CHAPTER I

INTRODUCTION

Cyanobacteria, the oxygenic photosynthetic prokaryotes from which plasmids of photosynthetic eukaryotes (algae and plants) derive, inhabit a variety of environments including those in which extreme conditions such as high temperature and/or high salinity are found. Cyanobacteria that are able to grow in high-salt concentration environments maintain their cell turgor by accumulation of potassium ions and by the synthesis and accumulation of low molecular weight organic osmoprotectants. Thus freshwater cyanobacteria accumulate disaccharides and glucosylglycerol in response to osmotic stress, whereas halotolerant forms accumulate glycine betaine (Table 1) (Blumwald *et al.*1983, Mackay *et al.*1984 and Reed *et al.*1986). Glycine betaine is synthesized and accumulated by a wide range of organisms including bacteria, higher plants and animals (Galinski and Truper 1982, Csonka and Hanson 1991 and Garcia-Perez and Barg 1991)

Glycine betaine(*N,N,N*-trimethylglycine), a derivative of amino acid glycine (Figure 1) is a nontoxic osmolyte accumulated by many prokaryotic and eukaryotic organisms that face saline or dry environment (Mackey *et al.* 1984, Wyn Jones and Storey 1981 and Yancey *et al.* 1982).

Table 1 Major organic osmoregulatory solutes of cyanobacteria (Mackey *et al.* 1984).

Taxonomic assignment	Sucrose	Glucose	Fructose	Glycosyl-glycerol	Glycine-betaine	Maximum salt tolerance (g NaCl/l)	Strain origin
Anabaena N115	+	-	-	-	-	14.3	Pond water
Synechococcus N158	+	-	-	-	-	23.8	Lake water
Synechocystic N104	+	-	-	-	-	28.5	Fresh water
Gloeothece N101	+	+	-	-	-	33.2	Fresh water
Calothrix N117	+	+	+	-	-	42.8	Soil
LPP group B N113	-	-	-	+	-	60.2	Marine plankton
Synechococcus N111	-	-	-	+	-	70.4	Alkaline pond
Synechococcus 353	-	-	-	-	+	> 150.7	Saline pond
Synechococcus 358	-	-	-	-	+	> 150.7	Saline pond
Spirulina 439	-	-	-	-	+	> 150.7	Salt lake

The role of glycine betaine

The role of glycine betaine is different in various organisms. Glycine betaine acts as an osmoprotectant in *E. coli*, *Aphanothece halophytica*, *S.typhimurium* and *K. pneumoniae* (Landfald and Strom 1986, Le Rudulier and Bouillard 1983 and Dragolovich 1994). In *Rhizobium meliloti*,the root nodule symbiont of alfalfa, glycine betaine has been shown to be a carbon and nitrogen source or as an osmoprotectant (Bernard *et al.* 1986). On the other hand the function of glycine betaine is to protect the growth of the free-living bacterium and to enhance symbiotic N_2 fixation of nodulated alfalfa seedings under osmotic stress (Bernard *et al.* 1986 and Le Rudulier *et al.*1983). Glycine betaine also stimulates the respiration of a moderately halophilic and heterophilic bacterium at a high NaCl concentration, and it accumulates in the halophilic, photosynthetic bacterium *Ectothiorhodospira halochloris* and in several hypersaline strains of cyanobacteria (Shkedy-Vinkler and Avi-Dor 1975, Galinski and Truper 1982 and Reed *et al.* 1984). Mamedov *et al.* (1991) reported that in cyanobacterium *Synechocystis* PCC 6803, glycine betaine was effective both in the stimulation and in the protection of the oxygen-evolving machinery and the synthesis of ATP.

In *A. halophytica*, a halotolerant cyanobacterium, glycine betaine was a major osmoticum accumulating inside the cells in response to changes in external salinity (Reed *et al.* 1984). In this organism, glycine betaine acts as an osmoprotectant. Moreover, glycine betaine can relieve the inhibition by Cl^- of the activity of RuBisCO from *A. halophytica*

(Incharoensakdi *et al.* 1985 , 1986 and Incharoensakdi and Takabe 1988).

The synthesis of glycine betaine

Despite the widespread occurrence of glycine betaine, the osmotic regulation of the synthesis is well understood only in several microbial systems and certain plants. The synthesis of glycine betaine can be achieved by the following 2 systems:

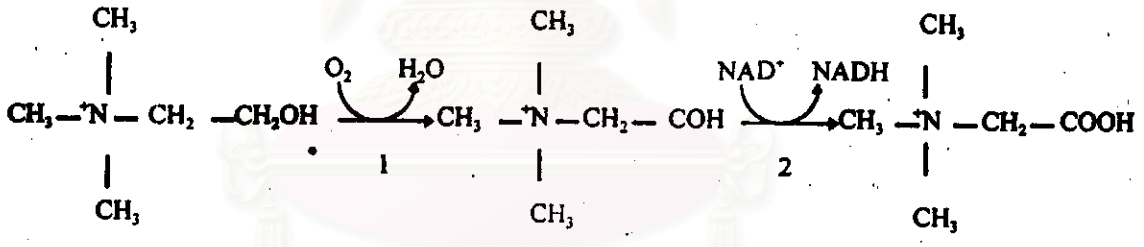
1) By import from the environment such as in *E. coli.*
2) By de novo synthesis from choline(called choline-betaine pathway) such as in spinach.

Choline is a precursor of several membrane phospholipids, of the methyl donor glycine betaine and of the neurotransmitter acetylcholine.

The glycine betaine synthesis has been studied in several bacteria, higher plants and marine animals (Landfald and Strom 1986, Reed *et al.* 1984 , Weigel *et al.*1986 and Dragolovich and Pierce 1994). In most organisms, glycine betaine was formed by the oxidation of choline to betaine aldehyde, which is then oxidized to glycine betaine (Figure 2). The first step (choline ⟶ betaine aldehyde) is catalyzed by different enzymes such as FAD-linked choline dehydrogenase (EC 1.1.99.1) in inner rat liver mitochondria membrane (Zhang *et al.* 1992). Choline oxidase(EC 1.1.3.17) is a new type of flavoprotein enzyme and was found in *Arthrobacter globiformis* (Ikuta *et al.* 1977). In all organisms studied, the final step in the synthesis of betaine from choline (betaine aldehyde ⟶ glycine betaine) is

Glycine Glycine betaine

Figure 1 **Structure of glycine and glycine betaine.**



Choline Betaine aldehyde Glycine betaine

Figure 2 **Choline-betaine pathway in the conversion of choline to glycine betaine. Two catalytic enzymes are 1) choline oxidase and 2) betaine aldehyde dehydrogenase.**

catalyzed by a NAD^+-dependent betaine aldehyde dehydrogenase; BADH (EC 1.2.1.8).

The determination of glycine betaine

The accumulation of glycine betaine was studied in several organisms. In spinach, glycine betaine was found to accumulate in response to water stress and salt stress. Simon *et al.*(1986) studied the effect of salt stress on the accumulation of glycine betaine in chloroplasts isolated from spinach leaves. They found that glycine betaine concentration was 26 and 289 mM in chloroplast isolated from control leaves and from salt-stressed leaves respectively. The effect of the reduction of external water potential was similar to salt stress. Ishitani *et al.*(1993) decreased the external water potential by adding organic osmoticum, sorbitol, to the *A. halophytica* culture and found that the amount of glycine betaine was increased in treated cells. This indicated that accumulation of glycine betaine occurs in the cells in response to changes in osmolarity of the culture medium. Because the accumulation of glycine betaine is an indication that the cells are adapting to the changes of external osmolarity, the determination of glycine betaine is important. There are many methods for the determination of glycine betaine.

At present different assay procedures are required to determine glycine betaine concentration in tissue extracts and most of the available methods are unsatisfactory both in terms of selectivity and convenience. Suitable methods for the determination of glycine betaine require various

levels of purification of the crude materials. The ion-exchange chromatography was essential to remove interfering amino acids prior to glycine betaine estimation. The available methods which are satisfactory both in terms of selectivity and convenience are:

1) the ^{1}H Nuclear Magnetic Resonance spectroscopy (NMR).

2) the tri-iodide assay with spectrophotometric method.

The ^{1}H-NMR method is used to quantitate the amount of compound by peak area. The NMR signals represent *N*-methyl protons of a compound. Peak identities are confirmed by addition of authentic standards and quantitation is obtained by comparing integrated peak intensities against standard curves. Peak positions are measured relative to internal reference such as sodium trimethylsilylpropionate (TSP) or sodium 2,2-dimethyl-2-silipentate-5 sulphonic acid (DSS). Jones *et al.*(1986) found that peak positions of glycine betaine and choline extracted from barley are 3.28 ppm and 3.20 ppm (relative to TSP), respectively. The glycine betaine and choline peaks were sharp and single. The advantages of this method are reliable, rapid, sensitive at low levels of compound and it can be used to detect and identify unknowns.

The tri-iodide assay is used to estimate quaternary ammonium compounds such as glycine betaine, choline, trigonelline and carnitine. The quaternary ammonium compounds form complex with iodine of potassium-iodide reagent in acidic condition. Iodine is a sensitive precipitating agent and yields similar water-insoluble complexes with these compounds. Ethylene dichloride can solubilize these complexes and exhibits an absorption maximum in the ultraviolet region at 365 nm. The glycine betaine estimation requires higher level of purification than

^{1}H -NMR method. The use of ion-exchange chromatography such as Dowex 50-W helps separate glycine betaine from the extract. The recovery of glycine betaine from the column must be evaluated.

Betaine aldehyde dehydrogenase (BADH) purification and characterization.

The purification method of BADH has been studied by several groups of workers. They used several steps such as ammonium sulfate precipitation, ion-exchange chromatography, gel filtration or affinity chromatography. Falkenberg *et al.* (1990) purified BADH of *E. coli* strain MC 4100(pFF 423) by using 35-60 % saturated ammonium sulfate precipitation and affinity chromatography (5 -AMP sepharore 4B). They found that the enzyme was purified 57-fold, giving a preparation with a specific activity of 46 μmol min^{-1} mg^{-1}. The enzyme was essentially pure after affinity chromatography, giving one single band by native gel electrophoresis as well as by SDS gel electrophoresis, even when overloading the gels with 20 to 30 μg of protein.

Pan *et al.* (1981) used 60-70% saturated ammonium sulfate precipitation, gel filtration (sephadex G-200) and hydroxyapatite column for BADH purification from spinach. They found that the enzyme was purified 175-fold, giving a preparation with a specific activity of 194 nmol min^{-1} mg^{-1}.

During the course of enzyme purification, BADH was assayed spectrophotometrically by monitoring the conversion of NAD^+ to NADH over 3 min at 340 nm, 25°C.

The characteristics of BADH have been studied in many organisms. Purified BADH has a broad pH optimum from about pH 7.5 to 9.5. The maximum enzyme activity occurs at temperatures from 25 to 30 °C (Table 2). The enzyme is relatively specific for NAD^+ as the electron acceptor and for betaine aldehyde as a substrate with a low K_m value. The equilibrium of the reaction is strongly in favor of betaine formation. The reducing agents, β-mercaptoethanol and dithiothreitol(DTT) both effectively protect the enzyme. For BADH purified from spinach preincubation of the enzyme with DTT could partially protect the enzyme from subsequent inhibition by *p*-chloromercuribenzoate(PCMB). Therefore, one or more sulfhydyl group in the enzyme is essential for activity (Shu-Mei *et al.* 1981). Falkenberg and Strom (1990) found that the purified BADH from *E. coli* strain MC 4100 (pFF423) was inhibited by analogs of betaine aldehyde. Two types of the analogs of betaine (aldehydes and *N*-methylated) were tested. Aldehydes with apolar groups such as isovaleraldehyde were the most effective inhibitors. The *N*-methylated compounds such as *N*-methylglycine,ethanolamine were also inhibitory, but to a much lesser degree than the aldehyde.

The salts could also affect BADH activity. The divalent cations such as Ca^{2+}, Mg^{2+} inhibited BADH from *E. coli* and chelating agents such as ethylenediamine tetraacetate(EDTA) could protect the enzyme against the divalent cation (Falkenberg and Strom 1990). The anions had little effect on the enzyme activity at low salt concentrations (Falkenberg and Strom 1990). Elevated Na^+ concentrations also

Table 2 The characterization of purified BADH from various organisms.

Kind of organisms	pI	MW. (kDa)	Optimum pH	Optimum Temp. (°C)	Inhibitor
Plant					
Spinach (*Spinacia oleracea* L.)	5.65	111	7.5-9.5	22	PCMB*
Microorganism					
E coli strain MC 4100 (pFF 423)	-	232	7.5-9.5	25	Ca^{2+}, Mg^{2+}
Animal cells					
Horseshoe Crab (*Limulus polyphemus*)	-	-	7.5-8.5	25	Na^{+}

* PCMB : *p*-chloromercuribenzoate

inhibited BADH activity in animal cells *Limulus polyphemus* (Dragolovich and Pierce 1994).

The molecular weights of BADH from several organisms are different. The BADH of *E. coli* constitute a tetramer with identical 55 kDa (Falkenberg and Strom 1990) . The BADH of spinach chloroplast is a dimer with subunits of 60 kDa (Arakawa *et al.* 1987) and 63 kDa (Weretilnyk and Hanson 1989).

In this study the unicellular halotolerant cyanobacterium, *A. halophytica* was chosen as a model organism to investigate the accumulation of glycine betaine as well as the enzyme that responsible for its synthesis. This cyanobacterium is classified into *Chroococcals* order, *Chroococcacean* cyanobacteria subgroup. This cyanobacterium is a rod-shaped organism which multiplies by binary fission. *A. halophytica* can be grown at high external NaCl concentration up to 3 M. It has been reported that glycine betaine is a major osmoticum accumulated inside *A. halophytica* in response to change in external salinity (Galick *et al.* 1973 and Reed *et al.*1984). In higher plants and bacteria but not in cyanobacteria (Hanson and Hitz 1982 and Weigel *et al.*1986,1988), the regulation of glycine betaine accumulation as well as the metabolism of glycine betaine,have been well studied . So far, neither the activity of choline dehydrogenase nor that of betaine aldehyde dehydrogenase has been detected in glycine betaine accumulating cyanobacteria.

The objectives of this project are the following:

1. To estimate glycine betaine content in *A. halophytica* by nuclear magnetic resonance method and spectrophotometric method.
2. To study the accumulation of glycine betaine in *A. halophytica* under salt stress.
3. To partially purify and characterize betaine aldehyde dehydrogenase from *A. halophytica* .
4. To study the relationship between salt stress and betaine aldehyde dehydrogenase activity in *A. halophytica* .