# $Na^+$-ACTIVATED ATPASE IN PLASMA MEMBRANE OF MARINE ALGA *HETEROSIGMA AKASHIWO*

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## ABBREVIATIONS

bp : base-pair

β-GP : Beta-Glycerophosphate

CsTFA : cesium trifluoroacetate

DCCD : dicyclohexylcarbodiimide

DEPC : diethyl propyl carbonate

ELISA : enzyme-linked immunosorbent assay

HEPES : N-2-hydoxyethylpiperazine-N'-2-ethanesulfonic acid

IAI : Isethionyl acetimidate

kbp : kilo-base-pair

kDa : kilo-dalton

PBS : phosphate buffer saline

PCR : polymerase chain reaction

PNP : para-nitro-phosphate

SDS-PAGE : sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Tris : 2-amino-2-hydromethyl-1,3-propanediol

## General Introduction

The Raphidophyceae(=Chloromonadophyceae) is a small group of little studied golden-brown flagellates. They were originally described from fresh waters, but are now also widely recognized in coastal waters(Loeblich and Fine 1977).

Basic features of the unicellular structure of raphidophytes include two unequal flagella arising from an apical depression: the forward flagellum bears two rows of fine hairs, while the trailing flagellum is smooth and lies close to the surface of the cell(Yokote et al. 1985).

One of marine raphidophycean, Heterosigma akashiwo is a dominant species of red tides in the coastal waters of Japan(Iizuka and Irie 1968, Honjo et al. 1978) and North America(Tomas 1980), and has the interesting physiological and structural characteristics as follows; 1) the cell division and chloroplast replication occur as separate synchronous events diurnally(Cattolico et al. 1976, Nemoto and Furuya 1985, Satoh et al. 1987), 2) they migrate diurnally between surface and bottom of sea water(Wada et al. 1985), 3) they have no structure like cell wall around plasma membrane(Yokote et al. 1985). The third character, lack of cell wall, offers great advantages to the investigation of organelles, especially to that of plasma membrane. In higher plants, cell walls are actually obstacles to isolate and investigate plasma membrane.

The common higher plants can not live in coastal areas whose soil contains sea water because they have no effective $Na^+$-pumping out systems to regulate ion concentration in the

cells. This means the possible presence of such systems in H. akashiwo, a kind of plant living in sea water. Moreover, according to Yamochi(personal communication), the red tide bloom of H. akashiwo is known to occur when the salinity of sea water decreases by the rainfall in the upper stream of rivers. The decrease of salinity should result in the decrease of both osmotic pressure and nutrient concentration. This means that H. akashiwo cells have the system to adapt the plasma membrane potential for nutrient absorption or osmotic pressure regulation under the wide range of salinity.

In sea water, most abundant ion is $Na^+$, which is common to the composition of animal body fluid. The animal cells make plasma membrane potential by $Na^+$ and $K^+$ gradient across the membrane under $Na^+$-rich environment. These gradients are formed by $Na^+,K^+$-ATPase in plasma membrane. The existence of similar selective $Na^+$ extrusion mechanism like animal $Na^+,K^+$-ATPase is supposed to be in marine algae for the regulation of osmotic pressure and/or formation of membrane potential. However,there have been only two reports(Sullivan and Volcani 1975, Sullivan 1978) about the presence of $Na^+,K^+$-ATPase activity in marine diatoms, in which the plasma membrane preparation was not reliable. Then, no further biochemical studies have been done.

In this thesis, I first detected the presence of $Na^+$-activated ATPase activity in the highly purified plasma membrane of H. akashiwo and characterized its enzymological characteristics(Part I) and then showed the immunological

cross-reactivity between H. akashiwo $Na^+$-activated ATPase and animal $Na^+,K^+$-ATPase(Part II). Finally, I cloned a part of cDNA of the $Na^+$-activated ATPase of H. akashiwo, and discussed about the homology to the other known ATPases and its functions(Part III).

# PART I

## CHARACTERIZATION OF $NA^+$-ACTIVATED ATPASE ACTIVITY ON THE PLASMA MEMBRANE OF THE MARINE RAPHIDOPHYCEAN HETEROSIGMA AKASHIWO

## Introduction

A major function of the plasma membrane is its involvement in the regulation of ion transport at the cell surface(Hodges 1976, Poole 1978, Quail 1979). In general, plants cells have been found not to have a $Na^+,K^+$-ATPase that acts as a $Na^+,K^+$ antiport across the plasma membrane. If such was also the case in marine plants, we must ask how these plants regulate the transport of $Na^+$ and $K^+$ across the membrane. A $Na^+$-pumping system must exist in those marine plants cells that grow in a $Na^+$-rich environment which bears a close resemblance to the body fluid of animals.

These have been few reports about ion transport systems in marine plant cells. ATPase activity, which may be related to ion transport, was found in a crude membrane fraction from a marine diatom(Sullivan and Volcani 1974, 1975). However, the impurity of the isolated membranes prevented further characterization of the enzyme.

H. akashiwo, a marine raphidophycean unicellular biflagellate, is a naturally occurring, wall-less organism(Yokote et al. 1985) which is useful for both the preparation of plasma membranes and investigations of its physiological functions. In this Part I, I describe the isolation of highly purified plasma membranes from H. akashiwo cells and the existence of a $Na^+$-activated ATPase in the purified plasma membrane.

## Materials and Methods

### Growth and Maintenance of Cells

Axenic clones of Heterosigma akashiwo (strain number OHE-1), were purchased from M. M. Watanabe of National Institute for Environmental Studies in Japan, were grown in 300 ml Erlenmeyer Flasks containing 150 ml of slightly modified ASP-7 which contained only vitamin $B_{12}$ as the vitamin source(Watanabe et al. 1982). All cultures were inoculated with living cells to a constant concentration of $10^3$ cells/ml and cultured at $20\pm1^{\circ}C$ in a growth chamber having a photoperiod of 12 hr light-12 hr dark. Light was given from above and the light intensity was adjusted at 4,000-6,000 lux with cool white fluorescent lamps(Toshiba FL 20-SW Tokyo Shibaura Electric Co. Kawasaki, Japan).

### Isolation of plasma membranes

The plasma membranes were isolated according to the method of Chaney(Chaney and Jacobson 1983, Schmidt et al. 1983, Wesserman et al. 1984) as follows;

1) Preparation of positive charged microbeads

Silica microbead(Sigma S5505; 0.014 μm) suspension(18 g in 182 ml of distilled water) was mixed with aluminum chlorohydroxide solution(15 g in 133 ml of distilled water). This mixture was stirred at $80^{\circ}C$ for 30 min, and incubated at room temperature for 16 hr. The pH of the solution was then adjusted to 5.0 with 1 N NaOH. Just prior to use, 1 ml of the silica suspension was added to 6.0 ml of coating buffer composed of 10 mM MES-NaOH(pH 6.5), 0.5 M sorbitol, 1 mM $MgSO_4$, and 2 mM KCl. The bead suspension was

centrifuged at 1,500xg for 10 min to remove aggregated materials, then diluted 8 times with the coating buffer.

2) Cell harvesting and bead coating

The culture solution(600 ml) of H. akashiwo cells in late log growth phase($8.0\text{-}15.0\times10^4$ cells/ml) was centrifuged at 300xg for 3 min at 20°C, and the precipitated cells were gently resuspended with 6.0 ml of the coating buffer. Six ml of colloidal silica suspension were mixed with the cell suspension in a beaker. After 5 min of stirring, 12 ml of neutralization solution containing 0.1 mg/ml polyacrylic acid(Mr 9,000; Aldrich Chemical Co.) in coating buffer were added. The mixture was placed on ice for 10 min, then centrifuged at 140xg for 3 min to remove the excess polyacrylic acid and microbeads. The bead-coated cells were collected as precipitate.

3) Cell lysis and fractionation

The bead-coated cells were suspended in 20 ml of a lysis buffer of 5 mM Tris-HCl(pH 8.0) with 1 mM EGTA, and homogenized with a glass-Teflon homogenizer at full speed for 5 strokes. The lysate was centrifuged at 1,000xg for 3 min at 4°C, and the resulting pellet was rinsed twice with 20 ml of lysis buffer, referred to the bead-bound membrane (BM) fraction. The supernatant was further centrifuged at 80,000xg for 1 hour at 4°C, and that resulting pellet and supernatant were referred to as the free membrane(FM) and supernatant(Sup) fractions, respectively. The obtained bead-bound membrane was suspended in 30 mM Tris-HEPES(pH 8.0) containing 0.5 M sorbitol.

**[$^{14}$C]-IAI labeling of plasma membrane**

[$^{14}$C]-isethionyl acetimidate(IAI; specific activity 50 μCi/ml) which specifically conjugates with amides on the cell surface(Whiteley and Berg 1974) was added to the cell suspension(10-13x$10^4$ cells/ml) to give a final concentration of 0.83 μCi/ml and allowed to stand for 2 hrs. After being rinsed for 3 times in 60 ml of ASP-7 at 4°C, the cells were collected by centrifugation at 300xg for 3 min and the plasma membranes by the silica microbead method mentioned above. An equal volume of 20 % TCA solution was added to each fraction. The resulting precipitates were collected by filtration through a Whatman GF/F glass filter paper, and thoroughly rinsed first with 5 % TCA, and then with 70 % ethanol. After drying, radioactivity on the filter papers were counted with a Beckman liquid scintillation spectrometer(Model LS-250). The scintillation liquid consisted of 0.4 % 2,5-diphenyloxasol and 0.01 % 1,4-bis-2-(5-phenyl-oxasolyl)-benzene in toluene.

**Organelle markers**

Cytochrome c oxidase, NADPH-dependent cytochrome c reductase, IDPase were determined spectrophotometorically as the respective markers of mitochondria, endoplasmic reticulum and Golgi body by the method of Hodges and Leonard(Hodges and Leonard 1974). The assay of NADPH cytochrome c reductase was performed at room temperature by measuring the cytochrome c content at 550 nm with a recording Shimazu spectrophotometer(UV-260 Shimazu, Co. Kyoto Japan). The 3 ml reaction system contained of 0.1 ml

of samples(10-50 μg of protein), 0.1 ml of 50 mM sodium cyanide, 0.2 ml of 0.45 mM cytochrome c, and 2.5 ml of 50 mM phosphate buffer(pH 7.5). The reaction was started with the addition of 0.1 ml of 3 mM NADPH. The assay of cytochrome c oxidase was performed as above except that the oxidation of reduced cytochrome c was measured. Cytochrome c was chemically reduced with addition of a few crystals of sodium dithionite. Excess dithionite was removed by passing air through the solution for a few minutes. A 3 ml reaction system consisted of 0.1 ml of sample(10-50 μg of protein), 0.1 ml of 0.3 % digitonin, and 2.7 ml of 50 mM phosphate buffer(pH 7.2). The reaction was started by the addition of 0.1 ml of reduced cytochrome c. Chlorophyll contents were calculated by Jeffrey's method(Jeffrey 1972) as the marker of chloroplast. Each 20 μl of samples was added to 1.8 ml of 100 % acetone. After heavy shaking by a vortex, acetone solutions were kept at -20°C for 30 min. Insoluble materials were precipitated by centrifugation at 3,000xg for 5 min, and then, the supernatant(1 ml) was measured with spectrophotometer at 630, 647 and 664 nm. Protein content was measured by the method of Bradford(Bradford 1976).

**Assay for ATPase activities**

The assay for ATPase activity was measured at 37°C for 30 min in a 0.5 ml reaction mixture containing 25 ul of isolated plasma membrane (ca 2.5 μg protein) in 30 mM Tris-HEPES(pH 8.0), 3 mM Tris-ATP, 5 mM $MgSO_4$, 100 mM NaCl, and 100 mM KCl. ATPase activity was calculated from the amount

of the release of inorganic phosphate by the method of Kasamo(Kasamo 1979). The Pi released from hydrolyzed ATP was measured as follows. An aliquot(2 ml) of the reacted mixture was pipetted into a test tube containing 2 ml of iso-buthanol, 0.5 ml of 1.5 N $H_2SO_4$ and 0.5 ml of 2 % sodium molybdate, then mixed vigorously for 10 sec. An aliquot(1 ml) of the iso-buthanol layer was pipetted into another test tube to which 1 ml of 0.5 % ascorbic acid and 0.5 ml of ethanol were added successively, and mixed vigorously for 10 sec. The test tubes were heated at 37°C for 30 min. Then, the absorbance at 720 nm was determined with spectrophotometer(UV-260 Shimazu, Co. Kyoto Japan). To characterize the ATPase activity on the bead-bound membrane, the composition of the reaction mixture was varied with respect to ions, pH, temperature, substrates and inhibitors as indicated in the legends of figures and tables.

**Phosphorylation**

Phosphorylation of the catalytic subunit of ATPases were performed according to the method of Briskin and Leonard with slight modification(Briskin and Leonard 1982a,b). The assay was carried out at 4°C in a 0.5 ml reaction mixture containing 40 μM [$\gamma$-$^{32}$P] ATP(3,000 Ci/mmol), 0.1 mM $MgSO_4$, 30 mM Tris-HEPES(pH 8.0) and of bead-bound membranes(80 μg protein). The reaction was started by the addition of [$\gamma$-$^{32}$P] ATP, and after the 40-sec phosphorylation, quenched by the addition of 10 ml ice cold 10 % TCA solution containing 40 mM $NaH_2PO_4$, 5 mM $Na_2P_2O_7$, and 1 mM ATP(disodium salt). The quenched reaction mixture was centrifuged at 3,000xg for

5 min at 4°C. To characterize the ATPase, each solution of monocations, cold nucleotides or EDTA was added to the reaction mixture. The obtained pellet was suspended in 10 ml of 10 % TCA solution mentioned above. After three additional cycles of suspension and centrifugation, the pellet was suspended in 100 µl of 50 mM $NaH_2PO_4/H_3PO_4$(pH 2.4), 1.0 %(w/v) SDS, 2 %(v/v) β-mercaptoethanol, 4 M urea, 20 %(v/v) glycerol, 10 µg/ml Pyronine Y and incubated at 4°C for 30 min. The phosphorylated intermediates of ATPases were detected by SDS electrophoresis. The electrode solution was composed of 50 mM $NaH_2PO_4$(pH 2.4) and 0.1 %(w/v) SDS, and the electrophoresis was conducted at 20 mA for 1.5-2.0 hr at room temperature. After electrophoresis, the gels were immediately dried in vacuo in two sheets of cellophane paper and subjected to autoradiography(4 to 6 hr) against Kodak AR X-ray film with intensifying screens at room temperature.

In some cases, the gels corresponding to ATPase intermediates were cut out and their radioactivities were measured by the Cerenkov radiation with a Beckman liquid scintillation spectrometer(Model LS-250).

## Results

### Isolation of plasma membrane

The distribution of organelle markers and radioactivity derived from [$^{14}$C]-IAI in each fraction which was obtained by the silica microbead method is shown in Table I-1. Almost all of NADPH cytochrome c reductase, cytochrome c oxidase and IDPase activities, and chlorophyll were detected in bead-free membrane fraction(FM), the remainder being in bead-bound membrane fraction (BM). On the other hand, more than 60 % of the total radioactivity derived from [$^{14}$C]-IAI, which specifically conjugates with amines on membrane surface membrane, was detected in bead-bound fraction(BM). These results indicate that the BM fraction contains highly purified plasma membrane, the recovery of plasma membranes being ca. 66 %.

### Effects of monovalent and/or divalent cations on the activity of plasma membrane bound ATPases

The effect of divalent cations on ATPase activities was tested in the absence of monovalent cations using the obtained plasma membrane. The results are shown in Table I-2. The ATPase was activated by the divalent cations, the order of activation being Mn>Ca>Mg at 5 mM. Data not given here indicated that maximum $Mg^{2+}$ activation was obtained at concentrations higher than 4 mM $Mg^{2+}$.

The monovalent cations activated the ATPase activities in the presence of 5 mM $Mg^{2+}$, the highest activation was obtained by the addition of $Na^{+}$(Table I-3). Under the presence of $Na^{+}$, the ATPase activity was increased by the

further addition of other monovalent cations, the order of increase being $NH_4$>Cs>Li>K>Rb. On the other hand, the co-application of KCl with other monovalent cations other than $Na^+$ was less effective in activating the ATPase activity. Data not given here indicated that the highest activity was obtained with 100 mM $Na^+$ and 100 mM $K^+$.

**Optimum pH and temperature on activities**

The ATPase activity in the plasma membrane were assayed at 37°C with 30 mM Tris-Hepes buffer(pH 6.3 to 8.8) containing 5 mM $MgSO_4$, 100 mM NaCl, and 100 mM KCl. The profile of pH dependency(Fig. I-1) showed the optimum pH at about 8.0. The optimum temperature was 37°C, when the activities were assayed at pH 8.0 in the presence of 5 mM $MgSO_4$, 100 mM NaCl, and 100 mM KCl(Fig. I-2).

**Substrate specificity on activity**

Substrate specificity of plasma membrane-bound ATPase was assayed with 30 mM Tris-HEPES buffer(pH 8.0) containing 5 mM $MgSO_4$, 100 mM NaCl, 100 mM KCl and 3 mM various substrates at 37°C(Table I-4). Phosphohydrolase activity showed a much higher specificity to ATP than to ADP, UTP, and CTP, but almost none to PNP and β-GP, both artificial substrates for phosphatase. Lineweaver-Burk's plot of ATPase activity gave a straight line, the apparent Michaelis constant(Km) was about 0.7 mM (Fig. I-3).

**Effects of inhibitors on ATPase activity**

The ATPase activity on bead-bound membrane was inhibited by 37 % and 75 % in addition of 1 mM DCCD and 100 uM vanadate respectively(Table I-5). No inhibition was

observed with Ouabain, ammonium molybdate, $NaN_3$, or $KNO_3$.

**Detection and characterization of ATPase intermediates**

Two phosphorylated polypeptide bands were detected on the plasma membrane when analyzed by acid SDS-PAGE(Fig. I-4). Phosphorylation of 95 kDa band occurred intensively without the addition of mono- or divalent cations(lane 1) and prevented by the treatment with 0.2 mM EDTA(lane 2). The addition of 0.3 mM $MgSO_4$ in the presence of 0.2 mM EDTA restored the phosphorylation of the 95 kDa band(lane 3). The 140 kDa band, however, was intensively phosphorylated only in the presence of both 0.3 mM $Mg^{2+}$ and 100 mM $Na^+$ when 0.2 mM EDTA(lane 4) was present, though its phosphorylation did not occur in the absence of $Mg^{2+}$(lane 5). Other monovalent cations, except for $Na^+$, had no effect on the phosphorylation of the 140 kDa band(data not shown).

In the next experiment, both polypeptides were phosphorylated for 40 sec in the presence of $Mg^{2+}$ with or without $Na^+$, and treated with $K^+$ or cold ATP for 20 second(Fig. I-5). Further addition of $K^+$ rapidly dephosphorylated the 140 kDa intermediate(lane 2). Dephosphorylation of 140 kDa polypeptide was also shown with an addition of other monocations except for $Na^+$. The addition of cold ATP decreased the amount of radioactivity of 140 kDa intermediate(lane 3). Table I-6 showed the effect of some nucleotides on the decrease of radioactivity of 140 kDa polypeptide. After 40-sec phosphorylation of the 140 kDa polypeptide in the presence of both 0.1 mM $Mg^{2+}$ and 100 mM $Na^+$, each nucleotide was added for following 40

sec. The addition of cold ATP was most effective in decreasing the radioactivity of 140 kDa polypeptide.

## Discussion

[$^{14}$C]-IAI has been well known as a cell surface labeling reagent which can not penetrate into cells through plasma membrane and converts cell surface amines to radioactive amidinines(Whiteley and Berg 1974). Thus, this reagent is thought to be useful for labeling of plasma membrane of the wall-less organism, H. akashiwo. Since the positive charged silica microbeads tightly bind with the negative charged plasma membrane, it would be expected that the radioactivity derived from [$^{14}$C]-IAI are found only in the bead-bound fraction. Nevertheless, ca. 30 % of the total radioactivity was found in the bead-free membrane and ca. 7 % in supernatant fraction (Table I-1). These could be explained by the supposition that the former derived from plasma membrane which could not bind with silica beads, and the latter from the soluble substances which contain amines and/or loosely bind to plasma membrane. If so, the results in Table I-1 indicate that the plasma membrane could be obtained at a recovery more than 66 % as a bead-bound fraction.

The bead-bound membrane fraction had little activities of NADPH cytochrome c reductase, a marker enzyme of endoplasmic reticulum, and cytochrome c oxidase, a marker enzyme of mitochondria, and IDPase, a marker enzyme of Golgi body, and allow content of chlorophyll, a marker of chloroplast (Table I-1)(Quail 1979). These results indicate that the bead-bound membrane is highly purified plasma membrane with little contamination from other organelles.

In the assay of ATPase activities with the bead-bound membrane fraction, the pH(Fig. I-1) and temperature(Fig. I-2) dependencies and Lineweaver-Burk's plot(Fig. I-3) suggested that a single ATPase exists in the obtained plasma membrane. However, two phosphorylated polypeptides having molecular weights of 140 kDa and 95 kDa were detected on this membrane fraction with acid SDS-PAGE(Fig. I-4). The former phosphorylation increased in the presence of both $Na^+$ and $Mg^{2+}$ and decreased by the further addition of $K^+$ or cold ATP(Fig. I-5). Substrate specificity on the phosphorylation also indicated that ATP was most effective(Table I-6). Moreover, Fig. I-4 shows that both $Mg^{2+}$ and $Na^+$ are indispensable for the phosphorylation of 140 kDa polypeptide. These results suggest that 140 kDa polypeptide is the intermediate of $Na^+$, $K^+$-ATPase(Fukushima and Post 1978, Robinson and Flashiner 1979). The $Na^+$, $K^+$-ATPase in animal tissues is known to be the same as $Na^+$, $K^+$ pump which translocates those ions across the plasma membrane(Robinson and Flashiner 1979). It can be considered that the 140 kDa polypeptide on plasma membrane of H. akashiwo cells is intermediate of ATPase which may plays the role of ions translocation to control the osmotic pressure. The phosphorylation of the latter, 95 kDa polypeptide, was inhibited by EDTA(Fig. I-4;lane 2), and the inhibition was recovered by the addition of $Mg^{2+}$(Fig. I-4;lane 3), but not other divalent cations(data not shown). These results indicate that $Mg^{2+}$ is indispensable for the phosphorylation of 95 kDa polypeptide. The decrease of the

phosphorylation of 95 kDa polypeptide occurred by the further addition of monovalent cations(data not shown). From these results, we believe that the 95 kDa polypeptide is $H^+$ translocating ATPase(Briskin and Leonard 1982a,b). The $H^+$, $K^+$-ATPase of higher plants has been known to make $H^+$ gradient for nutrient absorption or ion translocation(Hodges 1976, Sze 1984). Thus, the 95 kDa phosphorylated polypeptide may be thought to be a $H^+$ translocating ATPase for the absorption of nutrients in H. akashiwo cells. The optimum pH and temperatures, and substrate specificities of both ATPases seem to be closely similar to each other, because the experiments for activity in the bead-bound membrane have shown as if a simple ATPase exists in the obtained plasma membrane.

## Summary

Plasma membranes were isolated with a high purity from Heterosigma akashiwo cells, a marine raphidophycean unicellular biflagellate, according to the silica microbead method, and the characteristics of ATPase activity on the plasma membranes were tested. The ATPase activity was enhanced strongly by $Na^+$ and $K^+$ in the presence of $Mg^{2+}$. The apparent optimum pH and temperature of ATPase activity were 8.0 and 37°C, respectively. Lineweaver-Burk's plot of ATPase activity gave a straight line, the Km value being about 0.7 mM. The ATPase activity in the plasma membrane was much higher specificity to ATP than to ADP, UTP and CTP, but almost none to PNP and β-GP. The activity was inhibited strongly by vanadate and slightly by DCCD. Furthermore, we detected two phosphorylated intermediate forms of ATPases in the isolated plasma membranes by acid SDS-PAGE. The 140 kDa polypeptide phosphorylation increased by the addition of $Na^+$ in the presence of $Mg^{2+}$, and decreased by the further addition of other monovalent cations including $K^+$. The 95 kDa intermediate phosphorylation increased in the presence of $Mg^{2+}$ alone, and decreased by the further addition of monovalent cations. These results indicated that the former intermediate was a $Na^+$-activated ATPase, similar to $Na^+,K^+$-ATPase from animal, and the latter was similar to $H^+,K^+$-ATPase from higher plants and fungi.

Table I-1 Distribution of organelle markers in subcellular fractions obtained with microbead method

| Fractions | Relative activities of organelle markers( % )* | | | | |
|---|---|---|---|---|---|
| | Cyt c Oxidase | NADPH Cyt c Red. | IDPase | Chlorophyll | Radioactivity (cpm) |
| BM | 4.0 | 4.0 | 9.0 | 2.0 | 18130 |
| FM | 96.0 | 96.0 | 91.0 | 98.0 | 9160 |
| Sup | nd | nd | nd | nd | 2136 |

* Percent of total activity or content in cells

Bead-bound(BM) and free(FM) membrane and supernatant (Sup) fractions prepared as described in Materials and Methods were assayed for organelle markers, chlorophyll content and radioactivity. The relative activity of the organelle markers is given as percentage of the total.

Table I-2 Effect of divalent cations on the ATPase activity in the bead-bound membrane

| Divalent Cations | Specific activity (nmol Pi/mg prot./min) |
|---|---|
| None | 0.0 |
| $MgSO_4$ | 93.5 |
| $MnSO_4$ | 141.9 |
| $CaSO_4$ | 104.8 |
| $CoSO_4$ | 48.4 |
| $CuSO_4$ | 17.7 |
| $ZnSO_4$ | 22.6 |

ATPase activity was assayed at 37°C for 30 min in the reaction mixture composed of 0.5 ml of 30 mM Tris-HEPES buffer (pH 8.0) containing 3 mM Tris-ATP and 5 mM divalent cation and 25 µl of the sample(2.5 µg protein).

Table I-3 Effects of monovalent cations on the ATPase activity in the bead-bound membrane

| Monovalent Cations | Specific activity (nmol Pi/mg prot./min) |
|---|---|
| None | 105.0 |
| NaCl | 222.0 |
| KCl | 182.4 |
| LiCl | 180.0 |
| RbCl | 168.0 |
| CsCl | 165.0 |
| $NH_4Cl$ | 204.0 |
| NaCl+KCl | 295.1 |
| +LiCl | 299.5 |
| +RbCl | 255.2 |
| +CsCl | 321.6 |
| +$NH_4Cl$ | 370.0 |
| KCl+NaCl | 295.1 |
| +LiCl | 202.7 |
| +RbCl | 206.9 |
| +CsCl | 215.7 |
| +$NH_4Cl$ | 215.7 |

ATPase activity was assayed at 37°C for 30 min in the reaction mixture composed of 0.5 ml of 30 mM Tris-HEPES buffer (pH 8.0) containing 3 mM Tris-ATP, 5 mM $MgSO_4$ and 100 mM each monovalent cations, and 25 μl of the sample(2.5 μg protein).

Table I-4 Substrate specificity of the ATPase activities

| Substrate | Specific activity (nmol Pi/mg prot./min) |
|---|---|
| ATP | 588.7 |
| ADP | 145.2 |
| AMP | 32.3 |
| GTP | 48.4 |
| CTP | 137.1 |
| UTP | 217.7 |
| UDP | 24.2 |
| PNP | 32.3 |
| β-GP | 40.3 |

ATPase activity was assayed at 37 °C for 30 min in the reaction mixture composed of 0.5 ml of 30 mM Tris-HEPES(pH 8.0) buffer containing 3 mM of each disodium nucleotides or phosphatase substrates, 5 mM $MgSO_4$, 100 mM NaCl, 100 mM KCl and 25 μl of the sample(2.5 μg protein).

Table I-5 Effect of inhibitors on the ATPase activity

| Reagents | Conc. | Residual Activity ( % ) |
|---|---|---|
| None | | 100 |
| $(NH_4)_6Mo_7O_{24}$ | 50 μM | 120 |
| $NaN_3$ | 5 mM | 108 |
| $KNO_3$ | 50 mM | 116 |
| Ouabain | 100 μM | 113 |
| DCCD | 1 mM | 63 |
| Vanadate | 100 μM | 25 |

Each inhibitor was added to the reaction mixture just before the start of the reaction. The reaction condition was the same as mentioned in Table I-4 except that the substrate was 3 mM Tris-ATP.

Table I-6 Effect of cold nucleotides on the decrease in radioactivity of the labeled 150 kDa polypeptide

| Nucleotide (1 μmol) | Phosphorylation (cpm) |
|---|---|
| None | 1596 |
| ATP | 116 |
| ADP | 236 |
| AMP | 1796 |
| CTP | 623 |
| GTP | 532 |
| UTP | 546 |

The 150 kDa polypeptide was phosphorylated for 40 sec in 0.5 ml reaction mixture containing 5 μCi [$\gamma$-$^{32}$P]-ATP, 0.1 mM $MgSO_4$, 100 mM NaCl, 30 mM Tris-HEPES(pH 8.0) and the sample(80 μg protein) and then incubated with 1 μmol each of cold nucleotides for further 40 sec. After electrophoresis, the gels corresponding to 150 kDa were cut out and their radioactivity was counted.

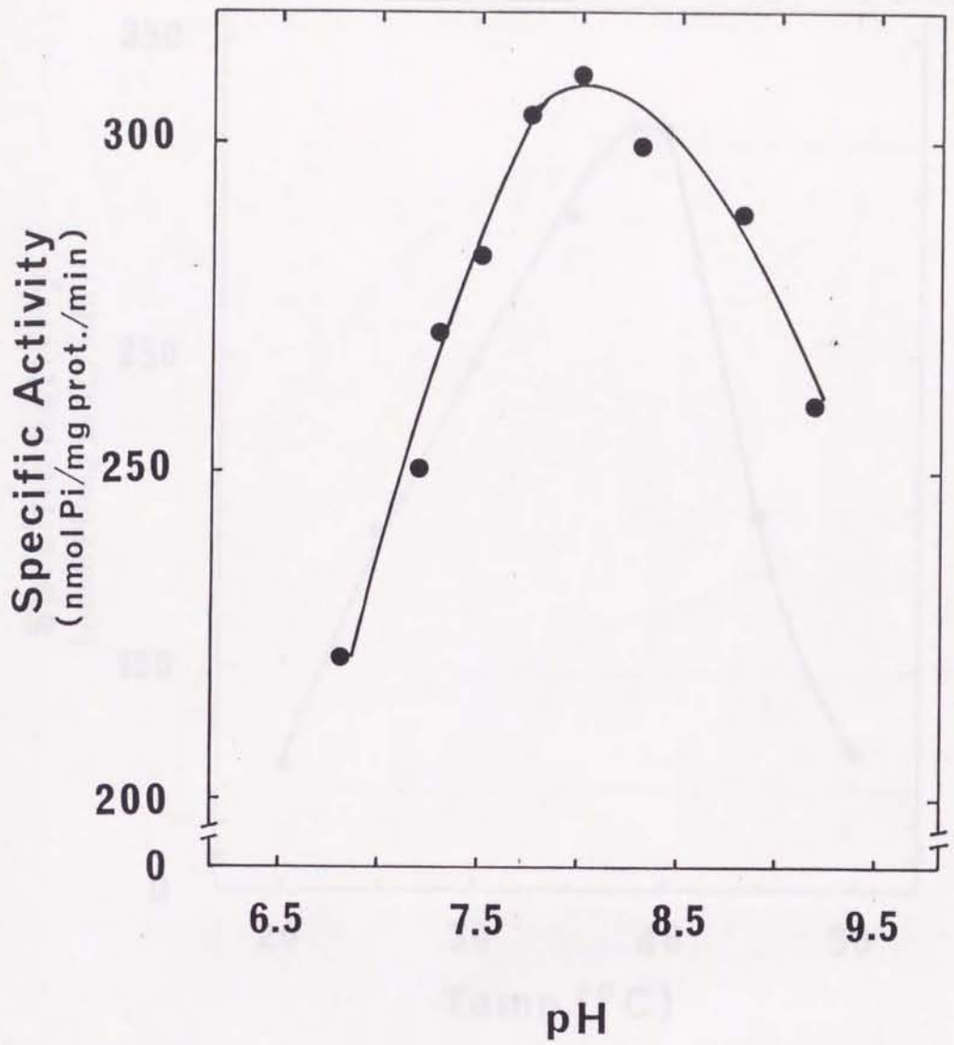


**Fig.I-1 PH dependency of ATPase activity in bead-bound membrane**

ATPase activity was assayed at 37°C for 30 min in reaction mixture composed of 0.5 ml of 30 mM Tris-HEPES buffer(pH 6.3 to 8.8) containing 3 mM Tris-ATP, 5 mM $MgSO_4$, 100 mM NaCl and 100 mM KCl, and 25 μl of sample(2.5 μg protein).

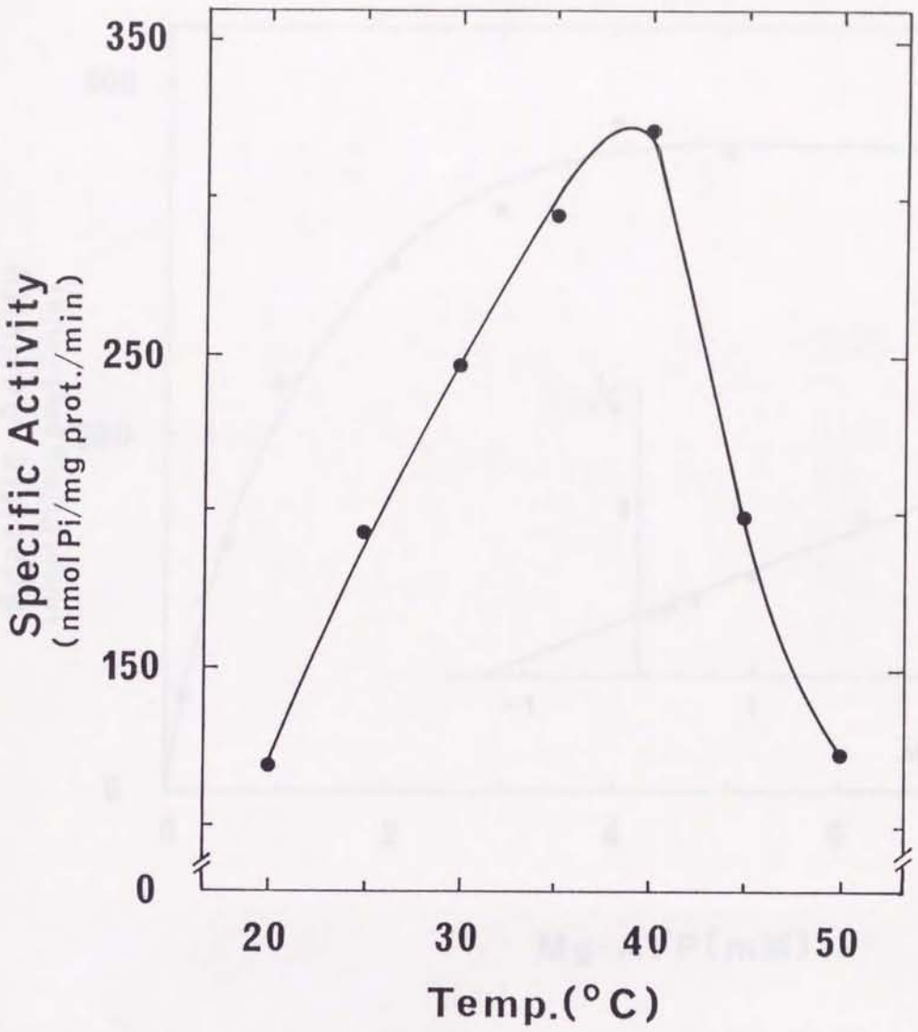


**Fig.I-2 Temperature dependency of ATPase activity in bead-bound membrane**

ATPase activity was assayed at various temperatures for 30 min in reaction mixture composed of 0.5 ml of 30 mM Tris-HEPES buffer(pH 8.0) containing 3 mM Tris-ATP, 5 mM $MgSO_4$, 100 mM NaCl and 100 mM KCl, and 25 μl of sample(2.5 μg protein).

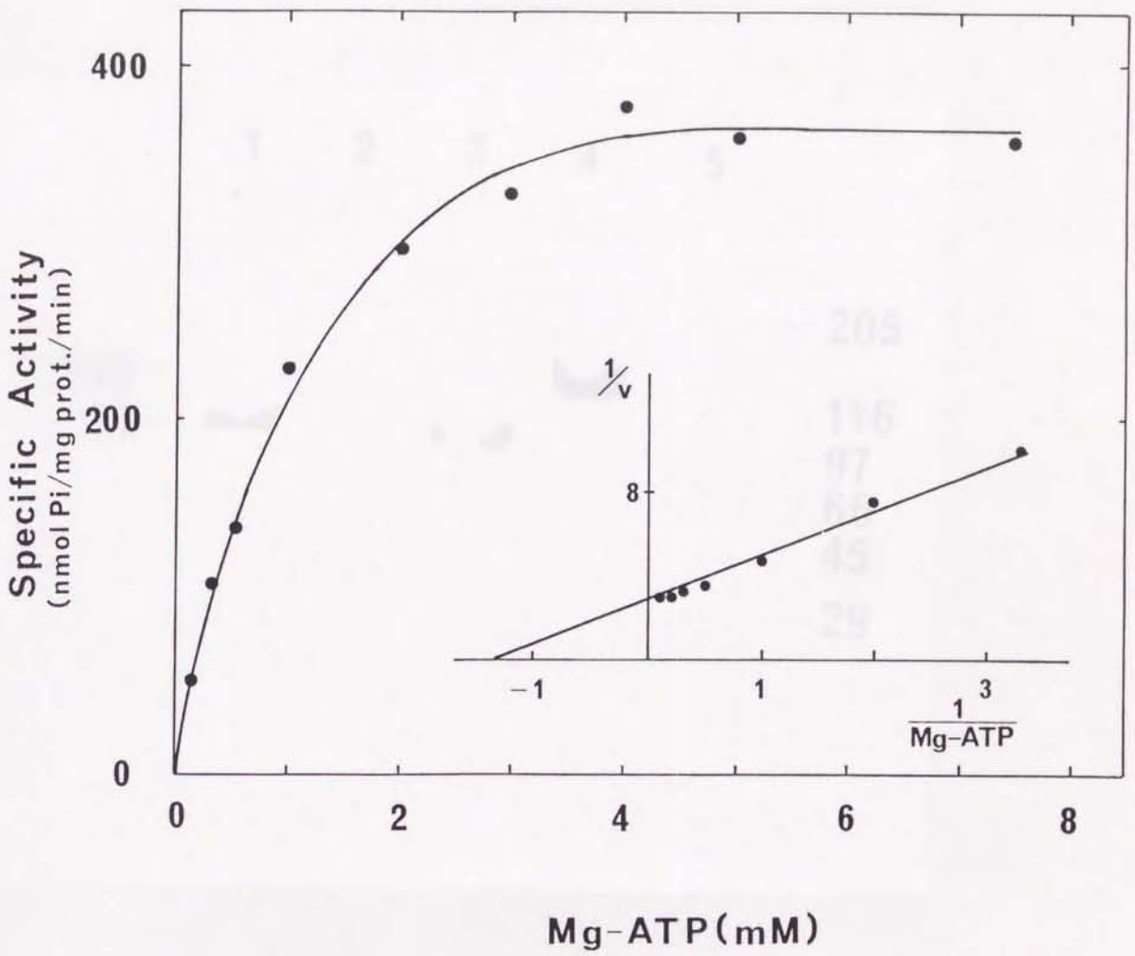


Fig.I-3 Effect of Mg-ATP concentrations on the ATPase activity

ATPase activity was assayed at 37°C for 30 min in reaction mixture composed of 0.5 ml of 30 mM Tris-HEPES(pH 8.0) buffer containing 100 mM NaCl, 100 mM KCl, 25 μl of sample(2.5 μg protein) and various concentration of Mg-ATP.

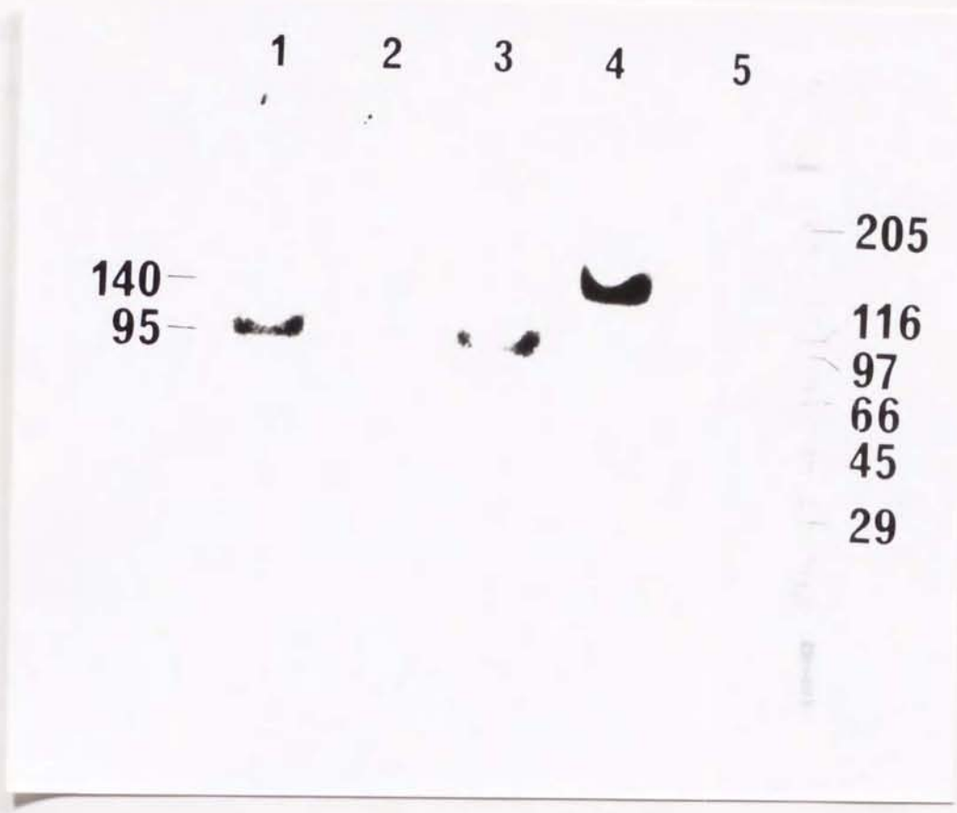


**Fig.I-4 Effect of EDTA on the phosphorylation of 95 kDa and 140 kDa polypeptides**

Phosphorylation was conducted with about 80 μg of membrane proteins in 0.5 ml reaction mixture. The dried gels were placed against X-ray film for 5 hr at room temperature. Numbers at the left side of the Figure indicate the apparent molecular weight of the two major bands. The molecular weight markers at right side of the Figure are 205; myosin, 116; β-galactosidase, 97; phosphorylase b, 66; bovine albumin, 45; egg albumin and 29; carbonic anhydrase. Lanes 1-5 show the phosphorylation patterns after 40-second incubation. The

reaction mixture contains no cations(lane 1), 0.2 mM EDTA(lane 2), 0.2 mM EDTA and 0.3 mM $MgSO_4$(lane 3), 0.2 mM EDTA, 0.3 mM $MgSO_4$ and 100 mM NaCl(lane 4) and 0.2 mM EDTA and 100 mM NaCl(lane 5).

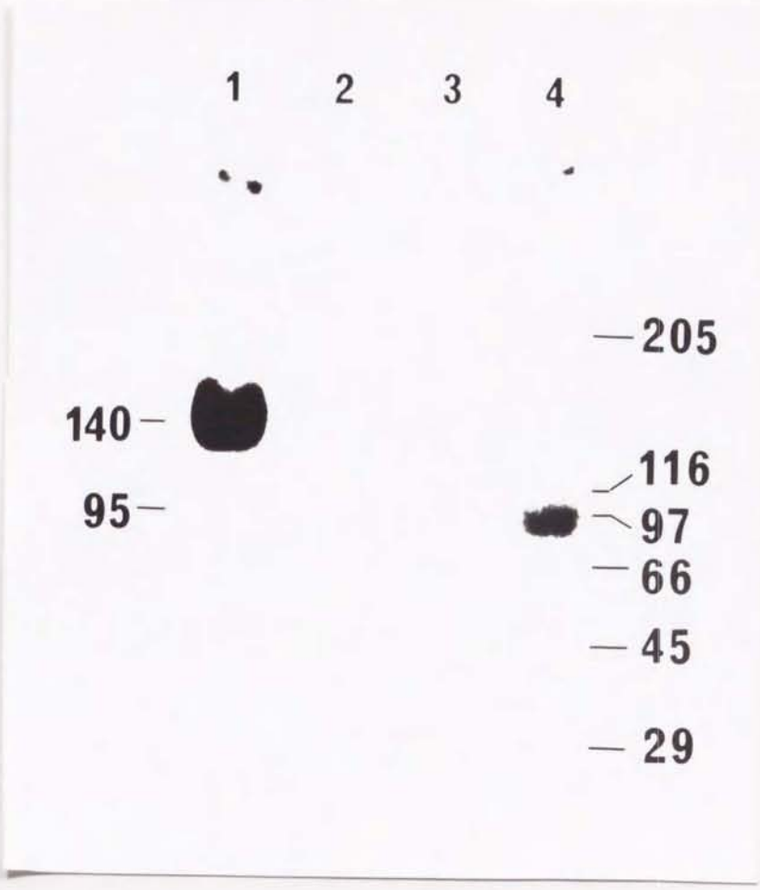


**Fig.I-5 Effect of $Na^+$ and $K^+$ or cold ATP on phosphorylation and/or dephosphorylation of membrane components**

Phosphorylation was conducted with about 80 μg of membrane protein in the 0.5 ml reaction mixture for 40-sec in the presence of 0.1 mM $Mg^{2+}$ and 100 mM $Na^+$(lane 1); 0.1 mM $Mg^{2+}$ and 100 mM $Na^+$ followed by a 20-sec chase with 100 mM $K^+$(lane 2) or unlabeled 1 μmol ATP(lane 3) and 0.1 mM $Mg^{2+}$(lane 4). The dried gels were placed against X-ray film for 5 hr at room temperature. Numbers at the left and right sides of the Figure are the same as Fig.4.

# PART II

## IMMUNOLOGICAL COMPARISON BETWEEN $NA^+$-ACTIVATED ATPASE FROM *HETEROSIGMA AKASHIWO* AND ANIMAL $NA^+,K^+$-ATPASE

## Introduction

The plasma membrane of marine raphidophycean H. akashiwo has been shown to have $Na^+$-activated ATPase activity in Part I(Wada et al. 1989). The characterization of the kinetics of the phosphorylation and the ATPase activity clearly indicated the similarity between H. akashiwo $Na^+$-activated ATPase and animal $Na^+,K^+$-ATPase.

The comparison is summarized in Table II-1. Only one big difference is the size of subunits. The molecular weight of the phosphorylated polypeptide(140 kDa) from H. akashiwo was much larger than the α subunit(100 kDa) of animal $Na^+,K^+$-ATPase, which was also phosphorylated. The ATPases, which phosphorylated intermediate during enzyme reaction, are grouped as P-type ATPase, and include $H^+$-ATPase, $Na^+,K^+$-ATPase and $Ca^{2+}$-ATPase. Recently, the cDNA cloning and the analysis of the sequence have been progressed in those ATPases of various organisms(Hesse et al. 1984, Kawakami et al. 1985, MacLennan et al. 1985, Schull et al. 1985, Serrano et al. 1986, Schull and Lingel 1986, Hara et al. 1987, Meade et al. 1987, Harper et al. 1989, Pardo and Serrano 1989) and the presence of two homologous parts in their molecule have been known beyond species. Those are phosphorylation site(P site) and ATP binding site(AB site) which concern ATP hydrolysis(Meade et al. 1987). These results strongly suggest that P-type ATPases had evolved from same origin. On the other hand, specific ion selectivity is thought to be owing to the other parts of ATPase molecule(Clark et al. 1989). It can be

supposed that the part of molecule owing to $Na^+$ selectivity is conserved in the H. akashiwo $Na^+$-activated ATPase and animal $Na^+,K^+$-ATPase. In this Part, I tried to make clear what homology exists between $Na^+$-activated ATPase from H. akashiwo and $Na^+,K^+$-ATPase from animal cells using immunological techniques.

## Materials and Methods

### Cell culture

Axenic clone of H. akashiwo(strain No. OHE-1) was purchased from National Institute for Environmental Studies in Japan. Culture conditions and medium content was described in Part I.

### Serum preparation

A rabbit anti-serum against $Na^+,K^+$-ATPase from pig kidney and pre-immune serum were raised by Dr. O. Urayama (Tokyo Medical and Dental University) as follows. The antigen, $Na^+,K^+$-ATPase, was purified from pig kidney by SDS extraction method(Jorgensen 1974). The samples whose specific activity was 1180 μmol Pi/mg prot./hour(0.8 mg/injection) were immunized as a mixture with Freund complete adjuvant(1:1) to rabbit in 2-week-intervals. The titer of the serum was determined with ELISA.

### Immunoblotting assay

Sodium-dodecyl-sulfate polyacrylamide gel electrophoresis(SDS-PAGE) was performed by the method of Laemmli(Laemmli 1970). The proteins on the gel were electrophoretically transferred to nitrocellulose filter(Gershoni and Palade 1983). The filters were incubated in 0.1 %(w/v) Tween-20 and 0.1 %(w/v) gelatin in phosphate buffer saline(PBS), pH7.6 for 1 hour. Then, the filters were incubated in 1,000-fold-diluted anti-$Na^+,K^+$-ATPase serum in 0.1 % gelatin PBS filter for 1 hour. After rinsed with PBS, the filters were incubated in 1,000-fold-diluted horseradish peroxidase conjugated goat anti-rabbit

IgG antibody(Jackson Immuno Research Laboratories, Inc.) in 0.1 % gelatin PBS for 1 hour. After rinsing with 0.1 % Tween 20 in PBS, antibody binding was visualized by reaction with diaminobendizine and hydrogen peroxide.

**Affinity purification of antibody**

For affinity purification of antibodies(Synder et al. 1987) to α or β subunit of $Na^+,K^+$-ATPase, the rabbit serum was adsorbed to western blots on nitrocellulose filters of sheep kidney purified $Na^+,K^+$-ATPase obtained from Y.Hara(Tokyo Medical and Dental University). After blocking the filter with 0.1 % gelatin PBS, reacted with anti-$Na^+,K^+$-ATPase serum at room temperature for 12 hours. Then, the filters were washed with 0.1 % Tween-20 in PBS 3 times for 20 min and rinsed with PBS 3 times. Then, the filters were incubated in 0.1 M glycine-HCl(pH 2.5) buffer for 2 min to dissociate each mono-specific antibody. Several drops of 1 molar Tris-HCl(pH 9.0) was immediately added to adjust pH around 7.0. These solutions were used as for α or β-monospecific antibody, respectively.

**Cell fractionation**

Cell fractionation was performed according to the silica microbead method as described in Part I. Bead-bound membrane(M+B), bead-unbound membrane(M-B) and supernatant(Sup) indicate the plasma membrane fraction, the membrane fraction which did not bind to beads and total supernatant fraction, respectively.

**Acid SDS-PAGE**

The Phosphorylated intermediate was identified in the

presence of $Na^+$ ion with acid SDS-PAGE as described in Part I.

## Results

### Immunological cross-reaction

Fig. II-1 shows the immunoblotting of plasma membrane proteins of H. akashiwo using anti-$Na^+,K^+$-ATPase antibody. Anti-sheep kidney $Na^+,K^+$-ATPase result to both α(98 kDa) and β(50 kDa) subunits of purified $Na^+,K^+$-ATPase from sheep kidney(lane 1) as positive control. Only about 140 kDa polypeptide of H. akashiwo in plasma membrane was detected by the serum(lane 2), not by the pre-immune serum(lane 3). These results show the presence of immunological homology between 140 kDa polypeptide from plasma membrane of H. akashiwo and animal $Na^+,K^+$-ATPase.

### Identification of the antigen

1) Evaluation of the antigen as phosphorylated intermediate and the antigen

As shown in Part I, the phosphorylated intermediate of the $Na^+$-activated ATPase of H. akashiwo was detected as 140 kDa polypeptide with acid SDS-PAGE(Fig. II-2, lane A). The phosphorylated polypeptides were electro-transferred to nitrocellulose filter and reacted with anti-$Na^+,K^+$-ATPase serum(Fig. II-2, lane B). The blot shows the immunological reaction in the same position as phosphorylated polypeptide.

2) Distribution of the antigen

Three subcellular fractions separated by the silica microbead method were subjected to SDS-PAGE and immunoblotting(Fig. II-3). The 140 kDa antigen was detected only in bead-bound(lane 2) and unbound(lane 3)

fractions, not in sup fractions. The distribution of 140 kDa antigen is quite similar to that of $Na^+$-activated ATPase activity(data not shown).

**Cross reactivity of anti α and β subunit mono-specific antibodies to 140 kDa polypeptide**

The mono-specific antibody against α or β subunit of the animal $Na^+,K^+$-ATPase was prepared and used for Immunoblotting analysis(Fig. II-4). The anti-α subunit antibody reacted to both α subunit(98 kDa) in animal $Na^+,K^+$-ATPase(A, lane 1) and 140 kDa polypeptide in the plasma membrane of H. akashiwo. On the other hand, no reaction was observed in 140 kDa polypeptide of H. akashiwo using anti-β subunit antibody(B, lane 2). This shows the presence of homology between 140 kDa polypeptide of the plasma membrane of H. akashiwo and α subunit of animal $Na^+,K^+$-ATPase.

## Discussion

Approximate 140 kDa polypeptide in plasma membrane of H. akashiwo reacted to the anti-serum against animal $Na^+,K^+$-ATPase(Fig. II-1) with SDS-PAGE. For comparison of the $Na^+$-activated ATPase of H. akashiwo with this antigen, phosphorylated intermediate of the $Na^+$-activated ATPase was investigated with acid SDS-PAGE and western blot. This antigen and phosphorylated intermediate were electrophoresed at same position(Fig. II-2, lane A,B). And the distribution of cell fractionation was consistent with the that of $Na^+$-activated ATPase activity(Fig. II-3). These results strongly indicated that this antigen is the $Na^+$-activated ATPase.

The serum against animal $Na^+,K^+$-ATPase in this research has been characterized that this serum cross-reacted $Na^+,K^+$-ATPase from other animal species(rat brain, kidney, pig kidney, sheep kidney and rabbit kidney), but didn't cross-react animal sarcoplasmic reticulum $Ca^{2+}$-ATPase(personal communication from Dr. Urayama). It has also been demonstrated that the epitope part of $Na^+,K^+$-ATPase for this serum was the N-terminal region of plasma membrane spanning domains(personal communication). This parts did not contain both the phosphorylation site(P site) and ATP binding site(AB site) as the conserved parts of ion transport ATPase. It is suggested that this serum recognizes the domain for selectivity to ions($Na^+$ or $K^+$).

The monospecific antibody to α or β subunit of animal $Na^+,K^+$-ATPase was isolated with purified animal $Na^+,K^+$-

ATPase and only the α monospecific antibody reacted the $Na^+$-activated ATPase from H. akashiwo(Fig. II-4). This result shows that both $Na^+$-activated ATPase from H. akashiwo and the α subunit of animal $Na^+,K^+$-ATPase have partially homological structure which is common to ion selectivity domain of animal $Na^+,K^+$-ATPase.

In Part I, the $Na^+$-activated ATPase from H. akashiwo shows the enzymatic similarity to animal $Na^+,K^+$-ATPase. Then, in this Part, the structural relatedness of ion selectivity is considered that the $Na^+$-activated ATPase from H. akashiwo could also anti-transport Na and K ions across plasma membrane like the $Na^+,K^+$-ATPase from animal cells. In Fig. II-4, the β monospecific antibody didn't cross-reacted the 140 kDa polypeptide nor other polypeptide from H. akashiwo plasma membrane. It was negative evidence that H. akashiwo didn't have similar polypeptide with β subunit. To tell the truth, the function of the β subunit has been unknown in even animal cells(Urayama et al. 1985). It possible that the $Na^+$-activated ATPase from H. akashiwo was a prototype molecule like linking α and β subunit from animal $Na^+,K^+$-ATPase, or that the $Na^+$-activated ATPase from H. akashiwo had no subunit form like $Ca^{2+}$-ATPase from animal cells. These questions could be answered to purify the $Na^+$-activated ATPase from H. akashiwo or to clone and sequence the DNA of the $Na^+$-activated ATPase to compare that of the $Na^+,K^+$-ATPase α or β subunit.

## Summary

Homology between $Na^+$-activated ATPase of marine raphidophycean H. akashiwo and animal $Na^+,K^+$-ATPase was studied immunologically. The rabbit anti-$Na^+,K^+$-ATPase from pig kidney serum reacted to 140 kDa polypeptide of plasma membrane fraction from H. akashiwo.

The 140 kDa antigen was phosphorylated intermediate of $Na^+$-activated ATPase of H. akashiwo. The 140 kDa polypeptide was reacted with α-subunit mono-specific antibody but not with β subunit mono-specific one. From those results, I discussed about the structure and function of $Na^+$-activated ATPase of H. akashiwo plasma membrane.

Table II-1 Comparison between $Na^+$-activated ATPase from *H. akashiwo* and animal $Na^+,K^+$-ATPase.

| | *H. akashiwo* $Na^+$-activated ATPase | Animal $Na^+,K^+$-ATPase |
|---|---|---|
| Localization | plasma membrane | Plasma membrane |
| Ion requirement | $Na^+$ and $K^+$ | $Na^+$ and $K^+$ |
| Optimum pH | around 8.0 | 7.2-7.6 |
| Optimum Temp. | 37°C | 37°C |
| Subunits | 140 kDa | α 100 kDa<br>β 50 kDa |
| Phosphorylated Intermediate | 140 kDa | α 100 kDa |

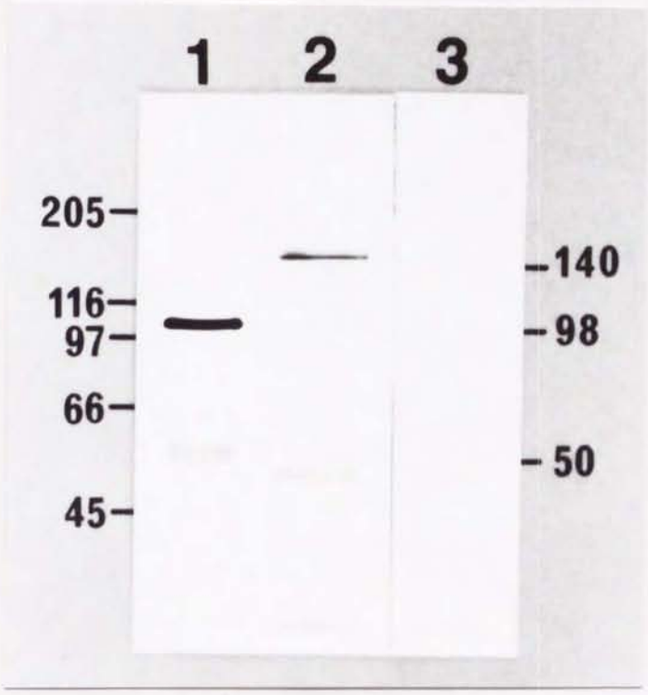


**Fig.II-1 Immunoblot of the proteins in plasma membrane of H. akashiwo and sheep kidney microsomes.**

The proteins of the membranes were electrophoresed on SDS-PAGE, transfered to nitrocellulose filter and reacted with anti-$Na^+$,$K^+$- ATPase serum(lane 1 and 2) or preimmune serum(lane 3). Lane 1, sheep kidney microsomes(50 ng). Lane 2 and 3, plasma membrane of H. akashiwo(10 μg).

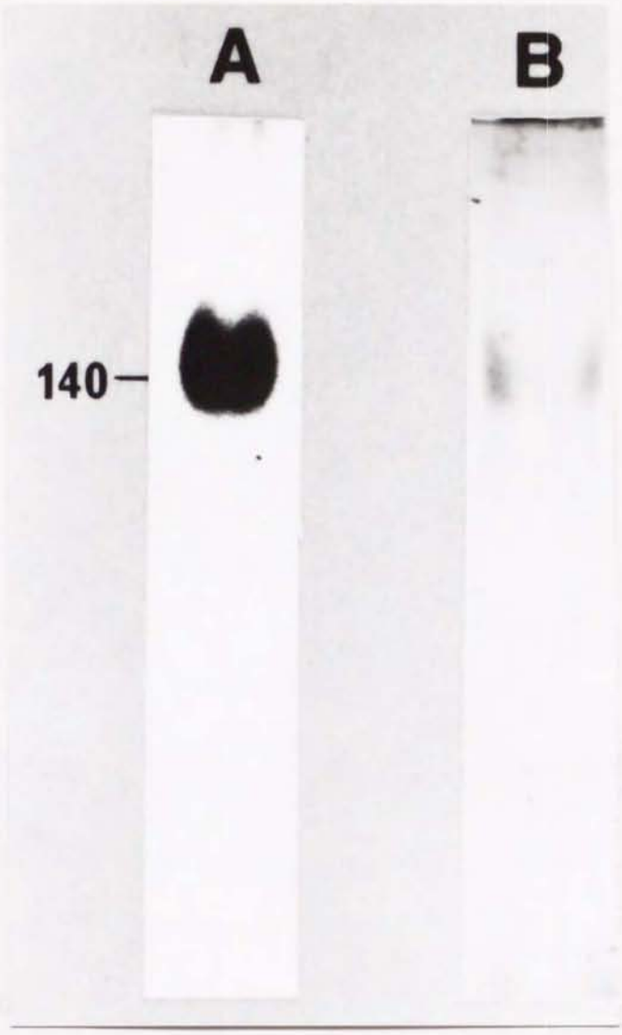


**Fig.II-2 Immunoblot of phosphorylated intermediate.**

The polypeptides of the H. akashiwo plasma membrane were phosphorylated in the presence of $Na^+$ ion as described in Part I. The phosphorylated polypeptide was visualized by autoradiography(lane A) or transfered to nitrocellulose filter followed by the immunological reaction with anti-$Na^+$,$K^+$-ATPase serum(lane B).

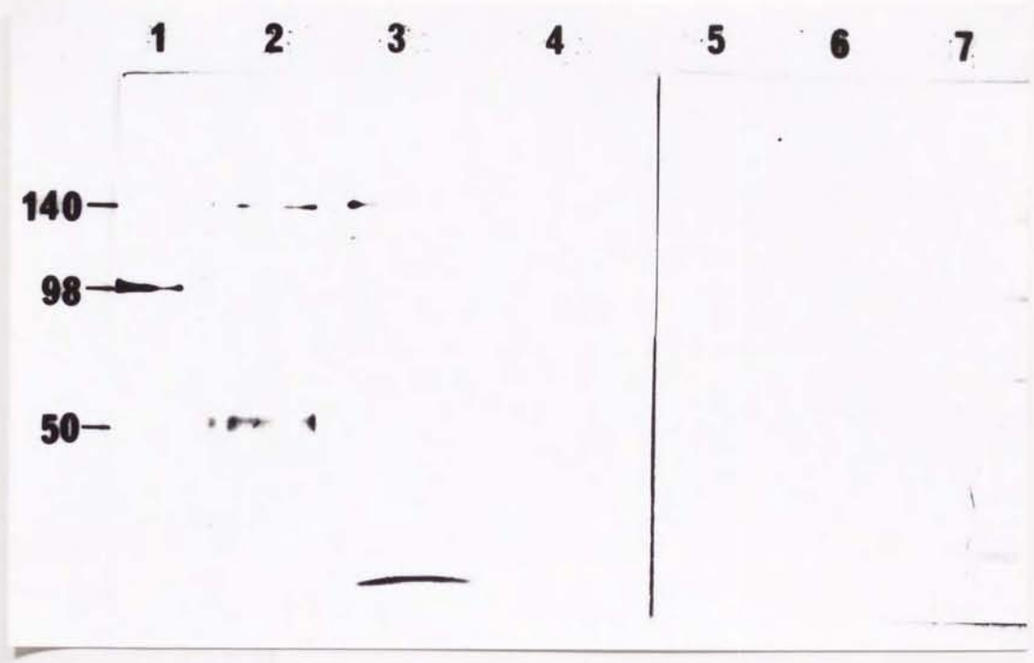


**Fig.II-3 Distribution of 140 kDa antigen in subcellular fractions of H. akashiwo**

Subcellular fractions separated by silica microbead method(described in Part I) was subjected to immunoblot analysis. Lane 1, sheep kidney microsomes. Lane 2 and 5, bead-bound fraction(plasma membrane fraction). Lane 3 and 6, bead-unbound fraction. Lane 4 and 7, soluble fraction. Nitrocellulose filters were incubated with rabbit anti-$Na^+,K^+$-ATPase(lane 1-4) or pre-immune rabbit serum(lane 5-7).

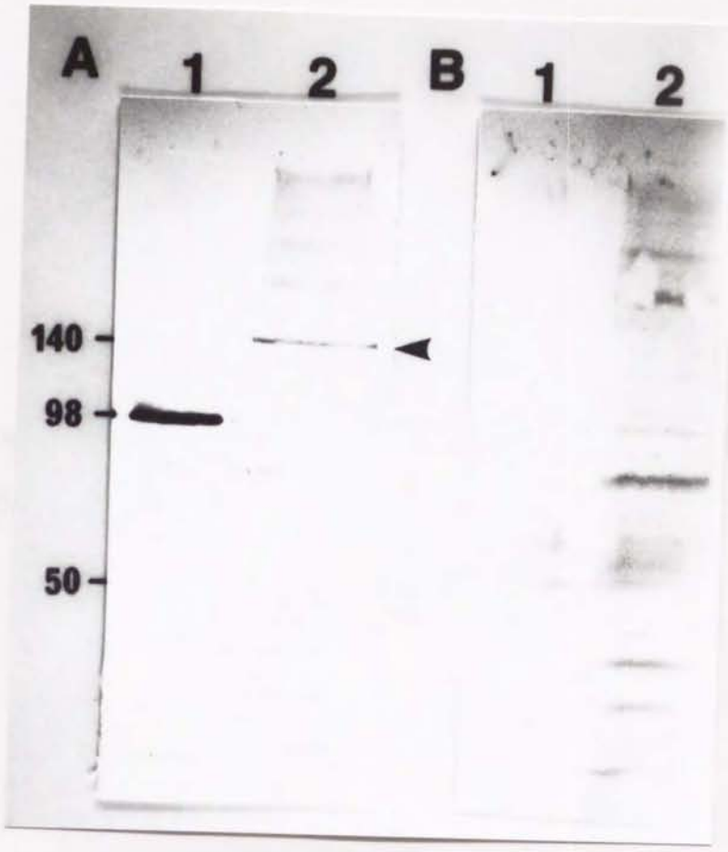


**Fig.II-4 Immunoblot analysis with affinity purified antibody.**

Proteins of sheep kidney microsomes(50 ng)(lane 1) and H. akashiwo plasma membrane(10 μg)(lane 2) were electrophoresed and subjected to immunoblot using α subunit(A) or β subunit(B) mono-specific antibody raised against animal $Na^+,K^+$-ATPase.

## PART III

## CLONING OF THE cDNA OF THE $NA^+$-ACTIVATED ATPASE FROM *HETEROSIGMA AKASHIWO*

## Introduction

$Na^+$-activated ATPase was first found in plant cells, H. akashiwo and the enzyme reaction mechanism of the $Na^+$-activated ATPase of H. akashiwo was known to be identical with that of animal $Na^+,K^+$-ATPase by the analysis of phosphorylated intermediates in Part I. Further investigations showed that the N-terminal region of α subunit of animal $Na^+,K^+$-ATPase may have a homologous structure to the $Na^+$-activated ATPase of H. akashiwo in Part II. These results suggest that $Na^+$-activated ATPase of H. akashiwo has similar functions and structures to animal $Na^+,K^+$-ATPase. This finding is quite novel because people has believed $Na^+,K^+$-ATPase must exist only in animal cells up to now. This discovery is thought to contribute to understand the evolution of $Na^+,K^+$-ATPase or other ATPases. The DNA sequences of animal $Na^+,K^+$-, $Ca^{2+}$- and $H^+,K^+$-ATPase have been already analyzed(Hesse et al. 1984, Maclennan et al. 1985, Schull et al. 1985, Meade et al. 1987, Pardo and Serrano 1989). The obtained sequence informations have facilitated to understand the ion transport mechanism, ion or ATP selectivity and ATP hydrolysis mechanism of these ATPases(Clark et al. 1989). In this Part, I described about the cloning of cDNA of $Na^+$-activated ATPase from H. akashiwo and the sequence analysis to have investigations about homology among ATPases.

## Materials and Methods

### RNA isolation

Heterosigma akashiwo cells at logarithmic growth phase($5x10^4$-$1x10^5$ cells/ml) were centrifuged at 300xg for 3 min. The resultant pellets were frozen immediately in liquid nitrogen and stocked in freezer at -80°C for RNA preparation. The collected cells($10^9$xcells) were lysed with Polytron(Kinematica, Luzen) in 50 ml of 5.5 M guanidine thiocyanate(Fluka Chemie A G, Buchs), 25 mM sodium citrate(pH 7.0 with NaOH) and 0.2 M 2-mercaptoethanol. The homogenates were centrifuged at 10,000xg for 10 min, and the each 8 ml supernatant was gently overlaid onto a 4 ml cushion of cesium trifluoroacetate(CsTFA, Pharmacia) solution(density 1.51±0.01 g/ml) containing 0.1 M EDTA(pH 7.0)(Okayama et al. 1987). The overlaid samples were centrifuged with SW 40.1Ti rotor(Beckman Co.,Ltd., California) at 27,000 rpm for 20-24 hrs at 20°C. After centrifugation, the upper guanidine thiocyanate layer was removed by aspiration. The tubes were quickly inverted on a paper towels to drain for 5 min, and then resulting RNA pellet was dissolved in a total of 0.4 ml of the 4 M guanidine thiocyanate solution. After insoluble materials are removed with microcentrifuge(Tomy Co.,Ltd., Tokyo) at 10,000 rpm for 5 min, each 400 µl RNA solution was added with 10 µl of 1 M acetic acid and 300 µl of ethanol, and chilled at -80°C for 3 hrs. The RNA was pelleted by centrifugation at 4°C for 10 min in a microcentrifuge and rinsed with 1 ml of 70 % ethanol. The RNA pellets were

dissolved in 200 µl of 10 mM Tris-HCl(pH 7.5) and 1 mM EDTA, and the insoluble materials was removed by centrifugation. Eight microliters of 5 M NaCl and 600 µl of ethanol were added to the solution. The RNA was precipitated by centrifugation after chilling at -20°C for several hrs.

**Poly A RNA preparation**

Prepared RNA sample was adjusted to 2.0 mg/ml concentration in 10 mM Tris-HCl(pH 7.5) and 1 mM EDTA. It was then incubated at 60°C for 5 min, quickly chilled on ice, and added 2 M NaCl and 1 M Tris-HCl(pH 7.6) up to the final concentration of 0.5 M and 10 mM, respectively. The RNA solution was incubated with oligo(dT) filters(MAP 1 mg RNA/2 $cm^2$; TAKARA Co.,Ltd., Kyoto)(Werner et al.) at room temperature for 15 min. The solution was decanted and the filters were washed 4 times in 1 ml of 0.5 M NaCl solution for each 5 min. The filters were rinsed in diethyl-pyrocarbonate-treated distilled water(DEPC water). Eight hundreds microliters of DEPC water were added to the filters and incubated at 70°C for 5 min. The released poly[A] RNA was added to 75 µl of 3 M sodium acetate(pH 5.6) and 1 ml of ethanol, and chilled at 20°C for overnight. Then poly[A] RNA was precipitated by centrifugation at 15,000 rpm for 10 min with microcentrifuge. The poly[A] RNA was dissolved in DEPC water and stocked in a freezer at -80°C.

**Preparation of cDNA library**

The synthesis of cDNA from the isolated poly[A] RNA of H. akashiwo cells was performed with cDNA synthesis system plus kit(Amersham). First strand of cDNA synthesis was on

isolated 1.0 μg poly[A] RNA using reverse transcriptase. The second strand was synthesized using the RNase H procedure(Gubler 1987). The amount of synthesized cDNA was calculated by that of radioactivities of [α-$^{32}$P]dCTP incorporated in cDNA.

**Oligonucleotide primers synthesis**

Oligonucleotide primers were synthesized with DNA synthesizer(Applied Biosystems, California). One kind of primer was designed according to the sequence Cys-Ser-Asp-Lys-Thr-Gly-Thr, which is a sequence in phosphorylation site of eukaryotic ion transporting ATPase. I synthesized two sorts of primers; Primer 1, 5' TG(T,C)AGIGA(T,C)AA(A,G)ACIG-GIAC 3' and Primer 2, 5' TG(T,C)TCIGA(T,C)AA(A,G)ACIGGIAC 3', which differed only in the codon selection of the Ser. The other, Primer 3, was designed according to the sequence Thr-Gly-Asp-Gly-Val-Asn-Asp, which is ATP binding site of eukaryotic ion transporting ATPase. The sequence is 5' ACIGGIGA(T,C)GGIGTIAA(T,C)GA 3'. All three primers were composed of 20 oligomers.

**Amplification of cDNA with Polymerase Chain Reaction(PCR)**

The amplification of the specific cDNA was performed by the polymerase chain reaction(PCR) method according to Gene Amp$^{TM}$ DNA amplification reagent kit(Perkin Elmer Cetus, Conneticut)(Ohara et al. 1989). The template of PCR method was 10 ng of the cDNA of H. akashiwo and the primers paired 1-3 or 2-3 were added at each 400 ng respectively. The annealing temperatures between the template and the primers were examined from 28 to 52°C and the DNA synthesis from the

primers was extended with thermo-resistant Taq I polymerase at 74°C. PCR amplification after 40 cycles was checked by 1.0 % agarose gel electrophoresis and ethidium bromide staining.

**Isolation of the DNA fragment produced by PCR**

The PCR synthesized DNA fragments were electrophoresed with 1.0 % agarose gel and detected with ethidium bromide staining. Then, a slice of the agarose gel containing the band was cut out and put into a dialysis bag in 250 µl of 1xTBE contained 0.089 M Tris-borate and 2.5 mM EDTA. After removing air bubbles, the bag sunk in a shallow layer of 1xTBE in an electrophoresis tank and passed electric current(100 V for 2-3 hours). The electroeluted DNA was recovered to pipet the buffer surrounding the gel. After microcentrifugation, the supernatant was added appropriate amounts of NaCl and ethanol and chilled at -80°C. DNA pellets were recovered by centrifugation at 15,000 rpm for 10 min and resuspended in TE buffer contained 10 mM Tris-HCl, pH7.5 and 1 mM EDTA as adjusted the concentration of 100 ng/µl. This DNA solution was used for the probe of Northern hybridization and for cloning in pUC118 vector.

**Sequencing of PCR fragment**

Isolated PCR fragment terminal ends was blunted with Klenow fragment and ligated in Sma I-cut and dephosphorylated pUC118 plasmid. A E. coli strain JM105 was used for the host. Screening density was Ca. 100 colonies on 9 cm plate with Isopropyl-b-D-thio-galactopyranoside(10 mM) and 5-bromo-4chloro-3-indoryl-b-

galactoside(2 %). The 10 individual colonies were picked up and used for the plasmid DNA preparation and digestion with restriction enzymes. Three clones were selected at random and digested with the combination of EcoR I and Sal I or only Bgl I. Two kinds of digested fragments were isolated with gel electroelution mentioned above, blunted with Klenow fragment or T4 polymerase, and cloned into phage M13mp18 for the template preparation. The sequencing was carried out 3 times in each clone by the dideoxynucleotide chain termination method according to Sanger et al.(Sanger et al. 1977) with DNA autosequencer 370A(Applied Biosystems). The sequence data were edited with Vectra(Applied Biosystems) and analyzed with Genetyx.

**Northern hybridization**

The poly [A] RNA of H. akashiwo was glyoxylated as described by McMaster and Carmichael(McMaster and Carmichael 1977), fractionated by electrophoresis through 1.0 % agarose gel, transferred to Hybond-N(Amersham), nylon filters, and prehybridized overnight at 42°C in 50 % formamide, 5xdenhart solution, 10.0 %(w/v) dextran sulfate, 5xSSPE contained 0.9 M NaCl, 0.5 mM EDTA and 50 mM phosphate buffer, pH 7.7 and 100 μg/ml of sonicated salmon sperm DNA. Blots were hybridized in the same buffer at 42°C for 16 to 20 hours with labeled probes($1x10^6$ cpm/ml) prepared by Multiprime DNA labeling systems(Amersham) with [$\alpha$-$^{32}$P]dCTP. Filters were washed first in 5xSSPE at 42°C for 15 min twice, and repeated in 1xSSPE and 0.1 % SDS for 30 min. Then, the filters were washed in 0.1xSSPE and 0.1 % SDS at room

temperature for 15 min, and exposed for autoradiography.

**Materials**

Restriction enzymes were obtained from Nippon Gene Co.,Ltd. and the PCR kit and plasmid or phages DNA were purchased from TAKARA Shuzo Co.,Ltd. [$\alpha$-$^{32}$P]dCTP was the product of Amersham Japan.,Ltd.

## Results

### Construction of primers for PCR method

Conserved amino acid sequences of ion transport ATPase were searched in 7 species of eukaryote and two highly conserved regions were found(Table III-1). The one sequence located in phosphorylation site(P site) of phosphorylated intermediate and 9 amino acids, Cys-Ser-Asp-Lys-Thr-Gly-Leu-Thr, were identical in all of them. The sequence of CSDKTGT was determined for synthetic oligonucleotide. Another sequence located in ATP binding site(AB site) and 7 amino acids, Thr-Gly-Asp-Gly-Val-Asn-Asp, being identical. Thus, the sequence of TGDGVND were determined for the synthesis. Because, the P site is located at the side of N-end in ATPase molecules, thereby two kinds of oligonucleotides were synthesized in sense direction. The AB site in the side of C-end is synthesized in anti-sense direction(Table III-2). Both primers contained deoxyinosine at ambiguous codon positions.

### DNA synthesis by PCR

Rat brain $Na^+,K^+$-ATPase cDNA was used as a PCR template to check the utility of the constructed PCR primers. The reaction was carried out on 1 ng of $Na^+,K^+$-ATPase cDNA at 37°C of annealing temperature in 25 cycles. As shown in Fig. III-1, shows 1.0 kbp DNA fragment is synthesized only paired 2-3 primers and the size was the same as expected. Then, this reaction is carried out on 10 ng of H. akashiwo cDNA(Fig. III-2). The annealing temperature was changed 34°C to 45°C with 40 cycles reaction. A single band of

0.75 kbp DNA is synthesized with paired 2-3 primers at 42 and 45°C of annealing temperature(lane 3).

**Confirmation of PCR-synthesized DNA as ATPase cDNA**

Northern blot analysis of H. akashiwo RNA with the 0.75 kbp PCR fragment was shown in Fig. III-3. When the total PCR-synthesized DNA was used for the probe, the two bands(3.8 and 1.7 kb) were detected(lane 1). Therefore, the PCR-synthesized DNA was cloned into pUC118 and the clones were used for the probe of Northern blot analysis. A clone shown up only a 3.8 kb band(lane 2). Then, I selected the other two clones which gave 3.8 kb band, and the three clones were sequenced with M13mp18 vector. The nucleotide sequence(Fig. III-4) and the deduced amino acid sequence(Fig. III-5) are determined. The three clones selected were identical completely. Both N- and C-ends of the amino acid sequence were consistent with the parts of other ATPase P site and AB site, respectively.

I compared the amino acid sequence from P site to AB site with those of $H^+$-ATPase from A. thaliana, L. donovani, N. crassa and S. cereviceae, $Na^+,K^+$-ATPase, $Ca^{2+}$-ATPase and $H^+,K^+$-ATPase from animal and KdpB ATPase from E. coli, and found that the 0.75 kbp cDNA fragment has about 40 % homology with that of L. donovani, about 30 % homology with those of A. thaliana, N. crassa and S. cereviceae about less than 20 % homology with those of animals. Fig. III-6 shows the Harr plots of amino acid sequence between H. akashiwo and L. donovani and high homology part is localized the side of N-end. Fig. III-7 shows that the Harr plots between

$H^+,K^+$-ATPase and $Na^+,K^+$-ATPase from animals demonstrates more than 90 % homology.

## Discussion

In the construction of oligonucleotides for PCR primers, I used deoxyinosines at wobbling positions of each codon(Table III-1,2). The deoxyinosines can be used as an appropriate base analogue that can pair with any of the four natural bases at the ambiguous positions with or without hydrogen bonding(Ohtsuka et al. 1984). This useful base has mainly been used for synthesis of DNA probe to screen cDNA or genomic DNA library. It was the first time that the primers containing deoxyinosine was used with PCR method. The utility of this base for PCR was first demonstrated in Fig. III-1.

All animal ATPase cDNA have about 1.0 kbp from P site to AB site from their sequencing data. Fig. III-1 strongly supports that the PCR-product is the same size as expected and indicate the reliability of this method. On the other hand, the nucleotide length from P site to AB site of higher plants, fungi and protista has been known to 0.75 kbp. The same length fragment was obtained in PCR amplification with H. akashiwo cDNA(Fig. III-2). These results indicate that the obtained 0.75 kbp product is the DNA fragment from P site to AB site of H. akashiwo $Na^+$-activated ATPase. The annealing temperature for the synthesis was also consistent with calculated temperature from the nucleotide sequence(Geoffrey et al. 1987).

In Northern hybridization analysis(Fig. III-3;lane 1), the isolated 0.75 kbp DNA hybridized with 3.8 and 1.7 kb poly [A] RNA from H. akashiwo. As shown in Part I and II,

H. akashiwo has two ATPases which can form phosphorylated intermediate, one is a $Na^+$-activated and 140 kDa polypeptide, another is a monocation activated and 95 kDa polypeptide. These results suggest that the 140 kDa polypeptide should be encoded in 3.8 kb RNA. The cloned 0.75 kbp PCR fragment with pUC118 vector selected to hybridize the 3.8 kb band(Fig. III-3;lane 2). It clearly shows that first synthesized PCR fragment was consisted of two kinds of cDNA from P site to AB site.

Ten clones of the PCR fragment inserted in pUC118 were digested by various restriction enzymes(multi cloning site;EcoR I,Sac I,Kpn I,Xba I,Sal I,Pst I,Sph I and Hind III or Pvu II,Bgl I,Ava I and Taq I), but those digest patterns except for Taq I were completely same. Taq I digestion patterns showed a little difference among 10 clones and were classified three groups. The sequence results of the three groups with difference of Taq I digestion patterns were caused by random partial loses of AB site primers(data not shown).

The deduced amino acids sequence(Fig. III-5) from the nucleotide sequence were compared with other ATPases from various species. And the most homologous organism(40 %) is the $H^+$-ATPase of protista Leishmania donovani on this corresponding part. This result is suspected that the cloned cDNA may be a part of a $H^+$-ATPase of H. akashiwo. But, the comparison of animal $H^+,K^+$- and $Na^+,K^+$-ATPase shows high homology on this part(Fig. III-7). This suggests that the part of ATPases is conserved among familiar species

rather than transported ion species.

## Summary

The partial cDNA of ion transport ATPase of H. akashiwo were amplified with polymerase chain reaction(PCR) method. Two primers were constructed on the conserved amino acid sequences of eukaryotic ion transport ATPases. The PCR amplification with H. akashiwo cDNA resulted in 0.75 kbp DNA synthesis. Northern hybridization to poly [A] RNA with 0.75 kbp cDNA probe showed two bands, 3.8 and 1.7 kb. Then, cloned 0.75 kbp cDNA with pUC118 was selected to hybridize the 3.8 kb band. The DNA was sequenced and the deduced amino acid sequence was analyzed the homology with other ion transport ATPase species. Analysis shows the highest homology with the $H^+$-ATPase of protista L. donovani(40 %), and with that of higher plants, A. thaliana, and fungi, S. cereviceae and N. crassa(30 %). But, animal $Na^+,K^+$-, $Ca^{2+}$- and $H^+,K^+$-ATPase were showed less than 20 % homology.

Table III-1 Conserved amino acid sequences in the ion transport ATPase

| | | | |
|---|---|---|---|
| **Phosphorylation Site(P site)** | | | |
| *A. thaliana* | 327 | VLCSDKTGTLTLNK | 340 |
| *L. donovani* | 347 | MLCSDKTGTLTKNK | 360 |
| *S. cereviceae* | 374 | ILCSDKTGTLTKNK | 387 |
| *N. crassa* | 374 | ILCSDKTGTLTKNK | 387 |
| Animal H | 382 | VICSDKTGTLTQNR | 395 |
| Na/K | 371 | TICSDKTGTLTTNQ | 384 |
| Ca | 347 | VICSDKTGTLTTNQ | 360 |
| *E. coli* KdpB | 303 | VLLLDKTGTITLGN | 316 |
| **ATP Binding Site(AB site)** | | | |
| *A. thaliana* | 585 | GMTGDGVNDAPALK | 598 |
| *L. donovani* | 602 | AMTGDGVNDAPALK | 615 |
| *S. cereviceae* | 631 | AMTGDGVNDAPSLK | 644 |
| *N. crassa* | 631 | AMTGDGVNDAPSLK | 644 |
| Animal H | 723 | AVTGDGVNDSPALK | 736 |
| Na/K | 712 | AVTGDGVNDSPALK | 725 |
| Ca | 699 | AMTGDGVNDAPALK | 712 |
| *E. coli* KdpB | 514 | AMTGDGTNDAPALA | 527 |

Conserved amino acid sequences(dotted area) both phosphorylation site and ATP binding site were determined in 7 species of eukaryotes, *A. thaliana*(higher plants), *L. donovani*(protista), *S. cereviceae*, *N. crassa*(fungi), $H^+,K^+$-, $Na^+,K^+$-, $Ca^{2+}$-ATPase(animal) and a prokaryote *E. coli*(prokaryote) as reference.

Table III-2 Oligonucleotides for PCR primers

site Primer -CSDKTGT-

1. 5' TG(T,C)AGIGA(T,C)AA(A,G)ACIGGIAC 3'
2. 5' TG(T,C)TCIGA(T,C)AA(A,G)ACIGGIAC 3'

site Primer -TGDGVND-

3. 5' ACIGGIGA(T,C)GGIGTIAA(T,C)GA 3'

The oligonucleotide of phosphorylation site(P site) was synthesized as same as mRNA. That of ATP binding site(AB site) was synthesized as complementary to mRNA. Each primer contains deoxyinosine(dot) at ambiguous codon positions.

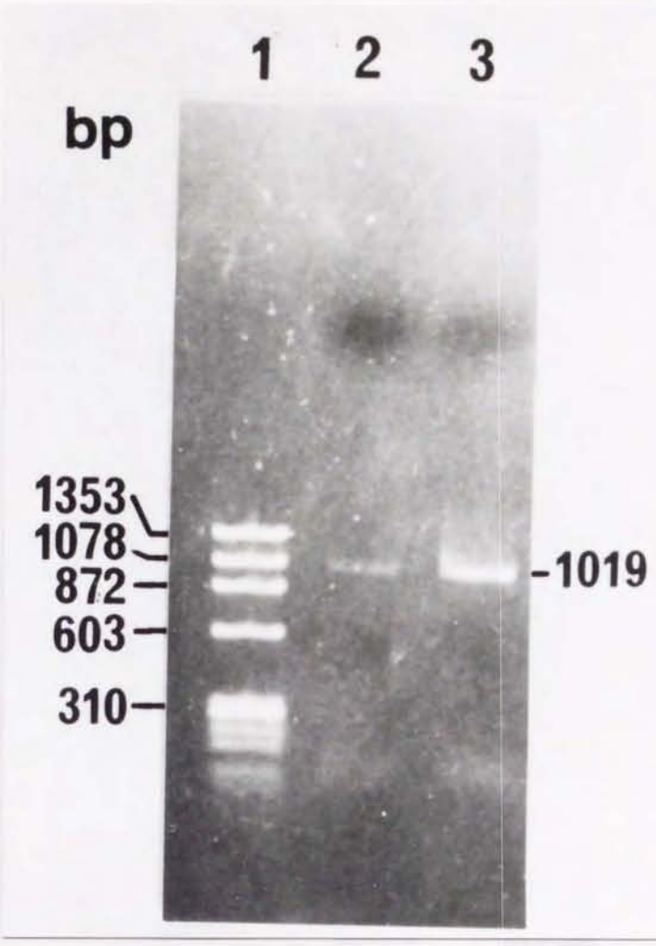


**Fig. III-1 DNA synthesis of animal $Na^+,K^+$-ATPase by PCR method**

The combination of Primer 1+3(lane 2) and Primer 2+3(lane 3) were used for synthesizing partial $Na^+,K^+$-ATPase DNA with PCR method. Both primers(1-3, 2-3) were added each 400 ng on one reaction, which contained 1.0 ng template animal $Na^+,K^+$-ATPase DNA, 1.25 mM dNTP and 2.5 unit Taq I polymerase and was carried out at 37°C for 25 cycles. After amplification, the synthesized DNA was checked by 1.0 % agarose gel electrophoresis and ethidium bromide staining. The numbers at both side indicate the size of DNA pairs.

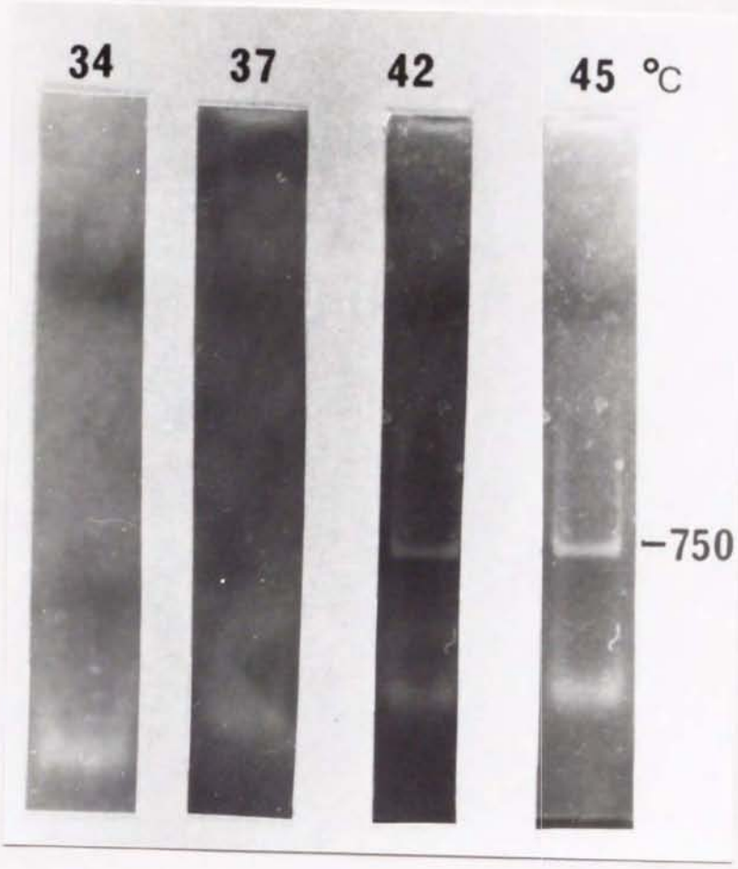


**Fig. III-2 PCR amplification of ATPase DNA of H. akashiwo**

PCR amplification was applied on the 10 ng of H. akashiwo cDNA. Reaction mixture was same as Fig. III-1 and condition was changed that annealing temperature was from 34°C to 45°C with 40 cycles. The numbers on the each lane indicate the annealing temperature and the number of right side indicates base pair.

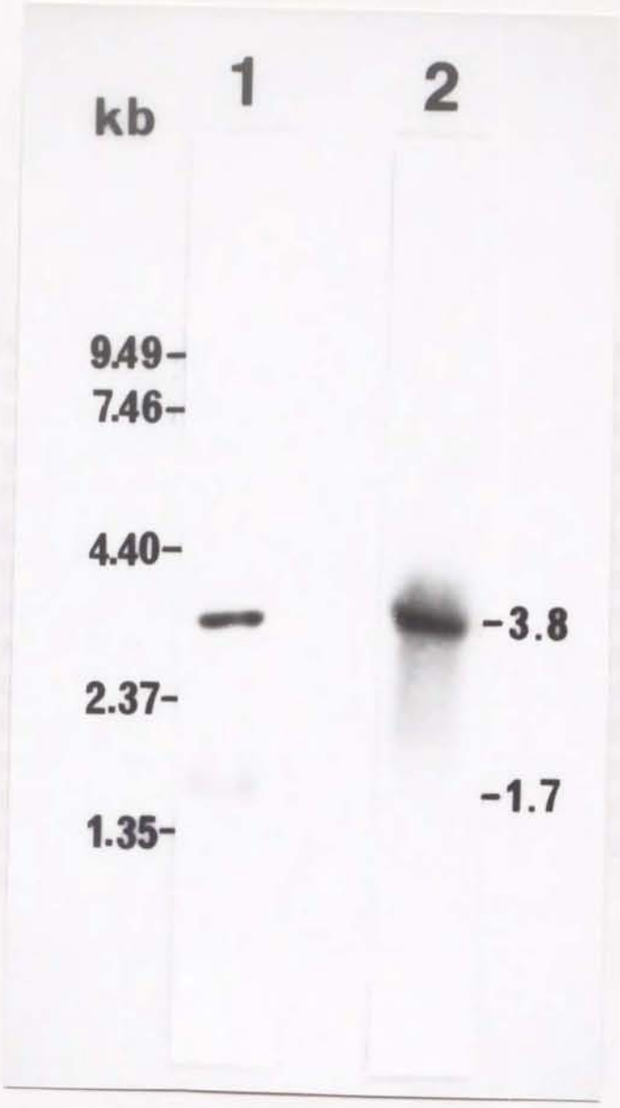


Fig. III-3 Northern blot analysis of H. akashiwo RNA with 0.75 PCR fragment.

H. akashiwo poly [A] RNA(10 μg) was applied on each lane and Northern hybridization was done with total 0.75 kbp PCR fragment(lane 1) and with a cloned one into pUC118(lane 2) as probes.

```
TGCATGC CTGCAGGTCG ACTCTAGAGG ATCCCCAATT CGAGCTCGGT ACCTGCTCGG  60
AAACGGG GACCTTGACC CTGAACAAGA TGGTGATCCA GGACGACTGT CCAATGTTTG 120
ATGGCAT CACCCCAGAG GACGTCATTC TCCATGCCGC GCTGGCTGCC AAGTGGAAAG 180
CCCCGAA GGACGCCTTG GACACCATGG TGCTCGGGGC CTGCGACGTG AGCCTGTGCA 240
CTTTCAC CCAGCTGGAC TACACCCCCT TTGACCCGAC CCTGAAGCGC ACGGAGGCTG 300
TGAAGGG CCCGGACGGG AAGACGTTCA AGGTGACGAA GGGCGCGCCG CACATCGTGC 360
ACCTTTG CCACGACAAG AAGCGCATCG AGGAGGCCGT TGGACTTCAA GGTGCTGGAG 420
GCCGAGC GGGCATCCGG TCCTGGCGGT GGCGAGCACC AACGCGAAGG GCCAGTGGTT 480
GCTGGGC ATCCAGACCT TCCTGGACCC CCGCGCCCGG ACACCAAGCT GACCATCGAG 540
GCCCGCG TGCACGGCGT GGAGGTGAAG ATGGTGACCG GCGACCACCA GGTCATCGCC 600
GAGACCG CGCGCGTGCT GGACGTGGGC ACCAACGTCC TGGGCTGCGA CGGCCTGCCC 660
CTGGATT CCGAGGGCAA GCTGCCCAGC GGCGCCGAGA TGGCCGACAT CTGCCAACGG 720
GTGGACT GCAACGGCTT TGCCCAGGTG TTCCCGGAGC ACAAGTTCGT CATCGTGGAG 780
TGCGCAT GGGCGGCTTT GAAGTGGGCA TGACCGGCGG TACCGAGCTC GAATTGGGGA 840
TCTAGAG TCGACCTGCA GGCATGCAAG C 871
```

Fig. III-4 Nucleotide sequence of H. akashiwo ATPase cDNA.

```
P site
1                                                              50
CSDKTGTLTLNKMVIQDDCPMFVDGITPEDVILHAALAAKWKEPPKDALD
51                                                             100
TMVLGACDVSLCNPFTQLDYTPFDPTLKRTEAELKGPDGKTFKVTKGAPP
101                                                            150
HIVLDLCHDKKRIEEAVGLQGAGAGRAGIRSWRWRAPTRRASGSCWASRP
151                                                            200
SWTPAPGHQADHRARPRARRGGEDGDRRPPGHRQGDRARAGHGHQRPGLR
201                                                            250
RPAHAGFRGQAAQRRRDGRHLPTGGGLQRLCPGVPGAQVRHRGGVRMGGF
251        261
EVGMTGDGVND
   AB site
```

Fig. III-5 Deduced amino acid sequence of H. akashiwo ATPase from Fig. III-4.

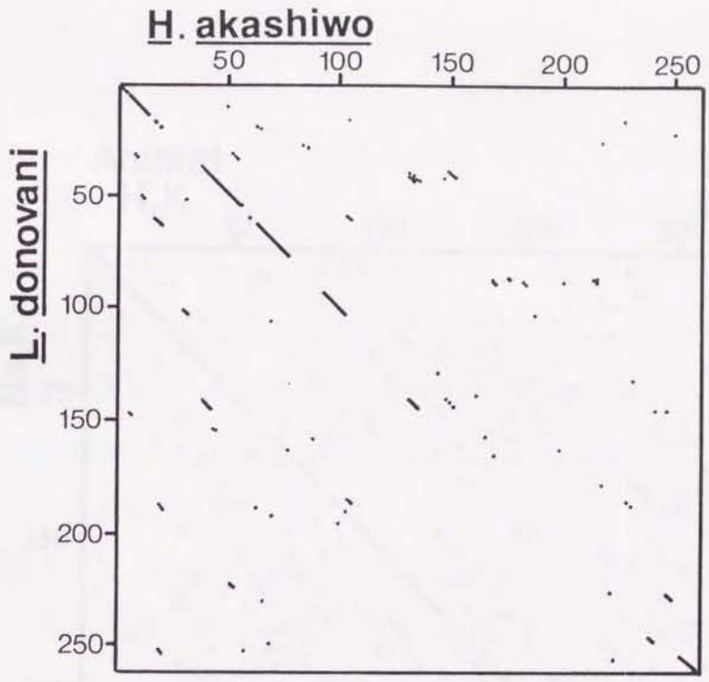


Fig III-6 Harr plot profiles comparing the H. akashiwo with $H^+$-ATPase of L. donovani.

This comparison was carried out at amino acid sequence between P site and AB site of the ATPases. The points were summed over a window of 30 amino acids, and if the total points were greater than 22.5, a dot was placed at the coordinates corresponding to the residue at the center of the window in each sequence.

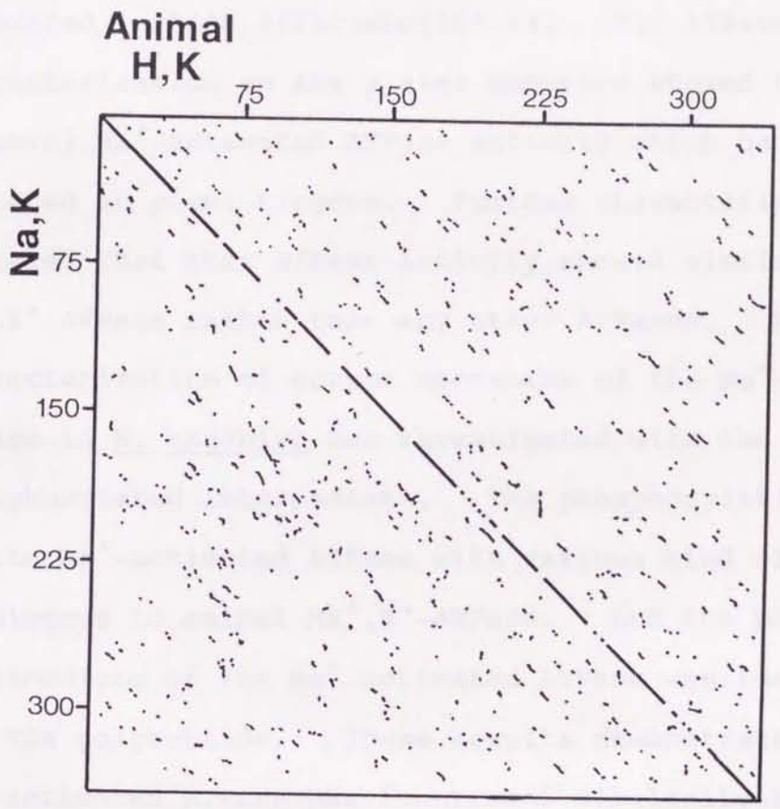


**Fig. III-7 Harr plot profiles comparing animal $H^+,K^+$- with $Na^+,K^+$-ATPase.**

The comparison of amino acid sequence was carried out as same as Fig. III-6 between animal gastric $H^+,K^+$- and kidney $Na^+,K^+$-ATPase.

## Concluding Remarks

In Part I, I applied a new method, silica microbead method, for isolation of plasma membrane from marine raphidophycean Heterosigma akashiwo. The isolated plasma membrane was demonstrated to be highly purified and recovered in high efficiency(66 %). The ATPase characterization on the plasma membrane showed the presence of novel $Na^+$-activated ATPase activity which had been seldom reported in plant kingdom. Further characterization revealed that this ATPase activity showed similar to animal $Na^+,K^+$-ATPase rather than any other ATPases. Further characterization of enzyme mechanism of the $Na^+$-activated ATPase in H. akashiwo was investigated with the detection of phosphorylated intermediate. The phosphorylation pattern of the $Na^+$-activated ATPase with various kind of ion showed homologous to animal $Na^+,K^+$-ATPase. And the phosphorylated intermediate of the $Na^+$-activated ATPase was identified with 140 kDa polypeptide. These results demonstrate that the $Na^+$-activated ATPase has functional similarity to animal $Na^+,K^+$-ATPase, which can pump $Na^+$ out cells.

In Part II, the $Na^+$-activated ATPase was compared with animal $Na^+,K^+$-ATPase immunologically. The aim of this approach was to examine the structural homology between two ATPases. The anti-serum to animal $Na^+,K^+$-ATPase was used for this detection. This serum was characterized that it recognized the $Na^+$ selectivity part of $Na^+,K^+$-ATPase molecule rather than the other common part of all ATPase molecules. The anti-$Na^+,K^+$-ATPase serum reacted only 140

kDa polypeptide of plasma membrane fraction of H. akashiwo. The molecular weight of cross-reacted polypeptide was consistent with $Na^+$-activated ATPase activity. Then, this suggested that this $Na^+$-activated ATPase(140 kDa antigen) of H. akashiwo has homologous structure to $Na^+$ selectivity of animal $Na^+,K^+$-ATPase molecule. These results strongly supported that the epitope of $Na^+$-activated ATPase had also homologous amino acid sequence with the $Na^+$ selectivity of animal $Na^+,K^+$-ATPase. The homology of amino acid sequence suggest that those ATPases evolved from common origin. But, it is necessary for further elucidation to compare nucleotide sequences of full length cDNA. Then, I tried to clone the $Na^+$-activated ATPase DNA for analyzing the nucleotide sequence. In Part III, DNA amplification technique of polymerase chain reaction(PCR) method was applied for the cloning of the $Na^+$-activated ATPase cDNA. The two primers, phosphorylation site and ATP binding site, for PCR method were used because these are conserved in eukaryotic ion transport ATPases. Partial cDNA, 0.75 kbp, of ATPase in H. akashiwo was amplified. Northern hybridization with this 0.75 kbp probe showed two bands, 3.8 and 1.7 kb. The $Na^+$-activated ATPase was 140 kDa polypeptide corresponded to 3.8 kb RNA. Then, the PCR clone was selected to hybridize with only 3.8 kb band. From these results, the selected clone was concluded the part of $Na^+$-activated ATPase cDNA in H. akashiwo. Deduced amino acid sequence from the nucleotide sequence of the cloned cDNA showed 40 % homology to the corresponding part

of $H^+$-ATPase from protista Leishmania donovani. The comparisons with other organism ATPases showed less homologous than the protista ATPase. This part from phosphorylation site to ATP binding site of ion transport ATPases is known to be more homologous among familiar species of organisms rather than transported ion species of ATPases. These views on the comparisons with ATPases show that marine raphidophycean H. akashiwo may be more familiar to the protista rather than higher plants. This work may be a clue to make clear the phylogenic relatedness of H. akashiwo to other organisms. Total conclusion of my experiments has suggested that the mechanism and structure of $Na^+$ selectivity and transport are common between $Na^+$-activated ATPase of H. akashiwo and animal $Na^+,K^+$-ATPase, and two ATPases have same origin. But, the analysis of full length cDNA of the $Na^+$-activated ATPase of H. akashiwo is needed for the clear elucidation.

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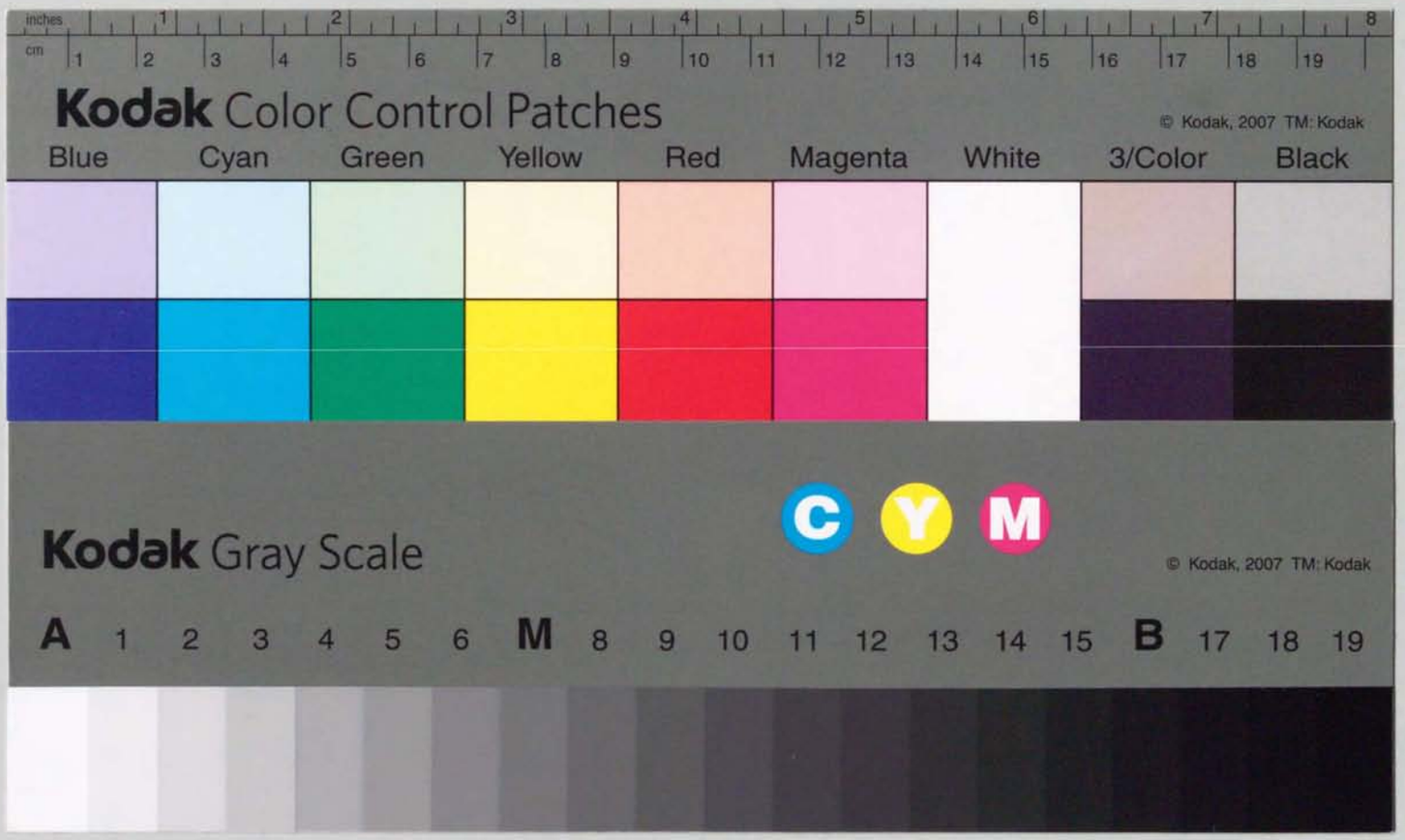
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Green
Yellow
Red
Magenta
White
3/Color
Black
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A 1 2 3 4 5 6 M 8 9 10 11 12 13 14 15 B 17 18 19