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Kinetic characterization of P-type membrane ATPase from *Streptococcus mutans*

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Abstract

The proton translocating membrane ATPase of oral streptococci has been implicated in cytoplasmatic pH regulation, acidurance and cariogenicity. Studies have confirmed that *Streptococcus mutans* is the most frequently detected species in dental caries. A P-type ATPase that can act together with F_1F_o-ATPase in *S. mutans* membrane has been recently described. The main objective of this work is to characterize the kinetic of ATP hydrolysis of this P-type ATPase. The optimum pH for ATP hydrolysis is around 6.0. The dependence of P-type ATPase activity on ATP concentration reveals high ($K_{0.5}$=0.27 mM) and low ($K_{0.5}$=3.31 mM) affinity sites for ATP, exhibiting positive cooperativity and a specific activity of about 74 U/mg. Equimolar concentrations of ATP and magnesium ions display a behavior similar to that described for ATP concentration in Mg^{2+} saturating condition (high affinity site, $K_{0.5}$=0.10 mM, and low affinity site, $K_{0.5}$=2.12 mM), exhibiting positive cooperativity and a specific activity of about 68 U/mg. Sodium, potassium, ammonium, calcium and magnesium ions stimulate the enzyme, showing a single saturation curve, all exhibiting positive cooperativities, whereas inhibition of ATPase activity is observed for zinc ions and EDTA. The kinetic characteristics reveal that this ATPase belongs to type IIIA, like the ones found in yeast and plants.

Keywords: *Streptococcus mutans*; H^+-ATPase; P-type ATPase; Oligomycin; Kinetic characterization

1. Introduction

Streptococcus mutans and other streptococci are of central importance for the formation of dental plaque and the establishment of conditions that can lead to the development of dental caries (Castilho et al., 2000). Dental caries is a multifactorial process, in which diet, the physical characteristics of the oral cavity, the pH of saliva and the types of microorganism present are all key determinants. Oral streptococci are the bacteria most frequently related to cariogenesis. Some streptococci are able to produce acids (acidogenesis), even at low pH values (aciduric capacity); grow in environments with an acidic pH (acidurance); and synthesize intra- and extracellular polysaccharides. Because all oral streptococci do not share these properties, cariogenic potential varies among species. Several studies confirm a relationship between dental caries and the *mutans* group streptococci, particularly *S. mutans* and *S. sobrinus* (Quivey et al., 2000).

It is known that the ATPases located in the plasma membrane are responsible for cytoplasmatic proton extrusion and regulate the internal pH. The F_1F_o-ATPase is described as the main enzyme responsible for this translocation activity, and studies have shown that the ATPase activity present in *S. mutans* plasma membrane increases when this organism grows in an acidic environment (Belli and Marquis, 1991; Kobayashi, 2003).

The F-ATPase ATP synthase is present in the membranes of bacteria, chloroplasts and mitochondria. In microorganisms that possess a respiratory chain, the F_1F_o-ATPase synthesizes ATP (Sabbert et al., 1996). In contrast, anaerobic bacteria (for example *S. mutans*) that do not possess a respiratory chain produce ATP by glycolysis,

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which results in lactic acid production (Suzuki et al., 2000). In this kind of organism, F_1F_o-ATPase hydrolyzes ATP, resulting in the electrogenic extrusion of protons, which generates a proton-motive force (Hamilton and Bowden, 1996). Also, the membrane-bound F_1F_o-ATPase is thought to support the survival of anaerobic bacteria under low pH conditions (Kobayashi et al., 1984).

In contrast, plasma membranes of plants and yeasts have P-type H^+-ATPase to maintain the intracellular pH and membrane potential. The P-type ATPases share the characteristic of having an aspartyl-phosphate intermediate state. A conserved aspartate (Asp^{378} in the *Neurospora* proton ATPase) is reversibly phosphorylated after the proton binds to the side of the membrane from the cytoplasmatic site. Aspartate phosphorylation results in a conformational change that reduces affinity of the binding site for the proton, which is then released to the outside (Auer et al., 1998; Kühlbrandt et al., 2002; Kühlbrandt, 2004).

P-type H^+-ATPases are well known in yeasts and plants, and they are constituted by one polypeptide with a 100 kDa chain (similar to the subunit α of Na,K-ATPase), with 10 transmembrane domains (Morsomme and Boutry, 2000). The H^+-ATPase can be active as a monomer (Goormaghtigh et al., 1986), and when isolated from *Neurospora crassa*, this enzyme forms stable hexamers (Auer et al., 1998; Toyoshima et al., 2000; Rhee et al., 2002; Kühlbrandt, 2004). From the conservation of the amino acid sequences responsible for the nucleotide binding and autophosphorylation, it is thought that the basic mechanism for coupling of ion transport to ATP hydrolysis may be common throughout the P-type family. Three of the most studied members are referred to as H^+-ATPase, Na^+,K^+-ATPase, or Ca^{2+}-ATPase, according to their selectivity for the ions to be pumped (Rhee et al., 2002).

The P-type ATPase family is a large, physiologically important family of membrane proteins that can be divided into two major groups based on cation specificity. Members of the P_1 group transport heavy metals, such as Cu^{2+}, Cd^{2+}, and Hg^{2+}, while the members of the P_2 group transport a wide array of monovalent and divalent cations, including H^+, Na^+, K^+, Mg^{2+} and Ca^{2+}. The KdpB-ATPase from *Escherichia coli*, which accumulates K^+ under conditions of potassium starvation, shares structural features with both groups and has been classified as a P_3-ATPase. Screening of the *Saccharomyces cerevisiae* genome has revealed a total of 16 P-type ATPases, including two P_1-type ATPases, nine P_2-type ATPases (including two H^+-ATPases) and two families of P-type ATPases called P_4 and P_5 (Lutsenko and Kaplan, 1995; Catty et al., 1997; Morsomme et al., 2000).

Based on the sequence homology of the P-type ATPase family, such family can be divided into five branches, which are referred to as types I–V. Within these branches, a total of 10 different subtypes or classes can be distinguished. The third branch Type, IIIA ATPases, are H^+ pumps that are found almost exclusively in the plasma membrane of plants and fungi. These ATPases are able to maintain an intracellular pH of ~6.6, against an extracellular pH of 3.5, which corresponds to a membrane potential of −180 mV (Axelsen and Palmgren, 1998; Kühlbrandt, 2004).

The recently published genome sequence of *S. mutans* UA159 (Ajdic et al., 2002) shows a putative P-type ATPase (24379191 putative calcium transport P-type ATPase), which displays high homology to H^+-ATPase of *N. crassa* (Blast Results: score=222 bits (566) Expect 3e-56, Magalhães et al., 2003). This possibility provides more convincing evidence that the proposed H^+ or H^+, ion antiport ATPase could exist, working in association with F_1F_o-ATPase and extruding H^+ during these bacteria growth. Moreover, the complete sequence of the *Streptococcus pneumoniae* genome has shown the presence of P-type ATPases, whose function is the transport of calcium, copper and metal/cation (Hoskins et al., 2001).

Specific inhibitors allow the classification of F-, V- and P-type ATPases. In general, the ATPase activity of the membrane bound ATP synthase is inhibited by typical F-type inhibitor such as oligomycin, which is a macrolide antibiotic that is well known as an inhibitor of the mitochondrial H^+-ATPase (Homareda et al., 2000). P-types ATPases, on the other hand, are potently inhibited by orthovanadate, an analogue of the phosphate transition state, which binds to the cytoplasmic side of the system and requires magnesium for binding (Dafnis and Sabatini, 1994; Fedosova et al., 1998; Rice et al., 2001). V-type ATPases are commonly inhibited by nitrate and bafilomycin A_1 (Hensel et al., 1996).

A preceding work (Magalhães et al., 2003) has described a 100 kDa vanadate and lanzoprazole-sensitive ATPase from the *S. mutans* membrane, with a possible H^+ transport or H^+/ion antiport activity that may be working in conjunction with the F_1F_o-ATPase, in the proportion of 60% and 40%, respectively, on the regulation of cytoplasmic pH in this microorganism.

In this paper, we report the kinetic behavior of the membrane fraction *S. mutans* P-type ATPase and study which ions may be involved in the mechanism of this new enzyme. Such work significantly contributes to this area, since the majority of the work related to proton extrusion in this microorganism has been done with purified F_1F_o-ATPase or with total membrane ATPase activity, where at least two proton ATPases are present (P- and F-types).

2. Materials and methods

2.1. Materials

All solutions were prepared by using Millipore MilliQ ultra pure apyrogenic water. Tris[hydroxymethyl] aminomethane (Tris), bis [2-hydroxyethyl]imino-tris[hydroxymethyl]methane (BIS-TRIS), imidazole; trichloroacetic acid (TCA), lysozyme; 2-*N*-morpholine ethanesulfonic acid (MES), 2-amino-2-methyl-propan-1-ol (AMPOL), oligomy-