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Structural and functional characterization of H^+,K^+-ATPase with bound fluorinated phosphate analogs

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ABSTRACT

Gastric H^+,K^+-ATPase is responsible for gastric acid secretion. In order to characterize the phosphorylation events on H^+,K^+-ATPase, the properties of fluorinated phosphate analogs [XFs, *e.g.* aluminum fluoride (AlF), beryllium fluoride (BeF) and magnesium fluoride (MgF)], and the structural differences induced by XFs were investigated. The addition of divalent cations to the XF-inhibited H^+,K^+-ATPase restores the activity of the AlF- or MgF-inhibited, but not of the BeF-inhibited enzyme, although limited trypsin digestion reveals that they assume the same E_2P-like state. To clarify the conformational differences induced by XFs, the structure of BeF-bound H^+,K^+-ATPase was analyzed at 8 Å resolution. The structure is almost identical to the previously reported AlF-bound E_2P structure, unlike the distinctive X-ray structure of BeF-bound SERCA, in which the luminal gate was observed to be widely opened. Since the analyzed structure of the H^+,K^+-ATPase revealed that both AlF and BeF-bound to the P domain were not exposed to the solvent, the dissociation of XFs induced by divalent cations could be interpreted in terms of stability against thermal fluctuations. Furthermore, the conformational differences found between the cytoplasmic domains of H^+,K^+-ATPase and SERCA provide a framework to understand the characteristic mechanism, by which divalent cations reactivate the XF-inhibited H^+,K^+-ATPase.

1. Introduction

The highly acidic environment (pH ~1) of the animal stomach is essential for digestion and also acts as a first barrier against bacterial and viral infection. Conversely, too much acid secretion induces gastric ulcer (Wolosin, 1985; Sachs et al., 2007). The gastric proton pump, H^+,K^+-ATPase is the major membrane protein responsible for the gastric acid (H^+) secretion (Ganser and Forte, 1973). The electroneutral exchange of two cytoplasmic protons for two luminal potassium ions is achieved by the hydrolysis of one ATP molecule (Rabon and Reuben, 1990). Like other members of the cation-transporting P-type ATPase family, such as the Na^+,K^+-ATPase and the sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA), the enzyme undergoes cyclical conformational changes between its main reaction states, E_1 and E_2, and their phosphorylated forms, E_1P and E_2P (Post and Kume, 1973, see Fig. 1). Conformations of the enzyme that bind cations for outward transport (H^+) are defined as E_1, whereas those that bind luminal cations (K^+) are termed E_2. Proton binding to E_1 activates autophosphorylation from Mg^{2+}-ATP to form E_1P, which is soon converted to E_2P in the H^+-transporting step. Binding of K^+ to the E_2P form stimulates dephosphorylation and the transition to the occluded form $(K^+)E_2$. Subsequently, a conformational change to E_1 conformation occurs and K^+ ions are released to the cytoplasm.

The H^+,K^+-ATPase consists of two subunits. Like other highly homologous P2-type ATPases (Palmgren and Axelsen, 1998), the catalytic α-subunit contains 10 transmembrane (TM) helices (M1–M10) in which cation binding sites are located, and three cytoplasmic domains, namely, a nucleotide-binding (N), a phosphorylation (P), and an actuator (A) domains. In addition to the α-subunit, H^+,K^+- and Na^+,K^+-ATPases require an accessory β-subunit, which is indispensable for the functional expression and the trafficking of the αβ-complex to the cell surface (Chow and Forte, 1995). Furthermore, recent structural and functional analyses of the H^+,K^+-ATPase revealed important contributions of the β-subunit to the E_1P/E_2P equilibrium, which might be indispensable for enzyme activity and the large H^+ gradient generated by this pump (Dürr et al., 2008; Abe et al., 2009).

Abbreviations: H^+,K^+-ATPase, potassium-activated adenosine triphosphatase; SERCA, sarco-endoplasmic reticulum Ca^{2+} adenosine triphosphatase; Na^+,K^+-ATPase, sodium and potassium-activated adenosine triphosphatase; EP, phosphoenzyme; cryo-EM, cryo-electron microscopy; XF, fluorinated complex; AlF, aluminum fluoride; BeF, beryllium fluoride; MgF, magnesium fluoride; HEPES, *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid; TM, transmembrane; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; MES, 4-morpholineethanesulfonic acid; SDS–PAGE, SDS–polyaclylamide gel electrophoresis.

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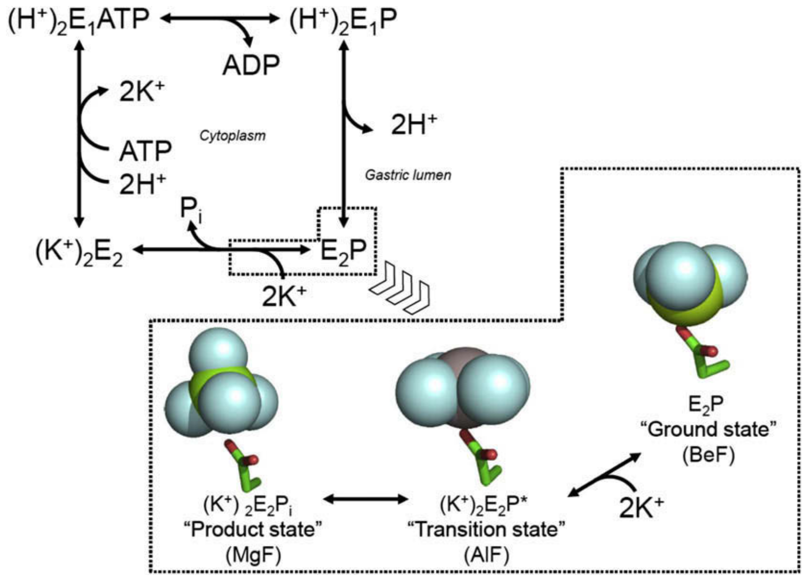

Fig. 1. Reaction scheme of H^+,K^+-ATPase and XF-induced reaction states proposed by SERCA crystal structure. Ion transport and ATP hydrolysis are coupled to the cyclic conformational conversion of the enzyme (abbreviated as "E") between its main states, E_1 and E_2, and their phosphorylation form, E_1P, E_2P. The hydrolysis of ATP generates the phosphoenzyme intermediate (E_1P, E_2P) by transfer of the γ-phosphate to the Asp386 residue of the invariant ^{386}DKTG motif in the presence of Mg^{2+} (omitted in the figure for simplicity). See Section 1 for more details. Extensive studies of SERCA with stable phosphate analogs (AlF, BeF and MgF) characterized their structural and functional properties. Namely, tetrahedral BeF shows the closest coordination of aspartylphosphate (ground state), trigonal bipyramidal AlF is assigned as a transition analog from aspartylphosphate to its hydrolysate, and tetrahedral MgF mimics inorganic phosphate produced by E_2P hydrolysis. Such subtle differences in their coordination environments generate a different type of cytoplasmic domain arrangement, thus causing distinct conformational changes of the enzyme.

Fluorinated complexes (XFs) such as aluminum fluoride (AlF), beryllium fluoride (BeF), and magnesium fluoride (MgF) are widely used to interfere with the activity of many types of enzymes. These small inorganic molecules mimic the chemical structure of phosphate (Bigay et al., 1987) and can therefore be employed for structural analysis of several isoforms of P-type ATPases (Toyoshima et al., 2004, 2007; Morth et al., 2007). A systematic comparison of XFs on SERCA (Danko et al., 2004) and their complex structures as determined by X-ray crystallography (Toyoshima et al., 2007; Olesen et al., 2007) suggested that the phosphate geometries mimicked by XFs were associated with specific conformational substates of the enzyme. When bound to the aspartyl residue in the P domain, the tetrahedral BeF stabilizes the E_2P "ground state", the trigonal bipyramidal AlF induces the E_2P^* "transition state", and the tetrahedral MgF induces the E_2P_i "product state" (Danko et al., 2004, see also Fig. 1). Furthermore, recent functional analyses regarding Ca^{2+}-induced reactivation of BeF-inhibited SERCA clearly illustrate the molecular mechanism upon reactivation (Danko et al., 2009). The binding of Ca^{2+} in the micromolar concentration range from the luminal Ca^{2+} binding site of SERCA E_2BeF induces the formation of an E_1P-like intermediate. Subsequent addition of Ca^{2+} in the millimolar concentration range to the cytoplasmic side is needed for the displacement of BeF at the phosphate binding site. However, extensive studies concerning the interaction of XFs with the H^+,K^+-ATPase remain sparse.

In order to understand conformational differences induced in the H^+,K^+-ATPase by each XF analog, we investigated their properties of inhibition, including stability and reversibility, and carried out structural analyses by employing electron crystallography of two-dimensional (2D) crystals. Although the structure of the BeF-inhibited H^+,K^+-ATPase was indistinguishable from the previously reported AlF-bound structure (Abe et al., 2009) at the currently achieved resolution level, our results clearly showed differences regarding the interaction of AlF and BeF with the H^+,K^+-ATPase.

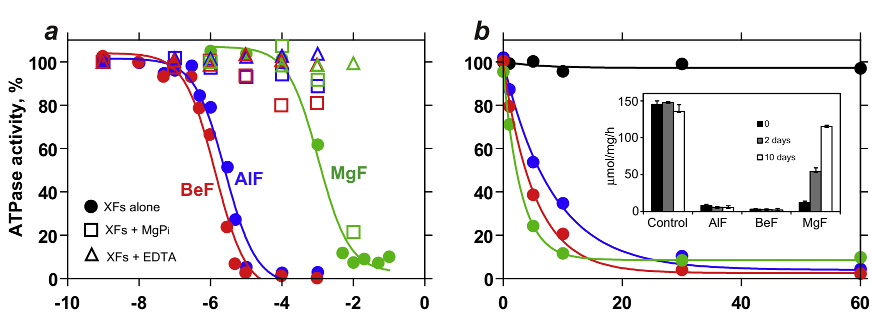

Fig. 2. Inhibition of H^+,K^+-ATPase activity by fluorinated phosphate analogs. (a) Dose-dependent inhibition of H^+,K^+-ATPase activity by XFs. SDS-purified membrane fractions (G2) were incubated at 37 °C for 1 h with indicated concentrations of AlF (blue symbols), BeF (red symbols) or MgF (green symbols), and ATPase activity was measured (filled circles and lines). Inhibition by XFs was also examined in the presence of MgP_i (open squares) and in the presence of EDTA but absence of $MgCl_2$ (open triangles). The values were plotted as a function of the logarithm of Al^{3+}, Be^{2+} and MgF concentrations, respectively. (b) Time course of inhibition by XFs. Membrane fractions were incubated without (control, black circles) or with 10 µM AlF (blue circles) or 10 µM BeF (red circles) or 10 mM MgF (green circles). At the indicated time, they were diluted 1000 times in sucrose buffer, and ATPase activity was measured. (Inset) Membrane fractions were treated with or without XFs indicated in the figure, and excess XF was removed by centrifugation. After suspending with sucrose buffer, they were stored at 3 °C, and their ATPase activities were measured at the indicated times (0–10 days).