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Isolation and characterization of Na^+/H^+ antiporter gene from a halotolerant cyanobacterium *Aphanothece halophytica*

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Introduction

The Na^+/H^+ antiporters catalyze the exchange of Na^+ for H^+ across membranes and play a variety of functions such as the regulation of internal pH, cell volume, and sodium level in the cytoplasm. In *E. coli*, three antiporters (NhaA, NhaB and ChaA) are known and their functional characteristics have been well described. Six kinds of Na^+/H^+ antiporters (exchangers) (NHE1-6) have been found in animals. In plants and yeast, the vacuole type (NHX1 and AtNHX1) and the plasma membrane type (SOD2 and SOS1) Na^+/H^+ antiporters have been reported. The homology between *E. coli* antiporters and eucaryotic ones was very low suggesting the independent evolution among Na^+/H^+ antiporter genes. To date, only a few functional residues, especially the residues involved in the cation transport, have been identified in Na^+/H^+ antiporter proteins. In a previous paper (Hamada et al, 2001), we showed that a cyanobacterium *Synechocystis* sp. PCC 6803 contains an Na^+/H^+ antiporter, SynNhaP, homologous to eucaryotic and procaryotic (NhaP from *Pseudomonas aeruginosa*) ones, but not to the NhaA, NhaB and ChaA. It was also shown that the SynNhaP contains a conserved Asp138 in trans-membrane (TM) spanning region and relatively long C-terminal hydrophilic tail important for the carrier activity. The long C-terminal tails are believed to play a role in the regulation of transport activity in animals. These facts suggest that the cyanobacterial antiporters would provide a model system for the study of structural and functional properties of eucaryotic Na^+/H^+ antiporters. *Aphanothece halophytica* (*A. halophytica*) is a halotolerant cyanobacterium which can grow in a wide range of salinity conditions from 0.25 to 3.0 M NaCl. *A. halophytica* accumulates an osmoprotectant glycine betaine at high salinity. DnaK protein of *A. halophytica* has been shown to contain the longer C-terminal segment than other DnaK/Hsp70 family members (Lee et al. 1997) and exhibit extremely high protein folding activity at high salinity (Hibino et al. 1999). Therefore, it was of interest to isolate the Na^+/H^+ antiporter from *A. halophytica* and compare its properties with other Na^+/H^+ antiporters.

Materials and methods

The basic culture medium for *A. halophytica* cells contained BG11 medium, 18 mM $NaNO_3$, and Turk Island salt. Using the partially-degenerate oligonucleotides, DNA fragment of an expected size were amplified (Waditee et al. 2001). Then, the adjacent unknown regions of DNA were amplified by the inverse polymerase chain reaction method. By repeating this, the

nucleotide sequence which covers the whole sequence of Na^+/H^+ antiporter gene (*apnhaP*) of *A. halophytica* was determined. For the construction of expression plasmid, the coding region of *apnhaP* was isolated by the PCR reaction and ligated into *Nco*I/*Hind*III sites of the pTrcHis2C plasmid. The resulting plasmid, pANhaP, encodes the ApNhaP fused in frame to six histidines, and was transferred to TO114 cells in which *nha*A, *nha*B, and *cha*A genes were deleted.

Quantification of ApNhaP mRNA expression was carried out using a TaqMan fluorescent chemical analysis method. Total RNA was extracted by using SDS-phenol method. Fifty microliters of reaction mixture were used, containing PCR products of 20 ng total RNA, 1x TaqMan buffer A, 5.5 mol/*l* $MgCl_2$, 300 mol/*l* dATP, dGTP, and dCTP, 600 mol/*l* dUTP, 0.05 mol/*l* forward and 0.9 mol/*l* reverse primers, 0.15 mol/*l* TaqMan probe, and 1.25 U Ampli Taq Gold. A computer algorithm was used for comparison of the amount of reporter dye emission with the quenching dye emission during the PCR amplification.

The Na^+/H^+ antiporter activity was examined on everted membrane vesicles prepared from the cells grown in LBK. The reaction mixture contained 10 mM Tris-HCl (titrated with HCl to the indicated pH), 5 mM $MgCl_2$, 0.14 M choline chloride, 1 M acridine orange, and membrane vesicles (50 g of protein) in a volume of 2 m *l*. The acridine orange fluorescence was monitored at an excitation wavelength of 492 nm and emission wavelength of 525 nm. At the onset of the experiment, Tris-DL-lactate (2 mM) was added and the fluorescence quenching was recorded. Salt (5 mM) was then added and the increase of fluorescence (dequenched fluorescence) was monitored.

Results

The Na^+/H^+ antiporter gene was isolated from a *A. halophytica* cells. The homology search revealed that ApNhaP is highly homologous to the Na^+/H^+ antiporters from eucaryotes (SOS1, NHEs, AtNHX1, and NHX1) and procaryotes (NhaP, SynNhaP). ApNhaP showed the highest homology to SynNhaP. Analysis of the Na^+/H^+ antiporters, hydropathy plot and the TM prediction program predicted 11 putative TM segments in ApNhaP. These data indicate that a halotolerant cyanobacterium *A. halophytica* contains an Na^+/H^+ antiporter homologous to eucaryotic ones.

To characterize the molecular properties of antiporter, the *E. coli* cells were transformed with pANhaP and pSNhaP, and their growth rates were examined. The *E. coli* TO114 cells transformed with pANhaP and pSNhaP can grow with a similar rate to that of TO114 cells transformed with pTrcHis2C in LBK medium at pH 7.0 and 37¡C. However, due to the absence of Na^+/H^+ antiporter genes (*nhaA*, *nhaB*, and *chaA*) in TO114 cells, the *E. coli* cells transformed with pTrcHis2C could not grow in the presence of 0.2 M NaCl. In contrast, the *E. coli* cells transformed with pANhaP or pSNhaP could grow. These results indicate that ApNhaP and SynNhaP could complement the salt sensitive phenotype of Na^+/H^+ antiporter deficient *E. coli* mutant TO114 cells.

Next, the everted membrane vesicles were prepared and their antiporter activities were monitored by measuring the dequenching of acridine orange fluorescence upon addition of NaCl. The dequenching of fluorescence by ApNhaP or SynNhaP was observed over a wide range of pH between 5 and 9 which is quite different from that by *E. coli* NhaA as shown in Fig. 1. In *E. coli* NhaA, the antiporter activity could not be observed below pH 7.5 while the activity increased with increasing pH.

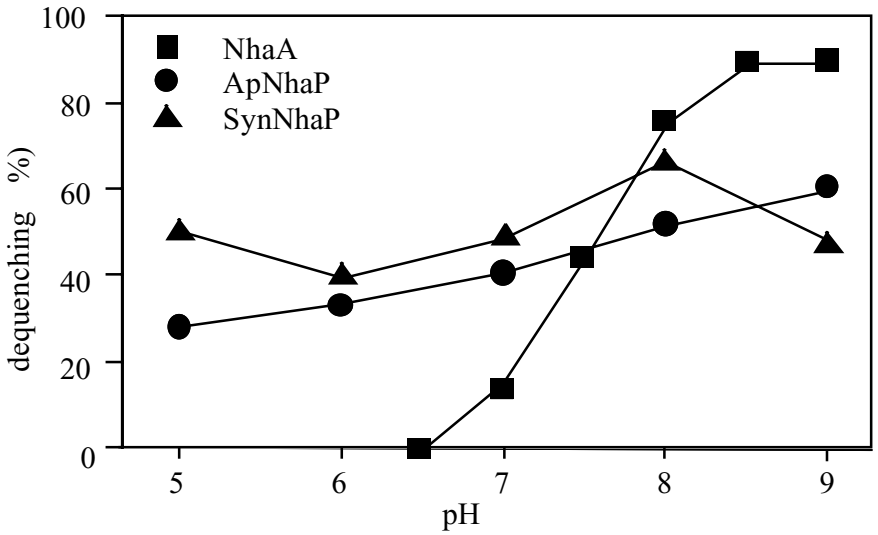

Fig. 1. pH dependence of Na^+/H^+ antiporter activity

To study the Na^+-induced regulation of Na^+/H^+ antiporter activity, the cellular levels of ApNhaP mRNA were directly measured using a TaqMan fluorescent analysis method. The total RNAs were extracted from the cells at various times after upshock from 0.5 M to 1.5 M NaCl and downshock from 1.5 M to 0.5 M NaCl. It was found that the level of ApNhaP mRNA increased upon upshock. The downshock also induced the increase of mRNA level for ApNhaP. These results suggest that the changes of NaCl concentrations in the growth medium induced the changes of Na^+/H^+ antiport activity at least by changing the levels of ApNhaP mRNA.

Next, we examined whether or not NaCl or glycine betaine activates the Na^+/H^+ antiporter molecules. The Na^+/H^+ antiporter activity of ApNhaP increased about 25% by addition of 1 M glycine betaine to the reaction mixture. In contrast, the same treatment caused a decrease of Na^+/H^+ antiporter activity in SynNhaP. Sucrose and KCl were essentially without effect on the Na^+/H^+ antiporter activity of both ApNhaP and SynNhaP. The inclusion of glycine betaine, NaCl, or sucrose during the preparation of everted membrane vesicles did not change the Na^+/H^+ antiporter activities of ApNhaP and SynNhaP. These results suggest that glycine betaine in the cytosolic side affects the antiporter activty,.

Discussion

We could isolate an Na^+/H^+ antiporter gene homologous to eucaryotic ones from a halotolerant cyanobacterium *A. halophytica*. It was found that the ApNhaP and SynNhaP exhibited the Na^+/H^+ antiporter activity over a wide range of pH between 5 and 9 and complemented the Na^+-sensitive phenotype of the antiporter deficient *E. coli* mutant. Since the overexpression of *nhaAv* gene from *Vibrio alginolyticus* in a freshwater cyanobacterium *Synechococcus* sp. PCC 7942 conferred lithium tolerance, but not sodium tolerance (Kaku et al. 1999), and the coexpression of two genes increased the tolerance for salt stress (Kaku et al. 2000), the overexpression of various genes including ApNhaP might be interesting. The NhaA Na^+/H^+ antiporter is activated by Na^+ ion via NhaR protein whereas mammalian NHEs are activated by binding Ca^{2+} to the long C-terminal tail. The recently discovered SOS1 antiporter has been shown to be up-regulated in response to NaCl stress via SOS2 and SOS3. The SOS3 is a Ca^{2+}-binding protein having sequence similarities with animal neuronal Ca^{2+} sensors and the yeast calcineurin B. The data showing increased Na^+/H^+ antiporter mRNA levels due to upshock and downshock of salt indicate that ApNhaP plays a role in osmoregulation of *A. halophytica*. The upshock effects on the antiporter mRNA was more pronounced than the downshock effect. However, both effects appeared to occur within a

relatively short time of 30 min, suggesting that an early event of osmoregulation in *A. halophytica* is due to the rapid efflux of Na^+ from the cells. This is in contrast to a much slower process of the accumulation and degradation of a compatible solute glycine betaine due to salt upshock and downshock *A. halophytica*. Glycine betaine was found to activate the Na^+/H^+ antiporter activity of *A. halophytica*. At present the mechanism by which glycine betaine activates or inhibits the Na^+/H^+ antiporter is not known but may be ascribed to its stabilization or destabilization effect as seen in many proteins. It should be noted that *A. halophytica*, but not *Synechocystis* sp. PCC 6803, accumulates glycine betaine intracellularly upon salt upshock. Since the enzymes involved for the synthesis of glycine betaine seems to have different properties in different organisms (Incharoensakdi et al., 2000, Hibino et al. 2001), it might be interesting to characterize the betaine synthesis enzymes in *A. halophytica*.

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