Identification and Function of a Cytoplasmic K\(^+\) Site of the Na\(^+\),K\(^+\)-ATPase*

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A cytoplasmic nontransport K\(^+/\)Rb\(^+\) site in the P-domain of the Na\(^+\),K\(^+\)-ATPase has been identified by anomalous difference Fourier map analysis of crystals of the [Rb\(_2\)].E\(_2\).MgP\(_{i}^{2-}\) form of the enzyme. The functional roles of this third K\(^+/\)Rb\(^+\) binding site were studied by site-directed mutagenesis, replacing the side chain of Asp\(_{74}^\) donating oxygen ligand(s) to the site with alanine, glutamate, and lysine. Unlike the wild-type Na\(^+\),K\(^+\)-ATPase, the mutants display a biphasic K\(^+\) concentration dependence of E\(_2\)P dephosphorylation, indicating that the cytoplasmic K\(^+\) site is involved in activation of dephosphorylation. The affinity of the site is lowered significantly (30–200-fold) by the mutations, the lysine mutation being most disruptive. Moreover, the mutations accelerate the E\(_2\) to E\(_1\) conformational transition, again with the lysine substitution resulting in the largest effect. Hence, occupation of the cytoplasmic K\(^+/\)/Rb\(^+\) site not only enhances E\(_2\)P dephosphorylation but also stabilizes the E\(_2\) dephosphoenzyme. These characteristics of the previously unrecognized nontransport site make it possible to account for the hitherto poorly understood trans-effects of cytoplasmic K\(^+\) by the consecutive transport model, without implicating a simultaneous exposure of the transport sites toward the cytoplasmic and extracellular sides of the membrane. The cytoplasmic K\(^+/\)/Rb\(^+\) site appears to be conserved among Na\(^+\),K\(^+\)-ATPases and P-type ATPases in general, and its mode of operation may be associated with stabilizing the loop structure at the C-terminal end of the P6 helix of the P-domain, thereby affecting the function of highly conserved catalytic residues and promoting helix-helix interactions between the P- and A-domains in the E\(_2\) state.

The essential gradients for Na\(^+\) and K\(^+\) across the plasma membranes of mammalian cells are created by the Na\(^+\),K\(^+\)-ATPase, a membranous enzyme that couples ATP hydrolysis to active extrusion of Na\(^+\) from the cells and uptake of K\(^+\) at a stoichiometry of three Na\(^+\) ions exchanged for two K\(^+\) ions per ATP molecule utilized (1, 2). The Na\(^+\),K\(^+\)-ATPase belongs to the family of P-type ATPases characterized by the formation of a phosphorylated enzyme intermediate through transfer of the γ-phosphate of ATP to a conserved aspartate residue in the enzyme. The ATP hydrolysis is tightly coupled to ion translocation, because Na\(^+\) binding from the cytoplasmic side triggers phosphorylation of the enzyme from ATP, whereas K\(^+\) binding from the extracellular side leads to rapid dephosphorylation (3). Recently, the structure of the Na\(^+\),K\(^+\)-ATPase was determined at 3.5 Å resolution (4), revealing two Rb\(^+\) ions bound as K\(^+\) congeners close together in a pocket formed by the transmembrane segments M4,2 M5, M6, and M8 of the catalytic α-subunit. The α-subunit consists of 10 transmembrane segments, M1–M10, and a large cytoplasmic part containing three distinct domains denoted A (actuator), N (nucleotide-binding), and P (phosphorylation), by analogy to the closely related sarcoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) (5). In contrast to the Ca\(^{2+}\)-ATPase, which is a single subunit enzyme, the Na\(^+\),K\(^+\)-ATPase complex crystallized consists of, in addition to the α-subunit, also a β-subunit, required for routing the α-subunit to the plasma membrane, and a γ-subunit known to have a regulatory role. The catalytic hydrolysis of ATP takes place in the cytoplasmic part of the α-subunit and appears to involve movements of the cytoplasmic domains that are transmitted to the transmembrane part, causing structural changes that expose the membranous cation binding sites alternatively toward the two sides of the membrane and switch their ion selectivity between Na\(^+\) and K\(^+\). Some of the residues involved in coordinating two of the three Na\(^+\) ions binding from the cytoplasmic side seem to be involved also in the coordination of the two K\(^+\) ions binding from the extracellular side (4, 6–12), and the functional cycle of the Na\(^+\),K\(^+\)-ATPase has generally been described in terms of a consecutive (“ping-pong”) mechanism, where Na\(^+\) is translocated before K\(^+\) (“Post-Albers model”; Scheme 1 (3)). The conformational states showing Na\(^+\) and K\(^+\) selectivity are usually denoted E\(_1\) and E\(_2\), respectively.

Although a large body of evidence seems to support the transport mechanism shown in Scheme 1, there are also observations that are not satisfactorily accounted for by the consecutive exposure of the ion binding sites toward the cytoplasmic and extracellular sides of the membrane. Hence, trans-effects requiring the simultaneous presence of ion-binding sites accessible from the cytoplasmic and the extracellular surface have

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2 The abbreviations used are: M1–M10, transmembrane segments numbered from the N terminus; K\(_{50}\), ligand concentration giving half-maximum effect; SERCA, sarcoplasmic reticulum Ca\(^{2+}\)-ATPase.

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been observed under certain conditions (13–15) and are sometimes interpreted in terms of simultaneous binding of Na\(^+\) and K\(^+\) at transport sites in two "out of phase" subunits of a dimeric pump protein. For the present study, it is of particular importance that cytoplasmic K\(^+\) has been shown to stimulate Na\(^+\) transport toward the extracellular side (16–18) as well as the processing of the phosphoenzyme involved in extracellular Na\(^+\) release and dephosphorylation activated by extracellular K\(^+\) (18). To account for these effects, it seems necessary to postulate the presence of at least one cytoplasmically facing K\(^+\) site existing simultaneously with the two extracellularly facing K\(^+\) transport sites. Such an additional K\(^+\) site with cytoplasmic access would also be consistent with the inhibitory effect of cytoplasmic K\(^+\) on the transition between \(E_2\) and \(E_1\) forms observed under some conditions (19). This site might in some way be involved in transport or could be a structural or regulatory site. Indeed, in SERCA, the activity is under the influence of a cytoplasmic K\(^+\) site in the P-domain (20–22), and a similar site has been identified in the plant plasma membrane H\(^+\)-ATPase by mutational analysis (23). However, the existence and functional role of a similar K\(^+\) site in the Na\(^+\),K\(^+\)-ATPase has remained unresolved.

In the present report, we reveal the existence of such an additional (third) Rb\(^+\)/K\(^+\) binding site in the structure of the Na\(^+\),K\(^+\)-ATPase \(\alpha\)-subunit. This site is located in the cytoplasmic part of the \(\alpha\)-subunit, in the P-domain, and is, thus, not directly involved in the binding of the translocated ions. By use of site-directed mutagenesis, we demonstrate its functional importance, both in connection with the dephosphorylation of \(E_2\)P induced by K\(^+\) binding to the membranous transport sites in their extracellularly facing configuration and in connection with the \(E_2 \rightarrow E_1\) transition of the dephosphoenzyme. The affinity of the site for cytoplasmic K\(^+\) is relatively high, and it is therefore likely to be permanently saturated at high internal K\(^+\) concentration present under physiological conditions.

**EXPERIMENTAL PROCEDURES**

**Crystallography**—The Na\(^+\),K\(^+\)-ATPase crystals were obtained with the pig \(\alpha\)-isoform in the presence of magnesium fluoride and Rb\(^+\) as described (4). Data for the anomalous difference Fourier map were collected at a wavelength of 0.8146 Å, at the absorption edge of rubidium, and were reprocessed in XDS (24) with the Friedel mates kept separate and with attention to avoid the inclusion of data suffering from radiation damage. Final model phases were used for the calculation of the anomalous difference Fourier map (25), based on data at 20 to 5 Å resolution.

**Mutagenesis and Expression**—Mutations D742A, D742E, and D742K were introduced into full-length cDNA encoding the rat kidney Na\(^+\),K\(^+\)-ATPase (\(\alpha\)-isoform) using the QuikChange site-directed mutagenesis kit (Stratagene), and the mutants and wild type were expressed in COS-1 cells, using the ouabain selection procedure (8, 26, 27).

**Functional Analysis**—The isolation of the plasma membrane fraction, made leaky prior to functional analysis, the measurement of ATPase activity by following the release of Pi at 37 °C, and the steady-state and transient kinetic measurements of phosphorylation from \([\gamma\text{-}^{32}\text{P}]\text{ATP}\) and dephosphorylation were carried out as previously (8, 28–31); the compositions of media are detailed in the figure legends. To determine the rate of \(E_2\)P dephosphorylation at 25 °C, a double mixing procedure was applied (28, 30, 31). The active site concentration was determined by phosphorylation at 0 °C in the presence of 2 mM [\(\gamma\text{-}^{32}\text{P}\text{ATP}\), 3 mM Mg\(^2\)+, 150 mM Na\(^+\), and oligomycin (20 \(\mu\)g/ml) to block dephosphorylation and thereby obtain stoichiometric phosphorylation. The contribution from endogenous Na\(^+\),K\(^+\)-ATPase in the functional assays was eliminated by including ouabain in the reaction media at a concentration sufficient to inhibit the endogenous enzyme but too low to affect the rat Na\(^+\),K\(^+\)-ATPase.

Each data point shown is the average value corresponding to at least two independent measurements. Data normalization, averaging, and nonlinear regression analysis were carried out as previously (29–31), and the results are reported ± S.E. Generally, the Hill equation was used for analysis of ligand concentration dependences.

**RESULTS**

**Localization and Structure of a Third, Cytoplasmic Rb\(^+\)/K\(^+\) Binding Site**—Rb\(^+\), a congener of K\(^+\), is transported by the Na\(^+\),K\(^+\)-ATPase into the cell at a stoichiometry of 2 Rb\(^+\) exchanged for 3 Na\(^+\), similar to the K\(^+\) transport. Recently, the Na\(^+\),K\(^+\)-ATPase was crystallized, and its structure was determined at 3.5 Å resolution in the [Rb\(_2\)]E\(_2\)MgF\(_4\) form, where two Rb\(^+\) ions are bound at the membranous transport sites in an occluded state (4). Here we report that the [Rb\(_2\)]E\(_2\)MgF\(_4\) crystal structure in fact contains an additional, third Rb\(^+\) ion, which is bound in the cytoplasmic part of the molecule about 30 Å above the two membranous transport sites. As seen in Fig. 1A, the third Rb\(^+\) ion is located in a binding cavity at the boundary between the P-domain (green) and the transmembrane domain (blue) with open access to the cytoplasmic medium. The two Rb\(^+\) ions bound in the transmembrane region are not seen in the surface representation of Fig. 1A, because they are buried in an occluded state between the membrane segments. In Fig. 1D, all of the three Rb\(^+\) sites are identified by the anomalous difference Fourier map contoured at a 5σ level. One elongated, major peak encompasses the two, closely spaced trans-
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FIGURE 1. Location of the cytoplasmic $K^+$ site. A, surface representation of the $Na^+,K^+$-ATPase structure (4) (Protein Data Bank code 3B8E) showing the localization of the $K^+$ site in the cytoplasmic part of the $\alpha$-subunit at the boundary between the P-domain (green) and the transmembrane domain (blue). The $Rb^+$ ion bound as $K^+$ congener is red. The A- and N-domains and the $\beta$-subunit are colored pink, yellow, and wheat, respectively. B, close up of the $K^+/Rb^+$ site with an indication of the residues thought to contribute back bone carbonyl groups (720, 721, 723, and 724) and side-chain oxygen atom(s) (D742) as $K^+/Rb^+$ ligands. The bound $Rb^+$ ion is shown as a red sphere (possible bonds indicated by dashed lines). C, sequence alignment showing species and isoform similarities and differences in the P-domain regions involved in formation of the $K^+$ site. Residues conserved within the P-type ATPase subfamily are indicated by capital letters. The proposed $K^+$ liganding residues are highlighted by gray (backbone carbonyl ligands) and yellow (side-chain ligand). D, a ribbon representation of the two $\alpha\beta$ complexes in the asymmetric unit is shown in light blue with overlay as red mesh of the anomalous difference Fourier map contoured at 5$\sigma$. E, close up of the cytoplasmic $Rb^+$ site with the anomalous difference density map shown as red mesh and residues 720–725 and Asp$^{742}$ indicated in stick representation. The small red sphere represents the refined position of the $Rb^+$. The surrounding P-domain helices P5, P6, and P7 are indicated.

port sites with bound $Rb^+$, and another peak is observed corresponding to the cytoplasmic surface. Both regions appear at the same position in the two $Na^+,K^+$-ATPase molecules of the asymmetric unit (Fig. 1D), and the corresponding peaks are related by noncrystallographic symmetry. At the concentration of 5 mM $Rb^+$ applied here, the electron density corresponding to the cytoplasmic site is somewhat weaker than that observed for the transport sites but nevertheless high enough to suggest an affinity for the third $Rb^+$ ion comparable to that of the transport sites. Under the present experimental conditions, $Rb^+$ is the only anomalous scatterer of significance. The identification of the third site through anomalous difference Fourier density allows us to unambiguously assign the peak density as a $Rb^+$ ion, as opposed to other monovalent cations present like $Na^+,K^+$ (both may be present in trace amounts) or choline$^+$ (present at 100 mM concentration). Under physiological conditions at $\sim$150 mM intracellular $K^+$, this site is probably occupied by $K^+$. The $Rb^+/K^+$ ion seems to interact with at least four backbone carbonyl groups (residues 720, 721, 723, and 724) and one or two side chain oxygen atoms from Asp$^{742}$ (see close up in Fig. 1, B and E, and the alignment highlighting the coordinating residues in Fig. 1C), either by direct coordination or indirectly through an intervening water molecule. These P-domain residues show high conservation among $Na^+,K^+$-ATPases and among P-type ATPases in general, the aspartate that contributes side chain oxygen(s) being replaced by glutamate only in the SERCA type of $Ca^{2+}$-ATPases (Fig. 1C). The backbone carbonyl oxygen ligands are donated by residues within the loop between the sixth helix of the P-domain ("P6 helix") and the following $\beta$-strand. Asp$^{742}$ is positioned in the loop between the seventh helix of the P-domain ("P7 helix") and a $\beta$-strand that leads into the M5 transmembrane segment (Fig. 1E). The distances between the putative oxygen ligands and the $Rb^+$ ion are indicated in Table 1 and compared with the corresponding distances in various $Ca^{2+}$-ATPase crystal structures, where either $K^+$ or $Na^+$ is bound at the equivalent site. It is of notice that generally the distances corresponding to the first three carbonyl oxygen groups (720/711, 721/712, and 723/714) where either $K^+$ or $Na^+$ is bound at the equivalent site.

3 All numbering of $Na^+,K^+$-ATPase residues in this article refers to the sequence of the rat $\alpha$1-isoform.
are longer in the Na\(^{+},\)K\(^{+}\)-ATPase than in the Ca\(^{2+}\)-ATPase, which may reflect the larger radius of the Rb\(^{+}\) ion (1.52 Å) relative to the radii of the K\(^{+}\) and Na\(^{+}\) ions (1.38 and 1.02 Å, respectively) found in the Ca\(^{2+}\)-ATPase crystal structures. The particularly large distance (4.92 Å) for Ser720 may imply second, aqueous sphere coordination.

Expression and Catalytic Turnover Rate—To study the functional importance of the third Rb\(^{+}/\)K\(^{+}\) binding site and of Asp742 in particular, we replaced the latter residue with alanine (to remove the side chain oxygen ligands), with glutamate (the residue present at the equivalent position in SERCA), and with lysine (to disrupt the site) and studied the effects on the overall and partial reactions of the Na\(^{+},\)K\(^{+}\)-ATPase, indicating that the Na\(^{+}\) and K\(^{+}\) transport rates of the mutants were sufficiently high to be compatible with cell viability.

The Na\(^{+}\) - and K\(^{+}\)-stimulated ATPase activity and the active site concentration (maximal phosphorylation capacity) were measured on isolated membranes containing the expressed exogenous enzyme, and the catalytic turnover rate was calculated as the ratio between these two numbers. Fig. 2 depicts the catalytic turnover rate and ATPase activity was determined at 37 °C in the presence of 30 mM histidine buffer (pH 7.4), 130 mM NaCl, 3 mM ATP, 3 mM MgCl\(_2\), 1 mM EGTA, ouabain to inhibit the endogenous enzyme and 20 or 100 mM KCl. The catalytic turnover rate was calculated as the ratio between the Na\(^{+},\)K\(^{+}\)-ATPase activity and the active site concentration (maximum phosphorylation from [γ-\(^{32}\)P]ATP measured at 0 °C in the presence of 150 mM NaCl and oligomycin) (29).

FIGURE 2. Catalytic turnover rate at 20 and 100 mM K\(^{+}\). The Na\(^{+},\)K\(^{+}\)-ATPase activity was determined at 37 °C in the presence of 30 mM histidine buffer (pH 7.4), 130 mM NaCl, 3 mM ATP, 3 mM MgCl\(_2\), 1 mM EGTA, ouabain to inhibit the endogenous enzyme and 20 or 100 mM KCl. The catalytic turnover rate was calculated as the ratio between the Na\(^{+},\)K\(^{+}\)-ATPase activity and the active site concentration (maximum phosphorylation from [γ-\(^{32}\)P]ATP measured at 0 °C in the presence of 150 mM NaCl and oligomycin) (29).
absence of Na\(^{+}\) and ATP, and the time course of phosphorylation with \([\gamma-\text{32P}]\text{ATP}\) in the presence of a saturating Na\(^{+}\) concentration and oligomycin was followed (10). For the Rb\(^{+}\) occluded enzyme, the reaction leading to phosphorylation proceeds through the steps [Rb\(_2\)]E\(_2\) \(\rightarrow\) E\(_1\) \(\rightarrow\) Na\(_3\)E\(_1\) \(\rightarrow\) [Na\(_3\)]E\(_1\)P (cf. Scheme 1, reactions 1–3), where [Rb\(_2\)]E\(_2\) \(\rightarrow\) E\(_1\) is the rate-limiting step. The observed rate constant of phosphorylation, reflecting the rate of [Rb\(_2\)]E\(_2\) \(\rightarrow\) E\(_1\), was significantly enhanced in all three mutants, 3-fold for D742A and D742E and 6-fold for D742K, relative to wild type (Table 2), thus indicating that the E\(_2\) form is destabilized in the mutants relative to the wild type and more so in D742K than in the other two mutants.

This conclusion was further substantiated by measurements of the vanadate concentration dependence of Na\(^{+}\), K\(^{+}\)-ATPase activity (Fig. 5). Vanadate acts as a phosphoryl transition state analog that specifically binds to the E\(_2\) form and inhibits the activity of the Na\(^{+}\), K\(^{+}\)-ATPase (32). Vanadate inhibition of the ATPase activity therefore provides information about the concentration of E\(_2\) accumulated at steady state; a reduced level of E\(_2\) caused, for example, by an increased rate of E\(_2\) \(\rightarrow\) E\(_1\) or a reduced rate of E\(_2\)P \(\rightarrow\) E\(_2\) dephosphorylation would be expected to reduce the apparent affinity for vanadate, which is exactly what was found for the D742 mutants, again most pronounced for D742K (more than 100-fold increase in K\(_{0.5}\); Fig. 5 and Table 2).

E\(_2\)P Dephosphorylation—The mutational effects on K\(^{+}\) interaction with the enzyme were investigated further by measuring the K\(^{+}\) concentration dependence of E\(_2\)P dephosphorylation (Scheme 1, reactions 5 and 6). To accumulate the E\(_2\)P intermediate, phosphorylation with \([\gamma-\text{32P}]\text{ATP}\) was carried out for 5 s at 25 °C in the presence of 20 mM Na\(^{+}\) and absence of K\(^{+}\), and subsequently K\(^{+}\) was added together with an excess of nonradioactive ATP to terminate the phosphorylation from \([\gamma-\text{32P}]\text{ATP}\), allowing dephosphorylation to be followed by acid quenching at sequential time intervals, using the quenched flow technique (28, 30, 31). Binding of K\(^{+}\) to the membranous transport sites in their extracellularly facing state of the E\(_2\)P form activates the Na\(^{+}\), K\(^{+}\)-ATPase by inducing dephosphorylation, leading to the E\(_2\) dephosphoenzyme form (Scheme 1). However, any effect on dephosphorylation of K\(^{+}\) binding at the site in the cytoplasmic domain should also be revealed, since the
membranes used here were permeabilized, such that the added
K\(^+\) had access to the cytoplasmic as well as the extracellularly
facing sites. The dephosphorylation data obtained are exempli-
cified in Fig. 6A for 5 mM added K\(^+\). The rate of dephosphoryla-
tion of D742K was found to be considerably reduced (to less
than one-third) relative to that of the wild type, whereas the
dephosphorylation rates of D742A and D742E are seen to be
much more wild type-like. However, the detailed examination
of the K\(^+\) concentration dependence (Fig. 6B) revealed signif-
cantly different characteristics of the D742A and D742E
mutants, as well as D742K, compared with the wild type, since
the mutants displayed a distinct biphasic K\(^+\) activation pattern.
Hence, a satisfactory fit of the wild-type data in Fig. 6B was
obtained using a single monophasic binding isotherm, whereas
for the mutants a biphasic function was required; for simplicity,
we used here the sum of two hyperbolic functions, thus
extracting two dissociation constants (K\(_{0.5}\) values). All three
mutants displayed a K\(_{0.5}\) value corresponding to the highest
affinity rather similar to that of the wild type (Table 3,
K\(_{0.5}\) = 1.5–3.4 mM), whereas the highest K\(_{0.5}\) value (corresponding
to the lowest affinity, K\(_{0.5}\)) differed among the mutants, being 99,
159, and 701 mM for D742E, D742A, and D742K, respectively.
The maximal rate corresponding to the sum of the amplitudes
of the two phases was wild type-like for D742A and D742E,
whereas it was reduced by one-third in D742K. The comparison
with the other two mutants shows that only the high affinity
phase has reduced amplitude in D742K (Table 3).

To further verify the absence of a low affinity phase in the K\(^+\)
dependence of E\(_{1}\)P dephosphorylation in the wild type at a
higher time resolution, dephosphorylation experiments were
also carried out at 10 °C in the presence of 100 mM K\(^+\) or 500
mM K\(^+\). Under these conditions, the rate constant was 103 \(\pm\) 5
and 108 \(\pm\) 11 s\(^{-1}\), respectively, thus again indicating that in
the wild type there is little further activation at K\(^+\) concentra-
tions above 100 mM. A nonspecific increase in ionic strength
obtained by the addition of 900 mM N-methyl-d-glucamine
to the wild type at 10 °C in the presence of 100 mM K\(^+\) was also
without significant effect on the dephosphorylation rate con-
stant (103 \(\pm\) 8 s\(^{-1}\)).

To make sure that the marked reduction of the rate of dephos-
phorylation in D742K is not caused by a block of the E\(_{1}\)P \(\rightarrow\) E\(_{2}\)P
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Table 3

Analysis of E₂P dephosphorylation

|        | High affinity phase | Low affinity phase | Total V<sub>max</sub> <sup>ab</sup> |
|--------|---------------------|--------------------|-----------------------------------|
|        | k<sub>off</sub> (K⁺) | V<sub>max</sub>     | k<sub>off</sub> (K⁺) | V<sub>max</sub>     |                     |
| Wild type | 3.4                  | 257                | 3.4                        | 257                |                        |
| D742A | 3.3                  | 182                | 3.3                        | 100                | 282                |
| D742E | 2.6                  | 152                | 2.6                        | 103                | 255                |
| D742K | 1.5                  | 49                 | 1.5                        | 711                | 165                |

<sup>a</sup> A hyperbolic function V<sub>max</sub>(K⁺) = (k<sub>off</sub> + [K⁺]) for wild type or the sum of two such functions V<sub>max</sub>(K⁺) = k<sub>off</sub> + (k<sub>off</sub> + [K⁺]) for D742A and D742E. Taking together these data indicate that the site is strongly affected by the role of the catalytic site asp in the ATPase activity. The catalytic site is needed to achieve the maximal rate of dephosphorylation. The K⁺ concentration dependence revealed two distinct phases of K⁺ activation of E₂P dephosphorylation in the mutants, corresponding to k<sub>off</sub> values of 2–3 mM and 100–700 mM, which may be interpreted as reflecting the extracellularly facing membranous transport sites and the cytoplasmic K⁺ site, respectively. The lower k<sub>off</sub> value being similar to the apparent affinity of 1.9–2.5 mM for binding of extracellular K⁺ to the transport sites in the Na⁺,K⁺-ATPase. Hence, the data indicate that the transport sites are undisturbed with normal affinity in the Asp<sup>742</sup> mutants. In Fig. 6B, as previous studies, only one phase with a k<sub>off</sub> of ~3 mM for K⁺ was distinguishable for the wild type, suggesting that the affinity of the cytoplasmic activating K⁺ site is close to that of the membranous transport sites. The proposition that the affinity of the cytoplasmic site is close to 3 mM in the wild type implies that the affinity is lowered ~30- and ~50-fold in D742E and D742A, respectively, and ~230-fold in D742K, which explains the finding that the ATPase activity increases in the mutants upon raising the K⁺ concentration from 20 to 100 mM, whereas the wild type is fully active already at 20 mM K⁺ (Fig. 2).

The functional requirement for the side chain of Asp<sup>742</sup> is consistent with the Na⁺,K⁺-ATPase structure, showing that one or possibly two side-chain oxygen atoms of Asp<sup>742</sup> are involved in the coordination of K⁺ at the cytoplasmic site (Fig. 1). The fact that not only the alanine substitution but even glutamate lowers the affinity substantially indicates that the exact distance between the negatively charged carboxylate group and the K⁺ ion is of utmost importance for high affinity K⁺ binding at this site. The very low affinity of D742K would be consistent with the rather bulky and positively charged side chain of the lysine competing with K⁺ for occupation of the site. The low affinity activating phase believed to reflect occupation of the cytoplasmic K⁺ site has similar amplitudes (V<sub>max</sub>) in the three mutants (Table 3), whereas only the lysine substitution causes a significant reduction of the maximal rate of dephosphorylation corresponding to the high affinity phase (V<sub>max</sub>). Hence, the extrapolated maximal rate of dephosphorylation corresponding to infinite K⁺ concentration is as high as two-thirds of the wild type in D742K and wild type-like in D742A and D742E (Table 3). Taken together, these data indicate that once the K⁺ ion is bound, it does activate the dephosphorylation, despite the various degrees of disturbance of the site introduced by the mutations. This seems to be in good accordance with the basic structure of the site being formed by a loop of backbone carboxyl groups at the C-terminal end of the P6 helix (Fig. 1, B and E), not touched by the mutations. Asp<sup>742</sup>, on the other hand, is not part of this loop, and the role of its side chain is mainly to ensure the high affinity of the site.

The functional importance of the K⁺ ion may be related to its ability to stabilize the loop structure, thereby causing effects on the P-domain enhancing the dephosphorylation. The P-domain helices P5, P6, and P7 together with the helices of the A-M3 and A-M2 linkers form a bundle of parallel helices (Fig. 7), which probably would tend to be destabilized by the dipole moments of the helices were it not for the presence of the compensating positive charge of the K⁺ ion at the C-terminal ends of P6 and P7. Hence, the K⁺ ion may be strongly influential on the position of the highly conserved residues of the catalytic site that are located right before the start of the P6 helix (Fig. 1, B and E), not touched by the mutations. Asp<sup>742</sup> is strongly influential on the position of the catalytic site that are located right before the start of the P6 helix (Fig. 1, B and E). The P-domain enhancing the dephosphorylation. The P-domain helices P5, P6, and P7 together with the helices of the A-M3 and A-M2 linkers form a bundle of parallel helices (Fig. 7), which probably would tend to be destabilized by the dipole moments of the helices were it not for the presence of the compensating positive charge of the K⁺ ion at the C-terminal ends of P6 and P7. Hence, the K⁺ ion may be strongly influential on the position of the highly conserved residues of the catalytic site that are located right before the start of the P6 helix (Fig. 1, B and E), not touched by the mutations. Asp<sup>742</sup> is strongly influential on the position of the catalytic site that are located right before the start of the P6 helix (Fig. 1, B and E).
The catalytic site is indicated by the magnesium fluoride \( \text{MgF}_4 \) the various \( \text{Ca}^{2+} \) crystal structure of the \( \text{Rb}^{+} \text{-ATPase} \), thought to be stabilized in the \( \text{E}_2 \) conformation by occupation of the cytoplasmic K\(^+\) site (the \( \text{Rb}^{+} \) ion bound as \( \text{K}^{+}\) sphere with the side chain of Asp\(^{742}\) (D742) seen behind). The A-, P-, and N-domains are colored yellow, dark blue, and red, respectively, and the membrane domain is light blue. The catalytic site is indicated by the magnesium fluoride \( \text{MgF}_4 \) (MgF) bound as phosphate analog and by the highly conserved catalytic residues Asp\(^{1271}\) (D371) (phosphorylated aspartate), Glu\(^{716}\) (E216) (A-domain TGES loop), Asp\(^{712}\) (D712), and Asn715 (N715), the last two P-domain residues being part of the loop between the P5 and P6 helices.

C-terminal end of the P6 helix and promoting helix-helix interactions. The N-terminal part of the P6 helix interacts directly with residues in the helix formed by the A-M3 linker, and the importance of the latter interaction for stabilization of the \( \text{E}_2 \) form was previously demonstrated in a mutagenesis study (36). We hypothesize that in the absence of K\(^+\) binding at the C-terminal ends of P6 and P7 the structure will be distorted, thereby interfering with the interaction between the P-domain and the A-M3 linker, thus leading to destabilization of the \( \text{E}_2 \) form. A corollary of this hypothesis is that the P-domain-A-M3 linker interaction should be absent in the \( \text{E}_1 \) form, which does not seem to be destabilized by the mutations. This is exactly what appears in the case of the closely related sarcoplasmic reticulum Ca\(^{2+}\)-ATPase, for which crystal structures have been solved both for \( \text{E}_1 \) and \( \text{E}_2 \) forms (5, 22, 37–40). In all of the crystallized Ca\(^{2+}\)-ATPase \( \text{E}_1 \) states, the A-M3 linker lacks secondary structure and has moved away from the P6 and P7 helices, whereas in the various Ca\(^{2+}\)-ATPase \( \text{E}_2 \) states, the A-M3 linker forms a helix approaching the P6 and P7 helices, just as seen for the crystal structure of the \( \text{Rb}^{+} \) occluded \( \text{E}_2 \) state of the Na\(^+\),K\(^+\)-ATPase (4). The cytoplasmic K\(^+\) binding site seems to be rather well conserved between the Na\(^+\),K\(^+\)-ATPase and the Ca\(^{2+}\)-ATPase, the latter having a similar monovalent cation binding loop structure at the C-terminal end of the P6 helix as the Na\(^+\),K\(^+\)-ATPase with contribution to cation coordination from a carboxylic acid residue at the C-terminal end of P7 (in this case a glutamate; see Fig. 1C and Table 1) (21). In the Ca\(^{2+}\)-ATPase, the affinity of this site for K\(^+\) seems to be ~15-fold lower than in the Na\(^+\),K\(^+\)-ATPase (20), which according to the present observations may be a consequence of the replacement of the aspartate with the larger glutamate. In this connection, it is interesting to note that in the \( \text{E}_2\)P analog structure of the Ca\(^{2+}\)-ATPase (Protein Data Bank code 3B9B), the side chain of Glu\(^{732}\) points away from the cation as indicated in Table 1 by long distances to the side chain oxygens. Occupation of the site nevertheless has a stimulating effect on \( \text{E}_2\)P dephosphorylation rather similar to that observed here for the Na\(^+\),K\(^+\)-ATPase (20, 21), and a stabilization of the interaction between the P- and A-domains may also be involved in the Ca\(^{2+}\)-ATPase (22). The activity of the plant plasma membrane H\(^+\)-ATPase is likewise stimulated by K\(^+\) (41), and a recent study attributes a major role to Asp\(^{742}\), corresponding to Asp\(^{742}\) in the Na\(^+\),K\(^+\)-ATPase (23), thus indicating that the cytoplasmic monovalent cation binding site is a general property of the P-type ATPases.

The high affinity of the cytoplasmic K\(^+\) site of the Na\(^+\),K\(^+\)-ATPase means that under normal physiological conditions, the site is permanently saturated. Apparently, the cytoplasmic K\(^+\) site has dual functional roles, since its occupation enhances \( \text{E}_2\)P dephosphorylation and at the same time stabilizes the \( \text{E}_2 \) form. The finding of this previously unrecognized nontransport site now for the first time makes it possible to account for the so-called trans-effects without having to replace the consecutive Post-Albers transport mechanism by a mechanism involving simultaneous exposure of the transport sites toward the cytoplasmic and extracellular side (13, 15, 33). Hence, stimulation of pump activity by cytoplasmic K\(^+\) (16, 17) may be explained by the activation of \( \text{E}_2\)P dephosphorylation. Yoda and Yoda (18), using a sided system consisting of reconstituted phospholipid vesicles, allowing independent variation of extracellular and cytoplasmic K\(^+\), were able to demonstrate acceleration of phosphoenzyme processing by K\(^+\) binding at a cytoplasmic site thought of as a transport site. Their finding is equivalent to the K\(^+\) stimulation of dephosphorylation reported in Fig. 6 of the present work and may be understood by reference to the cytoplasmic site described here. In fact, Yoda and Yoda (18) found an apparent affinity of ~5 nM for activation by cytoplasmic K\(^+\) (i.e. very similar to the present estimation for the wild type).

Under certain conditions, particularly at low ATP concentration, cytoplasmic K\(^+\) inhibits the pump activity (42), which often has been interpreted in terms of K\(^+\) binding at the transport sites in the cytoplasmically facing configuration in \( \text{E}_2 \), in competition with Na\(^+\), followed by a conformational transition to the K\(^+\) occluded \( \text{E}_2 \) form (43, 44). Cornelius and Skou (19), however, reported an anomalous inhibitory effect of cytoplasmic K\(^+\) observable at high ATP concentration or high temperature, where K\(^+\) binding in competition with Na\(^+\) could be excluded as an explanation. This type of inhibition may very well be caused by stabilization of \( \text{E}_1\), by K\(^+\) binding at the nontransport cytoplasmic K\(^+\) site, thus underscoring the point that several previously reported poorly understood observations now can be explained in a simple way.

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