Homology modeling of gastric H,K-ATPase based on the E2 model of sarcoplasmic reticulum Ca2+-ATPase (Toyoshima, C., and Nomura, H. (2002) Nature 392, 835–839) revealed the presence of a single high-affinity binding site for K+ and an E2 form-specific salt bridge between Glu820 (M6) and Lys791 (M5). In the E820Q mutant this salt bridge is no longer possible, and the head group of Lys791, together with a water molecule, fills the position of the K+ ion and apparently mimics the K+-filled cation binding pocket. This gives an explanation for the K+-independent ATPase activity and dephosphorylation step of the E820Q mutant (Swarts, H. G. P., Hermes, H. P. H., Koenderink, J. B., Schuurmans Stekhoven, F. M. A. H., and De Pont, J. J. H. M. (1998) EMBO J. 17, 3029–3035) and, indirectly, for its E1 preference. The model is strongly supported by a series of reported mutagenesis studies on charged and polar amino acid residues in the membrane domain. To further test this model, Lys791 was mutated alone and in combination with other crucial residues. In the K791A mutant, the K+ affinity was markedly reduced without altering the E2 preference of the enzyme. The K791A mutation prevented, in contrast to the K791R mutation, the spontaneous dephosphorylation of the E820Q mutant as well as its conformational equilibrium change toward E2. This indicates that the salt bridge is essential for high-affinity K+ binding and the E2 preference of H,K-ATPase. Moreover, its breakage (E820Q) can generate a K+-insensitive activity and an E1 preference. In addition, the study gives a molecular explanation for the electroneutrality of H,K-ATPases.

In the transmembrane domain of P-type ATPases there are a number of charged and other polar amino acid residues that are involved in cation binding and transport. Such a role was made likely by a large number of site-directed mutagenesis studies and confirmed by the crystal structure of sarcoplasmic reticulum Ca2+-ATPase (1). In gastric H,K-ATPase, most attention has been paid to a number of negatively charged residues present in transmembrane helices 4, 5, and 6. The central theme in studies with this enzyme was the binding site for K+, which ion stimulates the hydrolysis of the phosphorylated intermediate. Studies up to now suggest roles in K+ activation for at least Glu820 in M6 (2–4), Glu795 in M5 (4–6), and Glu343 in M4 (7–9). These glutamate residues play different roles. Glu343 could replace Glu795 without much change in properties. However, replacement of Glu795 by an Asp or Asn residue had marked effects on K+ affinity, suggesting that it is not the charge but the side-chain geometry of this residue that is important. Replacement of Glu795 by a Gln residue reduced the apparent K+ affinity by a factor of 10 (8, 9), whereas replacement of Glu435 by any other amino acid residue completely abolished K+ affinity and blocked dephosphorylation of the phosphorylated intermediate. Most interesting were mutations of Glu820. Replacement of this residue by an Asp had only minor effects, but replacement by a neutral amino acid abolished or markedly reduced the K+ affinity (3). The latter mutations resulted, surprisingly, in enzymes with a large K+-independent (constitutive) ATPase activity that could be inhibited by the specific H,K-ATPase inhibitor SCH 280801.

The E1/E2 conformational equilibrium of the wild type enzyme and mutants with K+-stimulated ATPase activity is in the direction of the E2 conformation. This can be concluded from the low concentrations of either vanadate or the specific inhibitor SCH 28080, needed to inhibit the ATPase activity (11). All mutants with K+-independent ATPase activity, however, had a preference for the E1 conformation (9, 11), as shown by the high vanadate concentration needed to inhibit the ATPase activity. This preference for the E1 form also explained why pre-incubation with the specific inhibitor SCH 28080 did not prevent ATP-phosphorylation or, in some cases, even enhanced the phosphorylation level of these mutants (12).

In the present study we used the information of molecular models of the E1 and E2 forms of the catalytic subunit of H,K-ATPase to investigate how the E820Q mutant can induce K+-independent ATPase activity. The model for the wild type enzyme predicts the presence of a salt bridge between the Glu820 and Lys791 exclusively in the E2 form of the wild type enzyme. Because of this salt bridge, there is only space for a single K+-binding site in the wild type enzyme. The model for the E820Q mutant explains its K+-independent ATPase activity. Mutational analysis of Lys791 confirmed the importance of the salt bridge and showed that, in the E820Q mutant, the presence of a positive charge at this position is necessary for the E1 preference of the E820Q mutant. The presence of a salt bridge also gives a molecular explanation for the electroneutrality of H,K-ATPases, in line with recent findings of Burnay et al. (13).

EXPERIMENTAL PROCEDURES

Molecular Modeling of H,K-ATPase—Initial alignments of the gastric H,K-ATPase α-subunit sequence (Swiss Prot accession number P09626)
to the known structures of the rabbit fast twitch skeletal muscle sarco/ endoplasmic reticulum Ca$^{2+}$-ATPase (SERCA1a) (Protein Data Bank identifications 1EUL for the E$_1$-state and 1IWO for the E$_2$-state to which thapsigargin was bound) were obtained from the 3D-PSSM fold recognition server (14). The catalytic subunits of Ca$^{2+}$-ATPase and gastric H,K-ATPase show similar hydrophathy plots and share 31% sequence identity in the aligned regions, which makes it practically certain that both adopt the same fold. This allows us to safely predict the structure of the latter using homology modeling techniques (15).

Gastric H,K-ATPase needs a $\beta$-subunit, but the interaction of this subunit with the catalytic subunit takes place in the M7/M8 region (16) and is assumed to have not much influence on structure of the cation binding sites. The WHAT IF program (17) was used to mutate the amino acid side chains based on a backbone-dependent rotamer library (18). Then the two models (E$_1$ and E$_2$) were refined with YASARA, which was shown to increase accuracy (19). Apart from a manual alignment correction at helix M7, the models were built fully automatically. The salt bridge between Lys791 and Glu820, shown in Fig. 1, was thus predicted in the absence of any additional data. The quality of the final models was evaluated using WHAT_CHECK (20), which compares structural features of the model to a database of high resolution $\alpha$-x-ray structures. The models were found to have about the same quality as the initial templates 1EUL and 1IWO. Coordinate files are available from the authors upon request.

**Identification of Potassium Binding Sites**—Valence calculations were done with YASARA on a grid with 0.2 Å spacing using an empirical formula (21) that has been applied to proteins previously (22). The parameters used to identify potassium sites were 2.276 Å for Ca$^{2+}$ and 2.410 Å for Na$^{+}$, as described previously (9). The new data were corrected for the levels of phosphorylated protein obtained with mock-infected cells. The ATP-dependent phosphorylation level was determined at 21°C as described previously (9). Data were corrected for the levels of phosphorylated protein obtained with mock-infected membranes. The rate of dephosphorylation was determined by measuring the residual phosphorylation level 5 s after 8.3-fold dilution with non-radioactive ATP.

**Chemicals**—SCH 28080 was kindly provided by Dr. C.D. Strader (Scherer-Plough, Kenilworth, NJ). The antibody HKB was a gift of Dr. Michael Caplan (Yale).

### RESULTS

**Modeling**—To investigate the structural basis of the K$^+$-independent ATPase activity of the gastric H,K-ATPase ES20Q mutant (10), molecular models of the E$_1$ and E$_2$ forms of the catalytic subunit of this ATPase were built using the known structures of sarcoplasmic reticulum Ca$^{2+}$-ATPase (1, 26).

With 31% sequence identity over 860 aligned residues, H,K- and Ca$^{2+}$-ATPase can safely be assumed to adopt the same fold (15). Nevertheless, the alignment had to be manually adjusted in difficult regions like helix M7 by considering multiple sequences and structural requirements.

Using the same valence scanning method as Ogawa and Toyoshima (27) in their recent modeling study of Na,K-ATPase, we found two weak signals for K$^+$ binding sites in the transmembrane region of the E$_2$ model. The addition of K$^+$ ions at both locations, followed by an energy minimization step, yielded one strong binding site, whereas the other site showed no improvement in the valence-scanning score. We therefore concentrated our analysis on the clearly identified binding site that is located at a similar position as the K$^+$ binding site II in the Na,K-ATPase model of Ogawa and Toyoshima (Table I). As shown in Fig. 1A, the K$^+$ ion is surrounded by six oxygen atoms contributed by the backbone carboxyl groups of Val338 and Val341 (M4), one side chain oxygen of Glu795 (M5) and Glu820 (M6), and both side chain oxygens of Glu831 (M4). A striking observation was the formation of a salt bridge between Lys791 and Glu820, shown in Fig. 1, which was shown to increase accuracy (19). Apart from a manual alignment correction at helix M7, the models were built fully automatically. The salt bridge between Lys791 and Glu820, shown in Fig. 1, was thus predicted in the absence of any additional data. The quality of the final models was evaluated using WHAT_CHECK (20), which compares structural features of the model to a database of high resolution $\alpha$-x-ray structures. The models were found to have about the same quality as the initial templates 1EUL and 1IWO. Coordinate files are available from the authors upon request.

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Analysis of Data—All data are presented as mean values for three to eight individual preparations with standard error of the mean. IC$_{50}$ and K$_{0.5}$ values were determined by analyzing the plots using the non-linear curve fitting program (Hill equation function) of Origin 6.1 (Microcal, Northampton, MA).
Lys791 plays an essential role. We therefore mutated Lys 791 above a certain temperature. It is likely that the above-described conformational rearrangements that ATP could still phosphorylate some mutants without K⁺-stimulated activity (29). The wild type enzyme was very K⁺-sensitive and that the E820Q mutant showed a high spontaneous dephosphorylation rate in the absence of K⁺. The E343D and E343D/E820Q mutants showed a low spontaneous dephosphorylation rate that was not increased by K⁺. The K791A mutant had a markedly reduced K⁺ sensitivity as compared with the wild type enzyme. The same was true for the K791S mutant (not shown). In contrast, the double K791A/E820Q and K791A/E343D as well as the triple K791A/E343D/E820Q mutants were completely K⁺-insensitive. Moreover, none of these mutants showed an enhanced K⁺-independent dephosphorylation rate as was found for the E820Q mutant. This finding is in agreement with the lack of (K⁺-stimulated) ATPase activity of these double and triple mutants (Fig. 2).

All K791R-containing mutants did not show any K⁺-stimulated dephosphorylation activity (Fig. 4). This was even true for the K791R single mutant that showed a very low K⁺-stimulated ATPase activity (Fig. 2). The spontaneous dephosphorylation rate of the K791R/E820Q mutant was significantly higher than that of the other K791R-containing mutant, but much less than that of the E820Q single mutant. This might explain the very minor K⁺-independent ATPase activity of the latter mutant.

In conclusion, although the K791A mutant is still K⁺-sensitive, the presence of this mutation prevents the K⁺-independent ATPase and dephosphorylation activity of the E820Q mutation. The K791R mutant, on the other hand, lost its K⁺-sensitivity but did not completely block the K⁺-independent ATPase and dephosphorylation activity of the E820Q mutation.

Deactivation Studies—Because of previous observations that ATP could still phosphorylate some mutants without ATPase activity, we determined the ATP-phosphorylation level of all these mutants. Fig. 3 shows that all mutants could indeed be phosphorylated, although to a variable level. The ATP phosphorylation levels of the K791A mutants and, in particular, of the K791R mutants were markedly lower than that of the control mutants. The difference in phosphorylation capacity cannot be due to differences in expression level of the catalytic subunit, as shown by Western blotting experiments. The fact, however, that all these mutants could be phosphorylated gave the possibility to study their spontaneous and K⁺-sensitive dephosphorylation steps.

None of the mutants or the wild type enzyme could be dephosphorylated by 1 mM ADP (not shown), indicating that the phosphorylated intermediates were all in the E₁-P form. Fig. 4 confirms that the wild type enzyme was very K⁺-sensitive and that the E820Q mutant showed a high spontaneous dephosphorylation rate in the absence of K⁺. The E343D and E343D/E820Q mutants showed a low spontaneous dephosphorylation rate that was not increased by K⁺. The K791A mutant had a markedly reduced K⁺ sensitivity as compared with the wild type enzyme. The same was true for the K791S mutant (not shown). In contrast, the double K791A/E820Q and K791A/E343D as well as the triple K791A/E343D/E820Q mutants were completely K⁺-insensitive. Moreover, none of these mutants showed an enhanced K⁺-independent dephosphorylation rate as was found for the E820Q mutant. This finding is in agreement with the lack of (K⁺-stimulated) ATPase activity of these double and triple mutants (Fig. 2).

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E₁/E₂ Equilibrium Determinations—One of the peculiar properties of the E820Q mutant is its tendency toward the E₁ form, whereas the wild type enzyme favors the E₂ form. This can be shown by the IC₅₀ value for vanadate on the ATPase activity (29). The wild type K⁺-dependent ATPase activity was
inhibited by very low (0.4 \muM) vanadate concentrations, whereas the K\(^+\)-independent ATPase activity of the E820Q mutant was only 50% inhibited at 1 mM vanadate (11). Swarts \textit{et al.} (9) showed that a measure for the direction of the E\(_1\)/E\(_2\) equilibrium can also be obtained by determining the effect of vanadate on the ATP phosphorylation level. This makes it possible to include mutants without ATPase activity. Similarly, determination of the effect of SCH 28080 on the phosphorylation level gives a measure for the direction of this equilibrium. The phosphorylation level of mutants with an E\(_2\) preference was decreased by low SCH 28080 concentrations, whereas it hardly changed with E\(_1\)-preferring mutants. In some cases, like the E820Q mutant, the phosphorylation level even increased when SCH 28080 was present (12).

Fig. 5 shows that low SCH 28080 concentrations decreased the phosphorylation level of all K791A-containing mutants with a similar IC\(_{50}\) value as that measured for the wild type enzyme and the E343D mutant. SCH 28080 also largely decreased the phosphorylation level of both the K791R and the K791R/E343D mutants. This indicates that these mutants have, like the wild type enzyme, a preference for the E\(_2\)-form. However, the phosphorylation level of the K791R/E343D/E820Q mutant was not lowered by SCH 28080, whereas that of the K791R/E820Q mutant was, like that of the E820Q mutant, even increased, indicating that the latter mutants have a preference for the E\(_1\) form (9).

High vanadate concentrations were needed to reduce the phosphorylation level of the latter two mutants as shown by the measured IC\(_{50}\) values (Fig. 6). All mutants in which the phosphorylation level was decreased by SCH 28080 had a low IC\(_{50}\) value for vanadate. Comparison of the results with mutants containing the K791A mutation with those lacking this
mutation shows that the K791A mutation prevents the conformational equilibrium shift toward E1 that is characteristic for the E820Q mutation.

**DISCUSSION**

**K⁺ Binding and Dephosphorylation**—The atomic model presented in this paper provides a structural basis for the intriguing finding that replacing Glu at position 820 by a neutral amino acid residue renders gastric H,K-ATPase active in the absence of K⁺ (10). Homology modeling of the α-subunit of H,K-ATPase was carried out to identify the K⁺ binding site(s) in the transmembrane region based on the two atomic structures of the sarcoplasmic reticulum Ca²⁺-ATPase, namely the Ca²⁺-bound E₁ state for H (1) and the Ca²⁺-free E₂ state for K⁺ (26). Valency scanning followed by energy minimization yielded one position at which high affinity for K⁺ is to be expected in the E₂ model (Fig. 1A). Comparison with a recently published atomic model for Na,K-ATPase (27) suggests that the K⁺ binding site identified in H,K-ATPase most likely resembles the K⁺ binding site II in Na,K-ATPase (Table I). Fig. 1A shows that the K⁺ ion is held in place by six oxygen atoms provided by surrounding amino acids, namely the backbone carbonyl groups of Val³³⁸ and Val³⁴¹ (M4), one side chain oxygen of Glu⁷⁹⁵ (M5) and Glu⁸²⁰ (M6), and both side chain oxygens of Glu³⁴³ (M4). Most remarkably, Lys⁷⁹¹ (M5) forms a salt bridge with Glu⁸²⁰. This salt bridge was absent in the E₁ model because of the large distance between these two residues (data not shown). Prevention of the formation of the salt bridge by replacing Lys at position 791 by Ala reduced both the K⁺ affinity and the ATPase activity level.

The model presented in this paper is supported by a vast amount of data obtained in mutagenesis studies. Thus, replacing Glu at position 795 by Gln (E795Q) did not affect the K⁺-affinity of the enzyme (4–6). This is in agreement with the involvement of a single side-chain oxygen in the binding of the K⁺ ion. In contrast, replacement of Glu⁷⁹⁵ with either Asp
or Asn (E795N) residues markedly reduced the K\textsuperscript{+} affinity, demonstrating the importance of the length of this side chain. As far as Glu 343 is concerned, replacement with a Gln (E343Q) caused a marked decrease in K\textsuperscript{+} affinity (7–9). This suggests that both oxygens are needed for optimal K\textsuperscript{+} binding. The mutants in which Glu343 is replaced by Asp (E343D) were inactive, demonstrating that the length of this side chain is crucial for enzyme activity (7–9).

The method used to determine the characteristics of the K\textsuperscript{+}-binding site in gastric H,K-ATPase is rather similar to that used by Ogawa and Toyoshima (27) for Na,K-ATPase. In their study, they found two K\textsuperscript{+}-binding sites in line with a plethora of kinetic evidence from the literature. Site-directed mutagenesis studies of the residues that are assumed to be directly involved in K\textsuperscript{+} binding in Na,K-ATPase partially support their model (30). The model assumes that both Glu 786 and Asp 811 (see Table I) are involved in binding of each of the two K\textsuperscript{+} ions, which must mean that both oxygen atoms of these carboxyl groups are involved in cation binding. However, mutant E786Q in Na,K-ATPase shows only a slightly reduced K\textsuperscript{+}-affinity (31).

The experiments of Koster et al. (32), which show that the properties of the E786K mutant in Na,K-ATPase are rather similar to that of the wild type, do not fit in the model for Na,K-ATPase (27). In the latter model Glu\textsuperscript{343} does not play a direct role, but mutagenesis experiments with Na,K-ATPase suggest that this residue is directly involved in K\textsuperscript{+}-binding (30, 33–35).

Because Na,K-ATPase binds two K\textsuperscript{+} ions and the model for H,K-ATPase presented in this paper shows only one K\textsuperscript{+}-binding site, the experimental results with these two related P-type ATPases cannot easily be compared. If a comparison is done, the K\textsuperscript{+}-binding site found in gastric H,K-ATPase corresponds to the K\textsuperscript{+}-binding site-II in Na,K-ATPase (Table I). In both binding sites there are roles for main chain carbonyls of M4 and for the Glu\textsuperscript{786}/Glu\textsuperscript{795} residues in M5, as well as for Asp\textsuperscript{811}/Glu\textsuperscript{820} in M6. In the model of Ogawa and Toyoshima (27), Asn\textsuperscript{782} is involved. No such role is found for Asn\textsuperscript{792} in the K\textsuperscript{+}-binding site in gastric H,K-ATPase. In the model this residue is involved in the hydrogen bond network outside the K\textsuperscript{+}-binding site, which explains the reduced K\textsuperscript{+} affinity of the N792A mutant (36). The prominent role of Glu\textsuperscript{343} in the model for H,K-ATPase is strongly supported by mutagenesis experi-

![Figure 5](image1.png)  
**Fig. 5.** Effect of SCH 28080 on the ATP-phosphorylation level of gastric H,K-ATPase mutants. ATP phosphorylation level was determined as described in Fig. 3 in the presence of the indicated concentration of SCH 28080. Values are the average from 3–4 experiments.

![Figure 6](image2.png)  
**Fig. 6.** IC\textsubscript{50} values for the effects of vanadate on the phosphorylation level of gastric H,K-ATPase mutants. The phosphorylation level of the indicated mutants was determined after pre-incubation with different vanadate concentrations by incubation with 100 nM radioactive ATP for 10 s. The IC\textsubscript{50} values, ± S.E., were determined from the average of dose-inhibition curves obtained from 3–8 experiments.
ments, and we assume that its counterpart, Glu\textsuperscript{343} in Na,K-ATPase, has a more important role than the model of Ogawa and Toyoshima (27) suggests.

The most important difference between gastric H,K-ATPase and Na,K-ATPase is that the Lys residue that can form a salt bridge in H,K-ATPase is a Ser in Na,K-ATPase. According to the model of Ogawa and Toyoshima (27) this Ser residue is involved in the K\textsuperscript{+}-binding site I. Such a role is supported by mutagenesis studies (37). However, the presence of the salt bridge between Lys\textsuperscript{791} and Glu\textsuperscript{820} in gastric H,K-ATPase probably prevents the formation of a similar K\textsuperscript{+} binding site in gastric H,K-ATPase.

The E820Q Mutant—Glu\textsuperscript{820} is involved in both the formation of the salt bridge and the binding of the K\textsuperscript{+} ion. Previous work has shown that replacing Glu\textsuperscript{820} with Gln (E820Q) yielded an enzyme with an ATPase activity in the absence of K\textsuperscript{+} that did not increase further upon increasing the K\textsuperscript{+} concentration (10). The atomic model presented in Fig. 1B gives a molecular explanation for this finding. Removal of the negative charge at position 820 leads to breakage of the salt bridge and repositioning of the side chain of Lys\textsuperscript{791}. In the case of E820Q, the remaining hole has precisely the size of a water molecule. This configuration increases the dephosphorylation rate, and, because the K\textsuperscript{+} ion can no longer enter this site, it cannot further stimulate this process. The structural model for the K\textsuperscript{+}-independent ATPase activity presented here is substantiated by the finding that the K\textsuperscript{+}-independent activity was completely lost when, in addition to the E820Q mutation, the Lys at position 791 was replaced by an Ala but not by the positively charged Arg. Moreover, the model predicts that shortening of the side chain of Glu\textsuperscript{20} by one CH\textsubscript{2} group does not affect the formation of the salt bridge. Accordingly, the E820D mutant showed a normal K\textsuperscript{+}-dependent activity in both the ATPase and dephosphorylation reaction (3, 4, 10).

All mutants in which Glu\textsuperscript{343} is replaced with an amino acid other than Gln, including those containing the E820Q mutation, could be phosphorylated but showed no ATPase activity (7–9). According to our model, Glu\textsuperscript{343}, which is present close to the unwounded part of M4, can adopt two different rotamers. One of these rotamers binds the K\textsuperscript{+} ion (Fig. 1A), and it is tempting to speculate that the formation of this rotamer is required for the dephosphorylation of Asp\textsuperscript{345}.

The model for the E820Q mutant (Fig 1B) is also supported by the fact that combined E795Q and/or E343Q mutations with E820Q still give K\textsuperscript{+}-independent ATPase activity (9). In contrast, a combined E343D/E820Q mutation does not show any ATPase or dephosphorylation activity. According to the model, one oxygen atom of both Glu\textsuperscript{795} and Glu\textsuperscript{343} is sufficient and required for hydrogen bonding to Lys\textsuperscript{791} and the water molecule, respectively. The fact that the E343D/E820Q mutant could not be dephosphorylated (9) indicates that the length of the side chain on position 343 is crucial for the dephosphorylation reaction.

Modeling studies (not shown) indicate that, despite the impossibility of forming a salt bridge, the cation-binding pocket in the K791A/E820Q mutant is again filled by K\textsuperscript{+}-ions, whereas it is empty in the E820Q single mutant. The K791A/E820Q mutant probably binds K\textsuperscript{+} but shows no K\textsuperscript{+}-stimulated ATPase activity. This can be explained by assuming that filling the cation pocket with K\textsuperscript{+} \textit{per se} is not sufficient for dephosphorylation. It is apparently necessary that the cation binding pocket should have a special structure so that it can transfer a signal to the phosphorylation domain. Such a structure is obtained with K\textsuperscript{+} in the native enzyme and with the E820Q mutant in the absence of K\textsuperscript{+}. However, the structure in the K791A/E820Q double mutant is rather similar to a lock in which a key fits but that still cannot be opened.

$E_1/E_2$ Equilibrium Shift—The wild type enzyme favors the $E_2$ form above the $E_1$ form as indicated by the low vanadate or SCH 28080 concentrations needed to inhibit the ATPase activity and lower the ATP phosphorylation level. The E820Q mutant, however, preferred the $E_1$ conformation. This property was kept in all double and triple mutants containing E820Q, except when, in addition, Lys\textsuperscript{791} was mutated into an Ala residue. We assume that the $E_2$ structure of the E820Q mutant, in contrast to that of the wild type enzyme, is energetically less favorable than the $E_1$ conformation, resulting in an equilibrium shift toward $E_1$. In all mutants that favor the $E_2$ conformation, the head group of Lys\textsuperscript{791} is either immobilized by a salt bridge with Glu\textsuperscript{820} or removed by mutation into an Ala residue. In the mutants that favor the $E_1$ conformation, the head group of Lys\textsuperscript{791} (or Arg) is located within the cation binding pocket. In the $E_1$ conformation, no salt bridge between Lys\textsuperscript{791} and Glu\textsuperscript{820} can be formed because of the large distance between these residues. Thus for $E_1$-preferring mutants like E820Q, the structure of the cation binding pocket in the $E_2$ form might be rather similar to that of the $E_1$ form, whereas it is very different in the wild type enzyme and $E_2$-preferring mutants.

Upon replacement of Lys\textsuperscript{791} by a positively charged Arg residue, a salt bridge with Glu\textsuperscript{820} in the $E_2$ form is still possible, but modeling shows that a considerable rearrangement around this residue is needed. The direction of the $E_1/E_2$ equilibrium of several double and triple mutants containing K791R is about the same as with the native Lys\textsuperscript{791} residue. Combined mutation of K791A with E820Q completely blocks all specific effects of the latter mutation. The conformational equilibrium is kept in the $E_2$ direction, as shown by the low concentrations of vanadate and SCH 28080 needed to decrease the phosphorylation level.

Stoichiometry—Gastric H,K-ATPase is, like non-gastric H,K-ATPase, an electroneutral cation pump. This means that the number of cation binding sites in the $E_1$ conformation is similar to that in the $E_2$ conformation. If the number of K\textsuperscript{+}-binding sites in the $E_1$ form is indeed one, as the modeling suggests, then the number of proton-binding sites would also be one. This would be in line with some literature findings that reported transport of one proton per ATP hydrolyzed (38), but not with other studies that reported transport of two cations in each direction per ATP hydrolyzed (39–41). One single binding site, however, could thermodynamically more easily explain the ability of the gastric proton pump to transport protons against gradients by a factor of 10\textsuperscript{6}. Moreover, the number of ion-binding sites in some other P-type ATPases also seems to be one. In yeast and plant H\textsuperscript{+}-ATPase, only one binding site for protons that corresponds to Ca\textsuperscript{2+}-binding site II has been identified (42). The amino acid in the plasma membrane Ca\textsuperscript{2+}-ATPase that corresponds to Glu residues in M5 of other P-type ATPases is an Ala, suggesting that also in this pump only ion binding site II is present (43).

Burnay et al. (13) recently observed that the electroneutral non-gastric \textit{Bufo marinus} H,K-ATPase became electrogenic upon mutation of the analogue of Lys\textsuperscript{791} in a neutral residue. Similarly, the electronegic Na,K-ATPase became electroneutral when the corresponding Ser was replaced by Lys residue. They postulated that, as in Na,K-ATPase, the number of putative cation-binding sites in the $E_1$ form in H,K-ATPase is one higher than in the $E_2$ form but that, because of the specific location of the Lys residue, one of the cation binding sites in the $E_1$ form is non-functional. The specific formation of an intramembrane salt bridge between the analogous residues of...
Lys<sup>791</sup> and Glu<sup>820</sup> in the E<sub>2</sub> form, but not in the E<sub>1</sub> form, gives a molecular explanation for the latter finding.

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