Effects of spermine and spermidine on the inorganic pyrophosphatase of *Streptococcus faecalis*

Interactions between polyamines and inorganic pyrophosphate

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We have shown a dual role for Mg^{2+} in the hydrolysis of PP_i catalysed by inorganic pyrophosphatase (PPase; EC 3.6.1.1) of *Streptococcus faecalis*; Mg^{2+} is necessary for the formation of the substrates, $Mg_1PP_i^{2-}$ and $Mg_2PP_i^0$, and it also acts as an allosteric activator [Lahti & Jokinen (1985) *Biochemistry* **24**, 3526–3530]. No activity can be observed with *S. faecalis* PPase in the absence of bivalent cations, which indicates that free PP_i cannot serve as a substrate for this enzyme. However, significant activities were observed in the presence of spermine and spermidine, even though no bivalent cations were present. It was shown by particle-induced gamma-ray emission and particle-induced X-ray-emission analysis that the polyamines used were not contaminated with Mg^{2+} or any other bivalent cations that could support PPase activity. Hence it is obvious that polyamines are able to form a complex with PP_i that serves as a substrate for PPase. The apparent stability constants for the 1:1 adducts of spermine and spermidine were estimated by a resin competition method. The values obtained at pH 7.5 were $2.7 \times 10^3 M^{-1}$ and $6.4 \times 10^2 M^{-1}$ respectively. Kinetic results further suggested that polyamines can also substitute for Mg^{2+} as an activator *in vitro*. The physiological significance of these polyamine effects were discussed.

INTRODUCTION

Inorganic pyrophosphatase (EC 3.6.1.1, PPase) is ubiquitous in Nature, and it catalyses the hydrolysis of PP_i to two molecules of P_i . This reaction is thermodynamically important, because it makes the PP_i -producing biosynthetic reactions, including the synthesis of proteins and nucleic acids, practically irreversible (Kornberg, 1962). PPase is highly efficient as a catalyst, enhancing the reaction rate by about 10 orders of magnitude (Cooperman, 1982). The structural and kinetic properties of various microbial PPases have been actively studied (Josse & Wong, 1971; Rapoport *et al.*, 1972; Unemoto *et al.*, 1973; Schreier & Höhne, 1978; Randahl, 1979; Terzyan *et al.*, 1984; Lahti & Jokinen, 1985; Gonzales & Cooperman, 1986). We have shown that PPase in *Streptococcus faecalis* is primarily regulated at the activity level (Lahti & Heinonen, 1981a) with GSH as an activator *in vivo* (Lahti & Suonpää, 1982). This enzyme exists in two forms which differ in activity. During the exponential phase of growth the enzyme is almost entirely in the high-activity form, whereas during the stationary phase the amounts of the highly active and less-active forms are similar (Lahti & Heinonen, 1981a).

The catalytic activity of PPase has an absolute requirement for Mg^{2+} ions (Lahti, 1983). With both forms of *S. faecalis* PPase, Mg^{2+} is necessary for the formation of the substrates, $Mg_1PP_i^{2-}$ and $Mg_2PP_i^0$, and it also acts as an allosteric activator (Lahti & Jokinen, 1985; Lahti & Lönnberg, 1985). Comparing the intracellular concentration of PP_i and Mg^{2+} , Klemme (1976) concluded that under certain conditions the intracellular concentration of Mg^{2+} available for PPase is sufficiently low to limit the enzymic hydrolysis of PP_i . Because

polyamines were known to be able to substitute for Mg^{2+} in various biological systems (Tabor & Tabor, 1985), it was of special interest to study the effects of polyamines on the activity of PPase. These studies were carried out with the less-active form of *S. faecalis* PPase. Part of the results has already been published as a Congress Abstract (Lahti, 1985).

MATERIALS AND METHODS

Streptococcus faecalis A.T.C.C. 8043 was grown and PPase was purified to homogeneity as described by Lahti & Niemi (1981). *S. faecalis* PPase is converted into the stable, less active, form by incubation at 37 °C (Lahti & Heinonen, 1981b). Accordingly, before each experiment the enzyme was preincubated for 2.5 h at 37 °C. Activity was measured at 25 °C, usually in 0.05 M-Tris/HCl buffer, pH 7.5, since the effects of polyamines on PPase activity were at this pH more marked than at pH 9.1 used in our previous work (Lahti & Jokinen, 1985; Lahti & Lönnberg, 1985). The activity was determined by measuring the release of $[^{32}P]P_i$ from $[^{32}P]PP_i$ as described by Heinonen (1970) with modifications presented by Lahti & Jokinen (1985). Each assay was performed in duplicate. In each reaction less than 15 % of the starting PP_i was allowed to be hydrolysed.

The apparent stability constants (K_s) for the complexes of pyrophosphate with polyamines (S) were determined by the method of Jenkins (1985), based on competition between a cationic polyamine and an anion-exchange resin (R) for free anionic PP_i in solution. K_s may be expressed by eqn. (1):

$$K_s = [SPP_i]/([PP_i][S]) \quad (1)$$

where $[PP_i]$, $[S]$ and $[SPP_i]$ stand for the sum concentrations of all different ionic forms of uncomplexed PP_i , uncomplexed polyamine and their adduct at a given pH, respectively. The distribution of the uncomplexed pyrophosphate between the solution and solid resin may be described by eqn. (2):

$$K_d = [PP_i]/[RPP_i] \quad (2)$$

This equation is valid when the binding capacity of the resin added is high compared with the amount of uncomplexed PP_i in solution, and competing anion-exchange reactions are negligible. Accordingly, the reciprocal stability constant, $1/K_s$, is equal to the negative abscissa intercept of the straight line defined by eqn. (3):

$$A_m/(A_0 - A_m) = K_d(1 + K_s[S]) \quad (3)$$

Here A_m and A_0 are the experimentally accessible PP_i concentrations in solution with both resin and polyamine present and with neither resin nor polyamine present, respectively.

The concentrations of uncomplexed polyamine can be calculated from that added ($[S]_{\text{total}}$) by the expression (4):

$$[S] = [S]_{\text{total}} - (A_m - A_r) \quad (4)$$

where A_r is the PP_i concentration in solution in the presence of resin before the addition of polyamine.

This technique relies on the measurement of PP_i distribution between the resin and solution, in both the absence and the presence of several concentrations of the polyamine. PP_i concentrations were measured spectrophotometrically at 650 nm as described by Heinonen *et al.* (1981). Analytical-grade anion-exchange resin Dowex-1 (Cl^- form, 250–100 mesh) was washed with water, and then allowed to dry in air at room temperature. To a 500 ml glass-stoppered flask containing 50 ml of 1 mM- PP_i in 0.05 M-Tris/HCl, pH 7.5, 160 mg of the resin was added. After shaking for 5 min in a rotary shaker at 25 °C, PP_i concentration was measured in a 5 ml sample, from which the resin was removed by centrifugation at 25 °C. This sample was resuspended by vortex mixing and added back to the flask. The resin was then titrated with small portions (20 μ l) of concentrated polyamine solution (0.25 M), and the PP_i concentration in the supernatant solution was measured as above. From the titration curve the stability constant was determined as described above.

That the commercial spermine and spermidine samples used did not contain as impurities Mg^{2+} or any other bivalent cation that could possibly support *S. faecalis* PPase activity (Lahti & Niemi, 1981) was checked by particle-induced gamma-ray emission ('PIGE') (Järnström & Pakarinen, 1984) for elements with $Z < 14$, and by particle-induced X-ray emission ('PIXE') (Järnström *et al.*, 1983) for elements with $Z \geq 14$ at the Accelerator Laboratory of Åbo Akademi, Turku, Finland. Samples were freeze-dried and the material was pressed into a pellet. Bovine liver standard material (National Board of Standards, SRM 1577) was treated similarly and served as a control. The limits of detection in both analyses were about 1 p.p.m.

RESULTS

Complexation of polyamines with PP_i was studied by a resin competition method as described by Jenkins (1985). In this procedure PP_i was bound to the resin

(Dowex 1) and then released by titration with polyamines. The PP_i released was determined by the method of Heinonen *et al.* (1981). The apparent stability constants were determined from the titration curves as described in Fig. 1, with the results of $6.4 \times 10^2 \text{ M}^{-1}$ and $2.7 \times 10^3 \text{ M}^{-1}$ (pH 7.5) for spermidine- PP_i and spermine- PP_i complexes respectively. On the basis of the acidity and stability constants reported in the literature (Moe & Butler, 1972; Frey & Stuehr, 1972), the corresponding apparent stability constant (K_{app}) for the Mg^{2+} complex of PP_i can be calculated to be $1 \times 10^4 \text{ M}^{-1}$ at pH 7.5.

Fig. 1. Determination of the stability constants for spermine- and spermidine- PP_i complexes

The negative abscissa intercepts of the straight lines are equal to the reciprocal stability constants ($1/K_s$) at pH 7.5. $\square$, Spermine; $\blacktriangle$, spermidine.

Fig. 2. Dependence of the activity of *S. faecalis* PPase on polyamine concentration in the absence of bivalent cations at a constant concentration (0.5 mM) of PP_i

$\blacksquare$, Spermine; $\blacktriangle$, spermidine. The proportions of PP_i complexed with spermine and spermidine are shown by broken curves (----- and ——— respectively). There was no activity in the absence of polyamines.

Accordingly, the complexing ability of polyamines is lower, but still of the same order as that of Mg^{2+} .

Free PP_i in the absence of bivalent cations cannot serve as a substrate for *S. faecalis* PPase (Lahti & Niemi, 1981). However, significant enzyme activities were observed in the presence of polyamines when no bivalent cations were present (Fig. 2). It was shown by particle-induced gamma-ray emission (Järnström & Pakarinen, 1984) and particle-induced X-ray emission analysis (Järnström *et al.*, 1983) (for details see the Materials and methods section) that the commercial spermine and spermidine samples used were not contaminated with Mg^{2+} or any other bivalent cation that could possibly support *S. faecalis* PPase activity (Lahti & Niemi, 1981).

As shown in Fig. 2, the activity of *S. faecalis* PPase increases markedly at those concentrations of polyamine for which the mole fractions of complexed and free PP_i become comparable. This suggests that the polyamine complexes of PP_i serve as substrates for PPase. Polyamines are positively charged molecules, and thus they may assist the catalysis as Mg^{2+} ions do in the $\text{Mg}_1\text{HPP}_i^{1-}$ complex (Cooperman, 1982; Welsh & Cooperman, 1984), which is the prevailing complex under the experimental conditions (pH 7.5). The activities induced by spermine and spermidine are, however, only 26 and 17% of that supported by Mg^{2+} (see Figs. 2 and 3), indicating that polyamine- PP_i adducts are poorer substrates than is $\text{Mg}_1\text{HPP}_i^{1-}$.

Fig. 3 shows the dependence of the activity of *S. faecalis* PPase on the concentration of spermine and spermidine at constant $[\text{PP}_i]_{\text{total}}$ and $[\text{Mg}]_{\text{total}}$ (0.5 mM). For comparison the dependence of activity on $[\text{Mg}]_{\text{total}}$ is also included. It is obvious that spermine, spermidine and Mg^{2+} will activate PPase, the maximal activities being obtained at 2 mM, 5 mM and 6 mM respectively. The degrees of activation were 3.2-, 2- and 2.5-fold as compared with the activity observed at 0.5 mM- MgCl_2 (Fig. 3). At these total concentrations of Mg^{2+} and PP_i

Fig. 3. Dependence of the activity of *S. faecalis* PPase on Mg^{2+} and polyamine concentrations at a constant concentration (0.5 mM) of PP_i .

●, Mg^{2+} alone; □, spermine plus 0.5 mM- Mg^{2+} ; △, spermidine plus 0.5 mM- Mg^{2+} .

Fig. 4. Dependence of the activity of *S. faecalis* PPase on Mg^{2+} concentration at constant concentrations of PP_i (0.5 mM) and polyamine (5 mM).

●, No polyamine; □, spermine; △, spermidine. There was no activity in the absence of Mg^{2+} and polyamines.

(0.5 mM), about 65% of PP_i is present as complexes with Mg^{2+} at pH 7.5 ($K_{\text{app}} \approx 1 \times 10^4 \text{ M}^{-1}$; Moe & Butler, 1972). At 1 mM-spermine a considerable rate enhancement is observed. Since spermine is a weaker complexing agent than Mg^{2+} , and the spermine- PP_i complex is a poorer substrate than $\text{Mg}_1\text{HPP}_i^{1-}$ (Figs. 2 and 3), the activity increase cannot be accounted for by spermine and Mg^{2+} competing for complexation. Hence the increase in activity under these conditions (Fig. 3) is due to the activation of PPase by free polyamines.

The activation of *S. faecalis* PPase by spermine, spermidine and Mg^{2+} is pH-dependent in the sense that the activation is decreased with increasing pH; at pH 9.1 maximal activation of PPase by spermine, spermidine and Mg^{2+} is 1.2-, 1.1- and 1.7-fold as compared with the activity with 0.5 mM- Mg_{total} and - PP_{total} respectively (results not shown). Tentatively, one might assume that the polyamine-protein interactions, which most probably are of electrostatic nature, become weaker at high pH, owing to partial deprotonation of the polyamines. For comparison, a similar change is observed in the stabilities of the polyamine complexes of organic anions (Wilson & Williams, 1987).

Fig. 4 shows the activity of PPase as a function of $[\text{Mg}]_{\text{total}}$ in the presence of fixed amounts of polyamines. The same activity is obtained with suboptimal $[\text{Mg}]_{\text{total}}$ and optimal spermine concentration as by having both Mg^{2+} and spermine at their optimal concentrations (Figs. 3 and 4). However, with spermidine the Mg^{2+} activation seems to be slightly cumulative; about 1.4-fold higher activity is observed when both spermidine and Mg^{2+} are at their optimal concentrations, compared with that with Mg^{2+} at suboptimal and spermidine at optimal concentrations (Figs. 3 and 4). In general, the activation of PPase by polyamines is most prominent when $[\text{Mg}]_{\text{total}}$ is suboptimal; at 6 mM- Mg_{total} activation by spermine and spermidine is only 1.3- and 1.1-fold at pH 7.5 respectively (Fig. 4).

DISCUSSION

Numerous studies have described interactions between polyamines and macromolecules, such as nucleic acids, ribosomes and proteins (Tabor & Tabor, 1985; Bratek-Wiewiorowska *et al.*, 1987). However, there are only a few studies on the interactions of polyamines with low-molecular-mass phosphate metabolites. In addition to inorganic polyphosphates (Wilson & Williams, 1987), nucleotides (Nakai & Glinsmann, 1977; Wilson & Williams, 1987), 5-phosphoribosyl 1-pyrophosphate (PRibPP) and 2,3-diphosphoglycerate (2,3-DPG) (Yip & Balis, 1980) have been shown to form stable complexes with polyamines. The PRibPP-spermine complex was further shown to act as a competitive inhibitor for hypoxanthine phosphoribosyltransferase, and the 2,3-DPG-spermine was an uncompetitive inhibitor for 2,3-DPG phosphatase (Yip & Balis, 1980).

In general, Mg^{2+} plays a dual role, analogous to that described above for *S. faecalis* PPase, in numerous enzymic reactions. Furthermore, obviously most ligands that form complexes with Mg^{2+} also form complexes with polyamines, and at least in some cases these complexes are about as stable as the corresponding Mg^{2+} complexes (Nakai & Glinsmann, 1977; Yip & Balis, 1980). Polyamine-ligand complexes cannot be inert to the reactions that modify the ligand; they act either as substrates or as inhibitors by decreasing the concentrations of the primary substrates, i.e. Mg^{2+} -ligand complexes. Hence it seems to us that, in addition to all the other functions of polyamines described so far (for a review see Tabor & Tabor, 1985), they also play an important role *in vivo* in many reactions that somehow require bivalent cations.

A large number of experiments have been reported on the effects of polyamines in a variety of enzymic systems isolated from prokaryotes (Tabor & Tabor, 1985). These studies are all of interest in showing the potential actions of polyamines. However, it is difficult to decide whether an observed effect of polyamines *in vitro* is physiologically significant. The physiological significance of the polyamine effects described in this paper is still unknown. In general, prokaryotes have rather high (several millimolar) concentrations of spermidine, but they lack spermine (Tabor & Tabor, 1985). Hence, spermidine might have some role in the regulation of PPase activity *in vivo*. As mentioned above, the intracellular concentration of Mg^{2+} available for PPase is under certain conditions so low ($[Mg]_{total}/[PP]_{total} \leq 2$; see Fig. 3, and Lahti & Jokinen, 1985) that it limits the enzymic hydrolysis of PP_i (Lahti & Lönnberg, 1985; Klemme, 1976). Therefore it is possible that free spermidine could act as an activator, and spermidine- PP_i adduct as a substrate, for *S. faecalis* PPase also *in vivo*. Furthermore, polyamine- PP_i complexes serve as interesting model systems when studying the detailed mechanism of the enzymic hydrolysis of PP_i .

A glutathionylspermidine conjugate has been isolated from *Escherichia coli* (Tabor & Tabor, 1975). The physiological role for this intriguing compound is completely unclear. A related glutathione-spermidine complex has been identified in *Crithidia fasciculata*, and shown to be a cofactor for the glutathione reductase of this organism (Fairlamb *et al.*, 1985). It is not known whether this conjugate exists in *S. faecalis*, but it is tempting to speculate that it might have some role in the regulation of *S. faecalis* PPase, since this enzyme has

already been shown to be regulated by glutathione *in vivo* (Lahti & Suonpää, 1982). In this way the glutathione-spermidine conjugate could mesh these two regulatory systems. The changes in glutathionylspermidine content in the cells during the batch culture of *Escherichia coli* (Tabor & Tabor, 1975, 1985) fit perfectly with those observed by us for glutathione and PPase activity in *S. faecalis* (Lahti & Suonpää, 1982), i.e. in the exponential phase of growth the concentration of glutathionylspermidine is low and glutathione content and PPase activity are high, whereas in the stationary phase the opposite is observed. Both activators (free glutathione and spermidine) of *S. faecalis* PPase are probably inactivated by the formation of glutathionylspermidine.

We gratefully acknowledge Stefan Järnström for carrying out the particle-induced gamma-ray-emission and X-ray-emission analysis, and Anne Kankaanpää for drawing the Figures. Furthermore, we thank Barry Cooperman, Jukka Heinonen and Pirjo Pakarinen for reading the manuscript and offering suggestions.

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Received 4 July 1988/18 October 1988; accepted 2 November 1988