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IMMUNOCHEMICAL STUDIES ON THE LARGE POLYPEPTIDE OF *ELECTROPHORUS* ELECTROPLAX ($Na^+ + K^+$)-ATPase

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Summary

Antibodies against Lubrol-solubilized *Electrophorus* electroplax ($Na^+ + K^+$)-ATPase (ATP phosphohydrolase, EC 3.6.1.3) and its 96 000-dalton polypeptide (P96) were raised in rabbits. The P96 antibody does not cross react with the ($Na^+ + K^+$)-ATPase from mammalian species and tissues, but it cross reacts with the ($Na^+ + K^+$)-ATPase from both *Electrophorus* electroplax and brain. The combination of enzyme with anti-P96 is found to inhibit phosphoryl enzyme formation to the same extent that it inhibits enzyme activity. The rate of K^+-sensitive dephosphorylation of phosphoryl enzyme appears to be unchanged. These are also found to be true with the antibody against the whole enzyme. Upon tryptic digestion of the enzyme · anti-P96 complex only the large polypeptide of the enzyme is protected. In the case of enzyme · anti-Lubrol-solubilized enzyme complex, both the large and small polypeptides are protected, whereas preimmune sera are without any protecting effect. The data indicate that the phosphoryl acceptor polypeptide and the Lubrol-solubilized electroplax ($Na^+ + K^+$)-ATPase from which the polypeptide is derived are phylogenetically distinct from those of the mammalian ($Na^+ + K^+$)-ATPases. The selective tryptic resistance of the enzyme-anti-P96 complex indicates that the two polypeptides are spatially well separated, possibly on opposite sides of the membrane.

Introduction

It is generally thought that ($Na^+ + K^+$)-ATPase (ATP phosphohydrolase, EC 3.6.1.3) isolated from broken cell homogenate is the enzymatic expression of active cation transport across intact cell membranes [1,2]. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of this transport ATPase consistently reveals two major polypeptides, regardless of the tissues from which the en-

zyme was obtained [3—5]. The large polypeptide (mol. wt. 96 000) is known as the phosphoryl acceptor protein [3—5]. The small polypeptide (mol. wt. 58 000) has been implicated as an integral part of the enzyme [6]: the antibody against the small polypeptide inhibits the enzyme activity, and forms a complex with the Lubrol-solubilized enzyme.

Although the antibodies raised against the whole enzyme invariably inhibit ($Na^+ + K^+$)-ATPase, there are reports that they do not inhibit K^+-*p*-nitrophenyl phosphatase [7], or that they inhibit Na^+ efflux only by interaction with the inner surface of the membrane [8]. McCans et al. reported that the antibody against canine heart ($Na^+ + K^+$)-ATPase can be resolved into an anticatalytic and an antidigitalis-binding fraction [9]. In this case it is apparent that different populations of antibodies were produced. This might indicate that different subunits with distinct functional roles exist. For instance, anti-large chain antibody was found only bound to the inner surface of the membrane [10].

In view of the potential applicabilities of the immunochemical approaches in structure-activity relationship and immunocytochemical localization of ($Na^+ + K^+$)-ATPase [11,12], it is important to find out whether an antibody raised against one ($Na^+ + K^+$)-ATPase will cross-react with the same enzyme from other species and tissues. Although the antibodies produced against pig kidney and canine heart enzyme were found to cross-react with the enzyme from other mammalian tissues, their cross reactivity with the enzyme from non-mammalian tissues was not known [13,14]. However, a highly specific antibody against glycoprotein of *Squalus acanthias* was reported [15].

We therefore undertook the present study to explore the function and localization of the large subunit of the ($Na^+ + K^+$)-ATPase and to determine if the ($Na^+ + K^+$)-ATPase from *Electrophorus electricus* and the large polypeptide derived from it are phylogenetically distinct from those of other species and tissues.

Materials and Methods

Materials

Lubrol WX was obtained from Savco, Fort Lauderdale, Florida; acrylamide and bisacrylamide from Bio-Rad; Freund's complete adjuvant and Noble agar from Difco. Other chemicals and reagents used were of reagent grade.

Methods

Preparation of enzyme. Membrane fragments enriched in ($Na^+ + K^+$)-ATPase activity were prepared from electroplax of *Electrophorus electricus* as previously described [16]. Lubrol-solubilized ($Na^+ + K^+$)-ATPase was prepared according to the method of Alber et al. [17]. Brain enzyme was prepared according to the method of Goldman [18].

The authors wish to acknowledge the kind gifts of various ($Na^+ + K^+$)-ATPase preparations from their colleagues: rabbit medulla from Drs. Takeguchi and Honegger; shark salt gland from Mr. George Koval; beef brain from Dr. Larry Rodichok; guinea pig kidney and brain from Dr. Alan Swann.

Immunization procedure. Lubrol-solubilized enzyme and large polypeptide (P96) were each used to immunize in rabbits. The preparation of P96 from Lu-

brol-solubilized enzyme, the immunization procedure, and the collection of sera have been described [6].

Testing inhibition of (Na^+ + K^+)-ATPase by antisera. Membranous (Na^+ + K^+)-ATPase or Lubrol-solubilized (Na^+ + K^+)-ATPase (180 μg) was preincubated with antisera (80 μl) in 25 mM Tris · HCl pH 7.4, 1 mM EGTA at 37°C for 15 min. A portion of the mixture was then assayed for the enzyme activity spectrophotometrically. Preimmune sera from each rabbit, assayed as above, served as its own control.

Testing the specificity of anti-Lubrol-solubilized enzyme by immunodiffusion. 1% agar gel in 50 mM Veronal · HCl pH 8.3 was prepared and run as described [6].

A constant amount of Anti-Lubrol-solubilized enzyme sera (20 μl) was incubated at 37°C for 15 min with 0, 10, 20 and 40 μl of each (Na^+ + K^+)-ATPase preparation to be tested in a final volume of 60 μl each. The above mixture (30 μl each) was then placed in the side wells Nos. 1, 2, 3 and 4 respectively of an immuno-diffusion plate. The central well contained 30 μl of Lubrol-solubilized electroplax (Na^+ + K^+)-ATPase.

Phosphorylation of (Na^+ + K^+)-ATPase in the presence of anti-Lubrol-solubilized enzyme and anti-P96. Membranous (Na^+ + K^+)-ATPase (about 180 μg) was preincubated at 37°C for 15 min with preimmune or anti-Lubrol-solibilized enzyme or anti-P96 sera (80 μl each) in the presence of 25 mM Tris · HCl pH 7.4, 1 mM EGTA. The enzyme · antibody complex was then centrifuged at 12 000 × *g* for 30 min and washed with 15 mM Tris · HCl pH 7.4, 3 times. The final, washed enzyme-antibody complex was labeled as described [19]. In case of testing the dephosphorylation in the presence of K^+, 25 mM KCl was included in the labeling medium.

Tryptic digestion of enzyme-antibody complex. Electroplax microsomal (Na^+ + K^+)-ATPase (180 μg each) was preincubated with preimmune sera (as control) or anti-Lubrol-solubilized enzyme or anti-P96 sera (80 μl each) at 37°C for 15 min in the presence of 25 mM Tris · HCl pH 7.4, 1 mM EGTA. The enzyme-antibody complex was centrifuged and washed three times with Buffer A (50 mM Tris · HCl pH 7.4/0.5 mM EGTA/0.5 mM dithiothreitol). Finally each was suspended in Buffer A and subjected to tryptic digestion at a ratio of 100 : 1 (protein : trypsin) at 26°C for the times specified. The digestion was terminated by soybean trypsin inhibitor.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis. 5% acrylamide gels with 0.1% sodium dodecyl sulfate were prepared as described [20]. The electrophoresis was run at 2 mA per tube for 10 min, followed at 7 mA per tube for 2.5 h. The gels were fixed, stained and destained as described before [20].

Results

Cross-reactivity of antibodies against Lubrol-solubilized enzyme and P96

Table I gives the degree of inhibition of (Na^+ + K^+)-ATPase from various species and tissues by anti-Lubrol-solibilized enzyme and anti-P96. Antibody against Lubrol-solubilized enzyme inhibits 80% of (Na^+ + K^+)-ATPase from *Electrophorus* electroplax, at the same time it inhibits only about 20% of that from *Electrophorus* brain. It does not inhibit the enzyme from cartilaginous

TABLE I

INHIBITION OF $(Na^+ + K^+)$-ATPase FROM VARIOUS SPECIES AND TISSUES BY ANTIBODIES AGAINST *ELECTROPHORUS* ELECTROPLAX LUBROL-SOLUBILIZED ENZYME AND P96

Each inhbition percentage represents an average of 2—3 independent determinations and was calculated from $1 - \frac{\text{activity in the presence of antisera}}{\text{activity in the presence of preimmune sera}} \times 100\%$.

Origin of $(Na^+ + K^+)$-ATPase	Percent inhibition by antibody against	
	Lubrol-solubilized enzyme	P96
Electrophorus electroplax	77.2	86.6
Electrophorus brain	19.8	25.4
Guinea pig brain	* (+) 0.9	7.0
Guinea pig kidney	0.4	5.6
Beef brain	1.6	7.0
Rabbit kidney	(+) 9.8	(+) 5.6
Shark salt gland	(+) 4.6	9.1

* (+) indicates stimulation.

fish, i.e. shark salt gland, to any significant degree, nor does it inhibit the enzyme from different tissues of various mammalian species (Table I). Immunodiffusion of Lubrol-solubilized enzyme against anti-Lubrol-solubilized enzyme in the presence of various enzyme preparations indicates that only the electroplax enzyme was bound to anti-Lubrol-solibilized enzyme to an appreciable extent, because there was no diminution in band intensity in any of the other tissues and species tested (Fig. 1). A second minor band that could be detected in well Nos. 2, 3 and/or 4 of Systems (C), (D), (E) and (F) probably results from proteolytic artifact, because these enzyme preparations had previously been thawed and refrozen and this minor band was observed about 3 days after the

TABLE II

EFFECT OF ANTIBODIES AGAINST LUBROL-SOLUBILIZED ENZYME AND AGAINST P96 ON THE PHOSPHORYLATION OF $(Na^+ + K^+)$-ATPase

Each inhibition percentage represents an average of at least 3 independent determinations.

Antibody against	Percent (mean ± S.E.M.) inhibition of		
	Enzyme activity *	$E\sim{}^{32}P$ *	$E\sim{}^{32}P$ in the presence of K^+ **
Lubrol-solubilized enzyme	77.1 ± 7.6	61.5 ± 5.3	65.2 ± 0.7
P96	63.4 ± 2.2	61.8 ± 5.6	66.7 ± 4.4
—	—	—	63.7 ± 3.5 ***

* Percent inhibition was calculated as $1 - \frac{\text{amounts remaining in the presence of antisera}}{\text{amounts remaining in the presence of preimmune sera}} \times 100\%$.

** $1 - \frac{\text{amounts labeled in the presence of } K^+}{\text{amounts labeled in the presence of } Na^+} \times 100\%$ in enzyme · anti-Lubrol-solubilized enzyme, enzyme · anti-P96 or *** enzyme · preimmune sera complex.

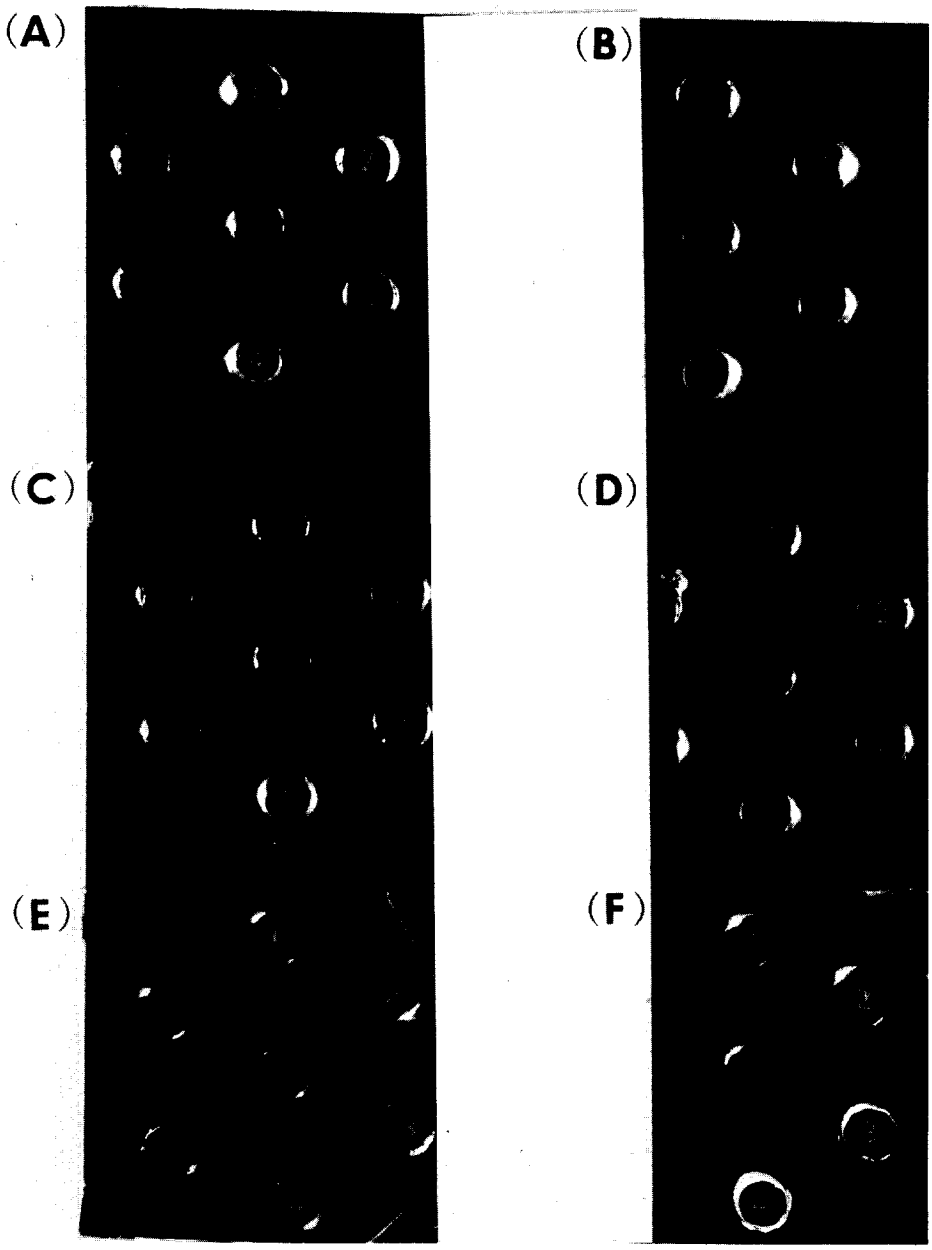

Fig. 1. Testing the specificity of anti-Lubrol-solubilized enzyme by immunodiffusion. Central well: 30 μl of Lubrol-solubilized enzyme each. Side wells: 30 μl each of the mixture containing 10 μl of anti-Lubrol-solubilized enzyme sera and varying amounts of enzyme (Well No. 1: 0 μl; No. 2: 5 μl; No. 3: 10 μl and No. 4: 20 μl). Anti-Lubrol-solubilized enzyme sera and enzyme were preincubated at 37°C for 15 min. The details were described in Methods. The $(Na^+ + K^+)$-ATPases were in microsomal fractions except (F): (A) *Electrophorus* electroplax (B) Shark salt gland (C) Guinea pig brain (D) Guinea pig kidney (E) Beef brain (F) Rabbit medulla (In Lubrol-solubilized form).

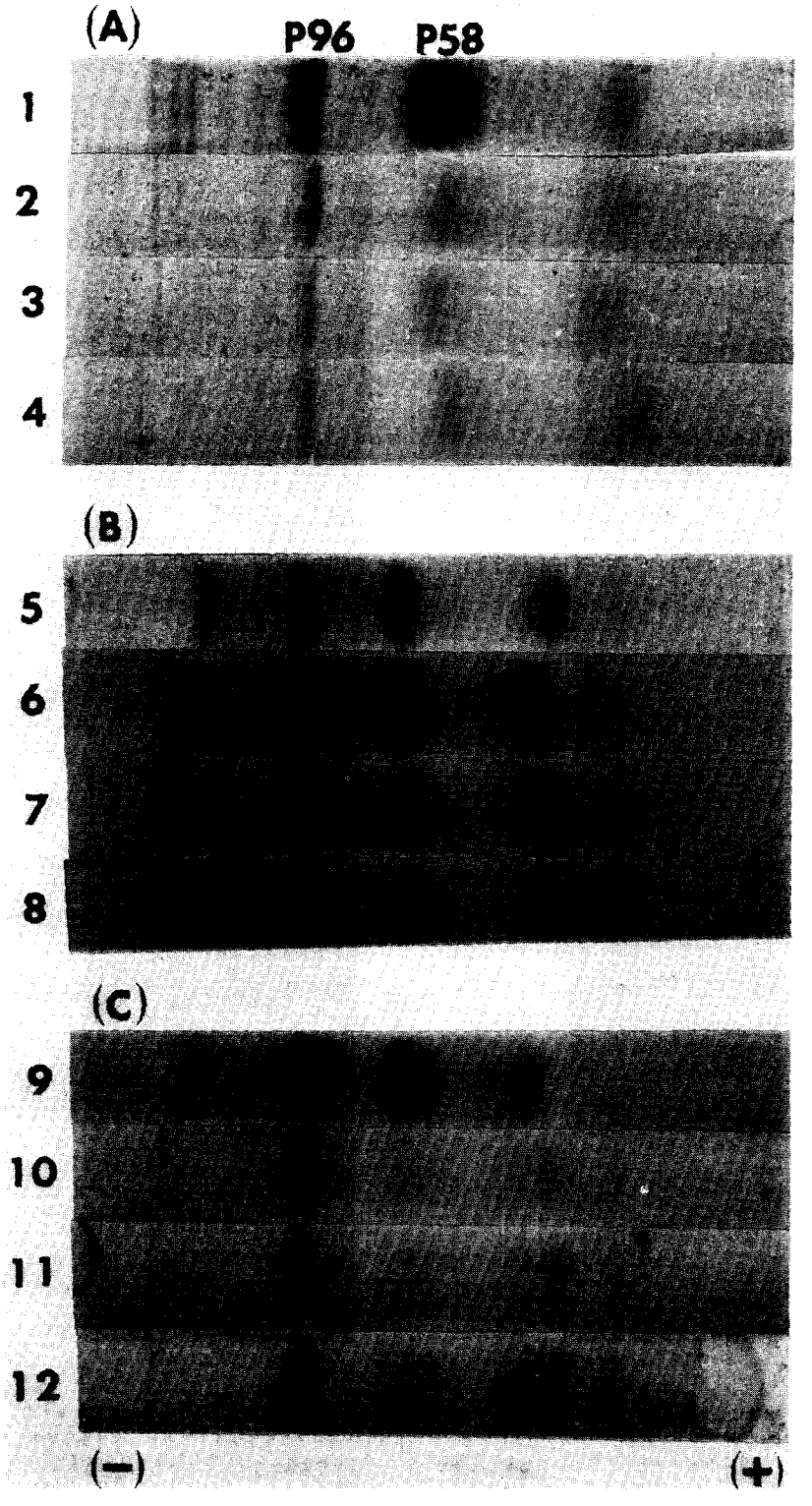

Fig. 2. Differential protective effects of anti-Lubrol-solubilized enzyme and anti-P96 against the tryptic degradation of the polypeptides of $(Na^+ + K^+)$-ATPase. The detailed tryptic digestive process was described in Methods. The direction of electrophoresis was from (−) to (+) and the amounts of protein applied on each gel was approximately 40—70 μg. (A) Enzyme · preimmune complex; (B) enzyme · anti-Lubro-solubilized enzyme complex; and (C) enzyme · anti-P96 complex. Tryptic digestion times: (1), (5) and (9): 0 time; (2), (6) and (10): 30 min; (3), (7) and (11): 1 h; (4), (8) and (12) 2 h.

major band had been developed. Likewise, antibody against the large subunit (P96) of the enzyme inhibits about 90% of electroplax and about 30% of brain ($Na^+ + K^+$)-ATPase from *Electrophorus* (Table I). It does not cross react with the enzyme from other tissues and species (Table I). The indirect immunodiffusion test was not possible in this case, because of the solubility of the complex formed from Lubrol-solubilized enzyme had anti-P96 [6].

Effect of anti-Lubrol-solubilized enzyme and anti-P96 on the phosphorylation of ($Na^+ + K^+$)-ATPase

Table II shows the degree of inhibition of ^{32}P incorporation into the enzyme by anti-Lubrol-solubilized enzyme and anti-P96. Both antibodies inhibit $E\sim{}^{32}P$ formation to about the same extent that they each inhibit ($Na^+ + K^+$)-ATPase (Table II). Yet in the presence of 25 mM K^+, the labeling is decreased by 60—70% regardless of the presence of pre-immune sera, anti-Lubrol-solubilized enzyme, or anti-P96 sera. Therefore, the rate of the K^+-sensitive dephosphorylation appears to be unchanged by either anti-Lubrol-solubilized enzyme or anti-P96 (Table II).

Anti-sera protection of ($Na^+ + K^+$)-ATPase against tryptic degradation

Membrane-bound ($Na^+ + K^+$)-ATPase is rapidly fragmented by trypsin. Sodium dodecyl sulfate polyacrylamide gel electrophoresis demonstrates that both polypeptide chains are attacked (Fig. 2A). Anti-Lubrol-solubilized enzyme sera protect both bands from trypsin (Fig. 2B). Under identical conditions only P-96 is protected by the anti-P96 sera (Fig. 2C). Pre-immune sera are without effect (Fig. 2A). This protection is not absolute, in that prolonged incubation (2 h) with trypsin will partially degrade both polypeptides, even in the form of the enzymes · antibody complex (Fig. 2, gel 8 and 12).

Discussion

In contrast to the antibodies raised against mammalian ($Na^+ + K^+$)-ATPase which cross-react with the enzyme from other mammalian tissues [13,14], the antibodies raised against Lubrol-solubilized electroplax ($Na^+ + K^+$)-ATPase are highly specific. They do not cross-react with the enzyme from mammalian tissues or with the enzyme from shark (Table I and Fig. 1). Similar high specificity is evident in the case of anti-P96 (Table I). Such high specificity suggests that *Electrophorus* electroplax ($Na^+ + K^+$)-ATPase, including its catalytic subunit, is phylogenetically distinct from the same enzyme of other species. A highly specific antiglycoprotein subunit of *Squalus acanthias* was also reported [15].

It is well documented that the phosphoryl acceptor and catalytic site reside on the larger polypeptide of the two major constituent polypeptides [3—5]. The fact that the antibody against P96 inhibits the degree of phosphorylation to about the same extent as it inhibits the enzyme activity provides additional evidence for this concept (Table II). The interaction of antisera and enzyme may have induced a conformational change that affects the sodium binding site (unpublished observations); a decreased rate of formation of phosphoryl enzyme may be the basis of the inhibited enzyme activity (Table II).

The protection of both large and small polypeptides to tryptic digestion by

anti-Lubrol-solubilized enzyme further demonstrates that antibodies against both subunits were produced in these sera (Fig. 2). This accounts for the parallel inhibition of phosphorylation and enzyme activity displayed by anti-Lubrol-solubilized enzyme and anti-P96 on the enzyme (Table II). In view of the size of γ-globulin (mol. wt. 150 000), the susceptibility of the small polypeptide to tryptic digestion in enzyme-anti-P96 complex suggests that large and small polypeptides are spatially well separated, possibly on the different sides of the membrane.

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