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Molecular biology of Na^+/H^+ antiporters: molecular devices that couple the Na^+ and H^+ circulation in cells

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1. Introduction

Circulation of H^+ and Na^+ ions is maintained across the cytoplasmic membrane of bacteria, animal and plant cells, and the membranes of various subcellular organelles. The coupling between these ion currents is catalyzed by Na^+/H^+ antiporters.

In most bacteria and plant cells (Reviews in 1–3), the Na^+/H^+ antiporters are involved in the pH-homeostasis of the cytoplasm as well as in the process of expelling Na^+, which is toxic to the cytoplasm as compared to K^+, the other abundant ion. In addition, Na^+-excretion is essential in bacteria for maintaining an inwardly-directed Na^+-gradient which serves as a driving force for many transport systems (reviews in Refs. [4] and [5]). In animal cells, the Na^+/H^+ antiporters are essential for pH-homeostasis and regulation of cell volume [6–8]. The physiological roles of the Na^+ and H^+ cycles share common properties in various cells but they also exhibit unique specializations related to the particular environment and metabolism of the organism [2].

In the present review we will focus on the Na^+/H^+ antiporters of *Escherichia coli* [1,2,4,10] as a paradigm for a coupling device of Na^+ and H^+ circulation across the cytoplasmic membrane.

2. Molecular biology of the Na^+/H^+ antiporters in E. coli

Escherichia coli has two specific Na^+/H^+ antiporter systems — *nha*A [11,12] and *nha*B [13], mapping at 0.3 min and 25.6 min, respectively. The deduced amino acid sequence of the respective genes shows that both proteins have extensive α-helical structures repetitively spanning the membrane as expected for transport proteins. When either of the genes is in a high copy number, on plasmids, the Na^+/H^+ (but not K^+/H^+ or Ca^{++}/H^+) antiporter activity dramatically increases.

Both the NhaA [14] and the NhaB (Pinner, E., Padan, E. and Schuldiner, S., unpublished results) proteins were purified in a functional form. This endeavor involved the identification of the proteins, over-expression, development of solubilization and purification procedures, and finally reconstitution of the purified proteins in proteoliposomes to test their activity. The purified proteins were found to be fully active. This achievement was crucial since it proved that *nha*A and *nha*B are structural genes and their product, a single polypeptide, is indeed an antiporter. Furthermore, since these are the first active Na^+/H^+ antiporters to be isolated, it allowed us to study aspects of the antiporter mechanism which could not easily or unequivocally be studied in the intact cells or membrane vesicles. A long-debated issue is the antiporter stoi-

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chiometry. With the purified system, it was found that both *nha*A and *nha*B are electrogenic and that the stoichiometry of NhaA is two protons for every Na^+ [15].

Another aspect, which bears upon the possible role of the antiporters in pH homeostasis, is the effect of pH on the activity of the antiporter. Working with isolated membrane vesicles, Leblanc and his colleagues [16] suggested that the Na^+/H^+ antiporter protein is highly sensitive to pH. Indeed, the purified NhaA protein was found to be sensitive to pH, changing its activity by three orders of magnitude over the pH-range from 7 to 9. These results imply that *nha*A can function simultaneously as a pH-sensor and a pH-titrator (Section 5).

In three aspects NhaB differs from NhaA. Its ion specificity is different, having a higher affinity to Na^+, but a lesser affinity to Li^+. It is sensitive to amiloride, an inhibitor of the eukaryotic antiporter, while NhaA is insensitive. Furthermore, whereas the activity of NhaA is dramatically affected by pH, NhaB is indifferent to it.

It is thus apparent that two very different antiporters exist in the cytoplasmic membrane of *E. coli* raising questions as to what is their differential role, and whether additional antiporters participate in the Na^+/H^+ antiporter activity of the cells. In order to answer these questions, *E. coli* mutants were constructed, *Δnha*A [17], *Δnha*B [34], or *Δnha*A*Δnha*B [34]. A comparison of the various mutants with the wild-type showed that NhaA is essential for adaptation to high salinity, growth at alkaline pH in the presence of Na^+ and for challenging Li^+ toxicity. NhaB confers low Na^+ tolerance and becomes essential when the activity of NhaA limits the growth [17,34]. As long as Na^+ is withheld from the medium, both genes are dispensable (Section 5).

Isolated membrane vesicles derived from the double mutant *Δnha*A*Δnha*B revealed no Na^+/H^+ antiporter activity. It can therefore be concluded that NhaA and NhaB are the exclusive specific Na^+/H^+ antiporters in the membrane of *E. coli*, except for the possible existence of silent antiporters.

3. A novel signal transduction to Na^+ regulates expression of the nhaA Na^+/H^+ antiporter

Construction of a strain containing *nha*A fused in frame to *lac*Z and monitoring β-galactosidase activity under various growth conditions showed that the extracellular signals stimulating the expression of *nha*A are Na^+ and Li^+ and that, although pH alone does not activate the system, it markedly affects the sensitivity of the expression system to Na^+ [18].

The inducibility of *nha*A implies that a regulatory protein or proteins of *nha*A must exist. *nha*R is located downstream of *nha*A [19]. Its deletion from the chromosome yields cells that, in spite of having *nha*A, are sensitive to Li^+ and Na^+. Furthermore, multicopy *nha*R increases expression of the *nha*A'-'*lac*Z fusion, and this increase is completely Na^+-dependent [19]. These results indicate that NhaR is a positive regulator of *nha*A, which works in *trans* and its effect is Na^+-dependent. Accordingly, partially purified NhaR protein binds specifically to the promoter region of *nha*A and retards its mobility in a gel retardation system [19].

Most interestingly, on the basis of protein homology NhaR belongs to a large family of positive regulatory proteins called the LysR family which contains in their N-terminal a helix-turn-helix motif that is believed to bind DNA [19,50]. Most importantly, several of these proteins are part of a signal transduction pathway involved in response to environmental stress [51]. We thus conclude that NhaR is part of a signal transduction which is essential for challenging Na^+ and pH stress.

4. Regulation of transcription of nhaA, the 'Na sensor'

The change in extracellular Na^+-concentration, must reach NhaR, since the effect of NhaR on *nha*A expression is Na^+-dependent. It is conceivable that a change in intracellular Na^+ which accompanies the change of the extracellular concentration of the ion, serves as the immediate signal for the NhaR-dependent expression of *nha*A. If intracellular Na^+ is indeed the immediate signal, then any means which decreases or increases intracelluar Na^+ should change the expression. The transformation of the fusion bearing strain with multicopy plasmids bearing either *nha*A or *nha*B inhibits the induction of *nha*A caused by Na^+ [4]. Since these genes share very little homology but common activity, Na^+ extrusion, we suggest that an increase in the intracellular Na^+ serves as the immediate on-signal for the NhaR dependent *nha*A expression. Accordingly, *nha*A-*lac*Z is fully expressed at a lower Na^+ concentration (10 mM, pH 7.5) in a *Δnha*A*Δnha*B mutant than in a *Δnha*A mutant (100 mM, pH 7.5) (Pinner, E., Padan, E., and Schuldiner, S., unpublished results).

Since the induction of *nha*A by Na^+ occurs via NhaR, it is conceivable that a 'Na^+ sensor'-site exists on NhaR which can be identified by mutations affecting the Na^+ sensitivity of the expression system. Should such a mutation increase the affinity to Na^+ of the expression system, then at a given Na^+ concentration, it may even increase the Na^+/H^+ antiporter activity in the membrane above the wild type level. A previously isolated mutation, designated *ant*up, was found to increase the Na^+/H^+ antiporter activity, thereby conferring Li^+ resistance upon wild type cells which are

otherwise Li^+-sensitive (Nha^{up} phenotype), [11,20]. We recently found that the Nha^{up} mutation resides in the C-terminus of NhaR and is Glu-134 to Ala-134 substitution in the protein [21]. This mutation increases the affinity to Na^+ of the *nha*R mediated *nha*A transcription. Although Na^+ may indirectly affect NhaR, it is compelling to speculate that Glu-134 is part of the Na^+ sensor of NhaR.

5. Regulation of NhaA activity, the 'pH sensor'

There is a strong pH homeostasis in *E. coli* as in other bacteria [22–26] and the Na^+/H^+ antiporters have been assigned an essential role in this homeostasis. It is therefore surprising that *Δnha*A*Δnha*B grows like the wild type as long as Na^+ is withheld. Hence, if homeostasis still exists, in this mutant, another system must be responsible for it.

Interestingly, the tolerance to Na^+ is linked very closely to pH. The sensitivity of *Δnha*A to Na^+ dramatically increases with the pH implying that the dependence of growth on *nha*A increases at alkaline pH. Accordingly, the inducibility of *nha*A increases with pH and its physiological importance is more pronounced at alkaline pH.

There are two possibilities to explain the increasing physiological importance of *nha*A with pH as observed with *Δnha*A [17]: given that *nha*A is involved only in excreting Na^+, pH homeostasis is not required at alkaline pH, Na^+-toxicity increases with pH, *nha*A is needed to alleviate the increased Na^+ toxicity. This increased toxicity at alkaline pH can be based on the interchangeability of Na^+ and H^+ shown in various systems (review in Ref. [4]). The other alternative is that *nha*A is involved both in the regulation of pH and Na^+, the pH homeostasis is required and an increased Na^+ load interferes with it. In both cases, NhaA must sense pH and its activity must increase with pH.

As mentioned above, the activity of purified NhaA is markedly dependent on pH [14], implying that NhaA is equiped with a 'pH sensor'. It is anticipated that a residue, which senses pH, has an ionizable H^+ with a pK at the sensing range. Histidine, having a pK at around pH 6.0, is a very good candidate. A histidine-specific modifier DEPC (diethyl pyrocarbonate) inhibits Na^+/H^+ antiporter activity in isolated membrane vesicles [27] and in proteoliposomes reconstituted with purified NhaA [15]. Furthermore, histidine 322 of the Lac permease has been implied to participate in coupling transport of lactose to the proton gradient [28,29].

NhaA has 8 histidines, each of which has been mutagenized by site-directed mutagenesis to arginine [30]. Seven of the mutants behaved exactly like the wild type, both at neutral and alkaline pH. However, the substitution of histidine 226 to arginine dramatically affected the phenotype. The pH profile of Arg-226 is shifted by half a pH unit towards the acidic range. Furthermore, we have recently changed this His-226 to aspartate and found that the pH profile of NhaA is shifted by about half a pH unit towards the basic range. It is therefore suggested that His-226 resides in the pH sensing domain of NhaA.

Revertants and second-site revertants of Arg-226 further substantiate the importance of His-226. They were selected after chemical mutagenesis of the plasmid bearing the H226R mutation, transformation into *Δnha*A*Δnha*B strain and growth of the transformants at alkaline pH in the presence of Na^+. These conditions are non-permissive unless H226R is reversed to H226 or second-site reversion occurs. Out of 37 independent isolates obtained 23 are true revertants to His-226 and the rest are second site revertants which will enable further identification of the pH-sensing domain (Rimon, A., Gerchman, Y., Schuldiner, S., and Padan, E., Unpublished results).

6. Cloning of new antiporters in search for common denominators among the antiporters

Cloning of novel antiporter genes is required to identify conserved sequences in this family of proteins. Two strategies for cloning of Na^+/H^+ antipoter genes have been advanced. Each based on one of the two substrates, Li^+ and Na^+, of all Na^+/H^+ antiporters [9,10].

On a concentration basis, Li^+ is 10-times more toxic than Na^+, both to wild-type *E. coli* [11] and fission yeast [31]. Therefore, Li^+ provides a screen for cells capable of maintaining low internal Na^+ or Li^+ levels without selecting for osmotolerance. Another advantage of Li^+ selection over that of Na^+ is that it can be applied directly to wild-type cells. Realizing this advantage of Li^+ selection, a mutant *nha*A^{up} which confers Li^+ resistance and increases the Na^+/H^+ antiporter activity of *E. coli* cells was isolated [20]. As described above (Section 4) this mutation is E134A substitution in NhaR, the positive regulator of *nha*A, which increases Na^+/H^+ activity by increasing the affinity to Na^+ of the NhaR system [21]. Mapping this mutation, utilizing the Li^+ selection and assuming that multicopy antiporter gene increases antiporter activity, we cloned *nha*A out of a multicopy plasmid DNA library containing sequences overlapping the *nha*A locus [11]. In a similar approach *sod*2 antiporter has been cloned from *Schizosaccharomyces pombe* [31].

Although wild type *E. coli* cells transformed with multicopy plasmids bearing *nha*A become Li^+ resistant as compared to the wild type their tolerance to Na^+ is unchanged. This implies that other factors, such

as adaptation to increased osmolarity, determine the upper level of resistance to Na^+. It also implies that, in contrast to wild-type cells, mutants *ΔnhaA* or *ΔnhaAΔnhaB*, which are Na^+ sensitive due to the lack of the antiporters, are most suitable to apply Na^+ selection and clone by complementation DNA inserts encoding Na^+/H^+ antiporter genes. With this approach and the *E. coli* mutants, various antiporter genes have been cloned from very different bacteria including *nhaB* from *E coli* [13] and *nhaC* from an alkaliphile *Bacillus firmus* OF4 [45]. A similar approach applied to *Enterococcus hirae* yielded *napA* antiporter [33].

7. Other systems conferring Na^+ resistance in *E. coli*

The Na^+ sensitivity revealed in the antiporter mutants (*ΔnhaA* or *ΔnhaAΔnhaB*) is consistent with the role assigned to the antiporters in Na^+ excretion from bacterial cells [17,34]. It can be anticipated that, unless other Na^+ expelling machinery exists in the absence of the antiporters, Na^+ will accumulate in the cells to growth inhibitory levels [4]. Primary Na^+ pumps were purified from various bacteria and have been shown to be involved in Na^+ excretion [35–39]. Furthermore, based on studies with intact cells [40–42] and isolated membranes vesicles [43,44], primary Na^+ pumps have also been implicated to exist in *E. coli*. However, the presence of the Na^+/H^+ antiporters in these preparations complicates the interpretation of these results.

Since the *ΔnhaAΔnhaB* mutant does not have the specific Na^+/H^+ antiporters it affords two approaches to search for the existence of Na^+ excreting machinery, other than the Na^+/H^+ antiporters. Given that there exist antiporter systems or pumps with very low affinity and/or V_{max} to Na^+, amplification of these systems on multicopy plasmid may confer some Na^+ resistance to *ΔnhaAΔnhaB*. Indeed, with this approach *chaA* has been cloned [32]. On the basis of its homology to calsequestrin and its promotion of Ca^{2+}/H^+ exchange activity in membranes, *chaA* appears to be a Ca^{2+}/H^+ antiporter with low affinity to Na^+.

The Na^+ sensitivity of the *ΔnhaAΔnhaB* strain provides yet another approach to look for systems conferring Na^+ resistance. It affords a powerful selection (growth on Na^+) of second site revertants resistant to Na^+. In this way we have recently isolated MH1, a second site revertant of *ΔnhaAΔnhaB*. It bears a mutation at around 27 min on the *E. coli* chromosome which confers Na^+ but not Li^+ resistance and restores partially the Na^+ excretion capacity in the absence of the Na^+/H^+ antiporters (Harel, M; Dibrov, P; Olami, Y; Pinner, E; Schuldiner, S; and Padan, E., unpublished results).

8. Concluding remarks

The Na^+/H^+ antiporters are widely distributed and are involved in homeostasis of pH and/or Na^+ in all cells. Accordingly, the human antiporter, Nhe1, also has a sensor but it functions as a mirror image of NhaA, reflecting the different pH ranges challenging the respective cells [46–48]. It is most intriguing to identify the amino acid residues in this sensor.

Can we find common molecular denominators in the Na^+/H^+ antiporter family? Interestingly, there is a sequence conservation among the Nhe family [49], but there is very little homology between it and NhaA, and even between NhaA and NhaB [1,2]. Do these results suggest that there is no universal Na^+ and H^+ recognition and exchange sites, and that instead, different sequences accomplish similar functions? Another possibility is that very few conserved residues are adequate to carry out these functions, even when they are dispersed throughout the protein. Identification of the domains neccesary for activity regulation and H^+- and Na^+-sensing is certainly one of the future challenges.

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