

Reversible Changes in the Activity of Inorganic Pyrophosphatase of *Streptococcus faecalis*. The Effect of Compounds Containing SH-Groups

REIJO LAHTI and JUKKA HEINONEN

Department of Biochemistry, University of Turku, SF-20500 Turku 50, Finland

The activity of inorganic pyrophosphatase (EC 3.6.1.1) from *Streptococcus faecalis* ATCC 8043 decreased rapidly, when the cell extracts prepared by lysozyme and osmotic shock were incubated in a buffer solution (pH 8.0) at 25–50 °C. Increase in pH (from 8.5 to 10.5) retarded the inactivation considerably. The low activity level was reached in 2.5 h at 37 °C (pH 8.0) and this residual activity was stable during prolonged incubation. The enzyme in the form of low activity was not rapidly denatured until at 70 °C.

The inactivation could be prevented and reversed by cysteine, dithiothreitol and 2-mercaptoethanol, but reductants, which do not contain SH-groups were not effective. Reduced glutathione slowed down and oxidized glutathione stimulated the loss of activity. SH inhibitors, such as *p*-hydroxymercuribenzoate and *N*-ethylmaleimide, stimulated the inactivation.

With pure enzyme it was shown that the low, stable activity level obtained with crude extracts during incubation is a property of inorganic pyrophosphatase and not due to another enzyme with a low capacity to hydrolyze inorganic pyrophosphate.

These findings suggest that inorganic pyrophosphatase from *S. faecalis* exists in two interconvertible forms which differ in activity.

Inorganic pyrophosphate (PP_i) is produced in various reversible NTP-dependent reactions, which presumably are pulled in the biosynthetic direction *in vivo* by enzymatic hydrolysis of PP_i to inorganic phosphate.¹ In addition PP_i is formed like ATP by photosynthetic,^{2–5} oxidative^{6–7} and glycolytic⁸ phosphorylation. Membrane-bound inorganic pyrophosphatase (pyrophosphate phosphohydrolase, EC 3.6.1.1., hereafter referred to as PPase)

catalyzes the production of PP_i energized by electron transfer reactions.^{5,9} So PPase is able to catalyze both the hydrolysis and the synthesis of PP_i.

PP_i serves as a source of energy and phosphate for several reactions in pro- and eucaryotes.^{10–13} It also regulates many enzymes without actually participating in the reactions. Almost invariably the effect of PP_i is then inhibitory.^{14–19} Contrary to common opinion it has been suggested that the ratio PP_i/P_i rather than the ratio ATP/ADP might control certain of the key reactions in carbohydrate metabolism.¹¹

The intracellular PP_i concentration depends mainly on the activity of PPase in the cells. Hence factors which affect the intracellular location or total activity of PPase influence secondarily all the reactions in which PP_i has a role as a substrate or regulator. Changes in the state of PPase thus have a wide effect on the general metabolism.

We are currently studying the role and regulation of PPase in bacteria.^{20–22} In the course of this research we observed that the activity of this enzyme decreased rapidly to a low, stable level, when the cell extract of *Streptococcus faecalis* was incubated at growth temperature 37 °C. The inactivation could be prevented or reversed by compounds containing SH-groups. The results of our studies concerning the phenomenon are presented in this paper.

MATERIALS AND METHODS

Chemicals. Radioactive pyrophosphate (marked with ³²P) was delivered by the Radiochemical Centre (Amersham, Bucks, England). Lysozyme, bovine

serum albumin, *N*-ethylmaleimide, *p*-hydroxymercuribenzoate and tris(hydroxymethyl)aminomethane (Tris) were obtained from Sigma Chemical Co. (St. Louis, Mo., USA), *L*-cystein hydrochloride was purchased from E. Merck AG. (Darmstadt, FRG), Reduced and oxidized glutathione were obtained from Boehringer Mannheim GmbH (Mannheim, FRG). DEAE-Sepharose 6B CL and Sepharose 4B CL were purchased from Pharmacia Fine Chemicals AB (Uppsala, Sweden). Ultrogel Aca 34 was obtained from LKB (Bromma, Sweden). The other chemicals were of the highest grade commercially available and were used without further purification.

Test organism and its culture. *Streptococcus faecalis* ATCC 8043 was grown in rich medium containing in one litre of distilled water: Fifteen grams of yeast extract (Difco), 5 g of trypsin hydrolyzed casein (Bacto tryptone, Difco), 10 g of *D*-glucose, and 2 g of K_2HPO_4 . *D*-glucose (as a 50% solution) was sterilized apart from the rest of the medium. Growth took place at 37 °C in a rotary shaker and was followed by measuring the turbidity of the culture with a Klett-Summerson colorimeter using filter 62 (590–650). Cell samples taken from the exponential growth phase were rapidly chilled by 1:2 dilution with ice cold 0.9% NaCl, harvested by centrifugation (5000 g, 10 min, 2 °C), and washed once in ice-cold saline. The cells were stored at –70 °C.

In case of purification, the final cultivation was carried out in a 150 l fermentor. The cells were collected when their wet weight reached 1.8 mg/ml and stored at –70 °C.

Disintegration of the cells. Cell samples (about 0.5 mg of dry weight) were suspended in 0.5 ml of incubation mixture, which contained 0.05 M Tris–HCl buffer pH 8.0, 0.5 M sucrose, and lysozyme, 1 mg/ml. After incubation (7 min, at 37 °C) osmotic shock broke down the spheroplasts, when they were suspended in 4.5 ml of ice-cold 0.02 M Tris–HCl pH 8.0. The method was adapted from that described by Roth *et al.*²³

Incubation of the cell extracts. The suspensions obtained by osmotic shock were diluted 1:2 with ice-cold 0.05 M Tris–HCl buffer (pH 8.0) containing the compounds to be tested. The incubation was performed in 175 μ l portions (which was the same as the amount of the enzyme in the reaction mixture) in open test tubes immersed in a thermostated water bath. After the desired incubation time the tubes were transferred into ice-water bath, $MgCl_2$ was added, and the reaction was started by labelled pyrophosphate as described by Heinonen.²⁴

When the enzyme in the form of low, stable activity was required the suspensions obtained by osmotic shock were incubated in 10–30 ml portions for 4 h at 37 °C, after which they were diluted 1:2 with appropriate solutions and 175 μ l portions were taken

for further incubations.

Enzyme assay. Inorganic pyrophosphatase was assayed by measuring the liberation of phosphate from labelled pyrophosphate.²⁴ The measured activity was a linear function of the amount of cell extract in the reaction mixture.

Purification of inorganic pyrophosphatase. The details of purification will be published elsewhere. Briefly: The cells were disrupted by lysozyme and a French press. Nucleic acids were removed by streptomycin sulfate precipitation. $(NH_4)_2SO_4$ treatment in Sepharose 4B CL column was a modification of the method presented by von der Haar.²⁵ Inorganic pyrophosphatase was eluted from the column (4.2 \times 21 cm) of Sepharose 4B CL by 1200 ml of linearly decreasing $(NH_4)_2SO_4$ concentration gradient (from 52 to 34% saturation) after which the elution was continued with the buffer (0.1 M Tris–HCl, pH 8.0) without $(NH_4)_2SO_4$. The column (3 \times 28 cm) of DEAE-Sepharose 6B CL was eluted with linearly increasing concentration gradient (from 0.1 to 0.5 M) KCl containing 0.05 M Tris–HCl buffer, pH 8.0. Gel filtration was carried out by two tandem columns (2 \times 2.5 \times 100 cm) of Ultrogel Aca 34 and the columns were eluted with 0.025 M Tris–HCl, pH 8.0. The final stage of purification was preparative slab gel electrophoresis. It was performed according to the method by Laemmli and Favre.²⁶

The purified preparation of inorganic pyrophosphatase was judged to be homogenous by slab gel electrophoresis in the presence and absence of SDS.

Protein determination. The method by Lowry *et al.*²⁷ was used to determine the protein concentration with bovine serum albumin as standard protein.

RESULTS

Effect of temperature. Inorganic pyrophosphatase from *Streptococcus faecalis* is inactivated spontaneously *in vitro* when the cell extracts prepared by lysozyme and osmotic shock are incubated in a buffer solution (pH 8.0) at 25–50 °C (Fig. 1 A). The rate of inactivation increases with temperature. The Arrhenius energy of activation for the inactivation reaction was calculated on the basis of the maximal rates observed at different temperatures (Fig. 1 A) with the result of 92 kJ/mol.

The low constant activity level is reached in about 2.5 h and it is stable at least for 20 h at 37 °C (Fig. 1 B). The enzyme in the form of low activity is not rapidly denatured until at 70 °C (Fig. 1 C).

Because of inactivation the rate of reaction decreased rapidly at 37 °C (Fig. 2). At 0 °C the more active form of enzyme is stable enough to be assayed accurately (Fig. 1 A and 2). Therefore kinetics and the

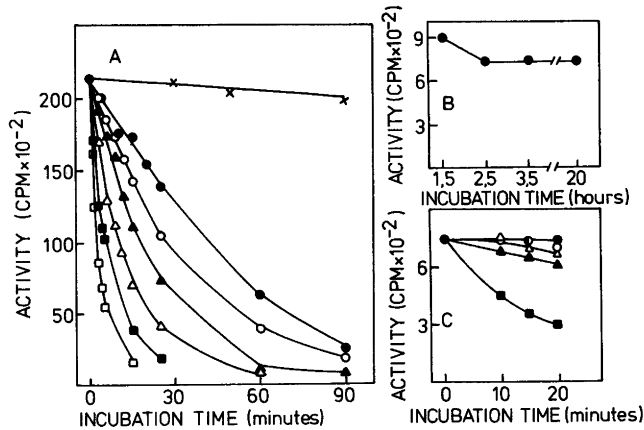


Fig. 1. A. Rate of inactivation at different temperatures. ×, 0 °C; ●, 25 °C; ○, 30 °C; ▲, 35 °C; △, 40 °C; ◻, 45 °C; ◼, 50 °C; B. Prolonged incubation at 37 °C; C. Heat stability of the activity reached after incubation for 4 h at 37 °C. ●, 37 °C; ○, 50 °C; △, 55 °C; ▲, 60 °C; ◼, 70 °C.

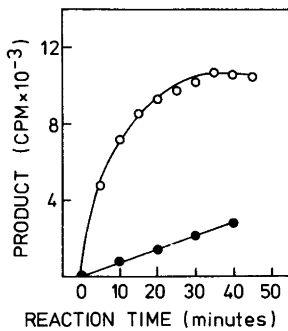


Fig. 2. Rate of reaction at 37 °C (○) and 0 °C (●).

activity before incubation were measured at 0 °C. On the other hand the enzyme in the form of low activity was determined at 37 °C, because its activity is too low 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-fold compared to that observed at 0 °C (Fig. 2). The protection of enzymes against thermal inactivation by ligands is a phenomenon often detected.²⁸ In this case, the effect of substrate (1:1 complex of Mg²⁺ and pyrophosphate) cannot be accurately studied because the substrate is hydrolysed during the incubation. However it is obvious that the substrate is unable to stabilize the enzyme because the reaction slows down rapidly at 37 °C (Fig. 2). Furthermore, Mg²⁺, which is both a part of substrate and, in some

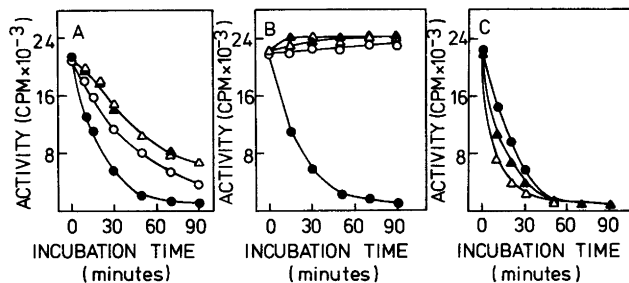


Fig. 3. Effects of reduced glutathione (A), cysteine (B), and oxidized glutathione (C) on the inactivation at 37 °C. ●, control; ○, 0.5 mM; ▲, 1 mM; △, 5 mM.

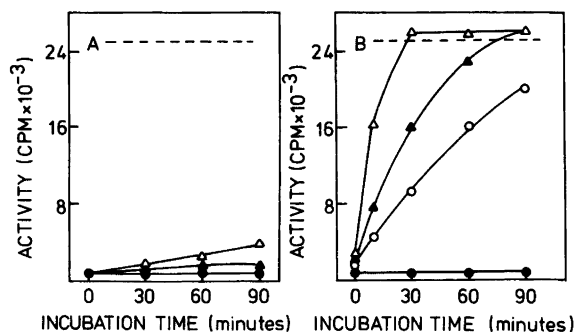


Fig. 4. Reactivation by reduced glutathione (A) and cysteine (B) at 37 °C after preincubation for 4 h at 37 °C. ●, control; ○, 0.5 mM; ▲, 1 mM; △, 5 mM. Dashed lines indicate the activity observed before preincubation.

cases, an activator,²⁹⁻³¹ exerts no effect on the inactivation (data not shown).

Effects of compounds interacting with SH-groups. Cysteine maintains the enzyme in the form of high activity, reduced glutathione (GSH) retards and oxidized glutathione stimulates the inactivation (Fig. 3). Inactivation is completely reversed by cysteine (Fig. 4). The initial rate of reactivation is linearly proportional to the concentration of cysteine. The apparent K_m value of cysteine is about 3.3 mM. GSH is not as efficient as cysteine to revert the inactivation (Fig. 4).

Nonphysiological SH-compounds, 2-mercaptoethanol and dithiothreitol, are as efficient as cysteine in preventing and reverting the inactivation. Reductants, which do not contain SH-groups (*e.g.*

NADH₂, Na₂S₂O₄, H₂) are without effect. Furthermore, oxygen bubbled through the extract does not stimulate the inactivation (data not shown). *p*-Hydroxymercuribenzoate and *N*-ethylmaleimide, which are known to bind to SH-groups, increase the initial rate of inactivation 1.9- and 1.6-fold at 1 mM concentrations, respectively, (data not shown).

Effect of pH. As with many other enzymes,^{30,32} changes in pH exert similar effects on the enzyme as specific ligands also in this case. Increase in pH (from 8.5 to 10.5) slows down the inactivation (Fig. 5 A). Furthermore at pH 8.5 inactivated enzyme can be partially reactivated (6.7-fold) by incubating the extract at pH 10.5 (Fig. 5 B).

Purification and reversible inactivation of highly

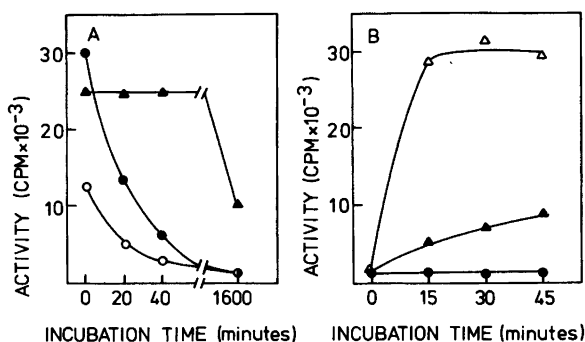


Fig. 5. A. Effects of pH on the inactivation at 37 °C. Buffer solution contained 25 mM morpholino-propanesulfonic acid and 25 mM glycine. PH was adjusted by KOH. ○, pH 6.5; ●, pH 8.5; ▲, pH 10.5; B. Effect of pH on the reactivation at 37 °C. The inactivation was carried out in a buffer solution (5 mM glycine-KOH, pH 8.5) for 4 h at 37 °C. Then the preparate was divided into three portions, which were diluted 1:2 by 50 mM glycine-KOH buffer at different pH. ●, pH 8.5; ▲, pH 10.5; △, pH 8.5+cysteine (final concentration 5 mM).

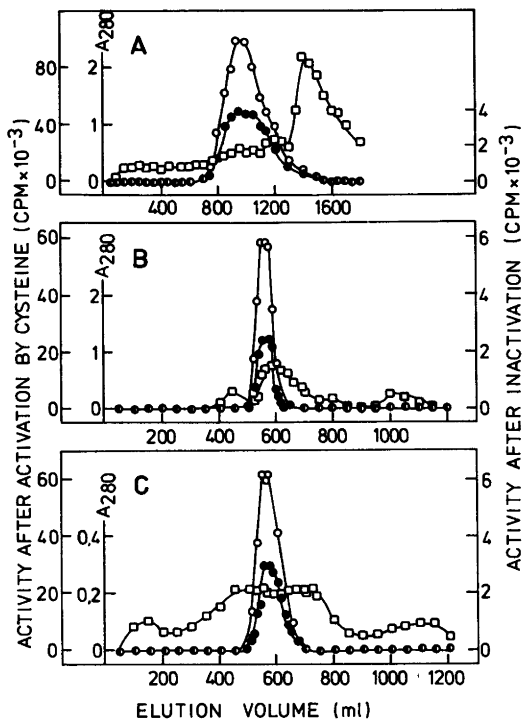


Fig. 6. Purification of inorganic pyrophosphatase by $(\text{NH}_4)_2\text{SO}_4$ treatment in Sepharose 4B CL column (A), chromatography on DEAE-Sepharose 6B CL (B) and gel filtration on Ultrogel AcA 34 (C). The elution conditions are described in Materials and Methods. \square , absorbance at 280 nm; The activity of inorganic pyrophosphatase was determined in two different ways, (O) after activation by cysteine (1 mM cysteine for 20 min at 37 °C) and (●) after inactivation to a low, stable activity level (incubation for 4 h at 37 °C).

purified inorganic pyrophosphatase. To find out if the two activity levels observed with crude extracts are properties of one or two enzymes, we purified inorganic pyrophosphatase to homogeneity. In the course of purification the activity was determined under two different conditions: After activation by cysteine (1 mM cysteine for 20 min at 37 °C) and after inactivation to a low, stable activity level (incubation for 4 h at 37 °C). Throughout the purification only one peak of inorganic pyrophosphate hydrolyzing activity could be seen and the two activities could not be separated at any stage of purification (Fig. 6). So it is evident that there is only one protein in *S. faecalis* which is able to hydrolyze inorganic pyrophosphate in our experimental conditions.

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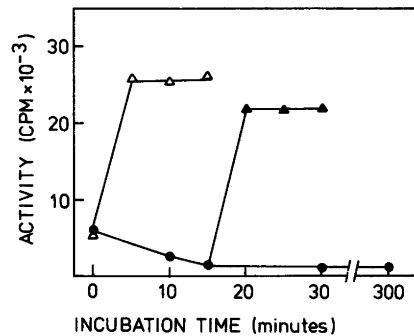


Fig. 7. Activation of the pure enzyme by cysteine during incubation at 37 °C. ●, control; Δ , 1 mM cysteine. After 15 min the control was divided into two portions and to the other cysteine (1 mM) was added (\blacktriangle).

Like the enzyme in crude extracts the pure enzyme is also inactivated to a low, stable activity level as a result of incubation in a buffer solution (0.05 M Tris-HCl, pH 8.0) at 37 °C. Inactivation can be prevented and reversed by cysteine (Fig. 7).

In the course of purification the enzyme is gradually inactivated and so the inactivation observed with pure enzyme is much smaller than with crude extracts. For this reason, it is not possible to study the inactivation phenomenon in its whole extent by highly purified preparations. However, the results presented in this chapter indicate that the reversible inactivation can be reliably studied with crude extracts.

During the purification the enzyme is to a small extent inactivated irreversibly. This is shown by somewhat lower degree of reactivation which is about 17 with pure enzyme and 27 with crude extracts.

DISCUSSION

As shown above inorganic pyrophosphatase of *Streptococcus faecalis* is inactivated spontaneously *in vitro*. Inactivation is completely reversed by cysteine (Fig. 4). This rules out proteolysis and denaturation as causes of inactivation. Furthermore, it was shown that the low, stable level of activity obtained during incubation is not due to another enzyme with low capacity to hydrolyze PP_i (Figs. 6 and 7). Hence it is obvious that inorganic pyrophosphatase of *S. faecalis* exists in two interconvertible forms, which differ in activity.

Most of this work was done with crude extracts because in the course of purification the enzyme is gradually inactivated and so the inactivation phenomenon cannot be studied in its whole extent using highly purified preparations.

We do not know the mechanism of the inactivation, but oxidation of 2 SH-groups seems to be the most plausible explanation. It is likely that the reacting SH-groups are not part of the active center, because the activity is not lost altogether, when they are oxidized. Based on the high activation energy of inactivation (92 kJ/mol) we suppose that the oxidation is connected with a change in the conformation of the enzyme protein. The mechanism of the inactivation is now under study.

The inactivation of inorganic pyrophosphatase, reversible by reductants has been observed earlier in the case of some strictly anaerobic bacteria.³³⁻³⁵ In these cases oxygen was the inactivating agent and several different reductants (NADH₂, Na₂S₂O₄ etc.) were able to revert its effect. Ware and Postgate³⁵ proposed that the mechanism was evolved to prevent the consumption of ATP in aerobic conditions, when strict anaerobes are unable to produce ATP. As far as we know, our paper is the first one describing the reversible inactivation of inorganic pyrophosphatase in the cell extracts of lactic acid bacteria, which are not strictly anaerobic, although they produce energy by fermentation not by oxidation. Contrary to the inorganic pyrophosphatases of strictly anaerobic bacteria the enzyme of *S. faecalis* is activated only by reductants containing SH-groups and is resistant to oxygen. So the mechanism must be somewhat different.

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