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EFFECTS OF WHEAT GERM AGGLUTININ AND CONCANAVALIN A ON PARAMETERS OF HIGHLY PURIFIED SODIUM-POTASSIUM ADENOSINE TRIPHOSPHATASES FROM *SQUALUS ACANTHIAS* AND *ELECTROPHORUS ELECTRICUS*

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Summary

The effects of two lectins, wheat germ agglutinin and concanavalin A, were studied on a variety of parameters of two highly purified ($Na^+ + K^+$)-ATPases (ATP phosphohydrolase, EC 3.6.1.3), from the rectal salt gland of *Squalus acanthias* and from the electroplax of *Electrophorus electricus*. Both lectins agglutinated the rectal gland enzyme equally, but wheat germ agglutinin inhibited ($Na^+ + K^+$)-ATPase activity much more. The electroplax enzyme was only marginally agglutinated and inhibited by the lectins. Neuraminidase treatment of the rectal gland ($Na^+ + K^+$)-ATPase had no effect on wheat germ agglutinin inhibition. The inhibition of the rectal gland ($Na^+ + K^+$)-ATPase by wheat germ agglutinin could be reversed by *N,N'*-diacetylchitobiose, which has a high affinity for wheat germ agglutinin. Neither ouabain inhibition nor ouabain binding to the rectal gland enzyme was affected by wheat germ agglutinin. The *p*-nitrophenylphosphatase activity of the rectal gland enzyme was not inhibited by wheat germ agglutinin. Na^+-ATPase activity, which reflects ATP binding and phosphorylation at the substrate site was inhibited by wheat germ agglutinin and this inhibition was reversed by potassium. Evidence is cited (Pennington, J. and Hokin, L.E., in preparation) that the inhibition of the ($Na^+ + K^+$)-ATPase by wheat germ agglutinin is due to binding to the glycoprotein subunit.

Introduction

The ($Na^+ + K^+$)-ATPase (ATP phosphohydrolase, EC 3.6.1.3) contains a large chain with a molecular weight of approx. 95 000, which is the catalytic subunit

† Deceased March 1, 1976.

of the enzyme, and a small glycoprotein chain with a molecular weight of approx. 50 000, whose function is unknown. The large chain appears to exist as a dimer, and the stoichiometry of the two chains is still unsettled. Studies with the purified kidney enzyme suggest an $\alpha_2\beta_2$ structure [1]. Previous studies [2] have shown that antibodies against the glycoprotein small chain inhibit ($Na^+ + K^+$)-ATPase activity, suggesting that the small chain plays some functional role in the ($Na^+ + K^+$)-ATPase. Plant lectins specifically bind to oligosaccharides on the surfaces of biological membranes because of their high affinity for specific sugars. It was thought that this specific interaction would be a useful tool in throwing light on the role of the glycoprotein subunit in the functioning of the ($Na^+ + K^+$)-ATPase. Two useful lectins are wheat germ agglutinin and concanavalin A, which have molecular weights of approx. 35 000 (the dimer at neutral pH [3]) and 50 000, respectively. Wheat germ agglutinin is directed against *N*-acetylglucosamine residues and concanavalin A is directed against α-mannoside or β-glucoside residues in oligosaccharides. An inhibition of the ($Na^+ + K^+$)-ATPase by one or both of these lectins and a reversal of this inhibition by the appropriate sugar would support studies which showed that antibody against the glycoprotein inhibited the ($Na^+ + K^+$)-ATPase [2]. In this study we have used two highly purified ($Na^+ + K^+$)-ATPase preparations, that from the electric organ of *Electrophorus electricus* and that from the rectal salt gland of *Squalus acanthias*, enzymes which show virtually identical amino acid compositions in their respective subunits but which show different carbohydrate compositions in their glycoprotein subunits. The effects of lectins on several parameters in addition to the overall ATPase reaction have been studied, i.e. K^+-dependent *p*-nitrophenylphosphatase, ouabain inhibition and ($Na^+ + K^+$)-ATPase. These studies have shown that binding of wheat germ agglutinin to the glycoprotein inhibits overall ($Na^+ + K^+$)-ATPase activity and Na^+-ATPase activity, providing further evidence for a role of the glycoprotein in the functioning of the enzyme.

Materials and Methods

Materials. Concanavalin A and wheat germ agglutinin were obtained from Miles-Yeda, Ltd. β-1,4-di-*N*-acetylglucosamine (*N*,*N'*-diacetylchitobiose) was kindly provided by Dr. Saul Roseman, McCollum-Pratt Institute, the Johns Hopkins University Medical School.

Methods. ($Na^+ + K^+$)-ATPase was purified from the rectal gland of *S. acanthias* and from the electric organ of *E. electricus* according to previously published procedures [4—6]. ($Na^+ + K^+$)-ATPase activity was assayed either by measurement of the liberation of inorganic phosphate as described by Hokin et al. [4] or by a radioactive assay. In the latter assay, 2.5 μg ($Na^+ + K^+$)-ATPase was diluted to 1 ml in 0.5% bovine serum albumin/2 mM [γ-^{32}P]ATP/30 mM imidazole · HCl (pH 7.0)/10 mM $MgCl_2$/120 mM NaCl. The reaction was started by making the solution 20 mM with respect to KCl. After 15 min incubation the reaction was stopped by adding 1 ml 14% $HClO_4$. 1 ml 12.5% suspension of activated charcoal in 14% $HClO_4$ was added to the reaction mixture. After 60 min at 0°C with occasional stirring, the charcoal was removed by filtration over Whatman No. 1 filter paper. 0.5 ml filtrate was counted in 5 ml toluene/Triton

X-100 scintillation fluid. The incubation temperature in the standard assay [4] and in the radioactive assay was 26°C. Units of enzyme activity are μmol P_i liberated per h.

p-Nitrophenolphosphatase activity was determined by the method of Interrusi and Titus [7].

The standard Na^+-ATPase activity was assayed by incubating 2.5 μg enzyme in 1 ml 40 mM NaCl/25 μM $MgCl_2$/30 mM imidazole · HCl buffer (pH 7.0)/10 μM [^{32}P]ATP for 20 min at 26°C. The reaction was stopped and radioactive inorganic phosphate was determined as described above for the radioactive assay.

Results

Effects of wheat germ agglutinin and concanavalin A on physical state and catalytic activity of ($Na^+ + K^+$)-ATPase of rectal gland and electroplax

Fig. 1a shows the effects of increasing concentrations of wheat germ agglutinin and concanavalin A on agglutination of the rectal gland enzyme as measured by absorbance at 420 nm. Both lectins produced comparable agglutination. Fig. 1b shows the effects of the two lectins on ($Na^+ + K^+$)-ATPase activity in the suspension of the rectal gland enzyme. There was a significant inhibition of ($Na^+ + K^+$)-ATPase activity on increasing the wheat germ agglutinin concentration. Concanavalin A showed a much smaller inhibition of ($Na^+ + K^+$)-ATPase activity over the same concentration range of lectin. These results with different lectins show the selectivity of the lectins in inhibiting ($Na^+ + K^+$)-ATPase and further demonstrate that inhibition of ($Na^+ + K^+$)-ATPase did not parallel agglutination. In studies on liposomes reconstituted with purified

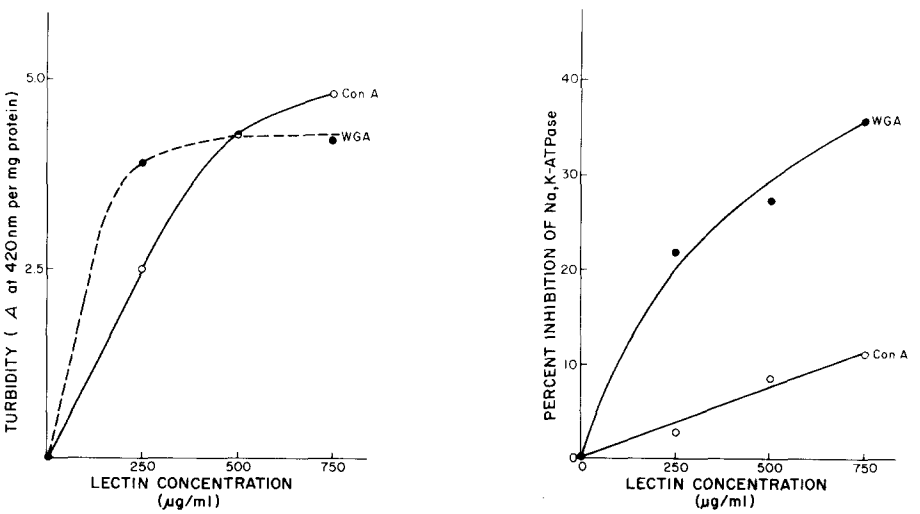

Fig. 1. Effects of lectins on agglutination and activity of rectal gland ($Na^+ + K^+$)-ATPase. 100 μg of rectal gland ($Na^+ + K^+$)-ATPase were incubated for 45 min at 25°C with varying amounts of wheat germ agglutinin and concanavalin A in 1 ml of 30 mM imidazole · HCl (pH 7.0). The absorbance of the solution was measured at 420 nm and appropriate corrections were made for pre-agglutination blank absorption. ($Na^+ + K^+$)-ATPase activity was measured by incubating an aliquot of the suspension containing 15 μg of enzyme for 10 min at 25°C in the standard assay medium. (A) Absorbance at 420 nm. (B) Percent inhibition of ($Na^+ + K^+$)-ATPase activity.

($Na^+ + K^+$)-ATPase agglutination occurred without any inhibition of enzyme activity (Pennington, J. and Hokin, L.E., unpublished data).

With these high levels of enzyme (250 μg/ml) neither lectin agglutinated nor inhibited the ($Na^+ + K^+$)-ATPase from the electroplax over the same concentration range used for the rectal gland enzyme (unpublished data).

Effects of neuraminidase

Although neither wheat germ agglutinin nor concanavalin A is specifically directed against the sialic acid residue, removal of part or all of the latter might alter agglutination or inhibition by wheat germ agglutinin for steric reasons. However, this was not found to be the case. Removal of 60% of the sialic acid from the rectal gland enzyme by digestion with neuraminidase did not affect agglutination or inhibition of ($Na^+ + K^+$)-ATPase activity by wheat germ agglutinin (unpublished data).

Reversal of inhibition of ($Na^+ + K^+$)-ATPase activity by N,N'-diacetylchitobiose

Lectins are known to specifically bind monosaccharides, disaccharides, oligosaccharides and polysaccharides. Wheat germ agglutinin binds the *N*-acetylglucosamine residues in carbohydrates or oligosaccharides. Various *N*-acetylglucosamine-containing sugars were therefore tested for their ability to reverse the inhibition of ($Na^+ + K^+$)-ATPase by wheat germ agglutinin. For this purpose, much lower concentrations of enzyme were used and the more sensitive radioactive ($Na^+ + K^+$)-ATPase assay as described under Materials and Methods was employed to avoid interference by the sugars in the standard spectrophotometric assay. The ratios by weight of lectin to enzyme were still within the 10 : 1 range used with the higher enzyme concentration. The effects of wheat germ agglutinin on ($Na^+ + K^+$)-ATPase activity of the rectal gland enzyme and the electroplax enzyme are shown in Fig. 2. 20% inhibition of the rectal gland

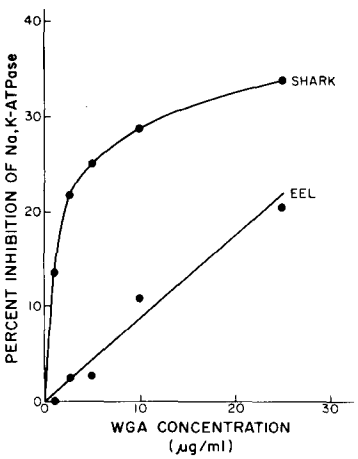

Fig. 2.Wheat germ agglutinin (WGA) inhibition at low concns. of ($Na^+ + K^+$)-ATPase. ($Na^+ + K^+$)-ATPase (2.5 μg) + varying amounts of WGA were preincubated for 15 min at 26°C in 1 ml of assay buffer as described under Materials and Methods. Potassium was excluded during preincubation. The ($Na^+ + K^+$)-ATPase reaction was initiated at the end of preincubation by the addition of 20 mM K^+. After incubation for 15 min at 26°C the reaction was stopped and radioactive inorganic phosphate was measured as described under Materials and Methods.

TABLE I

REVERSAL OF WHEAT GERM AGGLUTININ INHIBITION OF $(Na^+ + K^+)$-ATPase BY *N,N'*-DIACETYLCHITOBIOSE

2.5 μg of rectal gland $(Na^+ + K^+)$-ATPase was preincubated without and with wheat germ agglutinin and *N,N'*-diacetylchitobiose as indicated for 15 min at 26°C. Preincubation and assay conditions are described under Materials and Methods. The final volume was 1 ml.

Additions	Specific activity (μmol P_i/h per mg protein)	Percent inhibition
None	758	0
5 μmol *N,N'*-diacetylchitobiose	773	−2.0
5 μg wheat germ agglutinin	597	21.5
5 μg wheat germ agglutinin and 1 μmol *N,N'*-diacetylchitobiose	666	11.0
5 μg wheat germ agglutinin and 5 μmol *N,N'*-diacetylchitobiose	731	3.0

enzyme was seen with a concentration of wheat germ agglutinin as low as 2 μg/ml. The inhibition curve for wheat germ agglutinin appeared to be biphasic, rising sharply up to 20% inhibition and then rising rather slowly up to about a 35% inhibition at 22.5 μg/ml lectin. With the lower concentrations of $(Na^+ + K^+)$-ATPase and lectin some inhibition of the electroplax enzyme was seen with wheat germ agglutinin. The inhibition increased linearly up to about 20% as the lectin concentration rose to 25 μg/ml.

When 1 ml medium containing 2.5 μg rectal gland enzyme and 5 μg/ml wheat germ agglutinin was made 5 μmol/ml with respect to *N,N'*-diacetyl chitobiose (β-1,4-di-*N*-acetylglucosamine) essentially all of the inhibition by wheat germ agglutinin was reversed (Table I). The reversal was dependent on the sugar concentration, since only half-reversal was achieved with 1 μmol/ml *N,N'*-diacetylchitobiose.

It was difficult to demonstrate reversal of wheat germ agglutinin inhibition by sugars which interact less strongly with the lectin. *N*-Acetylglucosamine (up to 200 μM) gave marginal and inconsistent reversal of wheat germ agglutinin inhibition. Ovomucoid gave reversals of wheat germ agglutinin inhibition ranging from 7 to 68% (ratio by weight of ovomucoid to wheat germ agglutinin ranging from 7 to 500) and appeared to bear no relation to the ratio of ovomucoid to wheat germ agglutinin.

Effects of wheat germ agglutination on ouabain inhibition and binding

Wheat germ agglutinin and concanavalin A had no effects on ouabain inhibition of the rectal gland $(Na^+ + K^+)$-ATPase over a 5-fold range above the I_{50} of the cardiac glycoside (10^{-6}—$5 \cdot 10^{-6}$ M). Conditions for maximum sensitivity of lectin inhibition were used (2.5 μg $(Na^+ + K^+)$-ATPase). In these experiments a 4-fold excess (by weight) of either wheat germ agglutinin or concanavalin A preincubated with $(Na^+ + K^+)$-ATPase was used.

[^{3}H]Ouabain binding was studied in the presence of Na^+, Mg^{2+} and ATP, ligands known to promote binding. A sample not containing ATP was run as the control. The difference in binding may be taken as specific ouabain binding. Preincubation of rectal gland $(Na^+ + K^+)$-ATPase with a 2-fold excess of wheat germ agglutinin (by weight) for 30 min at 37°C did not affect ouabain binding (unpublished data).

Effects of lectins on K^+-dependent p-nitrophenylphosphatase activity

K^+-dependent *p*-nitrophenylphosphatase activity reflects the K^+-dependent dephosphorylation step in the overall ($Na^+ + K^+$)-ATPase reaction. Preincubation of the rectal gland ($Na^+ + K^+$)-ATPase with a 10-fold excess of either wheat germ agglutinin or concanavalin A failed to affect *p*-nitrophenylphosphatase activity (unpublished data). Under these conditions the rectal gland enzyme was agglutinated, and ($Na^+ + K^+$)-ATPase activity was partially inhibited by wheat germ agglutinin. These observations indicate that some step other than the dephosphorylation step of the ($Na^+ + K^+$)-ATPase was affected by wheat germ agglutinin.

Effect of wheat germ agglutinin on Na^+-ATPase activity

Another partial reaction of the ($Na^+ + K^+$)-ATPase which was studied was the Na^+-ATPase activity. This activity is seen at very low concentrations of ATP and reflects the initial phosphorylation step of the enzyme by MgATP in the presence of Na^+. As shown in Table II, 10—25 μg/ml wheat germ agglutinin inhibited Na^+-ATPase (2.5 μg protein ml) 18—31%. The inhibition of Na^+-ATPase by wheat germ agglutinin was not affected by increasing the NaCl concentration as high as 250 mM. Since Na^+-ATPase activity involves binding and spontaneous hydrolysis of ATP on the catalytic subunit at the inner surface of the membrane, while oligosaccharides which bind wheat germ agglutinin project from the outer surface on the glycoprotein subunit, the data suggest that wheat germ agglutinin inhibits the ATP site on the catalytic subunit indirectly by binding to the glycoprotein subunit.

Table II also shows the effects of K^+ on the inhibition of the Na^+-ATPase by wheat germ agglutinin. Interestingly, K^+ reversed the inhibition of Na^+-ATPase by wheat germ agglutinin. Since K^+ also binds to the outward facing surface of the enzyme, this reversal could be due to a reduction of binding of wheat germ agglutinin to the enzyme or to an indirect effect on the catalytic subunit.

Discussion

The salient observation in this paper is that wheat germ agglutinin readily agglutinated the purified ($Na^+ + K^+$)-ATPase from the rectal gland and inhibited ($Na^+ + K^+$)-ATPase activity. At high enzyme concentrations (100 μg/ml) the

TABLE II

INHIBITION BY WHEAT GERM AGGLUTININ OF Na^+-ATPase AND REVERSAL BY K^+

2.5 μg of rectal gland ($Na^+ + K^+$)-ATPase was assayed for Na^+-ATPase as described under Materials and Methods. The ATP concentration was 10 μM and the incubation time was 15 min. The reaction rate was linear over this period. The Mg^{2+}/ATP ratio was maintained constant at 2.5 : 1.

Additions	Specific activity of Na^+-ATPase (μmol P_i/mg protein per h)	
	−20 mM K^+	+20 mM K^+
None	8.13	13.9
10 μg wheat germ agglutinin	6.66	13.9
25 μg wheat germ agglutinin	6.33	13.8

purified electroplax enzyme was not agglutinated and was inhibited less than 10%. Under these conditions concanavalin A agglutinated the rectal gland enzyme, but not the electroplax enzyme. It inhibited the rectal gland enzyme 10% and had no effect on the electroplax enzyme. The lectins were present in excess of the enzymes by a factor as much as 7.5 times (by weight). The molar excesses would be even greater (the molecular weight of concanavalin A is 50 000 and that of dimeric wheat germ agglutinin is 35 000; the molecular weight of the $(Na^+ + K^+)$-ATPase is thought to be approx. 250 000 [6]). A 20% inhibition of the electroplax enzyme could be demonstrated by wheat germ agglutinin at very low enzyme concentrations (2.5 μg/ml) using comparable ratios of lectin to enzyme as were used with the higher enzyme concentrations. The inhibition of the rectal gland enzyme by wheat germ agglutinin was completely reversed by di-*N*-acetylchitobiose, which has a high affinity for wheat germ agglutinin. This indicates that the inhibition by wheat germ agglutinin is due to specific interaction with *N*-acetylglucosamine residues in the glycoprotein subunit. The relatively strong inhibition of the rectal gland enzyme and the rather weak inhibition of the electroplax enzyme by wheat germ agglutinin is consistent with their respective contents of *N*-acetylglucosamine. The rectal gland contains 7.0 mol *N*-acetylglucosamine per 100 mol of amino acid residues and the electroplax enzyme contains 1.5 mol [6]. The weak inhibition of $(Na^+ + K^+)$-ATPases from either the rectal gland or the electroplax by concanavalin A is of interest in the light of the mannose composition of the two enzymes. The rectal gland enzyme contains 4.1 mol mannose per 100 mol amino acid residues and the electroplax enzyme contains 2.2 mol. Although these levels are lower than that of *N*-acetylglucosamine in the rectal gland enzyme, they are not inordinately lower; it was possible to demonstrate some inhibition of the electroplax enzyme with wheat germ agglutinin at the low enzyme concentration (2.5 μg/ml), and the electroplax enzyme contains only 1.5 mol *N*-acetylglucosamine per 100 mol amino acid residues. It is possible that the lower molecular weight of wheat germ agglutinin makes it more accessible to critical sites on the $(Na^+ + K^+)$-ATPase.

Some of our data are at variance with observations of Swann et al. [8]. They found that concanavalin A inhibited the $(Na^+ + K^+)$-ATPase in a Lubrol extract of electroplax microsomes. This inhibition was only partly reversed by α-methylglucoside. Other lectins, including wheat germ agglutinin, were not inhibitory. Their protein concentration was 2 μg/ml, which is comparable to that used in our radioactive $(Na^+ + K^+)$-ATPase assay, and their lectin concentration ranged up to 10 μg. The *p*-nitrophenylphosphatase activity was inhibited by concanavalin A but this inhibition was not reversed by α-methylglucoside; the inhibition was therefore assumed to be non-specific. Possible explanations for the discrepancies are the impurity of the $(Na^+ + K^+)$-ATPase preparation of Swann et al. [8], which may contain other glycoproteins complexed with the $(Na^+ + K^+)$-ATPase in a large molecular weight aggregate in the Lubrol extract. It is also likely that very much more Lubrol was bound to the $(Na^+ + K^+)$-ATPase in their case than is the case with the purified fish enzymes, which contain no more than 5 mg Lubrol per 100 mg protein, when the Lubrol extract is purified by aminoethylcellulose chromatography followed by our usual ammonium sulfate fractionation [9] or by the earlier purification method

utilizing zonal centrifugation [4—6]. The purified enzymes used here are associated with phospholipids in a bilayer structure [4]. The accessibility of the oligosaccharides to lectins and the possible conformational changes brought about by lectin binding to the enzyme might be quite different in the two cases. Perhaps of critical importance is the uniquely high content of *N*-acetylglucosamine in the rectal gland enzyme, which may make it particularly susceptible to wheat germ agglutinin. Both the present studies and those of Swann et al. [8] show no effect of lectins on ouabain inhibition or [^{3}H]ouabain binding. It is of interest that concanavalin A agglutinated the rectal gland enzyme as much as wheat germ agglutinin did but it inhibited enzyme activity much less.

Marshall [10] demonstrated binding of concanavalin A to a partially purified shark rectal gland enzyme; the binding of concanavalin A to the shark enzyme had no effect on ($Na^+ + K^+$)-ATPase activity. This is not too different from our observations with comparable concentrations of concanavalin A and ($Na^+ + K^+$)-ATPase, where we observed at most inhibition of only 10%. Marshall further observed that binding of concanavalin A to the enzyme was completely reversed by α-methylmannoside, which also binds to concanavalin A.

The inhibition of ($Na^+ + K^+$)-ATPase activity by wheat germ agglutinin and its reversal by K^+ is of particular interest since Na^+-ATPase reflects the initial phosphorylation of the large chain on the inner surface of the enzyme. This suggests that the binding of wheat germ agglutinin to the glycoprotein may lead to a conformational change in the large chain affecting the substrate site.

It is unlikely that the reversal of the wheat germ agglutinin inhibition by K^+ was due to the superimposition of ($Na^+ + K^+$)-ATPase activity which was not inhibited by wheat germ agglutinin. In the first place, as reported here, when ($Na^+ + K^+$)-ATPase activity was assayed with the usual ATP concentrations the enzyme activity was consistently inhibited. In the second place, when Na^+-ATPase was assayed at sufficiently low ATP concentrations the superimposed ($Na^+ + K^+$)-ATPase activity in the presence of K^+ was low enough so that the inhibition of Na^+-ATPase by wheat germ agglutinin would not have been obscured.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis studies of Pennington and Hokin (in preparation) clearly show that wheat germ agglutinin specifically binds to the glycoprotein subunit, producing a high molecular weight complex which does not enter the gel (8.75% polyacrylamide). There is no change in the amount or position of the catalytic subunit band. There is no detectable *N*-acetylglucosamine in the lipid extract of the enzyme, ruling out lipids in the purified enzyme complex as a site for wheat germ agglutinin binding. The evidence is thus quite strong that the inhibition of the ($Na^+ + K^+$)-ATPase by wheat germ agglutinin is due to binding of the lectin to the glycoprotein. This inhibition may be brought about either by steric hindrance, by interference with normal conformational changes, or by blockage of an ionophore.

It should be noted that the inhibitory effects of wheat germ agglutinin on ($Na^+ + K^+$) -ATPase are only partial. This is similar to the observations of Swann et al. [8]. It is also similar to observations on the inhibition of the ($Na^+ + K^+$)-ATPase by antibody, which is only partial [2] (Rhee and Hokin, submitted for publication). Since electron micrographs [4] show various membranous forms

of the enzyme, large molecules such as lectins or antibodies may only be accessible to a fraction of the total enzyme molecules in the preparation.

Acknowledgements

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