SHORT COMMUNICATION

Activity Changes of Inorganic Pyrophosphatase of *Streptococcus faecalis* during Batch Culture

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The state of inorganic pyrophosphatase (EC 3.6.1.1) from *Streptococcus faecalis* ATCC 8043 was studied in different phases of batch culture. The degree of inactivation (i.e. the ratio of activities observed before and after incubation at 37 °C without cysteine) was highest, and the degree of activation (i.e. the ratio of activities after and before incubation in the presence of cysteine) was lowest, in samples taken during the early-exponential growth phase. During the various phases of batch culture, the specific activity before incubation and the degree of inactivation changed in parallel, whereas the specific activity observed after incubation remained nearly constant. During the early-exponential phase of growth almost all the enzyme was in the high-activity form, whereas during the stationary phase the highly active and the less active forms existed in equal amounts. These findings suggest that inorganic pyrophosphatase in *S. faecalis* is synthesized constitutively and is primarily regulated at the level of activity.

INTRODUCTION

Inorganic pyrophosphatase (EC 3.6.1.1) plays an important role in energy metabolism providing a 'thermodynamic pull' for biosynthetic reactions (Kornberg, 1962; Peller, 1976). However, the regulation of this enzyme has been little studied. In our laboratory we have examined the interrelationship of inorganic pyrophosphatase and macromolecular synthesis (Heinonen & Kärenlampi, 1973; Heinonen *et al.*, 1976; Heinonen & Kukko, 1977) and have shown that inorganic pyrophosphatase of *Streptococcus faecalis* exists in two interconvertible forms which differ in activity (Lahti & Heinonen, 1981). The highly active form of the enzyme is inactivated spontaneously in vitro to the stable, less active form. Inactivation can be prevented and reversed by compounds containing thiol groups, such as reduced glutathione and cysteine, and stimulated by oxidized glutathione and thiol inhibitors. We have also shown that there is only one protein in *S. faecalis* which is able to hydrolyse inorganic pyrophosphate in our experimental conditions, indicating that the reversible inactivation can be reliably studied with crude extracts (Lahti & Heinonen, 1981).

In the present paper we describe how the activity of inorganic pyrophosphatase from *S. faecalis* changes during batch culture.

METHODS

Test organism, culture conditions and sampling. *Streptococcus faecalis* ATCC 8043 was grown in a rich medium (Lahti & Heinonen, 1981) at 37 °C in a rotary shaker; growth was followed by measuring the turbidity of the culture with a Klett–Summerson colorimeter using filter 62 (590–650 nm). The volume of the sample to be taken was calculated by dividing 190 by the turbidity of the culture at the time of sampling. Thus, all the samples contained approximately equal amounts of cells (about 0.5 mg dry wt). Samples were rapidly chilled by 1:2 dilution with ice-cold 0.9% (w/v) NaCl, harvested by centrifuging (5000 g, 10 min, 2 °C), and washed once in ice-cold 0.9% (w/v) NaCl. The cells were stored at –70 °C.

Disintegration of the cells. The cells were disrupted by lysozyme treatment followed by osmotic shock as described previously (Lahti & Heinonen, 1981).

Incubation of crude extracts. Where indicated, crude extracts were preincubated with or without cysteine (to activate or inactivate the enzyme, respectively) before assay. Suspensions obtained by osmotic shock were diluted 1:1 with ice-cold 0.05 M-Tris/HCl buffer (pH 8.0) with or without 10 mM-cysteine, and 5 ml portions of these suspensions were incubated in open test tubes in a thermostated water bath at 37 °C. After the appropriate incubation time, 175 µl portions were removed from the tubes into an ice-water bath, MgCl₂ was added, and the assay reaction was started by adding [³²P]pyrophosphate.

Enzyme assay. Inorganic pyrophosphatase was assayed by measuring the liberation of phosphate from [³²P]pyrophosphate as described by Heinonen (1970a). At 0 °C the highly active form of the enzyme is sufficiently stable to be assayed accurately (Lahti & Heinonen, 1981). Therefore the activities observed before inactivation and after activation were measured at 0 °C. However, the enzyme in the form with low, stable activity was determined at 37 °C, because its activity is too low for accurate measurement at 0 °C. For comparison, the values obtained at 0 °C were multiplied by 14 because the activity of inorganic pyrophosphatase at 37 °C is 14 times that observed at 0 °C (Lahti & Heinonen, 1981).

Protein determination. Protein was measured as the turbidity caused by added sulphosalicylic acid (Heinonen, 1970b).

Chemicals. Tetrasodium [³²P]pyrophosphate was obtained from The Radiochemical Centre, Amersham. L-Cysteine hydrochloride was purchased from Merck. Commercially available chemicals of analytical grade were used in media and reaction mixtures.

RESULTS AND DISCUSSION

Because of the lability of inorganic pyrophosphatase from *S. faecalis* we had to look for as gentle a cell disintegration method as possible. The method finally chosen was lysozyme treatment followed by osmotic shock. Ultrasonic and toluene treatments gave considerably lower inorganic pyrophosphatase activities (5- and 14-fold lower, respectively) because the enzyme was partially inactivated during these procedures. When the cells were treated with hexadecyltrimethylammonium chloride as described by Heinonen *et al.* (1976) no activity could be detected. The maximal enzyme activity was obtained after incubation for 3 min at 37 °C in the presence of lysozyme; further incubation for up to 25 min did not decrease the activity. It was thus evident that the enzyme was somehow stabilized by our disintegration conditions. We routinely incubated the cells for 7 min at 37 °C in order to ensure complete disintegration.

The specific activity of inorganic pyrophosphatase (without preincubation) was maximal in the early-exponential phase of growth, whereas the specific activity observed after incubation for 4 h at 37 °C without cysteine showed little change during the various phases of batch culture (Fig. 1a). The degree of inactivation (i.e. the ratio of activities observed before and after incubation without cysteine) and the specific activity before incubation varied in parallel during growth (Fig. 1a, b). It was thus evident that inorganic pyrophosphatase of *S. faecalis* was synthesized constitutively and that the changes observed in the specific activity before incubation probably reflected regulation of the enzyme activity. This conclusion was supported by the fact that the degree of inactivation and the degree of activation (i.e. the ratio of activities observed after and before incubation in the presence of cysteine) changed in an antiparallel fashion (Fig. 1b). The higher the degree of inactivation, the greater was the proportion of inorganic pyrophosphatase in the more active form. That the proportion of activatable, less active form of the enzyme was small during the early-exponential growth phase was shown by the low degree of activation. The opposite situation existed during the stationary phase of growth, when the degree of inactivation was minimal (Fig. 1b).

Inorganic pyrophosphatase in extracts of cells in the early-exponential phase of growth could not be activated by cysteine above the activity found before incubation (Fig. 2); evidently practically all the enzyme was in the high-activity form during this growth phase. Oxidized glutathione, even at high concentrations, did not decrease the activity below that observed as a result of incubation without thiol compounds (Lahti & Heinonen, 1981). Hence, at this state of equilibrium all the inorganic pyrophosphatase is presumably in the

Fig. 1. Activity changes of inorganic pyrophosphatase of *Streptococcus faecalis* during batch culture. Inactivation was carried out by incubating crude extracts for 4 h at 37 °C in the absence of cysteine, as described in Methods; activation was performed by incubating crude extracts for 1 h at 37 °C in the presence of 5 mM-cysteine. Growth was followed turbidimetrically with a Klett–Summerson colorimeter, using filter 62 (590–650 nm; KU₆₂). ●, Growth; ▲, specific activity before inactivation; △, specific activity after inactivation; ■, degree of inactivation (the ratio of activities observed before and after inactivation); □, degree of activation (the ratio of activities observed after and before activation).

Fig. 2. Inactivation (●, ○) and activation (▲, △) of inorganic pyrophosphatase of *Streptococcus faecalis* in samples taken from the early-exponential phase of growth (KU₆₂ = 20; ●, ▲) and the stationary phase of growth (KU₆₂ = 185; ○, △). Activation and inactivation were performed by incubating the crude extracts at 37 °C in the presence or absence of 5 mM-cysteine.

low-activity form. Because the degree of inactivation in samples taken from cells in the stationary phase of growth was about half of that observed at the early-exponential phase (Fig. 1 b), both enzyme forms appear to be present in equal amounts during the stationary phase. The difference in activity between the two forms of enzyme is about 27-fold (the highest degree of inactivation – Fig. 1 b).

Starr & Oginsky (1972) found that the specific activity of inorganic pyrophosphatase from *S. faecium* F24 was maximal in the exponential phase of growth. They thought that activation in the exponential phase or inhibition in the stationary phase was unlikely since additive values were obtained on assaying mixtures of crude sonicates from different phases of growth. In the work reported here we have shown that the specific activity maximum

observed for inorganic pyrophosphatase from *S. faecalis* ATCC 8043 is probably due to the regulation of enzyme activity. It is not known whether the enzymes from these closely related strains are regulated in a different manner. The mixing experiments described by Starr & Oginsky (1972) may not be a reliable test for the presence or absence of activation, since during the preparation of crude extracts the effectors may be greatly diluted, their action remaining partly hidden. In our experiments, specific activity was analysed in terms of four different parameters (degrees of activation and of inactivation, and specific activity before and after incubation without thiol compounds); thus more information was obtained about the state of the enzyme. We emphasize that activation and inactivation, as defined by us, indicate changes in the activity and not changes in the amount of the enzyme.

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