Role of Cytoplasmic Thioltransferase in Cellular Regulation by Thiol–Disulphide Interchange

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Cytoplasmic thioltransferase purified from rat liver [Axelsson, Eriksson & Mannervik (1978) *Biochemistry* **17**, 2978–2984] catalyses the formation and decomposition of mixed disulphides of proteins and glutathione. The enzyme was found to catalyse the reversible thiol–disulphide interchange between glutathione disulphide and a crude thiol-containing protein fraction from rat liver. This finding indicates a role of the thioltransferase in the regulation of the 'glutathione status' of the cell. Specifically, it was found that thioltransferase catalyses the reactivation of pyruvate kinase from rat liver that had previously been inactivated by glutathione disulphide. It is suggested that thioltransferase may have a general role in regulatory processes involving thiol–disulphide interchange.

Regulation of cellular processes is dependent on enzymatic reactions governing the specific chemical changes which form the basis of the regulatory mechanisms. A major class of reactions involves reversible covalent modification of proteins such as the phosphorylation–dephosphorylation demonstrated in glycogen synthetase and phosphorylase, as well as in several additional systems (Krebs & Beavo, 1979). Little is known about corresponding regulatory mechanisms involving thiol–disulphide interchange but numerous examples show that the biological activity of a protein can be modulated by formation of a mixed disulphide of a protein thiol group and a low-molecular-weight thiol [see Kosower & Kosower (1976) and Table 1 for some examples]. The effect of thiol–disulphide interchange reactions may not be limited to an individual protein, but could as well extend to over-riding biochemical processes such as sporulation (in micro-organisms) and mitosis (Kosower & Kosower, 1978). This general function is coupled to the 'glutathione–glutathione disulphide status' of the cell (Kosower & Kosower, 1974), which in turn is governed by oxidative and reductive processes. Recent reports show diurnal variations in the amounts of reduced glutathione and mixed disulphides of proteins and glutathione in rat liver (Isaacs & Binkley, 1977a,b) and their possible regulation by cyclic nucleotides (Isaacs & Binkley, 1977b). The importance of these variations lies in the fact that a very substantial proportion of the cellular glutathione is bound to proteins (Modig, 1968; Harrap *et al.* 1973; Harisch & Schole, 1974; Isaacs & Binkley, 1977a,b). The amount of the mixed disulphides of proteins and glutathione in rat liver is about $10\,\mu$mol/g of protein, which is similar to the value for reduced glutathione (Isaacs & Binkley, 1977b).

Accordingly, the problem of cellular regulation by thiol–disulphide interchange reactions relates to a quite general process affecting the 'glutathione–glutathione disulphide status' as well as to modulation of the activities of individual proteins. The present paper addresses the question of whether the cytoplasmic thioltransferase (Axelsson *et al.*, 1978), which is known to catalyse thiol–disulphide interchange reactions between glutathione and protein mixed disulphides (Mannervik & Axelsson, 1975, 1978; Axelsson *et al.*, 1978), may be the catalyst required for the regulatory processes involving thiol–disulphide interchange. It is demonstrated that this enzyme may serve the suggested functions.

Experimental

Materials

Glutathione disulphide, NADH, and lactate dehydrogenase were obtained from Boehringer Mannheim, and reduced glutathione, dithioerythritol, ADP and phosphoenolpyruvate from Sigma. [*glycine*-2-^{3}H]Glutathione (250 Ci/mol), Protosol and Aquasol were purchased from New England Nuclear. Cytoplasmic thioltransferase from rat liver was purified and assayed as previously described (Axelsson *et al.*, 1978).

Preparation of cytosolic reduced-protein fraction (protein–SH)

A postmicrosomal supernatant fraction from rat liver (Axelsson *et al.*, 1978) was chromatographed on a column of Sephadex G-75 equilibrated with 10 mM-sodium phosphate (pH 6.1) in order to remove low-molecular-weight compounds and endogenous thioltransferase from the bulk of proteins (cf. Mannervik & Axelsson, 1975). The protein-containing fractions collected before the elution of thioltransferase were pooled. This crude protein fraction was adjusted to pH 8.2 with 0.1 M-NaOH and incubated with dithioerythritol (0.2 μmol/mg of protein) for 20 min to convert proteins into the 'reduced' thiol form. Dithioerythritol and its oxidized form were removed by gel filtration on Sephadex G-25. The protein fraction was concentrated by ultrafiltration with an Amicon PM 10 membrane to a concentration of 60 mg/ml and the stock solution was kept frozen (−20°C). The reduced protein contained 0.1 μmol of thiol groups/mg as measured with Ellman's reagent (Ellman, 1959).

Preparation of labelled mixed disulphides of glutathione and cytosolic proteins (protein–SS[³H]G)

Reduced protein (see above) was added dropwise to a solution of labelled glutathione disulphide at pH 8.5 and allowed to react for 2 h. The molar concentration of glutathione disulphide (2.5 mM) was in 4-fold excess over the thiol titre of the protein fraction. The excess of glutathione disulphide was removed by gel filtration on a Sephadex G-50 column, and the labelled protein was concentrated and stored frozen.

Assay procedure for the formation and decomposition of mixed disulphides of glutathione and proteins

The assay system for measuring formation of protein mixed disulphides (eqn. 1) contained: 0.31 mM-labelled glutathione disulphide (720 c.p.m./nmol), 11 mg of reduced cytosolic protein/ml, and 0.56 unit of purified thioltransferase/ml (Axelsson *et al.*, 1978) in 0.14 M-sodium phosphate buffer (pH 7.5) at 30°C. The volume of the reaction system was 0.4 ml. The reaction was started by addition of glutathione disulphide and stopped by addition of 0.6 ml of ice-cold 10% (w/v) trichloroacetic acid. After centrifugation for 1 min at $6000 \times g$ the supernatant fraction was mixed with Aquasol and the radioactivity was measured in a Beckman LS-100 liquid scintillation spectrometer. The pellet was dissolved in 0.8 ml of Protosol at 22°C overnight before addition of 10 ml of Aquasol and counting of radioactivity. The assay procedure for measuring decomposition of protein mixed disulphides (eqn. 2) was similar to that for formation of

mixed disulphides. The system (total volume 0.2 ml) contained: 0.5 mM-reduced glutathione, 1.0 mg of labelled protein mixed disulphides/ml and 1.2 units of purified thioltransferase/ml. The reaction was initiated by addition of reduced glutathione and stopped by addition of 0.8 ml of ice-cold 10% (w/v) trichloroacetic acid. In both types of assay the spontaneous reaction was measured by omission of thioltransferase from the assay mixture.

Assay of pyruvate kinase activity and preparation of disulphide-inhibited enzyme from rat liver

Pyruvate kinase activity was measured spectrophotometrically at 340 nm by coupling to lactate dehydrogenase (Bücher & Pfleiderer, 1955) in the following reaction system at 30°C: 2.7 ml of 50 mM-imidazole/HCl buffer (pH 7.6), 0.1 ml of 45 mM-ADP, 0.1 ml of 6.6 mM-NADH, 0.1 ml of 45 mM-phosphoenolpyruvate, and 10 μl (about 13 units) of lactate dehydrogenase. A postmicrosomal supernatant fraction from rat liver (see above) was chromatographed on Sephadex G-75 in 10 mM-sodium phosphate buffer (pH 6.1). A portion (200 μl) of the protein fraction, which contained pyruvate kinase, was mixed with an equal volume of 0.1 M-Tris/HCl buffer (pH 8.2) containing 10 mM-glutathione disulphide and was kept at 5°C overnight. The final protein concentration was 15 mg/ml. A control sample of pyruvate kinase was treated in the same way but in the absence of glutathione disulphide.

Reaction system for the reactivation of inhibited pyruvate kinase

The reactivation reaction was followed at 30°C in a system of 1 ml containing 5 mM-reduced glutathione in 70 mM-Tris/HCl buffer, pH 8.0. The thioltransferase-catalysed reactivation was usually measured in the presence of 0.15 unit of purified thioltransferase. Samples (200 μl) were taken at different times and assayed for pyruvate kinase activity. Proper controls (e.g. elimination of phosphoenolpyruvate) showed that the measured oxidation of NADH represented the pyruvate kinase activity in the sample.

Results

Thiol–disulphide interchange involving cytoplasmic proteins and glutathione

A crude cytosolic protein fraction from rat liver was reduced by dithioerythritol and freed from thioltransferase (see the Experimental section). This heterogeneous mixture of thiol-containing proteins (protein–SH) was found to react with labelled glutathione disulphide (GSS[³H]G):
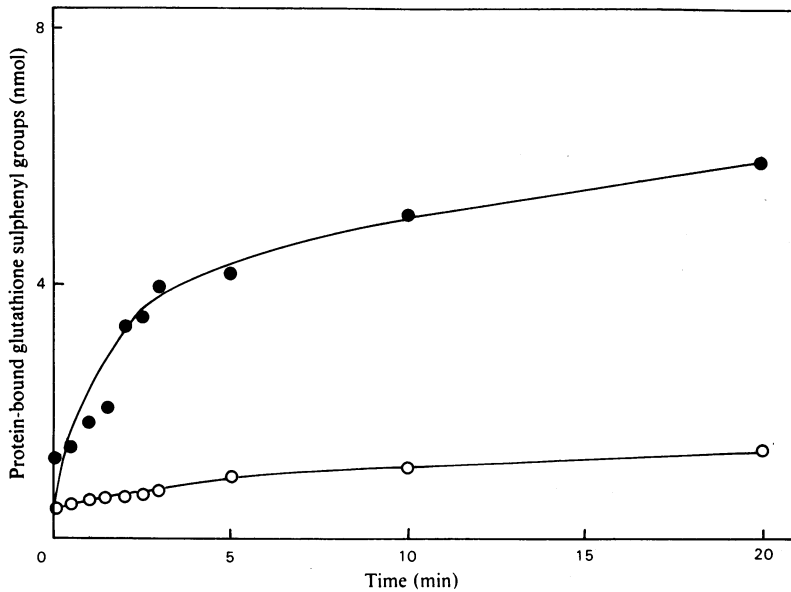
Protein–SH + GSS[³H]G → protein–SS[³H]G + GSH

(1)
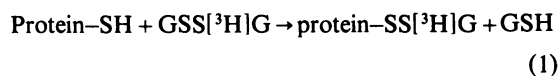
Fig. 1. *Progress curves for the formation of protein–glutathione mixed disulphides (protein–SS[³H]G) as monitored by incorporation of labelled glutathione sulphenyl groups (−S[³H]G)*

The protein was a reduced cytosolic fraction from rat liver (11 mg of protein/ml) which was allowed to react with 0.3 mM-labelled glutathione disulphide in the presence (●) or absence (○) of purified cytoplasmic thioltransferase from rat liver. For further details see the Experimental section.
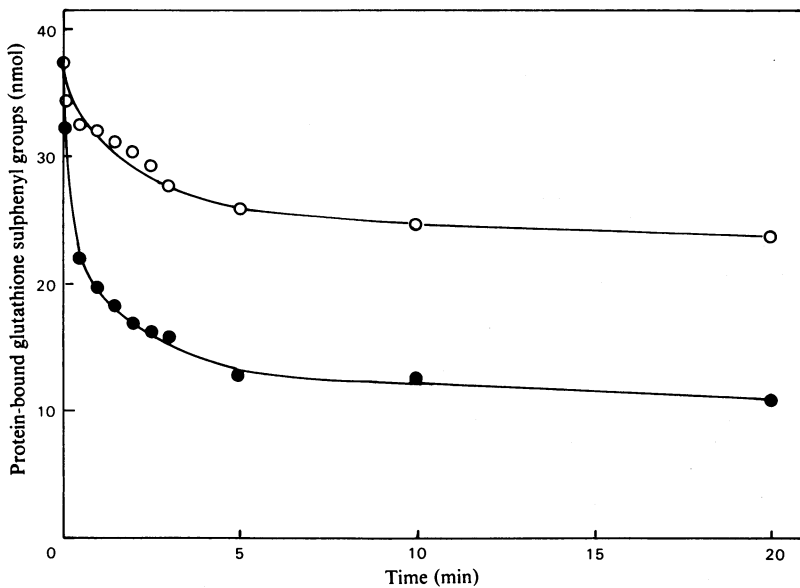

Fig. 2. *Progress curves for the decomposition of protein-glutathione mixed disulphides (protein–SS[³H]G) as monitored by protein-bound glutathione sulphenyl groups (−S[³H]G)*

The protein mixed disulphide was a cytosolic fraction from rat liver labelled with radioactive glutathione sulfenyl groups (0.18 μmol/mg of protein). The release of labelled glutathione was effected by 0.5 mM-reduced glutathione in the presence (●) or absence (○) of purified cytoplasmic thioltransferase from rat liver. For further details see the Experimental section.

Fig. 1 shows the progress curves for the incorporation of radioactivity into the trichloroacetic-acid-precipitable protein in the presence and absence of added purified thioltransferase. A corresponding progressive decrease in the concentration of glutathione was demonstrated in the non-precipitable fraction (data not shown). Fig. 2 demonstrates the reverse reaction in which the labelled mixed disulphides of glutathione and the protein fraction (protein–SS|^{3}H|G) react with reduced glutathione (GSH):

$$\text{Protein–SS}|^3\text{H}|\text{G} + \text{GSH} \rightarrow \text{protein–SH} + \text{GSS}|^3\text{H}|\text{G}$$
(2)
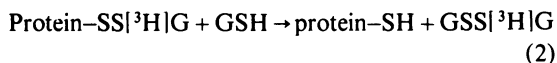
The loss of labelled glutathione from the protein mixed disulphide is shown in Fig. 2 and the concurrent stoichiometric increase of labelled glutathione disulphide was also demonstrated (data not shown). The rate of the reaction increased with the concentration of thioltransferase. It is evident that although the reactions take place spontaneously, a significant catalytic effect is obtained by addition of the purified thioltransferase. It should be emphasized that the estimated intracellular concentration of thioltransferase is approx. 10-fold higher than the concentration used in the assay system (approx. 1 unit/ml), which should result in a correspondingly more marked catalytic effect in the cell.

Activation of pyruvate kinase from rat liver by glutathione

It has been found that L-type pyruvate kinase from rat liver can be oxidized by glutathione disulphide and then can be reduced by glutathione (or mercaptoethanol) (van Berkel et al., 1973). The oxidized pyruvate kinase, which is probably a mixed disulphide, is strongly inhibited, and it was suggested that this modification of the enzyme serves a regulatory function in metabolism (van Berkel et al., 1973). Rat liver pyruvate kinase was inactivated by pre-incubation with glutathione disulphide and was then allowed to react with reduced glutathione in the presence and absence of purified thioltransferase (Fig. 3). The same experiment was made with untreated crude pyruvate kinase. A highly significant enzymatic effect was found with 0.15 unit of the thioltransferase/ml (more than 10-fold lower than the calculated intracellular concentration). With untreated pyruvate kinase no spontaneous reaction could be demonstrated, but a thioltransferase-catalysed increase of pyruvate kinase activity was clearly established (data not shown). The pretreated enzyme, which had about 40% of the activity of the untreated enzyme, was stimulated about 6-fold within 1 min in the presence of thioltransferase (Fig. 3). In the absence of reduced glutathione no reactivation took place. The rate of reactivation increased with the concentration of thioltransferase,
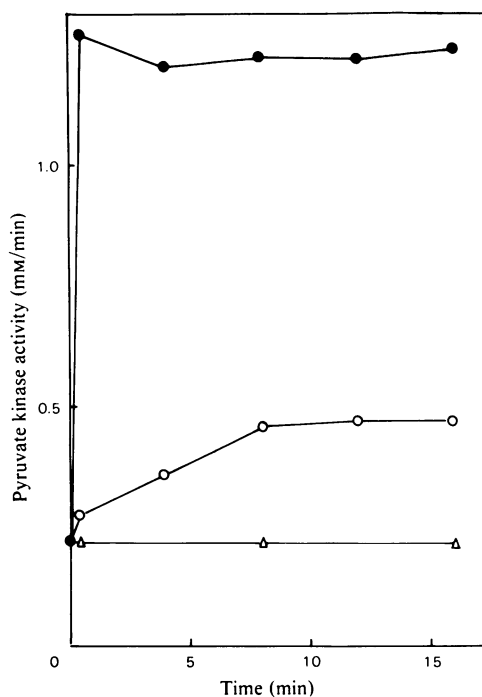

Fig. 3. *Activation of rat liver pyruvate kinase (L-type) with reduced glutathione*

A sample was inactivated by pretreatment (5°C, 18h) wtih glutathione disulphide before activation with 5 mM-reduced glutathione in the presence (●) and absence (O) of purified thioltransferase. A control containing thioltransferase but no glutathione showed no reactivation of inhibited pyruvate kinase (△). The pyruvate kinase activity, measured in aliquots from the reaction system, is expressed as change of substrate concentration in the assay system for pyruvate kinase. For further details see the Experimental section.

but the enzymatic effect was difficult to quantify because of the rapidity of the enzymic reaction. Half-maximal activation of pyruvate kinase was obtained in 30s with 0.03 unit of thioltransferase in the assay system.

Discussion

The data shown in Figs. 1 and 2 show clearly the catalytic effect of cytoplasmic thioltransferase on the formation and decomposition of mixed disulphides of glutathione and protein thiol groups according to eqns. (1) and (2). Previous results obtained with the mixed disulphide of egg-white lysozyme and glutathione have shown that thioltransferase could catalyse the scission of a specific protein mixed disulphide according to eqn. (2) (Mannervik & Axelsson, 1975). The significance of the new findings obtained with a crude protein fraction from liver

relates to the suggested rôles of the system of glutathione plus proteins containing thiol and disulphide groups as a redox buffer and a reservoir of glutathione (Axelsson & Mannervik, 1975) and to manifest the glutathione status of the cytosol (Kosower & Kosower, 1978). The diurnal variations in concentrations of protein mixed disulphides (Isaacs & Binkley, 1977a,b) have been observed in a protein fraction of rat liver, which corresponds closely to the material used in the present investigation, and the thioltransferase is evidently capable of catalysing such fluctuations.

The rôle of thioltransferase in catalysing the scission of disulphide bonds of specific proteins has previously been studied with a non-physiological model compound, the mixed disulphide of egg-white lysozyme and glutathione (Axelsson & Mannervik, 1975). However, the relevance of such a reaction for the regulation of the activity of a naturally occurring form of a protein has not been demonstrated before. The pyruvate kinase from rat liver used in the present investigation is inhibited by glutathione disulphide and reactivated by reduced glutathione (van Berkel et al., 1973). It is assumed that the modification of pyruvate kinase involves formation of a mixed disulphide with glutathione. Even if the oxidation of the enzyme takes place by a different mechanism (e.g. formation of an intracatenary disulphide bond, cf. Flasher et al., 1972), the importance of the present studies nevertheless lies in the finding that thioltransferase catalyses the regeneration of the active form. Studies on disulphide-containing proteins, such as serum albumin and ribonuclease, have shown that thioltransferase cata-lyses the reduction of protein disulphides provided that the disulphides are sterically available (Axelsson & Mannervik, 1980).

Numerous enzymes have been shown to respond to modification of thiol groups by low-molecular-weight disulphides. In most cases activity is lost or diminished, as expected from classical studies on blocking of the thiol groups of enzymes (Boyer, 1959). However, for some enzymes, e.g. fructose-1,6-bisphosphatase (Pontremoli et al., 1967; Nakashima & Horecker, 1970), the activity is increased. Consequently, metabolic networks could, in addition to other control parameters, be regulated by thiols and disulphides. Table 1 gives some examples of enzymes whose activities are modulated by reaction with naturally-occurring disulphides. It is proposed that for several of these enzymes regulation of activity is effected by thioltransferase.

Even if the extent of regulation by thiol–disulphide interchange and its molecular mechanisms have to be clarified by further studies, it appears as if thioltransferase could have a crucial role in the modification reactions. The enzyme is capable of promoting both formation and scission of protein mixed disulphides, and it will consequently be necessary to find out whether additional catalysts are involved or whether the availability of low-molecular-weight thiols and disulphides governs the direction of the modification reactions.

Table 1. *Some enzymes modified by naturally occurring disulphides*

Enzyme	Effect	Disulphide	Ref.
Fructose 1,6-bisphosphatase (rabbit liver)	Activation	Cystamine Homocystine	(Pontremoli et al. 1967) (Nakashima & Horecker, 1970)
Pyruvate kinase (rat liver)	Inhibition	Glutathione disulphide	(van Berkel et al., 1973)
Hexokinase (human erythrocytes)	Inhibition	Cystamine	(Eldjarn & Bremer, 1962)
Glycogen synthetase D (rat liver)	Inhibition	Glutathione disulphide	(Ernest & Kim, 1973)
Phosphorylase phosphatase (rabbit liver)	Inhibition	Glutathione disulphide	(Shimazu et al., 1978)
Adenylate cyclase (rat brain)	Inhibition	Glutathione disulphide	(Baba et al., 1978)
Acid phosphatase (spinach)	Activation	Glutathione disulphide	(Buchanan et al., 1979)
δ-Aminolaevulinate synthetase (Rhodopseudomonas spheroides)	Activation	Glutathione disulphide Homocystine	(Tuboi & Hayasaka, 1972)
Ribonucleotide reductase (Escherichia coli)	Inhibition	Glutathione disulphide	(Holmgren, 1979)
γ-Glutamylcysteine synthetase (rat kidney)	Inhibition	Cystamine	(Griffith et al., 1977)

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