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A PROTEOLIPID ASSOCIATED WITH Na,K-ATPase IS NOT ESSENTIAL FOR ATPase ACTIVITY

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Summary: 1. 11,000 dalton proteins were detected in membrane-bound preparations of Na,K-ATPase from shark rectal glands and avian nasal salt glands.

2. In shark rectal gland microsomal Na,K-ATPase the stoichiometry of the 11,000 dalton protein to the catalytic and glycoprotein subunits of the ATPase was 2.0 moles per mole $\alpha_2\beta_4$.

3. Non-ionic detergent treatment under non-denaturing conditions of the membrane-bound Na,K-ATPase from avian nasal salt glands and shark rectal glands simultaneously separated the small proteins from the α-β complex and the ATPase activity, indicating that the small proteins are not essential for the hydrolysis of ATP.

4. A fundamental role for the small proteins is suggested by the great similarity of the amino acid compositions of the 11,000 dalton protein from shark rectal gland Na,K-ATPase and the γ2 proteolipid from lamb kidney outer medulla reported by Reeves, A.S., Collins, J.H. and Schwartz, A. (1980) Biochem. Biophys. Res. Comm. 95, 1591-1598, which implies that there is a strong sequence homology between the two small proteins, i.e. that the primary structure is highly conserved.

Introduction: The existence of small proteins (proteolipids) of about 12,000 daltons associated with the ionic pumps of mitochondria, bacteria, chloroplasts and the sarcoplasmic reticulum is well established (1-6), and the possible role of such low molecular weight proteins in the active transport of ions has been discussed (7). However, whether any association exists between such a class of 12,000 dalton protein and the Na,K-ATPase[1] has been less certain. Goldin (8) failed to find any proteolipid in preparations of the

[1]Abbreviations: Na,K-ATPase, sodium plus potassium stimulated adenosine triphosphatase (E.C.3.6.1.3); EGTA, ethylene glycol bis (β-aminoethylether) N,N,N',N'-tetraacetic acid; Tes, N-tris (hydroxymethyl) methyl-2-aminoethane sulfonic acid; SDS, sodium dodecyl sulfate; α, catalytic subunit of Na,K-ATPase; β, glycoprotein subunit of Na,K-ATPase.

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Na,K-ATPase; but lately several groups have detected 12,000 dalton proteins which are labelled by photoaffinity derivatives of ouabain in membrane-bound Na,K-ATPase (9-12). Recently the amino acid compositions of two low molecular weight proteins in Na,K-ATPase preparations from lamb kidney outer medulla have been reported (13,14).

In ascribing the presence of low molecular weight proteins in highly purified membrane-bound Na,K-ATPase to some kind of association with the Na,K-ATPase, the stoichiometry of the small protein with the α^1 and β^1 subunits of the ATPase is an important consideration and requires investigation. The existence of small proteins of similar composition to those found in the mammalian sources in evolutionary lines widely separated from mammals would support the concept of a highly conserved and thus functionally important protein. Accordingly, we examined shark rectal gland and avian nasal salt gland membrane-bound Na,K-ATPase preparations for the presence of 11,000-12,000 dalton proteins. The stoichiometry of the small protein found in shark rectal gland microsomal Na,K-ATPase to the α and β subunits was determined and found to be very reasonable for a protein associated with the ATPase. The amino acid composition of this small protein was compared to that of a proteolipid from a mammalian source (13), when a strong resemblance was found. In the course of this work it was found possible to separate the small protein from the enzymatically active α-β complex under non-denaturing conditions by the use of non-ionic detergent in the presence of glycerol.

Materials and Methods: Avian nasal salt glands (common and royal tern) and shark rectal glands (Squalus acanthus) were provided by Dr. Joe Bonaventura of the Duke Marine Laboratory, Beaufort, North Carolina.

Crude microsomes were extracted with SDS[1] by the method of Jorgensen (15). Crude avian nasal salt gland microsomes were prepared in the presence of 0.1 mg/ml Trasylol (Bayer), 1 mM EDTA and 1 mM phenylmethylsulfonyl fluoride, followed by SDS extraction in the presence of 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA.

Solubilized ATPase was prepared from SDS-extracted microsomes by treating them with 2.6 mM Brij 58 (ICI) in the presence of 3 M glycerol at a total

protein concentration of 0.2 mg ml^{-1} (14.6 gm Brij 58 per gm protein) in a medium of 10 mM KCl, 0.5 mM EDTA, 0.5 mM dithiothreitol, 20 mM imidazole, pH 7.4, for 1/2 hour on ice. The preparation was then centrifuged at 10^5g for 1/2 hour, and the supernatant collected. In some cases the extract was passed over DEAE A.25 Sephadex as in the procedure of Hastings and Reynolds (16).

The small protein was prepared as follows: SDS was added to lyophilized microsomes to give a weight ratio of 40 mg SDS per mg protein, and 2-mercaptoethanol to 1% v/v. After heating the preparation at 90°C for 5 minutes and cooling, the solubilized material was applied to a 0.9 x 58 cm column of Sephacryl S-300 in 3.5 mM SDS, 10 mM sodium phosphate, pH 6.8. The small protein elutes with the SDS-phospholipid mixed micelles. Fractions from the trailing edge of the peak were pooled, and the protein concentrated by lyophilization.

Amino acid analysis was performed on a Beckman 120C analyser on undelipidated material. The small protein from SDS-extracted microsomes was hydrolysed *in vacuo* at 110°C for 24, 48 and 72 hours in constant boiling 6 N HCl, 10 mM phenol. No attempt was made to estimate serine. Enthanolamine was resolved from the basic amino acids. Values for threonine were extrapolated to zero time; and the values for valine and phenylalanine to infinite time; other values were averaged. N-terminal analysis was performed as described by Gray (17), using 5 x 5 cm Schleicher and Schuell micropolyamide plates. Protein determination was by the modified Lowry-Folin method of Bensdoun and Weinstein (18), with bovin serum albumin as standard. Organic phosphorus was determined by the method of Bartlett (19). Polyacrylamide gel electrophoresis was performed according to Weber and Osborn (20) in 7.5% gels with 0.1% w/v SDS and 50 mM sodium phosphate in the running buffer. Gels were stained in 0.1% w/v Coomasie Blue in 41 : 7 : 52, v/v/v, methanol/acetic acid/water, and destained by diffusion. Gels were scanned in a Gilford linear scanner at 565 nm. ATPase activities were determined in a coupled enzyme system (21). The medium contained 2.5 mM ATP, 2.5 mM $MgCl_2$, 1 mM EGTA[1], 0.26 mM β-NADH, 1.4 mM phosphoenolypyruvate, 20 mM KCl, 100 mM NaCl, 20 mM Tes[1], pH 7.4, with 10 IU pyruvate kinase and 20 IU lactic dehydrogenase in the presence and absence of 1 mM ouabain in a volume of 1 ml at 37°C.

Results and Discussion: Preparation of membrane-bound (Na,K)-ATPase from avian nasal salt glands in the presence of protease inhibitors yielded microsomes containing a low molecular weight protein (Figure 1a). This stained as a diffuse blue band with Coomasie Blue, as distinct from the pinkish color of phospholipid alone. Treatment of these microsomes with the non-ionic detergent Brij 58 at a weight ratio of 14.6 gm Brij per gm protein in 3 M glycerol for 1/2 hour followed by centrifugation clearly separated the preparation into two parts. Firstly, a Brij-insoluble pelleted residue lacking any ATPase activity (Table I) which contained the small protein (Figure 1b); and secondly, a supernatant possessing all the ATPase activity (Table I) in which only the α and β subunits were present (Figure 1c). If Brij extraction was carried out for an hour or longer, the small protein began

Table I. Separation of the small protein fron (Na,K)-ATPase activity. SDS-extracted avian nasal salt gland microsomes were prepared in the presence of protease inhibitors, and then treated on ice with Brij 58 in the presence of 3 M glycerol for 1/2 hour (see text). The preparation was centrifuged at 10^5 g and 1/2 hour, and the supernatant and pelleted residue collected.

				Na,K-ATPase			
	Volume	Protein conc.	Total protein	No ouabain	+ ouabain	Ouabain sens.	Total activity
	ml	mg/ml	mg	µmole/min/mg			µmole/min
Sealed microsomes	3.0	3.4	10.2	2.96	0.79	2.17	22.1
Microsomes + SDS just before cent.	7.3	1.4	10.2	13.52	0	13.52	138.0
SDS-extracted	1.7	1.67	2.8	24.9	0	24.9	70.7
Brij 58 extraction							
Before addition of Brij 58	1.0	0.2	0.2	24.5	0	24.5	4.9
+ Brij 58	1.0	0.2	0.2	23.0	0	23.0	4.6
Supernatant	1.0	0.14	0.14	26.93	0	26.93	3.8
Pellet	-	-	0.06	0	0	0	0

to be solubilized. A protein of low molecular weight was also seen in membrane-bound (Na,K)-ATPase preparations from shark rectal glands, and could be isolated from them by gel filtration in SDS on Sephacryl S-300, when the small protein emerged in the SDS-phospholipid mixed micelle peak (Figures 2 and 3). 90% of the small protein preparations from shark rectal glands was represented by a protein of 11,100 daltons. As expected from the asymmetry of the gel scan, two N-termini, Ala and Ileu/Leu were detected. Gel filtration in SDS of a known amount of shark rectal gland SDS-extracted microsomes indicated that 5.9% w/w of the total microsomal protein was represented by the 11,100 dalton small protein component when amino acid analysis was used to quantitate the recovery of small protein. This corresponds to a ratio of 1.62 moles of 11,100 dalton protein per mole of $\alpha_2\beta_2$, or 2.03 moles per mole $\alpha_2\beta_4$, using M_r = 106, 400 for α and M_r = 36,600 for the protein component of β (16). It was possible to separate the small protein of shark rectal gland microsomes from the α and β subunits by treatment with Brij 58, exactly as with the avian nasal salt gland small protein.

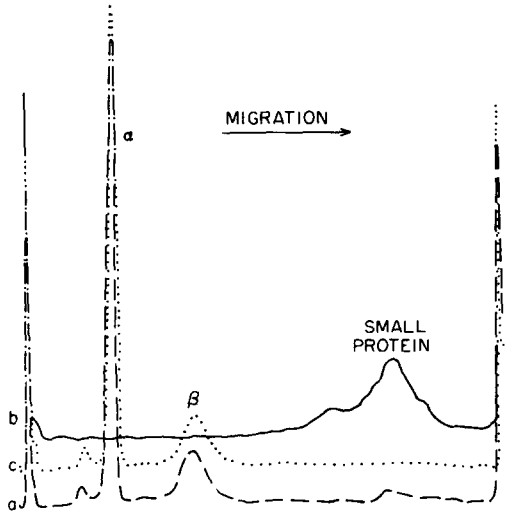

Figure 1. Presence of a small protein in avian nasal salt glands and its separation from the α and β subunits by treatment with non-ionic detergent. SDS-extracted microsomes were prepared in the presence of protease inhibitors and then extracted with Brij 58 (see text for details). The preparation was then centrifuged at 10^5g for 1/2 hour and the supernatant and the pelleted insoluble residue collected. 7.5% SDS polyacrylamide gels of: (a), SDS-extracted microsomes; (b), residue insoluble in Brij; (c), material solubilized in Brij 58.

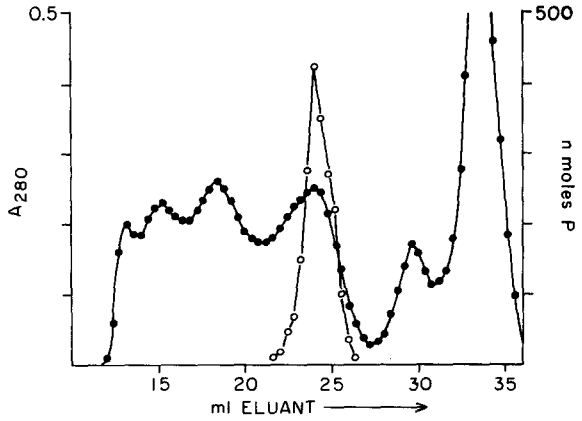

Figure 2. Purification of the small protein from shark rectal glands. 2 mg SDS-extracted microsomes were solubilized with 40 mg SDS per gm protein and 1% v/v 2-mercaptoethanol and then applied to a 0.9 x 58 cm column of Sephacryl S-300 in 3.5 mM SDS, 10 mM sodium phosphate, pH 6.8. Fractions from the trailing edge of the SDS-phospholipid mixed micelle peak were pooled and lyophilized. (●), A_{280}; (○), nmoles organic phosphorus.

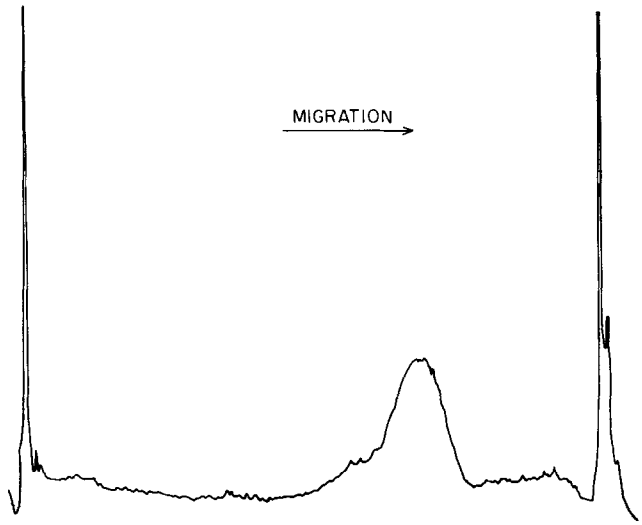

Figure 3. Small protein from shark rectal glands. 7.5% polyacrylamide gel of the column-purified small protein. Approximately 90% of the material is represented by the major peak of 11,100 daltons.

The amino acid composition of the small protein (90% 11,100 dalton component) isolated from shark rectal gland membrane-bound Na,K-ATPase is shown in Table II. The overall composition is not unusually hydrophobic, although the small protein was found to be soluble in chloroform-methanol. The absence of amino sugars in the analyses indicates no contamination of the small protein preparations by the glycoprotein subunit of the ATPase. The minimum molecular weight is 3672 (ignoring the Ser, Cys and Trp contents). Tryptophan was demonstrated by fluorescence emission at 330 nm by excitation at 295 nm. The Trp : Tyr ratio was estimated from the spectra in SDS to be 0.56 (average of two spectra). An interesting feature of the analyses of the shark rectal gland small protein is their similarity to the composition of the γ 2 proteolipid found in the membrane-bound (Na,K)-ATPase from lamb kidney outer medullar (13,14), the main difference being in the Ileu contents. When e.g. Preparation 3 of the shark gland small protein was compared to the γ 2 proteolipid by squaring the differences for each amino acid and summing these, a figure of 34.3 was obtained. Values below 50 indicate a high probability of

Table II. Amino acid analysis of the small protein from shark rectal glands compared the to the γ 2 proteolipid. Preparation 1 was hydrolyzed for 24, 48 and 72 hours and Preparations 2 and 3 for 24 hours only.

Moles per 100 moles	Preparation 1	Preparation 2	Preparation 3	γ2 proteolipid[a]
Lys	8.66	9.49	8.33	7.64
His	2.98	3.16	3.06	2.52
Arg	6.77	6.52	6.63	6.94
Axn	11.39	12.76	11.10	10.42
Thr	6.77	6.14	5.95	3.95
Glx	10.42	12.60	11.74	10.53
Pro	5.65	5.69	5.97	5.54
Gly	12.55	12.51	12.87	10.26
Ala	10.26	8.83	9.60	8.97
Val	5.44	3.10	3.69	5.14
Met	0.34	0	0.74	1.34
Ileu	2.69	2.55	2.83	6.11
Leu	8.16	7.93	7.43	10.05
Tyr	3.19	4.13	4.70	4.96
Phe	4.72	4.70	5.32	5.42

[a]The data of Reeves et al (13) have been adjusted to leave out Ser and Cys, which were not determined in this work, to allow direct comparison.

sequence homology (22). Such conservation between Chondrichthyes and Mammalia may mean that the small protein fulfills some basic function in the membrane.

The stoichiometric ratio of the small protein of shark rectal gland Na,K-ATPase to the α and β subunits in highly purified membrane-bound preparations is very suggestive of a relationship of the small protein to the Na-K pump. The separation from the α and β subunits when membrane-bound Na,K-ATPase is extracted with Brij 58 indicates that the small protein is not involved directly in ATP hydrolysis, and that the α and β subunits are sufficient for this. However, the small protein might function in some other aspect of ion transport, e.g. in an ion channel or as an ionophore.

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