral ATPase as shown in Table VI. About 50% inhibition was observed after irradiation of the enzyme with light in the presence of as little as 32.5 μM azido-

TABLE IV

Inhibition of ATP hydrolysis by other nucleotides

The enzyme was added to 0.2 ml of sucrose/Tris/EDTA solution (see Table II) containing 5 mM [^{32}P]ATP (1.6×10^5 cpm) in the presence or absence of 5 mM GTP, CTP, UTP, or ITP. The reaction was started by addition of 0.5 ml of a solution containing 50 mM Tris/Cl and 5 mM $CaCl_2$ (pH 8.0).

Additions	Specific activity	
	Virus	Purified enzyme
[^{32}P]ATP	30.9	86.0
+GTP	14.7	52.2
+CTP	14.7	48.4
+UTP	22.1	52.2
+ITP	13.5	59.2

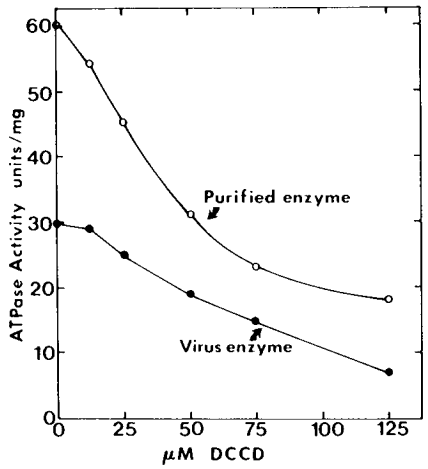

FIG. 4. Effect of DCCD on the ATPase activity of the virus and of purified enzyme. Virus or purified enzyme (2 μg) in 0.2 ml of sucrose/Tris/EDTA buffer was incubated with varying concentrations of DCCD at 37° for 5 min, followed by addition of 0.5 ml of reaction mixture to start the reaction.

TABLE V

Effect of inhibitors on ATPase activity

Samples (1.5 to 2.5 μg) of the virus or of the purified enzyme were incubated with the inhibitor at the concentration indicated in a final volume of 0.2 ml of sucrose/Tris/EDTA buffer (See Table II) at 37° for 5 min and then assayed for ATPase activity.

Additions	Specific activity	
	Virus	Purified enzyme
None	33.0	59.0
+Ouabain, 1 mM	32.0	59.0
+Sodium azide, 2 mM	32.6	59.0
+Quercetin, 20 μg/ml	22.0	59.0
+Mitochondrial inhibitor, 50 μg/ml	32.5	58.0
+*N*-Ethylmaleimide, 0.4 mM	32.0	56.0
+*p*-Hydroxymercuribenzoate, 0.4 mM	17.0	41.0
+Mersalyl, 1 mM	7.5	20.0
+Mersalyl + Dithiothreitol, 5 mM	25.0	40.0

TABLE VI

Inhibition of ATPase by azido-ATP and its protection by ATP

The virus or the purified enzyme (2 μg) was irradiated with azido-ATP in the presence or absence of ATP in a final volume of 0.2 ml of sucrose/Tris/EDTA buffer. The sample was irradiated five times for 1 min in the cold room (4°) with white light from a quartz-halogen DVY 650W lamp at a distance of 50 cm with a jar of cold water between the light and the sample.

Additions	Specific activity	
	Virus	Purified enzyme
Control	33.0	60
+Azido-ATP, 32.5 μM	16.0	58
+ATP, 10 μM + azido-ATP, 32.5 μM	17.0	
+ATP, 100 μM + azido-ATP, 32.5 μM	25.0	
+ATP, 1 mM + Azido-ATP, 32.5 μM	31.7	
+Irradiation without azido-ATP	33.0	
+Azido-ATP without irradiation	33.0	

TABLE VII

Effect of trypsin on virus ATPase and its protection by ATP or EDTA

The enzyme (2 μg) was incubated with or without 1 μg of trypsin in 0.2 ml of 0.25 M sucrose and 10 mM Tris/Cl (pH 8.0) buffer in the presence or absence of the indicated compounds at 37° for 2 h. The reaction of trypsin was terminated by addition of 1 μg of soy trypsin inhibitor.

Additions	Specific activity	
	Virus	Purified enzyme
Control	34	60
+ATP, 20 mM	33	80
+Trypsin	9	24
+ATP + trypsin	30	78
+EDTA, 1 mM	33	46
+EDTA + trypsin	30	36
+EGTA, 1 mM	32	56
+EGTA + trypsin	22	28
+Trypsin preincubated with soy trypsin inhibitor	33	62

TABLE VIII

Effect of phospholipase A on ATPase activity

The enzyme was incubated with varying concentrations of phospholipase A in the presence of 5 mM Ca^{2+} at 37° for 10 min in 0.2 ml of sucrose/Tris/EDTA buffer. It was further incubated with or without 125 μM DCCD for 5 min followed by assay of Ca^{2+}-ATPase activity.

Additions	Specific activity			
	Virus		Purified enzyme	
	−DCCD	+DCCD	−DCCD	+DCCD
Control	26.0	5.0	60	28
+Phospholipase A				
0.5 μg	26.6	8.0	60	28
1 μg	30.0	10.6	73	30
2 μg	32.0	15.0	75	31
5 μg	34.6	17.3	86	34

ATP. If the enzyme was first incubated with 1 mM ATP followed by irradiation in the presence of azido-ATP, the latter had little effect. Azido-ATP did not inhibit the purified enzyme probably because ATP remained bound to the enzyme even after dialysis as indicated by spectral analysis at 260 nm. Similar inhibition was also noted on mitochondrial ATPase (24).

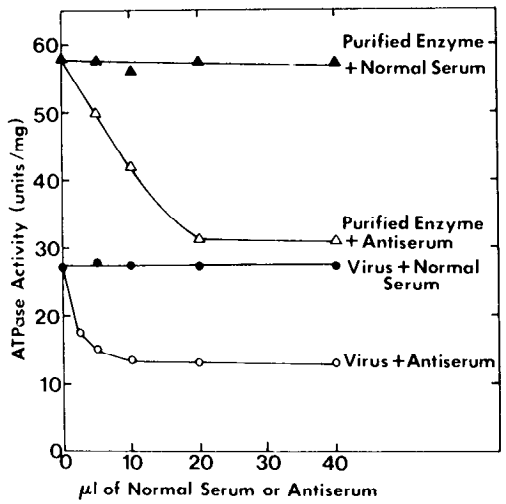

FIG. 5. Effect of antiserum on ATPase activity. The virus or the purified enzyme was incubated with varied amounts of normal or antiserum at 37° for 75 min followed by the assay of ATPase activity. After stopping the reaction with trichloroacetic acid, the precipitate was removed by centrifugation, and an aliquot of the supernatant was assayed for phosphate.

The enzyme was inactivated by every detergent that was tested, which accounts for previous failures to solubilize the activity from the virus. Cholate at 0.2% and deoxycholate at 0.025% concentration inhibited the ATPase activity of the virus by 50% or more. Triton X-100 at a concentration of 0.004% and NP-40 at 0.05% inhibited most of the activity. Lysolecithin at 12.5 μg/ml inhibited over 50%. Many other solubilizing agents tested such as Tween 80, lithium 3,5-diiodosalycilate, and sodium silicotungstate were found to be inhibitory at concentrations that do not affect many other membrane proteins. The enzyme lost 80 to 90% of the activity after exposure to 3.5 M urea for 10 min or 6 M guanidine for 30 min at 0°.

Effect of Trypsin and Phospholipase A – As shown in Table VII, the ATPase activity of the virus was lost on exposure to trypsin at 37° for 2 h. ATP or EDTA protected, while ADP or AMP were ineffective. EGTA was less effective than EDTA. In the case of purified enzyme, EDTA was less effective than ATP and appeared to be inhibitory by itself on incubation without ATP.

The ATPase activity of the virus as well as the purified enzyme was stimulated after exposure to phospholipase A from snake venom. At the same time, the enzyme became less sensitive to DCCD (Table VIII). Phospholipase A from pancreas and bee venom was also found to be effective; phospholipase C from *Bacillus cereus* had no effect (data not shown). The activating effect was observed only in presence of Ca^{2+} (2.5 mM). It was not due to liberation of lysolecithin or fatty acids since these products actually inhibited the enzyme at low concentrations. Moreover, the activation was observed in the presence of defatted bovine serum albumin.

Effect of Antiserum against Purified ATPase – Antiserum prepared against the purified ATPase inhibited the ATPase activity of both the virus and the purified enzyme (Fig. 5). The titration results show that the activity was maximally inhibited 40 to 50% when incubated at 37° for 75 min. The pellet which was formed after centrifugation of the antigen·antibody complex showed all the residual activity and there was no activity left in the supernatant (Table IX). The

TABLE IX

Demonstration of ATPase activity in the antigen-antibody precipitate

The enzyme (25 μg) was incubated with 100 μl of normal or antiserum at 37° for 90 min. It was kept overnight at 4°, diluted to 0.5 ml with 10 mM Tris/Cl and 0.15 M NaCl (pH 8.0), and centrifuged at 160,000 × *g* for 15 min. The pellet and the supernatant were then assayed for Ca^{2+}-ATPase activity.

Additions	Experiment I	Experiment II
	μmoles ATP cleaved/min	
Purified enzyme	0.39	0.29
+Normal serum	0.33	0.34
+Antiserum	0.18	0.22
Enzyme supernatant[a]	0.27	0.24
+Normal serum supernatant[a]	0.34	0.24
+Antiserum supernatant[a]	0.01	0.005
+Antiserum pellet[a]	0.18	0.21

[a] After centrifugation.

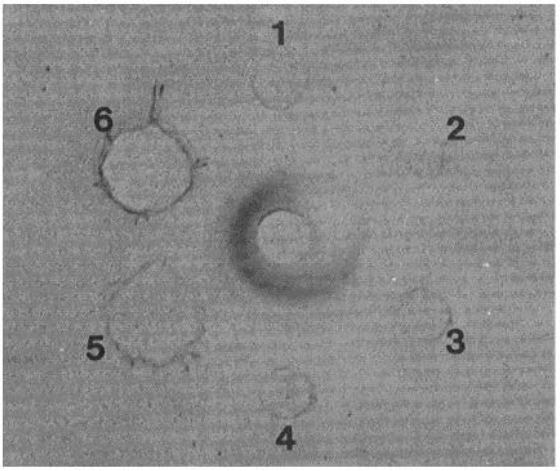

FIG. 6. Ouchterlony immunodiffusion. The antiserum of 1, 2, 5, 10, 20, and 40 μl was placed in the *outer wells* of *1* to *6*, respectively. The *central well* contained 9 μg of purified ATPase. The plates were incubated overnight in a container at room temperature in a moist atmosphere. The proteins were stained with Amido black after washing and drying.

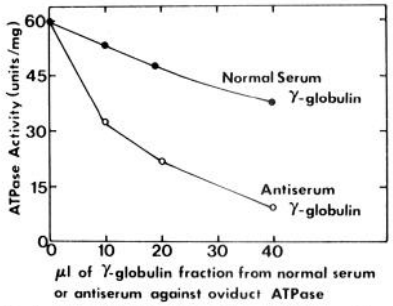

FIG. 7. Effect of γ-globulin fraction of the antiserum prepared against oviduct ATPase. The purified virus ATPase was incubated at 37° for 75 min with varied amounts of the antibody against oviduct ATPase followed by assay of ATPase activity. ●——●, ATPase activity when incubated with normal serum γ-globulin fraction; ○——○, ATPase activity when incubated with antiserum γ-globulin fraction.

antiserum was specific for the virus ATPase and did not inhibit the mitochondrial (F_1) or chloroplast (CF_1) ATPase. On Ouchterlony gel diffusion plates, the enzyme showed one strong precipitation line as well as a second thin line (Fig. 6).

The γ-globulin fraction, obtained from the antiserum against the chicken oviduct soluble ATPase (25) which was kindly provided by Dr. M. Rosenberg of the University of Minnesota, cross-reacted with the virus ATPase. Fig. 7 shows that the purified virus ATPase was inhibited 78% and a single precipitation line was seen in Ouchterlony plates.

DISCUSSION

It has been known for 25 years (1) that avian myeloblastosis virus catalyzes the hydrolysis of ATP, an activity which has been widely used for the rapid and convenient assay of virus during purification (9). What has not been fully appreciated is that this ATPase has rather remarkable properties.

The ATPase of avian myeloblastosis virus has a specific activity of 30. This is the same specific activity reported for the most active preparations of the (Na^+-K^+)-ATPase of the plasma membrane (26) or of the Ca^{2+}-ATPase of sarcoplasmic reticulum (27) or of the ATPase isolated from chloroplasts (10). It is several times greater than the specific activity of pure myosin and it is about one-third of the activity of the most active preparations of ATPase from mitochondria (28). Yet, it appears that in the virus the ATPase is only a minor component. Moreover, the concensus is that the enzyme is not coded by the virus, but incorporated from the host cell membrane during the process of budding (9). The evidence for the host cell origin of the enzyme appears quite persuasive, *e.g.* the finding that the presence of the ATPase is dependent on the cell type in which the virus is growing. The immunological cross-reaction reported in this paper between an ecto-ATPase from chick oviduct and the virus ATPase is further support for the host origin.

In view of these considerations, we have searched for a particularly potent ATPase in the plasma membrane of the host myeloblast cells. However, like plasma membranes from other cells, the specific activity of the ATPase in plasma membranes isolated from the myeloblast cells was 0.3 or less, which is only 1% of the activity of the virus. Thus, the high ATPase activity of the virus remains mysterious.

The second unusual feature of the virus ATPase is that the properties differ from all other ATPases described in the literature. It differs from the plasma membrane (Na^+-K^+)-ATPase in its resistance to ouabain and in its lack of stimulation by K^+ and Na^+. It differs from the mitochondrial ATPase by its resistance to azide and to the mitochondrial ATPase inhibitor. The pattern of susceptibility to DCCD, quercetin, and other inhibitors described in this paper is unlike that of any enzyme described in the literature.

The third remarkable property is the exquisite sensitivity of the enzyme to both ionic and nonionic detergents. Once again, we are not aware of any other ATPase which responds in this manner. This feature readily accounts for the repeated failures of previous attempts to solubilize the enzyme (*cf.* Ref. 9).

Although clearly different from the mitochondrial ATPase, the virus enzyme is more similar to it than to any enzyme from the plasma membrane. The tentative molecular weight of about 314,000 and the presence of five subunits are properties shared with the mitochondrial enzyme. However, the responses to inhibitors and detergents are strikingly different. Of particular interest is the DCCD sensitivity which is diminished after treatment with phospholipase. The allotopic sensitivity of the virus ATPase to quercetin which is lost on solubilization of the enzyme may help in the future to unravel the role of viral components in the induction of the remarkably high ATPase activity.

All these findings show that the enzyme in the virus is rather unique. It seems highly unlikely therefore that the enzyme is accidentally picked up from the host membrane and simply assimilated by the virus during the budding process. The high specific activity of the virus ATPase, the marked loss of activity after solubilization of the enzyme, and the observation that the purified enzyme has a specific activity only about 3 times greater than that of the virus all point to the notion that the virus holds the enzyme in a conformation that induces a high ATPase activity in the enzyme.

Could there be a selective advantage for the virus to sustain such a high ATPase activity? Perhaps we should reconsider the possibility that the ATPase is incorporated into the virus prior to the penetration through the plasma membrane and release from the host cell. If this is the case, the virus ATPase may, in the myeloblasts, contribute to the high aerobic glycolysis which is a characteristic feature of tumor cells. Alternatively, if the ATPase is never intracellular, an extracellular ATPase activity may modify the permeability properties of cells that respond to an external source of ATP (29).

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