An essential role of cytosolic thioltransferase in protection of pyruvate kinase from rabbit liver against oxidative inactivation

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Pyruvate kinase from rabbit liver is inactivated spontaneously in the presence of air. Glutathione in physiological concentrations gives partial protection against inactivation. Full protection is obtained with glutathione and purified cytosolic thioltransferase supplemented with a glutathione-regenerating system. It is suggested that thioltransferase plus glutathione serve a general function in protecting protein thiol groups against oxidation.

Thioltransferase *Glutathione* *Pyruvate kinase* *Protection* *Oxidative inactivation*

1. INTRODUCTION

Early work in biochemistry suggested a redox function of glutathione [1] and its possible participation in biological control and protection of enzymes against inactivation [2–4]. A protective role of thiols has repeatedly been demonstrated with many enzymes, and, in view of the abundance of glutathione in the cell, it appears logical that this thiol should be the most important protector. A requirement of a catalyst for glutathione to exert the protection has not previously been demonstrated. In this report it is shown that, in vitro, cytosolic thioltransferase [5] is necessary for full protection of hepatic pyruvate kinase by glutathione. The latter enzyme is known to be inactivated by oxidation and we have earlier shown that thioltransferase catalyzes the restoration of activity to partially inactivated pyruvate kinase [6].

2. MATERIALS AND METHODS

2.1. *Enzymes and chemicals*

Thioltransferase from rat liver cytosol was prepared as earlier described [5,7]. Pyruvate kinase from rabbit liver was purchased from Sigma and was used without further purification. Lactate dehydrogenase and yeast glutathione reductase were obtained from Boehringer-Mannheim and glucose-6-phosphate dehydrogenase from Sigma. Cofactors and other chemicals were standard commercial products of high purity. A unit of enzyme is the amount catalyzing the conversion of 1 μmol of substrate per min under standard assay conditions.

2.2. *Enzyme assays*

Pyruvate kinase activity was measured spectrophotometrically at 340 nm by use of lactate dehydrogenase as a coupling enzyme [8]. The assay system contained in 1 ml at 30°C: 5 mM ADP, 5 mM phosphoenolpyruvate, 160 μM NADH, 0.5 mM fructose-1,6-bisphosphate, 60 mM KCl, 10 mM $MgCl_2$, 30 units of lactate dehydrogenase, 50 mM imidazole/HCl (pH 7.6), and an aliquot of the pyruvate kinase to be assayed. Thioltransferase activity was tested with *S*-sulfocysteine and glutathione as substrates and was monitored by coupling with glutathione reductase [5,7].

2.3. *Inactivation studies*

Pyruvate kinase from stock was diluted 50–200-fold in buffer and the activity of the diluted enzyme was followed as a function of time by analysis of aliquots (50 μl) in the assay system described above. The incubation mixture of 1 ml

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of 0.12 M Tris/HCl (pH 8.3) or, in some cases of 0.12 M sodium phosphate (pH 7.3), was kept at 30°C. Reduced oxygen tension was maintained in a few experiments by flushing N_2 over the incubation mixture, which was kept in a sealed vial with two syringe needles pierced through the seal. The effect of various additions on the rate of inactivation of pyruvate kinase was studied under the conditions given in the legends to the figures. When thiols other than glutathione were used as protectors, they were used at 4 mM in combination with 0.1 mM glutathione, which in the presence of glutathione reductase and NADPH maintains the thiols in reduced form.

3. RESULTS

Pyruvate kinase from rabbit liver was found to loose catalytic activity with time when incubated in buffer at 30°C. The rate of inactivation increased with pH; 50% of the activity was lost in 150 min at pH 8.3 and about 20% was lost after the same time at pH 7.3. The mechanism of inactivation was not elucidated, but an oxidation of the enzyme was suggested by the finding that essentially no inactivation occurred under anaerobic conditions. Furthermore, pyruvate kinase from rat liver has previously been found to be inactivated by glutathione disulfide [6,9], supporting the interpretation of an oxidative process. In the present study it was also found that addition of glutathione disulfide or cystamine enhanced the rate but not the extent of inactivation.

The kinetics of partially inactivated pyruvate kinase were compared with those of the 'unmodified' enzyme. For these experiments pyruvate kinase was inactivated to approximately 17% residual activity in 0.12 M Tris/HCl (pH 8.3). The 'unmodified' enzyme was kept in 0.2 M sodium phosphate (pH 7.3) containing 0.1% bovine serum albumin, in which buffer it was essentially stable under the kinetic experiments. The rate-saturation curves for phosphoenolpyruvate at constant ADP concentration (5 mM) and for ADP at constant phosphoenolpyruvate concentration (5 mM) were obtained by measuring initial velocity as a function of varied substrate concentration. The concentrations for half-maximal velocity ($S_{0.5}$) under these conditions were 0.45 mM for phosphoenolpyruvate and 0.25 mM for ADP and did not change significantly upon inactivation of pyruvate kinase. Thus, the inactivation could be accounted for by a loss of catalytically active enzyme molecules or a decrease of the apparent k_{cat}-value of the enzyme (or both effects).

Several thiols were tried as protectors against inactivation of pyruvate kinase. These experiments were mostly carried out at pH 8.3, where the inactivation was more pronounced than at pH 7.3. It was found that 4 mM glutathione had a small protective effect that was significantly enhanced by addition of purified thioltransferase from rat liver. When glutathione reductase and NADPH were included in the incubation mixture, to regenerate glutathione from its disulfide, pyruvate kinase was essentially fully protected (fig.1). The protection was maintained over a longer period of time (6 h) in the presence of glucose-6-phosphate and

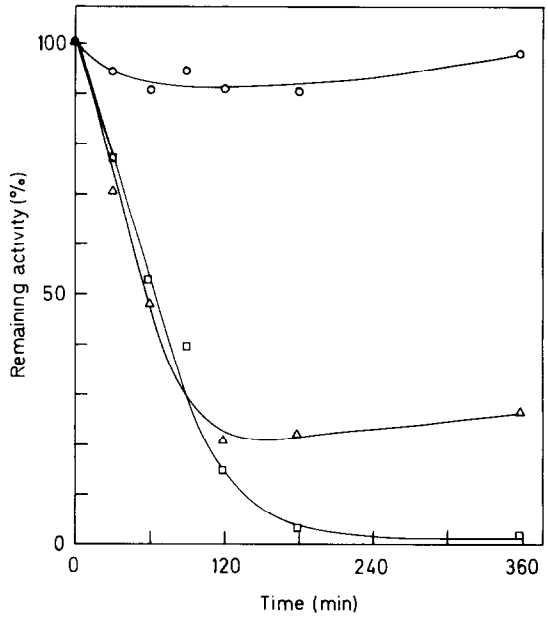

Fig.1. Effect of glutathione, thioltransferase, and a glutathione-reducing system on the inactivation of pyruvate kinase from rabbit liver. Pyruvate kinase (0.6 unit) was incubated aerobically in 0.12 M Tris/HCl (pH 8.3). Incubation without any additions (□); incubation in the presence of 4 mM glutathione, 3 units of glutathione reductase, 0.2 mM NADPH, 2 mM glucose-6-phosphate, and 17 units of glucose-6-phosphate dehydrogenase in the absence (△) and presence (○) of purified thioltransferase from rat liver (0.4 unit).

glucose-6-phosphate dehydrogenase, serving as an NADPH-regenerating system.

Earlier results have shown that thioltransferase reacts with oxidized pyruvate kinase [6] suggesting that the protective effect is caused by continuous reduction of oxidized enzyme. It was found that the effect was dependent on the concentration of thioltransferase (data not shown) as expected for enzymatic reactions.

Coenzyme A, cysteine, penicillamine, or cysteamine gave partial protection at 4 mM concentration; cysteamine and penicillamine being significantly less effective than coenzyme A or cysteine (fig.2). Only in the case of cysteine did addition of thioltransferase increase the protective effect of these thiols by a significant measure (fig.3) in spite of the fact that the thioltransferase is active with all these compounds as substrates [10]. Dithiothreitol (4 mM) by itself not only prevented inactivation of pyruvate kinase, but also stimulated its activity (fig.2). On the other hand, incubation with the same concentration of dithiothreitol in the presence of thioltransferase caused inactivation of pyruvate kinase (fig.3). In all experiments shown in fig.2 and 3, a catalytic amount of glutathione (0.1 mM) and glutathione reductase were added to secure that the added thiols remained in the reduced state.

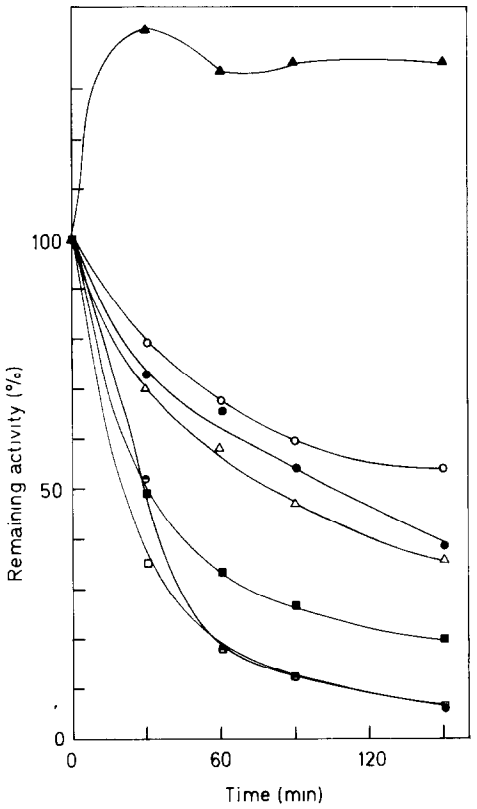

Fig.2. Effects of thiols on the inactivation of pyruvate kinase from rabbit liver. Pyruvate kinase (0.7 unit) was incubated aerobically in 0.12 M Tris/HCl (pH 8.3) with 3 units of glutathione reductase, 0.2 mM NADPH, and 0.1 mM glutathione in the absence of other thiols (control, □) or in the presence of 4 mM cysteamine (■), 4 mM coenzyme A (●), 4 mM dithiothreitol (▲), 4 mM glutathione (○), 4 mM cysteine (△), or 4 mM penicillamine (○).

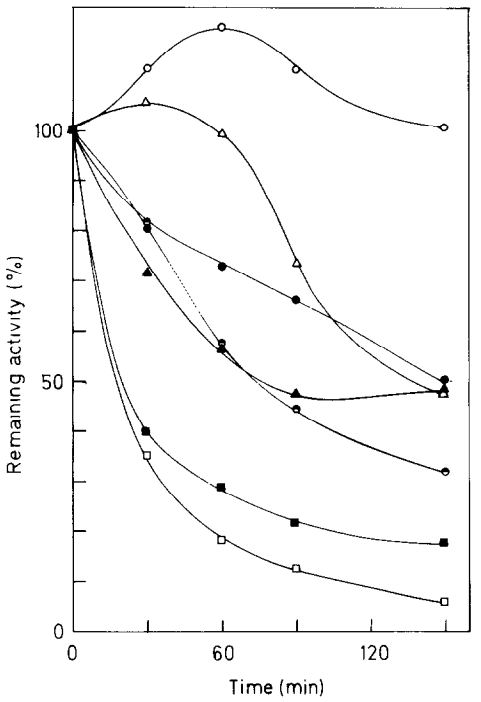

Fig.3. Effects of thioltransferase and thiols on the inactivation of pyruvate kinase from rabbit liver. Experimental conditions and symbols are the same as in fig.2 except for the addition of 0.4 unit of thioltransferase from rat liver. Note that only glutathione affords full protection in the presence of thioltransferase and that dithiothreitol is less protective in the presence than in the absence of thioltransferase (cf. fig.2).

4. DISCUSSION

Pyruvate kinase from liver has been found to be inactivated by oxidative processes [6,9], a finding confirmed in the present study. Glutathione protects against such inactivation, but even concentrations as high as 10 mM do not afford full protection (data not shown). The concentration of glutathione in mammalian cells is generally in the range of 1–10 mM; 9.35 ± 1.53 mM has been calculated for rat liver cytosol [11]. Thus, glutathione alone appears insufficient for complete protection against oxidation in vivo. Indeed, more effective protection was obtained in vitro by including purified thioltransferase from rat liver cytosol, and the further addition of glutathione reductase and an NADPH-generating system gave full protection (fig.1).

The effect of glutathione on pyruvate kinase could not be reproduced by other monothiols tested, even though some protection was obtained (fig.2 and 3). Thus, glutathione is probably the most important protective thiol in the cell owing to both its higher concentration and to its superior effectiveness. The difference between glutathione and the other thiols could not be explained by a more rapid autooxidation of the other thiols, because a small amount of glutathione plus glutathione reductase and NADPH were included in the inactivation system to maintain the thiols in the reduced state. The differences were apparent both in the absence and in the presence of thioltransferase, and consequently reflect differences in reactivity between thiols in the interaction with pyruvate kinase. The dithiol dithiothreitol gave full protection and in some cases even activation of pyruvate kinase in the absence of thioltransferase (fig.2) but only partial protection in the presence of thioltransferase. Presumably, the thioltransferase-catalyzed reaction in the latter case leads to 'over-reduction' and consequent inactivation of pyruvate kinase, e.g., by reducing an otherwise inaccessible disulfide bond in the protein. It has previously been demonstrated that thioltransferase has a catalytic effect on the reaction of dithiothreitol with proteins [10].

The results of the present study add to the examples of reactions with proteins that are catalyzed by cytosolic thioltransferase. It has previously been emphasized that the enzyme has a major role in reducing low-molecular-weight disulfides [12–14]. The chemical nature of the reaction with oxidized pyruvate kinase may be a reduction of a disulfide bond. However, any oxidation of a thiol group to the sulfenyl state ($R\text{-}S^+$), whether or not it is stabilized as a disulfide, sulfenic acid, or another derivative, would be expected to yield a substrate for thioltransferase provided that the modified group is sterically accessible [13,14].

The protective effect of glutathione and thioltransferase found with pyruvate kinase probably extends to other proteins as well. It represents a special case of the regulatory functions of thiols and disulfides discussed earlier [6,15]. Loss of the protective function by lowering the thioltransferase activity or the glutathione concentration may lead to pathological conditions in vivo. An extensively studied example is cataract formation in the lens of the eye [16], which is associated with the appearance of high concentrations of mixed disulfides of crystallins and glutathione [17]. We suggest that thioltransferase in combination with glutathione has a quite general biological role in catalyzing reactions involving protection of protein thiol groups.

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