# CHAPTER IV

## DISCUSSION

We could isolate an $Na^+/H^+$ antiporter gene homologous to eucaryotic ones from a halotolerant cyanobacterium *A. halophytica*. The *A. halophytica* antiporter gene exhibits highest homology to the *synnha*P and encodes a polypeptide consisting of 521 amino acids. The hydropathy plot and TM prediction analysis of ApNhaP suggested the presence of 11 TM segments and a relatively long C-terminal tail in cytosolic space. One of important ionic amino acids, Asp139 in ApNhaP, was conserved in membrane spanning region.

Based on the findings that the antiporter-deficient *E.coli* TO114 mutant cells became salt-tolerant by transformation with the *apnha*P gene (Fig. 3.6) and also by the direct observation of $Na^+/H^+$ antiporter activity in the transformant membrane vesicles (Fig. 3.8), it was concluded that the *apnha*P encodes the $Na^+/H^+$ antiporter ApNhaP. The most striking functional feature of ApNhaP is its novel ion specificity. The ApNhaP did not show the $Li^+/H^+$ antiporter activity (Fig. 3.8B), but did show the $Ca^{2+}/H^+$ antiporter activity (Fig. 3.8C). These conclusions were also substantiated by the observations that the ApNhaP did not complement the $Li^+$-sensitive phenotype of *E. coli* mutant (Fig. 3.10A), but complemented the $Ca^{2+}$-sensitive phenotype of *E. coli* mutant (Fig.3.10B).

It has been reported that the *E. coli* ChaA has the proton/cation exchange activity, with $Na^+$ or $Ca^{2+}$, but not $Li^+$ or $K^+$ (Ivey et al., 1994 ; Ohyama et al., 1995), which is essentially the same ion specificity as that of ApNhaP. However, the ChaA did not show any homology to the ApNhaP. The ChaA has the acidic motif Glu200-

His-Glu-Asp-Asp-Ser-Asp-Asp-Asp-Asp209 conserved in several $Ca^{2+}$-binding proteins such as calsequestrin, calreticulin, and $Na^{+}/Ca^{2+}$ exchanger (Ivey et al., 1994) whereas the ApNhaP lacks the acidic motif. The hydropathy plot of ChaA suggests the absence of a long hydrophilic C-terminal tail seen with the ApNhaP. The ApNhaP did not show any homology to the vacuolar $Ca^{2+}/H^{+}$ exchangers from yeast (Cunningham and Fink., 1996) and plants (Ueoka-Nakanishi et al., 1999). All these data suggest that the ApNhaP is an $Na^{+}/H^{+}$ antiporter with different structure from that of ChaA.

The data of Fig. 3.14 show that the exchange of long C-terminal tails of ApNhaP and SynNhaP with those of SynNhaP and ApNhaP greatly affected the ion specificity of the antiporter, especially that of $Li^{+}/H^{+}$ exchange activity. These data suggest that exchange of the long C-terminal tail could disrupt structural elements of the antiporter that are critical for ion-specific binding. A remarkable difference between the C-terminal tails of ApNhaP and SynNhaP is the net charges, 22 basic and 14 acidic amino acids in ApNhaP as compared to 15 basic and 24 acidic amino acids in SynNhaP. An intriguing possibility is that the negative charges on the C-terminal tail of SynNhaP help the binding of $Li^{+}$ which has smaller ion radius, consequently higher positive charge density than $Na^{+}$, on the membrane spanning region of the antiporter thereby allowing the $Li^{+}/H^{+}$ exchange activity. However, the data of ASNhaP chimera for $Ca^{2+}/H^{+}$ exchange activity and SANhaP chimera for $Na^{+}/H^{+}$ exchange activity are not consistent with the above viewpoint, suggesting the importance of TM region(s) for the ion specificity of $Na^{+}/H^{+}$ antiporters. In spite of high $Ca^{2+}/H^{+}$ exchange activity of SANhaP (Fig. 3.14C), the SANhaP chimera only partially complemented the $Ca^{2+}$-sensitive phenotype of *E.coli* mutant (Fig. 3.15C)

which is difficult to explain and remained to be clarified. The construction of various mutants with alteration in C-terminal hydrophilic region of ApNhaP is needed to elucidate the mechanism underlying ion specificity of ApNhaP. In addition, construction of additional chimera in which *chaA* from *E. coli* which lacks a C-terminal tail serving as a parental antiporter may further give insight into the role of the C-terminal tail.

It is worthwhile noting that there is an apparent disparity of the data between the exchange activity and the growth rate. The difference of growth rates between ApNhaP- and SynNhaP-expressing cells (Fig. 3.10B) was much greater than the difference of $Ca^{2+}/H^{+}$ antiporter activities between ApNhaP and SynNhaP (Fig.3.8C). In the case of complementation of $Ca^{2+}$-sensitive *E. coli* cells (Fig. 3.10B), $Ca^{2+}$ must be excluded from the cells against the concentration gradient of $Ca^{2+}$. This activity was much higher in ApNhaP expressing cells. In contrast, $Ca^{2+}/H^{+}$ exchange activity was measured using the everted membrane vesicles following the concentration gradient of $Ca^{2+}$ which was similar between ApNhaP and SynNhaP (Fig. 3.8C). Thus, the experimental conditons are different between the exchange activity and complementation test at least on the orientation of vesicles, $Ca^{2+}$ concentration gradient, and pH at the $H^{+}$ binding site. These different experimental conditions might be the cause, at least partly, for the apparent disparity between the exchange activity and the growth rate although the exact nature of this disparity is still unknown..

The NhaA $Na^{+}/H^{+}$ antiporter from *E. coli* is activated by the $Na^{+}$ ion via NhaR protein at the transcriptional level (Carmel et al., 1997) whereas the $Na^{+}/H^{+}$ exchange activity of mammalian NHEs are activated by binding $Ca^{2+}$ to the long C-terminal tail (Wakabayashi et al., 1994). The recently discovered SOS1 antiporter has been shown

to be up-regulated in response to NaCl stress via SOS2 and SOS3 (Shi et al., 2000; Halfter et al., 2000). The SOS3 is a $Ca^{2+}$-binding protein having sequence similarities with animal neuronal $Ca^{2+}$ sensors and the yeast calcineurin B (Halfter et al., 2000). The data of Fig. 3.17 show the increase of $Na^+/H^+$ antiporter mRNA levels upon upshock and downshock of salt which reached to the maximum within 30 min. This is in contrast to a much slower process of the accumulation and degradation of an osmoprotectant glycinebetaine due to the salt upshock and downshock in *A. halophytica* (Incharoensakdi and Waditee, 2000). Since the stop codon of *cyt. c550* gene is located at the 120 bp upstream of the first ATG codon of *apnha*P (Fig. 3.1), it will be interesting to analyze the promoter region of *apnha*P gene. Considering together with the fact that the relatively long C-terminal tail of SynNhaP plays an important role for $Na^+/H^+$ antiporter (Hamada et al., 2001), the C-terminal tail of ApNhaP now seems to be important for the ion specificity and activation of its antiporter whose mechanisms remained to be clarified.

The data of Figs. 3.6 and 3.7 show that the complementation ability of ApNhaP is more effective than that of SynNhaP. ApNhaP exhibited the $Na^+/H^+$ antiporter activity over a wide range of pH. Ion specificity of ApNhaP is unique. The ApNhaP did not show any activity of $Li^+/H^+$ antiporter, but had high $Ca^{2+}/H^+$ antiporter activity. This finding would suggest an interesting application of ApNhaP for the genetic engineering of salt tolerant plants. Previously, it was shown that the DnaK from *A. halophytica* exhibits *in vitro* much higher refolding activity than that of the DnaK from fresh water cyanobacterium (Hibino et al., 1999). Transformation of tobacco plants by *A. halophytica* DnaK conferred salt tolerance (Sugino et al., 1999) as well as high temperature tolerance (Ono et al., 2001). Since it has been reported that the

transformation of *Arabidopsis* by vacuole type $Na^+/H^+$ antiporter AtNHX1 from *Arabidopsis* could confer salt tolerance of *Arabidopsis* (Apse et al., 1999), further studies aimed at constructing the transgenic plants using ApNhaP would enable us to obtain improved salt tolerant plants. Transfer of multiple salt tolerant genes from *A. halophytica* into plants will provide an interesting example to construct the salt tolerant plants.

With regard to the compatible solute regulation in *A. halophytica*, it was previously shown this organism accumulates glycinebetaine upon the increase of external salinity (Takabe et al., 1988; Lee et al., 1997). Ishitani et al (1993) reported the decrease of glycinebetaine level when the cells were subjected to hypoosmotic stress but not examined in details. In this study, the focus was on the changing level of glycinebetaine in terms of its degradation. The effective treatment to induce the degradation of glycinebetaine was achieved by first subjecting the cells to a hyperosmotic condition (2.0 M NaCl) and followed by salt downshock of the cells to the normal 0.5 M NaCl. The intracellular glycinebetaine was decreased after salt downshock and the presence of glycinebetaine in the salt downshock medium was not detected, suggesting that the decreased level of intracellular glycinebetaine was not due to its being excreted out of the cells, but rather to its being catabolized.

The data of Fig 3.20 indicated that BHMT was at least one of the enzymes catabolizing glycinebetaine in *A. halophytica*. The observed decrease of enzyme activity after 3 h salt downshock accompanying a decrease in glycinebetaine level during this period was difficult to reconcile. There remained a possiblilty that the enzyme extract used in this experiment contained inhibitory sunstances. Previously, *N, N*-dimethylglycine, another product in the BHMT-catalyzed reaction, has been

reported to be a potent inhibitor of BHMT from many mammalian sources (Finkelstein et al., 1972; Garrow., 1996). High activity of BHMT after 3 h salt downshock meant that a considerable amount of N, N-dimethylglycine accumulated in the enzyme extract. Increased duration of salt downshock would result in more accumulation of *N, N*-dimethylglycine. This would lead to lower BHMT activity of *A. halophytica* observed in Fig. 3.20 The possiblity of the presence of some inhibitory substance of BHMT has also been reported for the enzyme extract from *Rhizobium meliloti* (Smith et al., 1988). Very low activity of BHMT was found in the enzyme extract in this bacterium when the cells are labeled with [methyl-$^{14}$C] glycinebetaine. Furthermore, BHMT activity was found to be higher with cells grown under low osmolarity than that under high osmolarity. The present study showing nondetectable activity of BHMT in cells grown under 2.0 M NaCl agree well with the results reported for the enzyme from *Rhizobium meliloti.*

The persistent decline in glycinebetaine level observed in Fig. 3.20 despite the possible inhibition of BHMT activity might be explained in part by the degradation of glycinebetaine through other routes. Demethylation of glycinebetaine to *N, N*-dimethylglycine and subsequent oxidation of methyl group to $CO_2$ has been reported in *Eubacterium limosum* (Muller et al., 1984). Indeed, it was found that *A. halophytica* could release $^{14}CO_2$ after labeling with [methyl-$^{14}$C] glycinebetaine (data not shown). This gave further evidence that glycinebetaine could be catabolized in *A. halophytica.* Another route of the degradation of glycinebetaine, which has been previously reported, involves a reductive cleavage of three molecules of glycinebetaine to form acetate and trimethylamine in *Sporomusa sphaeroids* and *S. ovata* (Moller et al., 1984).

*A. halophytica* appeared to utilize glycinebetaine as its carbon and nitrogen sources when these two elements were absent in the growth medium (Fig. 3.21). The detailed analysis of the reduction of BHMT during 12-36 h of treatment gave 64 nmol of methionine formed per hour per milligram protein as against the value of 84 nmol of glycinebetaine disappeared per hour per milligram protein. This indicated that BHMT accounted mainly for the degradation of glycinebetaine under carbon and nitrogen deprivation. Considering the effect of salt downshock observed in Fig. 3.20, methionine formed by BHMT during the first 3 h downshock (460 nmol $h^{-1}$ $mg^{-1}$) represent nearly one-half of the glycinebetaine disappeared (1038 nmol $h^{-1}$ $mg^{-1}$). The mechanism by which low osmolarity due to salt downshock stimulates the catabolism of glycinebetaine remains unclear. However, the rise in BHMT activity accompanying the drop in glycinebetaine after salt downshock indicated the important role for BHMT in the osmoregulation of *A. halophytica*. The purification of BHMT from this organism shows the activity enrichment of 24-fold with a final yield of 11%. The preparation was judged to be homogeneous by SDS-PAGE analysis yielding a single band of Mr 45 kDa (Fig. 3.27). The low specific activity of *A. halophytica* is similarly reported for human and rat liver BHMTs (Finkelstein and Mudd, 1967; Millian and Garrow, 1998). Although the subunit Mr of 45 kDa reported here for *A. halophytica* BHMT is consistent with those for rat, human and pig liver BHMTs (Lee et al., 1992; Skiba et al., 1982 ; Sunden et al., 1997), its molecular structure appeared to be an octamer as opposed to a hexamer found in mammalian BHMTs (Lee et al., 1992 ; Skiba et al., 1982).

The apperent Km values for glycinebetaine and L-Hcy of *A. halophytica* BHMT are much higher than those reported for rat and human enzymes (Finkelstein et

al., 1972; Lee et al., 1992 ; Skiba et al., 1982). The estimated high Km for L-Hcy would arise due to the oxidation of Hcy during the kinetic analysis of the enzyme. Another possible factor might be a result of incomplete hydroxide-dependent conversion of Hcy-thiolactone to Hcy (Finkelstein and Mudd, 1967), thus the concentration of Hcy used in the enzyme assay would represent the higher value than its actual concentration. Km for glycinebetaine of *A. halophytica* BHMT was relatively high (4.3 mM). The intracellular concentration of glycinebetaine of *A. halophytica* has been reported to be in range of several hundred millimolar especially when cells subjected to high osmolarity. This intracellular level of glycinebetaine is much higher than the Km (glycinebetaine). It is obvious that *A. halophytica* BHMT is at all times saturated with glycinebetaine. The fact that Vmax of *A. halophytica* BHMT is low suggested inefficient binding of glycinebetaine and/or Hcy to the enzyme. This is consistent with the observed high Km for both glycinebetaine and Hcy. At this stage the cause of low specificity activity of BHMT from *A. halophytica* is unknown. It is worth mentioning that in the case of human BHMT, the enzyme appeared to polymerize to integral aggregates in the ansence of thiol reagent (Skiba et al., 1982). The product of BHMT-catalyzed reaction, dimethylglycine, has been reported to be a potent inhibitor of pig, rat, human and bacterial BHMTs (Finkelstein et al., 1972; Garrow, 1996; Millian and Garrow, 1998 and Skiba et al., 1982). The present study also shows that dimethylglycine is a strong inhibitor of *A. halophytica* BHMT (Table 3). However, the most potent inhibitor is betaine aldehyde which is analog of glycinebetaine substrate. Sarcosine displays very little inhibition on the enzyme activity. The finding that betaine aldehyde, an intermediated of choline-glycinebetaine pathway, completely abolishes BHMT activity suggests that the

synthesis and degradation of glycinebetaine do not occur simultaneously. This is substantiated by the evidence that no BHMT activity was observed when *A. halophytica* cells were subjected to high osmolarity.

High level of NaCl could effectively inhibit BHMT activity of *A. halophytica* (Table 4). Reed et al (1988) found that the intracellular $Na^+$ of *A. halophytica* increased from 80 to 180 mM when the strength of the external medium was raised from 50% sea water (ca. 0.3 M NaCl) to 400% sea water (ca. 2.4 M NaCl). On the other hand, intracellular $Cl^-$ of *A. halophytica* was increased from 35 mM to 150 mM when NaCl in the growth medium was raised from 0.5 M to 2.0 M (Incharoensakdi and Takabe, 1988). It can be envisaged that high osmolarity causes an increase in the intracellular $Na^+$ and $Cl^-$ of *A. halophytica* which in turn prevents the degradation of glycinebetaine and therefore its high level can be maintained under high external osmolarity.