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Reduction of Disulphide Bonds in Proteins and Protein Mixed Disulphides Catalysed by a Thioltransferase in Rat Liver Cytosol

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The reduction of mixed disulphides of some proteins and GSH [Protein(–SSG)$_n$] is accomplished with GSH as a reductant and a thioltransferase from rat liver as a catalyst, thus:

$$\text{Protein(–SSG)}_n + \text{GSH} \rightleftharpoons \text{Protein}\begin{matrix}\text{(–SSG)}_{n-1} \\ \diagdown \\ \text{SH}\end{matrix} + \text{GSSG}$$

The spontaneous reaction is negligible in comparison with the enzymic reaction *in vivo*, and any direct reduction with glutathione reductase is not detectable with the substrates used. The reduction is only indirectly dependent on NADPH, which is required for the regeneration of GSH from GSSG. Other protein disulphides apparently are reduced via analogous GSH-dependent reactions.

Several mixed disulphides of GSH and proteins have been identified in biological tissues (Modig, 1968; Modig *et al.*, 1971; Harding, 1970; Harrap *et al.*, 1973). The function of formation of such mixed disulphides may be the modulation of the biological activity of specific proteins. They may also serve as a storage form of GSH, which in combination with mobile GSH has the capacity to act as a redox buffer in the cell. Independent of any specific functions of mixed disulphides of GSH and proteins, it is clear that such protein derivatives may arise *in vivo* under oxidizing conditions. It is consequently important to establish the biochemical processes which liberate GSH from these derivatives.

Materials and methods

The synthesis of a mixed disulphide of egg-white lysozyme and GSH was described in a recent paper from this laboratory (Axelsson & Mannervik, 1975). *S*-Sulphocysteine [Cys(SO_3H)] was synthesized as described by Segel & Johnson (1963). Other biochemicals were from Boehringer, Mannheim, Germany, or Sigma Chemical Co., St. Louis, Mo., U.S.A. Assays of the activities of glutathione reductase and thioltransferase were carried out as previously described (Eriksson *et al.*, 1974*a*). The thioltransferase activity (for nomenclature, see Askelöf *et al.*, 1974) was determined with GSH and either Cys(SO_3H) (0.5mM) or the mixed disulphide of lysozyme and GSH (5μM) as substrates; the production of GSSG was followed by the glutathione reductase-catalysed oxidation of NADPH.

Results

Postmicrosomal rat liver supernatants have been found to catalyse the reaction between GSH and low-molecular-weight mixed disulphides or thiosulphate esters (Eriksson & Mannervik, 1970; Mannervik & Eriksson, 1974; Mannervik *et al.*, 1974). The mixed disulphide of lysozyme and GSH was found to serve as a disulphide substrate in the system previously used (see Eriksson *et al.*, 1974*a*), which is based on the oxidation of NADPH by GSSG in the presence of added glutathione reductase. The catalytic effect of the supernatant was abolished by boiling, in agreement with the assumption that it was enzymic. The reaction between GSH and the mixed disulphide of lysozyme and GSH also took place spontaneously, but the rate of the spontaneous reaction was only about 10% of the velocity obtained with an amount of enzyme (5μg) giving an activity of 4nmol of GSSG formed/min (0.5mM and 2.5μM concentrations of GSH and the lysozyme derivative respectively).

The heat-lability of the catalytic activity of the supernatant could be explained as an inactivation of a thioltransferase, which might be similar to or identical with the enzyme active with low-molecular-weight disulphides and GSH. Another possibility might be the destruction of endogenous glutathione reductase, which reduces the lysozyme derivative directly in the presence of NADPH. To differentiate between alternative explanations of the catalytic effect of rat liver supernatant it was therefore desirable to separate the enzymes which might be involved in the reduction of the mixed disulphide of lysozyme and GSH.

Previous work in this laboratory has outlined a procedure for the purification of glutathione reductase from rat liver to near homogeneity (Carlberg & Mannervik, 1975). This procedure was followed in an attempt to separate glutathione reductase from

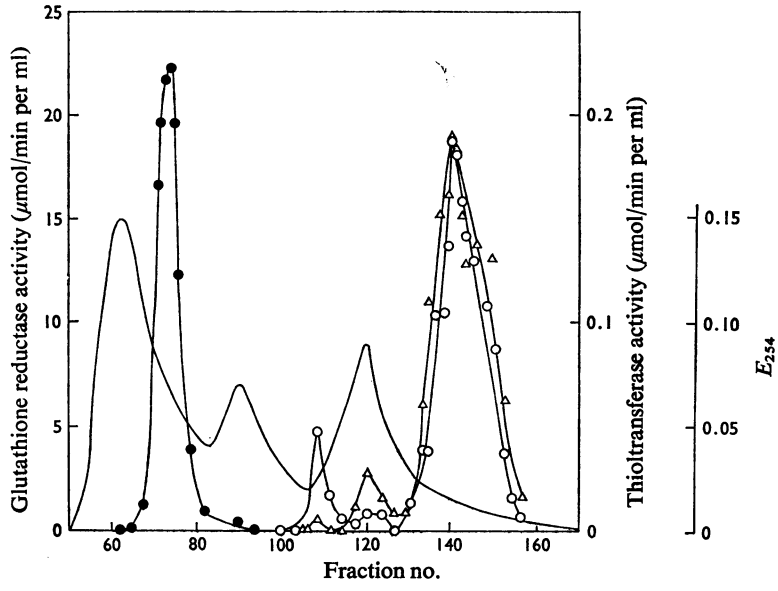

Fig. 1. *Separation of glutathione reductase and thioltransferase*

A sample (25ml) containing 1.5g of protein, obtained after chromatography on CM-cellulose of a rat liver supernatant, was applied to a Sephadex G-75 column (4cm×110cm) and eluted with 10mM-sodium phosphate (pH6.7); fractions (about 10ml) were collected. Symbols for activities: glutathione reductase (●); thioltransferase assayed with $Cys(SO_3H)$ (○) and with the mixed disulphide of lysozyme and GSH (△). ——, E_{254}. V_0 and V_0+V_1 correspond to fractions 61 and 120 respectively.

other proteins which might be involved in the reduction. The purification involved chromatography of a rat liver supernatant on Sephadex G-25, CM-cellulose and Sephadex G-75 columns as the initial purification steps (Carlberg & Mannervik, 1975). When the activity profiles of the reduction of the lysozyme derivative or $Cys(SO_3H)$ were determined they were found to overlap the peak of glutathione reductase activity in the effluents of the Sephadex G-25 and CM-cellulose columns. However, after the Sephadex G-75 step, glutathione reductase was completely separated from the thioltransferase activity (Fig. 1). This result shows that glutathione reductase in rat liver cannot directly reduce the lysozyme derivative [or $Cys(SO_3H)$]. This statement is supported by previous experiments with highly purified glutathione reductase from rat liver and from yeast (Axelsson & Mannervik, 1975). A still more important finding was that the thioltransferase activity obtained with the mixed disulphide of lysozyme and GSH coincided with the enzymic activity previously found with low-molecular-weight disulphides and thiosulphate esters [including $Cys(SO_3H)$ (Eriksson & Mannervik, 1970; Mannervik & Eriksson, 1974; Mannervik *et al.*, 1974)]. The thioltransferase appears in the effluent at an elution volume which is larger than that expected for proteins. Consequently, it is separated from the bulk of the proteins in the sample. The enzymic nature of the activity was established by heat-inactivation and staining for protein. The unusual elution characteristics of the activities with $Cys(SO_3H)$ and the lysozyme derivative and the high degree of purification of the protein indicate strongly that the two reactions studied can be ascribed to the same enzyme.

Some naturally occurring disulphides were tested as substrates for the purified thioltransferase (a pool of fractions 132–145 depicted in Fig. 1) to obtain some preliminary information on the specificity of the enzyme. Cystine gave about the same activity as $Cys(SO_3H)$, whereas lipoate was inactive (Table 1). Lysozyme and α-chymotrypsin gave no significant activity under the conditions tested, whereas trypsin was clearly a substrate. Evidently, the activities obtained with the proteins, trypsin and the lysozyme derivative, and with cysteine and $Cys(SO_3H)$, were of the same magnitude. Similar experiments performed with enzyme from another batch and under essentially the same conditions showed that the mixed disulphides of lysozyme and cysteine (Bradshaw *et al.*, 1967) and of trypsin and GSH were also substrates, giving activities similar to those of the above-mentioned compounds. None of the disulphides tested have been found to give a measurable activity with purified glutathione reductase.

Table 1. *Substrate specificity of the purified thioltransferase*

The thioltransferase activities were measured in the standard assay system with 0.5mM-GSH and 5 μg of enzyme.

Substrates	No. of S–S bonds per molecule	Molar concn. (μM)	Concn. of S–S bonds (μM)	Spontaneous reaction (nmol/min)	Thioltransferase activity (nmol/min)
Proteins					
Mixed disulphide of lysozyme and GSH	8	12	96	1.2	2.8
Lysozyme	4	69	276	0.2	0*
α-Chymotrypsin	5	41	205	0.2	0.1
Trypsin	6	42	252	0.5	1.6
Low-molecular-weight compounds					
Cystine	1	500	500	3.8	4.6
Cys(SO_3H)	1	500	500	3.4	4.7
Lipoate	1	500	500	0.2	0*

* Detection limit about 0.1 nmol/min.

Discussion

Several investigators have previously studied the enzymic reduction of mixed disulphides of proteins and GSH. A direct reduction involving NADPH and glutathione reductase has been proposed for the GSH derivatives of haemoglobin (Srivastava & Beutler, 1970), albumin (Harrap *et al.*, 1973) and lens crystallins (Srivastava & Beutler, 1973). An alternative reduction mechanism in which GSH is the donor of reducing equivalents has also been advocated for the haemoglobin mixed disulphide (Birchmeier *et al.*, 1973). The present investigation shows that only the latter mechanism is operative in the reduction of the proteins and protein mixed disulphides studied. Further, the reaction has been found to be catalysed by a thioltransferase from rat liver cytosol previously found to catalyse thiol–disulphide interchange of small molecules (Eriksson & Mannervik, 1970; Mannervik & Eriksson, 1974; Mannervik *et al.*, 1974). It can be calculated, as with the mixed disulphide of CoA and GSH (Eriksson *et al.*, 1974*b*), that the thioltransferase-catalysed reaction is at least 100 times as rapid as the spontaneous reaction when the enzyme concentration is adjusted to the conditions *in vivo*. The results obtained with the mixed disulphide of lysozyme and cysteine and with trypsin show that the enzyme is active also with protein disulphides which do not have GSH as a constituent.

We therefore conclude that the reduction of mixed disulphides of some proteins and GSH [Protein(–SSG)$_n$] is accomplished with GSH as a reductant and a thioltransferase from rat liver as a catalyst, thus:

$$\text{Protein(–SSG)}_n + \text{GSH} \rightleftharpoons \underset{\diagdown\,\text{SH}}{\text{Protein}}\text{(–SSG)}_{n-1} + \text{GSSG}$$

The spontaneous reaction is negligible in comparison with the enzymic reaction *in vivo*, and any direct reduction with glutathione reductase is not detectable with the substrates used. The reduction is only indirectly dependent on NADPH, which is required for the regeneration of GSH from GSSG. Other protein disulphides are apparently reduced via analogous GSH-dependent reactions.

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