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Phosphorylation of Lens Membrane: Identification of the Catalytic Subunit of Na^+, K^+-ATPase

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Summary: Phosphorylation of lens membrane in the presence [γ-P^{32}]ATP resulted in the radiolabeling of a protein doublet with an approximate molecular weight of 90,000. A doublet of the same relative mobility was radiolabeled after similar treatment of Na^+, K^+-ATPase from brain, and the phosphorylation of both doublets was inhibited in the presence of K^+. Verification that the radiolabeled lens doublet was the catalytic subunit of Na^+, K^+-ATPase was obtained by comparison of their phosphopeptides with these from Na^+, K^+-ATPase from brain.

Introduction

As an intrinsic component of the plasma membrane, Na^+, K^+-ATPase plays a key role in the regulation of intracellular Na^+ and K^+ levels. Its mechanism of action involves the translocation of Na^+ across the membrane with the concomitant formation of an acyl phosphate intermediate. Correspondingly, translocation of K^+ in the opposite direction is activated by dephosphorylation of this intermediate (1-7).

A decrease in Na^+, K^+-ATPase activity has been reported during cataract formation in the galactose-fed rat (8), the hereditary mouse (9), and the human senile lens (10). In the mouse model system, the decrease in Na^+, K^+-ATPase activity has been shown to be the initiating event in subsequent

Abbreviations: Buffer A; 1 mM ethylenediaminetetraacetic acid, 1 mM phenylmethylsulfonylfluoride, 5 mM Tris·HCl; pH 7.9: sample buffer; 1% (w/v) sucrose, 4% (w/v) sodium dodecyl sulfate, 0.063 M Tris·HCl, pH 6.8.

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cell swelling and lens opacification (9). The identification and characterization of lens Na^+, K^+-ATPase on a molecular level would therefore be an important prerequisite to understanding the mechanisms involved in loss of enzyme activity preceding lens opacification.

Because of its important regulatory role in various tissues of the body, extensive efforts have been directed towards the isolation of Na^+, K^+-ATPase. Highly purified preparations from the kidney (11-13), rectal gland (14), electric organ (15), salt gland (16) and brain (17) of various species have all indicated that the enzyme is comprised of a large subunit of approximately 90,000-100,000 daltons, and a smaller subunit of approximately 40,000-60,000 daltons. The larger subunit is radiolabeled in the presence of [γ-P^{32}]ATP, and therefore most likely possesses catalytic activity. The detection of this phosphorylated intermediate is dependent upon the presence of Na^+ in the reaction mixture, and is not present when K^+ is substituted for Na^+. In this report we have exploited these characteristics to identify the catalytic subunit of Na^+, K^+-ATPase in the plasma membrane of the lens, and have employed tryptic peptide mapping to verify this identification.

Methods

Preparation of lens fractions: Decapsulated lenses from adult chickens were homogenized in ten times volume of buffer A. A small amount of homogenate was saved for assay of Na^+, K^+-ATPase, and the remaining homogenate was pelleted at 27,000 x g for 10 min. The supernatant was retained for analysis of Na^+, K^+-ATPase activity. The pellet was used for the sucrose gradient purification of membrane (18). Protein of all fractions was determined using the Coomassie Blue assay (19).

ATPase assay: Na^+, K^+-ATPase of lens fractions was measured according to Muszbek et al. (20). The reported activities were calculated from the difference in phosphate produced in the presence and absence of 0.1 mM ouabain.

Phosphorylation and resolution of phosphoproteins and phosphopeptides: Phosphorylation of lens fractions in the presence of either Na^+ or K^+ was essentially according to Hokin et al. (14). Approximately 100 µg of protein in either 50 µl of Na^+ or K^+ buffer was incubated for 10 sec. at 0° with 0.008 µmoles [γ-P^{32}]ATP (New England Nuclear, 7.7 Curies/mmole), followed immediately by quenching with an equal volume of sample buffer. In a parallel experiment, approximately 10 µg of a highly enriched preparation of Na^+, K^+-ATPase from bovine brain (21) was phosphorylated in an identical manner.

Following addition of sample buffer, the volume (approximately 120 µl) was resolved on 6.0% polyacrylamide gels according to Laemmli (22). After staining and destaining, the gels were dried and exposed to XRP-1 film and Dupont Cronex intensifying screens. Phosphorylated bands were excised from the gel, digested with trypsin, and mapped in two dimensions as previously described (23). Molecular weights were approximated using human erthrocyte membrane proteins as markers (24).

Table I

Na^+, K^+-ATPase Activities of Lens Fractions

Lens Fraction	Protein Analyzed (µg)	Specific Activities* (nmoles/mg/hr ± S.D.)
membrane	15	29. ± 7.
supernatant	2500	0.47 ± 0.6
homogenate	60	1.10 ± 0.6

* Reported values are the average of three determinations.

Results

Previous histochemical analyses have localized Na^+, K^+-ATPase to the membrane of the lens (25). This conclusion is consistent with the results shown in Table I. The specific activity of this enzyme in the lens is approximately 26 times greater than that of the crude homogenate and approximately 62 times greater than that of the soluble proteins of the supernatant.

To identify the catalytic subunit of Na^+, K^+-ATPase from the lens membrane, the membrane fraction was phosphorylated with [γ-^{32}P]ATP, followed by resolution using gel electrophoresis. Autoradiography revealed the presence of a doublet of approximately 90,000 daltons (Figure 1a). Identical phosphorylation in the presence of K^+ resulted in the absence of any detectable phosphorylation in this molecular weight range (Figure 1b).

Phosphorylation of Na^+, K^+-ATPase from the rectal gland of the dogfish shark (14), and electric eel organ (15) have revealed the presence of only a single band of approximately 90,000-100,000 daltons, while similar studies of Na^+, K^+-ATPase from brain and brine shrimp have revealed a doublet (21,26-28). Figure 1c illustrates the phosphorylated doublet previously observed in bovine brain. This doublet is also not detected in the presence of K^+ (Figure 1d), and migrates with an identical relative mobility as the phosphorylated doublet from the lens (Figure 1a).

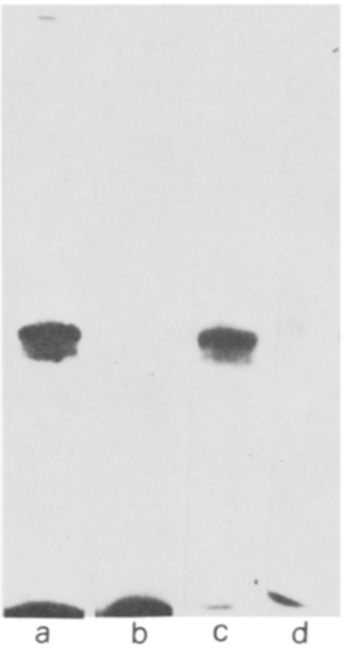

Figure 1: Phosphorylation of lens membrane and bovine brain Na^+, K^+-ATPase in the presence of Na^+ or K^+. See Methods for details of phosphorylation. a, lens membrane phosphorylated in the presence of Na^+, b, lens membrane phosphorylated in the presence of K^+, c, bovine brain Na^+, K^+-ATPase phosphorylated in the presence of Na^+; d, bovine brain Na^+, K^+-ATPase phosphorylated in the presence of K^+. All samples were run on the same slab gel, but lanes a and b were exposed for 6 days to X-ray film while c and d were exposed for 3 hrs.

Since we have not yet been able to purify the catalytic subunit of Na^+, K^+-ATPase from the lens membrane, we felt that further confirmatory data involving similarities in phosphorylation would be helpful in ascertaining the identity of the phosphorylated lens polypeptides as Na^+, K^+-ATPase. In Figure 2, the tryptic peptides of each of the phosphorylated species from lens and bovine brain Na^+, K^+-ATPase were resolved in two dimensions and visualized by autoradiography. Each species contained only one major phosphorylated peptide. Furthermore, the phosphopeptide from the components of bovine brain Na^+, K^+-ATPase were resolved in an identical manner both with respect to themselves (Figures 2a & 2b), as well as with respect to the phosphorylated species from lens membrane (Figure 2c and 2d).

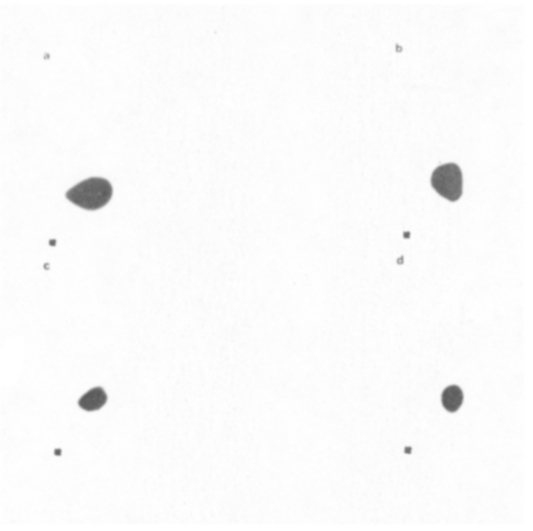

Figure 2: Tryptic peptide mapping of the phosphorylated doublet from lens membrane and bovine brain Na^+, K^+-ATPase. Approximately 1,000 cpm of phosphopeptides were resolved on a two-dimensional system as described in Methods, followed by exposure for 5 days. Heavy arrows designate sample origins. First dimension electrophoresis was from left to right and second dimension chromatography was from bottom to top. a, upper band of bovine brain Na^+, K^+-ATPase doublet; b, lower band of bovine brain Na^+, K^+-ATPase doublet; c, upper band of lens membrane doublet; d, lower band of lens membrane doublet.

Discussion

Although numerous reports have measured Na^+, K^+-ATPase levels in the lens (9,10,25), no one has yet identified the molecular component of the lens that possesses enzyme activity. This information is especially important when considering the key role Na^+, K^+-ATPase plays in lens cataract formation.

In regards to this objective, initial efforts were directed towards purification of the lens enzyme by previous methodology used in other tissues (13). The results were unsatifactory, principally because of the low level of Na^+, K^+-ATPase in lens membrane. These preparations contain less than 0.1% of the levels found in membranes from bovine brain (17) and rectal gland of dogfish (14). In addition, lens membrane contains large amounts of an intrinsic polypeptide called MIP (23) that is difficult to separate from the intrinsic subunits of Na^+, K^+-ATPase (results not shown).

Faced with these problems, we have exploited protein phosphorylation to identify the larger catalytic subunit of the enzyme. This phosphorylation

results in a doublet which migrates with an identical relative mobility with respect to the previously observed doublet from bovine brain. In addition, in both lens and bovine brain, the radiolabeled doublet is not detected when phosphorylation is done in the presence of K^+.

The identification of lens Na^+, K^+-ATPase has been further substantiated by comparison of the phosphorylated tryptic peptides from lens membrane and bovine brain. These radiolabeled peptides all migrate in an identical manner in two dimensions, a conclusion that has been verified by resolving a peptide mixture of equal counts from lens and brain together (results not shown). Furthermore, the migration of this phosphopeptide is not common to all types of protein phosphorylation since peptide analysis of tryptic fragments of phosphorylated protein kinases demonstrated no homology with those of Na^+, K^+-ATPase from brain or lens (results not shown).

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