Potassium as an Intrinsic Uncoupler of the Plasma Membrane H\(^{+}\)-ATPase*\(^{S}\)

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The plant plasma membrane proton pump (H\(^{+}\)-ATPase) is stimulated by potassium, but it has remained unclear whether potassium is actually transported by the pump or whether it serves other roles. We now show that K\(^{+}\) is bound to the proton pump at a site involving Asp\(^{687}\) in the cytoplasmic phosphorylation domain, from where it is unlikely to be transported. Binding of K\(^{+}\) to this site can induce dephosphorylation of the phosphorylated E\(_{1}\)P reaction cycle intermediate by a mechanism involving Glu\(^{184}\) in the conserved TGES motif of the pump activator domain. Our data identify K\(^{+}\) as an intrinsic uncoupler of the proton pump and suggest a mechanism for control of the H\(^{+}\)/ATP coupling ratio. K\(^{+}\)-induced dephosphorylation of E\(_{1}\)P may serve regulatory purposes and play a role in negative regulation of the transmembrane electrochemical gradient under cellular conditions where E\(_{1}\)P is accumulating.

P-type ATPases constitute a large ubiquitous family of cation pumps characterized by forming a phosphorylated intermediate as part of their catalytic cycle. In most organisms, except plants (1–3), members of this family are involved in active transport of K\(^{+}\) (4, 5). Examples of P-type K\(^{+}\) pumps are Kdp ATPases from bacterial systems (6), K\(^{+}\)-ATPases in fungi (7), and animal Na\(^{+}\)/K\(^{+}\)- and H\(^{+}\)/K\(^{+}\)-ATPases (8, 9), which exchange K\(^{+}\) with either Na\(^{+}\) or H\(^{+}\). The main primary transporter in the plant plasma membrane is the P-type plasma membrane H\(^{+}\)-ATPase, an electrogenic proton pump that generates the plasma membrane electrochemical gradient of H\(^{+}\) and the ATP hydrolytic activity of which is stimulated by K\(^{+}\) (10, 11). It has been speculated that the plasma membrane proton pump is an H\(^{+}\)/K\(^{+}\) counterion ATPase mechanistically similar to the animal Na\(^{+}\)/K\(^{+}\)-ATPase, H\(^{+}\)/K\(^{+}\)-ATPase, and Ca\(^{2+}\)/H\(^{+}\)-ATPases (12, 13), but decisive proof for active K\(^{+}\) transport has not been obtained. It therefore seems plausible that K\(^{+}\) could serve other role(s) in the plasma membrane H\(^{+}\)-ATPase.

During turnover, P-type ATPases alternate between different conformations that have very different structures. A simplified scheme operates with two basal conformations, Enzyme 1 (E\(_{1}\)) and Enzyme 2 (E\(_{2}\)) (14, 15) (Fig. 1A). Phosphorylation from ATP of E\(_{1}\), in which an ion binding site in the membranous region is facing the cytoplasm, results in formation of a phosphorylated pump intermediate, E\(_{1}\)P, that is spontaneously converted to E\(_{2}\)P, in which the ion is now exposed to the extracellular medium. E\(_{2}\)P will become dephosphorylated to produce E\(_{2}\) that spontaneously reverts to E\(_{1}\). According to this model, binding of H\(^{+}\) to plasma membrane H\(^{+}\)-ATPase triggers phosphorylation of E\(_{1}\) to form E\(_{1}\)P, whereas, in case it was a countereion, binding of K\(^{+}\) to E\(_{2}\)P would be expected to trigger dephosphorylation to E\(_{2}\) (16). In Na\(^{+}\)/K\(^{+}\)- and H\(^{+}\)/K\(^{+}\)-ATPases, uptake of K\(^{+}\) is associated with the E\(_{2}\) to E\(_{1}\) conformational step, which is preceded by dephosphorylation of E\(_{2}\)P (17, 18).

The D684N mutant of plasma membrane H\(^{+}\)-ATPase is H\(^{+}\)-transport-incompetent but can hydrolyze ATP and, as it is defective in the E\(_{1}\)P→E\(_{2}\)P conformational step, E\(_{1}\)P will start to accumulate (19, 20). The E\(_{2}\)P intermediate slowly dephosphorylates and returns to E\(_{1}\), which results in P, release not associated with proton transport (20). As no inhibitors of E\(_{1}\)P→E\(_{2}\)P partial reactions are known for plasma membrane H\(^{+}\)-ATPases, the D684N mutant serves as a unique tool to investigate partial reactions involving E\(_{1}\)P. Unexpectedly, we found that D684N H\(^{+}\)-ATPase is hypersensitive to K\(^{+}\). The D684N mutant proved effective for identifying new mutations that are completely insensitive to K\(^{+}\) but otherwise with normal catalytic properties. The study of these mutants has allowed for a detailed characterization of the role of K\(^{+}\) in the ATP hydrolytic mechanism of the proton pump. We demonstrate that K\(^{+}\) has the capacity to induce rapid dephosphorylation of the E\(_{1}\)P phosphoform. As transport takes place between the E\(_{1}\) and E\(_{2}\) conformational states, K\(^{+}\)-induced dephosphorylation of E\(_{1}\)P serves as a pump uncoupling mechanism.

**EXPERIMENTAL PROCEDURES**

*Site-directed Mutagenesis—*The construction of the wild-type H\(^{+}\)-ATPase vector for heterologous expression in the yeast *Saccharomyces cerevisiae*, as well as the construction of the D684N mutant, has been described (19). Other mutants were constructed by site-directed mutagenesis using an overlap extension polymerase chain reaction (21). All mutated sequences were verified by DNA sequencing. All proteins were expressed without the COOH-terminal 73-amino-acid residues but with a COOH-terminal Met-Arg-Gly-Ser-His\(_{6}\) (MRGSH\(_{6}\)) tag. The COOH-terminal deletion transforms the enzyme into a constitutively activated state, simplifying functional studies. The addition of the His\(_{6}\) tag allows affinity purification of recombinant H\(^{+}\)-ATPase (22).
**K⁺ Uncouples P-type Plasma Membrane H⁺-ATPase**

**Expression in Yeast and Protein Affinity Purification**—The *S. cerevisiae* strain RS-72 (23) was transformed and cultured essentially as described previously (24). Affinity purification of heterologously expressed wild-type and D684N mutant H⁺-ATPases was performed as described (20) except that purified proteins after purification were dialyzed against GMED20 (20% (v/v) glycerol, 100 mM Tris-ATP, pH 6.5 with N-methyl-D-glucamine), 1 mM MgSO₄, and 50 mM KCl. The ATP hydrolysis reaction was followed as described above. The activity remaining after 20 min at 46 °C was related to proteins incubated 20 min on ice (100% activity).

**Stability Measurements**—Purified proteins in reactivation buffer were incubated 46 °C for 20 min in 20 mM MOPS (pH adjusted to 6.5 with N-methyl-d-glucamine) and either in the absence or in the presence of 50 mM monovalent cation (LiCl, NaCl, or KCl). After heat treatment, proteins were diluted 50 times into buffer containing Tris-ATP (3 mM), 1 mM MgSO₄, and 50 mM KCl. The ATP hydrolysis reaction was followed as described above. The activity remaining after 20 min at 46 °C was related to proteins incubated 20 min on ice (100% activity).

**Reconstitution of H⁺-ATPases into Artificial Liposomes**—To determine the sidedness of the K⁺ effect, protein was reconstituted at a lipid to protein ratio of 200:1 as described (22) in 10 mM MES (adjusted to pH 6.5 with N-methyl-D-glucamine), 50 mM Na₂SO₄, or 50 mM K₂SO₄, and 20% (v/v) glycerol. Reconstituting wild-type protein with Na⁺ substituting for K⁺ consistently resulted in vesicles that were less transport-competent (data not shown). Immediately prior to use, the outside medium of K₂SO₄ containing vesicles was exchanged by spinning a 100-μl sample (12 μg of protein) through a 2-ml column of Sephadex G50 equilibrated in 10 mM MES (adjusted to pH 6.5 with N-methyl-D-glucamine), 50 mM Na₂SO₄, and 20% (v/v) glycerol.

**Phosphorylation and Dephosphorylation of the Phosphorylated Intermediate**—Phosphorylation by [γ-³²P]ATP and dephosphorylation initiated with 10 mM EDTA, 10 mM EDTA supplemented 2 mM ADP or various cations were performed as described (19).

**Protein Determination**—Protein concentrations were determined by the method of Bradford (25) employing bovine serum albumin as a standard.

**RESULTS**

The ATP Hydrolytic Activity of the D684N Mutant Is Stimulated to High Values by K⁺—The only conserved acidic residue in the proton coordination center of plasma membrane H⁺-ATPase is Asp⁶⁸⁴ in transmembrane segment 6 (16)(Fig. 1B). If this residue is coordinating K⁺ as a counter-transported ligand, a D684N substituted pump would be expected to have reduced K⁺ sensitivity. The ATP hydrolytic activities of affinity-purified wild-type and mutant D684N plasma membrane H⁺-ATPase proteins were measured in the presence of various concentrations of KCl (Fig. 2A). Although physiological concentrations of KCl (50 mM) stimulated ATP hydrolysis by the wild-type protein less than 2-fold, KCl stimulated the Asp⁶⁸⁴ substituted enzyme more than 4-fold when measured at standard conditions (pH = 6.5, 30 °C) (Fig. 2A). K⁺ stimulated ATPase activity of both the wild-type and the D684N substituted protein with the same apparent Kₘ (1 mM at pH 6.5). The

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2 The abbreviations used are: MES, 2-morpholinoethanesulfonic acid; MOPS, 3-(N-morpholino)propanesulfonic acid.
effect of K⁺ on the D684N substituted enzyme was specific for K⁺ as other physiologically relevant monovalent cations did not stimulate ATP hydrolysis to the same degree as K⁺ (Fig. 2B). The effect of monovalent cations on ATP hydrolytic activity of D684N was as the same order (K⁺ > Rb⁺ > Na⁺ > Li⁺) as seen for wild-type proteins (Fig. 2B) (26).

K⁺ Does Not Induce E₁–E₂ Transitions in the D684N Mutant—One explanation for the above finding could be that K⁺ accelerates transition of E₁P into the E₂P conformation that in turn is dephosphorylated. Vanadate is a transition state analogue of inorganic phosphate in the E₂ conformation of P-type ATPases, and for this reason, vanadate can be used as a conformational probe of P-type ATPases (4). The vanadate sensitivity of ATP hydrolysis was measured for the wild-type and D684N mutant proteins in the absence and in the presence of 50 mM KCl (Fig. 2C). K⁺ increased the vanadate sensitivity of the wild-type protein (Fig. 2C; Table 1; Kᵥ = 50 μM in the absence of KCl, Kᵥ = 10 μM at 50 mM KCl). However, K⁺ did not affect the vanadate insensitivity of the D684N mutant protein. Thus we conclude that the stimulatory effect of K⁺ is not due to an induced E₁–E₂ transition in the D684N mutant protein.

K⁺ Induces Dephosphorylation of the E₁P Phosphoform of the D684N Protein—In P-type ATPases, the E₁P phosphoform is capable of ATP synthesis from ADP and bound phosphate and dephosphorylates to E₁ in the presence of ADP (4). As expected for the E₁P phosphoform, all of the phosphoforms of P-type ATPases with K⁺ as an inorganic phosphate in the E₂ reaction cycle intermediate populations: one that is ADP-sensitive and another that is K⁺-sensitive. An increase in the KCl concentration from 50 to 1000 mM only resulted in a slightly increased degree of E₁P dephosphorylation (Fig. 3C), which indicates that K⁺-induced breakdown of E₁P in the D684N mutant is saturable. The remaining E₁P was still sensitive toward application of ADP, which suggests that it belongs to the E₁P pool (Fig. 3A). This indicates the presence of two E₁P reaction cycle intermediate populations: one that is K⁺- and ADP-sensitive and another that is K⁺-insensitive but ADP-sensitive.

### Table 1: Kinetic properties of plasma membrane H⁺-ATPase mutant proteins

| H⁺-ATPase | ATPase activity | Kᵥ (ATP) | Kᵥ (vanadate) | pH optimum |
|-----------|-----------------|----------|---------------|------------|
| WT        | 22.1 ± 1.9      | 64 μM    | 10 μM         | 6.8        |
| D617A     | 29.9 ± 1.7      | 50 μM    | 95 μM         | 6.8        |
| D684N     | 5.3 ± 1.0       | 56 μM    | Insensitive   | 6.4        |
| D617A/D684N | 4.8 ± 1.6   | 52 μM    | 5 Insensitive | 6.4        |

* Specific activity of ATP hydrolysis is expressed as μmol of Pᵢ produced/min/mg of protein in the absence of monovalent cations.
* The apparent Kᵥ for ATP hydrolysis.
* The Kᵥ for vanadate in the presence of 50 mM KCl.
* The pH optimum of the ATP hydrolysis reaction.

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**FIGURE 2.** The K⁺ stimulatory effect is potentiated in the D684N substituted plasma membrane H⁺-ATPase. A, KCl stimulation of ATP hydrolysis by wild-type (○, ●) and D684N substituted (□, △) pumps at 30 °C (○, ●; 3 mM ATP) or 0 °C (□, △; 1 μM ATP as in phosphorylation assays). The ATP hydrolytic activities in the absence of monovalent cations were taken as 0% and are indicated in Table 1. B, the effect of monovalent cations (25 mM) on ATP hydrolytic activity. Gray bars, LiCl; white bars, NaCl; black bars, KCl; light gray bars, RbCl. WT, wild type. C, insensitivity toward vanadate of the D684N substituted enzyme (□, △) when compared with wild-type (○, ●) and D617A (●, ▲). A substituted enzymes indicates that K⁺ does not induce E₁–E₂ transitions. KCl was either excluded from the ATPase assay (□, △, ○) or added at 50 mM (●, ▲, ○). In A–C, constant ionic strength (125 mM) was obtained by adding Tris-HCl. Error bars represent the standard deviation of at least three independent experiments.
The Effect of K⁺ on Other Asp684 Substituted Enzymes—Like D684N, other substitutions at this site (D684A, D684V, and D684R but not D684E) are blocked in the E₁P conformational state. The degree by which K⁺ stimulated the ATP hydrolysis reaction was in accordance with the degree of E₁P intermediate accumulation (Fig. 3D). This correlation indicates that K⁺ stimulation of ATP hydrolysis is not a general characteristic of E₁ accumulating mutants but rather could be related to the degree of accumulation of the E₁P conformational state.

K⁺ Exerts Its Effect from the Cytoplasmic Face of the Enzyme—K⁺ has been suggested to stimulate ATP hydrolysis from the cytoplasmic side of plasma membrane H⁺-ATPase (27). To test the sidedness of the K⁺ effect, detergent-solubilized and purified D684N protein was reconstituted into artificial proteoliposomes. As no ATP is included in the reconstitution buffer, only ATPase reconstituted with its cytoplasmic ATP binding site facing the outside of the vesicles will split ATP. D684N protein was reconstituted into proteoliposomes in the absence of K⁺, and the phosphorylation reaction was initiated by application of [γ-32P]ATP to the extravesicular medium. K⁺ efficiently promoted E₁P breakdown under these conditions (Fig. 4A), suggesting that binding of K⁺ occurs to the same part of the ATPase as does ATP, i.e. to the cytoplasmic side of the pump. Reconstitution in the presence of K⁺ with subsequent removal of K⁺ from the extravesicular medium resulted in relatively stable E₁P intermediate that could be triggered to dephosphorylate by the addition of K⁺ to the extravesicular medium (Fig. 4A).

Monovalent Cation Stimulation Is Lost in D617A/D684N Double Substituted Enzymes—The cytoplasmic part of P-type ATPases is subdivided into the A (actuator), N (nucleotide binding), and P (phosphorylation) domains (28, 29)(Fig. 1B). We hypothesized that K⁺ facilitates water attack in the P domain either by directly changing the geometry of the covalently bound phosphate or, alternatively, by indirectly influencing the phosphate geometry trough induction of a conformational change.

In a homology model of AHA2 plasma membrane H⁺-ATPase (30), the P domain is comprised of residues 308–337 and 491–622. Negatively charged amino acid moieties are often found in context with monovalent cation binding sites (31), and to investigate whether residues in the phosphorylation domain of the H⁺-ATPase mediate the K⁺ response, 8 conserved H⁺-ATPase acidic residues (Glu318, Asp492, Glu520, Glu556, Asp559, Asp600, Asp610, and Asp617) were selected for mutagenic analysis (Fig. 1B). Conserved residues that in related P-type pumps are essential for the catalytic mechanism had already been excluded. All mutations were introduced into the D684N mutant H⁺-ATPase background, and the corresponding proteins were expressed in yeast and affinity-purified (data not shown).

Monovalent cations (K⁺ > Na⁺ > Li⁺) stimulated release of P, in the ATPase assay from the E318A/D684N, D492A/D684N, E520A/D684N, E556A/D684N, D559A/D684N, D600A/D684N, and D610/D684N double mutants (Fig. 4B). However, in the D617A/D684N mutant, both the stimulation effect of K⁺ on ATP hydrolytic activity (Fig. 4B) and the K⁺-induced E₁P dephosphorylation (Fig. 4C) were completely lost. The D617A/D684N double mutant resembled the D684N single point substituted enzyme with respect to pH optimum for ATP hydrolysis and specific activity in the absence of K⁺ (Table 1). Furthermore, the lost K⁺ effect was apparent at all pH values investigated (pH 5.5, 6.5, and 7.2; Fig. 5), ruling out an effect of H⁺ competing out K⁺.
To test the hypothesis that Asp\textsuperscript{617} is essential for mediating the K\textsuperscript{+} effect, a single D617A substituted protein was produced. As expected, K\textsuperscript{+}/H\textsubscript{11001} stimulation of ATP hydrolytic activity was lost in the D617A mutant at all pH values determined (Fig. 5), and the pH optimum for ATP hydrolysis resembled that of the wild-type protein (Table 1). In contrast to the wild-type protein, K\textsuperscript{+} did not influence the vanadate sensitivity of the D617A substituted enzyme (Fig. 2C). The specific activity of the D617A single point substituted pump was found higher than the unmodified wild-type protein (Table 1).

**FIGURE 4.** Identification of a cytoplasmically located K\textsuperscript{+} binding site involving Asp\textsuperscript{617}. A, K\textsuperscript{+} affects D684N substituted pump from the cytoplasmic side. In reconstituted proteoliposomes harboring purified D684N, phosphorylation was initiated with [\textgamma\textsuperscript{32}P]ATP, and dephosphorylation (dephos.) of the phosphorylated intermediate was initiated after 8 s with EDTA only (− Addition) or EDTA including either KCl (50 mM) or ADP (2 mM). After either 0 s or 5 s of dephosphorylation, the EP remaining was determined (expressed as the percentage of the amount present at the t = 0) by acid quenching. B, mutating P-domain-associated acidic residues in the D684N background reveals that the monovalent cation effect is dependent on Asp\textsuperscript{617}. ATP hydrolysis was measured in the absence or in the presence of 50 mM LiCl (gray bars), 50 mM NaCl (white bars), or 50 mM KCl (black bars). Constant ionic strength was maintained with Tris-HCl (125 mM). Values are given relative to the measured ATP hydrolytic activity in the absence of added cation. C, EP of the D617A/D684N protein was phosphorylated, and the amount of EP was detected after 5 s of dephosphorylation initiated in the absence and in the presence of KCl (50 mM), NaCl (50 mM) or ADP (2 mM). The amount of EP at t = 0 is set to 100%. In A–C, error bars represent the standard deviation of at least three independent experiments.

**FIGURE 5.** D617A single substituted mutant protein is insensitive to KCl. ATP hydrolysis of wild-type (■), D684N (○), D617A (■), and D617A/D684N (□) proteins was measured in the presence of various concentrations of KCl and at pH = 5.5, pH = 6.5, and pH = 7.2. Error bars represent the standard deviation of at least three independent experiments.

\textbf{Glu\textsuperscript{184} in the A domain Is Necessary for Uncoupled Dephosphorylation of E\textsubscript{P}—Biochemical and structural data have demonstrated that the A domain of P-type ATPases undergoes substantial domain movements during catalysis (17, 32, 33). In the A domain of sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPase (SERCA1a), Glu\textsuperscript{183} in the conserved P-type ATPase signature motif TG\textsubscript{ES} is essential for dephosphorylation of E\textsubscript{P} (34–36). In the plasma membrane H\textsuperscript{+/-}ATPase, the corresponding residue is Glu\textsuperscript{184} (Fig. 1B). We generated E184A mutations in the wild-type and D684N mutant backgrounds to investigate whether the phosphatase function of the A domain is required in the plasma membrane H\textsuperscript{+/-}ATPase for dephosphorylation of E\textsubscript{P}. Neither E184A nor E184A/D684N substituted enzymes exhibited any detectable P\textsubscript{i} release in the ATPase assay (Fig. 6A), and both mutant proteins accumulated to a high degree in the E\textsubscript{P} phosphorylated state (Fig. 6A). This indicates the buildup of stable E\textsubscript{P} forms. A major fraction (~65%) of the E\textsubscript{P} pool of the E184A single substituted protein was insensitive to ADP, suggesting
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FIGURE 6. Glu₁⁸⁴ in the TGES phosphatase motif of the actuator domain is required for dephosphorylation of E₁P. A, ATPase activity (measured as Pᵢ release) (black bars) and steady state phosphorylation (white bars) of wild-type (WT), E₁₈⁴A, D₆₈⁴N, and E₁₈⁴A/D₆₈⁴N substituted enzymes. The Pᵢ release activity and phosphoenzyme level is given relative to the wild-type protein (100%). B, dephosphorylation of the phosphorylated intermediate measured after 1 s with dephosphorylation initiated in the absence (gray bars) or in the presence of ADP (dark gray) and in the presence of KCl (50 mM; white bars). The amount of EP remaining is given relative to at t = 0 (black bars). In A and B, error bars represent the standard deviation of at least three independent experiments.

FIGURE 7. K⁺ binding to Asp⁶¹⁷ provides protection to the H⁺-ATPase against thermal inactivation. Affinity-purified wild-type (WT), D₆₁⁷A, and D₆₈⁴N single substituted enzymes and D₆₁⁷A/D₆₈⁴N double substituted enzyme were incubated in the presence of 50 mM cation for 20 min at 46 °C. Gray bars, Li⁺; white bars, Na⁺; black bars, K⁺. Residual ATP hydrolytic activity was measured in the presence of excess potassium. Error bars represent the standard deviation of at least three independent experiments.

K⁺ Stimulation of Plasma Membrane H⁺-ATPase Involves Asp⁶¹⁷ Situated in a Cytoplasmic Domain—In this study, we show that in a E₁P-E₂P conformationally blocked plasma membrane H⁺-ATPase, K⁺ stimulates dephosphorylation of the E₁P phosphointermediate. Following the substitution of Asp⁶¹⁷ in the cytoplasmic phosphorylation domain, K⁺ stimulation of Pᵢ release and K⁺-induced E₁P dephosphorylation were completely lost. Asp⁶¹⁷ is not situated in transmembrane segments that facilitate ion transfer, but is predicted to be close to the aspartyl phosphate group at Asp⁶¹⁷ (30) (Fig. 1). The fact that the K⁺ effect is completely lost when substituting a single negatively charged residue, without having other measurable effects on H⁺-ATPase kinetics, strongly suggests a stoichiometry of 1 K⁺ bound per pump molecule. In a typical plant cell, the cytoplasmic concentration of K⁺ exceeds 50 mM, which indicates that the K⁺ binding site (apparent Kₘ = 1 mM) will be occupied under normal conditions.

Asp⁶¹⁷ Is Part of a Conserved P-type ATPase K⁺ Binding Site—The activities of Ca²⁺- and Na⁺/K⁺-ATPases are both stimulated by cytoplasmic monovalent cations (37–39). Further, using fast kinetic analysis, K⁺ has been demonstrated to affect the EP conformations of the plasma membrane Ca²⁺-ATPase (40) and the plasma membrane H⁺-ATPase (41). Although the mechanism is not clarified, it appears that cytoplasmic K⁺ promotes dephosphorylation of P-type Ca²⁺-, Na⁺/K⁺, and H⁺-ATPase phosphoenzymes. This demonstrates that a K⁺ effect on pump dephosphorylation is a general phenomenon in P-type ATPases.

In sequence alignments (the P-type ATPase data base), Asp⁶¹⁷ in the plant plasma membrane H⁺-ATPase AHA2 corresponds to Glu³⁷² in sarcoplasmic reticulum Ca²⁺-ATPase SERCA1a. A K⁺ binding site that involves Glu³⁷² (SERCA1a) has recently been identified in crystal structures of sarcoplasmic reticulum Ca²⁺-ATPase (42). K⁺ coordinating groups at

accumulation of the E₁P phosphointermediate (Fig. 6B). Accumulation of E₁P in response to mutations in the TGES motif is in accordance with results obtained in other pumps (35). In contrast herewith, the phosphoprotein that formed in the E₁₈⁴A/D₆₈⁴N double substituted enzyme was highly sensitive toward ADP (Fig. 6B), which indicates that the E₁P conformational state accumulates in this mutant. K⁺ had no effect on breakdown on EP in either E₁₈⁴A or E₁₈⁴A/D₆₈⁴N substituted proteins, which is strong evidence that the phosphatase activity of the A domain is required for K⁺-induced dephosphorylation of the E₁P phosphointermediate.

K⁺ Binding to Asp⁶¹⁷ Provides Protection to the H⁺-ATPase against Thermal Inactivation—If a role of K⁺ is to promote domain interactions in the pump protein, e.g. between the P domain and the A domain, it could be expected that K⁺ has a positive effect on enzyme stability. Indeed, it was found that monovalent cations protected wild-type and D₆₈⁴N H⁺-ATPase against thermal inactivation (Fig. 7). The substitution of Asp⁶¹⁷, to abolish the K⁺ binding site, lead to a loss of K⁺ protection against thermal inactivation (Fig. 7). This was seen for both D₆₁⁷A and D₆₁⁷A/D₆₈⁴N mutant proteins, indicating that K⁺ binding to Asp⁶¹⁷ not only influences enzyme turnover reactions but also affects overall protein stability. Na⁺ also influenced wild-type and D₆₈⁴N protein stability positively (Fig. 7), indicative that the protective effect of K⁺ against thermal inactivation is less specific than its effect on the ATP hydrolytic reaction (Fig. 2B).

DISCUSSION

K⁺ activity of the A domain is required for Ktided proteins, which is strong evidence that the phosphatase Ebreakdown on over reactions but also affects overall protein stability. Naalso has recently been identified in crystal structures of sarcoplasmic reticulum Ca²⁺-ATPase (42). K⁺ coordinating groups at
this site involves oxygen atoms derived from the negatively charged Glu\textsuperscript{722} (SERCA1a) and three oxygen atoms in backbone carbonyl residues (Leu\textsuperscript{711}, Lys\textsuperscript{712}, and Ala\textsuperscript{714} in SERCA1a). Mutation of Glu\textsuperscript{722} in the sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPase reduces the rate of phosphoenzymine dephosphorylation in the presence of K\textsuperscript{+}. A negative charge is present at an equivalent position in many P-type ATPases, which indicates the presence of a conserved K\textsuperscript{+} binding site in the P domain of P-type ATPases. It is an attractive hypothesis that binding of K\textsuperscript{+} to this site promotes pump dephosphorylation in the same P-type pumps.

**Mutational Evidence for Potassium-sensitive and Potassium-insensitive E\textsubscript{1}P Conformational States**—We found that the complete E\textsubscript{1}P pool is ADP-sensitive, whereas only part of the E\textsubscript{1}P pool is K\textsuperscript{+}-sensitive. This is compatible with the presence of two different E\textsubscript{1}P phosphoforms, in only one of which does K\textsuperscript{+} induce a rapid discharge of the covalently bound phosphate. In E\textsubscript{1}E\textsubscript{2} conformationally blocked Na\textsuperscript{+}/K\textsuperscript{+}-ATPase, an ADP- and K\textsuperscript{+}-sensitive E\textsubscript{1}P intermediate (termed E\textsubscript{1}P\textsuperscript{*}) has been described (39, 43). The E\textsubscript{1}P\textsuperscript{*} of this enzyme is sensitive toward application of cytoplasmic K\textsuperscript{+} and is a conversion product between E\textsubscript{1}P (ADP-sensitive) and E\textsubscript{1}P (ADP-insensitive, K\textsuperscript{+}-sensitive) (39, 43). The E\textsubscript{1}P\textsuperscript{*} intermediate of the Na\textsuperscript{+}/K\textsuperscript{+}-ATPase could resemble the K\textsuperscript{+}-sensitive form of E\textsubscript{1}P in the plasma membrane H\textsuperscript{+}-ATPase.

**Mechanism of the K\textsuperscript{+} Effect on Pump Dephosphorylation**—Our finding that K\textsuperscript{+} positively influences the stability of purified H\textsuperscript{+}-ATPase supports a model in which K\textsuperscript{+} induces a compact conformation of the protein. Crystal structures of sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPase have demonstrated that the A domain undergoes substantial movement and rotates during the E\textsubscript{1}E\textsubscript{2} conformational step (32, 33). Linker regions between the membranous region and the A domain direct this rotation, the purpose of which is to bring the TGES motif in the A domain in close proximity to the aspartyl phosphate group in the P domain. It has been proposed that K\textsuperscript{+} bound to the P domain assists in attracting the linker regions connected to the A domain (42).

In support of this model, the effect of K\textsuperscript{+} bound to the P domain at Asp\textsuperscript{645} was completely lost in mutants carrying substitutions at Glu\textsuperscript{184} (in the sequence TGES) in the A domain. The negative charge of the conserved TGES motif in the A domain has been suggested to interact with the water molecule that attacks the phosphoryl group (32, 35). The simplest hypothesis in accordance with our data is that K\textsuperscript{+} accelerates docking of the A domain to the P domain, and in this way, promotes A domain-catalyzed decay of the phosphoryl group situated in the P domain.

According to this model, K\textsuperscript{+} is not an uncoupler of the H\textsuperscript{+}-ATPase per se, which would require that it specifically dephosphorylates E\textsubscript{1}P, but rather, it has the capacity to stimulate dephosphorylation of both the E\textsubscript{1}P and the E\textsubscript{2}P phosphoenzymes. What determines whether K\textsuperscript{+} acts as an uncoupler or not is the time the enzyme spends in the E\textsubscript{1}P conformation before it proceeds to the E\textsubscript{2}P conformation. If the E\textsubscript{1}P–E\textsubscript{2}P transition is very fast, and faster than A domain movements, E\textsubscript{2}P will be dephosphorylated by the A domain, and the K\textsuperscript{+} effect will be seen as stimulation of coupled pump turnover. If the conformational transition is slow, the A domain moves in before the E\textsubscript{1}P–E\textsubscript{2}P transition, and K\textsuperscript{+} will act as a pump uncoupler. This could be the case if the membrane potential is close to the reversal potential of the pump. In such a situation, K\textsuperscript{+}-induced dephosphorylation of E\textsubscript{1}P would act as a safety valve.

**Regulation of P-type ATPases by Cytoplasmic K\textsuperscript{+}**—An important question that arises is why a mechanism has developed that potentially allows K\textsuperscript{+} to dephosphorylate E\textsubscript{1}P in a way that uncouples ATP hydrolysis from transport. Uncoupling has been described in several P-type ATPases and appears to be an inherent property of these pumps (44). Several lines of evidence suggest that proton transport and ATP hydrolysis in plasma membrane H\textsuperscript{+}-ATPase are partially uncoupled in the resting state (45–47). Yeast and plant plasma membrane H\textsuperscript{+}-ATPases are controlled by an autoinhibitory COOH-terminal regulatory domain (11) (Fig. 1B). Following relief of this constraint, e.g. by post-translational regulatory processes, proton transport is typically stimulated to a much higher degree than ATP hydrolysis (45–47). This would suggest that post-translational activation involving the regulatory domain is linked to the onset of strict coupling. The found connection between the K\textsuperscript{+} binding site and the phosphorylated aspartic acid could represent a mechanism controlling the coupling ratio depending on the activation state of the pump molecule. K\textsuperscript{+}-induced dephosphorylation of E\textsubscript{1}P may thus well serve regulatory purposes and play a role in negative regulation of the transmembrane electrochemical gradient under cellular conditions where E\textsubscript{1}P is accumulating. As a shift in conformational equilibrium between E\textsubscript{1} and E\textsubscript{2} is likely to determine the extent of the K\textsuperscript{+} effect, any effector that influences this equilibrium would in turn control the coupling state of the pump.

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