E2P-STATE STABILIZATION BY THE N-TERMINAL TAIL OF THE H,K-ATPASE 
β-SUBUNIT IS CRITICAL FOR EFFICIENT PROTON PUMPING UNDER IN VIVO CONDITIONS*

Katharina L. Dürr1, Kazuhiro Abe2, Neslihan N. Tavraz1, and Thomas Friedrich1

Running head: The βN-terminus stabilizes the E2P-state of gastric HK-ATPase

1 Technical University of Berlin, Institute of Chemistry, Secr. PC 14, Straße des 17. Juni 135, D-10623 Berlin, Germany.
2 Kyoto University, Faculty of Sciences, Department of Biophysics, Sakyo Ku, Kyoto 6068502, Japan. Address correspondence to: Katharina L. Dürr, Technical University of Berlin, Institute of Chemistry, Secr. PC 14, Straße des 17. Juni 135, D-10623 Berlin, Germany. Phone: +49-30-314-24128, Fax: +49-30-314-78600, E-mail: katharina.duerr@TU-berlin.de

The catalytic α-subunits of Na,K- and H,K-ATPase require an accessory β-subunit for proper folding, maturation and plasma membrane delivery, but also for cation transport. To investigate the functional significance of the β-N-terminus of the gastric H,K-ATPase in vivo, several N-terminally truncated β-variants were expressed in Xenopus oocytes, together with the S806C α-subunit variant. Upon labeling with the reporter fluorophore tetramethylrhodamine-6-maleimide, this construct can be used to determine the voltage-dependent distribution between E1P/E2P states. Whereas the E1P/E2P conformational equilibrium was unaffected for the shorter N-terminal deletions βΔ4 and βΔ8, we observed significant shifts towards E1P for the two larger deletions βΔ13 and βΔ29. Moreover, the reduced ΔF/F ratios of βΔ13 and βΔ29 indicated an increased reverse reaction via E1PÆE1P+ADPÆE1+ATP, since cell surface expression was completely unaffected. This interpretation is supported by the mutants’ reduced sensitivity towards the E2P-specific inhibitor SCH28080, which becomes especially apparent at high concentrations (100 μM). Despite unaltered apparent Rb⁺ affinities, the maximal Rb⁺ uptake of these mutants was also significantly lowered. Considering the two putative interaction sites between β-N-terminus and α-subunit revealed by the recent cryo-EM structure, the N-terminal tail of the H,K-ATPase β-subunit may stabilize the pump in the E2P-conformation, thereby increasing the efficiency of proton release against the million-fold proton gradient of the stomach lumen. Finally, we demonstrate that a similar truncation of the β-N-terminus of the closely related Na,K-ATPase does not affect the E1P/E2P distribution or pump activity indicating that the E2P-stabilizing effect by the β-N-terminus is apparently a unique property of the H,K-ATPase.

The gastric H,K-ATPase fulfills the remarkable task of pumping protons against a more than 10⁶-fold concentration gradient. H⁺ extrusion is coupled to counter transport of an equal number of K⁺ ions for each ATP molecule hydrolyzed, resulting in an electroneutral overall process (1). Characteristic for all P-type ATPases, the enzyme cycles between the two principal conformational states (E₁, E₂) and the corresponding phosphointermediates (E₁P, E₂P), which are formed by reversible phosphorylation of an aspartate residue in the highly conserved DKTGTLT motif. According to a Post-Albers-like reaction scheme (Figure 1 A), the conformational E₁PÆE₂P transition converts the high H⁺/low K⁺ affinity of the cation binding pocket into a low H⁺/high K⁺ affinity binding site, hence enabling proton release into the stomach lumen and subsequent binding of extracellular K⁺. Since the pump faces a lumenal proton concentration of about 150 mM (2), proton release is probably the energetically most demanding step in the reaction cycle. Thus, during the conformational E₁PÆE₂P transition, enormous pKₐ-changes of the H⁺ coordinating residues have to occur, which most likely involve the rearrangement of a positively charged lysine side chain (Lys-791 in rat H,K-ATPase) (3). All P₂-type ATPases share a common catalytic α-subunit, composed of 10 transmembrane domains harbouring the ion binding sites and a large cytoplasmic loop with the nucleotide binding domain (N-domain), the phosphorylation domain (P-domain) and the actuator domain (A-domain) (4). However, a
unique feature of K⁺ transporting Na,K- and H,K-ATPase enzymes is the requirement for an accessory β-subunit which is indispensable for proper folding, maturation and plasma membrane delivery (5,6). Despite only 20-30% overall sequence identity between the H,K-ATPase β-subunit and the Na,K β-isoforms, the topogenic structure is similar: a short N-terminal cytoplasmic tail, followed by a single transmembrane segment and a large extracellular C-terminal domain with glycosylation sites and disulfide bridging cysteines. Numerous studies have demonstrated that the β-subunit of the Na,K-ATPase is more than just a chaperone for the α-subunit, being also required for proper ion transport activity of the holoenzyme. In fact, it has been discovered that different cell- and tissue-specific β-isoforms have distinct effects on the cation affinities (7-9). Furthermore, it was shown that mutational changes in all three topogenic domains of the Na,K-ATPase β-subunit (10-19), as well as chemical interference with disulfide-forming cysteines in the Na,K-ATPase β-subunit’s ectodomain (20-22) affect the cation transport properties of the sodium pump. Finally, conformational changes in the β-subunit during the Na,K-ATPase reaction cycle were demonstrated by proteolytic digestion studies (23) and voltage-clamp fluorometry (24).

Far less is known about the functional significance of the single H,K-ATPase β-isoform, especially about its potential impact on cation transport (reviewed in (25) and (26)). We have proven recently that E₂P-state-specific transmembrane interactions between residues in the α7TM7 and two highly conserved tyrosines in the β7 of both Na,K- and H,K-ATPase significantly stabilize the E₂P-conformation (19). Mutational disruptions of this interaction resulted in substantial shifts towards E₁P and severely affected Η⁺ secretion, which highlighted the physiological relevance of this E₂P-state stabilization. Notably, according to the recently published cryo-EM structure of pig gastric H,K-ATPase in the pseudo-E₂P state (27), the N-terminal tail of the β-subunit makes direct contact with the phosphorylation domain of the α-subunit (Figure 1 B), thus indicating an additional E₂P-state stabilization mediated by the β-N-terminus. Although this idea was further supported by biochemical studies on N-terminally truncated mutants, direct evidence for this putative E₂P-stabilizing interaction and its potential significance for ion transport in intact cells is still lacking.

Here, we demonstrate for the first time the functional importance of the gastric H,K-ATPase β-subunit’s N-terminus in living cells under in vivo conditions: voltage-clamp fluorometry, Rb⁺ flux and SCH28080 sensitivity measurements revealed E₁P-shifted, ion transport-impaired phenotypes for two N-terminally truncated H,K β-variants, thus substantiating the E₂P-stabilizing effect of the β-N-terminus suggested by the recent cryo-EM structure.

EXPERIMENTAL PROCEDURES

Molecular biology and oocyte preparation. The cDNAs of the rat gastric H,K-ATPase β-subunit and a modified form of the α-subunit with a single cysteine replacement in the M5/M6 extracellular loop (S806C, see Figure 1 B) were subcloned into vector pTLN (28). This cysteine replacement enables environmentally-sensitive TMRM-labeling of gastric H,K-ATPase (29) without affecting its transport properties (30). For Na,K-ATPase studies, an ouabain-insensitive sheep α₁-Na,K-ATPase mutant (carrying mutations Q111R and N122D (31)) without extracellularly exposed cysteines (mutations C911S and C964A (32)) and a Na,K-ATPase β₁-subunit variant β₁S62C that can be site-specifically labeled with TMRM (24), were used as templates for mutagenesis and cRNA preparation.

N-terminally truncated β-contracts were generated by recombinant PCR and verified by DNA sequencing. Oocyte preparation, cRNA synthesis and injection were performed as described previously (30). Prior to experiments that were usually carried out 2-3 days after injection at 21-24 °C, oocytes were preincubated in solutions containing 100 μM ouabain (Sigma-Aldrich) to inhibit the endogenous Xenopus Na,K-ATPase.

Voltage-clamp fluorometry. Site-specific labeling of H,K- (or Na,K-) ATPase-expressing oocytes was achieved by incubating oocytes in HK₇.4 buffer (90 mM NaCl, 20 mM TEACl, 5 mM BaCl₂, 5 mM NiCl₂, 10 mM MOPS/TRIS, pH 7.4) containing 5 μM TMRM (tetramethylrhodamine-6-maleimide; Molecular Probes) for 5 min at room temperature in the dark, followed by extensive washes in dye-free HK₇.4 buffer. Voltage-clamp fluorometry measurements were carried out under high extracellular Na⁺/K⁺-free conditions, for the
characterization of H,K-ATPase mutants in HK$_{5,4}$ buffer (90 mM NaCl, 20 mM TEACL, 5 mM BaCl$_2$, 5 mM NiCl$_2$, 10 mM MES/TRIS, pH 5.5, 100 μM ouabain) and for Na,K-ATPase expressing oocytes in NaK$_{5,4}$ buffer (100 mM NaCl, 5 mM BaCl$_2$, 5 mM NiCl$_2$, 10 mM Hepes, pH 7.4, 100 μM ouabain), respectively. Details on setup components, data acquisition and analysis are given in (19).

Rb$^+$ uptake measurements. Oocytes were incubated for 15 min in Rb$^+$-flux-buffer (5 mM RbCl, 85 mM TMACI, 20 mM TEACl, 5 mM BaCl$_2$, 5 mM NiCl$_2$, 10 mM MES, pH 5.5, 100 μM ouabain). After 3 washing steps in Rb$^+$-free washing-buffer (90 mM TMACl or NaCl, 20 mM TEACl, 5 mM BaCl$_2$, 5 mM NiCl$_2$, 10 mM MES, pH 5.5) and one wash in water, each individual oocyte was homogenized in 1 ml of Millipore water. For SCH28080 inhibition experiments, the K$^+$-competitive inhibitor SCH28080 (Sigma-Aldrich) was added to the preincubation solution and Rb$^+$-flux-buffer (to a final concentration of either 10 μM or 100 μM), respectively. To determine the apparent constant K$_{1/2}$ for half-maximal activation of the H,K-ATPase by rubidium, the sum [TMACl] plus [RbCl] in the Rb$^+$-flux buffer was kept constant at 90 mM, e.g. 1 mM RbCl + 89 mM TMACl. After subtraction of average Rb$^+$ uptake into control oocytes from the same batches at a given [RbCl], data were fitted to a Michaelis-Menten type function: $v = v_{max} \cdot \frac{[S]}{K_{m} + [S]}$.

Aliquots of 20 μl from oocyte homogenates were analyzed by atomic absorption spectroscopy using an A Analyst800™ spectrometer (Perkin Elmer, Waltham, MA), equipped with a transversely heated graphite furnace using a temperature protocol according to manufacturer’s procedures (conditions available on request). Absorption was measured at 780 nm using a Rb hollow cathode lamp (Photron, Melbourne, Australia). After Zeeman-background correction, Rb$^+$ contents were calculated by comparison with standard calibration curves (measured between 0 and 50 μg/L Rb$^+$).

Western blot analysis of isolated plasma membranes. The procedures for isolation of plasma membranes and total cellular membranes from Xenopus oocytes, gel electrophoresis and immunoblotting were performed according to the protocols in (19). The polyclonal antibody HK12.18 (Merck)(6) and the monoclonal antibody 2B6 (MBL, Woburn, MA)(33) were used for detection of gastric H,K-ATPase α- and β-subunits, respectively.

RESULTS AND DISCUSSION

E$_1$P/E$_2$P conformational distribution of N-terminally deleted H,K-ATPase β-mutants. To investigate whether the β-subunit’s N-terminal domain indeed contributes to E$_2$P-state stabilization of the gastric H,K-ATPase, we expressed the wildtype β-subunit and four N-terminally deleted H,K β-mutants (Figure 2 A) together with an α-subunit variant S806C in Xenopus oocytes. After site-specific labeling of this cysteine with TMRM (see Figure 1 B), monoexponential fluorescence changes were observed upon voltage-jumps from a holding potential of -40 mV to values between -180 mV and +60 mV (Figure 2 B). Hence, this mutant α-subunit directly monitors the voltage-dependent distribution between E$_1$P/E$_2$P states of H,K-ATPase enzymes, as demonstrated previously (19,30). Figure 2 C shows the resulting voltage-dependence of steady-state fluorescence amplitudes (1-ΔF/F), which follow a Boltzmann-type distribution for both wildtype or β-N-terminally deleted H,K-ATPase enzymes. Whereas these curves were not changed for the shorter N-terminal deletions βΔ4 and βΔ8 compared to βWT, the distributions for the Δ13 and Δ29 truncated β-variants were significantly shifted towards more positive potentials (see Table I for Boltzmann parameters), indicating a relative destabilization of the E$_2$P-state in favor of E$_1$P. According to these distributions, about 65% of the βWT, βΔ4- and βΔ8-containing H,K-ATPase molecules occur in the E$_2$P-state at -60 mV (which is the physiologically relevant membrane potential determined for parietal cells (34)), but only 60% and 50% of the βΔ29 and βΔ13 mutant enzymes are present in E$_2$P, respectively. Furthermore, the E$_1$P-shift is also reflected by the voltage-dependent reciprocal time constants obtained from monoexponential fits to the fluorescence changes (Figure 2 D): of note, these are significantly smaller for the βΔ29 and βΔ13 mutants than for the wildtype only at positive potentials, which favor the forward reaction from E$_1$P to E$_2$P, but not at negative potentials, at which the enzyme is driven into the E$_1$P-state (via the backward reaction described by $k_{rev}$ in Figures 1 A and 2 D). Therefore, these N-terminal deletions apparently cause a shift towards E$_1$P by reducing the ‘forward’ rate constant (designated as $k_{fw}$) of the E$_1$P/E$_2$P
transition without changing the rate constant $k_{rev}$ for the 'reverse' reaction.

However, if only the results shown in Figure 2 C and D are considered, the conformational effect caused by the N-terminal deletions is probably underestimated, since compared to βWT, the two variants βΔ13 and βΔ29 also showed substantially smaller values for the enzyme-specific fluorescence changes ΔF/F (Figure 2 E). Western blot analysis of isolated plasma membranes excluded the possibility that the lower ΔF/F values observed for the βΔ13 and βΔ29 constructs were simply due to a reduced cell surface delivery of the α-subunits that are available for labeling with TMRM (see Figure 2 F and Supplementary Figure S1). Therefore, it is safe to assume that the shown ΔF/F ratio is directly proportional to the number of H,K-ATPase molecules that can be shifted between E1P/E2P-states by voltage-jumps (according to the reaction sequence highlighted in grey in Figure 1 A). The approximately two-fold lower ΔF/F values observed for the βΔ13 and βΔ29 mutants (compared to βWT, βΔ4 and βΔ8) most likely reflect a higher tendency of the mutant H,K-ATPase molecules (that accumulate in the E1P-state due to the reduced $k_{fw}$) to undergo further backward reactions via E1P (H+) → E1P + H+ + ADP → E1 + H+ + ATP. The resulting substantial depletion in the sum of E1P/E2P-states would therefore explain the observed smaller fluorescence changes. This interpretation is actually in line with previous results from pulse-chase experiments on purified membranes of H,K-ATPase-expressing HEK293 cells. Compared to the wildtype, the membrane fractions containing β-N-terminally truncated variants βΔ8, βΔ13 (27) or βΔ28 (K. Abe, unpublished results) showed increased reactivity of radiolabeled E232P phosphoenzymes with ADP to form [γ-32P]-ATP via the aforementioned reverse reaction. Of note, in these chase experiments, already the βΔ8 mutant exhibited a significantly reduced amount of 32P-phosphoenzyme after a 5-second chase with ADP. However, care has to be taken when comparing these and our VCF results due to the differences in experimental conditions. The pulse-chase experiments have been performed on membrane fractions at a ubiquitous pH of 6.4, lower ionic strength, and 0 °C, whereas VCF probes the enzyme within an intact membrane environment with physiological ion concentrations (and neutral intracellular pH) at room temperature. Differences in the intracellular ion concentration or pH may well affect intramolecular interactions, especially if these involve potentially charged residues (amino acids 6-8 in the N-terminal sequence MAALQEEKKSC...). Furthermore, the intracellular ATP and Mg2+ concentrations are by far higher in Xenopus oocytes than in the pulse-chase experiments (2.3 mM ATP (35), 0.5 mM Mg2+ versus 10 μM ATP, 20 μM Mg2+), thus favouring the forward phosphorylation reaction. Therefore, small effects on the E1P-E2P distribution that would reflect an enhanced ADP-dependent dephosphorylation of the E1P state of the βΔ8 mutant are possibly obscured under the conditions in living cells.

**Rb⁺ uptake and SCH28080 sensitivity of N-terminally truncated H,K-ATPase β-subunits.** To assess the impact on ion transport activity potentially caused by the conformational shifts of the N-terminally truncated β-variants, we determined Rb⁺ uptake at saturating extracellular concentrations. Whereas Rb⁺ uptake for βΔ4- and βΔ8-expressing oocytes was comparable to βWT, it was about 20% lower for βΔ13 and βΔ29 (Figure 3 A, Table II). This can be interpreted as a reduced turnover number (lowered $v_{max}$), since the apparent affinity for extracellular Rb⁺ was unaffected by the truncations (see Figure 3 B and Table II for apparent $K_{0.5}$ values). The affected turnover numbers of the βΔ13 and βΔ29 mutants demonstrate that obviously the transition which is characterized by the reduced rate constant $k_{fw}$ of these variants directly affects the rate-limiting step of the catalytic cycle. This illustrates how already small shifts in conformational equilibria can have significant functional consequences for the transport activity of the gastric H,K-ATPase.

In presence of 10 μM SCH28080, an E2P-specific inhibitor of the H,K-ATPase (36-38), the Rb⁺ uptake of βWT-, βΔ4- and βΔ8-expressing oocytes was reduced to approximately 20% (Figure 3 A inset, Table II), in agreement with the data of Mathews and colleagues (39). In contrast, the inhibition of ATPase complexes containing βΔ13 and βΔ29 was less efficient, resulting in significantly higher residual activities of about 30% at 10 μM SCH28080. Notably, 100 μM SCH28080 resulted in a suppression of Rb⁺ uptake to about 6% residual activity for H,KαS806C/βWT, whereas the effect was much smaller for any of the β-N-terminally deleted variants. As a possible consequence of the mutants' E2P-shifted phenotypes, the mean dwell time of molecules in
the SCH28080-sensitive E2P-state may be substantially shorter. Thus, increasing the inhibitor concentration would not result in enhanced binding of the compound, since the dwell time in E2P is not sufficient to reach binding equilibrium under turnover conditions. In contrast, the wildtype H,K-ATPase, which stays longer in E2P, is able to bind more inhibitor molecules if the SCH28080 concentration is increased. Interestingly, at the higher inhibitor concentration, a significantly reduced SCH28080 sensitivity was also observed for βΔ4- and βΔ8-expressing oocytes, which showed a more than two-fold higher residual activity compared to βWT (13-14% versus 6%). This suggests that already the shorter deletions cause an elevated preference for E1P, which raises the question, why no effect on the conformational distribution (data in Figure 2 C) was seen for these constructs. Two possibilities may account for this: i) minute shifts in the voltage-dependent E2P/E1P distribution may be difficult to resolve by VCF experiments, since low slope factors zq of the Boltzmann curves limit the accuracy of V0.5 determination. ii) E2-destabilizing effects that act on the relative distribution of pump molecules over all reaction intermediates may not be effective during the partial reaction sequence studied in pre-steady-state experiments (VCF), but rather become apparent under steady-state conditions (Rb+ uptake), in which the enzyme undergoes the full reaction cycle.

Although the reduction in Rb+ transport activity of βΔ13 and βΔ29 truncated mutants appears moderate, it is important to note that these subtle effects were observed already at a relatively mild proton gradient (pHint ~7.4 vs. pHext=5.5). However, in vivo the H,K-ATPase pumps protons against a 10,000-fold higher gradient of about 10^6. Under these physiological conditions (which are unfortunately not applicable to Xenopus oocytes), the high luminal H+ concentration would even more favor H+ reverse binding at the extracellular sites, thereby also stimulating the E2P→E1P ‘backward’ reaction (i.e. increasing krev). An enhanced krev, in addition to the reduced forward rate constant kfw (as observed for the N-terminally deleted β-mutants βΔ13 and βΔ29) is thus expected to have even more drastic effects on the turnover number. Therefore, albeit causing only small effects at the rather shallow pH gradient applied here, the E2P-state destabilization by the β-N-terminal truncations will almost certainly slow down H+ secretion under the pH conditions in the stomach.

A surprising finding from the current study is that the more extensive deletion βΔ29 caused a smaller shift towards the E1P-state than βΔ13 (see Figure 2C and Table I). The two putative interaction sites between the β-N-terminus and the α-subunit proposed by the recently published cryo-EM structure of the pig gastric H,K-ATPase (27) may provide a rationale to explain these rather unexpected effects: Apart from the aforementioned contact between the β-N-terminus and the P-domain (probably around Arg-716 to Ala-719, see red arrow in Figure 1 B), a stretch of the β-N-terminus located closer to the transmembrane domain also approaches the cytoplasmic stalk of αTM3, which is connected to the A-domain (Figure 1 B, black arrow). Therefore, a truncation of the first 12 amino acids in βΔ13 may only disrupt the interaction with the P-domain, and thereby cause the strong E1P shift, whereas the more extensive deletion in βΔ29 most likely affects both critical contacts, which might involve an additional compensatory mechanism (see below).

As outlined by Abe and colleagues, the contact between the β-subunit N-terminus and the P-domain probably stabilizes the enzyme in E2P, thereby minimizing the reverse reaction with ADP via E2P → E1P + ADP → E1 + ATP in terms of a ‘ratchet’-like mechanism (27). In the pseudo E2P-state revealed by the cryo-EM structure, the N-domain of the α-subunit is retracted far from the P-domain (see Supplementary Figure S2). Therefore, reverse transfer of the phosphate (bound to Asp-385 in the P-domain) to ADP (bound to the N-domain), would require a large movement of the P-domain, which is presumably prohibited by the tethering between P-domain and the β-N-terminus in the E2-state. In contrast, in a putative E1P-state derived from a SERCA-E1AIF4-ADP structure (40), the ADP at the N-domain is in close proximity to the phosphate analogue AlF4- (shown in Figure S2), which could thus explain the enhanced ADP-rephosphorylation of the E1P-shifted N-terminally deleted β-variants (27).

The interaction of the β-N-terminus with the A-domain via the movement of αTM3 may also be functionally significant, since the protrusion of the TGES-loop from the A-domain into the gap between N- and P-domain may have a segregating effect, too (see Supplementary Figure S2). Although the influence of the βN/αTM3 contact on positioning of the A-domain is not resolved yet, it seems possible that the missing contact to the β-N-terminus via
αTM3 (in βΔ29) may lead to unrestricted movement of the A-domain. This may promote the segregating effect of the TGES loop, which could partially compensate for the destabilization of E2P that arises from the missing interaction with the P-domain, thus explaining the less E1P-shifted phenotype of βΔ29 compared to βΔ13.

N-terminal truncation of the Na,K-ATPase β-subunit. Notably, according to the recently published crystal structure of the closely related Na,K-ATPase in the Rb⁺ occluded E2-state (41), there is no indication for similar interactions between β-N-terminus and α-subunit of the sodium pump. Although the β-N-terminus of the Na,K-ATPase was not completely resolved in this structure so that evidence for a similar interaction in the Na,K-ATPase is absent so far, we tried to explore whether the inter-subunit interaction mediated by the H,K-ATPase β-N-terminus is conserved among P2C-type ATPases. In order to determine the impact of β-N-terminal truncation on sheep Na,K-ATPase, the 8 most N-terminal amino acids of reporter construct β1S62C (24) of the β1-subunit were deleted (Figure 4 A). Since the cytoplasmic β-N-terminus of the Na,K-ATPase is shorter than that of the gastric H,K-ATPase, this truncation results in a N-terminal domain comparable in length to the βΔ13 variant of the H,K-ATPase. This N-terminally truncated β-variant NaKβ1S62CΔ8 was coexpressed with the wildtype sheep α1-subunit, labeled by TMRM and subjected to voltage jumps in a K⁺-free solution containing 100 mM Na⁺. The resulting voltage-dependent distribution of fluorescence amplitudes is not significantly different from the one observed for full-length β1S62C containing Na,K-ATPase complexes (see Figure 4 B and Table III). Furthermore, as inferred from the stationary pump currents at 10 mM K⁺, the ion transport activity of the Na,K-ATPase is completely unaffected by the β-N-terminal truncation (Figure 4 B, inset). Regarding the comparably shallow Na⁺ gradient under physiological conditions, an analogous ‘ratchet’-like E2P-stabilization is probably not necessary to assist Na⁺ transport of the sodium pump. This further supports our hypothesis that the E2P–state stabilization mediated by the H,K-ATPase β-N-terminus is unique to facilitate proton transport against the steep proton gradient across the parietal cell membrane, which is approximately 10,000-fold higher than the typical transmembrane gradient for Na⁺ ions. Therefore, the mechanism of E2P-state stabilization by the gastric H,K-ATPase β-N-terminus appears to be a distinctive property of this enzyme.

CONCLUSIONS

As a key observation, the recently published cryo-EM structure of the gastric H,K-ATPase highlighted the close proximity between the P-domain of the α-subunit and the short N-terminal tail of the β-subunit, suggesting an E2P-stabilizing interaction of the two subunits. The current study on N-terminally truncated β-variants provides direct evidence that the β-N-terminus assists in E2P-state stabilization and that this is critical for the enzyme’s transport efficiency under in vivo conditions. Since the effects of the mutants were already significant under the relatively shallow H⁺ gradient (ΔpH ≈ 2), they may even be more relevant for the enzyme in situ, where a strong E2P-preference is essential for efficient H⁺ release against a ΔpH of about 6 units. Moreover, since we recently demonstrated an E2P-stabilizing effect of interactions between residues in the transmembrane domain of the H,K-ATPase β-subunit and TM7 of the α-subunit, the two effects exerted by different regions of the β-subunit may synergistically contribute to the functional requirement of E2P-state stabilization.

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FOOTNOTES

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The abbreviations used are: TMRM, tetramethylrhodamine-6-maleimide; VCF, voltage-clamp fluorometry; TM, transmembrane domain; EM, electron microscopy; WT, wildtype, MES, 2-(N-morpholino)ethanesulfonic acid; TMACl, tetramethylammonium chloride; MOPS, 3-(N-morpholino)propanesulfonic acid, TEACL, tetraethylammonium chloride.

FIGURE LEGENDS

Figure 1. Post-Albers scheme (A) and cryo-EM structural representation of pig gastric H,K-ATPase in the fluoroaluminate-bound pseudo E2P-state (B).

A, Post-Albers-Scheme of the proposed reaction cycle of the gastric H,K-ATPase. E1P/E2P conformational states giving rise to voltage jump-induced fluorescence changes of TMRM-labeled H,K-ATPase molecules are highlighted (grey box).

B, Structural representation based on the cryo-EM structure of the pig gastric H,K-ATPase (surface or mesh, contoured at 1σ, EM Data Bank code 5104) and the corresponding homology model (cartoon, PDB code 3IXZ). Inset: a close-up view (from the right side of the molecule) showing the putative interaction sites of the β-subunit N-terminus with the P-domain (red arrow) and αTM3 (black arrow), respectively. Color coding is indicated in the figure.

Figure 2. Voltage-dependent E1P/E2P distribution and plasma membrane targeting of N-terminally deleted β-H,K-ATPase mutants.

A, Partial amino acid sequence of N-terminally deleted H,K-ATPase β-variants and the wildtype rat gastric H,K-ATPase β-subunit.

B, Voltage-pulse induced fluorescence signals of a TMRM-labeled oocyte expressing H,K-ATPase αS806C/βWT under high Na+/K+-free conditions at pH 5.5. Inset: voltage-protocol.

C, Voltage-dependent distributions of fluorescence amplitudes 1-ΔF/F for H,K-ATPase complexes consisting of the α-subunit reporter construct αS806C and either unmodified HKβWT (◼), or N-terminally deleted H,Kβ-variants βΔ4 (/rand), βΔ8 (△), βΔ13 (○) and βΔ29 (▲). Data are means ± S.E. of 15-30 oocytes from 2-3 different oocyte batches, normalized to saturating values obtained from fits of a Boltzmann function to the data (superimposed lines, see Table I for fit parameters).

D, reciprocal time constants obtained by monoexponential fits of the voltage jump-induced fluorescence changes for wildtype (◼) and N-terminally deleted H,K-ATPase β-variants βΔ13 (○) and βΔ29 (▲). Data are means ± S.E. of 10-16 oocytes.

E, ΔF/F values (in %) for TMRM-labeled oocytes expressing αS806C and either the wildtype H,K-ATPase β-subunit or N-terminally truncated β-variants. Data are means ± S.E. of 13-32 oocytes from 3 different oocyte batches. Inset: ΔF/F is calculated from the difference ΔF (represented by a black arrow) between the fluorescence at the most hyperpolarizing voltage (-180 mV, upper black trace) and the most depolarizing voltage (+60 mV, lower black trace), normalized to the background fluorescence F at -40 mV (grey trace).

F, Western blot analysis of plasma membrane (PP, upper panel) and total membrane (TP, lower panel) preparations from H,K-ATPase-expressing oocytes, using an anti-H,Kα antibody HK12.18 or anti-H,Kβ antibody 2B6 (See Supplementary Figure S1). The equivalent of two oocytes was loaded per lane, detection of the endogenous Xenopus Na,K-ATPase α1-isoform served as a loading standard, which is shown in Figure S1.
**Figure 3.** Rb⁺ uptake, SCH28080 sensitivity and apparent Rb⁺ affinity of N-terminally truncated H,K-variants.

A, Rb⁺ uptake was determined at pH 5.5 in extracellular Na⁺-free solutions containing 5 mM RbCl in absence (light grey bars) or presence of SCH28080 (dark grey bars: 10 μM, black bars: 100 μM). Inset: Residual Rb⁺ uptake activity (in %) in presence of 10 μM (dark grey bars) or 100 μM (black bars) SCH28080. Data were normalized to Rb⁺ uptake at 5 mM RbCl in absence of SCH28080 for each construct after subtraction of the mean Rb⁺ uptake of uninjected oocytes. Data are means ± S.E., n = 40-50 oocytes from 3-4 independent experiments.

B: Michaelis-Menten plots for concentration dependent Rb⁺ uptake by H,K-ATPase expressing oocytes. Oocytes were injected with cRNAs of the αS806C mutant and either the wildtype β-subunit (■) or N-terminally deleted β-variants βΔ4 (▲), βΔ8 (△), βΔ13 (○) and βΔ29 (▽) cRNA, respectively. Data are means ± S.E., n = 10-20 oocytes. One representative out of at least two independent experiments is shown. Apparent half-maximal activation constants K₀.₅ (see Table II) were obtained from a fit of a Michaelis-Menten-type function to the data (superimposed lines).

**Figure 4.** Conformational E₁P/E₂P distribution and pump currents of β-N-terminally truncated Na,K-ATPase.

A: Partial amino acid sequences of the N-terminally deleted Na,K-ATPase β-variant NaKβ₁Δ8 and the wildtype sheep Na,K-ATPase β₁-subunit (in black). For comparison, the wildtype and N-terminally truncated β-variants of the rat H,K-ATPase are also shown (in grey).

B: Voltage-dependent distribution of fluorescence amplitudes 1-ΔF/F for Na,K-ATPase complexes consisting of the sheep wildtype α₁-subunit and either the unmodified reporter construct β₁S62C (■), or the N-terminally deleted Na,Kβ-variant β₁S62CΔ8 (○). The same voltage-protocol as in Figure 2 B-D was applied in 100 mM [Na⁺] / K⁺-free solution at pH 7.4. Data are means ± S.D. of 10-15 oocytes, normalized to saturating values at -180 mV after subtracting the values for -60 mV. A curve corresponding to the fit of a Boltzmann function is superimposed, the resulting fit parameters V₀.₅ and zₗ are listed in Table III. Inset: Stationary pump currents of the two constructs at saturating K⁺ concentrations (10 mM). Data are means ± S.D. of 10-15 oocytes.
### TABLE I

Parameters from fits of a Boltzmann function to \((1-\Delta F/F)/V\) distributions of N-terminally deleted H,K-ATPase \(\beta\)-mutants (Figure 2 C).

| Boltzmann parameter \((1-\Delta F/F)/V\)-curves | \(V_{0.5}\) (mV) | \(z_q\) |
|-----------------------------------------------|------------------|-------|
| H,K\(\alpha\)S806C/\(\beta\)WT               | -110.1 ± 5.0     | 0.33 ± 0.01 |
| H,K\(\alpha\)S806C/\(\beta\)A4               | -115.3 ± 5.8     | 0.31 ± 0.01 |
| H,K\(\alpha\)S806C/\(\beta\)A8               | -108.7 ± 4.3     | 0.32 ± 0.02 |
| H,K\(\alpha\)S806C/\(\beta\)A13              | -56.3 ± 6.3      | 0.34 ± 0.02 |
| H,K\(\alpha\)S806C/\(\beta\)A29              | -89.9 ± 4.3      | 0.32 ± 0.01 |

Values are means ± S.E. of 15-30 oocytes from 2-3 different oocyte batches.

### TABLE II

Rb\(^+\) uptake activity, SCH28080 sensitivity and apparent Rb\(^+\) affinity of N-terminally deleted H,K-ATPase \(\beta\)-mutants (see Figure 3).

| Normalized Rb\(^+\) uptake activity (in %)\(^a\)\(^b\) | Residual Rb\(^+\) uptake activity in presence of SCH28080 (in %)\(^a\)^c | Apparent Rb\(^+\) affinity\(^d\) |
|-----------------------------------------------------|---------------------------------------------------------------------|------------------------|
| 5 mM RbCl                                           | 10 \(\mu\)M SCH28080                                              | 100 \(\mu\)M SCH28080 |
| H,K\(\alpha\)S806C/\(\beta\)WT                      | 100 ± 3.9                                                         | 21 ± 2.0               | 6 ± 0.7                | 0.66 ± 0.09 |
| H,K\(\alpha\)S806C/\(\beta\)A4                      | 96 ± 3.3                                                          | 21 ± 1.7               | 14 ± 1.8              | 0.62 ± 0.14 |
| H,K\(\alpha\)S806C/\(\beta\)A8                      | 95 ± 4.4                                                          | 18 ± 2.0               | 13 ± 2.3              | 0.70 ± 0.18 |
| H,K\(\alpha\)S806C/\(\beta\)A13                     | 77 ± 4.0                                                          | 28 ± 2.3               | 25 ± 2.6              | 0.65 ± 0.12 |
| H,K\(\alpha\)S806C/\(\beta\)A29                     | 83 ± 3.8                                                          | 27 ± 2.2               | 20 ± 1.7              | 0.52 ± 0.10 |
| uninjected                                         | 4 ± 0.4                                                           | -                      | -                     | -            |

\(^a\) Values are means ± S.E. of 40-50 oocytes from 3-4 different oocyte batches

\(^b\) Data were normalized to Rb\(^+\) uptake of H,K\(\alpha\)S806C/\(\beta\)WT, corresponding to 32 pmol min\(^{-1}\)/oocyte

\(^c\) Data were normalized to Rb\(^+\) uptake at 5 mM RbCl in absence of SCH28080 for each construct after subtraction of the mean Rb\(^+\) uptake of uninjected oocytes

\(^d\) Values are means ± S.E. of 10-20 oocytes

### TABLE III

Stationary pump currents and Parameters from fits of a Boltzmann function to \((1-\Delta F/F)/V\) distributions of \(\beta\)-N-terminal truncated Na,K-ATPase (Figure 4).

| Boltzmann parameter \((1-\Delta F/F)/V\)-curves | Stationary pump currents (nA) at 10 mM KCl |
|-----------------------------------------------|------------------------------------------|
| \(V_{0.5}\) (mV) | \(z_q\) |                                      |
| Na,K\(\alpha\)WT/\(\beta\)S62C                 | -92.7 ± 7.7                                | 221 ± 85                |
| Na,K\(\alpha\)WT/\(\beta\)S62C\(\Delta\)8      | -100.8 ± 7.5                               | 232 ± 38                |

Values are means ± S.D. of 10-15 oocytes.
Figure 1

A

ADP-\( (H^+) \) + EP1 \( \rightarrow \) ADP-E.P(H\( ^+ \))
Phosphorylation

Intracellular space
(pH ~ 7.4)

ATP-E\( _1 \) \( \rightarrow \) ADP-E.P(H\( ^+ \))

ATP-E\( _1 \)-H\( ^+ \) \( \rightarrow \) ADP-E.P(H\( ^+ \))

ATP-E\( _1 \)-K\( ^+ \) \( \rightarrow \) ATP

K\( ^+ \)

ATP

K\( ^+ \)

ATP-E\( _1 \)-H\( ^+ \) \( \rightarrow \) ADP-E.P(H\( ^+ \))

ATP-E\( _1 \)-H\( ^+ \)

E2(K\( ^+ \))

Dephosphorylation

E2 P-H\( ^+ \)

E2 P-K\( ^+ \)

ATP-

K\( ^+ \)

lumenal space
(pH ~ 1)

B

N-domain

A-domain

P-domain

Cytoplasm

Membrane

Lumen

β-subunit

N-terminus

TMRM-binding site

TM3

β-subunit

β-subunit

β-subunit
Figure 2

**Figure 2A** N-terminal truncated variants:

| Variant | Sequence |
|---------|----------|
| WT      | HKASQGK| |
| Δ14     | HKASQG| |
| Δ18     | HKASQG| |
| Δ29     | HKASQG| |

**Figure 2B**

Fluorescence increase

**Figure 2C**

Voltage (mV)

**Figure 2D**

Voltage (mV)

**Figure 2E**

Voltage (mV)

**Figure 2F**

PP HKα TP HKα
Figure 3

A

B

[Graph showing Rb$^+$ uptake in presence of SCH28080]

Figure 3

A

B

[Graph showing Rb$^+$ uptake with different constructs and [RbCl]]
Figure 4

A

HK\text{WT} M\text{AALQEKKSCSQRMABFRQYCWNPDQMLGRTPARWVWTM}
HK\text{A4} M\text{QEKSCSQRMABFRQYCWNPDQMLGRTPARWVWTM}
HK\text{A8} M\text{SCSQRMABFRQYCWNPDQMLGRTPARWVWTM}
HK\text{A13} M\text{AEFRQYCWNPDQMLGRTPARWVWTM}
HK\text{A29} M\text{LGRTPARWVWTM}
NaK\text{WT} M\text{ARGKAKEEGSWKKFIWNSEKKEFLGRTGGSFWFVWTM}
NaK\text{A8} M\text{GSSKKFIWNSEKKEFLGRTGGSFWFVWTM}

TM = transmembrane domain

B

Stationary currents (nA)

\text{Voltage (mV)}

\begin{align*}
&\text{Na,K-ATPase } \alpha_1\text{WT}\|\beta_{S62C} \\
&\text{Na,K-ATPase } \alpha_1\text{WT}\|\beta_{S62C}\Delta 8
\end{align*}
E2P-state stabilization by the N-terminal tail of the H,K-ATPase β-subunit is critical for efficient proton pumping under in vivo conditions
Katharina L. Dürr, Kazuhiro Abe, Neslihan N. Tavraz and Thomas Friedrich
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