Metal Fluoride Inhibition of a P-type H\textsuperscript{+} Pump

STABILIZATION OF THE PHOSPHOENZYME INTERMEDIATE CONTRIBUTES TO POST-TRANSLATIONAL PUMP ACTIVATION*

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Background: Plasma membrane H\textsuperscript{+}-ATPase proton pumps exist in basal and activated states.

Results: Metal fluoride phosphate analogs bind weakly to the proton pump in the basal state, which has a low H\textsuperscript{+}/ATP coupling ratio.

Conclusion: In the basal state, the phosphoenzyme intermediate is unstable.

Significance: A labile phosphoenzyme intermediate of the basal state uncouples ATP hydrolysis from H\textsuperscript{+} pumping.

The plasma membrane H\textsuperscript{+}-ATPase is a P-type ATPase responsible for establishing electrochemical gradients across the plasma membrane in fungi and plants. This essential proton pump exists in two activity states: an autoinhibited basal state with a low turnover rate and a low H\textsuperscript{+}/ATP coupling ratio and an activated state in which ATP hydrolysis is tightly coupled to proton transport. Here we characterize metal fluorides as inhibitors of the fungal enzyme in both states. In contrast to findings for other P-type ATPases, inhibition of the plasma membrane H\textsuperscript{+}-ATPase by metal fluorides was partly reversible, and the stability of the inhibited varied with the activation state. Thus, the stability of the ATPase inhibitor complex decreased significantly when the pump transitioned from the activated to the basal state, particularly when using beryllium fluoride, which mimics the bound phosphate in the E2P conformational state. Taken together, our results indicate that the phosphate bond of the phosphoenzyme intermediate of H\textsuperscript{+}-ATPases is labile in the basal state, which may provide an explanation for the low H\textsuperscript{+}/ATP coupling ratio of these pumps in the basal state.

The P-type plasma membrane (PM)\textsuperscript{3} H\textsuperscript{+}-ATPases of fungal and plant cells generate the essential electrochemical gradient across the plasma membrane and are as such the equivalent of Na\textsuperscript{+}/K\textsuperscript{+}-ATPases in animal cells (1–3). The PM H\textsuperscript{+}-ATPase transports a maximum of one proton per ATP hydrolyzed and pumps protons from the cytoplasm to the extracellular space apparently without any countertransport taking place (4). The PM H\textsuperscript{+}-ATPase belongs to the P-type ATPase family of biological pumps, the members of which share the same overall structural topology of three cytosolic domains and a transmembrane domain through which cations are transported (5). Upon ATP hydrolysis, the P-type ATPase is phosphorylated on a conserved aspartate residue in a cytoplasmic region of the protein. ATP hydrolysis and conformational changes in the cytoplasmic domains are coupled to ion transport through the transmembrane domain by extended transmembrane helices that move in response to events in the cytoplasmic domain and lead to the opening and closing of the ion binding sites. In P-type ATPases, these events are traditionally described by the E1-E2 reaction cycle (Fig. 1). In the E1 conformation, ions are bound to the cytoplasmic side of the pump, leading to pump phosphorylation and the formation of the E1P intermediate, which is converted to the E2P conformation with the concomitant release of ion(s) at the opposite side of the membrane. The E2P conformation is then dephosphorylated along with the countertransport of another ionic species, which for example in Ca\textsuperscript{2+}-ATPase is H\textsuperscript{+} (Fig. 1A), or as has been suggested for PM H\textsuperscript{+}-ATPases (6, 7), dephosphorylation is triggered by a built-in counterion.

The PM H\textsuperscript{+}-ATPase exists in at least two distinct biological states, a basal and an activated state, and similarly, plasma membrane Ca\textsuperscript{2+}-ATPase (8) and sarcoplasmic endoplasmic reticulum Ca\textsuperscript{2+}-ATPase (9) exist in different activity states. For all PM H\textsuperscript{+}-ATPases examined to date, terminal autoinhibitory regions appear to keep the PM H\textsuperscript{+}-ATPases in the low activity basal state (10–14), whereas the activated state is induced by post-translational modification of the autoinhibitory domains in a manner that is strictly dependent on the overall physiological status of the plant or fungal cell. The fungal PM H\textsuperscript{+}-ATPase is transformed from the basal to the activated state when glucose is supplied as a carbon source (15), whereas a multitude of environmental factors, such as blue light (16), microbial toxins (17), nutritional status (18), salt (19), signaling lipids (20), auxin (21), and peptide hormones (22), have been demonstrated to influence autoinhibition of the plant PM H\textsuperscript{+}-ATPase. In both systems, signal transduction cascades transform the pump from the basal state to its fully activated form, a process that typically involves cellular perception of the signal(s), phosphorylation of the PM H\textsuperscript{+}-ATPases at the terminal autoinhibitory
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regions, and structural rearrangements of the pump. The basal state is characterized by a lower affinity for ATP, an acidic pH optimum for ATP hydrolysis (indicating low H\(^+\) affinity), reduced sensitivity to vanadate, lower overall catalytic rate (15, 20), and lower suggested coupling rate (less than one H\(^+\) transported per ATP hydrolyzed) (23, 24). This could potentially indicate that autoinhibition in both fungal and plant H\(^+\)-ATPases shares basic mechanistic features.

The structural mechanism underlying the autoinhibition of PM H\(^+\)-ATPases remains largely speculative. The two-dimensional electron microscopy structure of a fungal PM H\(^+\)-ATPase at 8-Å resolution (25) and a full-length AMPPCP-inhibited plant PM H\(^+\)-ATPase at 5.5-Å resolution (6, 26) most likely reflect fully activated states of the pump, and no clearly defined terminal domains are traceable in these structures. Thus, important questions such as how the autoinhibitory domains interact with the main body of the enzyme, how the pump is transformed from the basal state into the fully activated state, and whether and how the transport coupling ratio varies in the basal versus the fully activated state remain to be addressed despite years of investigation.

A long term goal of our laboratory is to obtain three-dimensional protein crystals of plasma membrane H\(^+\)-ATPases in their various biological states and associated catalytic intermediates. Fluoride complexes of magnesium (MgF\(_3\)), beryllium (BeF\(_2\)), and aluminum (AlF\(_3\)) act as phosphate analogs (26) and inhibit P-type ATPases by interacting with the phosphorylation site, thereby stabilizing conformations that are analogous to specific phosphoenzyme intermediates of the reaction cycle (27–33). Therefore, the metal fluorides have been extensively used to isolate pumps in the E2 conformational state (33–38). However, studies of the interaction between the metal fluorides and the complexes is needed.

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Experimental Procedures

Yeast Strain and Growth Conditions—Strain YAK2 of Saccharomyces cerevisiae (MAT, ade2–101, leu2Δ1, his3Δ200, ura3–52, trplΔ63, lys2–801 pma1::HIS3, pma2Δ::TRPI) was used throughout this study (41). The yeast was grown at 30 °C in minimal medium containing 2% glucose (w/v) and harvested in the stationary phase. Glucose-metabolizing cells producing PM H\(^+\)-ATPases in the activated state and glucose-starved cells producing PM H\(^+\)-ATPases in the basal state were prepared as described (42).

Preparation of Plasma Membranes—The microsomal membranes were isolated as described (7), but 2 mM sodium molybdate was present in all buffers used for purification to inhibit phosphatases. Plasma membranes were isolated as described by Serrano (15), and proteins loosely bound to the plasma membranes were removed with a 15-min chaotropic wash at 4 °C in a buffer containing 50 mM MES (pH 6.5), 20% glycerol, 0.6 mM KCl, 1 mM EDTA, 1 mM dithiothreitol (DTT), 2 mM sodium molybdate, and 2 μg/ml pepstatin A. The fractions containing enriched plasma membranes were washed by ultracentrifugation at 1 h at 45,000 rpm (Beckman 70Ti rotor), and the PM was resuspended in 50 mM MES (pH 6.5), 20% glycerol, 1 mM EDTA, 1 mM DTT, 2 mM sodium molybdate, and 2 μg/ml pepstatin A; subsequently diluted to 2 mg/ml; aliquoted in 50-μl fractions; flash frozen in liquid nitrogen; and stored at −80 °C.

Reconstitution into Lecithin Liposomes—Pma1p in the activated and basal state was reconstituted into lecithin liposomes using the detergent octylglucoside as described earlier (23) except that 180 mg of Bio-Beads\(^{\text{TM}}\) SM2 from Bio-Rad was added to 250 μl of reconstituted Pma1p to remove excess detergent.

ATPase Activity Measurements—The ATPase activity was determined using the Baginski assay (43). Unless otherwise stated, the assays were carried out at 30 °C in a buffer containing 20 mM MES for pH 5.9, 20 mM MES-MOPS for pH 6.7, or 20 mM MOPS for pH 7.5, 10 mM MgSO\(_4\), 5 mM Na-ATP, 50 mM KNO\(_3\), 5 mM NaN\(_3\), 0.44 mg/ml phosphoenolpyruvate, 4 μg/ml pyruvate kinase, and 3.5 mM sodium molybdate. The assay buffers were equilibrated to 30 °C, and the assay was started by adding 150 ng of protein to 300 μl of ATPase buffer in Eppendorf tubes or 100 ng of protein to 60 μl of ATPase buffer in microtiter plates. The assays were stopped after 30–60 min as described (44) unless otherwise stated.

Inhibition of Pma1p—The inhibition of Pma1p by metal chlorides, metal fluorides, and vanadate was either tested directly using the Baginski assay or after 1 h of preincubation at 30 °C. For BeF\(_2\), MgF\(_3\), AlF\(_3\)-ADP, and AlF\(_3\), the BeCl\(_2\)/AlCl\(_3\) to sodium fluoride (NaF) ratio was 1:5 when run directly in the assay. For preincubation, the NaF concentration was maintained at a concentration 5 times that of the highest BeCl\(_2\), AlCl\(_3\), or MgCl\(_2\) concentration.

BeCl\(_2\), AlCl\(_3\), MgCl\(_2\), and orthovanadate were added directly to the assay buffer at concentrations as indicated on the x axis. For AlF\(_3\)-ADP inhibition, 1 mM ADP was added to the assay buffer.

BeCl\(_2\) and AlCl\(_3\) were also tested with preincubation for 1 h at 30 °C in a buffer containing 0.1 mg of plasma membrane/ml, 20 mM MES (pH 5.9), and 20% glycerol. For AlF\(_3\) and BeF\(_2\), the incubation buffer was supplemented with 0.1 mM MgCl\(_2\). For the AlF\(_3\)-ADP incubation, the incubation mixture was supplemented with 1 mM ADP and 0.5 mM MgCl\(_2\). The inhibitor concentrations in the preincubation mixture are indicated on the x axis. After preincubation, the plasma membrane-inhibitor mixtures were diluted 200-fold for the metal fluorides and 100-
fold for the metal chlorides, and the remaining activity was assayed as described above.

Test for Reversibility of Metal Fluoride Inhibition—The PMs were incubated for 1 h with either 0.5 mM AlF₃, 0.5 mM BeF₂, 5 mM MgF₂, or 0.25 mM AlF₃ supplemented with 1 mM ADP. The incubation buffer was supplemented with 0.1 mM MgCl₂ for AlF₃- and BeF₂-mediated inhibition and with 0.5 mM MgCl₂ for AlF₃-ADP before starting the assay by diluting the incubation mixture 200-fold in ATPase buffer. The residual activity was measured at the indicated time points. For time points up to 10 min, 1 mg/ml PM was used in the incubation buffer, and for higher time points, 0.1 mg/ml was used. The lowest time point included was 30 s, which was the lowest time point that showed measurable activity.

Measurement of Proton Pumping and Coupling Rate—Proton transport into vesicles was measured using fluorescence quenching of 9-amino-6-chloro-2-methoxyacridine (ACMA) simultaneously with measurement of ATP hydrolysis coupled to NADH oxidation (23). The assay was conducted in 96-well microtiter plates in a buffer containing 10 mM MES (pH 6.5), 50 mM KSO₄, 2 mM ATP, 2 mM phospho(enol)pyruvate, 30 μg/ml pyruvate kinase, 25 μg/ml lactate dehydrogenase, 0.5 μg/ml valinomycin, 0.25 mM NADH, 2 μM ACMA, and various protein concentrations in a final volume of 150 μl. The assay was started by adding a final concentration of 10 mM MgSO₄ and fluorescence quenching was monitored at excitation/emission wavelengths of 412/480 nm (ACMA) or 350/440 nm (NADH) to minimize spectral overlap. The proton gradient was collapsed by adding 5 μl of 3 mg/ml nigericin. BeF₂ inhibition was tested by directly adding BeF₂ to the assay medium at concentrations indicated on the x axis.

Protein Determination—The protein concentrations were determined using the Bradford reagent and bovine serum albumin as a standard (45).

Figures and Analysis—All figures were generated using GraphPad Prism® and analyzed using the analyzing tool nonlinear regression Michaelis-Menten, log(inhibitor) versus normalized response (variable slope), or sigmoidal dose response (variable slope) when full inhibition was not reached. The standard errors were calculated as S.E. The mode of inhibition was fitted to the following models: competitive, non-competitive, and mixed model inhibition. All experiments were conducted in at least triplicate with biologically independent membranes.

Homology Modeling—The homology model was built using SWISS-MODEL and visualized with PyMOL.

Results

Isolation of PM H⁺-ATPases in the Basal and Activated States—The aim of this study was to characterize metal fluoride-mediated inhibition of the yeast PM H⁺-ATPase Pma1p in two activation states, namely the low affinity basal state isolated from glucose-starved cells and the high affinity activated state isolated from glucose-metabolizing cells. In the yeast S. cerevisiae, two PM H⁺-ATPase genes are present (PMA1 and PMA2; Ref. 41). Here, we used a yeast strain in which PMA2 had been deleted to ensure that only Pma1p was expressed. As detergent solubilization may affect the activation state of the pump (46), we studied the enzyme directly in native plasma membranes except for the H⁺-pumping experiments, which utilized reconstituted pump. Compared with the PM H⁺-ATPase in its autoinhibited basal state, the activated PM H⁺-ATPase had a marked decrease in Km for ATP, an increase in Vmax an increase in vanadate sensitivity, and a pH optimum for ATPase activity shifted toward neutral pH, indicating increased H⁺ affinity (Table 1 and Ref. 15). When Pma1p was reconstituted into lecithin liposomes, specific activity dropped to about half of that in intact membranes, whereas Ki was unaffected. This is likely explained by the fact that a fraction of Pma1p is oriented with its ATP-binding domain toward the luminal side of liposomes following reconstitution and is thereby shielded from the Mg-ATP in the medium.

Effect of Metal Fluorides on PM H⁺-ATPase Activity in a Direct Assay—In the absence of added nucleotide or other ligands, metal fluorides have been reported to stabilize E2 conformations of other P-type ATPases (Refs. 27–33 and Fig. 1, A and B). We first examined the ability of fluorides of aluminum, beryllium, and magnesium to inhibit the PM H⁺-ATPase directly in an assay (Fig. 2). Because magnesium is an essential cofactor of the enzyme and the true substrate is Mg-ATP, magnesium had to be present in all assays and in excess of ATP. For this reason and so as not to increase the amount of magnesium further, we investigated the effect of MgF₂ simply by adding sodium fluoride to the standard assay already containing magnesium.

We found that fluorides of both aluminum and beryllium inhibited the PM H⁺-ATPase in the lower micromolar range at pH 5.9 (Fig. 2, A and B, and Table 1), whereas magnesium fluoride was less potent (Fig. 2C). The MgF₂ complex is expected to contain four fluoride ions per magnesium ion (32), meaning that the MgF₂ concentration is probably 4 times lower than that of sodium fluoride, which contains one fluoride ion per sodium ion. Aluminum fluoride added together with ADP has been reported to stabilize the E1 conformation of Ca²⁺- and Na⁺/K⁺-ATPases (Fig. 1A and Refs. 32 and 47) but failed to inhibit the PM H⁺-ATPase of S. pombe (39). We found that AlF₃-ADP inhibited the PM H⁺-ATPase with a potency of IC₅₀ ≈ 10 μM (Fig. 2D and Table 2).

Regardless of whether or not nucleotides were present, there was no significant difference in metal fluoride sensitivity between the basal state and the activated state of PM H⁺-ATPase (Fig. 2). Taken together, the interaction of PM H⁺-ATPase with metal fluorides appeared to be comparable with that of other P-type ATPases (27–33).

### TABLE 1

| Biological state | Plasma membrane | Reconstituted Pma1p |
|-----------------|-----------------|-------------------|
|                  | Km (ATP) | Vmax (μmol/mg/min) | Km (VO₄³⁻) | Vmax (μmol/mg/min) |
| Basal*           | 2.4 ± 0.7 | 40 ± 0.5 | 11.0 ± 1 | 2.3 ± 0.6 | 1.94 ± 0.2 |
| Activated*       | 0.6 ± 0.1 | 11.7 ± 0.3 | 3.8 ± 1 | 0.6 ± 0.1 | 4.47 ± 0.2 |

* PM H⁺-ATPase in the basal state was added directly to the assay with various concentrations of inhibitor or ATP.
* PM H⁺-ATPase in the activated state was added directly to the assay with various concentrations of inhibitor or ATP.
As controls for the above experiments, we next tested the effect of chloride salts of magnesium, aluminum, and beryllium on Pma1p pump activity. Magnesium is a necessary cofactor for Pma1p, but at high concentrations, MgCl$_2$ also acts as an inhibitor. As seen in Fig. 3A, MgCl$_2$ started to inhibit H$^+$-ATPase activity at concentrations above 10 mM but stimulated activity up to this concentration. When fluoride was substituted with chloride in the assay, both aluminum and beryllium also con-
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TABLE 2

Kinetic constants for the inhibition of PM H⁺-ATPase by metal fluorides in a direct and indirect assay (IC₅₀ values for inhibition are indicated in μM).

| pH   | Basal        | Activated   | MgF₂ direct | AIF₃ | BeF₂ direct | AIF₃-ADP | BeCl₂ | AICl₂ |
|------|--------------|-------------|--------------|------|-------------|---------|-------|-------|
| 5.9  | Basal ND     | Activated ND| 603 ± 12     | 14 ± 2 | 37 ± 1      | 85 ± 2  | 3119 ± 2 | 37 ± 2 | 1409 ± 1 |
|      | Activated    |             | 488 ± 10     | 13 ± 3 | 4 ± 1       | 119 ± 1 | 3159 ± 2 | 42 ± 2 | 1806 ± 1 |
| 6.7  | Basal ND     | Activated ND| 12 ± 1       | ND   | 52 ± 8      | 129 ± 2 | ND    | ND    | ND    |
|      | Activated    |             | 14 ± 1       | ND   | 4 ± 3       | 112 ± 1 | ND    | ND    | ND    |
| 7.5  | Basal ND     | Activated ND| 15 ± 1       | ND   | 154 ± 3     | 205 ± 10 | ND    | ND    | ND    |
|      | Activated    |             | 13 ± 1       | ND   | ND          | 20 ± 1  | ND    | ND    | ND    |

* PM H⁺-ATPase in the basal state was added directly to the assay with inhibitor.

* PM H⁺-ATPase in either the basal or activated state was preincubated with inhibitor for 1 h before dilution.

* ND, not determined. As complete inhibition could not be obtained, it was not possible to estimate an IC₅₀ value for these metal fluorides.

FIGURE 3. Metal chloride inhibition of PM H⁺-ATPase in a direct assay. Metal chloride concentrations were as indicated. The following metal chlorides were tested: magnesium chloride (A), aluminum chloride (B), and beryllium chloride (C). Plasma membrane containing pumps in either the activated (●, blue line) or basal (□, red line) state were used. Error bars refer to S.E. (n = 3).

logs, but the plant homolog of yeast PM H⁺-ATPase has several potential metal coordination sites (48). Binding to such sites might inhibit the PM H⁺-ATPase activity by a mechanism other than that of metal fluorides (48). To confirm that the metal fluoride-mediated inhibition of Pma1p is not strictly caused by the metal ions, we evaluated the inhibitory effects of AIF₃ and BeF₂ as well as aluminum chloride and BeCl₂ on Pma1p pump activity at higher pH in the direct assay. A higher pH is expected to stabilize the E2 conformation, which may increase AIF₃/BeF₂ potency (47). As seen in Table 2, both AIF₃ and BeF₂ have a stronger inhibitory effect on Pma1p pump activity at higher pH, whereas the opposite is the case for the chloride salts. We therefore conclude that metal fluoride complexes inhibit PMH⁺-ATPase activity with substantially higher potency than do fluorides or metals alone. However, we cannot rule out the possibility that, at least in the presence of either aluminum or beryllium fluorides, metal ions and metal fluorides inhibit the PM H⁺-ATPase simultaneously but by different mechanisms even at low concentrations.

Effect of Metal Fluorides on PM H⁺-ATPase Activity in an Indirect Assay—To minimize the effect of free metal and fluoride ions, we preincubated the PM H⁺-ATPase with inhibitor for 1 h at 30 °C and subsequently diluted the incubation mixture 200 times in ATPase buffer. This degree of dilution ensured that the free metal fluoride concentration in the assay was reduced to a level that did not influence PM H⁺-ATPase activity at higher pH, whereas the opposite is the case for the chloride salts. We chose an equilibration time of 1 h following dilution to ensure dissociation of loosely bound ions. Similar procedures have been used for other P-type ATPases (27–33), and this procedure successfully reversed the inhibitory effect of metal chlorides (Table 2).

When testing at pH 5.9 in the indirect assay using pumps in the activated state, AIF₃ appeared to inhibit the PM H⁺-ATPase completely in an irreversible manner both in the presence and absence of ADP (Fig. 4, A and J) and with potency somewhat equal to that seen in the direct assay (apparent IC₅₀ = 28 and 4.5 μM compared with apparent IC₅₀ ≈ 20 and 13 μM in the indirect assay, respectively; Fig. 4A and Table 2). Considering that pH is likely to affect the E1-E2 equilibrium of the PM H⁺-ATPase, we investigated the effect of decreasing the proton concentration. Again, the potency of AIF₃ increased with increasing pH, and at pH 7.5, we observed an apparent IC₅₀ of 11 μM. The opposite effect was seen when ADP was present, which is in agreement
FIGURE 4. Metal fluoride inhibition of PM H⁺-ATPase in an indirect assay. Following preincubation for 1 h with metal fluorides (pH 5.9), the inhibition mixture was diluted 100 times and allowed to equilibrate for 1 h before initiation of the PM H⁺-ATPase assay. Metal fluoride inhibition was determined after preincubation. The following metal fluorides were tested: aluminum fluoride (A–C), beryllium fluoride (D–F), magnesium fluoride (G–I), and aluminum fluoride (J–L) in combination with ADP. Preincubation was carried out at the following pH values: pH 5.9 (A, D, G, and J), pH 6.7 (B, E, H, and K), and pH 7.5 (C, F, I, and L). Plasma membranes containing pumps in either the activated (●, blue line) or basal (□, red line) state were used. Error bars refer to S.E. (n = 3).

When we used the indirect assay to investigate the effect of beryllium and magnesium fluorides, we were surprised to learn that these complexes failed to abolish PM H⁺-ATPase activity even at high concentrations. Thus, around 25% of the PM H⁺-ATPase population appeared to be resistant to BeF₂, and around 50% appeared to be resistant to MgF₂ (Fig. 4, D–I). However, we observed a slight potentiation of inhibition when the pH was raised from pH 5.9 to 7.5 (Fig. 4, D–I). Based on these observations, we conclude that AlF₃ with or without ADP is the most potent and stable metal fluoride inhibitor of yeast Pma1p. We thus used this compound for further analysis of the pump in the E1P or E2P conformation.

Inhibition of the PM H⁺-ATPase in the Basal State by Metal Fluorides—Next and still using the indirect assay, we tested the effect of metal fluorides on the PM H⁺-ATPase in its basal state. Strikingly and at all concentrations and pH values tested, the inhibitory effect was less potent than that observed with the activated protein (Fig. 4, A–I). Furthermore, a higher proportion of PM H⁺-ATPases appeared to be resistant to BeF₂, and around 50% appeared to be resistant to MgF₂ (Fig. 4, D–I). This was even true when AlF₃ was used as inhibitor. Thus, in the presence of this inhibitor, around 20% of the PM H⁺-ATPase population remained resistant, and half-maximal inhibition of the sensitive population required ~100 μM AlF₃. Around 50% of the population was resistant to BeF₂ (Fig. 4, D–F), and around 75% was resistant to MgF₂ (Fig. 4, G–I).

Taken together, the results obtained using the indirect assay suggest that inhibition with metal fluorides is reversible to varying degrees. Thus, following dilution and during the subsequent equilibration period, a substantial proportion of the inhibitor complexes dissociated (in the order AlF₃ (±ADP) ≪ BeF₂ ≪ MgF₂). Furthermore, the stability of inhibitor complexes involving PM H⁺-ATPases in the basal state was substantially lower than that involving pumps in the activated form.

PM H⁺-ATPase Inhibition by Metal Fluorides Is Reversible Depending on the Inhibitor and Activation State of the Protein—It was reported previously that the action of metal fluorides on some P-type ATPases is essentially irreversible (32, 33, 47). To further study the reversibility of metal fluoride inhibition, we kept a preincubation time of 1 h with a fixed amount of metal fluoride (0.5 mM BeF₂, 0.5 mM AlF₃, 0.25 mM AlF₃-ADP, and 5 mM MgF₂ (pH 5.9)) but varied the time allowed for equilibration after dilution. Thus, samples were equilibrated for between 30 s and 2 h before being subjected to an ATPase assay. Following equilibration period, the specific PM H⁺-ATPase activity was determined.

When the pump was in the activated state, we observed that inhibition of the PM H⁺-ATPase by AlF₃ was complete and irreversible after 1 h of preincubation (Fig. 5A). In the basal state, inhibition was complete following 30 min of equilibration after dilution, but the specific activity increased slightly with time, indicating that the effect was, at least to some degree, reversible (Fig. 5A).

A much higher degree of instability was observed for the complex between BeF₂ and PM H⁺-ATPase (Fig. 5B). The activated form of the protein was most stable, and 10% of the inhibitor complex had dissociated after 10 min of equilibration. However, when the protein was in the basal state, around 50% of the complexes appeared to have dissociated after just 30 s of

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equilibration (the first time point for which activity could be measured), and this proportion increased slowly to around 60% after 2 h (Fig. 5B). When the pumps were preincubated with MgF$_2$ around 25% of the inhibition remained following 30 s of equilibration, but this fraction remained constant after prolonged periods of equilibration (Fig. 5C). It appears that a fraction of inhibited PM H$^+$-ATPase cannot be reverted to the activated state even after prolonged equilibration times. Interestingly, inhibition by the only E1 inhibitor, AlF$_3$-ADP, appeared to be irreversible for both activation states after 2 h (Fig. 5D). This was unexpected as the IC$_{50}$ for the basal state is almost 10 times that of the activated state in the indirect assay at pH 5.9. As proton pumping rates using fluorescent probes are not directly quantifiable, the protein concentration of the activated state was reduced in the assay until it gave a signal equal to that of the basal state (Fig. 6B). Using 0.62 μg of membrane protein from the activated state in the assay, the signal equaled that of 5 μg of protein in the basal state, which corresponds to an ~8-fold difference in protein concentration. Taken together, the results indicate that in the activated state the coupling ratio between ATP hydrolysis and proton pumping is increased 8-fold. Assuming an ATP/H$^+$ stoichiometry of 1:1 in the activated state (23), this suggests that significantly less than one H$^+$ is translocated per ATP split in the basal state.

Finally, to test whether metal fluoride-mediated inhibition of proton pumping for the two activation states exhibits the same kinetics as that of ATP hydrolysis, we assayed BeF$_2$ inhibition directly in the H$^+$ pumping assay (Fig. 2E). There was no difference between the inhibitory effect of fluoride and ATP hydrolysis (Fig. 2E and Table 2).

Discussion

The Stability of Metal Fluoride Complexes Depends on Regulatory Post-translational Pump Modification—In this work, we used metal fluoride complexes as phosphate analogs to characterize the intermediary catalytic states of the PM H$^+$-ATPase. Different metal fluorides adopt unique geometries due to their different chemistries and are therefore expected to capture the pump in distinct conformational states (Fig. 1, A and B).

Surprisingly, our results show that the efficiency of irreversible inhibition of the PM H$^+$-ATPase by metal fluorides is strongly related to the activation status of the pump. Thus, for all the fluorinated complexes, the basal state of yeast PM H$^+$-ATPase was more labile than the activated state, and irreversible inhibition was only seen for the E1P inhibitor AlF$_3$-ADP. These results suggest that (i) phosphate is somehow restricted from binding tightly to the conserved aspartate (Asp378; covalently or non-covalently) of the basal state and (ii) the basal state prefers the E1 conformation. However, the observation that metal fluoride is somewhat able to inhibit the pump in the basal state is in agreement with the fact that ATP hydrolysis still takes place for this form and that activation stimulates proton pumping more than ATP hydrolysis.

Activation of the yeast PM H$^+$-ATPase has been linked to a number of events, including phosphorylation of Ser and Thr residues in the C terminus, which functions as an autoinhibitory domain of the pump (42, 49). In the crystal structure of a plant PM H$^+$-ATPase, several activating mutants map to the phosphorylation (P) domain close to the aspartyl phosphate residue (7). Mutational studies of the fungal PM H$^+$-ATPase have also identified residues that alter pump activation status, and these residues are mainly located in two regions: (i) the C-terminal domain (42, 49, 50) and (ii) the P-domain starting...
from stalk segment 5 and extending toward the N-domain close to the phosphorylated aspartate (mapped in Fig. 7 and Refs. 51 and 52). It is therefore likely that the autoinhibitory mechanism involves a conformational shift in the P-domain upon C-terminal binding, resulting in a decreased affinity for ATP and metal fluorides and a possible shift in the E1-E2 equilibrium when substrate is not present.

Metal Fluorides as Stabilizers of Conformational States of the PM H\(^{+}\)-ATPase—This study has shown that the complex between both activity states of the PM H\(^{+}\)-ATPase and metal fluoride varies in stability between the different metal fluorides. The complexes formed with BeF\(_2\) and in particular MgF\(_2\) were extremely unstable compared with those formed with AlF\(_3\) with or without ADP. A distinguishing feature of the PM H\(^{+}\)-ATPase is that so-called “backdoor phosphorylation” by P\(_i\) is barely detectable. Thus, direct phosphorylation from \(^{32}\)P (P\(_i\)) of a fungal PM H\(^{+}\)-ATPase is only observed when using a very sensitive method (53), and with an apparent \(K_m\) for P\(_i\) of 177 mM, the pump has an extraordinarily low affinity for P\(_i\) in comparison with the Ca\(^{2+}\)-ATPase (\(K_m = 4.1\); Ref. 54). At 6.85 mM P\(_i\) and in the absence of ATP, only 0.02% of the plasma membrane H\(^{+}\)-ATPase molecules are phosphorylated (53), whereas 50% of Ca\(^{2+}\)-ATPase molecules are phosphorylated under similar conditions (55). The PM H\(^{+}\)-ATPase is an electrogenic enzyme that catalyzes unidirectional proton transport against a proton gradient and strong membrane potential (56) caused by a substantial difference between the intra- and extracellular pH values of up to several units. The observed transient nature of the “product state” of the PM H\(^{+}\)-ATPase provides a mechanism for preventing the reversibility of proton pumping while simultaneously operating against steep electrochemical gradients. Thus, to prevent backflow of protons following proton translocation and release of the proton at the extracellular face of the membrane, rapid closing of the luminal gate is required. As MgF\(_2\) is expected to inhibit the E2 product state (Fig. 1B), our finding that the complex between the PM H\(^{+}\)-ATPase and
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MgF$_2$ is labile may be explained by the very low affinity of the pump for P$_v$ which may prevent backflow of protons.

A Working Model for the Regulation of the Yeast PM H$^+$/ATPase—In this work, we confirm that the basal state of yeast PM H$^+$/ATPases exhibits a strong reduction in coupling of ATP hydrolysis to protons pumped (Fig. 6). Similar changes in coupling rate are also seen elsewhere in the P-type family. Upon binding of the regulatory peptide sarcolipin, hydrolysis of ATP in the Ca$^{2+}$/ATPase is futile and generates heat only (57). In the structure of the sarcolipin-Ca$^{2+}$/ATPase complex, an open pathway to the phosphorylated aspartate is seen in the “ground state” (9), meaning that the hydrophobic environment that normally characterizes the E2P ground state is not present. Assuming a structural relationship between sarcolipin-inhibited Ca$^{2+}$/ATPase and autoinhibited PM H$^+$/ATPase, the C-terminal interaction may inhibit the pump from entering the fully closed E2P conformation. This would make water attack on the phosphorylated or BeF$_2$-bound aspartate possible and may explain why for the basal state and with the ground state inhibitor BeF$_2$, up to 50% of the inhibition is lost within the first 30 s in the indirect assay. If this model is correct, the aspartyl phosphate is likely to break before the pump undergoes the conformational change associated with proton translocation, which provides a mechanism for the lower coupling rate observed between ATP and H$^+$ pumped.

Metal Fluorides as Tools for Structural Studies of Yeast PM H$^+$/ATPase—We are still awaiting a high resolution crystal structure of a fungal PM H$^+$/ATPase. There may be several reasons for the slow progress toward this goal, but the lack of stable inhibitors that can lock the protein in only one conformation seems to be a significant obstacle. In this study, we have shown that metal fluorides inhibit Pma1p and that AlF$_3$ both in the presence and absence of ADP produces the most stable inhibition. We conclude that metal fluorides are potent inhibitors of the yeast PM H$^+$/ATPase and are thus useful tools for studying the different activation states of this pump. However, further studies are required to determine whether they are useful crystallization agents.

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