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Functional characterization of a NapA Na^+/H^+ antiporter from *Thermus thermophilus*

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Abstract **Na^+/H^+ antiporters are ubiquitous membrane proteins and play an important role in cell homeostasis. We amplified a gene encoding a member of the monovalent cation:proton antiporter-2 (CPA2) family (TC 2.A.37) from the *Thermus thermophilus* genome and expressed it in *Escherichia coli*. The gene product was identified as a member of the NapA subfamily and was found to be an active $Na^+(Li^+)/H^+$ antiporter as it conferred resistance to the Na^+ and Li^+ sensitive strain *E. coli* EP432 (Δ*nhaA*, Δ*nhaB*) upon exposure to high concentration of these salts in the growth medium. Fluorescence measurements using the pH sensitive dye 9-amino-6-chloro-2-methoxyacridine in everted membrane vesicles of complemented *E. coli* EP432 showed high Li^+/H^+ exchange activity at pH 6, but marginal Na^+/H^+ antiport activity. Towards more alkaline conditions, Na^+/H^+ exchange activity increased to a relative maximum at pH 8, where by contrast the Li^+/H^+ exchange activity reached its relative minimum. Substitution of conserved residues D156 and D157 (located in the putative transmembrane helix 6) with Ala resulted in the complete loss of Na^+/H^+ activity. Mutation of K305 (putative transmembrane helix 10) to Ala resulted in a compromised phenotype characterized by an increase in apparent K_m for Na^+ (36 vs. 7.6 mM for the wildtype) and Li^+ (17 vs. 0.22 mM), In summary, the Na^+/H^+ antiport activity profile of the NapA type transporter of *T. thermophilus* resembles that of NhaA from *E. coli*, whereas in contrast to NhaA the *T. thermophilus* NapA antiporter is characterized by high Li^+/H^+ antiport activity at acidic pH.**

Keywords: Na^+/H^+ antiporter; Membrane protein; Secondary transporter; *Thermus thermophilus*

1. Introduction

Na^+/H^+ antiporters are found in all domains of life and have been shown to be important for cell homeostasis including pH regulation, osmoregulation and Na^+/Li^+ tolerance, but also in cell cycle and cell proliferation control [1]. The understanding of the molecular mechanisms which provide the basis of Na^+/H^+ antiport made a huge leap due to the elucidation of the 3-dimensional structure of NhaA from *Escherichia coli* [2]. NhaA belongs to the NhaA family (TC 2.A.33) according to the transport database classification scheme [3], comprising orthologues from Bacteria and Archaea. However, recent evolutionary analysis [1] showed that bacterial NhaA shares ancestry with fungal NHA exchangers and fall within the monovalent cation:proton antiporter-2 (CPA2) family of the CPA superfamily (TC 2.A.37), which also comprises the CPA1 family. According to this analysis, subfamilies of the CPA1 family include bacterial/plant/protozoan NhaP-I/SOSl, NhaP-II, as well as the eukaryotic plasma membrane NHE and intracellular NHE subfamilies. The CPA2 family can be divided into two subfamilies: The NHA and CHX clades [1]. The latter clade has its origins in bacterial NapA and KefB (K^+/H^+) transporter genes. It also includes the plant AtCHX subfamily genes proposed to be involved in K^+ homeostasis in pollen development [4]. In line with our interest in structure function relationships of Na^+/H^+ antiporters, we searched for CPA1 and CPA2 homologues in the thermophilic bacterium *Thermus thermophilus*. Two genes, one encoding a NhaP and the other a NapA subfamily member were amplified from the *T. thermophilus* genome [5] and expressed in *E. coli*. The *T. thermophilus* NapA subfamily member, designated TtNapA, was found to be an active $Na^+(Li^+)/H^+$ antiporter and confers Na^+ and Li^+ resistance to the Na^+ and Li^+ sensitive strain *E. coli* EP432 (Δ*nhaA*,Δ*nhaB*) [6]. In this report, we describe the activity of TtNapA in everted membrane vesicles in relation to the concentration of its substrates Na^+, Li^+ and H^+ and the effect of alanine (Ala, A) substitution of residues E74, D156, D157 and K305.

2. Material and methods

2.1. Bacterial strains and growth condition

E. coli DH5α (Bethesda Research Laboratories) was routinely used as host for general cloning procedures. *T. thermophilus* HB27 was a kind gift from Prof. Dr. W. Boos, University of Konstanz. *T. thermophilus* was grown at 70 °C in TB medium (8 g l^{-1} trypticase, 4 g l^{-1} yeast extract, 3 g l^{-1} NaCl) [7] with shaking at 220 rpm. *E. coli* EP432 [6] was used for complementation with E*cnhaA*, Tt*napA* and Tt*napA* mutants. *E. coli* cells were grown at 37 °C with shaking at 220 rpm in LB medium (10 g l^{-1} tryptone, 5 g l^{-1} yeast extract, 10 g l^{-1} NaCl) or modified LB medium in which NaCl was replaced by the same concentration of KCl or LiCl. Ampicillin was used at 100 μg/ml (amp^{100}).

2.2. Recombinant DNA work

Tt*napA* was amplified from genomic *T. thermophilus* HB27 DNA (isolated using peqGOLD bacterial DNA kit, Peqlab Biotechnologies) by using the oligo nucleotides CPA2_for (5′AGGCCTTGCAGCTTTTCGGGGAGTG) and CPA2_rev (5′CCCGCTACCTCCGCTGGCTCAT). The PCR mixture contained in a total volume

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of 25 μl 500 ng of genomic *T. thermophilus* HB27 DNA, 0.8 μM of each primer, 0.4 mM deoxynucleoside triphosphate mix, 1.6% DMSO, 2.5 μl of PCR buffer (10×), and 1 U Taq polymerase (Eppendorf). Amplification was carried out for 40 cycles (94 °C, 20 s; 62 °C, 10 s; 72 °C, 150 s) in a thermocycler (Biometra). For construction of a His-tagged TtNapA with six consecutive His residues at the C-terminus, oligonucleotides CPA2cHis_for (5′GGAGGGGCATATGCACGGCGCGGAACA) and CPA2cHis_rev (5′CCGCCTACAAGCTTTACAAGGCGCTTCCTTAG) were used for amplification, cut with *Nde*I and *Hind*III and cloned into pET20, to obtain pET20_TtnapA-cHis. For construction of pTTQ18-TtNapA, Oligonucleotides FcCPA2_pTTQ18 (5′GAAGGAGAGAATTCTATGCACGGCGCGGAA) and cCPA2_pTTQ_rev (5′AAACCCCTGCAGACCCGTTTAGAGGCCCCAA) were used for amplification of Tt*napA* from pET20-TtnapA. The PCR product was digested with *Eco*RI and *Pst*I and the resulting 1.3 kb *Eco*RI–*Pst*I DNA fragment was cloned into the *Eco*RI–*Pst*I digested expression vector pTTQ18 [8], to obtain pTTQ 18-TtnapA. All constructs were sequenced by Microsynth (Balgach, Switzerland) to verify correct amplification and cloning of the Tt*napA* gene (TTC1108).

Site-directed mutagenesis was done by the method described by Fisher and Pei [9]. Primers used for the mutagenesis are described in Table 1. Each reaction contained in a final volume of 50 μl 50–100 ng pTTQ18-TtnapA, 125 ng of each primer, 0.2 mM dNTP mix, 5 μl PCR reaction buffer (10×), and 2.5 U of P*fu*Turbo polymerase (Stratagene). Thermal cycling was carried out for 18 cycles (95 °C, 30 s; 62 °C, 20 s; 72 °C, 12 min) in a thermocycler (Biometra). Parental strands were digested with *Dpn*I (10 U) for 2 h at 37 °C and 2 μl of the assay mixture was used to transform *E. coli* DH5α. Resistant clones (amp^{100}) were selected from LB agar plates and grown overnight at 37 °C and shaking at 220 rpm in LB amp^{100}. Plasmid DNA was isolated from these clones and sequenced to verify the presence of the mutation. Using this procedure, we obtained four additional pTTQ18_TtnapA clones encoding C-terminally His-tagged TtNapA including E74A, D156A, D157A, and K305A substitutions.

2.3. Detection and localization of TtNapA and TtNapA mutant derivatives

Membranes were isolated from *E. coli* EP432 harbouring pTTQ18-TtNapA (or mutant derivative E74A, D156A, D157A or K305A). Single colonies were cultured in LBK at 37 °C with shaking at 220 rpm and induced with 0.1 mM IPTG at OD_{600} of 0.6. After further growth for 3 h at 37 °C and 220 rpm, cells were harvested, washed and resuspended in 50 mM Tris, pH 8.0, containing 150 mM NaCl. After addition of MgCl (5 mM final), phenylmethylsulfonyl fluoride (0.1 mM final) and trace amounts of DNAse I, the cell suspension (ca. 7 ml per g cells) was passed twice through an Emulsiflex pressure chamber at 18 000 psi and unbroken cells and cell debris were removed by centrifugation at 8000 × *g* for 10 min at 4 °C. Everted membranes were collected by ultracentrifugation (100 000 × *g*, 1 h, 4 °C), washed and finally resuspended in 10 mM Tris, pH 7.5, 140 mM choline chloride, 250 mM sucrose, 0.5 mM DTT at a protein concentration of 10 mg ml^{-1}. Directly after preparation, aliquots of the membranes were frozen at −80 °C. Membranes (30 μg of protein) were subjected to SDS–PAGE (10% acrylamide) [10] and proteins were transferred electrophoretically onto polyvinylidine difluoride membranes (Immobilon-P, Millipore, Bedford, MA). The resulting Western blots were blocked with PBS/0.05% Tween-20/5% milk for 1 h at RT and then incubated with anti-His-Tag antibody (1:3000 in PBS/0.05% Tween-20/2.5% milk; Dianova, Germany) for 1 h at RT, three times washed with PBS/0.05% Tween-20 and incubated for 30 min with goat anti-mouse IgG-HRP antibody (1:5000 in PBS/Tween-20; Dianova, Germany). After multiple washing steps in PBS/0.05% Tween-20, antibody binding was detected with SuperSignal West Pico Substrate (Pierce Socochim, Lausanne, Switzerland). Chemiluminescence was detected with a DIANA III camera (Raytest Schweiz, Dietikon, Switzerland). Protein concentration was determined by the BCA method (Pierce) using BSA as standard.

2.4. Growth and complementation studies

E. coli EP432 harbouring pTTQ18-TtNapA or its mutant derivatives were grown in LBK until OD595 of 0.8, diluted to OD595 ∼ 0.05 in $LBamp^{100}$ containing 87 mM salt with different ratios of potassium and sodium/lithium. Cultures (150 μl) were incubated in triplicate in 96-well flat-bottom plates at 37 °C with shaking at 160 rpm. At given times, OD was measured in a microplate reader (Labsystems Multiskan RC) at 595 nm.

2.5. 9-Amino-6-chloro-2-methoxyacridine (ACMA) fluorescence assay to detect Na^+/H^+ antiport activity

ACMA fluorescence quenching and dequenching experiments were done essentially as described by Rosen [11]. Na^+/H^+ antiporter activity was measured in a thermostated cuvette containing a continuously stirred suspension of 2 ml of 10 mM Tris–HEPES at various pH (6.0–8.0), 140 mM choline chloride, 5 mM $MgCl_2$, 2 μM ACMA and membranes (200 μg of protein). The changes in pH inside the membranes were monitored by following the (de)quenching of ACMA fluorescence (excitation 410 nm; emission 480 nm). An electrochemical gradient of protons across the membrane (acid and positive inside) was established by addition of ATP (final 2 mM). Na^+/H^+ antiporter activity was initiated by addition of NaCl or LiCl to the assay mixture. Due to the presence of Na^+ or Li^+ at the outside, the Na^+/H^+ antiporter catalyzes $Na^+(Li^+)/H^+$ antiport, which leads to alkalanization of the buffer inside the vesicles, which causes ACMA fluorescence to increase (dequench). After addition of various concentrations of Na^+ or Li^+, a new steady-state level of ACMA fluorescence was observed. These steady-state levels were used to calculate the percentage of dequenching relative to the level of fluorescence before the addition of ATP to the membrane vesicles.

3. Results and discussion

3.1. Identification of a NapA-type Na^+/H^+ antiporter in T. thermophilus

A search for Na^+/H^+ exchanger sequences in a database comprising translated ORFs of the genome sequence of *T. thermophilus* resulted in the identification of a member of the CPAl family and of four putative cation/H^+ antiporters

Table 1
Primers used for site-directed mutagenesis of TtNapA. The substituted nucleotide is indicated in lower case

Mutation	Mutagenic primer	Codon change
E74A	for: TGGGCTTGGcGACCAGGCTTAAGGACA rev: TGTCCTTAAGCCTGGTCgCCAAGCCCA	GAG → GCG
D156A	for: GGCGGTGATTGcCGATGTCCTG rev: CAGGACATCGgCAATCACCGCC	GAC → GCC
D157A	for: GGCGGTGATTGACGcTGTCCTG rev: CAGGACAgCGTCAATCACCGCC	GAT → GCT
K305A	for: CCATCCTGGGCgcGGTCCTGGGCGGC rev: GCCGCCCAGGACCgcGCCCAGGATGG	AAG → GCG