

# Conserved arginine and aspartate residues are critical for function of MjNhaP1, a Na<sup>+</sup>/H<sup>+</sup> antiporter of *M. jannaschii*

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**Abstract** Recently MjNhaP1 was identified as a pH-regulated Na<sup>+</sup>/H<sup>+</sup> antiporter of *Methanococcus jannaschii* [Hellmer, J. et al. (2002) FEBS Lett. 527, 245–249]. The antiporter is active at pH 6.0 and displays continuously decreasing activity towards alkaline pH. We have performed a site-directed mutagenesis study on all histidines as well as on conserved Asp, Glu and Arg residues of MjNhaP1, and analyzed the mutated proteins for activity. The mutants fall into three classes, i.e. normally active mutants, mutants with intermediate activity and mutants which are completely inactive. None of the histidine residues appears to be essential unlike in the bacterial proteins. The results point at an important role of a number of aspartate and arginine residues.

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**Key words:** Na<sup>+</sup>/H<sup>+</sup> antiporter; pH regulation; Site-directed mutagenesis; *Methanococcus jannaschii*

## 1. Introduction

Na<sup>+</sup>/H<sup>+</sup> antiporters are ubiquitous membrane proteins that exchange sodium for protons. They play an important role in pH regulation, osmo-adaptation, cellular energetics and halo-tolerance [2,3].

Despite the increasing number of identified and cloned antiporters the mechanisms of pH regulation and ion transduction are not well understood. Crystals of NhaA (*Escherichia coli*) have been obtained, but the resolution of the X-ray data is not sufficient to gain insights into the mechanism of ion transport [4]. Therefore most information about the relationship between structure and function came from mutagenesis studies and antiporters of different families, like NhaA (*E. coli*), sod2 (*Schizosaccharomyces cerevisiae*), NhaD (*Vibrio parahaemolyticus*) and Nhe1 (*Homo sapiens*) have been studied by this technique.

Conserved Asp residues are indispensable for the function of NhaA and sod2 [5,6] and the role of the Asp and Glu residues for the pH regulation of NhaD has been demonstrated [7]. Asp residues are conserved in numerous ion transporters, and it was proposed that they bind cations in a crownether-like fashion, thereby mediating ion transport through the hydrophobic membrane [8].

His residues are able to induce conformational switches in

proteins, since their protonation changes under physiological conditions [9]. Their role for the pH-dependent regulation of the antiporters NhaA [10] and sod2 [6] has been demonstrated. No effect on pH regulation was found after mutating transmembrane His residues of Nhe1, but it should be mentioned that not all histidines of the protein were exchanged [11].

Besides the indispensability of acidic amino acids and His residues, the importance of basic (K<sup>300</sup>, NhaA) and neutral amino acids (T<sup>345</sup>, NhaD) has also been reported, but their function is still obscure [2,7].

Recently, we have shown that MjNhaP1 is a Na<sup>+</sup>/H<sup>+</sup> antiporter of *M. jannaschii* [1]. It is the first cloned prokaryotic Na<sup>+</sup>/H<sup>+</sup> antiporter that is activated at acid pH and therefore represents an interesting target for mutational analysis. Since the functional importance of histidines and charged residues has been well documented for other Na<sup>+</sup>/H<sup>+</sup> antiporters, we used site-directed mutagenesis to exchange conserved Asp, Glu and Arg residues, as well as all His residues of MjNhaP1. Subsequently the activities of the mutated proteins were assayed in everted vesicles.

## 2. Materials and methods

### 2.1. Bacterial strains and growth conditions

Everted vesicles were prepared from strain FRAG144 (ΔnhaA::Kan ΔnhaB::Cam). The cells were grown overnight at 30°C in LBK medium (10 g l<sup>-1</sup> tryptone, 5 g l<sup>-1</sup> yeast extract, 87 mM KCl) to A<sub>600nm</sub> = 1. After addition of 1 mM isopropyl thiogalactose (IPTG) incubation was continued for 1 h. Subsequently the cells were harvested by centrifugation at 4°C and 3500 × g for 10 min.

### 2.2. Preparation of everted vesicles and measurements of H<sup>+</sup> transport by fluorescence

Everted vesicles were prepared from strain FRAG144 by the French-Press method [12]. The fluorescence measurements were performed as described previously [12]. To initiate H<sup>+</sup> transport 20 mM NaCl was added to the assay.

### 2.3. Identification of fusion proteins

Everted vesicles (50 μg protein) were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The separated proteins were blotted to nitrocellulose (Biotra) and detected with anti-myc antibody coupled to alkaline phosphatase (Invitrogen), according to the manufacturers instructions.

### 2.4. Site-directed mutagenesis

Mutations were generated using a modified version of the method described before [13]. Instead of Taq polymerase, SAWADY Pwo-DNA-Polymerase (Peglab) was used for the generation of overlapping fragments and subsequent joining. A-tailing of the fusion product was performed with Neotherm Taq-Polymerase (Genecraft). The resulting constructs were cloned into the one-step cloning vector pTrcHis2TO-

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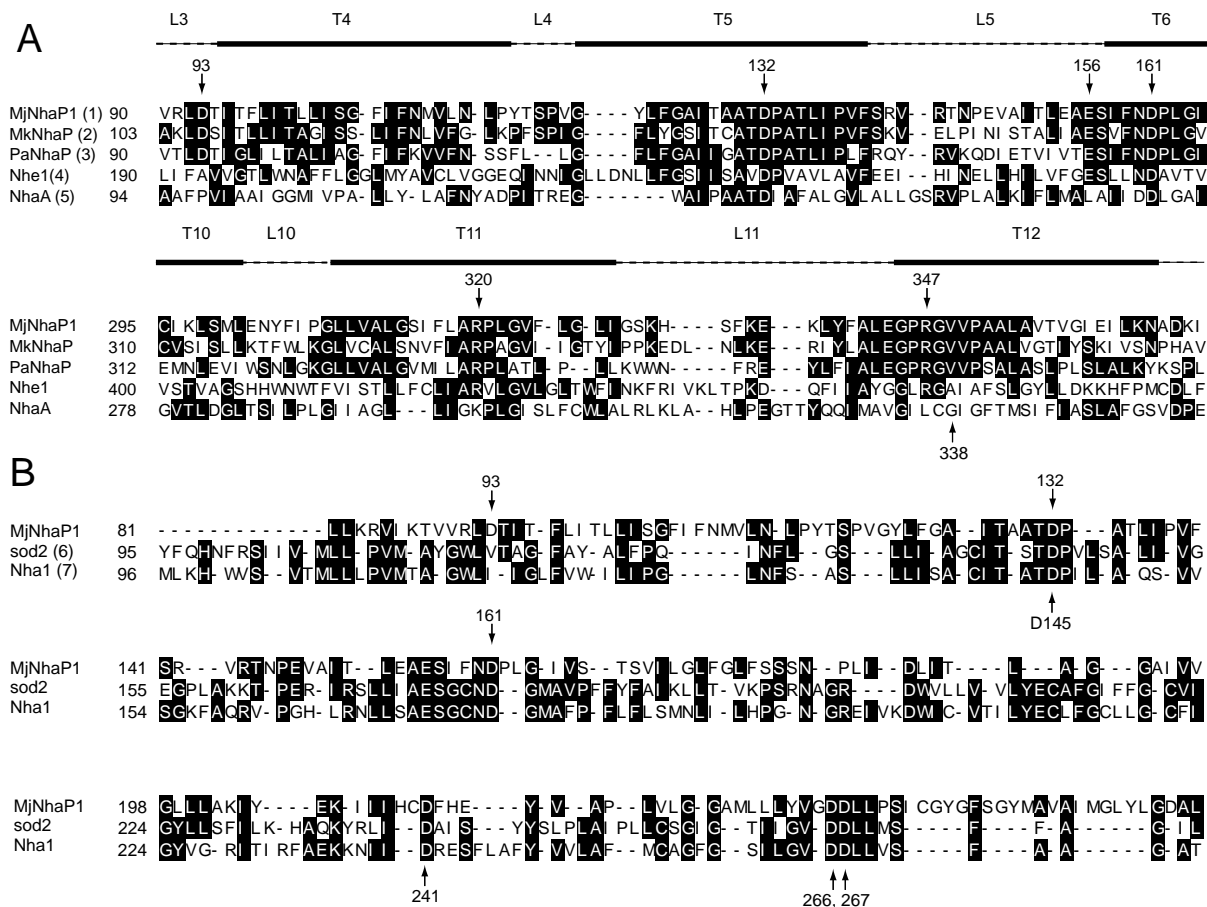


Fig. 1. Phylogenetic relationship between MjNhaP1 and various antiporters of pro- and eukaryotes. A: Alignment of MjNhaP1 with Nha1 (*H. sapiens*), NhaA (*E. coli*) and antiporters of the NhaP family. The putative transmembrane domains (T) and loops (L) are marked above the alignment. Conserved amino acids are highlighted in black. Exchanged amino acids and their positions are marked by arrows. Upper panel: conserved Asp and Glu residues of MjNhaP1 between amino acid positions 90 and 165. Lower panel: conserved Arg residues between amino acid positions 295 and 369. B: Alignment of MjNhaP1 with sod2 (*Schizosaccharomyces pombe*) and Nha1 (*Saccharomyces cerevisiae*), based on residues 81–262 of MjNhaP1. Functional important residues are marked above (MjNhaP1) and below (sod2) the sequences. The alignments were performed with Megalign (DNA-Star) using the CLUSTAL W algorithm. Predicted membrane spanning regions were calculated with Pro-tean (DNA-Star) using the algorithm of Kyte and Doolittle. The accession numbers refer to the arbitrary numeration of the antiporters in the alignment: (1) NP\_247021, (2) NP\_613837, (3) NP\_126974, (4) P19634, (5) NP\_285709, (6) P36606, (7) Q99271.

Table 1

Mutation (name of the clone)	Mutagenic primer	Codon change
H211R-H215R	A: CATACTCAcGGAAGTCACAAcGTATAAT B: ATTATACgTTGTGACTTCCgTGAGTATG	CAT → CGT
H333R	A: GTTTTTCTTTAAATGAAcGTTTTGAACCTATC B: GATAGGTTCAAACgTTCATTTAAAGAAAAAC	CAT → CGT
H425Δ-H426Δ	A: GAAGCTTTTAGGATCTCTCTTTATACTTTG	
D93A	A: GTAATTAATAATGTTATTGTAgCTAACCTC B: GAGGTTAGcTACAATAACATTTTAAATTAC	GAT → GCT
D132A	A: GTTGCTGGGgCTGTAGCAG B: CTGCTACAGcCCCAGCAAC	GAC → GCC
D161A	A: TATTCCTTAACGcCCCATTGGGAATA B: TATCTTTAAGcGCGCTGCTTAAAGATA	GAC → GCC
E156A	A: TTAAAGATACTCgCCGCTCTAAC B: GTTAGAGGCGGcGAGTATCTTTAA	GAG → GCG
R320A	A: CCAAGAGGTgTGCTAAGAATATAG B: CTATATTCTTAGCAgacACCTCTTGG	AGA → GCA
R320D	A: CCAAGAGGgTGCTAAGAATATAG B: CTATATTCTTAGCAgacCCTCTTGG	AGA → GAC
R320H	A: CCAAGAGGgTGCTAAGAATATAG B: CTATATTCTTAGCAcCCTCTTGG	AGA → CAC
R347A	A: ACAACACCTgTGCTCCCTC B: GAGGGACCAgAGGTGTTGT	AGA → GCA

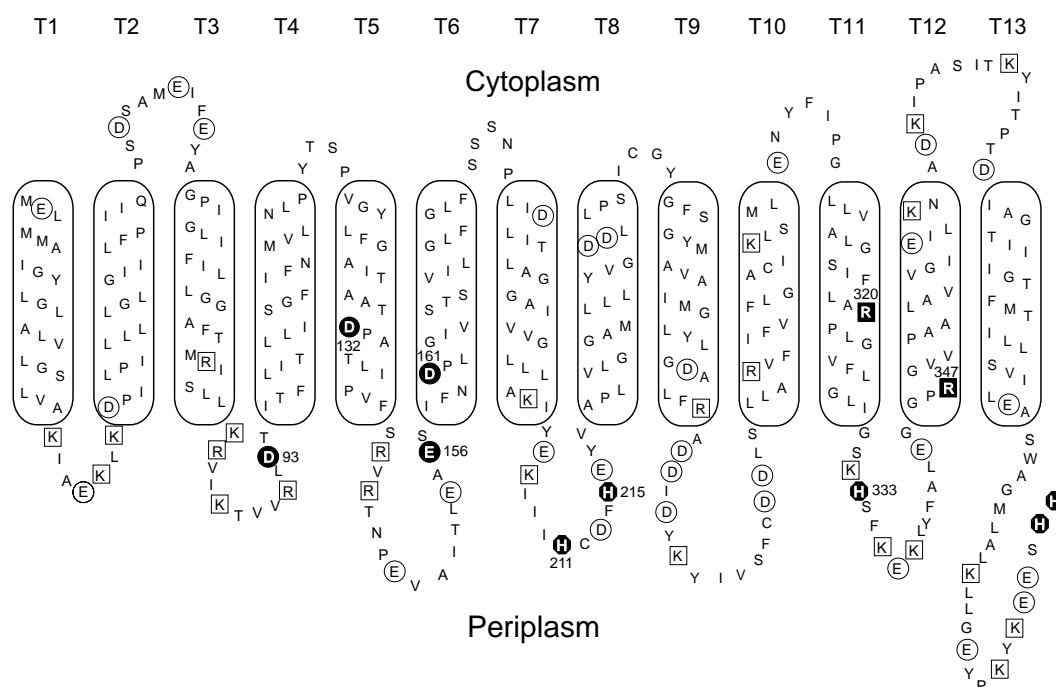


Fig. 2. Amino acids of MjNhaP1 selected for mutagenesis. The topological model was based on the secondary structure prediction using the Kyte and Doolittle algorithm of Protean (DNA-Star). In circles: strongly acidic residues (D, E). In squares: strongly basic residues (R, K). In octagons: histidine residues. Closed symbols indicate the residues selected for mutagenesis. Transmembrane domains (T) are numbered above the model.

PO (Invitrogen) and the mutations were confirmed by DNA sequencing. The primers used for mutagenesis are listed in Table 1. Primers designated with A were run against the forward primer 5'-ATG GAA CTT ATG ATG GCT ATT GGT TAC-3' to generate the former DNA fragment of Mj0057 and primers designated with B were run against the reverse primer 5'-ATG GTG GGA TTC TTC TTT ATA CTT TG-3' to generate the rear DNA fragment, respectively.

D<sup>93</sup>, D<sup>132</sup>, D<sup>161</sup> and E<sup>156</sup> were exchanged for Ala. The internal His residues were replaced by Arg and the codons for H<sup>425</sup> and H<sup>426</sup> were deleted from the gene. R<sup>320</sup> was replaced by Asp, His and Ala. The primers used for mutagenesis are summarized in Table 1. The clones were named after the introduced mutation.

### 3. Results and discussion

#### 3.1. Sequence analysis of MjNhaP1

Sequence analysis of MjNhaP1 revealed four acidic residues, D<sup>93</sup>, D<sup>132</sup> and D<sup>161</sup> and E<sup>156</sup>, which are highly conserved among the NhaP1 family. D<sup>93</sup> is located close to the membrane-liquid interface between the third loop (L3) and the fourth transmembrane domain (T4) and E<sup>156</sup> resides at the interface between L5 and T6. Assuming the secondary structure prediction obtained by the Kyte and Doolittle algorithm, E<sup>132</sup> and E<sup>161</sup> are located within the transmembrane domains T5 and T6 (Figs. 1A and 2).

D<sup>93</sup> is conserved among the NhaP family but homologous residues could not be identified in other classes of antiporters. In contrast, homologs of D<sup>132</sup> and D<sup>161</sup> are conserved from bacteria to human, since they are present in NhaA (*E. coli*), Nhe1 (*H. sapiens*) and sod2 (*Schizosaccharomyces cerevisiae*). A homolog of E<sup>156</sup> is not found in NhaA but occurs in Nhe1 and sod2, reflecting the closer relationship of MjNhaP1 to the eukaryotic antiporters.

Besides acid residues, there are two regions of high homology located in T11 and T12, which contain two conserved

basic residues, R<sup>320</sup> and R<sup>347</sup> (Fig. 1B). Homologues are even found in the human exchanger and it is interesting that K<sup>300</sup> of NhaA, which is required for the function of the *E. coli* antiporter [2], is homologous to R<sup>320</sup> of MjNhaP1.

MjNhaP1 contains five His residues, H<sup>211</sup>, H<sup>215</sup>, H<sup>333</sup>, H<sup>425</sup> and H<sup>426</sup>. Neither of these histidines is conserved among the NhaP family nor in other classes of antiporters. A model with 13 transmembrane domains locates H<sup>211</sup> and H<sup>215</sup> in L7 and H<sup>333</sup> in L11. H<sup>425</sup> and H<sup>426</sup> form the C-terminus of MjNhaP1 and face the extracellular space (Fig. 2).

For analysis of functional important amino acids of MjNhaP1, we focused on (1) residues that are conserved among the NhaP family and (2) residues which are homologous to NhaA or Nhe1.

#### 3.2. Site-directed mutagenesis

Mutated MjNhaP1 genes were cloned into the one-step cloning vector pTrcHis2TOPO, where expression is under control of a trc promoter. The plasmid provides a C-terminal fusion peptide, containing a myc-epitope tag for immune detection and a histidine hexamer allowing affinity purification. We cloned the wild-type gene both with and without tag and found no significant effects on the transport characteristics of MjNhaP1 (data not shown). Therefore all MjNhaP1 constructs, except for the clone H425Δ-H426Δ, contain the fusion peptide (Fig. 5). Interestingly, MjNhaP1 immunostains as two bands that migrate at 40 and 35 kDa. Neither the use of protease inhibitors nor the application of protease-deficient host cells prevented the formation of the 35 kDa protein. Possibly the second translation product appears due to an internal start codon, which was demonstrated for an archaeal potassium channel recently [14]. The mutants H211R-H215R, H333R and H425Δ-H426Δ did not exhibit significant changes

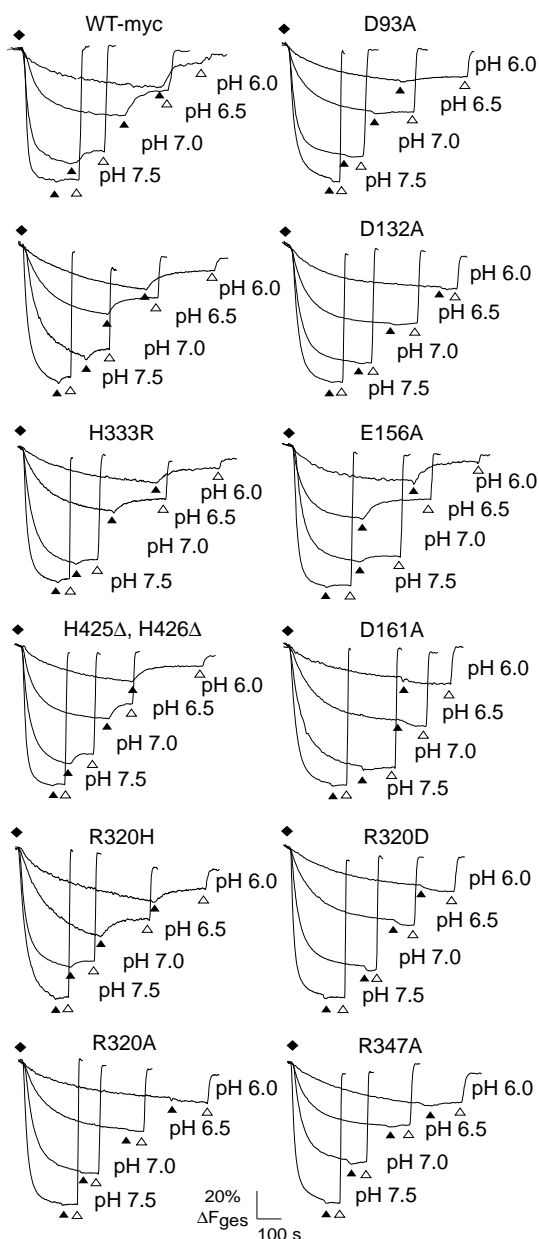


Fig. 3.  $\text{Na}^+/\text{H}^+$  antiport activities of MjNhaP1 mutants. Fluorescence measurements were performed with everted vesicles (50  $\mu\text{g}$  protein) in a thermo-stated (22°C), stirred cuvette containing 2 ml of MTCM buffer (10 mM MES/Tris, 140 mM choline chloride, 5 mM  $\text{MgCl}_2$ , 5 mM KCl, titrated to the indicated pH) and 2  $\mu\text{M}$  acridine orange. The vesicles were loaded with protons by addition of 2 mM Tris-DL-lactate (titrated to the pH of the MTCM buffer) (♦). Dequenching was initiated by adding 20 mM NaCl to the reaction mixture (▲). The transmembrane pH gradient was dissipated by the addition of 25 mM  $\text{NH}_4\text{Cl}$  (Δ). Fluorescence of the samples was excited at 495 nm and emission was recorded at 530 nm wavelength. Representative traces of each mutant are shown.

in the pH dependency compared to the wild type and also the activity of the mutants was not affected (Figs. 3 and 4). In contrast, the mutation H367R (sod2) led to complete loss of antiporter function and the mutation H225R (NhaA) shifted the pH optimum to more acidic values [15,16]. None of our His mutants displayed such properties, suggesting different mechanisms of pH regulation for each antiporter.

H367 (sod2) and H225 (NhaA) are well conserved among

their antiporter families. This is not the case for the His residues of MjNhaP1, maybe reflecting their insignificance for pH regulation.

To study the function of conserved basic residues we replaced R<sup>320</sup> and R<sup>347</sup> by Ala. The mutation R320A caused complete loss of activity, while the mutant R347A retained low activity at pH 6.0 (Figs. 3 and 4). R<sup>320</sup> was replaced by either His or Asp, which differ in the pK value of their side chains (Asp: 3.90, His: 6.04), to investigate the function of this residue in more detail. The mutant R320H displayed almost normal activity, but no antiporter activity could be detected for the R320D mutant. As mentioned before a positive charge at this position is found in many members of the

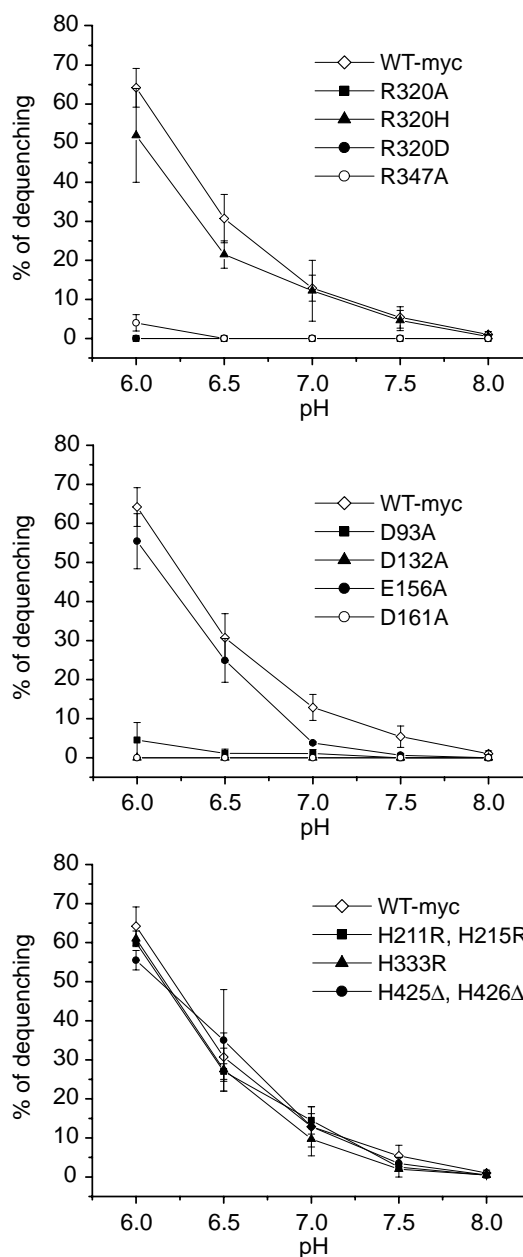


Fig. 4. pH-dependent  $\text{Na}^+/\text{H}^+$  antiporter activities of MjNhaP1 mutants. The activities of the mutants were assayed as described in Fig. 3 and expressed as percentage of dequenching after addition of lactate. Each point represents an average of at least three independent measurements.

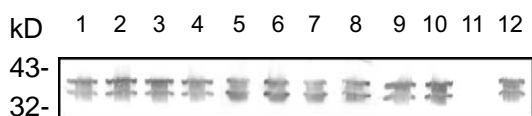


Fig. 5. Immunodetection of MjNhaP1 mutants in *E. coli* membranes. 50  $\mu$ g of total membrane protein was loaded per lane. The detection was performed with anti-myc antibody coupled to alkaline phosphatase, as described in Section 2. Lane: 1, D93A; 2, D132A; 3, D156A; 4, D161A; 5, R320A; 6, R320H; 7, R320D; 8, R347A; 9, H211R-H215R; 10, H333R; 11, H425 $\Delta$ -H426 $\Delta$  (no myc-tag); 12, WT-myc.

NhaP1 family, the human exchanger Nhe1 and NhaA, which suggests a function of the charge. Since Arg can be replaced by His, which is either neutral or negatively charged, our results indicate that rather a hydrogen bond could be responsible for the interactions, mediated by R<sup>320</sup>.

When assayed in everted vesicles, the mutants D132A and D161A did not show antiporter activity at any tested pH and the mutant D93A displayed low activity at pH 6.0. In contrast, activity of the E156A mutant was only slightly affected and the pH-dependent activation was similar to that of the wild type. Immunochemical detection shows that the strongly decreased activity of D93A, as well as the missing activities of the D132A and D161A mutants could not be attributed to an inability of antiporter expression (Fig. 5).

Because of the charged nature of Asp and Glu residues, their role in cation binding has been proposed. Alternatively, the participation in charge pairs, which are crucial for helix packing and maintenance of protein structure, is possible. Detailed studies on Cys mutants of NhaA did not confirm that the residues D<sup>63</sup>, D<sup>133</sup>, D<sup>163</sup> and D<sup>164</sup> are involved in salt bridges [2]. However, Cys mutants of MjNhaP1 are presently not available, but the high degree of homology to NhaA and the similar effects on antiporter activity suggest that at least D<sup>132</sup> and D<sup>161</sup> are involved in cation binding. D<sup>93</sup> may be functionally equivalent to D<sup>65</sup> (NhaA), since this mutation significantly reduced the activity of MjNhaP1.

Our results and those of Inoue et al. indicate that an Asp residue located in the conserved (A-T-D-X-A) sequence as well as one or two Asp residues located in the following transmembrane segment are crucial for antiporter function [5]. Dibrov and colleagues identified a pattern of a pair of acidic residues (Asp<sup>266</sup>, Asp<sup>267</sup>) preceded by an earlier acidic residue (Asp<sup>241</sup>), which is related to the pattern found in NhaA (Asp<sup>133</sup>, Asp<sup>163</sup>, Asp<sup>164</sup>) and required for antiporter function of sod2 (Fig. 1B). Interestingly, this motif is also present further downstream in MjNhaP1, which raises the question, whether (some) antiporters have two different ion conduction pathways. The crystal structure of NhaA shows two regions of low electron density, which could represent ion pores. Additionally, the different properties of hydronium and sodium ions with respect to charge density and ion radius would also suggest the existence of two pores.

Using site-directed mutagenesis to analyze the structure–

function relationship of amino acids of MjNhaP1, we demonstrated that the exchange of internal His residues by Arg and deletion of the C-terminal His residues do not affect the pH regulation of MjNhaP1. Therefore clear mechanistic differences seem to exist between the pH regulation of NhaA, sod2 and MjNhaP1.

We could also show that the exchange of conserved Arg residues influences the function of MjNhaP1 and that a positive charge at position R<sup>320</sup> is not required. To our knowledge, this is the first example of Arg residues being important for the function of Na<sup>+</sup>/H<sup>+</sup> antiporters. Sequence alignment revealed that homologous residues are also present in the human exchanger, which could be important for future mutagenesis studies on Nhe1.

Finally we demonstrated that MjNhaP1 contains conserved Asp residues, which are crucial for the activity. The remarkable similarity in function and location to residues of NhaA supports the theory that Asp residues are involved in ion binding. Sequence alignments revealed conserved acidic residues in five transmembrane domains of MjNhaP1, which may indicate the existence of two ion conduction pathways. This interesting point must be analyzed in future experiments.

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