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Review

Na^+/H^+ antiporters

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Abstract

Na^+/H^+ antiporters are membrane proteins that play a major role in pH and Na^+ homeostasis of cells throughout the biological kingdom, from bacteria to humans and higher plants. The emerging genomic sequence projects already have started to reveal that the Na^+/H^+ antiporters cluster in several families. Structure and function studies of a purified antiporter protein have as yet been conducted mainly with NhaA, the key Na^+/H^+ antiporter of *Escherichia coli*. This antiporter has been overexpressed, purified and reconstituted in a functional form in proteoliposomes. It has recently been crystallized in both 3D as well as 2D crystals. The NhaA 2D crystals were analyzed by cryoelectron microscopy and a density map at 4 Å resolution was obtained and a 3D map was reconstructed. NhaA is shown to exist in the 2D crystals as a dimer of monomers each composed of 12 transmembrane segments with an asymmetric helix packing. This is the first insight into the structure of a polytopic membrane protein. Many Na^+/H^+ antiporters are characterized by very dramatic sensitivity to pH, a property that corroborates their role in pH homeostasis. The molecular mechanism underlying this pH sensitivity has been studied in NhaA. Amino acid residues involved in the pH response have been identified. Conformational changes transducing the pH change into a change in activity were found in loop VIII–IX and at the N-terminus by probing trypsin digestion or binding of a specific monoclonal antibody respectively. Regulation by pH of the eukaryotic Na^+/H^+ antiporters involves an intricate signal transduction pathway (recently reviewed by Yun et al., Am. J. Physiol. 269 (1995) G1–G11). The transcription of NhaA has been shown to be regulated by a novel Na^+-specific regulatory network. It is envisaged that interdisciplinary approaches combining structure, molecular and cell biology as well as genomics should be applied in the future to the study of this important group of transporters.

Keywords: Sodium ion/proton antiporter; Active transport; Membrane protein; Homeostasis of Na^+ and H^+

1. Introduction

Na^+ and H^+ are the most common ions and they play primary roles in cell physiology: both are most important in cell bioenergetics and the concentration of protons within the cell is critical to the functioning of the cell and its proteins. Indeed, when the concentration of these ions becomes too high or too low they turn into potent stressors to all cells [1]. Hence, every cell has a very efficient homeostatic mechanism for these ions.

Proteins that play a primary role in this homeostatic mechanism are the Na^+/H^+ antiporters. These are membrane proteins that exchange Na^+ (or Li^+) for H^+. They were discovered by P. Mitchell and

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colleagues [2] and since then, as expected from their central role in cell physiology, were found to be widely spread. With only one known exception [3] they exist in the cytoplasmic membranes of all cells from bacteria to man and higher plants and in the membranes of many eukaryotic organelles [1,4–6].

Being involved in homeostasis implies that the Na^+/H^+ antiporters share unique properties in addition to their capacity to exchange the ions across the membrane: they must be equipped with sensors and transducers so as to be able to 'sense' the ion concentration of the environment and respond by modulating their activity to maintain homeostasis.

This review analyzes in molecular terms the unique properties of the Na^+/H^+ antiporters. Since NhaA, the key Na^+/H^+ antiporter of *Escherichia coli* and many other enterobacteria, has been most extensively studied, it serves as a model system for this analysis.

2. NhaA, the key Na^+/H^+ antiporter of *E. coli* and many other enterobacteria

Two genes encoding Na^+- and Li^+-specific Na^+/H^+ antiporters were identified in *E. coli*. These genes, *nha*A [7] and *nha*B [8], were cloned and deleted from the chromosome each separately and both together [9,10]. The strain deleted of *nha*A (NM81) showed that *nha*A is the main antiporter which is required to withstand the upper limit concentration of Na^+ for growth (0.9 M, pH 7.0) and to tolerate the upper pH limit for growth in the presence of Na^+ (0.7 M, pH 8.5). The strain deleted of *nha*B (EP431) showed that *nha*B is the housekeeping gene which becomes essential only when *nha*A is absent. The strain lacking both *nha*A and *nha*B (EP432) is therefore the most Na^+-sensitive strain and has been extensively used to clone by functional complementation and to express many Na^+/H^+ antiporter genes [4]. Recently, the genome project that made available the DNA sequences of genomes of many bacteria has revealed new *nha*A homologues including ones in most important pathogenic bacteria such as *Helicobacter pylori* [11], *Vibrio cholerae* [12], and *Vibrio parahaemolyticus* [13] (Fig. 1). Several of these genes were cloned and proven to encode a Na^+/H^+ antiporter by their functional expression in EP432 of *E. coli* or similar mutants [14,15] (Fig. 1). The genomics approach to identify the genes combined with the functional expression in antiporter-lacking mutants is envisaged as a most powerful tool to identify and study novel Na^+/H^+ antiporters. However, deletion of the gene from the original bacterial genome is an essential step to evaluate the role of the antiporter in its organism. This is as yet lacking for many of the new NhaA homologues.

3. Regulation of transcription

3.1. The response of *nhaA* to Na^+, an example of Na^+-specific signal transduction and regulation

Experiments with *nha*A′–′*lac*Z protein fusions [16,17] and Northern analysis [6,18] showed that the environmental signals which turn on *nha*A are $[Na^+]$ or $[Li^+]$ increases in the medium. Alkaline pH potentiates the effect of the ions but an increase in neither osmolarity nor ionic strength induces *nha*A. These results demonstrated for the first time that *E. coli* has a unique regulatory network responding specifically to Na^+ (and Li^+). Interestingly, a similar role has recently been assigned to Na^+ in the regulation of expression of the Na^+/ATPase of *Enterococcus hirae* [19].

To determine whether a change in $[Na^+]_{in}$ rather than $[Na^+]_{out}$ is the immediate signal, $[Na^+]_{in}$ was changed in the *nha*A′–′*lac*Z strain while maintaining $[Na^+]_{out}$ constant. An increase in $[Na^+]_{in}$, achieved by deleting both antiporter genes, *nha*A and *nha*B, led to a high expression of *nha*A′–′*lac*Z [18]. A decrease in $[Na^+]_{in}$, obtained by introducing *nha*A on a multicopy plasmid, led to a drastic reduction in *nha*A expression [17]. These results imply that $[Na^+]_{in}$ is the direct signal for induction of *nha*A. The relative contributions of various possible Na^+ entry routes into the cells have not yet been determined.

3.2. The NhaR regulator

A regulator of *nha*A is encoded by *nha*R which maps downstream of *nha*A [20]. A *nha*R deletion strain is hypersensitive to Na^+ and Li^+ in spite of the presence of *nha*A [17]; NhaR is a positive regulator which works *in trans*, since *nha*R supplied on a multicopy plasmid dramatically increases expression

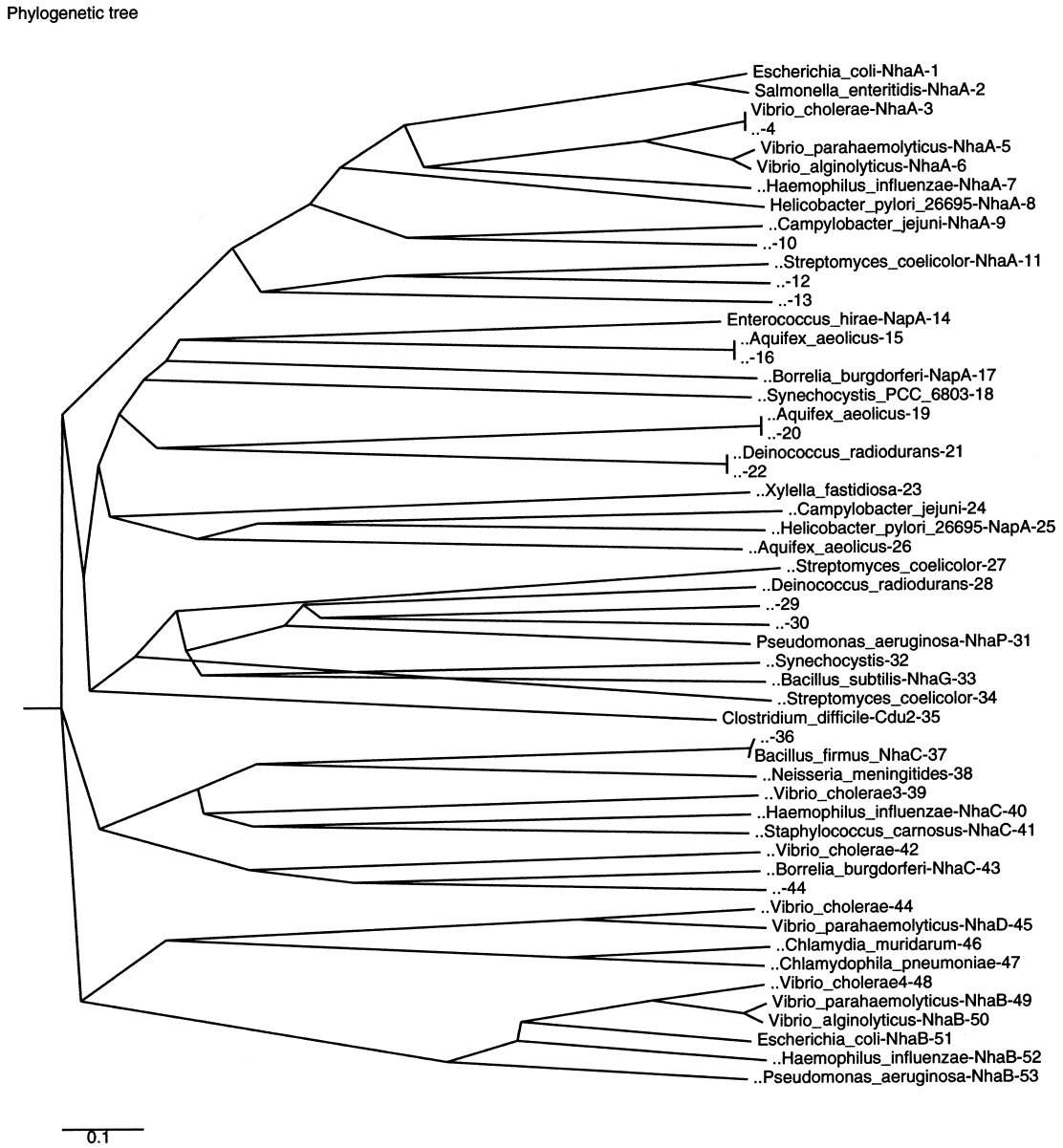

Fig. 1. Prokaryotic Na^+/H^+ antiporters, phylogenetic relationship. Phylogenetic relationships were analyzed with the CLUSTAL W multiple sequence alignment algorithm ([76], http:www.ebi.ac.uk/CLUSTALW). The tree was drawn by Phylodendron, D.G. Gilbert (http://iubio.bio.indiana.edu/treeapp7treeprint-form.html). When neither phenotype nor antiporter activity has been documented the strain name is preceded by a double dot. The accession numbers refer to the strains shown on the tree by arbitrary numbers. (1) P13738; (2) 1817175A; (3) AAC33562; (4)AAF94778; (5) JX0360; (6) BAA04944; (7) P44581; (8) H64713; (9) CAB73643; (10) CAB73642; (11) T36685; (12) T37078; (13) T36473; (14) A42111; (15) E70380; (16) AAC07034; (17) AAC66821; (18) S74951: (19) C70474; (20) AAC07780; (21) G75431; (22) AAF10721; (23) AAF84207; (24) CAB73670; (25) AAD08229; (26) AAC06696; (27) CAC05973; (28) H75278; (29) AAF12552; (30) AAF10227; (31) BAA31695; (32) BAA18490; (33) BAA89487; (34) CAB90969; 35) JC5342; (36) A41594; (37) AAC45432; (38) AAF40965; (39) AAF95185; (40) AAC22762; (41) AAB94653; (42) AAF96106; (43)E70179; (44) D70179; (45) AAF96911; (46) BAA25994; (47) AAF39116; (48) AAF38631; (49) AAF95049; (50) JC4814; (51) BAA12086; (52) BAA36041; (53) AAC22086; (54) AAG05209.

of the *nha*A′–′*lac*Z protein fusion [17]. The protein is homologous to the LysR family of positive regulators which are involved in the response of bacteria to various environmental stresses [17,21–23].

To purify NhaR a His-tagged derivative of NhaR (His-tagged NhaR) was constructed [24]. The His-tagged protein, which was found to be as active as the wild-type protein in vivo, was purified to homogeneity on a Ni^{2+}-NTA affinity column. Separation by gel filtration on a HPLC column showed one homogeneous peak at 72.5 kDa suggesting that the 36.2 kDa His-tagged NhaR protein exists as a dimer in solution [24].

3.3. The cognate binding site and the Na^+-specific footprint of NhaR on nhaA

A genetic approach undertaken previously [25] showed that NhaR may have a Na^+ 'sensor' activity; E134A, a point mutation in *nha*R, increased the affinity of the regulator to Na^+ and conferred resistance to Li^+. These results suggested that the interaction between Na^+, NhaR, and the target regulatory sequences of *nha*A is direct. Hence, a biochemical approach was undertaken to study this interaction using purified components.

DNA mobility shift and DNase I protection assays showed that both wild-type NhaR [17] and its purified His-tagged derivative [24] bind specifically to DNA sequences overlapping the promoter region of *nha*A [24]. The smallest fragment, overlapping all binding sequences of *nha*A, was located between bp −120 and +14 (of the *nha*A initiation codon). It contains three copies of the LysR consensus motif (T-N_{11}-A) [26–28]. Na^+ had no effect on the gel retardation patterns suggesting that Na^+ causes a change in the footprint rather than a change in the affinity. This behavior is characteristic to the LysR family of regulators [27,28].

[Na^+] up to 100 mM had no effect on the DNase I footprint. This negative result, however, was found to be related to the limitations of the DNase I protection assay employed, i.e. it is not sensitive and is limited to the minor groove of the DNA [29]. Thus when dimethyl sulfate (DMS) methylation protection footprint analysis was used, both in vitro and in vivo experiments revealed a specific effect of Na^+ [24]. Whereas the NhaR-dependent protection of bases −24, −29 and −92 observed in vitro was not affected by either 100 mM K^+ or 100 mM Na^+, protection of base −60 was differentially affected by the two ions. Na^+ but not K^+ specifically exposed this base to methylation. The concentration of Na^+ affecting the footprint was found to be within the range expected for the intracellular concentration (10–20 mM). Remarkably in line with the finding that the expression is potentiated in vivo by alkaline pH [16], the Na^+ interaction with base −60, but not with either bases −24 or −29, was dramatically affected by pH. At alkaline pH (7.5–8.5), but not at acidic pH (6.5), Na^+ affected the NhaR/*nha*A interaction at base −60 [24].

DMS methylation protection assays were also conducted in vivo. As expected from the in vitro results bases −24 and −29 were protected but not affected by the type of ion; base −60 was protected in the presence of K^+ but not in the presence of Na^+. Most interestingly, base −92, which in vitro was protected by the regulator but unaffected by the presence of Na^+, in vivo was exposed to methylation by Na^+ but not by K^+. The Na^+-specific effects on the footprint of NhaR on *nha*A required NhaR since they were not observed in a Δ*nha*R strain [24]. Taken together there data suggest that NhaR is both the sensor and the transducer of the Na^+ signal and undergoes a conformational change upon binding Na^+. The G^{-92}/NhaR interaction observed only in vivo suggests an involvement of another factor(s) in the regulation of *nha*A [24]. A model describing the Na^+-specific NhaR-dependent regulation of transcription of *nha*A is shown in Fig. 2.

3.4. Global regulation of nhaA, the involvement of H-NS

Recently, several global regulators have been shown to have an important impact on the expression of many unlinked genes. We have established a connection between the Na^+-specific, NhaR-dependent regulation of *nha*A and H-NS, a DNA binding protein and a global regulator [18,24]. Thus the expression of *nha*A′–′*lac*Z was derepressed in strains bearing an *hns* mutation, and transformation with a low-copy-number plasmid carrying hns^+ repressed expression and restored Na^+ induction. The derepression in *hns* strains was *nha*R-independent. Most

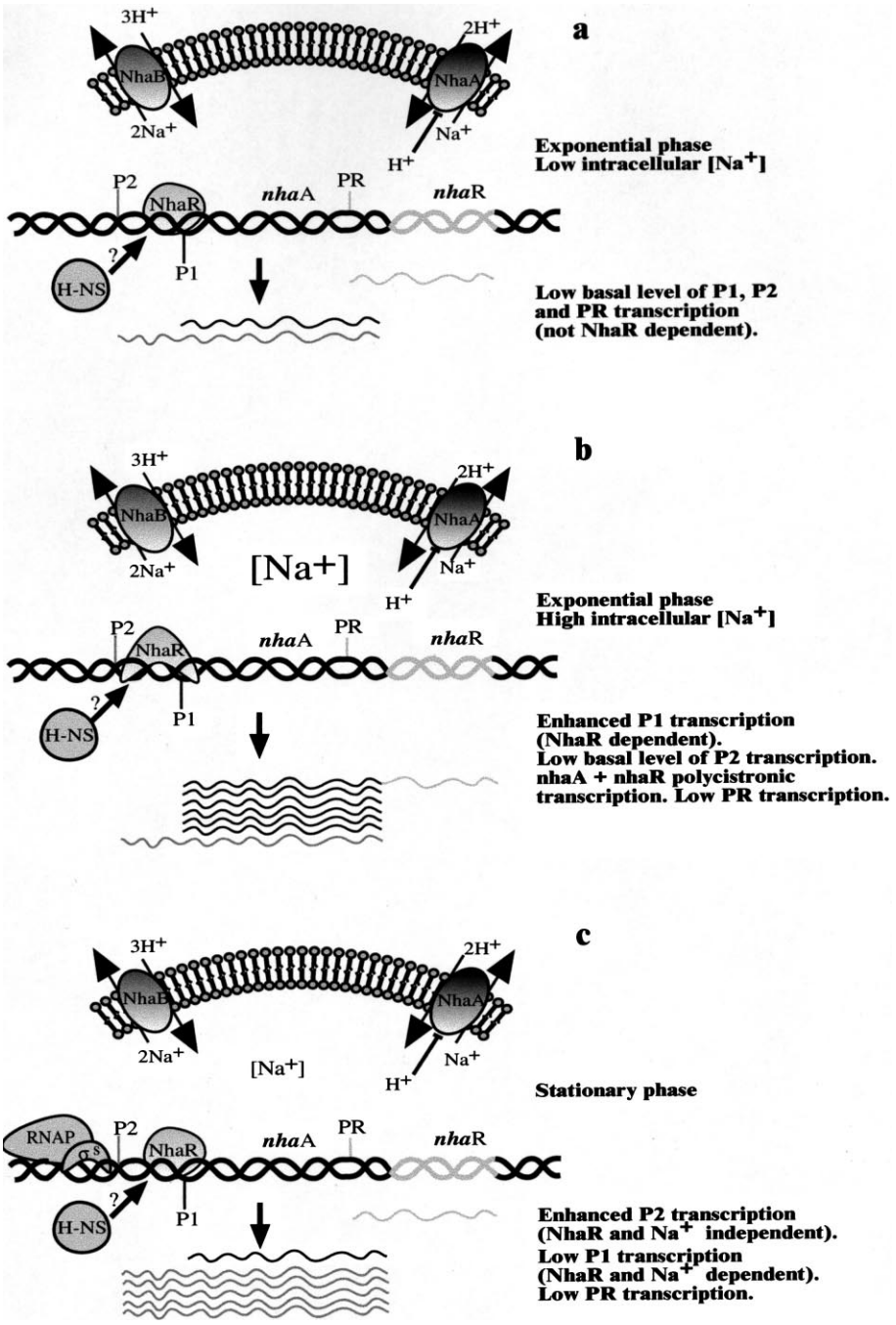

Fig. 2. *nha*A is regulated by Na^+ and growth phase via separate promoters.

interestingly, multicopy *nha*R, which in an *hns*$^+$ background acted only as a Na^+-dependent positive regulator, acted as a repressor in an *hns* strain in the absence of Na^+, but was activated in the presence of the ion. Hence an interplay between *nha*R, Na^+ and *hns* in the regulation of *nha*A was suggested (Fig. 2). Although the mechanism of regulation mediated by H-NS is not known it has been suggested to involve a change in the topology of the DNA [30].

3.5. *P1 is the Na^+-specific promoter of nhaA which is induced by NhaR and repressed by H-NS*

Two promoters, P1 and P2, located 31 bp and 172

bp upstream of the initiation codon of *nha*A respectively, have been identified previously [16]. Surprisingly, the binding region of NhaR on *nha*A contains only P1. Therefore it has become crucial to establish which is the Na^+-dependent promoter of *nha*A. Primer extension assays (N. Dover, E. Padan, unpublished results) conducted on RNA isolated from cells grown in the absence of Na^+ show a weak transcript band of a size expected from the P1 promoter which is accompanied by a transcript 8 bp shorter [1]. The latter is most probably due to premature fall-off of the reverse transcriptase. However, the possible involvement of another promoter cannot yet be ruled out. The level of both transcripts was drastically increased (more than 10-fold) when the assay was conducted on RNA isolated from Na^+-induced cells. On the other hand, the transcript corresponding to P2 was hardly detected and it was not affected by the presence of Na^+. These results suggest that P1 is the Na^+-specific promoter of *nha*A.

3.6. P2 and the role of NhaA in the stationary phase

It is well established today that when entering the stationary phase the bacteria become more resistant to various stress conditions [31], including high Na^+ [32]. We therefore recently studied (N. Dover, E. Padan, unpublished results) the pattern of *nha*A transcription in the stationary phase. In marked contrast to P1, P2 is induced in the stationary phase to become the major promoter of *nha*A. Furthermore while P1 is activated by σ^{70}, P2 is activated by σ^s and is neither induced by Na^+ nor dependent on NhaR or affected by H-NS. Most importantly, P2 via σ^s was found to be responsible for the survival of the cells in the stationary phase in the presence of high Na^+, alkaline pH, and the combination of high Na^+ and alkaline pH, the most stressful condition. These results strongly suggest that in addition to its role during exponential growth, NhaA is of paramount importance for the survival of *E. coli* in a stationary phase mode in its natural habitats such as the sea. A model describing the regulation of NhaA in the stationary phase is shown in Fig. 2.

These results should intrigue the study of Na^+/pH regulation of *nha*A homologues and their role in the life cycle and virulence of pathogenic enterobacteria. Such a study has recently been initiated with *nha*A of *V. cholerae* (*nha*A-V_C). Using *nha*A′–′*lac*Z transcription fusion Na^+ and Li^+ induction was observed [12]. A *nha*R homologue was also found in *V. cholerae* [33]. However, unlike *E. coli*, the growth of the *nha*A-V_C-inactivated mutant of *V. cholerae* was not affected at various pHs and Na^+ concentrations [12]. Nevertheless, as in *E. coli*, the gene may be crucial for survival of *V. cholerae* in the stationary phase under conditions prevailing in its natural estuarine habitat, a harsh environment in terms of pH and Na^+ concentration [12].

4. Structure of the NhaA protein

4.1. Expression and purification

NhaA was the first Na^+/H^+ antiporter that was purified and reconstituted in a functional form in proteoliposomes [34]. Recently a very efficient expression system was constructed for NhaA [35]. In this system it is drastically overexpressed from the Tac promoter fused to six histidines (His tag) at its C-terminus. The His tag allows a very efficient purification on Ni^{2+}-NTA columns in amounts as high as 5 mg/l cells and as concentrated as 40 mg/ml. This protein is fully active when reconstituted into proteoliposomes. This high yield of purified active NhaA opened many avenues of research aiming at understanding the structure and function of NhaA.

4.2. Two-dimensional model of NhaA

A two-dimensional model of NhaA was predicted from the amino acid sequence of the protein and substantiated by various approaches, including *pho*A and *lac*Z fusions, mapping of antibody epitope, measurement of accessibility of the protein to proteases [36], and Cys accessibility scanning [35]. These studies showed that NhaA is similar to many other secondary transporters in being composed of 12 transmembrane segments (TMS) linked with hydrophilic loops (Fig. 3). Interestingly, two TMSs (VII and VIII) of NhaA appear shorter than the others (16 and 14 amino acids respectively) [36].

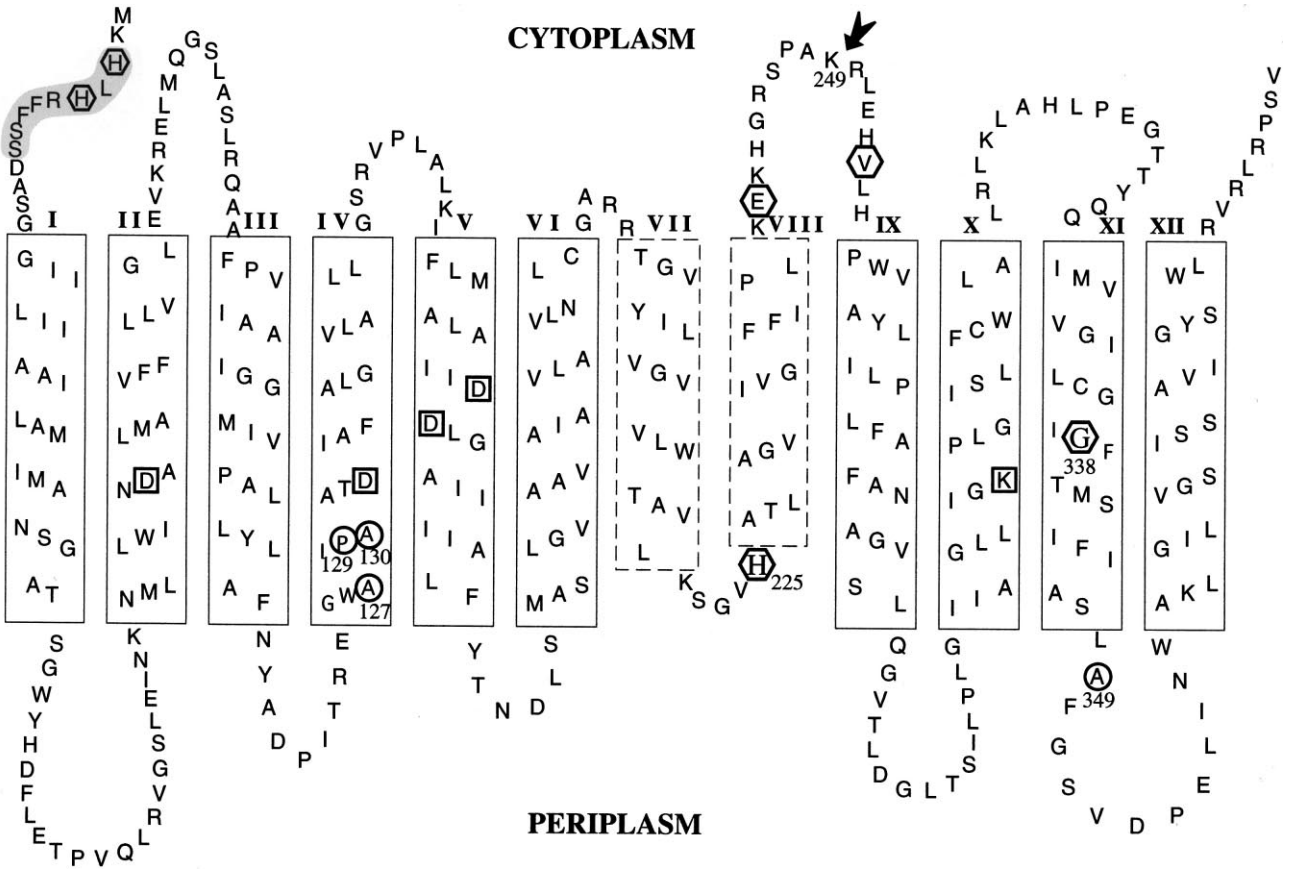

Fig. 3. Amino acid residues and domains that are involved in the activity of NhaA and/or its response to pH. In hexagons, residues important for the pH response. Encircled, residues identified by suppression mutations, to affect the pH response. In rectangles, residues involved in the transport activity. Arrow, the site of the pH-dependent split by trypsin. Gray background, the pH-dependent binding site of mAb 1F6.

4.3. Three-dimensional crystals of NhaA

The availability of highly concentrated purified NhaA opened the way to study directly the structure of NhaA. 3D crystals of NhaA were obtained. These are very reproducible, 1 mm long and 0.1 mm wide crystals that diffract X-rays to above 10 Å and therefore are not suitable for atomic structure analysis [37]. Two main reasons most probably account for this behavior: the antiporter exists in various conformations which introduce structural heterogeneity interfering with the production of a highly ordered crystal lattice, and the very limited hydrophilic domains of the proteins are not enough to form uniform and stable crystal contacts. To overcome these limitations, shared by many membrane proteins, H. Michel introduced the crystallization of membrane proteins in complex with F_V fragments of specific monoclonal antibodies (mAbs) [38]. The F_V fragment fixes one conformation and provides an extensive hydrophilic domain to form the crystal contacts.

We have already produced four anti-NhaA mAbs [39], three (2C5, 5H4, 6F9) recognize the native conformation since they are negative in Western blot analysis but bind to dodecyl-β-D-maltoside (DM)-solubilized NhaA. The fourth mAb (1F6) is positive in Western blot analysis but still binds native NhaA at alkaline pH. Except for mAb 6F9 all mAbs inhibit the antiporter activity in proteoliposomes.

The DNA encoding F_V fragment 2C5 has been cloned, overexpressed in *E. coli*, purified and used for co-crystallization of NhaA. New 3D crystal forms were obtained and these diffract at 7–8 Å resolution [37]. Although this is not sufficient to resolve the structure of NhaA, it should be emphasized that these are the first crystals of a transporter which

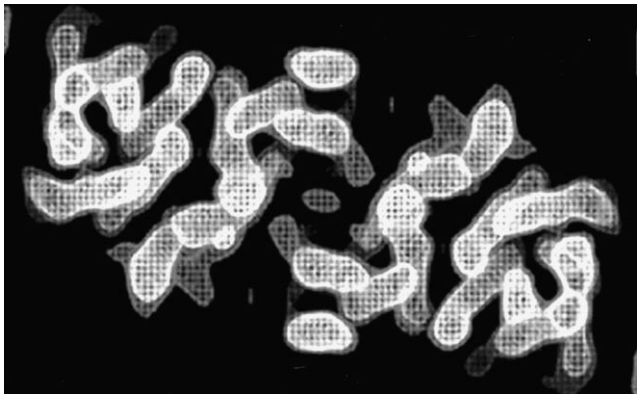

Fig. 4. The NhaA dimer. The 3D map of NhaA at 7 Å resolution viewed normal to the membrane plan. Twelve rod-shaped density features corresponding to transmembrane helices are seen in each monomer. The figure was kindly provided by Karen Williams [48,49] and Christine Ziegler, Max-Planck Institut für Biophysik, Frankfurt/Main, Germany).

diffract X-rays. Therefore these crystals both challenge the dogmatic suggestion that polytopic transporters are not amenable to crystallization and serve as a promising starting point to resolve the 3D structure of NhaA.

4.4. Structure of 2D crystals of NhaA

Electron crystallography has emerged as a powerful tool to determine the structure of membrane proteins from 2D crystals [40], including several close to atomic resolution [41–43]. 2D crystals of a number of secondary transporters have been obtained previously, including the membrane domain of human erythrocyte band 3 [44], full-length band 3 [45], homologously overexpressed *E. coli* melibiose permease [46], and lactose permease [47]. All of these studies were conducted in negative stain and as a result provide low-resolution insights into the overall size and shape of these transport proteins.

The 2D crystallization of NhaA was undertaken in an effort to obtain large, well-ordered crystals so that electron cryomicroscopy could be used to uncover internal structural details of this representative secondary transport protein. Large and ordered 2D crystals of NhaA were obtained [48]. These made it possible to apply electron cryomicroscopy and to obtain a projection map of NhaA at 4 Å resolution. The projection map reveals that NhaA has a highly asymmetric structure in projection with an overall dimension of 38×48 Å. Two crystal forms with $p22_12_1$ symmetry strongly suggest that NhaA in these 2D crystals is a dimer ([48] and Fig. 4). These crystals also made it possible to reconstruct a three-dimensional map of NhaA at 7 Å resolution in the membrane plane and at 14 Å vertical resolution [49]. This map reveals 12 tilted bilayer-spanning helices. A roughly linear arrangement of six helices is adjacent to a compact bundle of six helices. Hence these results substantiate the proposed 12 TMS model of NhaA. Furthermore, the molecular organization of NhaA represents a new membrane protein structural motif and offers the first insight into the architecture of an ion-coupled transport protein [49].

5. Function

As yet NhaA [34] and NhaB [8] are the only specific Na^+/H^+ antiporters which have been purified in a functional form. These studies opened the way to biochemical studies some of which have already been extensively summarized [5,50–52]. Therefore we will refer here only to new data and these were obtained with NhaA.

5.1. Aspartates involved in the binding of Na^+ by NhaA

Since the 3D structure of NhaA and its Na^+ binding site is not yet known the residues binding Na^+ can only be indirectly identified. One approach is site-directed mutagenesis of residues which have the chemical capacity to bind cations. The mutants are then analyzed for their ion transport properties such as ion specificity. Another approach is random mutagenesis, selecting phenotypes with a change in ion specificity and then identifying the amino acid residues involved. The importance of charged residues in transmembrane domains has been well documented in many membrane transporters. One important role is the participation in charge pairs, within and between helices. These are crucial for helix packing and maintenance of the native structure of the protein [53–55]. The existence of such pairs in transporters has been identified genetically on the basis of two criteria: (i) neutral replacement of one partner of the pair leads to transport arrest which is repaired

by a second neutral replacement of the remaining uncompensated charged partner; (ii) exchanging the location of the partners has no effect on activity or expression of the transporter.

The functional importance of charged residues located in hydrophobic transmembrane regions has been postulated for many ion-coupled transporters. Anions such as aspartate and glutamate have been implied or shown to bind cations in many cases (review in [56]), whereas positively charged amino acids were shown to bind anions [57].

According to the model proposed for the secondary structure of NhaA (Fig. 3 and [36]), six charged residues are located in putative transmembrane α-helices: four Asp residues in the N-terminal region (Asp-65, Asp-133, Asp-163 and Asp-164) and two Lys residues in the C-terminal region (Lys-300 and Lys-362). With the exception of Lys-362, which can be replaced with arginine, these residues are conserved in all members of the family of NhaA antiporters (Fig. 1).

Each of the four conserved aspartate residues in NhaA of *E. coli* [58] or *Vibrio alginolyticus* [59] has been replaced by asparagine in plasmid-encoded *nha*A, and the constructs expressed in an *E. coli* mutant devoid of both *nha*A and *nha*B. The findings have suggested that Asp-133, Asp-163, and Asp-164 are essential for the function of NhaA and appear to play a role in cation binding or transport. Alternatively, as discussed above, these residues might be structurally important and involved in charge pairs within the protein. We have therefore substituted with cysteine both positively and negatively charged residues which are conserved and present in putative transmembrane regions of NhaA (L. Galili, A. Rothman, S. Schuldiner, E. Padan, unpublished results). The effect of these mutations was studied on the growth of EP432 in high-NaCl or high-LiCl medium, and on the Na^+/H^+ and Li^+/H^+ antiporter activities of NhaA. We found that substitution of Asp-163, Asp-164, or Lys-300 for Cys impairs host growth in high-salt medium and is detrimental for both Na^+/H^+ and Li^+/H^+ antiporter activities. According to the criteria described above, none of these residues is involved in charge pairing. Thus these residues appear to be essential for the function of NhaA. Since they are located in the middle of TMS V (Fig. 3) it is conceivable that both anionic residues participate in the Na^+ binding site. In contrast, substitution of Asp-65, Asp-133, and Lys-362 for Cys does not impair host growth in high-salt medium or Na^+/H^+ and Li^+/H^+ antiporter activities. While Lys-362 does not appear to be important for the function of NhaA, Asp-65 and in particular Asp-133 change the ion specificity of the antiporter. Hence, though replaceable, we suggest that both residues Asp-65 and Asp-133 also affect the binding site of the cations.

Given that the 3D structure of NhaA has not been solved none of these suggestions can be proven; we cannot rule out the possibility that all these effects of both replaceable and non-replaceable residues occur by a conformational change even at a long distance from the cationic binding site.

5.2. The pH response of NhaA

In accordance with its role in pH homeostasis NhaA is highly sensitive to pH throughout the pH range in which it is essential [34,60,61]. It is shut off below pH 7 and increases its V_{max} over three orders of magnitudes up to pH 8.5. This high sensitivity to pH is exhibited by the pure NhaA reconstituted in proteoliposomes. It was therefore implied that amino acid residues in NhaA act as pH 'sensors', while others or the same ones transduce the pH signal into a conformational change leading to the dramatic change in the activity of NhaA.

5.2.1. Amino acid residues involved in the pH response of NhaA

A genetic approach has been undertaken to identify the residues involved in the NhaA response to pH. This involved site-directed mutagenesis of amino acids, such as histidine, with a pK corresponding to the apparent pK of the response [60,62]. With this approach His-225, which is conserved in all members of the NhaA family (Fig. 1), was found to play a primary role in the pH response. In addition it was demonstrated that the 'on' part of the pH response of NhaA is essential to withstand alkaline pH in the presence of Na^+. The mutation H225R caused an acidic shift in the pH response of NhaA by half a pH unit and shuts off its activity at pH 8.5. It therefore produced conditional lethal cells that grow at alkaline pH without Na^+ but not in the presence

of the ion. These conditional lethal mutants which are Na^+-sensitive at alkaline pH initiated the application of random mutagenesis to identify novel residues involved in the pH response of NhaA [62]. Following random mutagenesis of a plasmid encoding H225R-NhaA and plating the transformed cells on high pH in the presence of Na^+, revertants and suppressor mutations were sought. Only first-site reversions were obtained even though the H225R mutation was constructed with double mismatches to reduce the frequency of first-site reversion ([64] and Y. Gerchman, E. Padan, unpublished results). These results therefore emphasize the importance of residue His-225 in the pH response of the protein.

Random mutagenesis of the wild-type NhaA plasmid and selecting for cells with the H225R phenotype, i.e. conditional lethal phenotype at alkaline pH in the presence of Na^+, yielded a new mutation, G338S, which changed the pH response [63]. It rendered the protein insensitive to pH and very active throughout the pH range 6–8.5 and incapable of shutting off. Surprisingly, these cells also died at alkaline pH in the presence of Na^+ implying that in addition to the 'turning on' of NhaA the 'shutting off' is essential for growth at alkaline pH in the presence of Na^+. In this case the cause of death is most probably over-acidification of the cells.

This new phenotype of Na^+ conditional lethal at alkaline pH made it possible to select revertants or suppressor mutations by growing G338S at alkaline pH in the presence of Na^+. Several suppressor mutations were found and strikingly all shut off the activity at pH 6 and all cluster in helix IV (Fig. 3 and [63]).

5.2.2. *pH-dependent conformational change(s) involving loop VIII–IX*

NhaA undergoes a conformational change upon its activation by pH which can be probed by trypsin [65]. At acidic pH the protein in everted membrane vesicles is completely resistant to trypsin while at alkaline pH it is digested in a pattern reflecting the pH profile of the antiporter activity. Furthermore, two mutants with a modified pH profile are susceptible to trypsin in isolated membrane vesicles only at the pH range where they are active and reflecting the level of activity [65]; H225R, the mutant with a pH profile shifted toward acidic pH, is digested at the pH where it is active; G338S, which lost pH control, is active and exposed to trypsin throughout the entire pH range of activity [61].

Although NhaA has many trypsin-cleavable sites only two main fragments were observed following digestion of isolated membrane vesicles at alkaline pH [65]. This observation suggests that only one cleavage site is exposed by pH while all the other sites are masked. It was therefore inferred that the trypsin cleavage site is located inside and therefore serves as a tag of that part of the protein which undergoes a conformational change in response to pH.

Digestion with trypsin of purified His-tagged NhaA in a solution of DM yields two fragments at alkaline pH but only one fragment at acidic pH [61]. Determination of the amino acid sequence of the N-terminus of the cleavage products shows that the pH-sensitive cleavage site of NhaA, both in isolated everted membrane vesicles and in the pure protein in detergent, is Lys-249 in loop VIII–IX which connects transmembrane segments VIII and IX. Interestingly, the two polypeptide products of the split antiporter remain complexed and co-purify on Ni^{2+}-NTA column. Loop VIII–IX has also been found to play a role in the pH regulation of NhaA; three mutations introduced into it shift the pH profile of the Na^+/H^+ antiporter activity as measured in everted membrane vesicles [61]. Taken together these results imply the involvement of loop VIII–IX in the pH-induced conformational change which leads to activation of NhaA at alkaline pH.

Recently a NhaA homologue (named HNhaA) was cloned from *Helicobacter pylori* ([11] and Fig. 1). Transformation of an *E. coli* mutant deleted of its antiporter genes with H*nha*A complemented the salt-sensitive phenotype of the mutant and everted membrane vesicles isolated from these transformants exhibited Na^+/H^+ antiporter activity differing in pH profile from *E. coli* NhaA (ENhaA): it was active at acidic and neutral pH where ENhaA is inactive. Remarkably, the primary sequences and the hydropathy profiles of ENhaA and HNhaA are very homologous except for one additional region found in HNhaA. This sequence has about 40 hydrophilic amino acid residues inserted at the position next to residue 235 of ENhaA (245 of HNhaA) and includes loop VIII–IX (Fig. 3). Taken together these results corroborate

our results suggesting that loop VIII–IX has an important role in the pH response of NhaA.

It is remarkable that the pH-dependent conformational change as probed by trypsin is maintained by the purified NhaA in a solution of DM. Furthermore, even the two tryptic NhaA polypeptide products remained complexed in DM and copurified on Ni^{2+}-NTA column. Similarly, the *lac* permease in DM maintains a native conformation [66]; its ligand-induced conformational changes in DM were denatured reversibly in vitro. These results are in line with previous results showing that DM is a most suitable detergent for purification and subsequent reconstitution in proteoliposomes of many transporters including NhaA [34].

5.2.3. *pH-dependent conformational change(s) involving the N-terminal domain*

Monoclonal antibodies have been raised against numerous membrane proteins, and they are a versatile tool for their biochemical and structural characterization. Most importantly, if they recognize a native epitope of a protein, they can be used to probe conformational changes in this epitope [67,68]. Modifying the standard ELISA (His-tag ELISA) we have recently isolated four NhaA-specific mAbs [39]. In this assay the antigen is presented in its native form when bound on Ni^{2+}-NTA-coated polystyrene plates via its His tag in the presence of the detergent DM and under physiological conditions. Three of the mAbs obtained recognize only the native antiporter and are Western blot-negative [39]. The fourth mAb (1F6), which is Western blot-positive, showed a unique behavior. In His-tag ELISA it gave rise to much higher signals at alkaline as opposed to acidic pH.

We found that mAb 1F6 recognizes yet another domain of NhaA in a pH-dependent manner [69]. This antibody binds NhaA at pH 8.5 but not at pH 4.5, whereas two other mAbs bind to NhaA independently of pH. The epitope of mAb 1F6 was located at the NH_2-terminus of NhaA by probing proteolytic fragments in Western blot analysis and amino acid sequencing. The antibody bound to the peptide HLHRFFSS, starting at the third amino acid of NhaA (Fig. 3). A synthetic peptide with this sequence was shown to bind mAb 1F6 at both acidic and alkaline pH, suggesting that this peptide is accessible to mAb 1F6 in the native protein only at alkaline pH. Although slightly shifted to acidic pH, the profile of the binding of mAb 1F6 to the antiporter is similar both to that of the Na^+/H^+ antiporter activity and to its sensitivity to trypsin. We thus suggest that these pH profiles reflect a pH-dependent conformational change, which leads to activation of the antiporter. Indeed, a replacement of Gly-338 by Ser (G338S), which alleviates the pH dependence of both the NhaA activity [63] and its sensitivity to trypsin [61], affects in a similar pattern the binding of mAb 1F6 to NhaA. Furthermore, the binding site of mAb 1F6 is involved in the functioning of the antiporter as follows: a double Cys replacement H3C/H5C causes an acidic shift by half a pH unit in the pH dependence of the antiporter; *N*-ethylmaleimide, which does not inhibit the wild-type protein, inhibits H3C/H5C antiporter to an extent similar to that exerted by mAb 1F6 [69].

6. The Na^+/H^+ antiporter families

6.1. *The prokaryotic families of Na^+/H^+ antiporters*

In addition to the NhaA family, other families of Na^+/H^+ antiporters with little or no homology to NhaA can already be discerned in prokaryotes (Fig. 1). These include NapA, NhaP, NhaC, NhaD and NhaB. Many DNA sequences that are now becoming available fall into one of these clusters on the basis of the homology of their putative open reading frame (see also the review of T.A. Krulwich in this volume). It is already very clear from Fig. 1 that we are seeing only the tip of the forthcoming contributions of the genome project to the understanding of the Na^+/H^+ antiporters. However, it is also very clear that structural, biochemical, physiological, and ecological studies must be carried out at the same pace so as to be able to exploit this vast information to the understanding of these interesting groups of transporters.

6.2. *The eukaryotic Na^+/H^+ antiporters*

The Na^+/H^+ antiporters of higher eukaryotes (from *Caenorhabditis elegans* to human) show distinct homology between them [4]. The yeast and

mammalian Na^+/H^+ antiporter gene families have recently been reviewed ([70,71] respectively).

An exciting new development is the recent cloning of the Na^+/H^+ antiporters from plants [72–75]. Soil salinity is a major abiotic stress for plant agriculture. Na^+ ions have adverse effects on K^+ nutrition, cytosolic enzyme activities, photosynthesis and metabolism. Three mechanisms function cooperatively to prevent the accumulation of Na^+ in the cytoplasm, i.e. restriction of Na^+ influx, active Na^+ efflux and compartmentation of Na^+ into the vacuole. The *Arabidopsis thaliana* AtNHX1 gene has been cloned, shown to encode a tonoplast Na^+/H^+ antiporter and to function in compartmentation of Na^+ into the vacuole [72]. Overexpression of AtNHX1 enhances the salt tolerance of *Arabidopsis* plants [73].

Most recently SOS1 has been cloned from *A. thaliana* and the locus was found to be essential for Na^+ and K^+ homeostasis [75]. The gene is predicted to encode a 127 kDa protein with 12 transmembrane domains in the N-terminal part and a long hydrophilic cytoplasmic tail in the C-terminal part. The transmembrane region of SOS1 has significant sequence similarity to plasma membrane Na^+/H^+ antiporters from animals and bacteria (NhaP, NhaA) and fungi (SOD2, Nha1). It has therefore been suggested to play a major role in excreting Na^+ from the plant cells [75]. These findings form the basis for the understanding of the Na^+ cycle in plants and the molecular basis of salt tolerance.

7. Concluding remarks

The universality of the Na^+/H^+ antiporters in the cytoplasmic membranes of cells and organellar membranes throughout the prokaryotic and eukaryotic kingdoms is apparent. This wide occurrence is irrespective of the mode of energization of the membranes: the antiporters are engaged in maintaining a secondary cycle of either Na^+ or H^+, depending on the conditions, the two cycles can even alternate in a single cell. The phylogenetic analysis that has become possible with the genome project already makes it possible to identify several families of antiporters. This is certainly only the beginning. This study already showed that, although a single polypeptide comprises an antiporter, there are many different polypeptides with Na^+/H^+ exchange activity. Does this mean that the residues essential for activity are very few and can be fulfilled by various different combinations? Most interestingly many of the Na^+/H^+ antiporters are already known to be pH-regulated. Hence their versatility poses another intriguing question: is the mechanism underlying the pH response of this group of transporters common or different? It is very clear that answers to these questions will be obtained only when structure and function studies of the antiporters will keep up with the flow of genetic information.

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References

[1] E. Padan, T. Krulwich, in: G. Storz, R. Hengge-Aronis (Eds.), Bacterial Stress Responses, ASM Press, Washington, DC, 2000, pp. 117–130
[2] I.C. West, P. Mitchell, Biochem. J. 144 (1974) 87–90.
[3] G. Speelmans, B. Poolman, T. Abee, W.N. Konings, Proc. Natl. Acad. Sci. USA 90 (1993) 7975–7979.
[4] E. Padan, S. Schuldiner, Biochim. Biophys. Acta 1185 (1994) 129–151.
[5] E. Padan, S. Schuldiner, in: E. Bakker (Ed.), Alkali Cation Transport Systems in Procaryotes, CRC Press, Boca Raton, FL, 1992, pp. 3–24.
[6] E. Padan, in: A. Oren (Ed.), Microbiology and Biochemistry of Hypersaline Environments, CRC Press, Boca Raton, FL, 1998, pp. 163–175.
[7] B.G. Goldberg, T. Arbel, J. Chen, R. Karpel, G.A. Mackie, S. Schuldiner, E. Padan, Proc. Natl. Acad. Sci. USA 84 (1987) 2615–2619.
[8] E. Pinner, E. Padan, S. Schuldiner, J. Biol. Chem. 267 (1992) 11064–11068.

[9] E. Padan, N. Maisler, D. Taglicht, R. Karpel, S. Schuldiner, J. Biol. Chem. 264 (1989) 20297–20302.
[10] E. Pinner, Y. Kotler, E. Padan, S. Schuldiner, J. Biol. Chem. 268 (1993) 1729–1734.
[11] H. Inoue, T. Sakurai, S. Ujike, T. Tsuchiya, H. Murakami, H. Kanazawa, FEBS Lett. 443 (1999) 6–11.
[12] S. Vimont, P. Berche, J. Bacteriol. 182 (2000) 2937–2944.
[13] T. Kuroda, T. Shimamoto, K. Inaba, M. Tsuda, T. Tsuchiya, J. Biochem. (Tokyo) 116 (1994) 1030–1038.
[14] K. Nozaki, K. Inaba, T. Kuroda, M. Tsuda, T. Tsuchiya, Biochem. Biophys. Res. Commun. 222 (1996) 774–779.
[15] P. Thelen, T. Tsuchiya, E.B. Goldberg, J. Bacteriol. 173 (1991) 6553–6557.
[16] R. Karpel, T. Alon, G. Glaser, S. Schuldiner, E. Padan, J. Biol. Chem. 266 (1991) 21753–21759.
[17] O. Rahav-Manor, O. Carmel, R. Karpel, D. Taglicht, G. Glaser, S. Schuldiner, E. Padan, J. Biol. Chem. 267 (1992) 10433–10438.
[18] N. Dover, C. Higgins, O. Carmel, A. Rimon, E. Pinner, E. Padan, J. Bacteriol. 178 (1996) 6508–6517.
[19] T.I. Murata, K. Yamato, K. Igarashi, Y. Kakinuma, J. Biol. Chem. 271 (1996) 23661–23666.
[20] R. Karpel, Y. Olami, D. Taglicht, S. Schuldiner, E. Padan, J. Biol. Chem. 263 (1988) 10408–10414.
[21] M. Christman, G. Storz, B.N. Ames, Proc. Natl. Acad. Sci. USA 86 (1989) 3484–3488.
[22] S. Henikoff, G. Haughn, J. Calvo, J. Wallace, Proc. Natl. Acad. Sci. USA 85 (1988) 6602–6606.
[23] G. Storz, L.A. Tartaglia, B.N. Ames, Science 248 (1990) 189–194.
[24] O. Carmel, O. Rahav-Manor, N. Dover, B. Shaanan, E. Padan, EMBO J. 16 (1997) 5922–5929.
[25] O. Carmel, N. Dover, O. Rahav-Manor, P. Dibrov, D. Kirsch, S. Schuldiner, E. Padan, EMBO J. 13 (1994) 1981–1989.
[26] K. Goethals, M. Van Motagu, M. Holsters, Proc. Natl. Acad. Sci. USA 89 (1992) 1646–1650.
[27] M. Schell, Annu. Rev. Microbiol. 47 (1993) 597–626.
[28] M. Toledano, I. Kullik, F. Trinh, P. Baird, T. Schneider, G. Storz, Cell 78 (1994) 897–909.
[29] S. Sasse-Dwight, J. Gralla, Methods Enzymol. 208 (1991) 146–168.
[30] T.A. Owen-Huges, G.D. Pavitt, D.S. Santos, J.M. Sidebotham, C.S. Hulton, J.C. Hinton, C.F. Higgins, Cell 71 (1992) 255–265.
[31] R. Kolter, D.A. Siegele, A. Tormo, Annu. Rev. Microbiol. 47 (1993) 855–874.
[32] R. Hengge-Aronis, Mol. Microbiol. 21 (1996) 887–893.
[33] S.G. Williams, O. Carmel-Harel, P.A. Manning, J. Bacteriol. 180 (1998) 762–765.
[34] D. Taglicht, E. Padan, S. Schuldiner, J. Biol. Chem. 266 (1991) 11289–11294.
[35] Y. Olami, A. Rimon, Y. Gerchman, A. Rothman, E. Padan, J. Biol. Chem. 272/3 (1998) 1761–1768.
[36] A. Rothman, E. Padan, S. Schuldiner, J. Biol. Chem. 271 (1996) 32288–32292.
[37] M. Venturi, Ph.D. Thesis, Max-Planck Institut für Biophysik, Frankfurt/Main, 2000.
[38] S. Iwata, C. Ostermeier, B. Ludwig, H. Michel, Nature 376 (1995) 660–669.
[39] E. Padan, M. Venturi, H. Michel, C. Hunte, FEBS Lett. 441 (1998) 53–58.
[40] T. Walz, N. Grigorieff, J. Struct. Biol. 1 (1998) 142–161.
[41] R. Henderson, J.M. Baldwin, T.A. Ceska, F. Zemlin, E. Beckmann, K.H. Downing, J. Mol. Biol. 213 (1990) 899–929.
[42] W. Kuhlbrandt, D.N. Wang, Y. Fujiyoshi, Nature 367 (1994) 614–621.
[43] Y. Kimura, D.G. Vassylyev, A. Miyazawa, A. Kidera, M. Matsushima, K. Mitsuoka, K. Murata, T. Hirai, Y. Fujiyoshi, Nature 389 (1997) 206–211.
[44] D.N. Wang, V.E. Sarabia, R.A.F. Reighmeier, W. Kuhlbrandt, EMBO J. 13 (1994) 3230–3235.
[45] M. Dolder, T. Walz, A. Hefti, A. Engel, J. Mol. Biol. 231 (1993) 119–132.
[46] J.L. Rigaud, G. Mosser, J.J. Lacapere, A. Olofsson, D. Levy, J.L. Ranck, J. Struct. Biol. 118 (1997) 226–235.
[47] J. Zhuang, G.G. Privé, G.E. Werner, P. Kingler, H.R. Kaback, A. Engel, J. Struct. Biol. 125 (1999) 63–75.
[48] K.A. Williams, Nature 403 (2000) 112–115.
[49] K.A. Williams, U.G. Kaufer, E. Padan, S. Schuldiner, W. Kühlbrandt, EMBO J. 18 (1999) 3558–3563.
[50] S. Schuldiner, E. Padan, in: E. Bakker (Ed.), Alkali Cation Transport Systems in Procaryotes, CRC Press, Boca Raton, FL, 1992, pp. 25–51.
[51] E. Padan, S. Schuldiner, J. Bioenerg. Biomembr. 25 (1993) 647–669.
[52] E. Padan, S. Schuldiner, in: W.N. Konings, H.R. Kaback, J. Lolkema (Eds.), The Handbook of Biological Physics, Vol II. Transport Processes in Membranes, Elsevier Science, Amsterdam, 1996, pp. 25–51.
[53] J.I. Lee, P.P. Hwang, T.H. Wilson, J. Biol. Chem. 268 (1993) 20007–20015.
[54] H.R. Kaback, in: W.N. Konings, H.R. Kaback, J.S. Lolkema (Eds.), Handbook of Biological Physics, Elsevier Science, Amsterdam, 1996, pp. 203–229.
[55] R.L. Dunten, T.M. Sahin, H.R. Kaback, Biochemistry 32 (1993) 3139–3145.
[56] H. Yerushalmi, S. Schuldiner, FEBS Lett. 476 (2000) 93–97.
[57] M. Kolbe, H. Besir, L.O. Essen, D. Oesterhelt, Science 288 (2000) 1390–1396.
[58] H. Inoue, T. Noumi, T. Tsuchiya, H. Kanazawa, FEBS Lett. 363 (1995) 264–268.
[59] T. Nakamura, Y. Komano, T. Unemoto, Biochim. Biophys. Acta 1230 (1995) 170–176.
[60] Y. Gerchman, Y. Olami, A. Rimon, D. Taglicht, S. Schuldiner, E. Padan, Proc. Natl. Acad. Sci. USA 90 (1993) 1212–1216.
[61] Y. Gerchman, A. Rimon, E. Padan, J. Biol. Chem. 274 (1999) 24617–24624.
[62] A. Rimon, Y. Gerchman, Y. Olami, S. Schuldiner, E. Padan, J. Biol. Chem. 270 (1995) 26813–26817.

[63] A. Rimon, Y. Gerchman, Z. Kariv, E. Padan, J. Biol. Chem. 273 (1998) 26470–26476.
[64] Y. Gerchman, Ph.D. Thesis, The Hebrew University of Jerusalem, 2000.
[65] A. Rothman, Y. Gerchman, E. Padan, S. Schuldiner, Biochemistry 36 (1997) 14572–14576.
[66] M.M. He, H.R. Kaback, Mol. Membr. Biol. 15 (1998) 15–20.
[67] S. Belli, A. Sali, J.W. Goding, Biochem. J. 304 (1994) 75–80.
[68] A. Wolf, E.W. Shaw, K. Nikaido, G. Ames, J. Biol. Chem. 269 (1994) 23051–23058.
[69] M. Venturi, A. Rimon, Y. Gerchman, C. Hunte, E. Padan, H. Michel, J. Biol. Chem. 275 (2000) 4734–4742.
[70] C.H. Yun, C.M. Tse, S.K. Nath, S.A. Levine, S.R. Brant, M. Donowitz, Am. J. Physiol. 269 (1995) G1–G11.
[71] P. Dibrov, L. Fliegel, FEBS Lett. 424 (1998) 1–5.
[72] R.A. Glaxiola, R. Rao, A. Sherman, P. Grisafi, S.L. Alper, G.R. Fink, Proc. Natl. Acad. Sci. USA 96 (1999) 1480–1485.
[73] M.P. Apse, G.S. Aharon, W.A. Snedden, E. Blumwald, Science 285 (1999) 1256–1258.
[74] E. Blumwald, Curr. Opin. Cell Biol. 12 (2000) 431–434.
[75] H. Shi, M. Ishitani, C. Kim, J.K. Zhu, Proc. Natl. Acad. Sci. USA 97 (2000) 6896–6901.
[76] J.D. Thompson, D.G. Higgins, T.J. Gibson, Nucleic Acids Res. 22 (1994) 4673–4680.