ERYTHROCYTE MEMBRANE PROTEINS AND ADENOSINE TRIPHOSPHATASE ACTIVITY

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SUMMARY Erythrocyte membrane was treated from outside with crude mold protease, and the membrane proteins were analyzed on acrylamide-gel electrophoresis in the presence of sodium dodecyl sulfate. Band-III protein, in the region of an intermediate of Na-K-ATPase, was digested almost completely, although approximately 80% of the ATPase activity was retained by the treated membranes. It is concluded that band-III protein is not the Na-K-ATPase intermediate, although they co-electrophorese.

INTRODUCTION A number of investigators[1-5] have used the results of acrylamide-gel electrophoresis in SDS* of solubilized erythrocyte membranes to develop models for the arrangement of the proteins in the lipid layer of the membrane. One of the major proteins of the membrane, band-III protein[5,6], extends from the outer membrane surface to the inner membrane surface of the cell[3,5]. The molecular weight of band-III protein has been reported to be 89,000 to 105,000 [2,6]. This protein is a single polypeptide[3].

During the course of ATP hydrolysis by Na-K-ATPase(ATP phosphohydrolase EC 3.6.1.3), a peptide component of the enzyme can be labeled with [γ-^{32}P]ATP, in the presence of Na^+ and Mg^{++} [7]. The radioactive phosphorylated peptide is detected on the region of the band-III protein[8]. Trypsin digestion of the erythrocyte membrane causes inactivation of the ATPase and loss of structure[9]. With milder digestion conditions, band-III protein is attacked selectively[10], and it was suggested that band-III protein is one of the components of Na-K-ATPase molecule.

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*The abbreviation used is: SDS, sodium dodecyl sulfate.

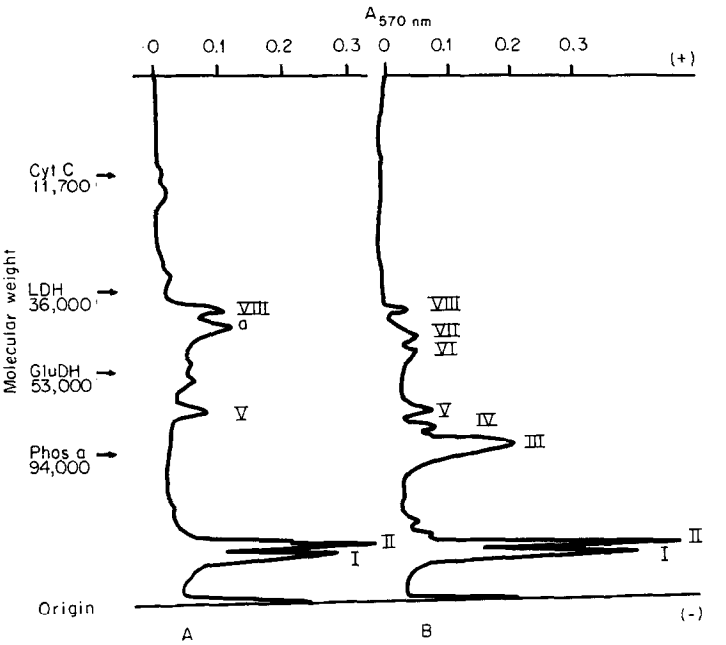

Fig. 1 Electrophorogram of erythrocyte membrane proteins, from erythrocytes treated(A) and not treated(B) with "lytic enzyme-3". Membrane samples were prepared and electrophoresed in 0.1% SDS on 7.5 % acrylamide gel as described in MATERIALS AND METHODS. The gel was loaded with about 100μg of membrane protein. Gel were scanned at 570 nm after staining. Arrows show the mobility of proteins with known molecular weight; cytochrome c(Cyt.C), glutamate dehydrogenase(GluDH), lactate dehydrogenase(LDH) and phosphorylase-a(Phos-a).

In an attempt to separate the enzymes from the erythrocyte membrane, proteases were applied to the intact membrane. It was possible to digest the band-III protein without significant loss of Na-K-ATPase activity.

MATERIALS AND METHODS Crude enzyme containing proteases and β-1,3-glucanase, "lytic enzyme-3", was purchased from Kyowa Hakko Co. (Tokyo). The crude enzyme was obtained by ammonium sulfate fractionation and acetone fractionation from mold.

MEMBRANE PREPARATION. Human blood (50 ml) was centrifuged, and the plasma and buffy coat were removed. The cells were washed three times with 0.155 M sodium phosphate buffer, pH 7.4, and

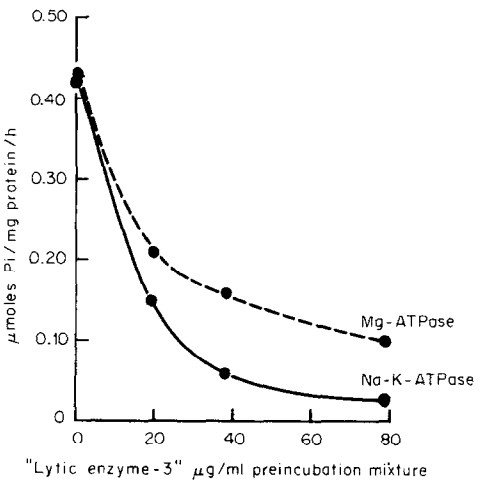

Fig. 2 Effects of hydrolysis of membrane preparation on ATPase activity. Erythrocyte membrane suspension (3.2 mg protein/ml) was mixed with 60 μl of "lytic enzyme-3" (0~80 μg), and incubated for 20 minutes at 37°, followed by centrifugation at 156,500×g for 30 minutes. The precipitate was suspended in 1 ml of 1 mM EDTA, pH 7.0. 0.1 ml of each was used for the ATPase assay. The assay method is described in the legend of Table 1.

finally suspended in 40 ml of the phosphate buffer. The washed cells (10 ml) were treated with the "lytic enzyme-3" (1 mg) for 3 h at 37°. The treated cells were centrifuged off and washed once with the phosphate buffer. Hemolysis and membrane preparation were performed by the method of Dodge and his co-workers[11]. The isolated membranes were washed twice with 0.5 mM EDTA-Tris, pH 7.5, and suspended in the EDTA-buffer solution to give 1~2 mg of membrane protein per ml.

SOLUBILIZATION OF MEMBRANE. The membrane suspension was added an equal volume of 2% SDS solution containing 0.4% mercaptoethanol, and incubated in boiling water for 2 minutes.

SDS ACRYLAMIDE-GEL ELECTROPHORESIS. The method of Weber and Osborn[12] was employed. Calibration curves were obtained with proteins of known molecular weights with disulfide bonds reduced. Coomassie brilliant blue was used for the staining of protein.

Table 1. Effects of Hydrolysis of Intact Cells on ATPases.

Pretreated with	Activity Na-K-ATPase	Mg-ATPase
	(μmoles Pi/mg/h)	
None	0.34	0.85
"lytic enzyme-3"	0.31	0.82
None	0.46	0.73
"lytic enzyme-3"	0.32	0.88
None	0.28	0.27
pronase	0.30	0.30
None	0.34	0.25
trypsin	0.06	0.14

ATPase activity was assayed in a final volume of 1 ml, containing 140mM NaCl, 14mM KCl, 5mM $MgCl_2$, 0.5mM EDTA, 3mM ATP, and 50mM Tris buffer, pH 7.5, in the presence or the absence of 0.2mM ouabain. Incubation was for 1 hr at 37°. Mg-ATPase: the activity measured with ouabain, Na-K-ATPase: activity without ouabain minus activity with ouabain.

INORGANIC PHOSPHATE DETERMINATION. In order to eliminate the deproteinization step, 0.1 ml of 5% SDS solution was added to each assay medium after incubation, followed by analysis by the method of Fiske and SubbaRow[13]. No turbidity was observed. This method was successful for the determination of inorganic phosphate even in the presence of large amounts of protein (Tashima, manuscript in preparation).

PROTEIN DETERMINATION. The method of Lowry and his co-workers[14] was used. The specific activity of ATPase was defined as μmoles Pi per mg protein per hour.

RESULTS AND DESCUSSION

After treatment of intact cells with the "lytic enzyme-3", the erythrocyte membrane fraction was prepared, solubilized, and analyzed by SDS-acrylamide gel electrophoresis. As shown in Fig. 1, the control sample gave approximately same pattern as reported previously[1,2,5]. Band-III and -IV disappeared almost completely in the treated samples. Band-a was the most prominent of the new bands.

Its molecular weight was approximately 43,000. Bands-I, -II and -V were not changed remarkably. The pretreated sample yielded no distinct bands in the region between bands-II and -V. It appeared that the proteins on bands-III and -IV were digested to peptides with molecular weights smaller than band-V.

After treatment of erythrocytes with the "lytic enzyme-3", the remaining Na-K-ATPase activity averaged 80% of the control values, as shown in Table 1. The p-nitrophenylphosphatase activity depending on K^+ was not changed remarkably by the pretreatment (Data are not shown).

The average specific activity of Na-K-ATPase was from 0.3 to 0.8 units per mg membrane protein. Band-III protein represents 15% [2] or 30% [5] of the whole membrane proteins stained on the gel. The specific activity of the ATPase as counts per mg band-III protein is less than 5.6, which is lower than Na-K-ATPase activity of kidney[15, 16] or brain[17, 18]. If all of band-III protein were a component of Na-K-ATPase, specific digestion of this protein would cause some change in the characteristics of the ATPase activity. The "lytic enzyme-3" did not change the effects of Na^+ and K^+ concentration or of ouabain on the ATPase activity, and did not alter the stability of the ATPase in alkaline solution (pH 8.5) or in 1.6 M NaI. Thus, the major component of band-III appears to be unrelated to the Na-K-ATPase.

If the Na-K-ATPases from erythrocyte, kidney and brain are assumed to have similar specific activity, as counts per mg protein (above 500[15~18]), it can be estimated that less than 1.1 % of the band-III protein would be responsible for the ATPase activity. This quantity would constitute a negligible band on the SDS-acrylamide gel electrophorogram.

In contrast to the intact cells, digestion of erythrocyte membrane preparations was found to cause a rapid loss of Na-K-ATPase

activity, as shown in Fig. 2, and a loss of distinct bands on the gel electrophorograms.

The specificity of the outer surface of erythrocytes to proteases was observed under identical conditions (See Table 1). The effects of pronase were similar to those of the "lytic enzyme-3". Trypsin inactivated Na-K-ATPase strongly. In all cases, mainly bands-III and -IV were attacked. The resistance of the ATPase activity to nonspecific proteases might suggest that a very small area of Na-K-ATPase protein faces the outside of the cell.

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