Comparative kinetic studies on the two interconvertible forms of *Streptococcus faecalis* inorganic pyrophosphatase

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In this work the two interconvertible forms of inorganic pyrophosphatase (EC 3.6.1.1) of *Streptococcus faecalis* were shown to differ in kinetics. The highly active form of the enzyme was more sensitive to the changes in the Mg^{2+} concentration, and thus also more sensitive to the inhibition caused by ATP, which competes with PP_i for the chelation of Mg^{2+} ions. We have previously described a kinetic model for the less-active form of *S. faecalis* inorganic pyrophosphatase [Lahti & Jokinen (1985) *Biochemistry* **24**, 3526–3530]. The kinetic model of the highly active enzyme form is proposed to be a modification of the model of the less-active form in which enzyme activation by free Mg^{2+} is necessary for the reaction to occur. In this model the enzyme exists in two states, referred to as R- and T-states. In the absence of ligands the enzyme is in the T-state. R-state, i.e. the catalytically active state, exists only in the presence of free Mg^{2+} . $Mg_1PP_i^{2-}$ is the primary substrate, and free pyrophosphate is a weak inhibitor that cannot serve as a substrate for the highly active form of *S. faecalis* inorganic pyrophosphatase. This model closely resembles that previously presented for yeast inorganic pyrophosphatase.

INTRODUCTION

Inorganic pyrophosphatase (EC 3.6.1.1, PPase) is ubiquitous in Nature and plays an important role in energy metabolism, providing a thermodynamic pull for biosynthetic reactions (Kornberg, 1962). According to Peller (1976), nucleic acid syntheses would be energetically impossible *in vivo* if they were not coupled to enzymic hydrolysis of inorganic pyrophosphate (PP_i), catalysed by PPase. In addition, various findings suggest that PPase might have important roles not only in the regulation of macromolecular synthesis and growth (Heinonen *et al.*, 1976; Heinonen & Kukko, 1977; Dignam & Deutscher, 1979) but also in evolutionary events by affecting the accuracy by which DNA molecules are copied during chromosome duplication (Herbomel & Ninio, 1980; Bourguignon-van Horen *et al.*, 1982).

We have for some years studied the role and regulation of PPase in microbes (Lahti & Raudaskoski, 1983; Lahti, 1983), and we have shown that PPase of *Streptococcus faecalis* is primarily regulated at the activity level (Lahti & Heinonen, 1981a) with the ratio of reduced glutathione to oxidized glutathione as an effector *in vivo* (Lahti & Suonpää, 1982). The enzyme exists in two forms, which differ in activity. During the exponential phase of growth almost all the enzyme is the high-activity form, whereas during the stationary phase the highly active and the less-active forms exist in equal amounts (Lahti & Heinonen, 1981a).

We have further studied the kinetics of the less-active form of *S. faecalis* PPase and found that co-operativity in ligand binding contributes in a significant way to the behaviour of this enzyme form. Both $Mg_1PP_i^{2-}$ and $Mg_2PP_i^0$ are substrates for this enzyme form, the latter being less reactive than the former. Mg^{2+} is an activator for $Mg_1PP_i^{2-}$ hydrolysis and an inhibitor of $Mg_2PP_i^0$ hydrolysis (Lahti & Jokinen, 1985).

In the present work the kinetics of the highly active form of *S. faecalis* PPase was studied, and it was found that the two enzyme forms differ significantly in their kinetic behaviour.

MATERIALS AND METHODS

The cells of *Streptococcus faecalis* A.T.C.C. 8043 were grown and PPase was purified to homogeneity as described by Lahti & Niemi (1981).

PPase of *S. faecalis* is converted into the stable, less-active, form by incubation at 37 °C (Lahti & Heinonen, 1981b). Hence when the kinetics of the less-active enzyme form was studied the enzyme was preincubated for 2.5 h at 37 °C. Furthermore, before the studies on the kinetics of the highly active enzyme form, PPase was activated by L-cysteine (0.5 mM) for 20 min at 37 °C (Lahti & Heinonen, 1981b).

The enzyme activity was measured at 25 °C in 0.05 M-piperazine/HCl buffer, pH 9.1, containing 0.05 M-tetramethylammonium chloride as described by Lahti & Jokinen (1985). Each assay was performed in duplicate. In no case was more than 15% of the starting PP_i hydrolysed during the reaction. The stock solution of $MgCl_2$ was titrated with 8-hydroxyquinoline (Guibault, 1973) for the determination of exact concentration. The concentrations of Mg^{2+} , PP_i^{4-} , $Mg_1PP_i^{2-}$ and $Mg_2PP_i^0$ in the reaction mixture were calculated as described by Rapoport *et al.* (1972) by making use of a DECsystem-20 computer.

A potentiometric technique with an electrode sensitive for bivalent cations was used for the determination of the apparent stability constant of the Mg^{2+} -cysteine complex. The titration procedure was as follows. To a thermostatically controlled vessel containing an Orion 9332 bivalent-ion electrode and a Metrohm EA 404 calomel

Abbreviation used: PPase, inorganic pyrophosphatase.

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reference electrode were added 10 ml of 0.05 M-piperazine/HCl buffer, pH 9.1, containing 0.05 M-tetramethylammonium chloride, and 0.05 ml of 0.2 M-MgCl₂. The mixture was kept under N₂, and agitated with a magnetic stirrer. After the meter readings had settled, a known amount of L-cysteine was added; there was a fall in the potential when complex-formation with Mg²⁺ occurred. Then 0.2 M-MgCl₂ was added from a calibrated micrometer syringe until the potential reached its initial value. The apparent stability constant ($K_{app.}$) was calculated from eqn. (1):

$$K_{app.} = [MgC]/[Mg^{2+}][C]_{tot.} - [MgC] \quad (1)$$

where [MgC], [Mg²⁺] and [C]_{tot.} are the concentrations of the Mg²⁺-cysteine complex, free Mg²⁺ ion and total cysteine respectively.

RESULTS

The measured activities of the two enzyme forms of *S. faecalis* PPase and the calculated concentrations of Mg²⁺, PP_i⁴⁻, Mg₁PP_i²⁻ and Mg₂PP_i⁰ at pH 9.1 are shown in Fig. 1 as a function of [Mg]_{tot.} at constant [PP_i]_{tot.}. The activity curve of the less-active form has already been analysed in detail by Lahti & Jokinen (1985), and it is shown in Fig. 1 only for comparison. The two interconvertible enzyme forms seem to be quite different with respect to kinetics. The kinetics of the less-active form is clearly more complicated than that of the highly active form. The activity of the highly active form decreased more rapidly as the concentration of free Mg²⁺ was decreased. In addition, much higher activities were observed in the absence of free Mg²⁺ with the less-active form than with the highly active form (Fig. 1). Maximal activities were reached when [Mg]_{tot.}/[PP_i]_{tot.} was about 4 and 13 in the case of the highly active and the less-active forms of the enzyme respectively (Fig. 1), indicating that both enzyme forms are activated by free Mg²⁺. As in the studies of the less-active enzyme form (Lahti & Jokinen, 1985), it was shown that the decrease in the activity of the highly active enzyme form observed with increase in [Mg]_{tot.} at high [Mg]_{tot.} is due to the changes in the concentration of the reaction components, and not due to the general ionic effects. This suggests that Mg₂PP_i⁰ is a less-active substrate than Mg₁PP_i²⁻ for the enzyme in the highly active form, as is also the case with the less-active enzyme form (Lahti & Jokinen, 1985).

In the presence of 3 mM-[PP_i]_{tot.} the activity of the highly active enzyme form was 86% of that observed at 0.5 mM-[PP_i]_{tot.} when [Mg]_{tot.} was 0.5 mM. Furthermore, there was no detectable activity with the highly active enzyme form in the absence of bivalent cations. Hence it is obvious that free PP_i is a rather weak inhibitor that cannot serve as a substrate for the highly active form of *S. faecalis* PPase, as has been observed also for the enzyme in the less-active form (Lahti & Jokinen, 1985).

The highly active enzyme form was more sensitive than the form of low activity to changes in [Mg]_{tot.} when [Mg]_{tot.}/[PP_i]_{tot.} < 2 (Fig. 1). Hence this enzyme form was also more sensitive to the inhibitory effect of ATP, which competes with PP_i for complex-formation with Mg²⁺ (Fig. 2).

In this highly purified enzyme preparation the maximal activity of the highly active enzyme was 18.5-fold greater than that of the less-active enzyme form. However, for the sake of clarity the activities of both enzyme forms are

Fig. 1. Dependence of the activity of the highly active form of *S. faecalis* PPase on the concentrations of ionic species in the reaction mixture as a function of [Mg]_{tot.} at constant (0.5 mM) [PP_i]_{tot.} and (0.39 mM) [L-cysteine]

The activity curve of the less-active form of *S. faecalis* PPase taken from Lahti & Jokinen (1985) is shown (—) for comparison. For illustrative purposes the activities of both enzyme forms in Figs. 1 and 2 are presented as relative activities. However, it has to be recognized that with this highly purified enzyme preparation the maximal activity of the highly active enzyme form is 18.5-fold greater than that of the less-active enzyme form (see the text). ○, Rate measurements; broken lines, variation in the concentrations of different ionic species (— · — · —, Mg²⁺; · · · · ·, Mg₁PP_i²⁻; — — —, Mg₂PP_i⁰; — — —, PP_i⁴⁻).

Fig. 2. Dependence of the activities of the highly active (○) and less-active (●) forms of *S. faecalis* PPase on the concentration of ATP at constant (0.5 mM) [Mg]_{tot.} and [PP_i]_{tot.}

presented in Figs. 1 and 2 as relative activities. The less-active enzyme form was generally used in 18.5-fold concentration compared with the highly active enzyme form in order to ensure that approximately the same amount of the substrate was consumed by both enzyme

forms during the reactions. That the observed differences in the kinetics of the two interconvertible forms of *S. faecalis* PPase were not due to the different concentrations of the enzyme protein is supported by three observations: (1) there was a linear correlation between the initial velocity and the amount of enzyme at the enzyme concentrations used at 0.1 mM-, 5 mM- and 20 mM-[Mg]_{tot.} when [PP_i]_{tot.} was 0.5 mM; (2) an 18.5-fold dilution of the less-active form did not alter the kinetic pattern shown in Fig. 1; (3) the M_r of the highly active enzyme form determined by Ultrogel AcA-34 in the presence of 1 mM-L-cysteine was similar (128 000) to that of the less-active enzyme form described by Lahti & Niemi (1981), i.e. the degree of subunit aggregation was not altered during the interconversion of these two enzyme forms.

To find out the possible effect of L-cysteine on the concentration of the ionic species in the reaction mixture, the apparent stability constant ($K_{app.}$) for Mg²⁺-cysteine complex was determined under the reaction conditions used by an electrode sensitive for bivalent cations (see the Materials and methods section). A value of $K_{app.} \approx 10 \text{ M}^{-1}$ was obtained. Albert (1952) determined the stability constant (K') for the complex between Mg²⁺ and the dianion of cysteine with the approximate result $K' < 10^4 \text{ M}^{-1}$. This is in agreement with our result, as only a minor portion (about 4%) of total cysteine is in its dianion form under our experimental conditions. Hence it is evident that Mg²⁺ is bound about 25 000-fold and 22-fold more tightly by PP_i⁴⁻ and Mg₁PP_i²⁻ respectively than by cysteine (the stability constants for Mg₁PP_i²⁻ and Mg₂PP_i⁰ are $10^{5.41} \text{ M}^{-1}$ and $10^{2.34} \text{ M}^{-1}$ respectively; Lambert & Watters, 1957), and thus the possible weak interactions between cysteine and free Mg²⁺ can be disregarded in our system. Furthermore, it seems very unlikely that under our experimental conditions cysteine would interact electrostatically with pyrophosphate and/or with its Mg²⁺ complexes. In conclusion, it seems to us that cysteine acts only to activate the enzyme, and does not exert any effect on the concentrations of the reaction components, and thus the observed different kinetics of the two interconvertible enzyme forms are real.

DISCUSSION

The two interconvertible forms of *S. faecalis* PPase are similar with respect to M_r and high specificity for PP_i, but they differ from each other with respect to kinetics. The activity curve of the highly active form of *S. faecalis* PPase is not so complicated as that of the less-active form (Fig. 1); in fact, it closely resembles that of yeast PPase presented by Moe & Butler (1972). In the case of yeast PPase activation of the enzyme by free Mg²⁺ is necessary for the reaction to occur (Moe & Butler, 1972; Rapoport *et al.*, 1972), whereas in the case of the less-active form of *S. faecalis* PPase it is not (Lahti & Jokinen, 1985). The greatest qualitative difference between the kinetics of the two forms of *S. faecalis* PPase is actually in the response of the activity of the changes in [Mg²⁺]; the presence of free Mg²⁺ seems to be necessary for the activity of the highly active form, whereas in the case of the less-active form it is not.

To understand the proposed model of the highly active enzyme form we describe briefly the model of the less-active form of *S. faecalis* PPase described by Lahti

& Jokinen (1985). It is a Monod-Wyman-Changeux (Monod *et al.*, 1965)-type allosteric model, in which the enzyme is proposed to exist in two states, referred to as R- and T-states. In that model 94% of the enzyme is in the T-state in the absence of ligands. Mg₁PP_i²⁻ is the only substrate for the enzyme in the R-form. This substrate is bound equally well by both enzyme forms, but it is hydrolysed 5-fold more efficiently by the R-form than it is by the T-form. Mg₂PP_i⁰ is bound exclusively to the T-state of the enzyme, and it is hydrolysed 25% as rapidly as Mg₁PP_i²⁻ by the T-form. Mg²⁺ stabilizes the R-state, thus activating the hydrolysis of Mg₁PP_i²⁻ and inhibiting that of Mg₂PP_i⁰ (Lahti & Jokinen, 1985). In this model significant activities (due to the Mg₁PP_i²⁻-hydrolysing activity of the enzyme in T-state) are possible when no free Mg²⁺ is present (Lahti & Jokinen, 1985).

It seems reasonable to us to suppose that the kinetic model of the highly active enzyme form is a derivative of the model of the less-active enzyme form presented above. To derive the model of the highly active enzyme form, which emphasizes the role of free Mg²⁺ and accounts for the rather low activities observed when no free Mg²⁺ is present, we suppose that the T-state of the enzyme is less reactive in the case of the highly active enzyme form than it is with the less-active form. In other words, we think that the ratio of the Mg₁PP_i²⁻-hydrolysing activity in the R-state to that in the T-state is higher in the highly active form than it is in the less-active form. It is further proposed that in the case of the highly active enzyme form Mg²⁺ is necessary for the transition from the T-state to the R-state; the binding constant of free Mg²⁺ to the R-form is higher in the case of the highly active enzyme form than it is in the case of the less-active enzyme form, so that low concentrations of free Mg²⁺ are able to shift drastically the equilibrium between the two enzyme states in favour of the R-state. Thus in the model of the highly active enzyme form the equilibrium in the presence of the ligands (= free Mg²⁺) is more in favour of the R-state than it is in the case of the less-active form, and so the relative activity of Mg₂PP_i⁰ hydrolysis is lower with the highly active form than it is with the less-active form (Fig. 1).

The kinetic model of the highly active enzyme form presented above closely resembles that of yeast PPase, because the R-state in the case of the highly active form does not occur in the absence of free Mg²⁺, and thus the R-state in this model can be considered to be the same as the enzyme activated by Mg²⁺ in the model of yeast PPase (Moe & Butler, 1972).

On the basis of the calculations presented by Klemme (1976) it is obvious that the intracellular concentration of Mg²⁺ available for PPase is less than 1 mM. In addition, it has been shown that the cytoplasmic concentration of PP_i is about 0.6 mM in *Escherichia coli* (Kukko & Heinonen, 1982). Under these conditions the highly active enzyme form is more sensitive to the inhibitory effect of ATP than is the less-active form (Fig. 2). It has been suggested that adenylate nucleotides might regulate PPase activity in the cells by competing with PP_i for Mg²⁺ (Klemme & Gest, 1971); thus the activity of PPase can be adjusted to either anabolic or catabolic reactions via the relation between the adenylate charge and Mg²⁺. The sensitivity of *S. faecalis* PPase to this metabolic regulation would be highest in the exponential growth phase, when practically all the enzyme is in the highly active form (Lahti & Heinonen, 1981a).

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