ลักษณะสมบัติของโซเดียมไอออนเอทีพีเอสในไซยาโนแบคทีเรีย *Aphanothece halophytica* ที่ทนเค็ม

นางสาว กาญจนา เวียงนนท์

CHARACTERIZATION OF Na$^+$ - ATPASE IN THE HALOTOLERANT CYANOBACTERIUM

Aphanothece halophytica
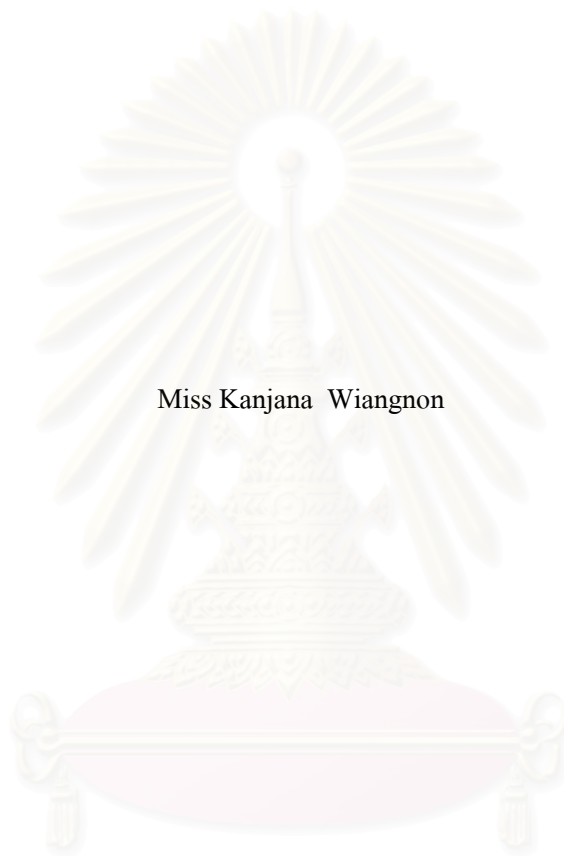
Miss Kanjana Wiangnon

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science Program in Biochemistry

Department of Biochemistry

Faculty of Science

Chulalongkorn University
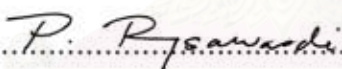
Academic Year 2005

Thesis Title   CHARACTERIZATION OF Na$^+$ - ATPASE IN THE HALOTOLERANT

                                   CYANOBACTERIUM *Aphanothece halophytica*

By   Miss Kanjana Wiangnon

Field of study   Biochemistry

Thesis Advisor   Associate Professor Aran Incharoensakdi, Ph.D.

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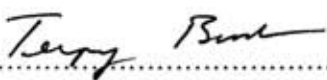
                                                      Deputy Dean for Administrative Affairs,

....………. Acting Dean, The Faculty of Science

(Associate Professor Tharapong Vitidsant, Ph.D.)

THESIS COMMITTEE

....……… Chairman

(Associate Professor Piamsook Pongsawasdi, Ph.D.)

....……….. Thesis Advisor

(Associate Professor Aran Incharoensakdi, Ph.D.)

.... Member

(Associate Professor Nattaya Ngamrotnavanich, Ph.D.)

.... Member

(Assistant Professor Teerapong Buaboocha, Ph.D.)

กาญจนา เวียงนนท์ : ลักษณะสมบัติของโซเดียมไอออนเอทีพีเอสในไซยาโนแบคทีเรีย *Aphanothece halophytica* ที่ทนเค็ม (CHARACTERIZATION OF Na$^+$ - ATPASE IN THE HALOTOLERANT CYANOBACTERIUM *Aphanothece haolphytica*) อาจารย์ที่ปรึกษา: รศ. ดร. อรัญ อินเจริญศักดิ์, 101 หน้า. ISBN 974-53-2778-6

สิ่งมีชีวิตที่อยู่ภายใต้สภาวะแวดล้อมที่มีความเข้มข้นของเกลือโซเดียมคลอไรด์สูง สามารถเจริญเติบโตได้ เนื่องจากเซลล์มีกลไกในการขับโซเดียมออกจากเซลล์เป็นการรักษาระดับโซเดียมภายในเซลล์ให้เหมาะสมเพื่อไม่ให้เป็น อันตรายต่อกระบวนการเมแทบอลิซึม งานวิจัยนี้จึงทำการศึกษาผลของความเค็มที่มีต่อแอกทิวิตีและลักษณะสมบัติทาง ชีวเคมีบางประการของโซเดียมไอออนเอทีพีเอสในไซยาโนแบคทีเรียทนเค็ม *Aphanothece halophytica* โดยพบว่า เมื่อนำ เซลล์มาบ่มด้วย ^{22}NaCl เซลล์จะมีการขับโซเดียมไอออนในรูปของ [^{22}Na$^+$] ออกภายนอกเซลล์เมื่อให้พลังงานด้วยการเติม กลูโคส และเพื่อเป็นการตรวจสอบว่าการขับโซเดียมไอออนออกนอกเซลล์นั้น ผ่านทางโซเดียมไอออนเอทีพีเอสจึงทำการ สกัดแยกพลาสมาเมมเบรนโดยวิธี aqueous polymer two - phase partitioning และนำมาทดสอบแอกทิวิตี พบว่า มี แอกทิวิตีเพิ่มขึ้นเมื่อมีโซเดียมคลอไรด์ 100 มิลลิโมลาร์ และ แมกนีเซียมคลอไรด์ 5 มิลลิโมลาร์ ในภาวะที่ค่าความเป็นกรด ด่างเท่ากับ 7.6 การศึกษากลไกทางจลนพลศาสตร์ของเอทีพีเอส พบว่าค่า K_m ของ ATP และ Na$^+$ เท่ากับ 1.66 และ 25 มิลลิโมลาร์ ตามลำดับ และมีค่า V_{max} เท่ากับ 0.66 และ 0.50 ไมโครโมล นาที$^{-1}$ มิลลิกรัมโปรตีน$^{-1}$ การศึกษาผลของตัว ยับยั้ง พบว่า vanadate และ gramicidin มีผลยับยั้งการทำงานของเอทีพีเอสโดยลดลง 50 % ที่ความเข้มข้น 0.01 และ 0.20 มิลลิโมลาร์ ตามลำดับ ซึ่ง vanadate เป็นตัวยับยั้งของ P-type ATPase และในการศึกษาการนำเข้าของโซเดียมจากภายนอก ในรูปของ [^{22}NaCl] เข้าสู่เมมเบรนเวสซิเคิล พบว่า มีการนำเข้าเมื่อมีการเติม ATP และถูกยับยั้งด้วย sodium ionophore และ gramicidin จึงสรุปได้ว่า เอทีพีเอสเกี่ยวข้องกับการขนส่งโซเดียมไอออน ทั้งการนำเข้าและการขับออก นอกจากนั้น ในภาวะเซลล์ที่เจริญเติบโตในอาหารที่มีความเครียดของเกลือ (2.0 โมลาร์ NaCl) กับสภาวะปกติ (0.5 โมลาร์ NaCl) พบว่า แอกทิวิตีของเอทีพีเอสเพิ่มขึ้น โดยมีค่าแอกทิวิตีจำเพาะเท่ากับ 0.80 และ 0.50 ไมโครโมล นาที$^{-1}$ มิลลิกรัมโปรตีน$^{-1}$ ตามลำดับ

ภาควิชา.................ชีวเคมี..................	ลายมือชื่อนิสิต................................
สาขาวิชา.................ชีวเคมี..................	ลายมือชื่ออาจารย์ที่ปรึกษา...................
ปีการศึกษา...............2548..................	ลายมือชื่ออาจารย์ที่ปรึกษาร่วม...............

KANJANA WIANGNON: CHARACTERIZATION OF Na^+ - ATPASE IN THE HALOTOLERANT CYANOBACTERIUM *Aphanothece haolphytica*. THESIS ADVISOR: ASSOC.PROF. ARAN INCHAROENSAKDI, Ph. D., 101 pp. ISBN 974-53-2778-6.

Extrusion of sodium ion is needed to balance and maintain sodium level for microorganisms to survive in hypersaline environment. Activity of Na^+-ATPase in halotolerant cyanobacterium *Aphanothece haolphytica* was investigated. Cells were preloaded with $^{22}Na^+$ and the extrusion of $^{22}Na^+$ from cells was followed by energizing the cells with glucose. To ascertain that this Na^+ extrusion is Na^+-ATPase, the plasma membranes vesicles were prepared by aqueous polymer two-phase partitioning with dextran and polyethylene glycol and used for the assay of Na^+-ATPase activity by measuring ATP hydrolysis. The ATPase activities were stimulated to greatest extent in the presence of 100 mM Na^+ and 5 mM Mg^{2+} ions, pH 7.6. The K_m value for ATP and Na^+ ions were 1.66 mM and 25 mM respectively. The maximum velocities (V_{max}) were 0.66 and 0.50 μmol/min/mg protein using ATP and Na^+ as variable substrate respectively. The activity was inhibited by vanadate, a potent inhibitor of P-type ATPase. The activity was decreased about half of original value by 0.01 mM sodium vanadate and 0.20 mM gramicidin. Alternatively, the membrane vesicle containing Na^+-ATPase, exhibited $^{22}Na^+$ transport by the addition of ATP, which was inhibited by sodium ionophore and gramicidin. It is likely that the Na^+-ATPase belongs to P-type and is involved in Na^+ transport. The enzyme activity was enhanced by the high salinity of growth medium.

Department………..Biochemistry..........   Student's signature..............................

Field of study………Biochemistry..........   Advisor's signature........................

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Finally, I wish to express my deepest gratitude to my family for their support, infinite love and understanding.

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LIST OF ABBREVIATIONS

A	absorbance
BSA	bovine serum albumin
^{o}C	degree celsius
CCCP	Carbonyl cyanide *m*-chlorophenylhydrazone
DCCD	*N,N'*-Dicyclohexylcarbodiimide
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid
g	relative centrifugal force = $1.12r$ (RPM/1000)^2
h	hour
Hepes	*N*-2-hydroxyethylpiperazine-*N*-ethanesulfonic acid
HCl	hydrochloric acid
kDa	kilodalton
Km	Michealis constant

l	litre
lux	photometric (light density)
M	molar
mA	milliampare
mg	milligram
min	minute
ml	millilitre
mM	millimolar
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
μCi	microcurie
μl	microlitre
μM	micromolar
OD	optical density

rpm   revolution per minute

Tris-HCl   Tris hydrochloride

W   watt

CHAPTER I

INTRODUCTION

Salinity is one the important limiting factors in crop productivity and much effort has been made to understand salt tolerance. Although the ability to adapt to fluctuations in the external salinity is fundamental to survival of organisms, the mechanisms responsible for osmotic adaptation have been largely unknown. Upon the changing salinity of environment, all microorganisms need to balance their cytoplasm with the osmotic pressure exerted by the external medium. To adjust the internal osmotic status that enables organisms to survive in hypersaline environment. Upon the exposure to high salinity, extrusion of sodium ion is needed to balance and maintain sodium level (Bohnert and Jenzen, 1996; Zhu et al., 1997).

Halotolerant are organisms that inhabit hypersaline environments. They include mainly prokaryotic and eukaryotic microorganisms with the capacity to balance the osmotic pressure of the environment and resist the denaturing effects of salts. Examples of well-adapted and widely distributed extremely halotolerant microorganisms, cyanobacteria such as *Aphanothece halophytica*, and the green algae *Dunaliella salina*. Among multicellular eukaryotes, species of brine shrimp and brine flies are commonly found in hypersaline environments. The determination of saline spectrum is very important for the characterization of the type of bacteria to be regarded as halotolerant or extreme, moderate and slight halophiles. According to the salt concentration required for optimum growth, the bacteria were classified as non halophilic (grow below 0.2 M NaCl), slightly halophile (grow at 0.2 to about 1.0-1.2

M NaCl), moderately halophilic (grow at about 1.0-1.2 to 2.0-2.5 M NaCl) and extremely halophilic bacteria (grow at 2.0-2.5 M NaCl or more). Halophile is demonstrated by the requirement of a high salt concentration for optimum growth; however, halotolerance, qualitatively and quantitatively, describes the ability to grow at a salt concentration higher than optimum. However, the degree of tolerance of bacteria depends on the composition of the growth medium (Zahran, 1997). High osmolarity in hypersaline conditions can be deleterious to cells since water is lost to the external medium until osmotic equilibrium is achieved.

Cells have shown that one mechanism for grown under high salinity is to accumulate high solute concentrations as an osmoprotectant within the cytoplasm (Galinski, 1994). When an isoosmotic balance with the medium is achieved, cell volume is maintained. The compatible solutes or osmolytes that accumulate in halotolerant are usually amino acids and polyols, e.g. glycine betaine, ectoine, sucrose, trehalose and glycerol, which do not disrupt metabolic processes and have no net charge at physiological pH. A major exception is for the halobacteria and some other extreme halotolerant, which accumulate KCl equal to the external concentration of NaCl and this is rapidly taken up from the environment via turgor-responsive mechanism. Potassium ion is the prominent compatible solutes found in bacteria, transiently accumulated and is surperseded by the cocommitted other compatible solute such as praline, polyols (Kempf and Bremer, 1998), while many halotolerant bacteria accumulate glycine betaine and ectoine.

The accumulation of organic molecule (compatible solute)

Compatible solutes are generally defined as organic compound of low molecular weight that have no inhibitory effects on metabolism when present at high concentration. There are several types of compatible solutes : sugar (e.g. trehalose), polyols (e.g. glycerol, arabitol, manitol, glycosylglycerol), free amino acids (e.g. praline and glutamate), derivatives thereof (e.g. prolinebetaine, ectoine), quaternary amines and their sulfonium analogs (e.g. glycinebetaine, carnitine and dimethylsulfanipropionate), low-molecular weight nonionic carbohydrates (sucrose, trehalose), free amino acids and their derivatives (praline, glutamate, glycine, γ-amonobutyrate, taurine), unique organic zwitter ion (tetrahydropyrimidine such as ectoine), methylamines (glycinebetaine and trimethylamine-N-oxide) (Yancey, 1982).

Accumulation of compatible solutes is a particularly ubiquitous response. It serves a dual function in osmoregulating cells and also serves as stabilizer of proteins and cells components against the denaturing effects of high ionic strength (Kempf and Bremer, 1998). The current understanding is that these compatible solutes maintain and equilibrium between macromolecule surface areas and the water phase by resisting drastic changes in intracellular water density (Martin et al., 1999). These compatible solutes are accumulated by many organisms through synthesis or uptake from the environment to counteract the outflow of water under hypertonic growth condition (Galinski and Truper, 1994). Most species of green algae are moderate halotolerant, with only a few extremely halotolerant species, e.g. *Dunaliella salina* and *Asteromonas gracilis*, which can grow even in saturated NaCl. Algae predominantly use polyols as compatible solutes. In *Dunaliella salina*, glycerol is

synthesized in response to osmotic stress. The cytoplasmic concentration of glycerol can reach 7 M when grown in medium containing 5 M NaCl. The intracellular sodium concentration has been reported to be less than 100 mM over a wide range of external salt concentrations. Diatoms include *Amphora coffeaeformis* and *Nitzschia* and *Navicula* species., accumulation of proline and oligosaccharides has been reported in some species. Cyanobacteria, *Aphanothece halophytica* is a high salt tolerant cyanobacterium capable of growth in media containing NaCl concentration as high as 3.0 M (Takabe, 1988). The natural habitat of *A. halophytica* used in the present study is in Solar Lake, Israel. Previously we have shown that exogenously provided choline could confer salt tolerance on *A. halophytica* via the accumulation of glycine betaine (Incharoensakdi and Wutipraditkul, 1999). Glycine betaine transport has been shown to be increased by A. halophytica grown in media of elevated osmotic strength (Moor, 1987). For the cyanobacteria, the type of compatible solutes synthesized or accumulated in the cells has been used to differentiate the degree of tolerance to external salinity. For example, strains with low salt tolerance (max 0.7 M) synthesize sucrose or trehalose, strains with moderate salt tolerance (max 1.8 M) synthesize glucosylglycerol and strains with high salt tolerance synthesize glycine betaine. The accumulation of glycine betaine occurs either via transport from the environment or via biosynthesis from a two-step oxidation of choline with betaine aldehyde as an intermediate. It can grow over a wide range of salt concentrations, from 2–5 M NaCl, is an extreme halotolerant with a salt optimum of 3.5 M, and lyses in distilled water. It uses glycine betaine as the major compatible solute, which it can take up from the medium or synthesize from choline (Incharoensakdi and Karnchanatat, 2003).

Another mechanism involves the extrusion of Na^+ from cells. Active sodium export is a well-investigated phenomenon in bacteria, fungi and animals. Whenever in a saline environment the passive Na^+ flux into the cell increases the cytoplasmic Na^+ concentration above a critical level, Na^+ re-export into the environment is initiated (Na^+ homeostasis). One obvious possibility of Na^+ retranslocation is the coupling to inverse H^+-gradients created by H^+-ATPases. The well-known Na^+/H^+ antiporter utilizes these gradients by exchanging external H^+ for internal Na^+ (secondary energized Na^+ export). Recently, we have reported that Na^+/H^+ antiporters from *A. halophytica* can make *E. Coli* and a freshwater cyanobacterium *Synechococcus* PCC 7942 tolerant to high salinity. (Waditee et al., 2002).

Inorganic Cation Transport and Energy Transduction

It is well established that bacteria conserve and transduce metabolic energy by means of an electrochemical gradient of hydrogen ions across the cytoplasmic membrane ($\Delta\mu H^+$), in accordance with the chemiosmotic theory of Peter Mitchell (Mitchell, 1961, 1973). According to this theory, extrusion of protons via one primary transport system or another establishes a proton potential. A primary transport system, or primary pump, is defined as active transport directly linked to a metabolic reaction; examples include electron transport by a redox chain, a proton-translocating ATPase (Fig.1), and a light-driven reaction such as the photosynthetic reaction center and bacteriorhodopsin (Harold, 1977). The electrochemical gradient of protons $\Delta\mu H^+$ (proton potential, Δp) across the plasma membrane is the sum of two components, an

electrical potential ($\Delta\psi$, interior negative) and a pH gradient (ΔpH, interior alkaline). The relationship of these parameters is described by $\Delta p = \Delta\psi - Z\Delta$pH, where ΔpH is the difference between the pH of the bulk medium and that of the cytosol and the factor Z is $2.303RT/F$ and is 59 mV at 25 °C. The proton potential (proton motive force) can then be used by the cells to drive proton-linked energy-consuming processes. Most important, it is employed in the synthesis of ATP from ADP and inorganic phosphate by the $F_O F_1$-ATP synthase and in active transport by secondary transport systems which are not associated with a concurrent chemical reaction. Porters perform osmotic work by coupling the flux of one solute to that of another, for example protons. The linkage of coupled fluxes with the same direction in space is called symport, and the linkage of those with the opposite direction is called antiport (Fig. 1). Exergonic and endergonic reactions are thus coupled via the circulation of protons across the membrane (Harold, 1970).

There are least three general types of transport ATPase. Several distinct types of ATP-dependent active transports have arisen, differing in structure, mechanism, and localization in specific tissues and intracellular compartments (Nelson and Cox, 2000).

(i) P-type ATPase are ATP-driven cation transporters that are reversibly phosphorylated by ATP as part of the transport cycle. All P-type transport ATPase have similarities in amino acid sequence, especially near the Asp residue that undergoes phosphorylation, and all are sensitive to inhibition by phosphate analog vanadate. Each is an integral protein with multiple membrane - spanning regions in a single polypeptide; some also have a second subunit (Fig. 1). P-type transporters are very widely distributed. It animal tissues, the Na^+ K^+ ATPase an antiporter for Na^+

and K^+, and the Ca^{2+} ATPase, a uniporter for Ca^{2+}, are ubiquitous, well-understood P-type ATPase that maintain disequilibrium in the ionic composition between the cytosol and the extracellular medium. Parietal cells in the lining of the mammalian stomach have a P-type ATPase that pumps H^+ and K^+ across the plasma membrane, thereby acidifying the stomach contents. In higher plants, a P-type ATPase pumps protons out of the cell, establishing a difference of as much as 2 pH units and 250 mV across the plasma membrane. A similar P-type ATPase in the bread mold *Neurospora* pumps protons out of cells to establish an insidenegative membrane potential, which is used to drive the uptake of substrates and ions from the surrounding medium by secondary active transport. Bacteria use P-type ATPase to pump out toxic heavy metal ions such as Cd^{2+} and Cu^{2+} (Nelson and Cox, 2000; Kakinuma and Unemoto, 1985).

(ii) A distinctly different class of proton-transporting ATPases is responsible for acidifying intracellular compartments in many organisms. The vacuoles of fungi and higher plants maintain a pH between 3 and 6, well below that of the surrounding cytosol (pH 7.5), by the action of V-type ATPase proton pumps. V-type ATPase (V for vacuolar) are also responsible for the acidicfication of lysosomes, endosomes, the Golgi complex, and secretory vesicles in animal cells. Structurally unrelated to P-type ATPase, the V-type ATPase do not undergo cyclic phosphorylation and de phosphorylation and are not inhibited by vanadate. All V-type ATPase have a similar complex structure, with an integral (transmembrane) domain (V_0) that serves as a proton channel and a peripheral domain (V_1) that contains the ATP-binding site and the ATPase activity. The mechanism by which V-type ATPase couple ATP hydrolysis to the uphill transport of protons is not understood in detail.
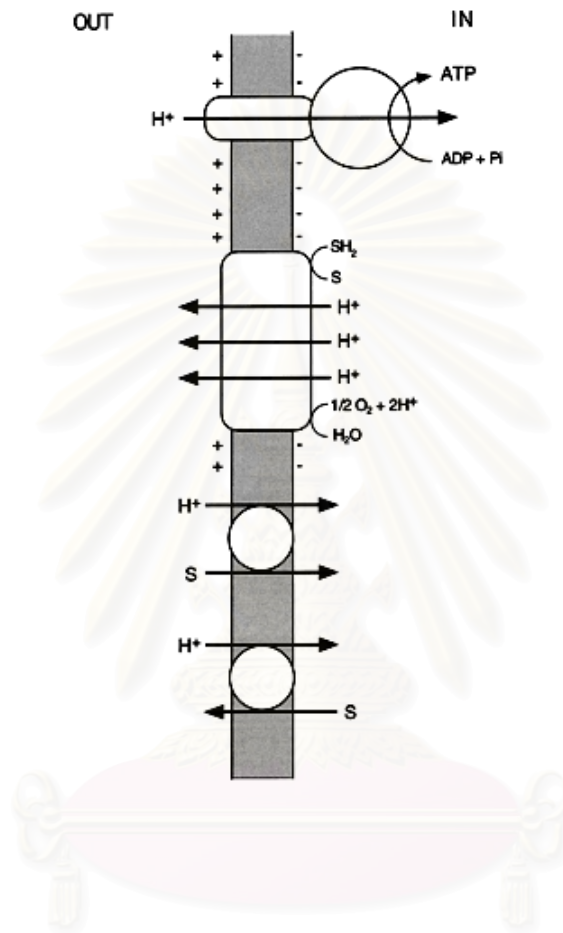
Figure 1 Chemiosmotic energy coupling. Electrogenic proton extrusion by the respiratory chain generates an electrochemical gradient of protons $\Delta\mu H^+$ (proton potential), composed of a pH gradient (inside alkaline) and a membrane potential (inside negative). Proton flow into the cytoplasm via F_OF_1-ATP synthase energizes formation of ATP from ADP and inorganic phosphate (Pi) and, via cotransport systems, drives active uptake (symport) or extrusion (antiport) of various substrates (S).

The V-type proton pumps are related in structure, and probably in mechanism, to a third family of proton pump, the F-type ATPases (Hilpert, 1984; Kakinuma and Unemoto, 1985).

(iii) F-type ATPases play a central role in energy-conserving reactions in bacteria, mitochondria, and chloroplasts; that role will be discussed in detail when we describe oxidative phosphorylation and photophosphorylation. They catalyze the uphill transmembrane passage of protons driven by ATP hydrolysis, as well as the reverse reaction, in which downhill proton flow drives ATP synthesis. In the second case, The F-type ATPases are more appropriately name ATP synthases. The proton gradient in oxidative phosphorylation and photophosphorylation is established by other types of proton pumps powered by substrate oxidation or sunlight. The F-type ATPase/ATP synthases are multisubunit complexes that provide a transmembrane pore (the integral protein F_0) for protons and a molecular machine (The peripheral protein F_1) that uses the energy released by downhill proton flow through F_0 to from the phosphoanhydride bonds of ATP. The ATP-synthesizing and ATPase activities reside in the F_1 protein (Nelson and Cox, 2000).

Sodium transport system

Bacteria actively extrude sodium ions and maintain the electrochemical concentration gradient of sodium directed inward. The significance of the sodium gradient in bacteria is well know; the sodium current is frequently linked with cotransport systems and can serve as the driving force for flagella rotation. The maintenance of a constant internal ion composition is indispensable to all living cells.

Bacteria tend to maintain the cytoplasmic pH within a narrow range and to establish gradients of K^+ and Na^+ ions between their cytoplasm and the surrounding medium such that the cytoplasmic K^+ concentration is higher than and the Na^+ concentration is lower than that of the environment. It is accepted that secondary transport systems coupled to protons mediate the movements of K^+ and Na^+ ions. Proton movement across the membrane is the primary event not only for energy metabolism but also for performing this homeostatic work. Microorganisms living in aquatic habitats are directly exposed to the outside world through a cell surface layer. Their habitats commonly encompass a wide range of physical conditions: oxygen, pH, salinity, temperature, light, etc. Bacteria that cannot cope with and survive in severe environments by depending on their H^+-linked machinery alone have evolved a variety of ancillary energy conversion mechanisms. It is now recognized that Na^+ ions supplement the role of protons in energy transduction across the bacterial membrane (Lanyi, 1979). We know of diverse sodium pumps, such as

(i) Na^+-translocating membrane-bound decarboxylases in *Klebsiella pneumoniae*, *Salmonella typhimurium*, *Veillonella alcalescens*, *Propionigenium modestum*, etc. (Dimroth, 1987).

(ii) Na^+-translocating NADH oxidoreductase in various marine bacteria such as *Vibrio alginolyticus*. (Tokuda and Unemoto,1982).

(iii) Na^+-translocating ATPase in *Enterococcus hirae*. (Kakinuma, 1998).

All these generate an electrochemical gradient of sodium ions, which is used by the cells to drive secondary Na^+-linked processes such as solute transport (Kakinuma and Unemoto, 1985) and flagellar rotation (Hirota and Imae, 1983).

In *P. modestum*, the Na^+ gradient generated by the decarboxylation of organic acids is used for ATP synthesis by the Na^+-ATPase (Hilpert, 1984). In marine bacteria, a variety of transport systems are linked to Na^+ rather than H^+; in this case, the sodium circulation is primary rather than supplemental to the proton circulation. The mechanism of sodium extrusion is generally thought to be secondary antiport of sodium ions for protons, energized by the protons potential, just as Mitchell envisaged. However, the activity on the Na^+/ H^+ antiporter is supplemented by a variety of primary transport systems energized by ATP hydrolysis, redox potential, or decarboxylation. Sodium transport in streptococci has been extensively studied in *E. hirae*. In of Mitchell's antiport hypothesis but now illustrates the interplay between the primary and secondary modes of energy linked transport. Alkaliphilic bacteria use an Na^+ gradient as the driving force for solute transport and flagellar rotation at high pH (Imae and Atsume, 1989), when the proton concentration is too low. On the other hand, halorhodopsin functions as an electrogenic chloride pump in *Halobacterium halobium*, generating a membrane potential. These specialized energy-transducing systems are again very important for ion homeostasis in particular cases. In these bacteria, which lack a respiratory chain (Fig. 1), ATP produced by substrate-level phosphorylation is hydrolyzed by the F_OF_1-ATPase with accompanying translocation of protons; the resulting proton potential is utilized for various proton-coupled transport reactions. It is noteworthy that among streptococci, enterococci are particularly tolerant to external stresses including high temperature, high salt concentration, alkaline pH, and the presence of bile salts. *Lactococcus lactis* is also known to be moderately tolerant to these factors. Facing harsh conditions such as high salinity and alkaline pH, enterococci and probably also *L. lactis* have evolved special

energy conservation mechanisms for cation transport and homeostasis, which other streptococci may not have. *E. hirae* (formerly *Streptococcus faecalis*), which is found in the intestine of higher animals, proved to be a useful system for unraveling the energetics of active transport, particularly the role of the F_OF_1-ATPase in chemiosmotic energy transduction (Harold et al., 1972,1969). First, *E. hirae*, like other streptococci, lacks respiratory chains. It can generate a proton potential only by the hydrolysis of ATP, effected by the proton-translocating F_OF_1-ATPase. Second, its simple metabolic pathways allow the precise calculation of ATP yields from the few compounds that it can metabolize, such as glucose and arginine. Third, the cells are easily depleted of energy, because the organism does not make energy reserve polymers. Finally, like other gram-positive organisms, it is sensitive to ionophores and inhibitors that act on the cell membrane. Because of these advantages, research related to inorganic cation transport processes has been carried out primarily with enterococci, although work on other species such as *L. lactis* has recently begun to be published. One of the major findings with *E. hirae* and other streptococci is that ATP plays a much more important role in membrane transport than it does in nonfermentative organisms; streptococci are unable to generate a large proton potential because they lack respiratory chains (Harold and Kakinuma, 1985). Streptococci cope with their limited proton potential by expressing a variety of primary transport systems. Na^+/H^+ antiporter. Harold and his colleagues contributed fundamental information on Na^+ transport in *E. hirae.* Early studies on Na^+ transport in *E. hirae* were interpreted as providing support for Na^+/H^+ antiporter driven by the proton potential. Sodium extrusion from the cells against the Na^+ gradient was blocked by DCCD, an inhibitor of proton translocating F_0F_1- ATPase, suggesting that

sodium efflux requires a proton potential. Furthermore, H^+ influx accompanying Na^+ efflux was observed in alkalinized Na^+- loading cells metabolizing in Na^+- free buffer. In this experiment, the driving force for Na^+ efflux was presumably the Na^+ gradient directed outward, since DCCD prevented establishment of proton potential. These finding provided the first confirmation of the proposal bacteria contain Na^+/ H^+ antiporter. For some year, this antiporter activity was held to be an artifact cennected with the newly discovered Na^+- translocating ATPase. Briefly, the hypothesis was that the Na^+ pump of *E. hirae* catalyzes an ATP-driven exchange of H^+ for Na^+. The antiporter activity, visible only in membrane vesicles or in a mutant deficient in the Na^+ pump, was considered to be the antiporter moiety of a modular ATP–driven exchanger of Na^+ for H^+. This interpretation, however, proved incorrect. In the wild type strain cultured on Na^+-limited medium, in which the Na^+-inducible Na^+- ATPase level was minimal, the Na^+/ H^+ antiporter activity was clearly observed. In response to an artificially imposed pH gradient (with exterior acid), energy depleted cells exhibited a transient sodium extrusion which as unaffected by treatments that dissipated the membrane potential but was blocked by proton conductors. One most conclude that *E. hirae* has two separate Na^+ extrusion systems: Na^+- ATPase and Na^+/ H^+ antiporter (Kakinuma, 1998).
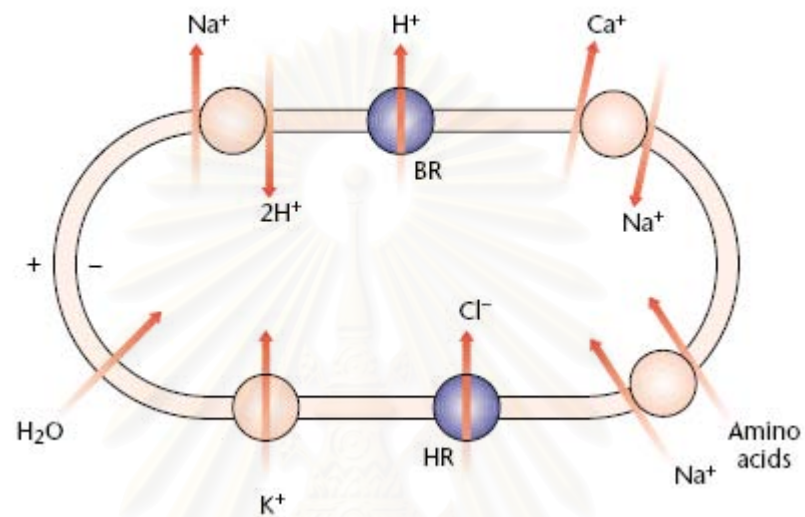
Figure 2 Flux of ions and molecules across the membrane of halobacteria. The light-driven proton and chloride pumps, bacteriorhodopsin (BR) and halorhodopsin (HR) (respectively) and the sodium/proton antiporter, potassium uniporter, sodium/amino acid symporter and calcium/sodium antiporter.

Na$^+$/H$^+$ antiporters

Hence all cells have sodium extrusion via the Na$^+$/H$^+$ antiporters which catalyze the exchange of Na$^+$ for H$^+$ across membranes. The primary energy source for this system in most organisms is the proton electrochemical gradient ($\geq\mu_{H+}$) across the cytoplasmic membrane is the proton electrochemical gradient is derived ether from respiratory electron transport or at the expense of ATP formed during substrate-level phosphorylation by activity of the membrane ATPase (Oren, 1999). The Na$^+$/H$^+$ antiporters are membrane proteins that are essential for maintenance of the balance between Na$^+$ and K$^+$ in plant, Fungal and bacterial cells, in particular when the organism lacks primary Na$^+$ pumps (Inaba et al., 2001). In cyanobacteria, the existence of a concentration gradient of Na$^+$ directed inward which is dependent on the photosynthetic or respiratory metabolism, has been demonstrated (Padan and Vitterbo,1986). These result indicate the existence of an active transport system extruding Na$^+$ from cyanobacterium cells. From the complete nucleotide of cyanobacterium *Synechocystis* sp. PCC 6803, it was suggested that *Synechocystis* sp. PCC 6803 contain at least five Na$^+$/H$^+$ antiporters. The Na$^+$/H$^+$ antiporters play a variety of functions such as:

(i)   Establish of an electrochemical potential of Na$^+$ across the cytoplasmic membrane, this being the driving forces for Na$^+$-coupled processes such as Na$^+$/solute symport and Na$^+$/driven flagella rotation (Vimont and Borche, 2000).

(ii)   Extrusion of Na$^+$ and Li$^+$, which are toxic if they accumulate to high concentrations in cells (Dover and Padan, 2001).

(iii)   A role in pH homeostasis, i.e., regulate intracellular pH under alkaline pH condition. In the mutant lacking Na^+/H^+ antiporters av\ctivity the capacity to regulate its intracellular pH (pH_i) is lost (Vimont and Borche, 2000).

(iv)   Cell volume regulation (Orlowski and Grinstein, 1997).

(v)   Morphogenesis, it has been reported in yeast cells, *Canadida albicans* that the deletion of *chn1* which encoded Na^+/H^+ antiporters resulted in the retardation of growth and highly elongation morphology in a signification fraction of cells under conditions that normal support yeast growth (Soong et al., 2000).

Another possible factor that can modulate the exclusion of from cells is the so-called "sodium pump" which is a Na^+- stimulated ATPase (primary energized Na^+ export) is a membrane protein found in a number of organisms including cyanobacteria. Reviewing the literature of the last ten years one gets the impression that only bacteria, cyanobacteria, fungi, and mammalia, but not algae or higher plants, possess Na^+-ATPases in their plasma membranes (PM). This is surprising, since algae and halophytic higher plants also survive high salinity. In fact, algae such as *Dunaliella* species which can grow in the saturated salt solution in the Dead Sea. Marine algae, seaweeds, and sea grasses are exposed to NaCl concentration between 450 and 500 mM at pH values between 8 and 9.
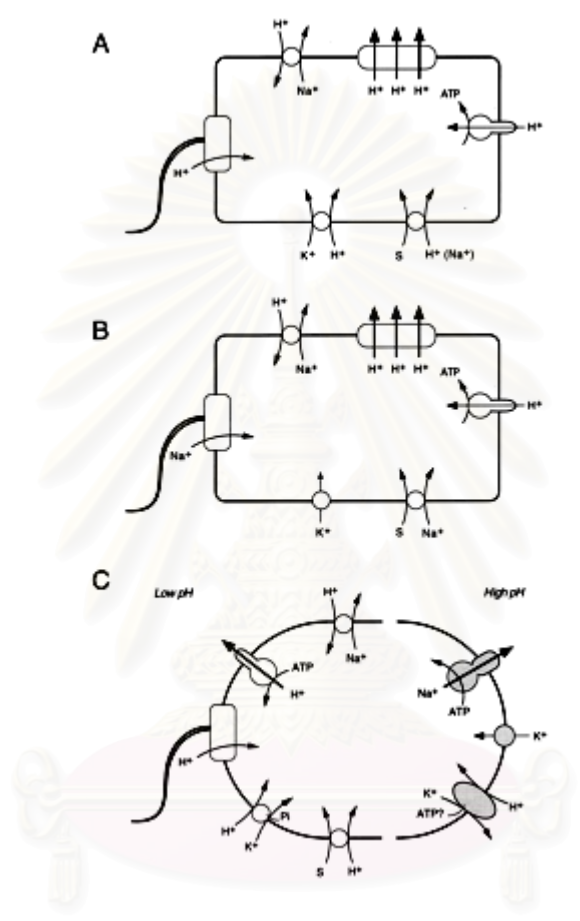
Figure 3 Circulation of H^+, Na^+, and K^+ in bacteria. (A) *E. coli*. (B) Alkaliphilic bacteria. (C) *E. hirae*.

Na$^+$ dependent ATP-hydrolysis (Na$^+$- ATPase)

Plasma membrane vesicles of *Tetraselmis viridis* and *Heterosigma akashiwo* catalyse a Mg^{2+} dependent ATP-hydrolysis stimulated by Na$^+$ (Wada et al., 1989; Shono et al., 1995). ATP-hydrolysis is maximal at pH values between 8 and 9, it is inhibited by vanadate, but not inhibited by ouabain. The K$_m$ values for ATP is 0.88 mM and that for Na$^+$ 12 mM (Shono et al., 1995). However, it is striking that in contrast to the pH profile of vanadate-sensitive ATP hydrolysis of plasma membrane vesicles isolated from *Dunaliella acidophila*, which exhibits a peak at pH 6, the saline *Dunaliella* species exhibit either a broad optimal range between pH 6 and 9 (Gimmler et al., 1989; Weiss et al., 1992) or a narrow peak at pH 8.5 (Smahel et al., 1990). These data might indicate the overlapping of ATP-hydrolysis catalysed by two distinct ATPases. At a pH of 7.6, vanadate-sensitive ATP hydrolysis of *Dunaliella parva* plasma membrane vesicles is slightly, but significantly, stimulated by Na$^+$ and activity is higher in the presence of permeant anions. Although most of the early data on Na$^+$ extrusion by *E. hirae* cells fit the sodium/proton antiport model, there was one important observation which could not be easily explained by this model: net Na$^+$ movement and ^{22}Na$^+$-Na$^+$ exchange were seen only in cells capable of generating ATP (Harold, 1972). The search for an answer to this question led to the discovery of the sodium-translocating ATPase in *E. hirae*. Sodium extrusion against a concentration gradient, under conditions such that the proton potential has been totally dissipated by the presence of DCCD or protonophores and valinomycin, was readily induced by the addition of glucose or arginine. Since the energy donor common to the metabolism of glucose and arginine is ATP, Na$^+$ extrusion was attributed to an ATP-

driven Na^+ pump (Heefner, 1980). ATP-driven $^{22}Na^+$ uptake and Na^+-stimulated ATP hydrolysis were observed in everted membrane vesicles in the presence of DCCD and the ionophores (Heefner, 1982). No Na^+-pumping activity was detected in vesicles of mutant 7683 (Harold, 1970); this mutant is now considered to be a double mutant defective in both Na^+-ATPase and the Na^+/H^+ antiporter (Kakinuma and Igarashi, 1989; Solioz and Davies, 1994). All these results suggest that a Na^+-translocating ATPase exists in the cell membrane of *E. hirae*, which was the first bacterium in which a Na^+-ATPase was discovered. Na^+ movements unconnected to a proton potential have recently been reported in *S. bovis* (Strobel, 1989); an Na^+-ATPase is likely to be responsible. Ion-motive ATPases are divided into two categories: one which forms phosphorylated intermediates (E-P enzyme; P-ATPase) and the other which does not. P-ATPases are exemplified by the Na^+, K^+-ATPase, H^+,K^+-ATPase and Ca^{2+}-ATPase of higher organisms and by a variety of ion-translocating ATPases in bacteria (Pedersen, 1987; 1987). The ATPases which do not form E-P intermediates are now divided into two types: F_OF_1-ATPase (F-ATPase) and vacuolar ATPase (V-ATPase). F-ATPase functions as an ATP synthase in oxidative bacterial membranes and as the proton pump in the membranes of fermentative bacteria such as streptococci. On the other hand, the V-ATPase is known as the proton pump of acidic organelles, such as the vacuoles of fungi and plants and various endosomes of animal cells (Forgac, 1989; Nelson, 1988; 1992). Although the "modular pump model" has been excluded, we expected the *E. hirae* Na^+-ATPase to be distinct from other ion-motive ATPases, because of its resistance to both vanadate, an inhibitor of P-ATPase, and DCCD, an inhibitor of F- and V-ATPases. An antibody to purified *E. hirae* F_1-ATPase did not inhibit the Na^+-ATPase (Kakinuma and Igarashi, 1989). A decade

after the discovery of the Na^+-ATPase, attempts at purification showed that the enzyme is complex, containing both peripheral and membrane-embedded subunits. Everted vesicles were treated with EDTA for the purpose of peeling off the unrelated peripheral proteins from the membranes, especially F_1-ATPase. Contrary to expectations, Na^+-stimulated ATP hydrolytic activity of the membranes was easily removed by this treatment. However, Na^+-ATPase activity was fully restored by addition of an excess of Mg^{2+} to this EDTA-treated membrane suspension. The component essential for the activity of this enzyme was released and detected by polyacrylamide gel electrophoresis. The amount of Na^+-ATPase in the membranes increases in cells cultured in high-Na^+ medium (Kakinuma and Harold, 1985; Kinoshita et al., 1984). The amount of this protein in the EDTA extract altered in parallel with the activity of the Na^+-ATPase. The ATPase was subsequently purified; its molecular mass was about 400 kDa, consisting of 65-, 56-, and 29-kDa polypeptides with a probable stoichiometry of 3:3:1. Linkage with K^+ movement, before finding the structural information on the Na^+-ATPase by gene cloning, Harold and Kakinuma suggested that this ATPase may be a $Na^+(K^+)$-ATPase (Kakinuma and Harold, 1985). Potassium uptake via the K^+ transport system is somehow linked to the Na^+-ATPase, which, in intact cells, expels Na^+ ions by exchange for K^+. In 1984, Kinoshita et al. reported that the Na^+-ATPase level of *E. hirae* is not constant. They measured the Na^+-stimulated ATPase in mutant AS25, deficient in the H^+-ATPase, and observed much higher levels of Na^+-ATPase in this mutant than those found by Heefner and Harold (Heefner, 1982) in the wild type. Moreover, both the rate of sodium transport by intact cells and the activity of Na^+-ATPase in vesicles were altered by the culture conditions. Mutant cells grown in high-Na^+ (0.12 M)

media exhibited high activities, while those grown in low-Na$^+$ (5 to 10 mM) media exhibited much lower enzymatic and transport activities. Sodium transoort and Na$^+$-ATPase activity in the wild-type strain were much lower than those in the mutant strain. When the wild-type cells were grown in the presence of a protonophore, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), both Na$^+$ transport and Na$^+$-ATPase activity were elevated. Furthermore, when the wild-type cells were grown at alkaline pH, both activities increased significantly (Murata et al., 1996). The sodium ATPase is thus induced when cells are grown on media rich in sodium, particularly under conditions that limit the generation of a proton potential, indicating that an increase in the cytoplasmic sodium level serves as the signal. Western blotting and Northern blotting experiments revealed substantial correlation between the amount of this enzyme and expression of the *ntp* operon. Even when the cells were grown on low-Na$^+$ medium, the ionophores monensin and gramicidin D, which render the membrane permeable to Na$^+$, significantly increased the amounts of Na$^+$-ATPase and of the mRNAs for the operon (Murata et al., 1996). All these data are explained by the hypothesis that the Na$^+$-ATPase is induced at the transcriptional level by an increase in the cytoplasmic Na$^+$ concentration. Whether a vacuolar-type Na$^+$-ATPase occurs in other streptococci is not clear. Based on their tolerance to salinity and alkaline pH, some related streptococci such as *L. lactis* and *S. bovis* appear to possess a Na$^+$-ATPase, although it may be less prominent. We have already found that everted membrane vesicles of *L. lactis* contain a Na$^+$-stimulated ATPase; reactions with antibodies directed against components of the *E. hirae* enzyme suggest the presence of homologs to NtpA and NtpB. An Na$^+$-translocating ATPase, recently discovered in the thermophilic *Clostridium fervidus* (Speelmans et al.,1993), is very likely to be of

the vacuolar type as judged by the enzymatic and biochemical properties of the purified enzyme (Honer et al., 1997). Furthermore, the enzymatic features of an Na^+-stimulated-ATPase observed in *Mycoplasma mycoides* (a parasitic glycolytic organism) are virtually identical to those of the *E. hirae* enzyme (Benyoucef et al., 1982), and an Na^+-stimulated ATPase from *Acholeplasma laidlawii* seems to be of the V type as judged by its subunit composition (Jinks et al., 1978; Lewis and Maelhaney, 1983). On the other hand, the electrogenic Na^+-ATPases found in *P. modestum*, *Acetobacterium woodii* (Reidliner and Muller, 1994), *Methanosarcina mazei* (Becher and Muller, 1994) and other organisms belong to the F-ATPase class. It is proposed that these ATPases perform ATP synthesis, energized by the Na^+ gradient generated by a metabolic sodium pump. The *P. modestum* Na^+-ATPase has contributed powerfully to analysis of the mechanism of F-ATPases (Kaim and Dimoroth, 1995; Laubinger and Dimoroth, 1987). To date, no P-type Na^+-ATPase has been found in bacteria. *E. hirae* is the organism that has clearly proved that functional F- and V-ATPases can coexist on the same plasma membrane; there is no particular similarity between the subunits of these ATPases found in the same organism. Coexistence of V- and F-ATPases in a single bacterial cell is no longer exceptional. Most marine algae are able to maintain lower cytosolic concentrations of Na^+ ions than those in the seawater in which they live. Two systems can be postulated to achieve such a difference: a system for extrusion of Na^+ ions on the plasma membrane and a system for sequestration of Na^+ ions into an organelle such as the vacuole. Some evidence indicates that intracellular Na^+ ions are compartmented into the vacuoles of marine algae. Using the unicellular marine alga *H. akashiwo,* Wada et al. (1989) reported the presence of a plasma membrane bound ATPase whose activity was inhibited by

vanadate and activated by Na^+ and/or K^+ with Mg^{2+} ions; the highest activity was obtained in the presence of both 100 mM Na^+ and K^+ with 5 mM Mg^{2+} ions at pH 8.0. These characteristics strongly suggested that the Na^+-activated ATPase from *H. akashiwo* must be a Na^+ plus K^+ synergistically stimulated ATPase. The ion requirements of the Na^+-ATPase were very similar to those of Na^+/K^+-ATPases from animal cells, and the activity was stimulated to the greatest extent in the presence of more than 100 mM NaCI and 100 mM KC1 with 5 mM Mg^{2+} ions. All vanadate-sensitive ATPases or P-type ATPases, such as animal Na^+/K^+-ATPases, form a phosphorylated intermediate (Kakinuma, 1987) that is easily detectable by acid SDS-PAGE.

At least one route for Na^+ entry must exist, since a Na^+ circulation is important for the growth of streptococci. The nature of this pathway is still unknown. The limited data at hand suggest that the Na^+/H^+ antiporter mentioned above may allow Na^+ ions to enter cells whose membrane potential has been collapsed (Kakinuma, 1987). A different pathway was observed by Heefner and Harold, who showed that when a membrane potential (inside negative) was imposed by K^+ efflux, the cells took up Na^+ ions by exchange for K^+. The process involved has low affinity but high capacity ($K_m > 20$ mM; $V_{max} > 50$ nmol/min/mg of cells) and apparently responds to both the concentration gradient and the electrical potential. Because of its low affinity, we suspect that this pathway is relatively nonspecific and reflects some kind of leakage down the electrochemical potential gradient. Streptococci exclude Na^+ ions and accumulate K^+ ions. There is no simple answer to the question why cells expend energy on generating this ion gradient. Generation of a sodium gradient is an essential

aspect of any sodium circulation. The reason for the obligatory role of sodium circulation in streptococci is not evident, except for the accumulation of K^+ at alkaline pH, which is somehow linked to Na^+ extrusion. Na^+-dependent secondary transport has been reported only for *S. bovis* (Russell et al., 1988). There is also a body of evidence to suggest that a high Na^+ concentration in the cytoplasm is generally inhibitory to cell physiology, although specific targets have not been identified (Harold et al., 1970). On the other hand, it has long been known that K^+ ions are the major cations specifically required for protein synthesis

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            ┌─────────────────────────┐
            │ Cyanobacteria in fresh water │
            └─────────────────────────┘
                        ↓
        Hypersaline treatment (e.g.addition of NaCl)
                        and
      ↙                                              ↘
osmoticstress                                   Ionic stress
      ↓                                              ↓
Loss of water                              Influx of inorganic
                                                  ions
                                             (e.g. Na⁺, Cl⁻)
```

Immediate responses, Protein synthesis independent

- Accumulation of osmoprotective Compounds Sucrose, trehalose, glucosyl-glycerol, glycine-betaine

Active export of inorganic ions

Long term adaptation, Protein synthesis dependent

- Expression of salt stress proteins

- Synthesis of osmoprotective compounds

- Enhanced energetic activities cytochrome oxidase and PSI cyclic photosynthetic electron flow
- Changed membrane composition increased P-ATPase activity
- Increase Na^+/H^+ antiporter activity

Cyanobacteria adapted to saline media

- Low internal content of toxic inorganic ions

- High internal amount of osmoprotective compounds

- Decrease internal water potential

Figure 4 Schematic overview of the strategy developed by cyanobacteria to adapt to

high salinity (Joset et al, 1996).

The organism used in this study is the unicellular cyanobacterium *A. halophytica.* This organism is a extremely halotolerant organism that can grow in a wide range of salinity conditions from 0.25-0.5 M NaCl (Takabe et al., 1988). In general, there are a number of protective mechanisms for cells thriving in changing salinity environment. Previously, *A. halophytica* was shown to accumulate a compatible solute, glycinebetain, upon the increase in the external salinity (Takabe et al., 1998). Recently, it has reported that Na^+/H^+ antiporters from *A. halophytica* can make *E. Coli* and a freshwater cyanobacterium *Synechococcus* PCC 7942 tolerant to high salinity. (Waditee et al., 2002) tolerant to high salinity. Another possible factor that can modulate the exclusion of Na^+ from cells is the so-called "sodium pump" which is a Na^+-stimulates ATPase. This Na^+-stimulates ATPase is a membrane protein found in a number of organisms including cyanobacteria.

The objective of this thesis is isolation of the plasma membrane from *A. halophytica* and characterizing Na^+-stimulates ATPase in the plasma membrane with the emphasis on the effect of salinity.

CHAPTER II

MATERIALS AND METHODS

Materials

2.1 Equipments

Autoclave	:	Hirayama Manufacturing Cooperation, Japan
Autopipette	:	Pipetman, Gilson, France
Centrifuge, Refrigerated	:	Model J-21C, Beckman Instrument Inc, USA
French Pressure	:	American Instrument Company, USA
Auto Gamma Counter	:	Cobra II, Packard a Canberra Company
Incubator	:	Model G76D, Scientific co.inc., USA
Larminar flow	:	BVT-124, International Scientific Supply Co.Ltd., Thailand
Microscope	:	Olympus, USA
Microcentrifuge	:	Kubota, Japan
Peristaltic pump	:	Pharmacia LKB, Sweden
pH meter	:	PHM 83 Autocal pH meter, Radiometer, Denmark
Power supply	:	Pharmacia, England
Pump (Vacuum Dry)	:	Taitech, Japan
Rotary shaker	:	New Brunswick Scientific Edison, USA

Spectrophotometer	:	UV-240 :Shimadzu, Japan and du series 650,
Ultracentrifuge	:	Hitachi, Japan
Vortex	:	Model K-550-GE, Scientific Industries, USA
Water bath	:	Charles Hearson, England
Water bath shaking	:	Heto Lab Equipment, Denmark

2.2 Chemicals

Acetic acid	:	Merck Ag Darmstadt, Germany
Ammonium chloride	:	Sigma, USA
Ascorbic acid	:	Sigma, USA
ATP	:	Sigma, USA
Benzamidine	:	Sigma, USA
Calcium chloride	:	Merck Ag Darmstadt, Germany
CCCP	:	Sigma, USA
Coomassie brillant blue G-250	:	Sigma, USA
DCCD	:	Sigma, USA
Dextran T- 500	:	Amersham Bioscience, USA
DTT (Dithiothreitol)	:	Sigma, USA
EDTA (Ethylenediamine tetraacetic acid)	:	Sigma, USA
Ethanol	:	Katayama Chem, Japan
Glycerol	:	Merck Ag Darmstadt, Germany
Glycine	:	Sigma, USA
Hepes	:	Sigma, USA

Gramicidin D	:	Sigma, USA
Lysozyme	:	Sigma, USA
Magnesiam sulfate	:	Merck Ag Darmstadt, Germany
Magnesium chloride	:	Merck Ag Darmstadt, Germany
Methanol	:	Merck Ag Darmstadt, Germany
N-Ethylmaleimide	:	Sigma, USA
Membrane filter	:	Schleicher & Schuell Microscience, Germany
Ouabain	:	Sigma, USA
Phosphoric acid	:	Sigma, USA
Polyethylene glycol 3350	:	Sigma, USA
Potassium chloride	:	Merck Ag Darmstadt, Germany
Sodium chloride	:	BDH, England
Sodium dodecyl sulfate	:	Sigma, USA
Sodium fluoride	:	Merck Ag Darmstadt, Germany
Sodium molybdate	:	BDH, England
Sodium nitrate	:	BDH, England
Sodium nitrite	:	Fluka AG, Switzerland
Sodium dithionite	:	M&B, England
Sodium hydrogencarbonate	:	Sigma, USA
Sodium ionophore	:	Fluka AG, Switzerland
Sodium *p*-chloromercuribenzoate	:	Sigma, USA
Sodium vanadate	:	Fluka AG, Switzerland
Sodium - 22	:	Amersham Biosciences, USA

Sucrose	:	Katayama Chem, Japan
Toluene	:	BDH, England
Tris – hydrochloride	:	Katayama Chem, Japan

2.3 Bacterial strains

Aphanothece halophytica was initially isolated from Solar lake in Israel. The organism was kindly provided by Dr. T. Takabe of Nagoya University, Japan.

Methods

2.4 Growth of *A. halophytica* in various NaCl concentrations

A. halophytica was grown photoautotrophically in a BG_{11} medium plus 18 mM $NaNO_3$ and Turk Island Salt Solution, pH of medium was adjusted to 7.6 (see Appendix A). Ten percents inoculum of cells was inoculated into 250 ml flasks containing 100 ml of medium on a rotary shaker with 160 rpm, three 20 w fluorescent lamps placed overhead provided the flasks with 2,000 lux of continuous illumination at 30 ^{o}C without CO_2 supplement. The concentration of NaCl in the culture medium was adjusted by adding NaCl as required (0.25 M, 0.5 M, 1.0 M and 2 M). At various time intervals an aliquot of the culture was withdrawn and its turbidity was measured by a spectrophotometer at 750 nm.

2.5 Detection of Na$^+$-ATPase activity in *A. halophytica* cells

2.5.1 Effect of ^{22}NaCl concentration on sodium uptake in cells

Ten percent inoculum of *A. halophytica* was inoculated into a flask containing 100 ml of Turk Island Salt Solution plus modified BG$_{11}$ medium with 0.5 M NaCl, pH of the medium was adjusted to 7.6. Cells were grown on a rotary shaker with 160 rpm at 30 ^{o}C, 1,800 lux of continuous illumination. After fifteen days, cell culture of 100 ml was harvested at late exponential growth phase. The cells were harvested by centrifugugation at 8,000 rpm for 20 min, washed three times in 20 mM Tris-HCl buffer pH 7.6, containing 1.0 M sucrose to protect the cells from lysis. The cells (2 mg protein/ml) were incubated in 20 mM Tris-HCl buffer pH 7.6 containing with various concentration of ^{22}NaCl (0.45 µCi/ µmole, 2-20 mM). At intervals, samples (50 µl) were filtered through nitrocellulose membrane (pore size 0.45 µm); the cells were washed twice with the same buffer and counted in a gamma counter.

2.5.2 Sodium extrusion from cells

Na$^+$ extrusion was measured by a method similar to that of Kawano et al. (1998). Ten percents inoculum of *A. halophytica* was innoculated into a flask containing 100 ml of Turk Island Salt Solution plus modified BG$_{11}$ medium with 0.5 M NaCl, the pH of the medium was adjusted to 7.6. Cells were grown on a rotary shaker with 160 rpm at 30 ^{o}C, 1,800 lux of continuous illumination. After fifteen

days, cell culture of 100 ml was harvested at late exponential growth phase. The cells were harvested by centrifugugation at 8,000 rpm for 20 min, washed three times in the 20 mM Tris-HCl buffer pH 7.6, containing 1.0 M sucrose to protect the cells from lysis. Uptake was carried out by adding ^{22}NaCl at 0.45μCi/ μmol with a final concentration 20 mM. The cells were suspended in the same buffer at room temperature. The suspension was divided into two portions, one with 10 mM glucose added at 10 min and the other without glucose. At various time intervals for 0-60 min, 50 μl reaction mixture was withdrawn and terminated by rapid filtration through a membrane filters (nitrocellulose membrane; 0.45 um pore size, 25 mm diameter). The cells were then washed twice with the same buffer. The membrane filter was transferred to tube. The radioactive sodium accumulated by the cells were determined by gamma counter. The protein content of cell suspensions was determined by Bradford's method (see Appendix E) (Bradford, 1976).

2.6 Preparation of plasma membrane in *A. halophytica* cells

A. halophytica was grown in BG$_{11}$ medium containing either 0.5 M or 2.0 M NaCl. The concentration of NaCl in the culture medium was 0.5 M for normal condition and 2.0 M for salt-stress condition. After fifteen days, cells attained late exponential growth phase. The cells were harvested by centrifugation at 8,000 rpm for 20 min and washed twice with 20 mM Tris-HCl buffer, pH 7.6, containing 1.0 M sucrose to protect the cells from lysis. The cells of culture medium were collected and the plasma membranes were prepared according to the method by Murata and Omata (1988). The cells were resuspended in lysis buffer (see Appendix B)

containing 1.0 M sucrose. Solid lysozyme was added to a concentration 0.2% (w/v) and the suspension was incubate at 37 ^{o}C under room light for 30 min and gentle agitation. The lysozyme treated cell was collected by centrifugation at 8,000 rpm for 20 min. For a thorough removal of lysozyme, cells were washed twice with lysis buffer (pH 7.6) containing 1.0 M sucrose by resuspension and centrifugation. All the above mentioned procedures were carried out at room temperature, and subsequent steps at ice temperature. The pelleted cells were suspended in lysis buffer and passed through a precooled French press cell operating at 900 psi (MPa) followed by centrifugation at 8,000 rpm for 20 min to remove unbroken cells and cell debris. The resulting dark blue green supernatant contained a crude membrane mixture and was used as starting material for two phase partitioning.

2.6.1 Aqueous polymer two-phase partitioning

The procedure was carried out according to Norling, et al. (1994). Crude membranes prepared as described in section 2.6 were centrifuged at 100,000 g for 1 hr. This pellet was suspended to a total volume of 5 ml in 0.25 M sucrose, 5 mM Tris-HCl buffer,pH 7.6, and 4 g (10-20 mg protein) of the suspension was added to the 10 g phase mixture (see Appendix D) to give a 15 g phase system with a final concentration of 5.6 % (w/w) Dextran T-500, 5.6 % (w/w) polyethylene glycol, 0.25 M sucrose and 5 mM Tris-HCl buffer, pH 7.6. The partitioning was performed by turning the tube gently upside-down 20-30 times at 4 ^{o}C. After mixing, phase setting was facilitated by centrifugation for 5 min at 3,000 g and the upper and lower phases were collected separately. The lower phase was collected and repartitioned with a

pure upper phase obtained from a phase system (see Appendix D) with the same final concentration but without added membrane sample (volumes of fresh phases are not critical and can thus be adjusted by eye). This was repeated three times, yielding a final lower phase. The final lower phase was diluted 2-fold with lysis buffer and plasma membranes were collected by ultra centrifugation at 100,000 g for 1 hr. The high speed supernatant was collected and the pellet (membrane vesicle) was washed twice by lysis buffer and centrifuged again. Plasma membranes were obtained by suspending the pellet in 20 mM Tris-HCl buffer (pH 7.6) and 4 mM benzamidine. The undissolved material were removed by centrifugation (12,000 rpm for 20 min), then stored at -80 $^{\circ}$C. The plasma membrane was used for the assay of ATPase activity. Protein content was estimated by the Bradford' s method (Bradford, 1976).

2.6.2 Absorption spectra

As a purity criterion for plasma membrane, the ratio between the absorption at 485 nm (carotenoid) and at 680 nm (chlorophyll) was used, since plasma membranes contain carotenoids absorbing at 485 nm but do not contain chlorophyll which absorbs at 680 nm (Murata and Omata, 1988). Absorption spectra of the different membrane fractions were measured at room temperature with spectrophotometer. The samples were measured for the absorption ratio between carotenoid absorption (485 nm) and chlorophyll absorption (680 nm) after increasing number of partitioning steps. For comparison, the 485/680 absorption ratio for the total mixture of cyanobacterial membranes (crude membrane) before partitioning was also determined (Norling et al., 1994).

2.6.3 Chlorophyll determination

Chlorophyll-a was determined using spectrophotometer after acetone extraction utilizing the absorption coefficient factor as described by Mackinney (1941). The different membrane fractions during the isolation were analyzed with respect to chlorophyll/protein ratio. For comparison, the chlorophyll/protein ratio for the total mixture of cyanobacteria membranes (crude membrane) before partitioning was also determined. For measurements, aliquots of plasma membrane (40 μl) and 2 ml 80% acetone was subjected to centrifugation to remove protein and the supernatant was measured at 663 nm ($\varepsilon_{chl\ a}$ =82.04 ml mg^{-1} cm^{-1} at 663 nm).

2.6.4 Electron micrographs of plasma membranes

Plasma membrane was analyzed the nature of membrane vesicle by electron microscopy.

2.7 Characterization of ATPase activity in plasma membrane

After fifteen days, *A. halophytica* culture in BG$_{11}$ medium plus 0.5 M NaCl attained late exponential growth phase. The cells were harvested by centrifugation at 8,000 rpm for 20 min, washed twice in 20 mM Tris-HCl buffer, pH 7.6, containing 1.0 M sucrose to protect the cells from lysis. Plasma membranes were prepared as described in section 2.6.1. For the estimation of membrane vesicle

Na$^+$-ATPase activity levels an in situ assay was used. The composition of the reaction mixture was varied with substrates (ATP). The ATPase activity was the measure of inorganic phosphate by the hydrolysis of ATP was done according to the method by Koyama (1980). The reaction mixture (in a final volume of 1 ml) contained Tris-HCl buffer (20 mM, pH 7.6), MgCl$_2$ (5 mM), NaCl (100 mM) and ammonium molybdate (0.2 M). 100 μl of the plasma membranes (1 mg protein/ml) was added to the reaction mixture and the mixture was shaken rigorously for 5 min at 30 ^{o}C. The reaction was then started with the addition of 4 mM ATP and stopped after 10 min at 30 ^{o}C with 1 ml of phosphate reagent (see Appendix C). Then this mixture was centrifuged at 12,000 rpm for 10 min to give a clear supernatant. Color was developed with 20 μl of ascorbic acid and the absorbance at 750 nm read after 10 min. The absorbance at 750 nm was read against H$_2$O blank, and the amount of inorganic phosphate produced is estimated from a calibration curve of an inorganic phosphate standard in the range 1 to 10 μmol phosphate per sample (see Appendix E). Correction for the amount of inorganic phosphate present at zero time was also done. The plasma membrane without added ATP was the control condition. The values are the average of three replicates of independent cultures. One unit of enzyme activity was defined as 1 μmol of ATP hydrolyzed/min under the assay condition.

2.7.1 Integrity of plasma membrane

Prior to the investigation of ATP-dependent sodium transport in crude vesicles preparations of *A. halophytica*, the integrity of the membrane vesicles, as well as the optimum conditions for hydrolysis of ATP, had to be determined (Heise

et al., 1996). Plasma membrane (about 1 mg/ml) in 20 mM Tris-HCl buffer (pH 7.6), 100 mM NaCl, 4 mM ATP and 5 mM $MgCl_2$ with the following amounts of toluene 0.01 %- 3 %, was stirred gently for 1 hr at 4 ^{o}C. Reaction was centrifuged at 100,000 g for 1 hr and resuspend pellet in an equal volume of buffer containing the same detergent concentration. Determined the ATPase activity in each fraction as described in 2.7 (Bollag et al., 1996).

2.7.2 Effect of various ions on the ATPase activity

After fifteen days, *A. halophytica* culture in BG_{11} medium plus 0.5 M NaCl attained late exponential growth phase. Plasma membrane was prepared by as described in section 2.6.1. Dependence of ATPase activity on the concentrations of NaCl, KCl, $CaCl_2$, and $LiCl_2$ was tested by varying the concentrations of NaCl, KCl, $CaCl_2$, and $LiCl_2$ and measuring ATPase activity as described in section 2.7.

2.7.3 Effect of various concentrations of ATP on the Na^+-ATPase activity

A. halophytica was grown in the medium plus 0.5 M NaCl to late exponential growth phase. After fifteen days, the cells were harvested and isolated plasma membrane was prepared by aqueous polymer two-phase partitioning as described in section 2.6.1. ATPase assay was conducted at 30 ^{o}C by measuring the amount of inorganic phosphate released by the hydrolysis of ATP at various concentrations as described in section 2.7.

2.7.4 Kinetics of ATPase in plasma membrane

2.7.4.1 Kinetics of ATPase with variable ATP concentration

A. halophytica was grown in the medium plus 0.5 M NaCl to late exponential growth phase. After fifteen days, the cells were harvested and isolated plasma membrane was prepared as described in section 2.6.1. To determine the kinetic constants of the Na^+-ATPase, initial rates of ATP hydrolysis were determined over a wide range of ATP concentration (0.5 to 12 mM). The dependence of the Na^+-ATPase activity on ATP concentration followed Michaelis-Menten kinetics. The Michaelis constant (K_m) and maximum velocities (V_{max}) were calculated from x-axis and y-axis intercepts respectively of the double-reciprocal plot of activity against the ATP concentration, i.e., 1/[s] vs 1/[v].

2.7.4.2 Kinetics of ATPase with variable Na^+ concentration

A. halophytica was grown in the medium plus 0.5 M NaCl to the late exponential growth phase. After fifteen days, the cells were harvested and plasma membrane was prepared as described in section 2.6.1. To determine the kinetic constants of the Na^+-ATPase, initial rates of Na^+ catalysis were determined over a wide range of Na^+ concentration (0 to 200 mM). The dependence of the Na^+-ATPase activity on Na^+ concentration followed Michaelis-Menten kinetics. The Michaelis constant (K_m) and maximum velocities (V_{max}) were calculated from x-axis

and y-axis intercepts respectively of the double-reciprocal plot of activity against the Na$^+$ concentration, i.e., 1/[s] vs 1/[v].

2.7.5 Effect of various concentrations of MgCl$_2$ on the Na$^+$- ATPase activity

After fifteen days, *A. halophytica* culture in BG$_{11}$ medium plus 0.5 M NaCl attained late exponential growth phase. Plasma membrane was prepared by as described in section 2.6.1. Dependence of ATPase activity on the concentration of MgCl$_2$ was tested by varying the concentration of MgCl$_2$ and measuring Na$^+$-ATPase activity as described in section 2.7.

2.7.6 Effect of cations on the Na$^+$- ATPase activity

A. halophytica was grown in the medium plus 0.5 M NaCl to the exponential growth phase. After fifteen days, the cells were harvested and isolated plasma membrane was done as described in section 2.6.1. Dependence of Na$^+$-ATPase activity on the concentration of KCl, CaCl$_2$, and LiCl$_2$ was tested by varying the concentration of KCl, CaCl$_2$, and LiCl$_2$ and measuring Na$^+$-ATPase activity as described in section 2.7. The reaction mixture contained 100 mM NaCl, 4 mM ATP, 5 mM MgCl$_2$ and varying the concentration of KCl, CaCl$_2$, and LiCl$_2$.

2.7.7 Effect of pH on the Na$^+$- ATPase activity

A. halophytica was grown in the medium plus 0.5 M NaCl to the exponential growth phase. After fifteen days, the cells were harvested and plasma membrane was prepared as described in section 2.6.1. Dependence of ATPase activity on the pH was tested by varying the pH, 20 mM MES buffer (pH 6-7); 20 mM Hepes-NaOH and 20 mM Tris-HCl buffer (pH 7-9) was used as the buffer and measuring Na$^+$-ATPase activity as described in section 2.7.

2.7.8 Effect of various inhibitors on the Na$^+$- ATPase activity

The inhibition of the enzyme Na$^+$-ATPase was studied by observing the initial rates of Na$^+$-ATPase activity. The Na$^+$-ATPase activity was measured as described in section 2.7. Each inhibitor was added to the reaction mixture and incubated 10 min before the start of the reaction. The Na$^+$-ATPase activity was expressed as the percentage of remaining Na$^+$-ATPase activity compared to the control.

2.7.9 Effect of salt stress on the Na$^+$- ATPase activity

After fifteen days, *A. halophytica* culture in BG$_{11}$ medium plus 0.5 M or 2.0 M NaCl attained late exponential growth phase. The cells were harvested by centrifugation at 8,000 rpm for 20 min, washed twice in 20 mM Tris-HCl buffer, pH 7.6, containing 1.0 M sucrose to protect the cells from lysis.

Membrane fractions from cells grown in two concentration NaCl (0.5 or 2.0 M) were prepared as described in 2.6.1 and measuring Na^+-ATPase activity as described in section 2.7.

2.8 Determination of Na^+ uptake in plasma membrane

Ten percents inoculum of *A. halophytica* was inoculated into a flask containing 100 ml of Turk Island Salt Solution plus modified BG_{11} medium with 0.5 M NaCl, pH of the medium was adjusted to 7.6 Cells were grown on a rotary shaker with 160 rpm at 30 $^{\circ}$C, 1,800 lux of continuous illumination. After fifteen days, cell culture of 100 ml was harvested at late exponential growth phase. The cells were harvested by centrifugation at 8,000 rpm for 20 min, washed twice in the 20 mM Tris-HCl pH 7.6. Plasma membranes were prepares as described in section 2.6.1. The membrane vesicles were suspended in buffer pH 7.6 (1 mg of protein/ml); 20 mM ^{22}NaCl (0.45 µCi/ µmol) was added to the mixture and allowed to equilibrate for 30 min. The uptake reaction was started by the addition of 4 mM ATP. Sodium ionophore (N,N,N′,N′,-tetracyclohexyl-1, 2-phenyl-enedioxydiacetamide, ETH 2120) and gramicidin were added at 10 min before the addition of ATP. At intervals, 50 µl of the reaction mixture was filtered on a nitrocellulose filter (0.2 µm pore size) with suction and washed twice with 1 ml of buffer. The radioactivity trapped on the filter was measured with a gamma counter.

2.9 Determination of protein

Protein content was determined by Bradford᾽ s method (Bradford, 1976). The protein sample (maximum 100 μl) was aliquoted into a tube and distilled H_2O was added to make a total volume of 100 μl. This was followed by 1 ml of Bradford working solution. The mixture was mixed well by vortex. Absorbance at 595 nm was read after 2 min but before 1 hr. The standard curve was constructed using 1 mg/ml bovine serum albumin as protein sample.

The composition of Bradford stock solution and Bradford working solution are as follows:

Bradford stock solution   : 100 ml 95%ethanol

200 ml 85%phosphoric acid

350 mg serva Blue G

Stable indefinitely at room temperature.

Bradford working solution   : 425 ml distilled water

15 ml 95%ethanol

30 ml 85%phosphoric acid

30 ml Bradford stock solution

Filter through Whatman No.1 paper,store at room temperature in brown glass bottle. Usable for several weeks, but may need to be refiltered.

CHAPTER III

RESULTS

3.1 Growth of *A. halophytica* in various NaCl concentrations

A. halophytica is a short cylindrical shape cyanobacterium surrounded with mucous membrane (Figure 5). The cells multiply by binary fission. The cells were grown photoautotrophically in a BG_{11} medium plus 18 mM $NaNO_3$ and Turk Island Salt Solution, pH of medium was adjusted to 7.6 at different NaCl concentrations, i.e., 0.25 M, 0.5 M, 1.0 M and 2.0 M NaCl. Bacterial growth was monitored spectrophotometrically every two days (30 days) by the increase of the optical density at 750 nm. Figure 6 shows that maximal growth occurred in the medium containing 0.5 M NaCl. At 0.25 M, 1.0 M and 2.0 M NaCl, a reduction in growth was observed.

3.2 Detection of Na^+-ATPase activity in *A. halophytica* cells

3.2.1 Effect of ^{22}NaCl concentration on sodium uptake in cells

Cells were grown in 0.5 M NaCl media for fifteen days and washed cells were suspended at 2 mg protein per ml in 20 mM Tris-HCl buffer (pH 7.6) containing various concentrations of ^{22}NaCl (0.45 µCi / µmol, 2-20 mM) were then added and the suspension was incubated for 60 min. We measured the initial

rates of sodium uptake in cells. There was a substantial sodium uptake by *A. halophytica* which was stimulated by the increase of NaCl (Fig. 7). The 20 mM ^{22}NaCl was used for the experiment on sodium extrusion.

3.2.2 Sodium extrusion from *A. halophytica*

The assay of Na^+- ATPase activity in *A. halophytica*. Cells were grown in 0.5 M NaCl media for is fifteen days and washed cells were suspended at 1 mg protein per ml in 20 mM Tris-HCl buffer (pH 7.6) containing 20 mM ^{22}NaCl (0.45 μCi / μmol) was then added and the suspension was incubated for 60 min. The suspension was future incubated for 10 min and then divided into two portions in the presence or absence of glucose. Cells were uptake $^{22}Na^+$ reached saturation within 40 min and extrusion of $^{22}Na^+$ from cells when added glucose. The result indicate that cells were loaded with $^{22}Na^+$ which extrusion was followed by energizing the cells with glucose. $^{22}Na^+$ remaining in cell reflects Na^+ extrusion capacity as shown in Figure. 8.

Figure 5 Microscopic picture of *Aphanothece halophytica* grown in Turk Island Salt
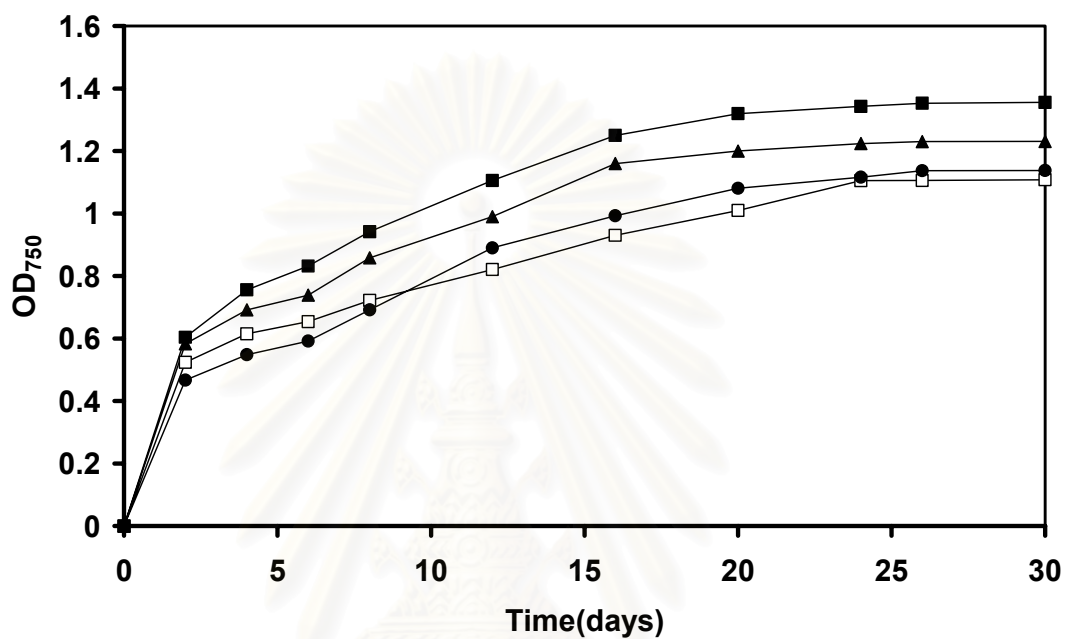Solution plus BG$_{11}$ at 14 days (x 2250).

Figure 6 Growth of *A. halophytica* in Turk Island Salt Solution plus modified BG11

medium containing various NaCl concentrations. Symbol: □, 0.25 M NaCl;
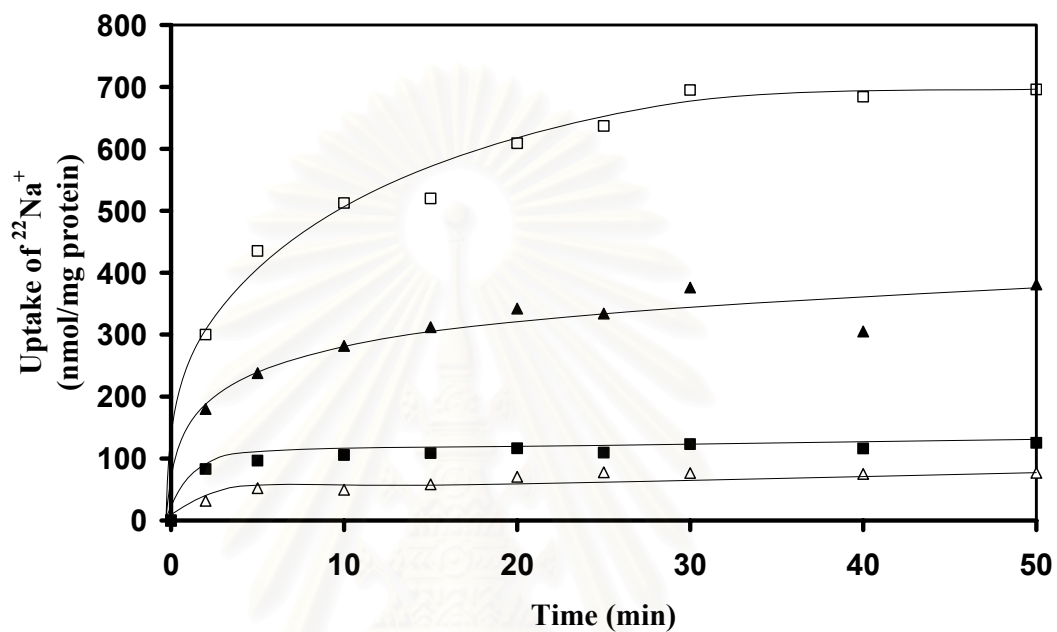
■, 0.5 M NaCl; ▲, 1.0 M NaCl ; ●,2.0 M NaCl.

Figure 7 Sodium uptake by *A. halophytica*. Cells were incubated with various ²²NaCl

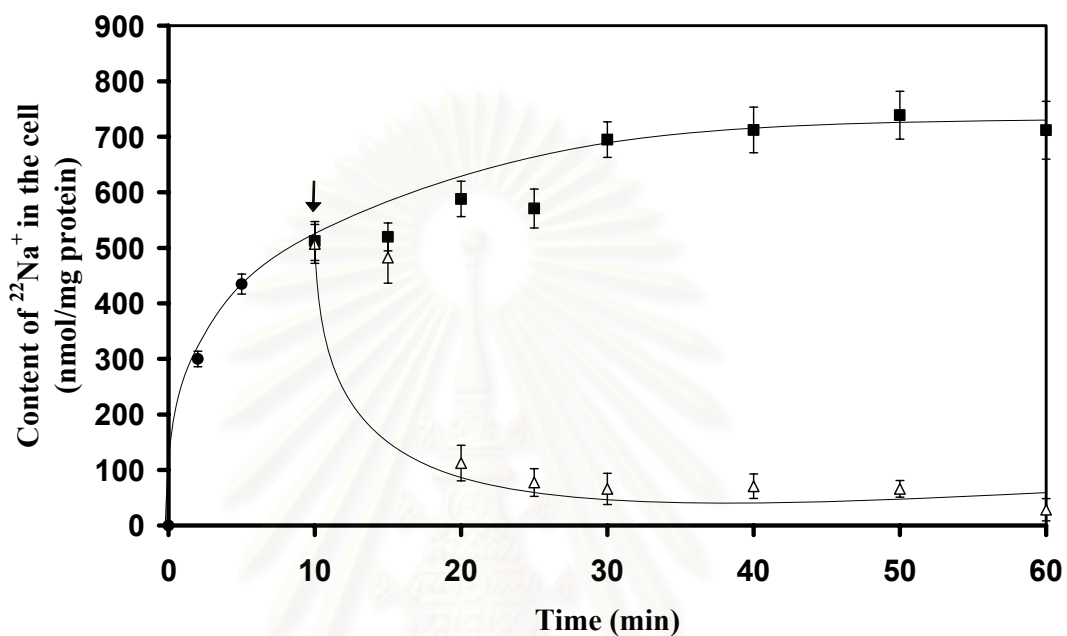concentration. Symbol: Δ, 2 mM NaCl; ■, 5 mM NaCl; ▲, 10 mM NaCl ;

□, 20 mM NaCl.

Figure 8 Sodium extrusion from *A. halophytica*. Cell were preloaded with 20 mM

^{22}NaCl (0.45 µCi/µmol) for 60 min. The suspension was further incubated

for 10 min (●) and then divided into two portions with additions as follows;

(■) without glucose; (Δ) 20 mM glucose at 10 min.

3.3 Preparation of plasma membrane in *A. halophytica* cells

3.3.1 Aqueous polymer two-phase partitioning

Crude membranes was applied to polymer mixure yielding two phase system of a final concentration of 5.6 %(w/w) Dextran T-500, 5.6 %(w/w) polyethylene glycol, 0.25 M sucrose and 5 mM Tris-HCl buffer, pH 7.6. A phase system of 5.6 % dextran and 5.6 % polyethylene glycol was used (see Appendix D). In the color photograph shown in Figure 9 the result of three partitioning was observed. Partitioning of total cyanobacterial membranes (crude membranes) (tube 1) gave rise to dark green upper phase (containing most of thylakoid membranes) and a less green lower phase (containing most of plasma membranes). The thylakoid containing upper phase was removed and replaced by a fresh upper phase and a second partitioning was performed. This resulted in a distribution of the green thylakoid membranes present in the original lower phase into the upper phase, while the yellow carotenoid containing plasma membranes remained in the lower phase (tube 2). By two repartitioning of the lower phase with fresh upper phase, pure plasma membranes were obtained. In certain preparations, three partitions, yielding the third lower phase fraction (B3), were needed for optimal purification of the plasma membranes.

3.3.2 Absorption spectra

The plasma membranes were obtained during the isolation procedure and measured for absorption ratio between carotenoid absorption (485 nm) and chlorophyll absorption (680 nm) after increasing number of partitioning steps. For comparison, the 485/680 absorption ratio for the total mixture of cyanobacterial membranes before partitioning was also done. As seen in Figure 10, three partition steps resulted in maximal increase of the carotenoid/chlorophyll ratio of the lower phase. As seen in Table 1 the absorption ratio at 485/680 nm was increased from 0.20 in total membranes to 4.52 in purified plasma membrane (B3), indicating a large degree of the plasma membranes in the B3 fraction after three-phase partition.

3.3.3 Chlorophyll determination

The different membrane fractions obtained during the isolation procedure were analyzed with respect to chlorophyll/protein ratio of different membrane fractions isolated by aqueous two-phase partitioning (Table 1). Plasma membranes (B3) contain 0.08 μg chlorophyll/mg protein and thylakoid membranes recovered in the first upper phase (T1) 4.28 μg chlorophyll/ mg protein.

3.3.4 Electron micrograph of isolated plasma membranes

Figure 11 shows an electronmicrograph obtained from the plasma membranes preparation (B3). The sample has been vesicle diameter in the range of 0.1-0.22 μm. The electrodense, amorphous structures seen in the electron micrograph are probably aggregates of lipophilic compounds often seen in membrane preparations.
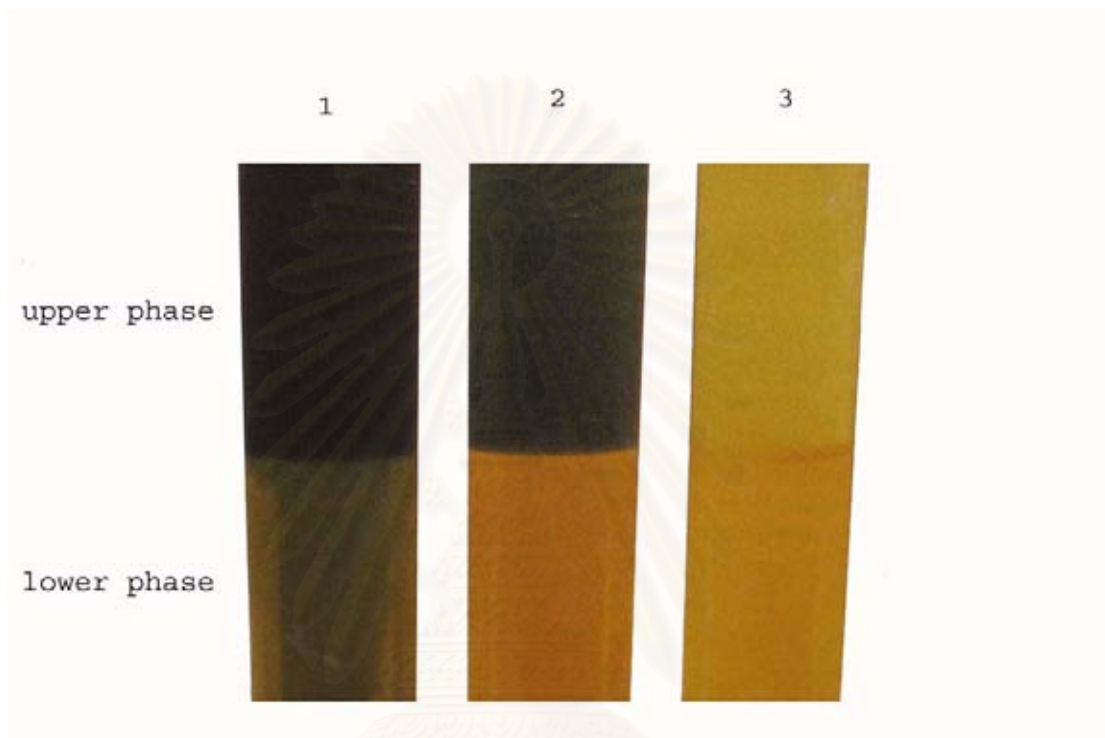
Figure 9 Presentation of the partition of total cyanobacteria membrane in two-phase system (5.6 % dextran/5.6 % PEG) (tube 1) and repartition of the bottom phase with fresh top phase once (tube 2), and twice (tube 3).
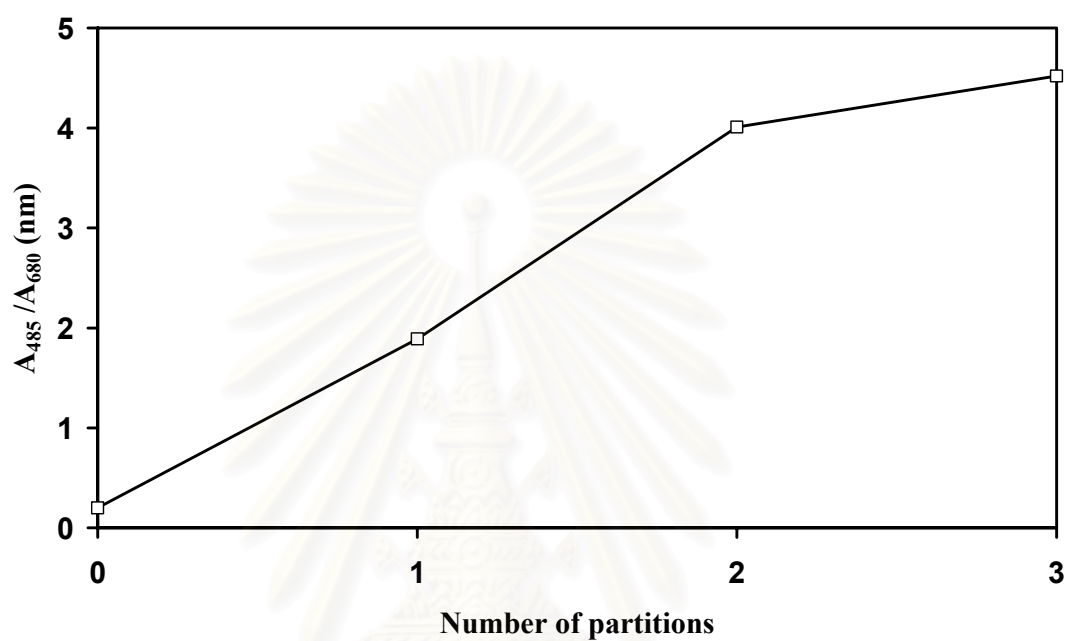
Figure 10 Ratio between carotenoid absorption (485 nm) and chlorophyll absorption

(680 nm) after increasing number of partitioning steps.

Table 1 Characterization of different membrane fractions isolated by aqueous two-
phase partion.

Fraction	Chlorophyll a/protein (μg/mg)	A_{485}/A_{680}
Total membrane	5.36	0.20
First upper phase (T1)	4.28	0.23
First lower phase (B1)	2.23	4.01
Third upper phase (T3)	1.33	0.25
Third lower phase (B3)	0.08	4.52

Note. Total membranes (10-20 mg protein) were partitioned in three times by phase system of 5.6 % dextran and 5.6 % polyethylene glycol.

Figure 11 Electron micrograph of the plasma membrane preparation. Scale bar

= 1 μm.

3.4 Characterization of ATPase activity in plasma membrane
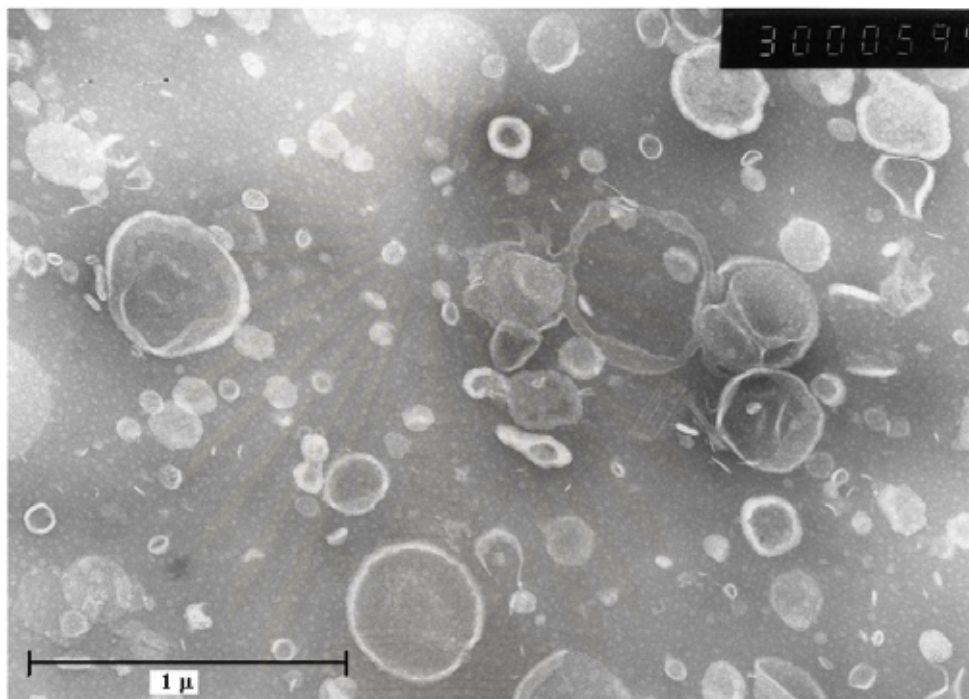
3.4.1 Integrity of plasma membrane

The integrity membrane vesicle, as well as the optimal conditions of hydrolysis ATP, had to be determined. ATP hydrolysis can only be catalyzed by integrity of membrane vesicles. The membrane integrity of plasma membrane preparations of *A. halophytica* was abolished by toluene but the rate of ATP hydrolysis was not stimulated indicating that the preparations consisted entirely of integrity membrane vesicles for ATPase activity (Figure 12) (Heise, 1992).

3.4.2 Effect of various ions on the ATPase activity

ATPase activity was assayed at 30 $^{\circ}$C for 10 min in a reaction mixture composed of 20 mM Tris-HCl (pH 7.6) buffer that contained 100 μl of plasma membrane (protein 1 mg/ml), 5 mM $MgCl_2$, 4 mM ATP and the various concentrations of NaCl, KCl, $CaCl_2$ and $LiCl_2$ as shown in the Figure 13. In the absence of ions, an activity of 0.1 U/mg protein was obtained, which could be stimulated 4-5 fold by NaCl. Maximal hydrolysis was obtained at higher than 100 mM NaCl; addition of KCl, $CaCl_2$ and $LiCl_2$ did not stimulate ATP hydrolysis.

3.4.3 Effect various concentrations of ATP on the Na$^+$-ATPase activity

The membrane vesicles isolated from cells grown in medium with 0.5 M NaCl was assayed for ATPase activity. The plasma membranes were incubated in Tris-HCl buffer (pH 7.6) containing various concentrations of NaCl and various concentrations of ATP at 5 mM MgCl$_2$, and experiment was done as described in 2.7. Figure 14 shown dependence of ATP hydrolysis on ATP at 100 mM NaCl. The maximal ATP hydrolysis rate of plasma membrane was obtained at 4-8 mM ATP.

3.4.4 Kinetics of ATPase in plasma membrane

3.4.4.1 Kinetics of ATPase with variable ATP concentration

We also determined the concentration of ATP needed to saturate ATPase activity using membrane vesicles of *A. halophytica*. Membrane vesicles were assayed with Tris-HCl buffer (pH 7.6) containing 100 mM NaCl and various concentrations of ATP at 5 mM MgCl$_2$.The dependence of the reaction on ATP concentration followed Michaelis-Menten kinetics; The maximal velocity (V_{max}) was 0.66 μmol Pi/min/mg protein (U/mg protein). The Km value for ATP was 1.66 mM. (Figure 15).

3.4.4.2 Kinetics of ATPase with variable Na$^+$ concentration

We also determined the concentration of Na$^+$ needed to saturate ATPase activity using membrane vesicles of *A. halophytica*. Membrane vesicles were assayed with Tris-HCl buffer (pH 7.6) containing 4 mM ATP and various concentrations of NaCl at 5 mM MgCl$_2$.The dependence of the reaction on NaCl concentration followed Michaelis-Menten kinetics; The maximal velocity was 0.50 U/mg protein and K_m value for NaCl was 25 mM (Figure 16).

3.4.5 Effect of various concentrations of MgCl$_2$ on the Na$^+$- ATPase activity

We tested whether the reaction of Na$^+$-ATPase was dependent on MgCl$_2$. Optimal ATP hydrolysis was obtained at 5 mM MgCl$_2$ (Fig. 17), whereas concentrations above 5 mM reduced the enzyme activity.
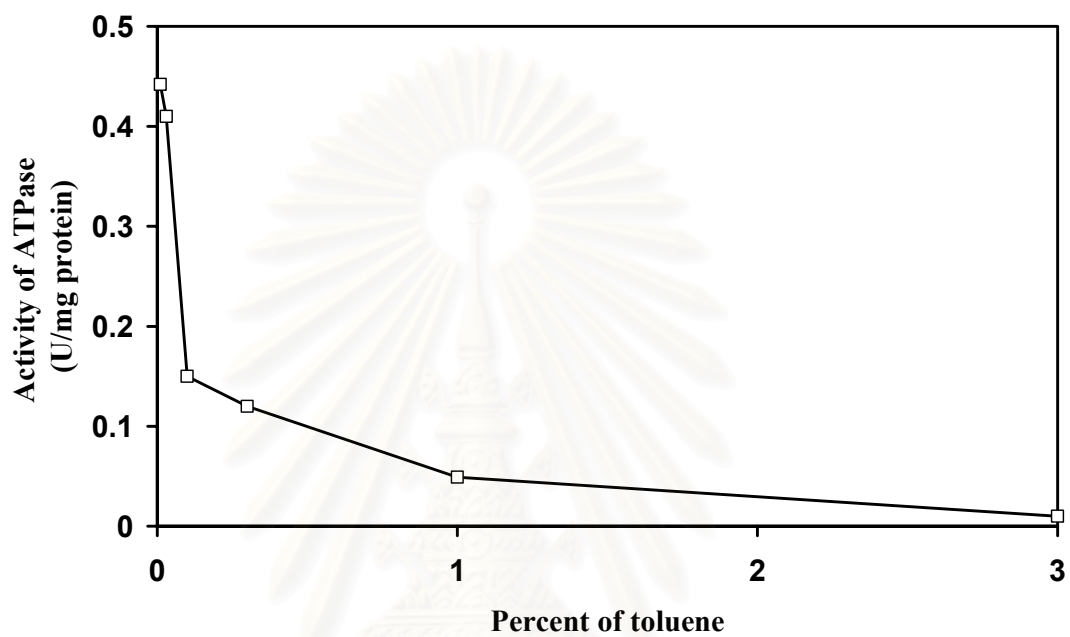
Figure 12 The ATPase activity of plasma membrane fraction (about 1 mg/ml) in 20

mM Tris-HCl buffer (pH 7.6) containing 100 mM NaCl, 5 mM $MgCl_2$, 4

mM ATP and various toluene concentrations.
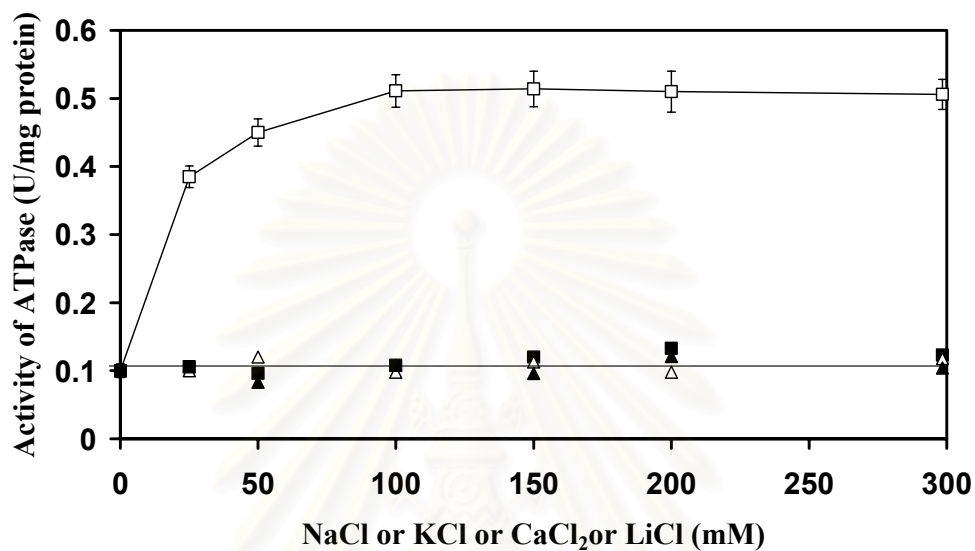
Figure 13 Effect of increasing Na^+, K^+, Ca^{2+} and Li^{2+} concentrations on the rate of

ATP hydrolysis. Plasma membranes (protein 1 mg/ml) were incubated in

20 mM Tris-HCl buffer pH 7.6 containing 4 mM ATP, 5 mM $MgCl_2$. The

with increasing Na^+(□), K^+(■), Ca^{2+}(▲) and Li^{2+}(Δ) concentration.
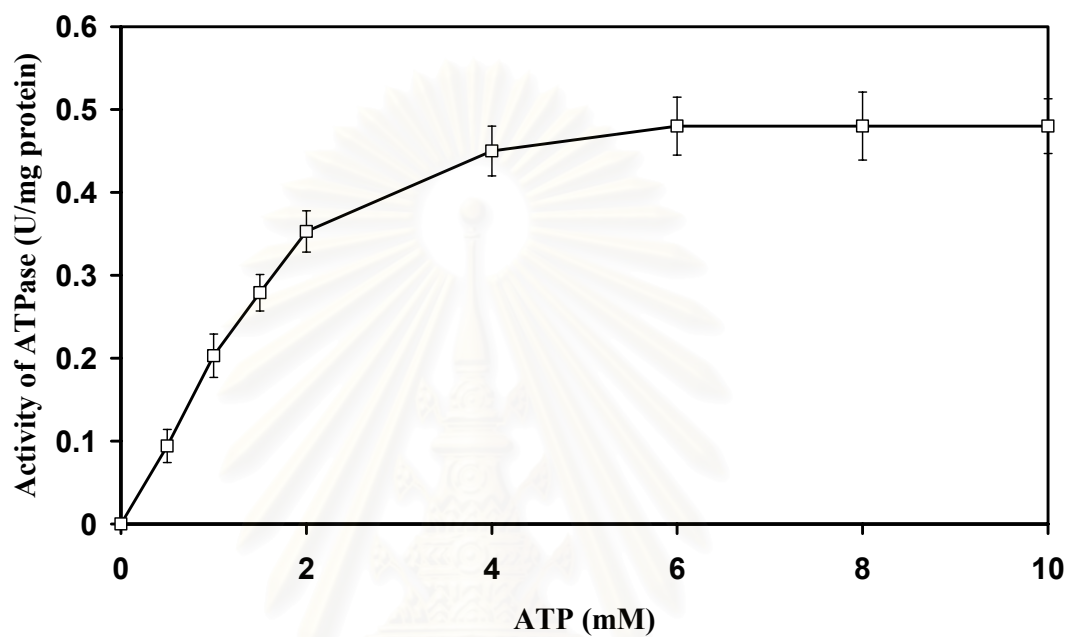
Figure 14 Dependence of ATP hydrolysis on concentration of ATP. Plasma membrane (protein 1 mg/ml) was incubated in Tris-HCl buffer (pH 7.6) containing 100 mM NaCl and various concentrations of ATP at 5 mM $MgCl_2$.
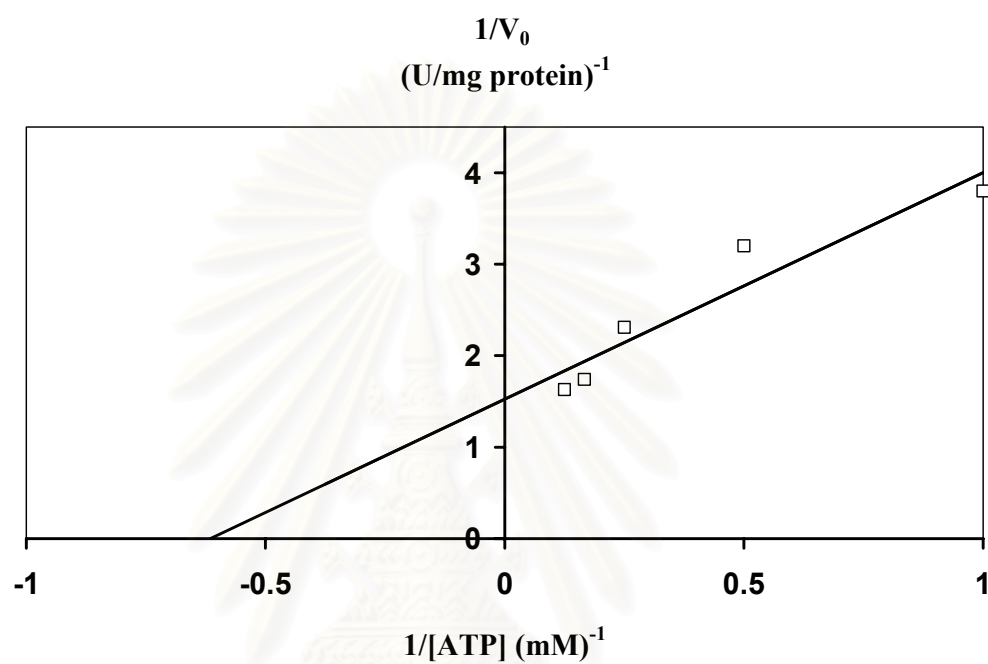
Figure 15 Lineweaver-Burk plot of the activity against the ATP concentration. The
apparent K_m values were 1.66 mM, the maximum velocity (V_{max}) was 0.66
U/mg protein.

Figure 16 Lineweaver-Burk plot of the activity against the NaCl concentration. The apparent K_m value was 25 mM, the maximum velocity (V_{max}) was 0.50 U/mg protein.
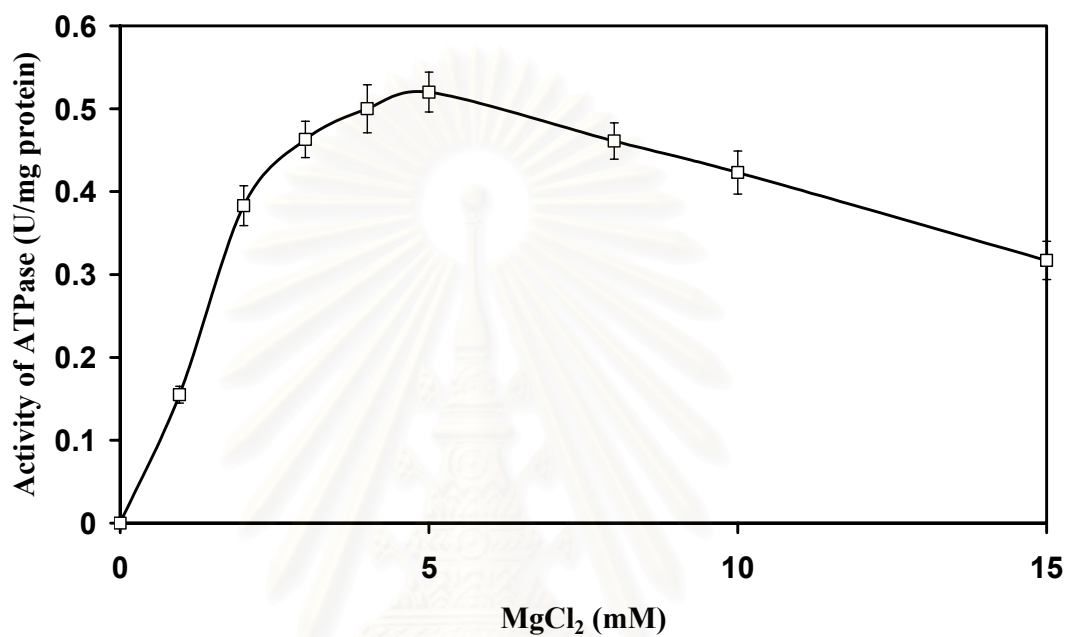
Figure 17 Dependence of ATP hydrolysis on $MgCl_2$. Plasma membrane (protein content 1 mg/ml) was incubated in Tris-HCl buffer (pH 7.6) containing 100 mM NaCl and various concentrations of $MgCl_2$ at 4 mM ATP.
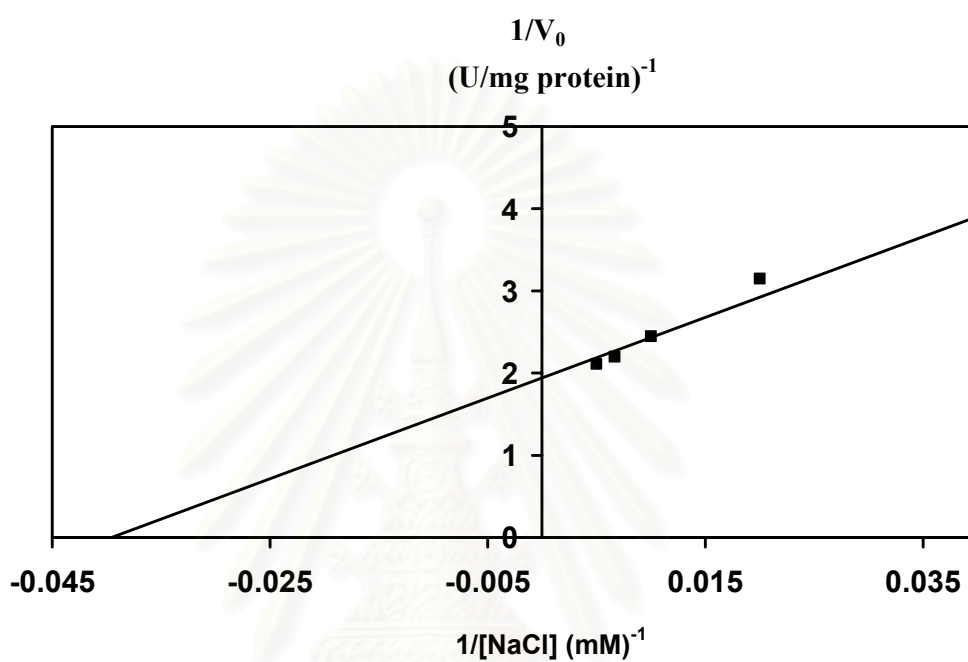
3.4.6 Effect of cations on the Na$^+$- ATPase activity

ATPase activity was assayed at 30 $^{\circ}$C for 10 min in a reaction mixture composed of 20 mM Tris-HCl (pH 7.6) buffer that contained 100 μl of sample (protein 1 mg/ml), MgCl$_2$ 5 mM and various concentrations of KCl, CaCl$_2$ and LiCl$_2$ as shown in the Figure 18. In the presence of 100 mM NaCl, the ATPase activity was further increased by the addition of KCl. The highest activity obtained with co-application of Na$^+$ and K$^+$ was seen at more than 100 mM Na$^+$ with 100 mM K$^+$. Addition of CaCl$_2$ and LiCl$_2$ did not stimulate Na$^+$- ATP hydrolysis (data not shown).

3.4.7 Effect of pH on the Na$^+$- ATPase activity

Fig. 19 depicts the effect of pH on the ATPase activity. The enzyme exhibited an optimum pH for activity at around 7-7.5.

3.4.8 Effect of various inhibitors on Na$^+$- ATPase activity

The Na$^+$- ATPase activity of plasma membrane was determined after a 10 min incubation of the plasma membrane at 30 $^{\circ}$C with the inhibitor at the concentrations indicated (100 % activity corresponds to 0.5 unit). Table 2, shows that the membrane-bound ATPase activity from *A .halophytica* in the presence of 100 mM NaCl, 4 mM ATP and 5 mM MgCl$_2$ was decreased to 63 % of original value by addition of 1 mM N, N$^/$- dicyclohexylcarbodiimide (DCCD) and exhibited 50 % of

original activity by the treatment with 0.01 mM sodium vanadate and 0.20 mM gramicidin. No inhibition was observed with 50 mM KNO_3, 0.5 mM *N*-Ethylmaleimide, 5 mM azide and 1 mM CCCP, ouabain, sodium fluoride.

3.4.9 Effect of salt stress on Na^+- ATPase activity

We investigated the influence of high osmolarity media on the growth of the *A. halophytica*. When the osmolarity of the medium was increase by addition of 0.5 M and 2.0 M NaCl. We tested the activity of Na^+- ATPase using plasma membrane prepared from cells grown under different stress conditions. Na^+- ATPase activity was stimulated by high osmolarity of growth medium (Fig. 20).

3.5 Determination of Na^+ uptake in plasma membrane

The plasma membrane of *A. halophytica* was prepared by aqueous polymer two-phase partitioning method. Fig. 21 shows the time course of $^{22}Na^+$ transport into membrane vesicles. Virtually no $^{22}Na^+$ was transported into the vesicles in the absence of ATP. Upon its addition, $^{22}Na^+$ rapidly accumulated inside the vesicles, reaching a steady state in about 10 min. Accumulation of $^{22}Na^+$ ions was prevented by incubation of the membrane vesicles with sodium ionophore (ETH 2120) or gramicidin.
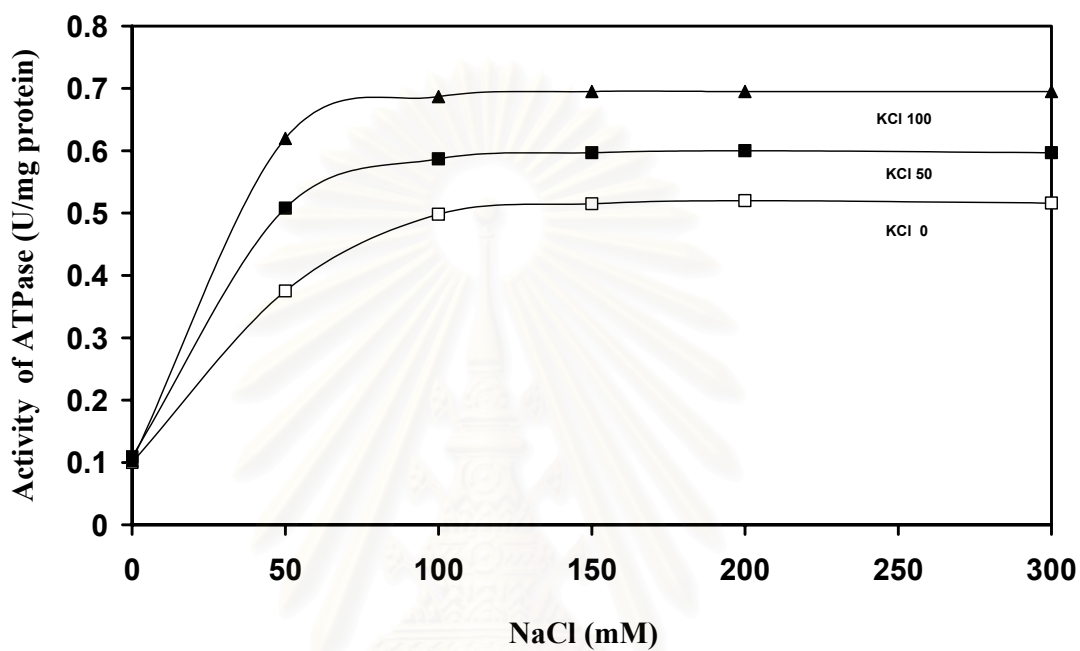
Figure 18 Effect of the concentration of NaCl and KCl on the ATPase activity in the plasma membrane. ATPase activity was assayed at 30 $^{\circ}$C in a reaction mixture composed of 20 mM Tris-HCl (pH 7.6) buffer that contained 5 mM $MgCl_2$, 4 mM ATP and the various concentrations of NaCl and KCl.
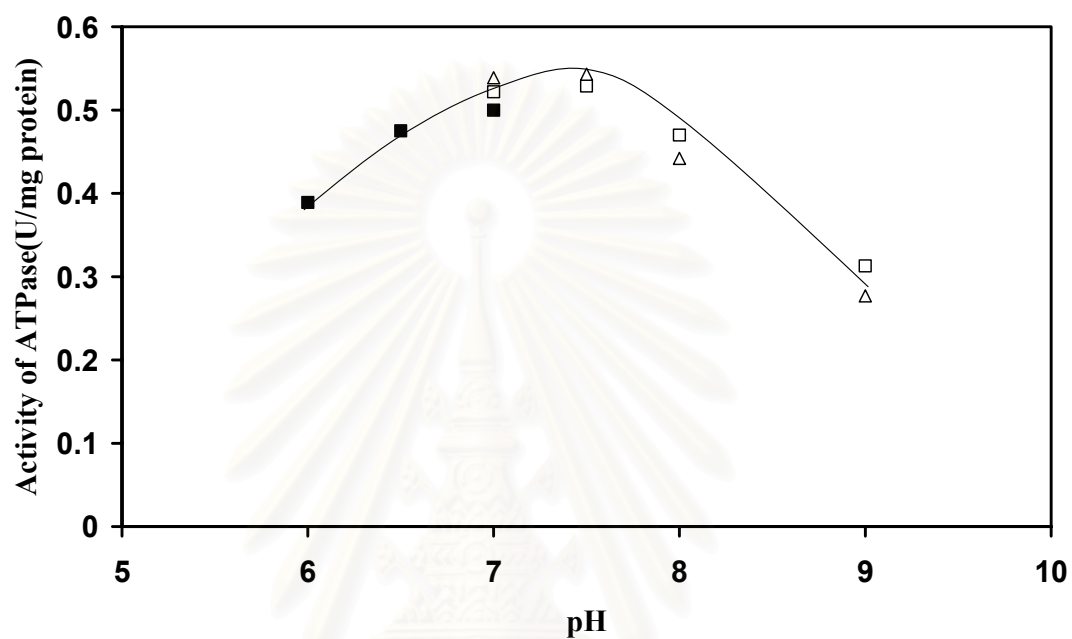
Figure 19 Effect of pH on the ATPase activity. Assay was conducted at 30 $^{\circ}$C in a

reaction consisting of (■) 20 mM MES buffer, pH 6-7. For pH 7-9, (Δ) 20

mM Hepes-NaOH and (□) 20 mM Tris-HCl buffer was used.

Table 2 Effect of various inhibitors on ATP hydrolytic activity in the plasma membrane fraction.

Reagent	Conc.	Remaining activity (%)
None	-	100
CCCP	1 mM	95
DCCD	1 mM	63
Gramicidin	200 μM	45
KNO_3	50 mM	100
N-Ethylmaleimide	0.5 mM	100
Sodium fluoride	1 mM	98
Azide (NaN_3)	5 mM	100
Ouabain	1 mM	100
Vanadate	10 μM	52

Note Each inhibitor was added to the reaction mixture 10 min before the start of the reaction. The activities were assayed at 30 $^{\circ}$C in the 1 ml of the reaction mixture that contained 20 mM Tris-HCl (pH 7.6) buffer, 4 mM ATP, 5 mM $MgCl_2$, 100 mM NaCl, and 100 μl of the sample (1 mg/ml protein).
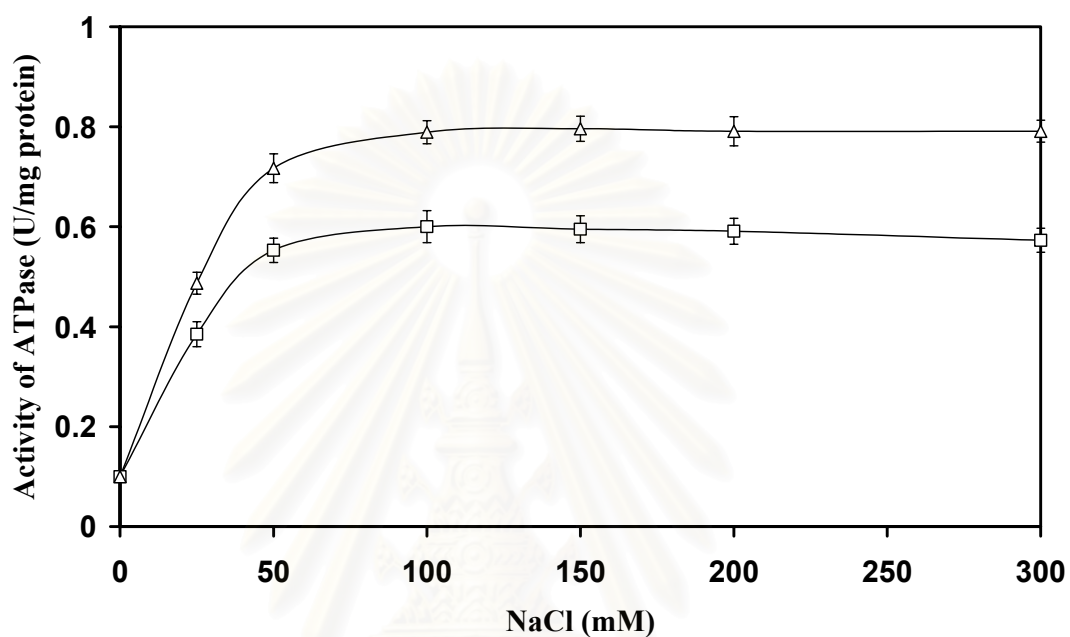
Figure 20 Effect of osmolarity of growth medium on Na^+- ATPase activity of plasma membranes (protein 1 mg/ml) were incubated in Tris-HCl buffer (pH 7.6) containing various concentrations of NaCl and 4 mM ATP at 5 mM $MgCl_2$ Plasma membranes were prepared from cells grown under stress (Δ, 2.0 M NaCl) and control condition ($\square$, 0.5 M NaCl).
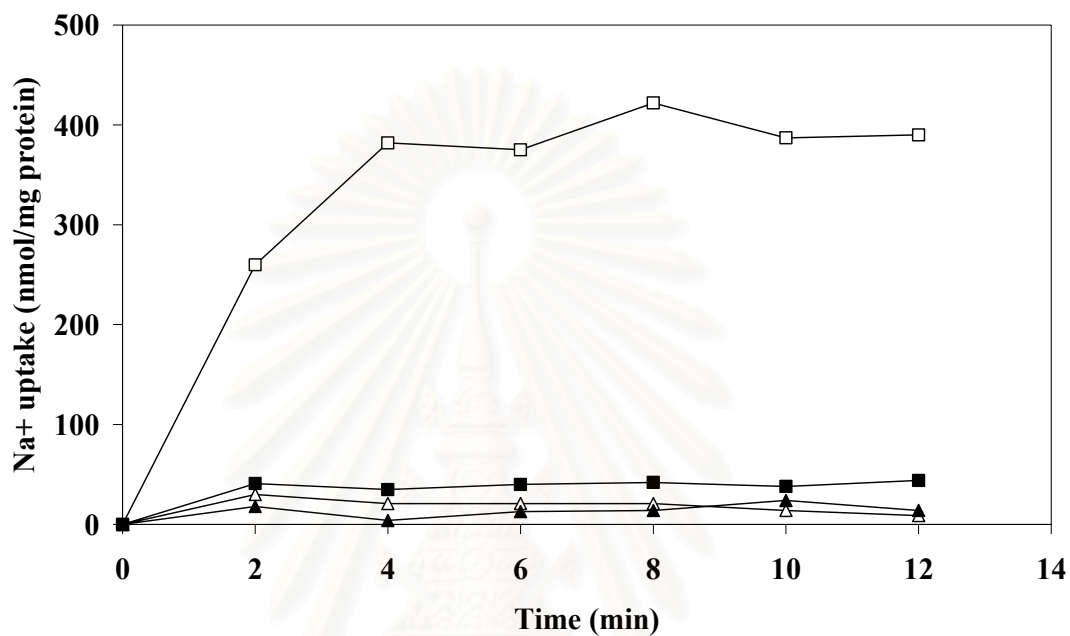
Figure 21 Na^+ uptake into membrane vesicles. The membrane vesicles were
suspended in buffer containing 20 mM $^{22}Na^+$ (0.45 µCi /µmol), and
uptake was started by the addition of 4 mM ATP at 0 min. Sodium
ionophore and gramicidin was added at 10 min before the addition of
ATP; ▲, no ATP; □, ATP; ■, ATP plus 25 µM sodium ionophores; △,
ATP plus10 µM gramicidin.

CHAPTER IV

DISCUSSION

4.1 Preparation of plasma membrane of in *A. halophytica* by aqueous polymer two-phase partitioning

Cyanobacteria are prokaryotes that are capable of oxygenic phothosynthesis. Unlike most prokaryotic organisms, the cyanobacterial cell contains two different types of biomembranes: the plasma membrane, and the thylakoid membranes. Several components of the respiratory chain have been identified and found to be located in both the thylakoid membrane and the plasma membrane (Norling et al., 1994). Functional plasma membranes have been isolated from several species of cyanobacteria by sucrose density centrifugation. These preparation procedures of cyanobacterial plasma membranes, however display low yields, involve time-consuming gradient centrifugation steps, and cannot easily be performed in large scale (Murata and Omata, 1988).

Plasma membranes have previously been isolated from *A. halophytica* by sucrose gradient centrifugation. However, it has been difficult to design a method for preparation of plasma membranes in any large quantities. This has been a limiting factor on the biochemical studies on these membranes. In previously research by Norling has been isolate plasma membrane from plants by partitioning in aqueous polymer two phase systems based on dextran and polyethylene glycol. This method separates membranes according to differences in surface properties. Aqueous polymer

two-phase partition has also been used for subfractionation of thylakoid membranes including the isolation of inside-out vesicles from higher plant chloroplasts and from cyanobacteria (Norling et al., 1994)

We isolated plasma membranes from a halotolerant cyanobacterium *A. halophytica* by using aqueous polymer two-phase partitioning. The usefulness of this method depends largely on three properties of polymer two-phase systems;

(i)   The partition behavior of a membrane particle is determined by its surface properties such as charge and hydrophobicity, a unique dimension for fraction of biomembranes. Two-phase partitioning can therefore be used to separate particles of the same size and density, e.g., membrane vesicles with different membrane orientation.

(ii)   The high water content, together with the fact that suitable buffer, salts, and osmoticum may be added, makes the method very biocompatible.

(iii)   Two-phase partitioning can easily be scaled up and performed in a few rapid partition steps.

Two factors contribute to these difficulties, the limited bulk of material that may be purified using sucrose gradient centrifugation and the fact that cyanobacterial cells contain very little plasma membrane compared to the amount of thylakoid membrane. Moreover, the time required for the centrifugation step is quite long, usually 16 hr. Two-phase partitioning allows for a rapid, large-scale preparation which can handle any bulk of starting material. In this research we separated plasma membranes from thylakoid membranes of cyanobacteria in aqueous polymer two-phase systems.

The results demonstrate that the cyanobacterial plasma membranes have a high affinity for the lower phase, on the other hand, a low affinity for the upper phase (Figure 9), Inspection for purity of plasma membrane was done by measuring absorption spectra (ratio between the absorption at 485 nm; carotenoid and at 680 nm; chlorophyll). As seen in Figure 10, three partition steps resulted in maximal increase of the carotenoid/chlorophyll ratio (A_{485}/A_{680}) of the lower phase. The different membrane fractions obtained during the isolation procedure were analyzed with respect to chlorophyll/protein ratio (Table 1). Total membranes contain 5.36 μg chlorophyll/mg protein and thylakoid membranes recovered in the upper phase contain 4.28 μg chlorophyll/mg proteins. The third lower phase has the lowest chlorophyll/protein ratio (0.08 μg chlorophyll/mg proteins), but the primary lower phase, and third upper phase fractions also have very low chlorophyll/protein ratios, revealing a high degree of purification of plasma membranes from thylakoid membranes. From this result, it was observed that the A_{485}/A_{680} of the first lower phase (B1) and third lower phase partition (B3) is about 4.01 and 4.52 respectively. These values are not significant different from each other. However, chlorophyll content after the first lower phase and third lower phase partition shown significant different amount; ranging from 2.23 to 0.08. This might be explained by the lost of carotenoid during the partition process. Figure 11 shows an electronmicrograph obtained from the plasma membrane preparation (Third lower phase, B3). The plasma membranes are appearing as spherical vesicles of variable diameter about 0.22 μm. The integrity of membrane vesicles, as well as the optimal conditions for hydrolysis of ATP, had to be determined. ATP hydrolysis can only be catalyzed by integrity membrane vesicles. The membrane vesicle of *A. halophytica* was abolished by

toluene but the rate of ATP hydrolysis was not stimulated, indicating that the preparations consisted almost entirely of integrity membrane vesicles (Figure 12). The orientation of the vesicles of *Acetobacterium woodii*, as well as the optimal conditions for hydrolysis of ATP, which is impermeable to membranes, ATP hydrolysis can only be catalyzed by inverted vesicles (Heise et al., 1992).

In conclusion, aqueous polymer two-phase systems offer a rapid method for separation of thylakoid, plasma membranes from cyanobacteria *A. halophytica*.

4.2 Characterization of ATPase activity in *A. halophytica*

4.2.1 Characterization of ATPase in cells

Prokaryotic and eukaryotic microorganisms must be able to adapt to changes in the high salinity of their environment. To adapt to these changes, extrusion of sodium ion from cells is needed to balance and maintain sodium level. The well-known Na^+/H^+ antiporter utilizes these gradients by exchanging external H^+ for internal Na^+ (secondary energized Na^+ export). Recently, it has been reported that Na^+/H^+ antiporters from *A. halophytica* can make *E. coli* and a freshwater cyanobacterium *Synechococcus* PCC 7942 tolerant to high salinity. (Waditee et al., 2002). Another possible factor that can modulate the extrusion of Na^+ from cells is sodium pump which is a Na^+- stimulated ATPase (primary energized Na^+ export). This is a membrane protein found in a number of organisms including cyanobacteria.

The sodium extrusion was studied in *A. halophytica* cells by loading the cells with $^{22}Na^+$ and the extrusion of $^{22}Na^+$ from cells was followed by energizing the cell with glucose. Figure 8 shown that cells with glucose exhibited sodium extrusion whereas in cells without glucose, no sodium extrusion was apparent. *A. halophytica* can also use glucose for the generation of ATP for extrusion of sodium ions in cells. This suggests that *A. halophyt*ica may have Na^+-ATPase. In support of this, it was found that sodium dependent ATPase activity was observed in plasma membrane.

4.2.2 Characterization of ATPase in plasma membranes

Bacteria generally tend to expel sodium ions from their cytoplasm by Na^+-ATPase and Na^+/H^+ antiporter; that is achieved by primary and secondary mechanisms, respectively. The sodium gradient established is used for different energetic purposes such as solute transport, pH regulation, motility and ATP synthesis (Heise et al., 1992).

Figure 13 shows the concentration effect of NaCl on the membrane-bound ATPase activity. ATPase activity was remarkably increased with increasing NaCl concentration. By the addition of 100 mM NaCl, the activity was enhanced fivefold. The membrane-bound ATPase was activated by Na^+ with Mg^{2+} ions. The activity was stimulated to the greatest extent in the presence of 100 to 200 mM Na^+ with 5 mM Mg^{2+} ions (Figure 13), but was not stimulated by the addition of K^+ alone. ATPase activity was not increased by LiCl, KCl and $CaCl_2$, suggesting a specific effect of Na^+ on stimulation of ATPase (Figure 13). In the presence of Na^+

ions, the ATPase activity was further increased by the addition of K^+. The highest activity was obtained in the presence of both more than 100 mM Na^+ and 100 K^+ with 5 mM Mg^{2+} ions at pH 7.6 (Fig. 18), In the absence of NaCl, the ATPase activity of membranes was 0.10 U/mg protein. Upon addition of 100 mM NaCl, the activity was increased to 0.51 U/mg protein and activity was increased to 0.68 U/mg protein when added with 100 mM KCl. These characteristics suggested that the Na^+-activated ATPase from *A. halophytica* may be a Na^+ plus K^+ synergistically stimulated ATPase. Optimum pH was 7.5 (Figure 19). The kinetics of ATPase activation by ATP and Na^+ ion are shown in Figure 15 and 16, respectively. The K_m value for ATP and Na^+ ions were estimated to be 1.66 mM and 25 mM, respectively. The V_{max} using variable ATP and Na^+ ions were 0.66 and 0.50 U/mg protein, respectively. Because of the low affinity for Na^+, it is likely that Na^+-ATPase has ATP-dependent affinity change of Na^+ binding sites of ATPase. V-type Na^+-ATPase of *Enterococcus hirae* Na^+ binding site increased depending on the decrease of ATP hydrolysis rate. These suggest that the affinity of Na^+ binding sites is lowered in the present of high concentration ATP (Murata et al., 2001). Other reason for the low affinity of Na^+ might be because of the ATPase is not purify and electrochemical potential gradient has been estimated with membrane-bound of the ATP hydrolytic activity for Na^+ suspect that this pathway is relatively nonspecific and reflects some kind of leakage down the ATPase (Heefner and Harold, 1980).

A. halophytica* grown in the presence of 0.5 M and 2.0 M NaCl shown ATPase activity of 0.50 U/mg protein and 0.80 U/mg protein, respectively (Figure 20). This suggested that high NaCl concentration of the medium could induce the activity of ATPase. This might be explained by the increase of biosynthesis of

ATPase (Kakinuma, 1998) or increase affinity of Na^+ with ATPase. Figure 21 shown the ATP-dependent movement of $^{22}Na^+$ across *A. halophytica* membrane vesicles upon addition of ATP to membrane vesicles. By the addition of ATP, the membrane vesicles exhibited a significant uptake of $^{22}Na^+$. The uptake was inhibited by sodium ionorphore and gramicidin. The Na^+-stimulated may be P-type ATPase and involved in Na^+ transport. That this transport is electrogenic can be concluded from the results that the $^{22}Na^+$ transport is inhibited by dissipating the transmembrane electrical gradient.

Table 2 shows that the ATPase activity in the presence of NaCl and KCl was decreased to 50 % of original value by addition of 0.01 mM sodium vanadate, whereas it was not essentially affected by azide and KNO_3. Azide, vanadate, and NO_3^- are known as the inhibitors of F-, P-, and V-type ATPases, respectively (Koyama, 1999) and that vanadate - sensivitive ATPase is stimulated by Na^+. Several Na^+-dependent F-type and V-type ATPases are stimulated not only by NaCl but also by LiCl. ATPase activity of the membranes was not increased by LiCl (Figure 16), suggesting a specific effect of Na^+ on the stimulation of vanadate-sensitive ATPase. Inhibition by micromolar vanadate may suggest that the Na^+-stimulated ATPase is P-type. The Na^+-stimulated P-type ATPase may be involved in Na^+ transport. All vanadate-sensitive ATPases or P-type ATPases, such as animal Na^+/K^+- ATPases, form a phosphorylated intermediate (Post et al., 1973) that is easily detectable by acid SDS- PAGE.

In summary, the results demonstrate the presence of an ATPase in *A. halophytica* which uses Na^+ as the coupling ion. Although the inhibitor studies suggest an enzyme of P - type ATPase, a final conclusion has to await more data.

Expression of the Na^+-ATPase in these cells must be confirmed by Western blot analysis, using an antiserum against the Na^+-ATPase (Kawano et al., 1998). Together with a yet to be identified primary Na^+ pump, this enzyme would enable the organism to synthesize ATP during the operation of the Wood pathway with the electrochemical Na^+ gradient as driving force.

4.3 Effects of inhibitors on Na uptake and ATPase activity

Table 2, shows that the membrane-bound ATPase activity from *A.halophytica* in the presence of 100 mM NaCl, 4 mM ATP and 5 mM $MgCl_2$ was decreased to 63 % of original value by addition of 1 mM (DCCD) N,N$^/$-dicyclohexylcarbodiimide, a modifier of carboxyl group. It has been suggested that both F-type and V-type ATPase were sensitive to DCCD, and its inactivation effect was blocked by addition of NaCl, indicating that the modifiable carboxyl groups of these ATPases are Na^+- binding sites (Hirano and Koyama, 2001). These studies to demonstrate inactivation of the ATPase from *A.halophytica* by DCCD was the same irrespective of the presence of NaCl to indicate DCCD inhibited the Na^+-ATPase activity of membrane vesicles, but it did not affect the Na^+ binding. The ATPase from *A.halophytica* exhibited 50 % of original activity by the treatment with 0.01 mM sodium vanadate, P-type ATPase is characterized by sequentially phosphorylated intermediate during the ATP hydrolysis reaction that distinguishes P-type from V- and F- type ATPase. P-type ATPase is very sensitive to inhibition by phosphate analog vanadate (Nelson and Cox, 2000). ATPase activity was decreased to 45 % of original value by addition of 200 μM gramicidin. However, no inhibition was

observed with 50 mM KNO_3, 0.5 mM N-Ethylmaleimide, 5 mM azide and 1 mM CCCP, ouabain, sodium fluoride. CCCP is protonophore. Nitrate (KNO_3) and N-Ethylmaleimide (NEM) are known to inhibit V-type ATPase probably by dissociating the V_1 complex of the ATPase. Azide and ouabain are known to inhibit F-type ATPase and Na^+/K^+ ATPase, respectively.

CHAPTER V

CONCLUSION

The results of the present investigation can be summarized as follows:

1. Aqueous polymer two-phase partitioning is a rapid method for isolation of plasma membrane from cyanobacteria *A. halophytica*.

2. Sodium extrusion from cells can be followed by energizing the cell with glucose.

3. ATPase is present in membrane and enzyme activity is stimulated about fivefold in the presence of 100 mM NaCl and 5 mM $MgCl_2$ at 4 mM ATP.

4. Using lineweaver-Burk transformation of the data, the apparent K_m of Na^+ ion and ATP were 25 and 1.66 mM respectively, the V_{max} of both were 0.50 and 0.66 μmol/min/mg protein, respectively.

5. The inhibition by various agents reveals that vanadate, gramicidin, and DCCD, can inhibit more than 50 % ATPase activity.

6. ATPase activity was enhanced by high salinity of growth medium.

7. Sodium uptake in membrane vesicle is stimulated by addition of ATP.

8. The uptake of sodium by *A.halophytica* was inhibited by sodium ionophore and gramicidin.

9. It was demonstrated that the Na^+-stimulated ATPase was present in the membrane of cyanobacteria, *A. halophytica* and involved in Na^+ transport.

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APPENDICES

APPENDIX A

Turk Island Salt Solution + modified BG11 medium contained the following components:

1. Preparation of Turk Island Salt Solution

Stock solution A: KCl   33.3   g

MgCl$_2$.6H$_2$0   275.0 g

CaCl$_2$.2H$_2$0   73.3   g

and made up to 5 litres with distilled water .

Stock solution B: MgSO$_4$.7 H$_2$0   347.0 g

and then made up to 5 litres with distilled water .

To make Turk Island Salt Solution, 500 ml of Stock solution A was added to 500 ml of Stock solution B. To this mixture 140.8 g NaCl was added and then final volume was made to 5 litres with distilled water .

2. Composition of modified BG$_{11}$ medium (BG$_{11}$ medium + NaNO$_3$ Solution)

NaNO$_3$ (75g / 500 ml)   50   ml

KH$_2$PO$_4$ (8g / 200 ml)   5   ml

MgSO$_4$.7H$_2$0 (15g / 200 ml)   5   ml

CaCl$_2$.2H$_2$0   (7.2g/ 200 ml)   5   ml

Na$_2$CO$_3$ (4g/ 200 ml)   5   ml

Citric acid   (1.2g / 200 ml)   5   ml

EDTA.Na$_2$ (0.2g / 200 ml)   5   ml

FeSO$_4$.7H$_2$0 (1.2g / 200 ml)   5   ml

*Trace element A_5 solution + Co   5   ml

          *Trace element A_5 solution + Co contained the following component in gram per litres H_3PO_4 : 2.86 ; $ZnSO_4.7H_2O$: 0.2 ; $CuSO_4.5H_2O$: 0.08 ; $MnCl_2.4H_2O$: 1.81 ; $Na_2MnO_4.2H_2O$: 0.39 ; $Co(NO_3)_2.6H_2O$: 0.049.

          Culture medium of *Aphanothece halophytica* was prepared by adding all solution of item 2 at indicated volume to 5 litres of Turk Island Salt Solution and the pH was adjusted to 7.6 by slowly adding 2.0 M NaOH. The medium was sterilized by autoclaving at 15 lb/in^2 for 15 minutes.

APPENDIX B

Preparation for lysis buffer

20 mM Tris-HCl (pH 7.6)

      Tris(hydroxymethyl)-aminomethane   24.2   g

2 mM EDTA

      EDTA   0.07   g

4 mM benzamidine

      benzamidine   0.06   g

Adjusted pH to 7.6 with 1.0 M HCl and adjusted volume to 100 ml with distilled water.

APPENDIX C

Preparation of reagent for assay ATPase activity

Incubation buffer

(0.2 M Tris-HCl, 0.2mM Ammonium molybdate)

    0.2 M Tris-HCl (pH 7.6)

    Tris(hydroxymethyl)-aminomethane   2.42   g

    Ammonium molybdate   0.025 g

    Adjusted pH to 7.6 with 1.0 M HCl and adjusted volume to 100 ml with

    distilled water.

Phosphate reagent

    (0.5% Ammonium molybdate, 2% (v/v) sulfuric acid)

    Ammonium molybdate   10   g

    sulfuric acid   2   ml

    Added distilled water to a total volume of 100 ml.

10% Ascorbic acid

    Ascorbic acid   0.1   g

    Added distilled water to a total volume of 1 ml and stored frozen.

APPENDIX D

Preparation of dextran and polyethylene glycol

Phase mixture and phase system used in plasma membrane purification

(Larsson, et al., 1987)

Step	Phase mixture	Phase system
20% (w/w) Dextran T-500	4.20 g	14.00 g
40% (w/w) Polyethylene glycol 3350	2.10 g	7.00 g
Sucrose (solid)	1.28 g	4.28 g
0.2 M Tris-HCl, pH 7.6	0.38 ml	1.25 ml
Add water to a final weight of:	10.00 g	50.00 g

APPENDIX E

Calibration curve for protein determination by Bradford's method

$y = 0.0069x$
$R^2 = 0.9838$
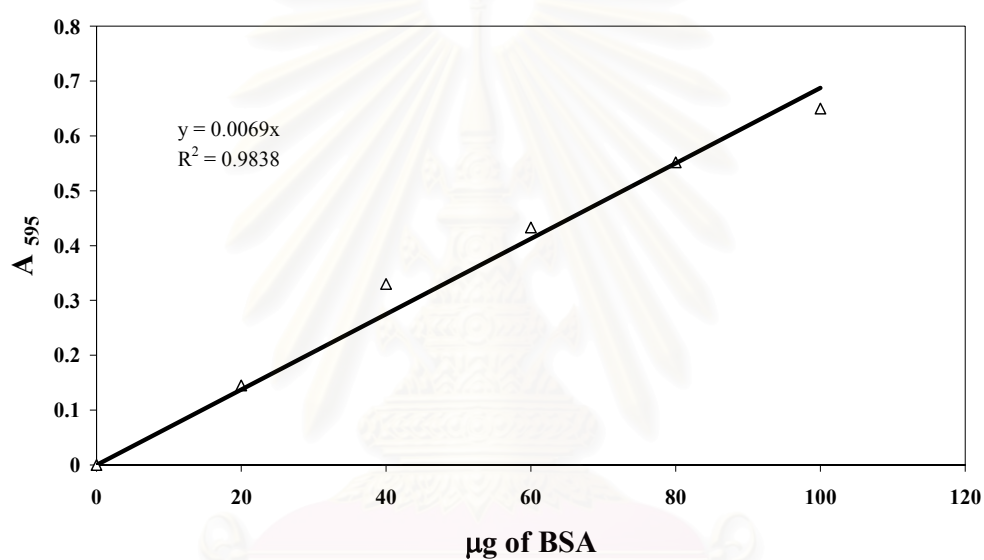
APPENDIX F

Standard curve for phosphate

$y = 0.2208x$
$R^2 = 0.9945$
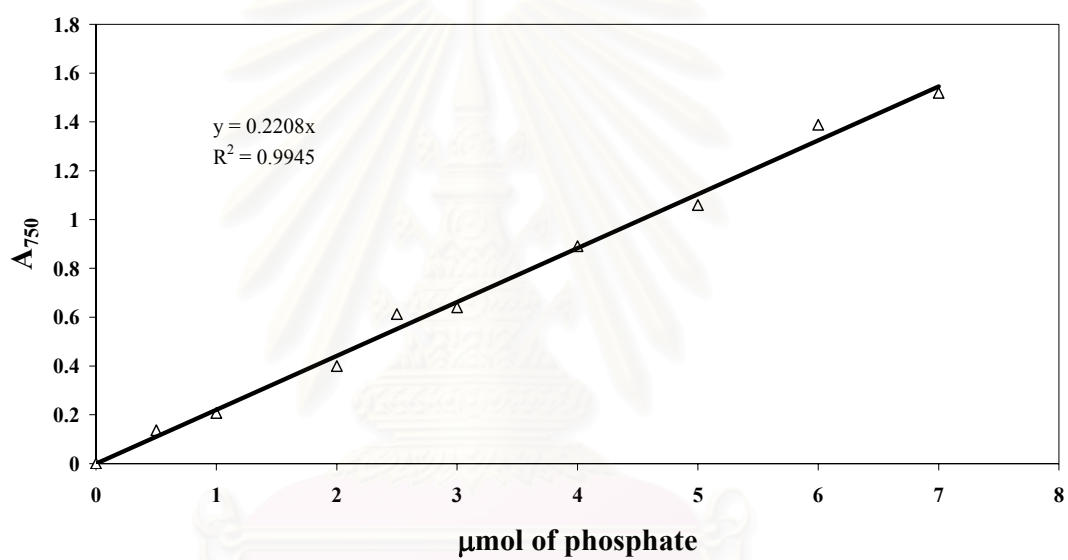
APPENDIX G

Sodium-22

Description

100 µCi Activity Date 1 November 2004

3.70 MBq

74 MBq/ml

2 mCi/ml

3.41 µg Na/ml

Specification

Chemical form	Sodium chloride in aqueous solution
Specific activity	>3.7 GBq/mg sodium
	>100 mCi/mg protein
Radioactive concentration:	Refer to vial/plot lable
Chemical purity:	<10 µg/ml of any cation impurity
	<50 µg/ml of total cation impurities
Radionuclidic purity:	No γ impurities detected
pH:	3.0-8.0

Nuclear Data

Production process:	$^{24}Mg\,(d,\alpha)\,^{22}Na$
Half-life	2.6 years
Type of decay:	β^+, electron capture
β^+ energy (maximum)	1.83 MeV
γ energy (maximum)	1.28 MeV
	0.51 MeV (from β^+)

APPENDIX H

Efficiency of instrument

Convert cpm to dpm by

% Efficiency of gamma counter   = $\dfrac{Cpm \times 100}{Dpm}$

= $\dfrac{67 \times 100}{100}$

=   67%

BIOGRAPHY

Miss Kanjana Wiangnon was born on January 04, 1980 in Samutsongkram, Thailand. She graduated with a Bachelor Degree of Science in Biology from Faculty of Science, Silapakorn University, Nakornprathom, Thailand in 2002 and studied for a Master Degree in Biochemistry program since 2005.