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STUDIES ON THE GLYCOPROTEIN COMPONENT OF $(Na^+ + K^+)$-ATPase FROM DOG FISH SALT GLAND

BINDING TO CONCANAVALIN A AND REMOVAL OF SIALIC ACID BY NEURAMINIDASE

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SUMMARY

1. The presence of concanavalin A binding sugars in the glycoprotein component of a partially purified $(Na^+ + K^+)$ ATPase preparation from dog fish salt gland was demonstrated by binding of a Triton X-100 extract of the enzyme and isolated glycoprotein to concanavalin A-Sepharose, and by binding of membrane-associated enzyme to free concanavalin A.

2. The binding of concanavalin A to the glycoprotein in both membrane-associated enzyme and a Lubrol extract of the enzyme had no effect on $(Na^+ + K^+)$-ATPase activity. Binding was completely inhibited by methyl-α-mannoside. Also, enzyme activity was not affected by removal of 50 % of glycoprotein sialic acid by neuraminidase. These results suggest that the carbohydrate moiety of the glycoprotein does not play a catalytic role in the $(Na^+ + K^+)$-ATPase.

3. When a Triton X-100 extract of $(Na^+ + K^+)$-ATPase was chromatographed on concanavalin A-Sepharose, 37 % of total protein was bound to the column and eluted by methyl-α-mannoside. The bound fraction was free of lipid, and contained not only the glycoprotein but also the large protein which is the catalytic subunit of the enzyme, and small amounts of other membrane derived proteins. The ratio of large protein to glycoprotein, as measured by the relative Coomassie blue absorbance of the two proteins separated by gel electrophoresis, was the same in the bound fraction as in the membrane. These results suggest that the glycoprotein and large protein are either associated together in the membrane or become associated during lipid replacement by Triton.

INTRODUCTION

Purified $(Na^+ + K^+)$-ATPase from a wide variety of sources can be resolved by sodium dodecyl sulphate-gel electrophoresis into two protein components, a

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protein of molecular weight 84 000–97 000, and a sialoglycoprotein of apparent molecular weight, 47 000–57 000 [1–3]. There is strong evidence for the catalytic role of the large protein, but the role of the glycoprotein is far from understood. The ratios of large protein to glycoprotein from different sources are similar [4–7], which suggests that the glycoprotein is an integral part of the $(Na^+ + K^+)$-ATPase. More positive evidence is that antibody raised against the isolated glycoprotein inhibits a Lubrol extract of the enzyme from electric eel [8] and membrane bound enzyme from dog fish salt gland [9]. Furthermore, there is evidence suggesting the presence of a Na^+ ionophore in the glycoprotein [10]. On the other hand, the isolation of $(Na^+ + K^+)$-ATPase with high activity from a Lubrol extract of brain microsomes, in which the glycoprotein was claimed to be absent, has been described [11, 12].

The aim of the present work was to investigate some properties of the glycoprotein in relation to its possible function in the $(Na^+ + K^+)$-ATPase. The source of the enzyme was a partially purified membrane preparation from dog fish salt gland. Firstly, the question of whether the glycoprotein carbohydrate moiety plays a role in the enzyme was investigated by studying the effect of modifying the carbohydrate structure on enzyme activity. The structure was modified by binding of concanavalin A, a plant lectin specific for α-D-mannosyl and α-D-glucosyl groups [13, 14], to the glycoprotein in both membrane-associated enzyme and a Lubrol extract, and by partial removal of sialic acid with neuriminidase. While this work was in progress, a report appeared by Swann et al. [15] claiming that concanavalin A inhibited Lubrol extracted enzyme from eel electric organ, and by Perrone et al. [7] that removal of 37 % of sialic acid from the dog fish glycoprotein did not affect enzyme activity. Secondly, the degree of association of the glycoprotein with the large protein after replacement of membrane lipids by Triton X-100 was investigated. In these experiments, the protein composition of lipid-free Triton protein complexes bound to concanavalin A-Sepharose from Triton extracts of the enzyme was analyzed. The only information so far published on the protein composition of lipid-free detergent complexes of $(Na^+ + K^+)$-ATPase is that by Clarke [16], who found that the large protein and the glycoprotein in a Triton extract of kidney $(Na^+ + K^+)$-ATPase migrated together during gel filtration and sucrose density centrifugation as a lipid-free Triton protein complex. The molecular weight of the protein component of this complex was 140 000, which suggested that the two proteins were associated together in the Triton complex.

METHODS

Preparation of membrane-associated $(Na^+ + K^+)$*-ATPase.* The method was similar to that used by Jørgensen and Skou [17] for the kidney enzyme. Microsomes from the salt gland of the spiny dog fish were prepared in a suspension containing 30 mM histidine, pH 6.8, 250 mM sucrose, 5 mM EDTA as described elsewhere [18]. To 60 ml of microsomes (2.4 mg protein/ml), was added 60 ml of 0.2 % sodium deoxycholate (Merck) in 20 mM imidazole, pH 7.0, 0.8 mM EDTA, 0.08 % β-mercaptoethanol. After 15 min at 22 °C, the mixture was centrifuged at $6800 \times g$ for 15 min at 0 °C in a Sorval RC2B (SS 34 rotor, 15 ml per tube), the pellets discarded, and the supernatants centrifuged at $32\,000 \times g$ for 30 min. The pellets were suspended in 25 mM imidazole, pH 7.0; the suspensions were

combined, and centrifuged at 100 000 × *g* for 15 min. The pellet was washed again, and suspended to 3–4 mg/ml in 10 ml of 25 % glycerol, 20 mM imidazole, pH 7.0, 0.45 mM EDTA, 0.08 % β-mercaptoethanol, and stored at 0 °C. The specific activity was 1150–1500 μmol $P_i \cdot h^{-1} \cdot mg^{-1}$, as compared with 360 for the microsomes. Experiments were carried out within 5 days of preparation.

Preparation of Lubrol and Triton X-100 extracts of ($Na^+ + K^+$)*-ATPase.* ($Na^+ + K^+$)-ATPase was extracted by Lubrol as follows. To a suspension of membrane-associated enzyme (4 mg protein/ml) in 25 mM imidazole (pH 7.3), 180 mM KCl at 0 °C, was added an equal volume of a solution of Lubrol WX (I.C.I.) (25 mg/ml) in same buffer. After incubation for 20 min, the mixture was centrifuged at 150 000 × *g* for 15 min and the supernatant retained. The extract contained 45 and 50 % of membrane ($Na^+ + K^+$)-ATPase activity and protein respectively. The method of extraction with Triton X-100 was the same except that one volume of a solution of Triton X-100 (Koch-Light) (24 mg/ml) was added to three volumes of membrane suspension. Extraction of activity and protein was 56 and 62 % respectively.

Binding of concanavalin A to membrane-associated ($Na^+ + K^+$)*-ATPase and Lubrol extract.* Membrane-associated enzyme was suspended in 25 mM imidazole, pH 7.0, 180 mM KCl to 1.9 mg protein/ml. Solutions of concanavalin A (Pharmacia) in the concentration range 0.6–6.5 mg/ml were freshly prepared in the same buffer and clarified by centrifugation at 150 000 × *g* for 15 min. The membrane suspension (0.1 ml) was added to the concanavalin A (0.1 ml) at 0 °C, and after 15 min aliquots were assayed for enzyme activity and the mixture centrifuged at 150 000 × *g* for 15 min. Control tubes contained methyl-α-D-mannoside (Fluka) (30 mg/ml) preincubated with concanavalin A. An aliquot of the supernatant was assayed for protein, and bound concanavalin A was calculated from the difference in supernatant protein with that of control. There was no contribution to supernatant protein by unsedimented membrane, nor was binding increased with longer incubation times.

Incubations of concanavalin A with a Lubrol extract were carried out by addition at 0 °C of 0.4 ml of Lubrol extract to 0.4 ml of concanavalin A solution (concentration range 0.16–6.5 mg/ml). After 15 min, a fraction of the mixture was centrifuged at 150 000 × *g* for 15 min and the pellet suspended in 25 mM imidazole, pH 7.0, 180 mM KCl.

Concanavalin A-Sepharose chromatography of Triton X-100 extract. A column (3 × 1 cm) of concanavalin A-Sepharose (Pharmacia) was prewashed with 10 ml of 2 % methyl-α-mannoside in solution A (Triton 6 mg/ml, 25 mM imidazole, pH 7.3, 180 mM KCl) followed by 50 ml of solution A. Freshly prepared Triton extract was applied at 5 °C and unbound protein was eluted with solution A and bound protein by 2 % mannoside in solution A. 1 ml fractions were collected at a flow rate of 0.16 ml/min.

Incubation of ($Na^+ + K^+$)*-ATPase with neuraminidase.* Membrane-associated ($Na^+ + K$)-ATPase was washed once in H_2O, and suspended in H_2O. The incubation medium (total volume, 250 μl) contained ($Na^+ + K^+$)-ATPase (1 mg protein), 3 mM ATP, 25 units of neuraminidase (*Vibrio cholerae*; Behringwerke AG) and 50 mM of buffer, either Na^+ acetate, pH 5.5, histidine, pH 6.5, or imidazole, pH 7.5. After 30 min at 37 °C, the membrane was isolated by centrifugation, suspended in H_2O to 400 μl, and assayed for ($Na^+ + K^+$)-ATPase activity and protein. Sialic acid in membrane protein was measured as follows. 250 μl of membrane suspension was

freeze dried, the lipids extracted by $CHCl_3$/MeOH (2 : 1), and the protein residue dissolved in 150 μl of 2 % sodium dodecyl sulphate. Protein was recovered in 90–100 % yield and was free of glycolipid. The protein fraction was then hydrolyzed in 0.1 N H_2SO_4 at 80 °C for 30 min and an aliquot of hydrolysate (containing about 2 nmol sialic acid) was assayed for sialic acid by a scaled down modification of Warren's procedure [19]. Measurements were also made of the total sialic acid content of the membrane, following acid hydrolysis of the membrane for 60 min.

Gel electrophoresis. This was carried out essentially as described by Fairbanks et al. [20], as modified by Steck and Yu [21]. Gels were stained with Coomassie blue (Gurr). Destained gels were scanned at 580 nm in a Beckmann Acta-spectrophotometer with scanning attachment (aperture 0.1 mm), and scanning and chart speeds 1.5 and 3 cm/min respectively. Peak areas were measured using a Haff planimeter.

Enzyme assays. $(Na^+ + K^+)$-ATPase activity was measured at 37 °C in a medium (2 ml) containing 3 mM ATP (Tris salt), 3 mM $MgCl_2$, 130 mM Na^+, 20 mM K^+, 30 mM histidine, pH 7.4, 0.033 % bovine serum albumin and 0.2 mM EGTA. The reaction was stopped by 200 μl 50 % trichloroacetic acid, and released phosphate measured in the supernatant after centrifugation [22]. Control tubes contained 1 mM ouabain. *p*-Nitrophenylphosphatase was assayed as described by Ottolenghi [18]. In both assays, membranes were diluted in 30 mM histidine, pH 7.4, prior to assay, but detergent extracts were assayed directly.

Protein and phospholipid measurement. Protein was measured by the procedure of Lowry et al. [23], using bovine serum albumin as standard. Triton caused the formation of a yellow precipitate during colour development which could be removed by centrifugation without loss of absorbance. Phospholipid in lipid extracts [24] of membrane and Triton fractions were measured by the procedure of Bartlett [25] as modified by Kates [26].

RESULTS

The membrane-associated $(Na^+ + K^+)$-ATPase used in this study was enriched in the large protein and catalytic subunit component A, and the glycoprotein component B (Fig. 1). Together these two proteins accounted for 66 % of total protein (see below). The molecular weights of A and B, determined by callibration of three gels with standards of known molecular weight [27] were 95 000 and 58 000 respectivly, which agrees with previous values [4–7]. It should be pointed out that the value for the glycoprotein is only an apparent value since sodium dodecyl sulphate-gel electrophoresis does not give absolute values for glycoproteins [28].

Experiments were carried out to determine if concanavalin A would bind to the glycoprotein component of $(Na^+ + K^+)$-ATPase and if so, the effect of such binding on enzyme activity. The results in Fig. 2 show that concanavalin A could bind to the membrane-associated enzyme, but at both low and saturating concentrations had no effect on either $(Na^+ + K^+)$-ATPase or *p*-nitrophenylphosphatase activities. Binding was completely inhibited by methyl-α-mannoside (Fig. 3), suggesting that the concanavalin A was binding to terminal mannose or glucose residues in the glycoprotein. Binding to membrane glycolipid was unlikely, since glycolipids containing terminal mannose are absent and those containing terminal glucose are very minor components of the lipids of dog fish salt gland [29]. Concanavalin A was

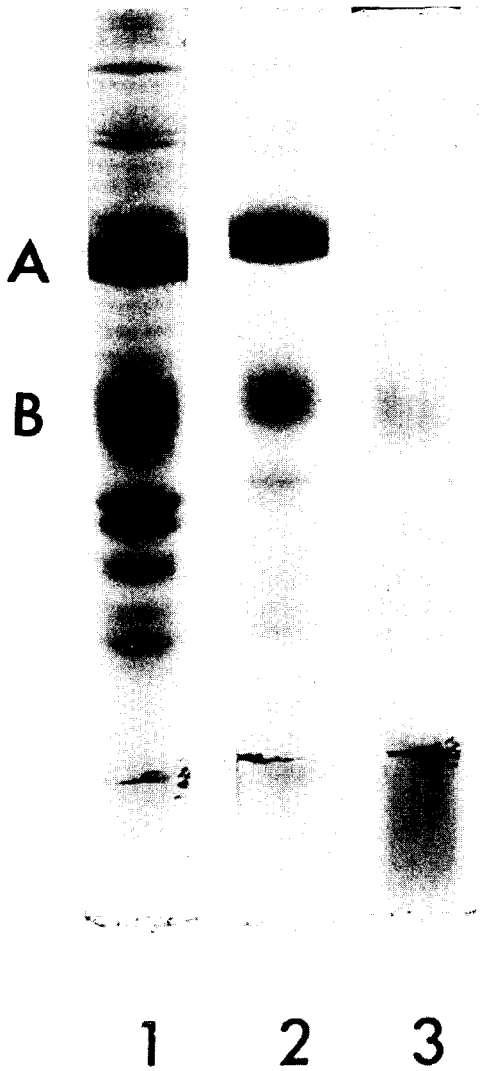

Fig. 1. Sodium dodecyl sulphate-gel electrophoresis of $(Na^+ + K^+)$-ATPase from dog fish salt gland. Gel 1, microsomes; gels 2 and 3, membrane-associated enzyme. Gels 1 and 2 were stained with Coomassie blue, gel 3 with Periodic acid-Schiffs sugar detecting reagent [20]. Electrophoresis was carried out as described in Methods. The gel front was marked with ink. The sugar positive band running ahead of the front in gel 3 was glycolipid.

also without effect on the total $(Na^+ + K^+)$-ATPase activity in a Lubrol extract of the enzyme (Fig. 4). It is of interest that the binding of concanavalin A to the glycoprotein precipitated a fraction (38 %) of the enzyme in the extract. Binding was again specific since this enzyme precipitation was completely inhibited by mannoside. In separate experiments, the effect of KCl on concanavalin A binding was studied. Binding in the absence of KCl had no effect on $(Na^+ + K^+)$-ATPase activities in membrane and

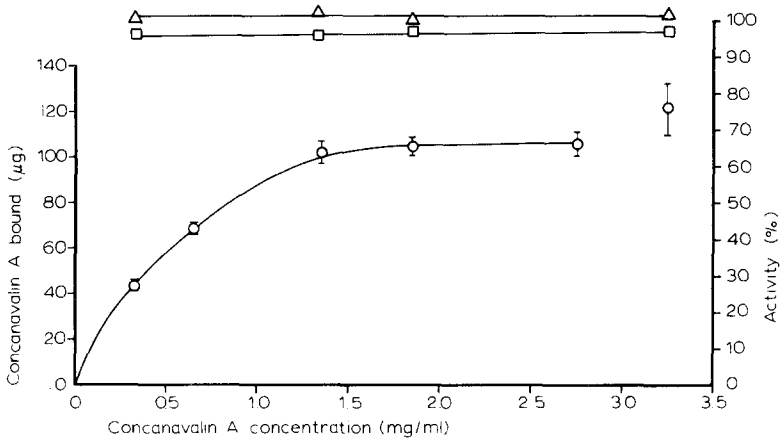

Fig. 2. Incubation of concanavalin A with membrane-associated $(Na^+ + K^+)$-ATPase. Incubations as described in Methods with concentration of concanavalin A varied as shown. □—□, $(Na^+ + K^+)$-ATPase activity of membrane concanavalin A mixture; △--△, *p*-nitrophenylphosphatase activity of membrane concanavalin A mixture; ○···○, concanavalin A bound to membrane. Values for binding are mean ± S.E. from four incubations. The enzyme was freshly prepared with specific activity 1150 μmol $P_i \cdot h^{-1} \cdot mg^{-1}$. Incubation tubes contained 190 μg membrane protein.

extract. However, an increase (40 %) in the amount bound to the membrane-associated enzyme was observed. This extra binding could not be inhibited by mannoside, and most likely resulted from non-specific ionic interaction of concanavalin A with the membrane proteins.

To investigate whether removal of glycoprotein sialic acid would affect enzyme activity, membrane-associated $(Na^+ + K^+)$-ATPase was incubated with neuramini-

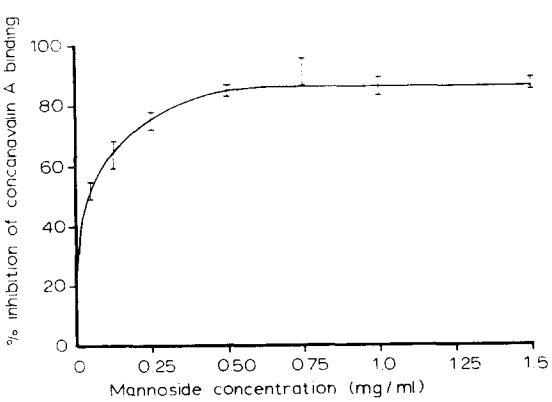

Fig. 3. Inhibition of concanavalin A binding to membrane-associated $(Na^+ + K^+)$-ATPase by methyl-α-mannoside. Incubations with concanavalin A (0.46 mg/ml) were as described in Methods, except that the amount of concanavalin A bound was calculated from the difference in supernatant protein between incubations containing concanavalin A, membranes and mannoside, and incubations with concanavalin A alone. The horizontal bars denote standard error from four incubations.

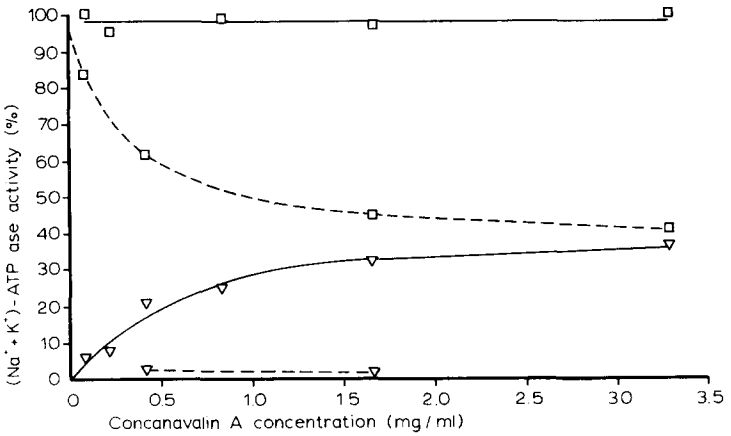

Fig. 4. Incubation of concanavalin A with Lubrol extract of $(Na^+ + K^+)$-ATPase. Incubation as described in Methods, with concentration of concanavalin A varied as shown. □—□, $(Na^+ + K^+)$-ATPase activity in extract concanavalin A mixture; □---□, activity in supernatant; ▽—▽, activity in pellet; ▽---▽, activity in pellet in presence of mannoside. Activities are expressed as % of activity in the extract. The concentration and activity of the enzyme in the incubation mixture was 0.54 mg/ml and 515 μmol $P_i \cdot h^{-1} \cdot ml^{-1}$, respectively.

dase. Incubation for 30 min at different pH values removed 41–49 % of the glycoprotein sialic acid, but with no significant affect on $(Na^+ + K^+)$-ATPase activity (Table I). There was not further release of sialic acid when incubation time was increased to 1 h (data not shown). The difference between the total sialic acid content of the membrane (61 nmol/mg) and membrane protein (18.4 nmol/mg) was found in separate experiments to be due to the presence of sialic acid in glycolipid. Since neither partial removal of sialic acid, nor binding of concanavalin A to mannose or

TABLE I

THE EFFECT OF TREATMENT OF MEMBRANE-ASSOCIATED $(Na^+ + K^+)$-ATPase WITH NEURAMINIDASE ON ENZYME ACTIVITY AND SIALIC ACID CONTENT OF GLYCOPROTEIN

Incubation with neuraminidase and measurement of sialic acid were as described in Methods. Incubation was for 30 min. The values for sialic acid in membrane and protein, minus neuraminidase, were the mean ±S.E. from five different preparations of membrane (mean specific activity 1210 μmol $P_i \cdot h^{-1} \cdot mg^{-1}$); all other values were from single incubations with neuraminidase on the same membrane preparation (1200 μmol $P_i \cdot h^{-1} \cdot mg^{-1}$). n.d., not determined

Incubation conditions	Sialic acid				$(Na^+ + K^+)$-ATPase activity remaining (%)
	Membrane		Protein		
	(nmol/mg)	(%)	(nmol/mg)	(%)	
−neuraminidase	61 ±0.5	100	18.4 ±0.3	100	100
+neuraminidase, pH 5.5	28.0	46	10.9	59	100
+neuraminidase, pH 6.5	n.d.	n.d.	10.4	57	103
+neuraminidase, pH 7.5	n.d.	n.d.	9.4	51	94

glucose residues in the glycoprotein had any effect on $(Na^+ + K^+)$-ATPase activity, it was concluded that the carbohydrate moiety of the glycoprotein does not play a catalytic role in the enzyme.

The presence of concanavalin A binding sugars in the glycoprotein was supported by results from concanavalin A-Sepharose chromatography of an isolated glycoprotein fraction. Gel filtration of sodium dodecyl sulphate dissolved membrane on Sepharose 6B, followed by removal of sodium dodecyl sulphate [30] from the glycoprotein rich fraction, gave a product composed of 70 % glycoprotein and 30 % low molecular weight proteins. When this fraction was chromatographed on concanavalin A-Sepharose in 25 mM Tris, pH 7.4, 200 mM KCl, all of the glycoprotein bound to the column (results not shown). Binding was specific, since no glycoprotein was bound when it was applied to the column in the presence of 2 % mannoside. However, the glycoprotein bound in the absence of mannoside was irreversibly bound since it could not be eluted by mannoside. No attempt was made to elute the glycoprotein with detergents. Irreversible binding to concanavalin A-Sepharose has been observed for other glycoproteins [31–33].

The presence of concanavalin A binding sugars in the glycoprotein was utilized to isolate a lipid-free glycoprotein Triton complex by chromatography of a Triton X-100 extract of the enzyme on concanavalin A-Sepharose. The extract was resolved into two protein fractions (Fig. 5 and Table II). The first did not bind to the column and contained 52 % of applied protein (63 % with protein in tail), only 3 % of $(Na^+ + K^+)$-ATPase activity, but all of the phospholipid. The second fraction was bound to the column and could be eluted with mannoside. It contained 30 % of applied protein (37 % with protein in tail), but neither activity nor phospholipid. Chromatography was repeated three times with identical results. Binding was optimal since only a small amount (15 %) of the unbound fraction was bound when rechromatographed

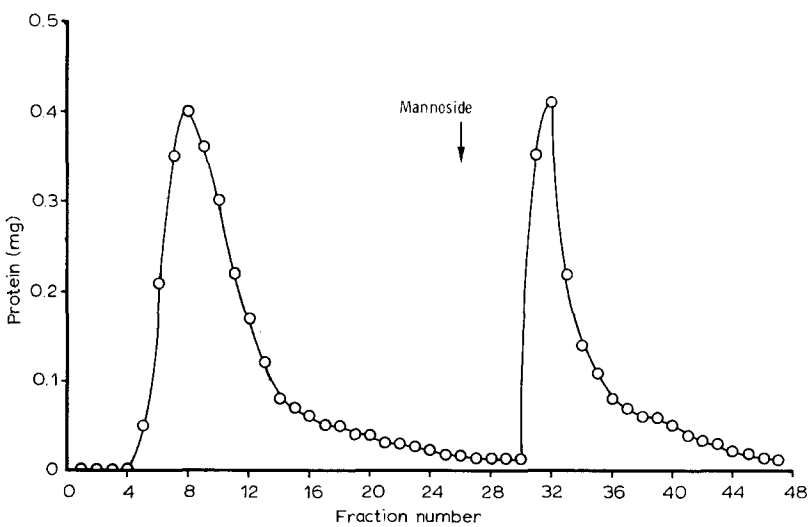

Fig. 5. Concanavalin A-Sepharose chromatography of a Triton X-100 extract of $(Na^+ + K^+)$-ATPase. Chromatography was carried out as described in Methods, using 2.5 ml of Triton extract (4.8 mg protein).

TABLE II

RECOVERY OF $(Na^+ + K^+)$-ATPase ACTIVITIES, PROTEIN AND PHOSPHOLIPID IN FRACTIONS FROM CONCANAVALIN A-SEPHAROSE CHROMATOGRAPHY OF TRITON X-100 EXTRACT

Unbound and bound fractions were tubes 5–14 and 30–38 respectively from the experiment described in Fig. 5. Aliquots of fraction taken and reaction time in $(Na^+ + K^+)$-ATPase assay were 10 μl and 1 min for Triton extract, and 40 μl and 10 min for unbound and bound fractions. The Triton extract was stored at 5 °C during chromatography and assayed simultaneously with column fractions. Protein and phospholipid were measured as described in Methods.

Fraction	$(Na^+ + K^+)$-ATPase activity		Protein		Phospholipid	
	(μmol P_i/h)	(%)	(mg)	(%)	(mg)	(%)
Triton extract	4255	100	4.82	100	3.37	100
Unbound	108	3	2.48*	52	3.34	99
Bound	0	0	1.44**	30	0	0

* Yield in tail (tubes 15–24) was 0.52 mg (11 %)
** Yield in tail (tubes 39–47) was 0.33 mg (7 %)

on a fresh column. It is not clear why the total recovery of enzyme activity from the column was so low. Activities were not increased by prior mixing of the unbound and bound fractions, and of bound fraction with a salt gland phospholipid extract.

The protein composition of the column fractions was calculated from the relative peak areas of scanned gels (Fig. 6, Table III). The amount of protein applied

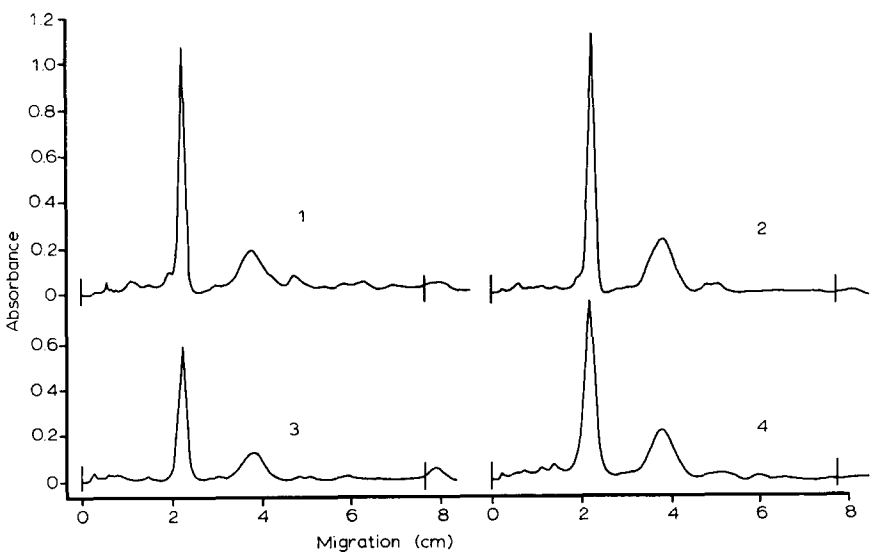

Fig. 6. Sodium dodecyl sulphate-gel electrophoresis of membrane-associated enzyme, Triton X-100 extract and concanavalin A-Sepharose fractions. 1, membrane-associated enzyme; 2, Triton X-100 extract; 3, unbound fraction; 4, bound fraction. The bound and unbound fractions were stored for 1 day at 5 °C before electrophoresis. Electrophoresis and scanning were carried out as described in Methods.

TABLE III

PROTEIN COMPOSITION OF MEMBRANE-ASSOCIATED ENZYME, TRITON EXTRACT AND FRACTIONS FROM CONCANAVALIN A-SEPHAROSE CHROMATOGRAPHY

The protein composition was calculated from the relative areas of Coomassie blue stained protein bands separated by gel electrophoresis (see Fig. 6). Total protein per gel was 6 μg. Scanning and measurement of peak areas were as described in Methods. Values are the mean ±S.E. obtained from three gels.

Fraction	Protein composition (%)			Ratio A/B
	A	B	Other	
Membrane-associated enzyme	40.3 ± 0.3	25.5 ± 0.4	34.2 ± 0.7	1.58 ± 0.01
Triton extract	46.2 ± 1.3	29.7 ± 0.6	24.2 ± 1.9	1.56 ± 0.02
Concanavalin A-Sepharose unbound	49.1 ± 1.1	26.6 ± 1.0	24.4 ± 1.9	1.85 ± 0.06
Concanavalin A-Sepharose bound	49.2 ± 1.0	30.0 ± 1.6	20.8 ± 2.0	1.65 ± 0.09

per gel was 6 μg, which was found in separate experiments to be in the range in which absorbance peak area for both proteins A and B was linearly related to the amount of applied protein. The bound fraction contained not only protein B, the glycoprotein, through which the fraction must have been bound to the column, but also protein A. The same results were obtained when chromatography was carried out in the presence of 1 M NaCl and 25 mM β-mercaptoethanol (the latter also present during extraction). The relative ratio A/B in the bound fraction was essentially the same as in the membrane and the extract. The bound fraction also contained small amounts of other proteins, but at a lower concentration than in the membrane. These results suggest that proteins A and B were associated together in the lipid-free Triton complex bound to the concanavalin A. It should be pointed out that the ratio A/B measured does not represent an absolute mass ratio since the relation of Coomassie blue absorbance to mass for each protein was not determined. However, the ratio remains a valid measurement for detecting differences in the relative amounts of A and B in the various fractions.

It is of interest that a significant amount of the glycoprotein in the Triton extract did not bind to concanavalin A-Sepharose. The enzyme not precipitated by free concanavalin A in a Lubrol extract (Fig. 4) may also have contained glycoprotein not bound to concanavalin A. These results should be contrasted with the finding that all of the water soluble isolated glycoprotein could bind to concanavalin A-Sepharose (see above). It is possible that the lower binding of the glycoprotein in detergent medium was due to heterogeneity in the physical state of the glycoprotein in this medium.

Concanavalin A-Sepharose chromatography was also carried out on sodium deoxycholate extracts of the enzyme (results not shown). Though the yield of bound protein was somewhat lower than with Triton, the bound fraction also contained both proteins A and B, thus ruling out the possibility that the above results were due to a specific Triton effect. Chromatography was also carried out with membrane-associated enzyme in the absence of detergent, but no adsorption to the column was observed. This lack of adsorption, in contrast to the ready binding of membrane-associated enzyme to free concanavalin A, was presumably due to the exclusion of the large membrane fragments from the Sepharose beads.

DISCUSSION

The present results showed that saturation binding of concanavalin A to the glycoprotein, most likely to terminal mannose or glucose residues [13, 14], did not affect $(Na^+ + K^+)$-ATPase or *p*-nitrophenylphosphatase activities in membrane-associated enzyme, or $(Na^+ + K^+)$-ATPase activities in a Lubrol extract of the enzyme. Furthermore, 50 % of glycoprotein sialic acid could be removed by neuraminidase with no effect on enzyme activity. These results taken together suggest that the carbohydrate moiety of the glycoprotein does not play a catalytic role in $(Na^+ + K^+)$-ATPase. However, no conclusions can be made from these results regarding a possible role of the polypeptide moiety of the glycoprotein in the enzyme. The results from sialic acid removal essentially confirm the recent findings of Perrone et al. [7] that a maximum of 37 % of sialic acid could be removed from the dog fish glycoprotein and 100 % from the eel electric organ glycoprotein by neuraminidase with no effect on enzyme activity. It may be noted, however, that the present results differ from those of these workers in the content of sialic acid in the dog fish glycoprotein (18.4 and 79 nmol/mg membrane protein respectively), and glycolipid (43 and 0 nmol/mg respectively), which cannot be accounted for by the relatively small difference in the purity of the enzyme preparations used. The results from concanavalin A binding are in contrast to the report by Swann et al. [15] that concanavalin A binding to the glycoprotein in a Lubrol extract of the enzyme from eel electric organ caused a 50 % inhibition of $(Na^+ + K^+)$-ATPase, but no inhibition of *p*-nitrophenylphosphatase. However, the results of Swann et al. [15] are difficult to interprete since concanavalin A inhibition of the $(Na^+ + K^+)$-ATPase was found to be only partially reversed by glycoside at low concanavalin A concentrations, and not at all at high concentrations, whereas in the present results concanavalin A binding was specific since it was completely inhibited by glycoside.

The results from concanavalin A-Sepharose chromatography of Triton extracts of the enzyme gave useful information regarding the degree of association of the glycoprotein with the large protein. The presence of both proteins in the lipid-free Triton protein complex bound to concanavalin A-Sepharose suggested that they were associated together in this complex. Evidence for association of the two proteins in a lipid-free Triton complex was first obtained by Clarke [16] who used the kidney enzyme and gel filtration and centrifugation techniques to isolate the complex. The present results essentially confirm this earlier work using instead a different source of enzyme and concanavalin A-Sepharose chromatography to isolate the complex. However, no firm conclusions can be made in the present study as to whether the two proteins were originally associated in the membrane, or alternatively, became associated only after lipid replacement by Triton. Since other membrane derived proteins, not likely part of the enzyme, were also present in the concanavalin A bound fraction, the latter hypothesis cannot be ruled out. However, the finding that the ratio of large protein to glycoprotein in the bound fraction was the same as that in the membrane suggests the former hypothesis to be the more likely. The finding by Kyte [34] that the two proteins in a membrane preparation from kidney can be specifically cross-linked by dimethyl suberimidate is also consistent with this hypothesis. However, more direct evidence is required before firm conclusions can be made regarding the association of the glycoprotein with the large protein in the membrane.

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