Deceleration of the $E_1P$-$E_2P$ Transition and Ion Transport by Mutation of Potentially Salt Bridge-forming Residues Lys-791 and Glu-820 in Gastric H$^+$/K$^+$-ATPase*§

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A lysine residue within the highly conserved center of the fifth transmembrane segment in P$\text{II}_\text{C}$-type ATPase $\alpha$-subunits is uniquely found in H,K-ATPases instead of a serine in all Na,K-ATPase isoforms. Because previous studies suggested a prominent role of this residue in determining the electro-kinetic activity of non-gastric H,K-ATPase and in $E_2p$ modulation of the proton-translocating residues in the gastric H,K-ATPases as well, we investigated its functional significance for ion transport by expressing several Lys-791 variants of the gastric H,K-ATPase in Xenopus oocytes. Although the mutant proteins were all detected at the cell surface, none of the investigated mutants displayed any measurable K$^+$-induced stationary currents. In Rb$^+$ uptake measurements, replacement of Lys-791 by Arg, Ala, Ser, and Glu substantially impaired transport activity and reduced the sensitivity toward the $E_2$-specific inhibitor SCH28080. Furthermore, voltage clamp fluorometry using a reporter site in the TM5/TM6 loop for labeling with tetramethylrhodamine-6-maleimide revealed markedly changed proton pumping by expressing several Lys-791 variants of the gastric H,K-ATPase in Xenopus oocytes. The electrogenicity during the $E_1P$-$E_2P$ reaction of reversed electrogenicity in the K$^+$-branch of the cycle is most likely counterbalanced by another partial reaction of reversed electrogenicity in the K$^+$-branch to bring about net electroneutrality.

Despite these common features, there are also important differences between the two enzymes. For example, the asymmetrical transport stoichiometry of Na$^+$ and K$^+$ exchange (3 versus 2) by all known Na,K-ATPase isoforms results in a net electrogenic transport (2), whereas gastric and non-gastric H,K-ATPases operate strictly electroneutral with 2:2 (or 1:1) stoichiometry (3, 4). Although the transport cycle of the H,K-ATPase is net electroneutral, evidence has been accumulated that several partial reactions of the cycle are electrogenic. Electrophysiologic experiments using purified H$_2$K$_{-}$ATPase-containing membrane fragments on planar lipid bilayers (5, 6) have shown that the proton branch of the cycle, which involves the $E_1P$-$E_2P$ conformational change investigated in the current study, includes an electrogenic step. As it is the case for the electrogenic Na,K-ATPase, the $E_1P$-$E_2P$ distribution might be driven by the redistribution of cations within intracellularly oriented high-field access channels to the transport sites. The electrogenicity during the H$^+$ branch of the cycle is most likely counterbalanced by another partial reaction of reversed electrogenicity in the K$^+$ branch to bring about net electroneutrality (7).

Notably, mutagenesis studies have shown that Na,K-ATPase and the gastric H,K-ATPase belong to the P$\text{II}_\text{C}$-subgroup of the extensive class of P-type ATPases, which use ATP hydrolysis for active transport of cations. The reversible phosphorylation of a highly conserved Asp residue, a hallmark of all P-type ATPases, is uniquely found in H,K-ATPases instead of a serine in all Na,K-ATPase isoforms. Because previous studies suggested a prominent role of this residue in determining the electrokinetic activity of non-gastric H,K-ATPase and in $E_2p$ modulation of the proton-translocating residues in the gastric H,K-ATPases as well, we investigated its functional significance for ion transport by expressing several Lys-791 variants of the gastric H,K-ATPase in Xenopus oocytes. Although the mutant proteins were all detected at the cell surface, none of the investigated mutants displayed any measurable K$^+$-induced stationary currents. In Rb$^+$ uptake measurements, replacement of Lys-791 by Arg, Ala, Ser, and Glu substantially impaired transport activity and reduced the sensitivity toward the $E_2$-specific inhibitor SCH28080. Furthermore, voltage clamp fluorometry using a reporter site in the TM5/TM6 loop for labeling with tetramethylrhodamine-6-maleimide revealed markedly changed fluorescence signals. All four investigated mutants exhibited a strong shift toward the $E_1P$ state, in agreement with their reduced SCH28080 sensitivity, and an about 5–10-fold decreased forward rate constant of the $E_1P$ ↔ $E_2P$ conformational transition, thus explaining the $E_1P$ shift and the reduced Rb$^+$ transport activity. When Glu-820 in TM6 adjacent to Lys-791 was replaced by non-charged or positively charged amino acids, severe effects on fluorescence signals and Rb$^+$ transport were also observed, whereas substitution by aspartate was less disturbing. These results suggest that formation of an $E_2P$-stabilizing interhelical salt bridge is essential to prevent futile proton exchange cycles of H$^+$ pumping P-type ATPases.

The ubiquitous Na,K-ATPase and the gastric H,K-ATPase belong to the P$\text{II}_\text{C}$ subgroup of the extensive class of P-type ATPases, which use ATP hydrolysis for active transport of cations. The reversible phosphorylation of a highly conserved Asp residue, a hallmark of all P-type ATPases, is uniquely found in H,K-ATPases instead of a serine in all Na,K-ATPase isoforms. Because previous studies suggested a prominent role of this residue in determining the electrokinetic activity of non-gastric H,K-ATPase and in $E_2p$ modulation of the proton-translocating residues in the gastric H,K-ATPases as well, we investigated its functional significance for ion transport by expressing several Lys-791 variants of the gastric H,K-ATPase in Xenopus oocytes. Although the mutant proteins were all detected at the cell surface, none of the investigated mutants displayed any measurable K$^+$-induced stationary currents. In Rb$^+$ uptake measurements, replacement of Lys-791 by Arg, Ala, Ser, and Glu substantially impaired transport activity and reduced the sensitivity toward the $E_2$-specific inhibitor SCH28080. Furthermore, voltage clamp fluorometry using a reporter site in the TM5/TM6 loop for labeling with tetramethylrhodamine-6-maleimide revealed markedly changed fluorescence signals. All four investigated mutants exhibited a strong shift toward the $E_1P$ state, in agreement with their reduced SCH28080 sensitivity, and an about 5–10-fold decreased forward rate constant of the $E_1P$ ↔ $E_2P$ conformational transition, thus explaining the $E_1P$ shift and the reduced Rb$^+$ transport activity. When Glu-820 in TM6 adjacent to Lys-791 was replaced by non-charged or positively charged amino acids, severe effects on fluorescence signals and Rb$^+$ transport were also observed, whereas substitution by aspartate was less disturbing. These results suggest that formation of an $E_2P$-stabilizing interhelical salt bridge is essential to prevent futile proton exchange cycles of H$^+$ pumping P-type ATPases.

*§This work was supported by the Deutsche Forschungsgemeinschaft (SFB 740 and Cluster of Excellence Unifying Concepts in Catalysis). The online version of this article (available at http://www.jbc.org) contains supplemental Appendices A and B. Present address: Vollum Institute, Oregon Health and Science University, 3181 SW Sam Jackson Park Rd., Portland, OR 97239. To whom correspondence should be addressed. Tel.: 503-494-6721; Fax: 503-494-1700; E-mail: duerrr@ohsu.edu.

2 The abbreviations used are: TM, transmembrane domain; TMRM, tetramethylrhodamine-6-maleimide; VCF, voltage clamp fluorometry.
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(17–20) under sufficiently steep ion gradients and high ADP/ATP ratios in presence of P.

Notably, all these unique H,K-ATPase properties have been linked to the presence of a lysine residue in the fifth transmembrane segment of the catalytic α-subunit (Lys-791 in gastric H,K-ATPase (shown in blue in Fig. 1 and in the sequence alignments in Fig. 9). This lysine in the otherwise highly conserved (K/S)NIPEIT sequence motif is replaced by an uncharged serine in Na,K-ATPases (see P-type ATPase alignments in Fig. 9). Remarkably, it is the only positively charged amino acid in the whole TM region of the H,K-ATPase α-subunit. Homology modeling of the cation binding pocket based on the SERCA (sarcoplasmic reticulum calcium ATPase) structure in the $E_1$-state together with mutagenesis studies have predicted an $E_2$-conformation-specific salt bridge between the side chains of Lys-791 (in TM5) and Glu-820 (in TM6, highlighted in brown in Figs. 1 and 9) of the cation binding pocket (21). Koenderink et al. (21) concluded that this interhelical interaction could contribute to the inherent preference of the gastric H,K-ATPase, which in turn could be relevant for preventing a backward running of the pump (see “Discussion”). Moreover, molecular dynamics simulations by Munson et al. (22) have shown that the positively charged side chain of Lys-791 probably reorients during the $E_1P \leftrightarrow E_2P$ transition as a consequence of a change in the relative positions of the TM5 and TM6 helices. The reorientation may move the NH$_3^+$ group of the side chain closer to the plane of the ion binding site in $E_2P$ (Fig. 1), thereby lowering the effective pK$_a$ of the putative H$_3$O$^+$-coordinating carboxylates. This change in pK$_a$ could enable proton release at low luminal pH (22, 23).

Apart from this proposed function as a pK$_a$-modulating molecular device, Lys-791 is possibly also a major determinant for the electroneutral transport mode characteristic for H,K-ATPases, as suggested by mutagenesis studies on the non-gastric H,K-ATPase expressed in Xenopus oocytes. Upon mutation of the corresponding Lys-800 to Ala or Glu, the normally electroneutral Na$^+$/K$^+$ exchange of wild-type toad bladder H,K-ATPase (sometimes also termed X,K-ATPase to differentiate it from the “gastric” H,K-ATPase) becomes electrogenic, thus, generating positive pump currents in the presence of extracellular K$^+$. Vice versa, a charge-introducing mutation of the respective serine to a positively charged arginine in toad Na,K-ATPase resulted in a loss of pump currents with minor effects on Rb$^+$ transport activity (24). However, the interpretation of these ion transport studies on the non-gastric H,K-ATPase was complicated by the fact that both Na$^+$ and H$^+$ ions are transported in exchange for K$^+$ and that the H$^+$/Na$^+$ exchange stoichiometry is not known. Because the non-gastric H,K-ATPase is more similar to Na,K-ATPases in terms of transported ions (25, 26) and the sensitivity towards ouabain (27) and SCH28080 (28, 29), the implications of these findings for the gastric enzyme are unclear so far.

These limitations prompted us to investigate the functional relevance of Lys-791 for electroneutral transport of a bona fide H$^+$-transporting H,K-ATPase, the rat gastric H,K-ATPase expressed in Xenopus oocytes. We took advantage of a variant gastric H,K-ATPase α-subunit with a single cysteine replacement S806C in the extracellular TM5/TM6 loop (see Fig. 1) that enabled us to study several Lys-791 mutants also by voltage clamp fluorometry (VCF) upon site-specific labeling with tetramethylrhodamine-6-maleimide (TMRM), as described previously (30–32). In addition to the information regarding steady-state transport, which was provided by Rb$^+$ uptake measurements, this method allows the study of voltage-dependent conformational changes under presteady-state conditions. Moreover, VCF not only reveals the voltage-dependent distribution between $E_1P/E_2P$ states but also the kinetics of the $E_1P \leftrightarrow E_2P$ conformational transition.

**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 TM5/TM6 extracellular loop (S806C, see Fig. 1) were subcloned into vector pTLN (33). This cysteine replacement is homologous to the N790C mutation in the TM5/TM6 loop of the Na,K-ATPase α-subunit (34, 35). It has been shown previously that the S806C mutation enables site-specific labeling of H,K-ATPase with the environmentally sensitive fluorophore...
TMRM, and rubidium uptake measurements confirmed that the αS806C mutation did not affect the transport properties of gastric H,K-ATPase (32). Additional amino acid replacements at positions Lys-791 and Glu-820 were introduced into the αS806C reference construct (which is referred as “wild-type” or WT throughout the current study) using the QuikChange XL site-directed mutagenesis kit (Agilent Technologies) and verified by DNA sequencing (Eurofins MWG Operon).

Xenopus oocytes were obtained by collagenase treatment after partial ovariectomy from Xenopus laevis females. cRNAs were prepared using the SP6 mMessage mMachine kit (Applied Biosystems). A 50-nl aliquot containing 20–25 ng of H,K-ATPase α-subunit cRNA and 5 ng of H,K-ATPase β-subunit was injected into each cell. After injection, oocytes were kept in ORI buffer (110 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 5 mM NiCl₂, 10 mM MES, pH 5.5) and 1 wash in water, each individual oocyte was homogenized in 1 ml of Millipore water. For inhibition experiments, the respective oocytes were injected with 50 nl of a solution containing 100 μM ouabain (Sigma) to inhibit the endogenous Xenopus Na,K-ATPase.

Rb⁺ Uptake Measurements—Oocytes were incubated for 15 min in Rb⁺ flux buffer (5 mM RbCl, 85 mM tetramethylammonium chloride, 20 mM tetraethylammonium chloride, 5 mM BaCl₂, 5 mM NiCl₂, 10 mM MES, pH 5.5, 100 μM ouabain). After 3 washing steps in Rb⁺-free washing buffer (90 mM tetramethylammonium chloride or NaCl, 20 mM tetraethylammonium chloride, 5 mM BaCl₂, 5 mM NiCl₂, 10 mM MES, pH 5.5) and 1 wash in water, each individual oocyte was homogenized in 1 ml of Millipore water. For inhibition experiments, the K⁺-competitive inhibitor SCH28080 (Sigma) was added to the preincubation solution and Rb⁺ flux buffer (to final concentrations as indicated by the respective figure legends to Figs. 3, 6, and 8). For vanadate inhibition experiments, the respective oocytes were injected with 50 nl of a solution containing 100 mM sodium orthovanadate (buffered with 10 mM Hepes at pH 7.4) ~30–40 min before Rb⁺ uptake measurements. Assuming an oocyte volume of about 1 μl, this corresponds to a final intracellular concentration of ~5 mM. Whereas the inhibitor vanadate interacts specifically with the E₂ conformational state, SCH28080 is specific for both E₂ and E₃P conformations (36–38). Therefore, testing the inhibition efficiency by these compounds can be used to probe conformational preferences of H,K-ATPase variants (38, 39).

Atomic Absorption Spectrometry—Aliquots of 20 μl from oocyte homogenates were analyzed by atomic absorption spectrometry using an AAnalyst800™ spectrometer (PerkinElmer Life Sciences) 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 rubidium 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/liter 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 Dür et al. (31). The polyclonal antibody HK12.18 (Merck) (40) was used for detection of gastric H,K-ATPase α-subunit. We have shown previously that the plasma membrane fraction obtained by this procedure does not contain protein from intracellular membranes, as the H,K-ATPase α-subunit was not detected in this fraction when it had been co-expressed without β-subunit, although the α-subunit protein was clearly present in the total cellular membrane fraction (32). Furthermore, this study also demonstrated that the characteristic glycosylation pattern of the H,K-ATPase β-subunit present in isolated plasma membranes is another supportive indicator for the purity of the preparation (see Fig. 2A in Ref. 32).

Voltage Clamp Fluorometry—Site-specific labeling of H,K-ATPase-expressing oocytes was achieved by incubating oocytes in HK₇.₄ buffer (90 mM NaCl, 20 mM tetraethylammonium chloride, 5 mM BaCl₂, 5 mM NiCl₂, 10 mM MOPS/Tris, pH 7.4) containing 5 μM TMRM (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 HK₅.₅ buffer (90 mM NaCl, 20 mM tetraethylammonium chloride, 5 mM BaCl₂, 5 mM NiCl₂, 10 mM MES/Tris, pH 5.5, 100 μM ouabain). Due to the aforementioned electrogenicity of the H⁺ branch of the catalytic cycle, characteristic fluorescence changes are observed in response to voltage pulses under these conditions, reflecting the voltage dependence of the E₃P-E₃P conformational transition (30–32). Several control measurements demonstrating the specificity of the voltage jump-induced fluorescence signals are presented in Fig. 2. Details on setup components, data acquisition, and analysis are given in Dür et al. (31). For analysis of the voltage-dependent fluorescence signals, the fluorescence data traces were fitted with a single exponential function, and the stationary amplitudes from the fits were plotted against the membrane potential to be approximated with a Boltzmann-type function,

\[
F(V) = F_{\text{min}} + \frac{F_{\text{max}} - F_{\text{min}}}{1 + \exp\left(\frac{z q (V - V_{0.5})}{R T}\right)} \tag{1}
\]

in which \(F_{\text{min}}\) and \(F_{\text{max}}\) are the minimal and maximal fluorescence amplitude from the fit, \(z_q\) is the equivalent charge (slope factor), \(V_{0.5}\) is the half-maximal voltage, \(F\) is the Faraday constant, \(R\) is the molar gas constant, and \(T\) is the absolute temperature in K. The limited voltage range accessible in two-electrode voltage clamp experiments on oocytes together with the low voltage dependence of the H,K-ATPase did not allow direct determination of the saturation values of the voltage-dependent fluorescence amplitudes. However, already the slight deviations of the voltage-dependent distributions from linearity impose sufficient constraints on fits of a Boltzmann function to the data that consistent saturation values are obtained independent of the choice of start parameters. This is also valid for data sets, which cover the Boltzmann distribu-
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FIGURE 2. Control measurements on TMRM-labeled oocytes expressing construct αS806C, demonstrating the specificity of the voltage-jump induced fluorescence changes. A–D, shown are fluorescence signals of TMRM-labeled oocytes in response to voltage jumps from a holding potential of −40 mV to values between −180 and −60 mV in 20-mV increments. A and B, shown are fluorescence signals of an individual oocyte expressing the αS806C construct together with the H,K-ATPase β-subunit before (A) and after (B) the addition of the specific inhibitor SCH28080 (100 μM). C and D, shown are voltage jump-induced fluorescence responses from two oocytes from the same batch, which were either injected with the cRNA of the αS806C construct alone (D) or co-injected with the H,K-ATPase β-subunit (C). E, shown is normalized cell fluorescence of TMRM-labeled oocytes, which were either un.injected or expressed the H,K-ATPase αS806C construct together with the wild-type β-subunit. Data were obtained from three batches of cells, and the fluorescence intensity was normalized to the mean fluorescence of un injected oocytes in each batch. For the measurement of cellular fluorescence, all cells from each batch of oocytes were placed into the perfusion chamber of the experimental microscope and illuminated with constant excitation light intensity. A constant area of interest was chosen for all cells using a circular iris aperture that allowed measurement of the fluorescence of about 90% of the illuminated cell surface. a.u., arbitrary units.

FIGURE 3. Rb⁺ uptake and cell surface expression of Xenopus oocytes expressing H,K-ATPase wild-type or Lys-791 variants. A, H,K-ATPase-mediated Rb⁺ uptake at 5 mM RbCl in the absence (hatched bars) or presence (black bars) of 10 μM SCH28080 is shown. Results from uninjected control oocytes, oocytes injected with the reference construct HKαS806C/βWT, or constructs with the indicated Lys-791 point mutations are shown. Rb⁺ uptake was measured on individual cells by atomic absorption spectrometry (see “Experimental Procedures”). Data are the means ± S.E. from two individual experiments with 15–20 oocytes each. B, shown is Western blot analysis of plasma membrane (PP, upper panel) and total membrane fractions (TP, lower panel) isolated from H,K-ATPase-expressing oocytes. Detection used anti-H,Kα antibody HK12.18. One representative Western blot is shown. C, densitometric analysis is shown of Western blot bands from corresponding total membrane or plasma membrane fraction preparations as shown in B, normalized to the band intensity of the TP fraction of the WT protein. Data are expressed as the means ± S.D. from four to five experiments using oocytes from different Xenopus females. a.u., arbitrary units.

Homology Model of the Rat Gastric H,K-ATPase in the E₂ State—A rough molecular model of the gastric H,K-ATPase structure in the E₂ state was built using the crystal structure of the pig renal Na,K-ATPase (PDB structure entry 3B8E) in the K⁺-occluded E₂ state (41) to illustrate the location of re-
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A

-180 mV

-40 mV

+60 mV

+40 mV

500 msec

\( \frac{\Delta F}{F} = 1\% \)

WT

B

C

-40 mV

-180 mV

+60 mV

+40 mV

500 msec

\( \frac{\Delta F}{F} = 1\% \)

K791S

K791A

F

-200 mV

-40 mV

+40 mV

+40 mV

2 sec

\( \frac{\Delta F}{F} = 0.1\% \)

K791E

K791R

H

\( \mu \) (sec)

Voltage (mV)

\( \sigma \)

I

J

\( z_i = 0.30 \pm 0.02 \)

\( V_{0.5} = -127.3 \pm 8.2 \) mV

K791S

\( z_i = 0.43 \pm 0.05 \)

\( V_{0.5} = +36.2 \pm 14.7 \) mV

K791S

K791A

\( z_i = 0.54 \pm 0.07 \)

\( V_{0.5} = +23.9 \pm 12.8 \) mV

K791A

K791E

\( z_i = 0.67 \pm 0.08 \)

\( V_{0.5} = -18.0 \pm 6.1 \) mV

K791E

K791R

\( z_i = 0.38 \pm 0.05 \)

\( V_{0.5} = 26.8 \pm 24.0 \) mV

K791R
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RESULTS

Rb+ Uptake and Cell Surface Expression of Lys-791 Mutants—Because mutation of Lys-791 in the non-gastric H,K-ATPase produced interesting effects on pump stoichiometry resulting in electrogenic pump activity, we first examined whether the corresponding amino acid replacements in rat gastric H,K-ATPase would give rise to K+ -induced pump currents upon expression in Xenopus oocytes. However, we were unable to measure any K+ -induced pump currents for all investigated Lys-791 mutants (data not shown). Therefore, we also assessed the Rb+ uptake activity of these mutants under saturating Rb+ concentrations (5 mM) by atomic absorption spectrometry. As shown in Fig. 3A, all Lys-791 variants (including the charge-conserving K791R mutant) exhibited substantially reduced transport activities in Rb+ uptake measurements, reaching only about 15–30% of the wild-type activity. Of note, the Lys-791 mutants also displayed a largely reduced sensitivity toward the E2-specific H,K-ATPase inhibitor SCH28080 (37), in agreement with previous observations on crude membrane preparations from HEK293 cells expressing the K791A mutant gastric H,K-ATPase (45). Unfortunately, however, because of the low Rb+ uptake activities of these mutants, a more detailed quantification of the effect (e.g. determination of IC50 values) was impossible.

Because none of the previous studies on Lys-791 variants addressed the question of whether the plasma membrane targeting of the H,K-ATPase mutants is affected, we had to exclude that the reduced Rb+ uptake activities could be a simple consequence of an impaired cell surface delivery. Western blot analysis of isolated plasma membranes from Xenopus oocytes expressing these Lys-791 variants showed similar protein levels for all Lys-791 mutants compared with the wild-type, except for the charge-inverting K791E mutant, for which the amount of protein in plasma membrane fractions was reduced (Fig. 3, B, upper panel, and C). However, the reduced cell surface expression of the K791E protein cannot fully account for the strongly reduced Rb+ uptake of this mutant, and also for the other Lys-791 mutants the strongly reduced Rb+ transport activity cannot be due to impaired plasma membrane targeting.

E1P/E2P Conformational Distribution and Kinetics of E1P ↔ E2P Transition of Lys-791 Mutants—To investigate whether these mutations influence the kinetics or voltage dependence of the E1P/E2P conformational distribution, we inserted the Lys-791 mutations into the backbone of H,K-ATPase mutant S806C, which carries a reporter cysteine for site-specific fluorescence labeling. This strategy enabled us to measure conformation-dependent fluorescence changes of mutant H,K-ATPases in response to voltage pulse protocols (insets in Fig. 4 A and F). Notably, all Lys-791 mutants showed substantially altered fluorescence signals compared with the wild-type pump (Fig. 4, A–G). Voltage jumps to hyperpolarizing potentials, which drive the enzyme into the E2P state, resulted in smaller relative fluorescence changes for all four mutants compared with the wild-type (Fig. 4A), whereas the fluorescence responses induced by jumps to positive potentials (favoring conversion to E1P) were larger and profoundly slower than the corresponding signals of the wild-type protein. A noticeable feature of the fluorescence changes observed for these variant pumps is the presence of small sharp peaks that occur immediately after an abrupt change in the membrane potential (for both on and off pulses, see the small arrows in Fig. 4, B and C). Most likely, this peak reflects a rapid initial change in the microenvironment of the fluorophore that cannot be resolved due to the limited time resolution in two-electrode voltage clamp experiments.

This environmental change, which is not observed in the S806C wild-type H,K-ATPase, is possibly a specific consequence of the charge-neutralizing Lys-791 mutations in the vicinity of the TMRM labeling site S806C in the TM5/TM6 loop (see Fig. 1). Of note, this effect was much less pronounced for replacements by charged amino acids (K791E and K791R in Fig. 4, F and G).

The above described alterations in the fluorescence signals of the Lys-791 mutants indicate a conformational shift toward the E2P-state, which can be further quantified by plotting the steady-state amplitudes (from monoexponential fits to the fluorescence changes) at each membrane potential (Fig. 4, I–L). In fact, the resulting Boltzmann curves of all investigated mutants are strongly shifted to more positive potentials compared with the wild-type (V0.5 values from fits of a Boltzmann function to the shown curves are given in each panel of Fig. 4, I–L). Interestingly, the Boltzmann parameters obtained for the two charge-neutralizing amino acid replacements (K791S and K791A) and the charge-inverting K791E mutant showed substantially higher slope factors zq (values between 0.43 and 0.67 compared with ~0.30 for the reference construct S806C), whereas this parameter was not significantly changed for the charge-conserving K791R variant. The higher zq values for the former mutants hint at an increased voltage dependence of the E1P ↔ E2P conformational transition.
more detailed kinetic analysis of the Lys-791 mutants (see supplemental Appendix B for details) revealed that the increased \( z_4 \) values of these mutants can be directly attributed to a significantly increased voltage dependence \( z_4 \) of the forward rate constant \( k_f \) for the \( E_1 P \rightarrow E_2 P \) conformational transition (see calculated \( z_4 \) and \( z_2 \) values in Table 1).

The aforementioned changes in the time course of the fluorescence signals at jumps to positive membrane potentials likely reflect severely altered kinetics of the \( E_1 P \rightarrow E_2 P \) conformational transition. To quantify the effect, we also determined the voltage-dependent reciprocal time constants from fits of a single exponential function to the fluorescence traces (Fig. 4H). This panel shows that for all investigated Lys-791 mutants the reciprocal time constants were significantly smaller than the wild-type values. Although the whole voltage range is affected, the most pronounced reductions of the \( 1/\tau \) values occur at positive potentials, which readily explains the strong shifts of the \( E_1 P/E_2 P \) conformational distribution toward the \( E_1 P \) state (as follows). The observed reciprocal time constants \( 1/\tau \) are the sum \( (k_{\text{on}}) \) of the rate constants for the forward \( (k_f) \) and the backward reaction \( (k_b) \) of the \( E_1 P \leftrightarrow E_2 P \) conformational transition. Therefore, the individual contributions of the forward and reverse rate constant can be calculated at each membrane potential using a simple two-state kinetic model to describe the \( E_1 P \leftrightarrow E_2 P \) conformational transition (for details, see supplemental Appendix A). As shown in Table 1, the rate constants \( k_f (E_1 P \rightarrow E_2 P, \text{ red squares in Fig. 5, B–E}) \) obtained by this procedure are between 5- and 20-fold decreased for all four Lys-791 variants compared with the wild-type (red squares in Fig. 5A). In contrast, the reverse rate constants \( k_b (E_1 P \rightarrow E_2 P, \text{ blue squares in Fig. 5, B–E}) \) are much less affected by the mutations, with the remarkable exception of mutant K791R, which showed a strongly reduced \( k_b \) as well. Notably, for all Lys-791 variant pumps, the observed shift toward \( E_2 P \) (destabilization of \( E_2 P \)) is the consequence of a reduced rate constant for \( E_2 P \) formation but not an accelerated \( E_2 P \) decay toward \( E_1 P \).

Importantly, the forward rate constant \( k_f \) of the \( E_1 P \leftrightarrow E_2 P \) conformational transition might be rate-limiting for steady-state ion transport of the gastric H,K-ATPase under saturating K\(^{+}\)/Rb\(^{+}\) concentrations.\(^3\) Accordingly, the markedly decreased forward rate constant of the \( E_1 P \leftrightarrow E_2 P \) conformational transition rationalizes the substantially reduced Rb\(^{+}\) uptake activity of all Lys-791 substitutions (Fig. 3A).

**TABLE 1**

Calculated \( z_4 \) values for the forward \( (k_f) \) and reverse \( (k_b) \) reaction of the \( E_1 P \rightarrow E_2 P \) conformational transition (see supplemental Appendix B for details) and their sum \( z_{\text{tot}} \) in comparison to the experimentally obtained values \( z_4 \)

| Construct | \( z_4 \) from Boltzmann fits to \( (1−\Delta F)/V \) distributions | \( z_4 \) from Boltzmann fits to \( (1−\Delta F)/V \) distributions |
|-----------|---------------------------------------------------------------|---------------------------------------------------------------|
| WT        | \( 0.067 \pm 0.006 \)                                      | \( 0.315 \pm 0.029 \)                                      |
| K791S     | \( 0.158 \pm 0.011 \)                                      | \( 0.422 \pm 0.032 \)                                      |
| K791A     | \( 0.183 \pm 0.019 \)                                      | \( 0.463 \pm 0.056 \)                                      |
| K791E     | \( 0.238 \pm 0.033 \)                                      | \( 0.587 \pm 0.086 \)                                      |
| K791R     | \( 0.200 \pm 0.004 \)                                      | \( 0.339 \pm 0.034 \)                                      |
| E820D     | \( 0.087 \pm 0.004 \)                                      | \( 0.296 \pm 0.015 \)                                      |
| E820K     | \( 0.001 \pm 0.023 \)                                      | \( 0.397 \pm 0.070 \)                                      |

Note that the \( z_4 \) values (which describe the voltage dependence of the forward rate constant \( k_f \)) of mutants K791A, K791S, K791E, and K791R are by a factor of 2–4 increased compared to the wild-type, whereas the respective \( z_2 \) values are much less affected by the mutations. Therefore, the observed change in \( z_{\text{tot}} \) of the Boltzmann distributions (shown in Fig. 4, I–L) can be mainly attributed to an increased electrogenicity of the forward rate constant \( k_f \) of the two variant pumps.

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3 K. Dürr and T. Friedrich, unpublished observations.
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FIGURE 5. Calculated voltage dependence of the forward and reverse rate constants of the $E_1P \leftrightarrow E_2P$ conformational transition for wild-type H,K-ATPase and several Lys-791 variants. Shown is calculated voltage dependence of the forward ($k_f$) and reverse ($k_r$) rate constant of the $E_1P:E_2P$ conformational transition in comparison to the experimentally obtained reciprocal time constants ($k_r^{-1}$) from voltage clamp fluorometric measurements (using the H,K-ATPase α806C/βWT as a background) for the wild-type (A) and mutants K791S (B), K791A (C), K791E (D), and K791R (E). Values were calculated using a simple two-state kinetic model; see supplemental Appendix A for details.

Notably, the observed differences in the $k_r$ values are qualitatively in good agreement with the mutants’ reduced Rb$^+$ uptake activities, which are probably rate-limited by the $E_1P \rightarrow E_2P$ partial reaction. Whereas the E820D mutant still retains 50–60% of the wild-type transport activity (Fig. 6C), the charge inversion of the E820K mutant results in a residual activity of ~25% (Fig. 8C).

As shown in Fig. 6, C and D, charge-neutralizing substitutions by glutamine or alanine not only eliminated voltage-induced fluorescence signals but also had strong effects on the enzyme Rb$^+$ uptake activities. An interesting property of these two mutants is revealed by comparing Rb$^+$ uptake measurements at two different extracellular pH values. Whereas Rb$^+$ uptake of the wild-type and E820D mutant are significantly increased upon a change in extracellular pH from 7.4 to 5.5, the behavior is inverted for pumps carrying the mutation E820A or E820Q (compare light gray and gray bars in Fig. 6D).

We have demonstrated recently that the increased Rb$^+$ uptake activity observed for the gastric H,K-ATPase at pH$_{ex}$ = 5.5 is caused by a slight intracellular acidification (~0.5 pH units) as a consequence of the acidic extracellular pH, because a similar pH change of the cell interior, which can be achieved at pH$_{ex}$ = 7.4 by adding 40 mM butyrate to the extracellular solution, results in very similar Rb$^+$ uptake in the case of the wild-type protein (compare light gray and gray-shaded bars in Fig. 6D). The increased availability of intracellular protons at the cytosolic cation binding site presumably accelerates the phosphorylation reaction and the subsequent $E_1P \rightarrow E_2P$ conformational transition, which is rate-limiting for the transport activity at saturating Rb$^+$ concentrations. Notably, this stimulation of Rb$^+$ transport activity by intracellular acidification is maintained for E820D, but it can no longer be observed when Glu-820 is replaced by neutral residues (compare gray and gray-shaded bars in Fig. 6D). Compared with the Rb$^+$ uptake activity observed at pH$_{ex}$ = 7.4 in the presence of butyrate (which generates a small outwardly directed H$^+$ gradient, ΔpH = 0.5), the transport activity of mutant E820Q and E820A is significantly diminished in the presence of a larger, inwardly directed pH gradient at pH$_{ex}$ = 5.4 (~ΔpH 1.5–2). In contrast, the magnitude of the ΔpH has apparently no effect on ion transport of the wild-type and E820D variant pumps. These observations indicate that the charge-neutralizing mutations result in an increased competition of extracellular H$^+$ (or H$_3$O$^+$) with Rb$^+$ ions at the extracellular binding sites. Therefore, Glu-820 could be crucial for determining K$^+$ (hence, also Rb$^+$) selectivity in the $E_2P$ state, which is especially important at steep H$^+$ gradients.

Charge-inverting Lys-791/Glu-820 Amino acid Replacements—A famous example for an interhelical salt bridge in a membrane transport protein, which was initially proposed by biochemical studies (46–48) and later confirmed by x-ray crystallography (49), is residue Asp-237 and residue Lys-358 in the lactose permease LacY from Escherichia coli. Notably, neutral substitutions for either of the two charged residues resulted in transport-defective carriers (46). How-
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Western blot of at least three from different oocyte batches is shown. B, shown is a densitometric analysis of Western blot bands from corresponding total membrane or plasma membrane fraction preparations as shown in A, normalized to the band intensity of the TP fraction of the WT protein. Data are expressed as the means ± S.D. from 2–5 experiments. a.u., arbitrary units. C, H,K-ATPase-mediated Rb⁺ uptake at 5 mM RbCl in the absence (hatched bars) or presence of 100 μM SCH28080 (black bars) is shown. D, H,K-ATPase-mediated Rb⁺ uptake at 5 mM RbCl and pH₇.₄ = 7.₄ (dark gray bars), pH₇.₄ = 5.₅ (light gray bars), or at pH₇.₄ = 7.₄ in the presence of 40 mM butyrate, which causes a slight intracellular acidification (by ~0.5 pH units, hatched dark gray bars), is shown. Results from uninjected control oocytes or oocytes injected with the reference construct HKs8060C/S806C/E820X (X = Ala, Gln, Asp) are shown. Rb⁺ uptake was measured on individual cells by atomic absorption spectrometry (see "Experimental Procedures"). Data are the means ± S.E. from four individual experiments with 15–20 oocytes, normalized to Rb⁺ uptake of the wild-type construct HKs8060C/S806C/E820X (corresponding to 23.9, 22.5, 21.2, and 24.9 pmol/oocyte/min, respectively). Inset, shown is inhibition of Rb⁺ uptake by sodium orthovanadate for oocytes expressing either the wild-type or the E820Q mutant proton pump at 5 mM RbCl and pH 7.₄ in presence of 40 mM butyrate (black bars, see "Experimental Procedures" for details). One representative experiment is shown.

DISCUSSION

In this study we addressed the functional significance of a positively charged lysine in the fifth transmembrane segment ever, inversion of the polarity by mutual exchange of both residues maintained substantial transport activity (47). Similarly, for yeast plasma membrane H⁻/ATPase, single charge-neutralizing mutations of Arg-695 and Asp-730 resulted in defects in folding and biogenesis and reduced ATPase activity, a phenotype that could be overcome when both residues were replaced by neutral amino acids or mutually exchanged (50).

To test whether the inactive phenotypes of the here-investigated charge-neutralizing Lys-791 and Glu-820 mutants could be “rescued” by an analogous mechanism, we characterized the respective charge-inverting K791E/E820K double mutant. Yet, the Rb⁺ uptake of this variant was not significantly higher than that of the single replacements E820K (Fig. 8C) and K791E (Fig. 3A). Again, we confirmed that the pronounced reduction in Rb⁺ uptake activity of these mutants was not due to a reduced cell surface expression (Fig. 8A). Unfortunately, however, the double mutant displayed no discernable fluorescence changes in response to voltage jumps, not even with largely expanded pulse durations. This might imply that (possibly for kinetic reasons) the E₃P/E₄P equilibrium of the K791E/E820K mutant is no longer voltage-sensitive or that the deleterious effects of the single mutations on the conformational dynamics are additive rather than compensatory. Yet, the fact that inversion of the salt bridge polarity does not rescue function does not necessarily exclude that Lys-791 and Glu-820 in the wild-type proton pump interact in an E₃P-stabilizing manner. Apart from the presence of opposite charges, structural constraints regarding side-chain length and geometry might be critical for the interaction, too. Notably, already the charge-conserving mutations E820D (Fig. 7C) and even more so K791R (Fig. 4H and L) led to significant functional changes. The mutual exchange of the two charged residues might cause steric clashes, or the charge inversion could be unfavorable within the local electrostatic environment, thus, preventing reversed salt bridge formation in the double mutant.

FIGURE 6. Cell surface expression and Rb⁺ uptake of Xenopus oocytes expressing H,K-ATPase wild-type or several Glu-820 variants. A, shown is a Western blot analysis of plasma membrane (PP, upper panel) and total membrane fractions (TP, lower panel) isolated from H,K-ATPase-expressing oocytes. Detection used anti-H,Kα antibody HK12.18. One representative
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\[ z_q = 0.31 \pm 0.02 \]
\[ V_{0.5} = -145.6 \pm 9.0 \text{ mV} \]

\[ z_q = 0.30 \pm 0.02 \]
\[ V_{0.5} = -127.3 \pm 8.2 \text{ mV} \]

\[ z_q = 0.32 \pm 0.06 \]
\[ V_{0.5} = -151.5 \pm 7.8 \text{ mV} \]
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of the gastric H,K-ATPase, Lys-791, as it was shown previously that the homologous residue in non-gastric H,K-ATPase is crucial for electrogenicity (24). In contrast to a study on a X,K-ATPase from toad bladder in which ouabain-sensitive, K+ -stimulated stationary currents for two different amino acid replacements of the aforementioned lysine in TM5 (24) were reported, we did not observe any pump currents for the corresponding Lys-791 mutants of the gastric H,K-ATPase (K791A and K791E). However, our voltage clamp fluorometric characterization for several Lys-791 mutants revealed significant changes in the \( z_q \) parameter, which reflects the electrogenicity of a partial reaction that occurs in conjunction with the \( E_1^-P/E_2^-P \) conformational change of the pump cycle. Of note, all Lys-791 replacements, which neutralized or inverted the charge of the side chain, resulted in a significantly increased \( z_q \) whereas the parameter was unaffected for the charge-conserving K791R mutant (Fig. 4, I–L).

Furthermore, the mutants-altered voltage dependence of fluorescence amplitudes indicated a strong shift of the \( E_1^-P/E_2^-P \) distribution toward \( E_1^-P \). This is in agreement with their reduced sensitivity toward the \( E_2^- \)-specific inhibitor SCH28080 in Rh+ uptake measurements (Fig. 3A) and might provide an alternative mechanistic explanation for the severely reduced SCH28080 sensitivity of the K791A mutant found in biochemical studies (45), in contrast to a direct effect on the inhibitor binding site.

Additional kinetic analysis of the fluorescence signals showed that the observed \( E_1^-P \)-shift of all these mutants is apparently a consequence of a markedly decreased forward rate constant of the \( E_1^-P \leftrightarrow E_2^-P \) conformational transition (Figs. 4H and 5). The concomitantly reduced Rh+ transport activity suggests that this partial reaction step might critically limit the turnover rate of the enzyme.

The \( E_1^-P \)-shifted phenotypes of the here-investigated Lys-791 mutants corroborate results from earlier mutagenesis studies on the gastric H,K-ATPase, which predicted an \( E_2^- \)-specific, interhelical salt bridge between Lys-791 in TM5 and Glu-820 in TM6 (21). Homology modeling of the cation binding pocket (Fig. 1) suggested that the putative salt bridge is an exclusive feature of the \( E_2^- \)-form due to the large distance between the two residues in an \( E_1^- \)-state model of the gastric H,K-ATPase. If the salt bridge would energetically contribute to the stability of the \( E_2^- \) or \( E_2^-P \) states, a disruption of the interaction by introduction of charge-neutralizing or sterically unfavorable amino acids in either of the two positions is expected to selectively destabilize the \( E_2^- \) (D) conformation. This notion is indeed supported by the alterations in the voltage-dependent fluorescence changes and the reduced rate constants (\( k_2^- \)) for the formation of \( E_2^-P \), as observed for the Lys-791 and Glu-820 variants studied in this work.

From the observed shifts of the \( E_1^-P/E_2^-P \) distributions (\( \Delta V_{1,2} \), ~160 mV) with a \( z_q \) of about 0.5, the relative destabilization of \( E_2^-P \) would be by more than \(-3 kT \) (= 0.08 electron volt (1 \( kT = 0.025 \) electron volt)), which is well in the order of magnitude of strong H-bond interactions in proteins. Similar values (3–5 kcal/mol ~ 5–8 \( kT \)) were reported for the energy contribution of salt bridges in other proteins (e.g. T4 Lysozyme (51, 52)).

Unfortunately, the lack of fluorescence signals did not allow us to reveal similar \( E_1^-P \)-shifted phenotypes for charge-eliminating replacements of the partner residue (Glu-820) within this putative charge pair (mutants E820A and E320Q). However, it should be noted that these two mutations were shown to result in a constitutive (\( K^+ \)-independent) ATPase activity of the proton pump expressed in Sf9 cells (53). This finding can actually provide an explanation for the small fluorescence changes observed for the Glu-820 mutants, as they are expected to undergo full ATPase cycles even under the \( K^+ \)-free conditions of our VCF experiments. Under these conditions the wild-type pumps usually accumulate in \( E_1^-P \), a situation that allows a switch of the conformational equilibrium by voltage jumps, resulting in changes of fluorescence amplitudes. Due to the constitutive activity of these two Glu-820 mutants, the redistribution of the enzyme molecules over all reaction cycle intermediates apparently creates a situation in which the enzyme conformation is insensitive to changes in the transmembrane potential.

According to the afore-stated Sf9 cell studies, both constitutively active mutants also exhibited a significantly reduced sensitivity toward the \( E_2^- \)-specific inhibitors SCH28080 and vanadate (38, 54, 55), which is in agreement with the results from our Rh+ uptake experiments (Fig. 6C and the inset in D). Again, these findings hint at \( E_1^-P \)-shifted phenotypes, which are possibly obscured by the constitutive activity of these mutants in the VCF experiments. Importantly, in the current study a charge-conserving replacement of Glu-820 by asparagine maintained the \( E_1^-P \) preference of the proton pump. This further corroborates the notion of an \( E_1^-P \)-stabilizing salt bridge between Lys-791 and Glu-820, although the individual energy barriers for the forward and backward transition, as inferred from the changes in the reciprocal time constants, are also modified by the Glu to Asp substitution.

FIGURE 7. Voltage dependence of the \( E_1^-P/E_2^-P \) distribution and kinetics of \( E_1^-P/E_2^-P \) transitions of H,K-ATPase mutants E820D and E820K. A and B, shown are fluorescence responses of site-specifically labeled gastric H,K-ATPase under \( K^- \)-free conditions (90 mm NaCl, pH 5.5) upon voltage jumps from a holding potential of ~40 mV to voltages between 180 and ~60 mV (same voltage protocols as in Fig. 4, A and F). Recordings originated from a representative oocyte coexpressing the wild-type H,K-Pi subunit with Hku5806C.E820D in A or Hku5806C.E820K in B, respectively. C and D, shown is voltage dependence of fluorescence amplitudes (1 – \( \Delta F/F \)) under \( K^- \)-free conditions for mutants Hku5806C.E820D (open red circle in C) and Hku5806C.E820K (open blue circle in D) compared with the reference construct Hku5806 (open square). Data are the means ± S.E. of 9–14 oocytes. A curve resulting from a fit of a Boltzmann function is superimposed. The fluorescence amplitudes 1 – \( \Delta F/F \) were normalized to saturation values from the fits. Midpoint potentials \( V_{1,2} \) and slope factors \( z_q \) derived from the fits are also shown. E and F, shown are reciprocal time constants from monoeponential fits to voltage jump-induced fluorescence changes under \( K^- \)-free conditions for the mutants Hku5806C.E820D ([open red circle in E] and Hku5806C.E820K [open blue circle in F], each in comparison to aS806C [filled square]). Data are means ± S.E. from 7–9 oocytes. G and H, shown is the calculated voltage dependence of the forward (\( k_2^- \)) and reverse (\( k_2^+ \)) rate constant of the \( E_1^-P/E_2^-P \) conformational transition in comparison to the experimentally obtained reciprocal time constants (\( k_{2^-} \)) from voltage clamp fluorometric measurements for the mutants E820D (G) and E820K (H). Values were calculated using a simple two-state kinetic model; see supplemental Appendix A for details.
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Unlike the observations for the aforementioned salt bridge in LacY, a charge-inverting double mutation of the proton pump (K791E/E820K) did not restore the wild-type phenotype but behaved rather similarly to the single amino acid replacements in Rb\textsuperscript+ uptake experiments. However, the altered side-chain geometry or the charge inversion itself could be incompatible with salt bridge formation in the context of the local environment. In addition, it must be noted that in the case of the LacY, neither of the individual residues nor the salt bridge between them plays an important role in the transport mechanism, as simultaneous neutral substitutions of both residues that remove the salt bridge do not cause inactivation of the enzyme (47). This means that the two charges are only required for neutralizing each other, which is still possible if the residues are mutually exchanged. This is in strong contrast to the roles of Lys-791 and Glu-820, which are both part of the cation binding pocket of gastric H,K-ATPase. Thus, interchanging the residues might even allow the formation of an inverted salt bridge but at the same time interfere with other functions of the two side chains for ion transport (e.g. proton storage of Glu-820, see below).

On the other hand, one could argue that the pronounced effects of the Lys-791 and Glu-820 mutations on Rb\textsuperscript+ transport activity might be a direct consequence of the involvement of the residues in cation binding. However, in case of the E822D mutant of the rabbit gastric proton pump (corresponding to mutation E820D here), Asano et al. (56) found a ~60\% reduction in K\textsuperscript+ -stimulated ATPase activity but at the same time demonstrated that the K\textsuperscript+ affinity was unaffected. Notably, our VCF data revealed a significantly reduced forward rate constant of the $E_1P\cdot E_2P$ conformational transition for this mutant (Fig. 7E), therefore, nicely explaining the phenotype and corroborating our assumption that this partial reaction is rate-limiting under saturating K\textsuperscript+ concentrations.

Apart from its possible participation in an interhelical salt bridge, our Rb\textsuperscript+ uptake experiments hint at a potential role of the Glu-820 side chain in intracellular proton binding, as the sensitivity of the proton pump toward intracellular acidification is eliminated upon replacements by non-protonatable amino acids. A rather conservative replacement of Glu-820 by a protonatable aspartate, on the other hand, maintains the stimulatory sensitivity of the proton pump toward intracellular acidification but results in slightly reduced Rb\textsuperscript+ uptake activity under all investigated pH conditions (Fig. 6D). This is in agreement with previous results from S9 cells, which revealed a reduced apparent ATP affinity for the E820D variant in ATP phosphorylation experiments. Because a pH change from 7.0 to 6.0 increased the apparent ATP affinity, which in turn affects the phosphorylation kinetics (57). This is again in line with the reduced forward rate constant of the $E_1P\leftrightarrow E_2P$ conformational transition observed for the E820D variant in our VCF experiments (Fig. 7E) and its diminished Rb\textsuperscript+ uptake activity (Fig. 6D). Apparently, the normalized to Rb\textsuperscript+ uptake of the wild-type construct HK\textsubscript{a}S806C/\textsubscript{b}wt (corresponding to 14.4 and 29.8 pmol/oocyte/min, respectively). a.u., arbitrary units.
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**FIGURE 9. Alignment of TM5 and TM6 from P-type ATPases of the P_{Nc} and P_{Na} subfamilies.** Sequence alignments were adapted from Axelsen and Palmgren (60) and adjusted manually for comparison of P_{Nc} and P_{Na} type ATPases. The sequence of the rat gastric H,K-ATPase used for mutagenesis in the present study, and sequences from other P-type ATPases for which individual residues are explicitly discussed here are framed by red boxes. TM5/TM6 loops are underlaid in black, and charged residues that are involved in putative interhelical salt bridges between TM5 and TM6 are highlighted in different colors (color coding is analogous to Fig. 1). The position (τSS06C) used for site-specific TMRR-labeling of the gastric H,K-ATPase is highlighted in magenta. Residues of the rat gastric H,K-ATPases that are shown in Fig. 1 are underscored.

Microenvironment around the acidic group (which is critical for its effective pK_a) is slightly altered due to the shorter side chain of the aspartate. Of note, the corresponding residue of Glu-820 in all sodium potassium ATPases is an aspartate (e.g. Asp-804 in the sheep α_1 isoform; see the alignments in Fig. 9). The reduced proton affinity of the E820D mutant described by Hermsen et al. (57) might, thus, provide an explanation for the fact that, in contrast to the case of the gastric H,K-ATPase, the acidic residues involved in ion binding of the Na,K-ATPase do not coordinate protons, at least not to a significant extent at physiological conditions (58). Therefore, the slightly longer side chain of Glu-820 together with a different local electrostatic environment (e.g. Lys-791, see below) seem to be important factors in determining H^+ affinity of the gastric H,K-ATPase. The acidic side chain of Glu-820 could represent a site where protons are bound before being expelled to the extracellular space. The immediate formation of a salt bridge with Lys-791 after expulsion of the proton from this site could be crucial for preventing reprotonation of the site from the lumenal space at a physiological pH of ~1. The site probably stays occupied by the positive side chain of Lys-791 until the catalytic group is no longer exposed to the gastric lumen, i.e. after closure of the extracellular gate. To enable subsequent reprotonation of the site with H^+ from the cytosolic space, the salt bridge must be transiently disrupted. This most likely occurs during the E_2 to E_1 conformational transition, causing a rearrangement of the Lys-791 side chain. The here-proposed pumping model would provide a rationale for the concept that the salt bridge is E_2 conformation-specific.

Remarkably, similar interhelical salt bridges between charged residues in TM5 and TM6 have been observed in other proton pumping P-type ATPases as well. For example, mutagenesis studies on the plasma membrane H^+ ATPase PMA1 from Saccharomyces cerevisiae hint at a charge pair in this area (50), which however involves other residues, i.e. Arg-695 in TM5 and Asp-730 in TM6 (highlighted in the sequence alignment in Fig. 9 in yellow and green, respectively). Of note, the corresponding Asp-684 (also highlighted in green in Fig. 9) of the plant plasma membrane H^+ ATPase from Arabidopsis thaliana (AH2) probably interacts with yet another arginine in TM5 (Arg-665, highlighted in yellow in Fig. 9) according to the recent crystal structure (59). The authors suggested that this arginine could serve as a built-in counterion during phosphorylation, as the pump does not countertransport any other ion in exchange for protons. Furthermore, similar to the here proposed role of Lys-791 in the gastric H,K-ATPase, the arginine may serve as a positive plug that prevents extracellular protons from re-protonating Asp-684 (presumably the central proton donor/acceptor of the pump) in the E_1P state, when the proton exit pathway opens to the extracellular space (59).

Although there is currently no experimental proof available that the aforementioned putative salt bridges in other H^+-transporting P-type ATPases are also specific for the E_1P-state, the close similarity regarding their respective location is striking. Because putative salt bridge-forming charges in M5 and M6 are conserved among other H^+-translocating P-type ATPases (Fig. 9), a common functional importance of these salt bridges for H^+ transport seems reasonable. Of note, all these H^+ ATPases release their transported protons in the E_2P state. Hence, the conserved salt bridge as a potential device for preventing reprotonation of the proton release site would be required in the E_1P state but must be removed in E_2 to enable protonation from the cytoplasmic space.

**CONCLUSIONS**

According to the current study, mutations of Lys-791 in TM5 of gastric H,K-ATPase do not change the overall electronegativity of ion transport. Rather, this lysine is essential for a rate-limiting partial reaction of the transport cycle, the E_1P → E_2P conformational transition, and thus, is crucial for transport activity. Our results suggest that Glu-820 in TM6 is a critical residue for intracellular proton binding and might serve as a putative partner for an electrostatic interaction to Lys-791, a salt bridge that could contribute to the inherent E_1P preference of the gastric H,K-ATPase and prevent reprotonation of Glu-820 after proton release to the extracellular space. This salt bridge between TM5 and TM6 might be a universal feature of H^+-translocating P-type ATPases to avoid futile pump cycling, unlike other P-type ATPases, which can...
be readily forced to run backward. This might be an essential requirement for efficient ion transport of H⁺-transporting ATPases, which face the steepest electrochemical gradients of all P-type ATPases.

Acknowledgment—We thank Neslihan Tavraz for critically reading the manuscript.

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