Role of Glutathione in the Regulation of Inorganic Pyrophosphatase Activity in *Streptococcus faecalis*

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When the thiol-oxidizing agent diamide was added to a batch culture of *Streptococcus faecalis* during exponential growth, the specific activity of inorganic pyrophosphatase (EC 3.6.1.1) decreased to the level observed in the stationary phase. The effect of diamide was completely reversed by reduced glutathione. Furthermore, the ratio of reduced to oxidized glutathione and the specific activity of inorganic pyrophosphatase changed in a very similar fashion during batch culture. These findings, together with our earlier results, suggest that the activity of inorganic pyrophosphatase in *S. faecalis* is regulated by glutathione via the ratio of reduced to oxidized glutathione.

INTRODUCTION

We have recently shown that inorganic pyrophosphatase (EC 3.6.1.1) of *Streptococcus faecalis* exists in two interconvertible forms which differ in activity (Lahti & Heinonen, 1981*a*). During the early-exponential phase of growth almost all the enzyme is in the high-activity form, whereas during the stationary phase the highly active and the less active forms exist in equal amounts (Lahti & Heinonen, 1981*b*).

In this paper we describe the factors which regulate the equilibrium between these two enzyme forms *in vivo*.

METHODS

Organism and culture conditions. *Streptococcus faecalis* ATCC 8043 was grown in a rich medium at 37 °C in a rotary shaker and growth was followed by measuring the turbidity of the culture with a Klett–Summerson colorimeter as described previously (Lahti & Heinonen, 1981*a*).

Enzyme assay. The activity of inorganic pyrophosphatase was determined by measuring the liberation of labelled phosphate from [³²P]pyrophosphate (Heinonen, 1970*a*). Details of the reaction conditions, the collection of the cell samples and their treatment have been described previously (Lahti & Heinonen, 1981*b*).

Glutathione assay. Samples (2.5 ml) taken from the culture were rapidly chilled by 1:1 dilution with ice-cold 0.15 M-NaCl containing 10 mM-sodium azide. Cells were harvested by centrifugation (5000 g, 10 min, 4 °C) and washed once with ice-cold 0.15 M-NaCl containing 10 mM-sodium azide. The cells were stored at –70 °C. Glutathione was extracted by shaking the cells (20 min, 37 °C) in a buffer/toluene mixture (0.1 ml toluene in 5 ml of 0.1 M-potassium phosphate buffer, pH 7.5, containing 5 mM-Na₂EDTA). The extract was centrifuged (5000 g, 10 min, 4 °C) and glutathione was measured in the supernatant.

Total glutathione was determined as described by Tietze (1969). In this method oxidized glutathione (GSSG) is reduced to reduced glutathione (GSH) by glutathione reductase and NADH, and a yellow GSH–5,5'-dithiobis-(2-nitrobenzoic acid) complex is formed which can be measured with a Klett–Summerson colorimeter using filter 42 (390–440 nm). The reaction mixture was as follows: 4 ml of diluted extract (dilution with 0.1 M-potassium phosphate buffer, pH 7.5, containing 5 mM-Na₂EDTA) containing up to 0.3 and 0.2 nmol glutathione per reaction mixture for total and oxidized glutathione measurements, respectively; 1 ml of 1.1 mM-Na₂NADH; 0.025 ml (about 3.5 units) of glutathione reductase and 0.1 ml of 10 mM-5,5'-dithiobis-(2-nitrobenzoic acid).

GSSG was determined in the same way after precipitation of GSH with CuCl (Lahti & Vuorinen, 1981). CuCl (20 mg powder) was added to 5 ml of diluted extract containing up to 0.25 nmol glutathione. After 30 s of vigorous mixing with a vortex mixer at room temperature (about 20 °C) the suspension was centrifuged (5000 g, 10 min, 4 °C) and GSSG was determined in the supernatant. As only 95 % of GSH was precipitated by CuCl, this had to be taken into account in the calculations. Furthermore, CuCl was slightly oxidized under our experimental conditions (the solution became blue), resulting in a partial inhibition of glutathione reductase (Lahti & Vuorinen, 1981). To overcome this effect a GSSG standard was determined in the presence of CuCl. For the estimation of intracellular glutathione the volume of the bacterial cells in each sample was calculated as described by Moses & Sharp (1972).

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

Chemicals. Tetrasodium [^{32}P]pyrophosphate was obtained from Amersham. L-Cysteine hydrochloride was purchased from Merck. GSH and GSSG, NADH, 5,5'-dithiobis-(2-nitrobenzoic acid), tetramethylazoformamide, glutathione reductase and lysozyme were obtained from Sigma. Commercially available chemicals of analytical grade were used in media and reaction mixtures.

RESULTS

The results obtained *in vitro* suggested that compounds containing thiol groups might regulate inorganic pyrophosphatase activity in *S. faecalis* (Lahti & Heinonen, 1981a). It was of interest to discover what happens when the intracellular redox state of the glutathione system is artificially disturbed. For this purpose tetramethylazoformamide (diamide) was used which has been shown to oxidize intracellular GSH quickly and efficiently (Kosower & Kosower, 1969). When diamide was added to cultures during the early-exponential phase of growth the specific activity of inorganic pyrophosphatase decreased to the level observed in the stationary phase (Fig. 1). The effect of diamide was completely reversed by adding GSH or cysteine (Fig. 1). When diamide was added to crude extracts the initial rate of inactivation of inorganic pyrophosphatase increased 1.6- and 3.6-fold in the presence of 1 and 5 mM-diamide, respectively (data not shown).

To obtain further evidence for the possible role of glutathione in the regulation of inorganic pyrophosphatase we determined the intracellular glutathione content of *S. faecalis*. The glutathione content varied considerably during batch culture (Fig. 1) and was close to that observed in other microbes (Fahey *et al.*, 1978; Loewen, 1979), except that in this case a considerable amount of glutathione was in its oxidized form. It was significant, with respect to the regulation of inorganic pyrophosphatase, that the glutathione content, especially the ratio GSH/GSSG, changed in a very similar manner to the specific activity of inorganic pyrophosphatase during batch culture (Fig. 1).

DISCUSSION

The highly active form of inorganic pyrophosphatase of *S. faecalis* is inactivated spontaneously *in vitro* to the stable, less active form. *In vitro*, S-S-compounds stimulate and SH-compounds prevent or reverse the inactivation (Lahti & Heinonen, 1981a), whereas *in vivo* they regulate the state of equilibrium between these two enzyme forms. Hence, the effects *in vivo* are qualitatively but not quantitatively identical to those observed *in vitro*. For example, GSH reactivates inorganic pyrophosphatase rather slowly *in vitro* (Lahti & Heinonen, 1981a), whereas *in vivo* it rapidly causes complete reactivation of the enzyme (Fig. 1). The effect of diamide is also rather small *in vitro*, whereas *in vivo* it produces the maximal change in the equilibrium observed during the batch culture (Fig. 1). *In vitro*, cysteine is more efficient than GSH as a reactivator and activator of inorganic pyrophosphatase (Lahti & Heinonen, 1981a), but *in vivo* they are equally effective in this respect (Fig. 1). Because the intracellular concentration of cysteine is at most one-tenth of that of GSH (Jocelyn, 1972) it seems reasonable to suppose that glutathione, not cysteine, regulates the activity of inorganic pyrophosphatase in *S. faecalis*. This is further supported by

Fig. 1. Glutathione content and the effects of diamide, GSH and L-cysteine on the specific activity of inorganic pyrophosphatase during batch culture of *Streptococcus faecalis*. A culture in the early-exponential phase of growth ($KU_{62} = 20$) was divided into two portions. One served as a control and diamide (5 mM) was added to the other. After 5 min the culture containing diamide was divided into three portions. One served as a diamide control, to the second GSH (10 mM) was added, while the third received L-cysteine (10 mM). ●, Specific activity of inorganic pyrophosphatase; □, specific activity after addition of diamide; ■, specific activity after addition of diamide and GSH; ▲, specific activity after addition of diamide and L-cysteine; ▲, intracellular GSH concentration; △, intracellular GSSG concentration; ○, ratio GSH/GSSG; ---, growth (KU_{62}). Inactivation of inorganic pyrophosphatase by diamide is indicated by the solid arrow, and its reactivation caused by GSH and L-cysteine is shown by the dashed arrow.

the fact that the ratio GSH/GSSG and the specific activity of inorganic pyrophosphatase changed in a very similar fashion during batch culture (Fig. 1).

Several bacterial inorganic pyrophosphatases are stabilized by reductants *in vitro* (D'Eustachio *et al.*, 1965; Ware & Postgate, 1970; Lahti & Heinonen, 1981a). As far as we know our results provide the first evidence that this stabilization reflects the regulation of the enzyme at the activity level, with GSH/GSSG as an effector *in vivo*. Rapoport & Scheuch (1960) have suggested that glutathione might regulate inorganic pyrophosphatase of rabbit reticulocyte.

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