INHIBITION OF THE PURIFIED SODIUM-POTASSIUM ACTIVATED ADENOSINETRIPHOSPHATASE FROM THE RECTAL GLAND OF SQUALUS ACANTHIAS BY ANTIBODY AGAINST THE GLYCOPROTEIN SUBUNIT*

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Rabbits have been immunized with purified shark rectal gland NaK ATPase and its glycoprotein component. Serum, globulin from this serum, and a purified antibody fraction from rabbits immunized either with holoenzyme or with glycoprotein inhibited NaK ATPase activity. These antibodies also inhibited a purified NaK ATPase from the electric organ of the electric eel, but to a lesser extent, suggesting some cross reactivity. Ouchterlony double diffusion showed precipitation bands between shark NaK ATPase and serum or globulin containing antibodies against the holoenzyme or the glycoprotein. The inhibition of the NaK ATPase by antibody directed against the purified glycoprotein provides some direct evidence that the glycoprotein is a subunit of the NaK ATPase.

INTRODUCTION

Previous studies have indicated that NaK ATPases[1] purified from several sources contain a catalytic subunit and a glycoprotein, which, based on SDS-polyacrylamide gel electrophoresis have molecular weights of approximately 95,000 and 47,000-55,000, respectively (1). There is direct evidence that the catalytic subunit is a component of the enzyme in that the β-carboxyl of an aspartyl residue becomes phosphorylated in the presence of Na^+, Mg^{++}, and ATP and dephosphorylated if K^+ is added (1). In addition, photoaffinity labelling with the ethyl diazomalonyl derivative of cymarin indicates that the cardiac glycoside binding site is also on the large subunit (2). Evidence that the glycoprotein is an integral component of the NaK ATPase is indirect. This evidence consists of the following: 1) Sodium dodecylphosphate gel electrophoresis indicates that the catalytic subunit and the glycoprotein enrich in

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[1]Abbreviations: NaK ATPase, Na^+-K^+ activated adenosinetriphosphatase (E. C. 3. 6. 1. 3); SDS, sodium dodecyl sulfate.

parallel on purification (1). 2) With the exception of one report (3), the catalytic subunit and the glycoprotein are always present on SDS-polyacrylamide gels. 3) The amino acid composition and the N-terminal amino acid (alanine) of the glycoprotein are the same throughout vertebrate evolution (4). 4) Cross linking with dimethyl suberimidate indicates that the glycoprotein and the catalytic subunit are close together in the membrane (5).

Obviously, more direct evidence for a role of the glycoprotein in the NaK ATPase is required, because it can be argued that the glycoprotein is a membrane constituent which binds to the NaK ATPase but which is not directly involved in the activity of the enzyme. A more direct line of evidence than those listed above would be inhibition of the activity of the NaK ATPase by antibody raised against the glycoprotein. This communication reports that such an inhibition does in fact occur.

MATERIALS AND METHODS

The NaK ATPase was purified from the rectal gland of Squalus acanthias as described previously (6). Pure glycoprotein from the NaK ATPase was prepared by preparative SDS-polyacrylamide gel electrophoresis (6). Antibodies to the holoenzyme and to the glycoprotein were raised in female white New Zealand rabbits. One to three mg of holoenzyme or glycoprotein were suspended in physiological saline and mixed thoroughly with an equal volume of Freund's complete adjuvant. Initially this mixture was injected into the hind toe-pads and also subcutaneously in the neck region. Subsequent injections were made in the neck region weekly for 5-6 weeks. Blood was obtained from the jugular vein or the lateral vein of the ear one week after the last injection. Subsequently, booster injections of 1 to 2 mg of antigen were given one week prior to bleeding. Control serum was obtained from non-immunized animals or from animals prior to immunization. Complement was destroyed in all animals by heating at 56° for 30 min. The globulin fraction was purified by ammonium sulfate fractionation by the method of Cann et al. (7). In some experiments the antibodies were further purified by adsorption on the insoluble holoenzyme by the method of McCans et al. (8). If antiserum itself was used the serum was dialyzed against 50 to 100 volumes of 0.15 M imidazole buffer (pH 7.0) for 3 days at 4° with frequent changes of dialyzing medium. This removed inorganic phosphate which interfered with the NaK ATPase assays. Protein was determined by the method of Lowry et al. and NaK ATPase was assayed essentially by our previously described procedure (6).

Immunodiffusion studies were carried out on commercial immunodiffusion discs (Miles Laboratories) containing 0.9% agarose, 0.175 μ borate buffer (pH 8.5), and 0.01% merthiolate. Sephadex G-50 and agarose which were used for some double diffusion tests. They were obtained from Sigma. Ouabain was obtained from Aldrich Chemical Co. and sodium ATP from Calbiochem. All other chemicals were reagent grade.

RESULTS

Globulin purified from antiserum from rabbits immunized against purified NaK ATPase from the rectal gland of the spiny dogfish shark, Squalus acanthias, and its

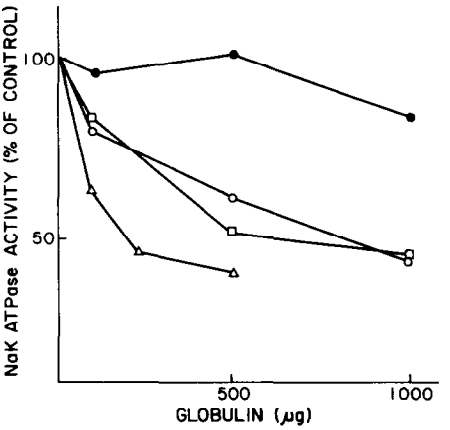

Figure 1. Inhibition of shark rectal gland NaK ATPase by antibodies. Twenty μg of purified shark NaK ATPase were preincubated 30 min at room temperature with globulin or purified antibody in assay medium containing all ingredients except ATP. The NaK ATPase assay was initiated by addition of 5 mM ATP. ●, enzyme preincubated with globulin from nonimmunized rabbit; O, enzyme preincubated with globulin from rabbit immunized with holoenzyme; □, enzyme preincubated with globulin from rabbit immunized with glycoprotein; △, enzyme preincubated with purified antibody from rabbit immunized with holoenzyme. Each point is a mean of at least three determinations.

glycoprotein subunit inhibited enzyme activity in a concentration dependent manner (Fig. 1). Antibody to holoenzyme purified by adsorption to and elution from holoenzyme (8) also inhibited holoenzyme, and, as to be expected, was more potent than the globulin fraction. Serum and globulin fractions from nonimmunized rabbits did not inhibit the NaK ATPase. The amount of globulin fraction required to inhibit the NaK ATPase 50% was, on a protein weight basis, approximately one-fifth of the amount of serum required for an equal inhibition.

Our laboratory has also purified the NaK ATPase from the electric organ of the electric eel, Electrophorus electricus (9). It was of interest to study the cross reactivity of antibodies prepared against the shark enzyme with the NaK ATPase from the electric eel. Table I summarizes the effect of antibody against the shark holoenzyme and its glycoprotein on the NaK ATPases from the shark and the eel. Control sera or control globulin had no effect on the NaK ATPases from either species. Antiholoenzyme and antiglycoprotein globulins inhibited the enzymes from both species; however, the inhibition of the eel enzyme was far less than the inhibition of the shark

TABLE I

Cross-reactivity of antibodies against shark NaK ATPase

and its glycoprotein with the NaK ATPase from the electric eel

Twenty μg of purified enzyme was assayed as reported previously. Enzyme was incubated with antiserum as described in Fig. 1. Each value is the mean of at least two independent determinations.

Experiments	Percent Inhibition of NaK ATPase Activity	
	Shark NaK ATPase	Eel NaK ATPase
Control (20 μg)	0	0
Control globulin (3 mg)	-0.2	0.5
AntiNaK ATPase globulin (1 mg)	51.0	16.9
AntiNaK ATPase globulin (3 mg)	57.4	27.7
Antiglycoprotein globulin (3 mg)	37.3	3.3
Purified AntiNaK ATPase (0.1 mg)	17.0	---
Purified AntiNaK ATPase (0.3 mg)	54.8	10.0
Control sera (0.3 ml)	-5.3	12.3
AntiNaK ATPase sera (0.3 ml)	65.4	25.0
Antiglycoprotein sera (0.3 ml)	29.0	11.8

enzyme. For example, 1 mg of globulin containing antiholoenzyme against the shark enzyme inhibited the shark NaK ATPase 51% and the eel enzyme 17%. This difference was even more marked for the antiglycoprotein antibody. Serum and globulin containing antibody against the shark glycoprotein gave only marginal inhibition of the eel NaK ATPase, suggesting little cross-reactivity with the glycoproteins from the two species.

Using equivalent units of enzyme, with the exception of the membrane fraction, 0.6 ml of antiserum against the shark NaK ATPase inhibited enzyme activity 90% at different stages of purification. The enzyme in the native membrane fraction was

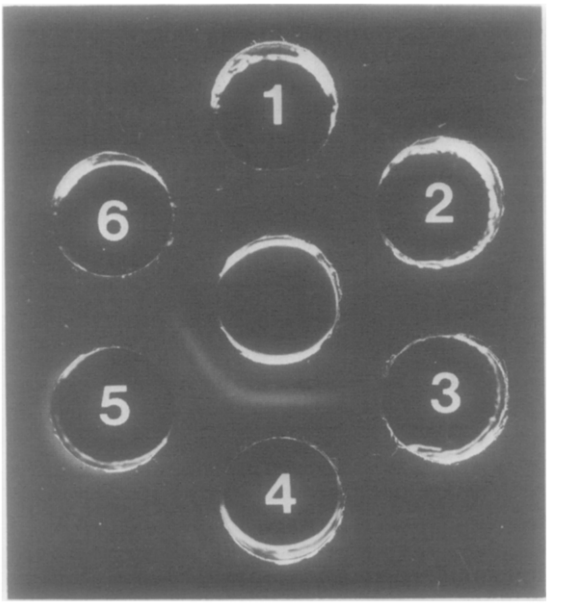

Figure 2. Ouchterlony double diffusion pattern of antibodies against shark NaK ATPase and the holoenzyme. Each well contained 30 μl. Well 1 contained 0.15 M imidazole buffer; 2, control serum; 3, control globulin (300 μg); 4, antiserum to holoenzyme; 5, globulin from antiserum to holoenzyme (300 μg); and 6, no additions. The center well contained 100 μg of purified shark NaK ATPase. Diffusion bands were visible after 24 hours at room temperature and were photographed after 48 hours.

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inhibited 70%. Ouchterlony double diffusion showed that antiserum against the rectal gland NaK ATPase or globulin purified from this antiserum gave precipitation bands with the purified NaK ATPase from the rectal gland (Fig. 2). Serum and globulin from nonimmunized animals did not give precipitation bands. Since the NaK ATPase was placed in the center well as the insoluble membranous enzyme without detergent, it can be concluded that the enzyme was able to diffuse in the gel in the insoluble form. Fig. 3 shows that antiserum prepared against the glycoprotein from the rectal gland NaK ATPase formed a precipitation band with rectal gland NaK ATPase solubilized with 2.5% Lubrol. Thus, Ouchterlony double diffusion gels are in agreement with the enzyme inhibition data reported above.

DISCUSSION

This report demonstrates that antibody raised against the glycoprotein chain isolated from the purified NaK ATPase from the rectal salt gland of the spiny dogfish

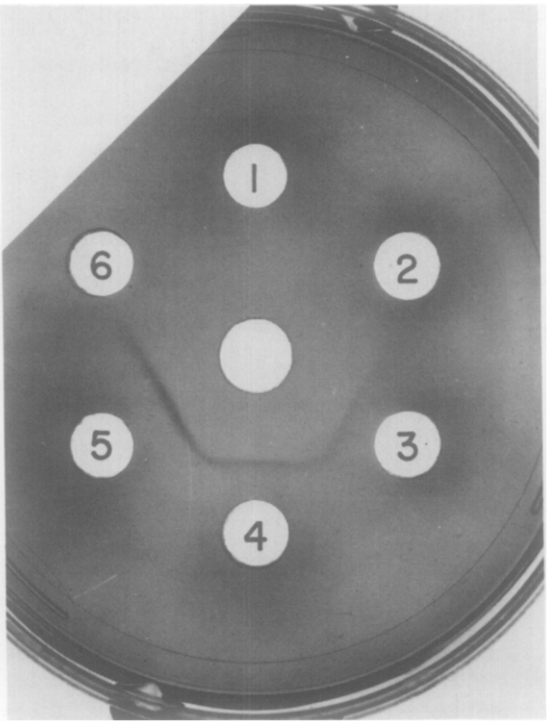

Figure 3. Ouchterlony double diffusion pattern of antibodies against the glycoprotein from the shark NaK ATPase and the Lubrol-solubilized holoenzyme. Well 1 contained 40 μl of 0.15 M imidazole buffer; 2, 500 μg of control globulin, 3, 4, and 5, 10, 20 and 45 μl of antiserum against the glycoprotein; and 6, no additions. The center well contained 100 μg of purified shark NaK ATPase solubilized in 2.5% Lubrol. The gel was photographed after diffusion for 72 hours at room temperature.

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shark, Squalus acanthias, inhibits NaK ATPase activity. Inhibition occurs with immune serum and with globulin isolated from immune serum. The potency of the antibody, on a protein basis, increases with purification. Serum and globulin fractions from nonimmunized animals do not inhibit the NaK ATPase. As reported by others (10-15), antibody against holoenzyme inhibits enzyme activity. Antibody against shark rectal gland holoenzyme shows some inhibition of NaK ATPase activity of the purified enzyme from the electric organ of the electric eel, Electrophorus electricus, indicating cross reactivity. Cross reactivity with antibody against holoenzyme has been observed with mammalian NaK ATPases (11). With the exception of the native enzyme fraction all stages of purification of the NaK ATPase are inhibited to the same extent

by antiserum. The enzyme in the native membrane fraction is inhibited less, suggesting inaccessibility of antibody to some of the antigenic sites when the enzyme is embedded in the native membrane.

There have been several indirect lines of evidence, cited in the Introduction, that the glycoprotein chain seen in the most highly purified preparations of NaK ATPase is a subunit of the enzyme. However, to date there has been no direct evidence. The salient observation of this paper, namely that antibody raised against purified glycoprotein inhibits NaK ATPase activity, is strong evidence that the glycoprotein is indeed a subunit of the enzyme. There is one reservation which must be made, however: it is possible that because of the proximity of the glycoprotein to the NaK ATPase, the binding of the antibody to the glycoprotein covers crucial sites on the catalytic subunits thus inhibiting the enzyme indirectly. This possibility can only be tested by other approaches such as enzyme reconstitution experiments.

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