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Relative contributions of thioltransferase- and thioredoxin-dependent systems in reduction of low-molecular-mass and protein disulphides

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Two enzyme systems capable of reducing disulphide bonds both in low-M_r compounds and in polypeptides and proteins exist. One consists of thioltransferase in combination with reduced glutathione and glutathione reductase, and the second consists of thioredoxin in combination with thioredoxin reductase. Their relative effectiveness in catalysing disulphide reduction of various substrates in rat liver cytosol was evaluated in the present study. The thioltransferase-dependent system was found to be more efficient in reducing small molecules. Insulin was most effectively reduced by the thioredoxin system. Bovine trypsin was a better substrate for thioltransferase, and partially proteolysed bovine serum albumin was equally good for the two systems. Thus, in the case of protein disulphide bonds, the nature of the particular substrate used determines which of the two reducing systems is the more important.

Two well-characterized disulphide-reducing systems exist in the cytosol fraction of cells of various types. One makes use of GSH as a reductant and effects disulphide reduction by way of thiol–disulphide interchange catalysed by thioltransferase (Mannervik & Eriksson, 1974; Mannervik & Axelsson, 1978; Mannervik, 1980). GSH is regenerated by the action of glutathione reductase and NADPH. The second system involves thioredoxin, which in its reduced form may react with disulphide bonds without any auxiliary protein. Reduced thioredoxin is produced by thioredoxin reductase and NADPH (Holmgren, 1977, 1979). However, the importance of the two disulphide-reducing systems in relation to each other has not previously been studied. The present investigation was undertaken to clarify this relationship for both low-M_r and protein disulphide substrates.

Materials

Enzymes and chemicals

Thioltransferase (Axelsson *et al.*, 1978; Mannervik *et al.*, 1981) and glutathione reductase (Carlberg & Mannervik, 1975) from rat liver cytosol were prepared as previously described. Thioredoxin and thioredoxin reductase from rat liver were purified by using published procedures (Engström *et al.*, 1974; Holmgren, 1977). Antibodies against rat liver thioredoxin (cf. Holmgren & Luthman, 1978) and glutathione reductase (Carlberg *et al.*, 1981) were also available. The immunoglobulin G fraction of the antisera was used. Cystine, cystamine and homocystine were from Sigma Chemical Co. *S*-Sulphocysteine and *S*-sulphoglutathione were synthesized by previously described procedures (Segel & Johnson, 1963; Eriksson & Rundfelt, 1968), which was also the case with the mixed disulphide of L-cysteine and GSH (Eriksson & Eriksson, 1967). All the substrates synthesized were homogeneous when analysed by electrophoresis. Trypsin was from Boehringer Mannheim (Stockholm, Sweden); insulin (pig) was purchased from KABI–Vitrum (Stockholm, Sweden). Partially proteolysed bovine serum albumin was produced by the method described previously (Axelsson & Mannervik, 1980). NADPH and GSH were obtained from Sigma Chemical Co.

Preparation of rat liver cytosol fraction

Livers from male specific-pathogen-free Sprague–Dawley rats (150–200g) were homogenized in ice-cold 0.25M-sucrose with a blender. The preparation was subsequently maintained at 4°C. The homogenate was diluted with the sucrose solution to a final concentration of 20% (w/v), and centrifuged at 19200*g* for 45min. The microsomal fragments in the resulting supernatant fraction were precipitated by lowering the pH to 5.5 with ice-cold 0.2M-acetic acid and removed by centrifugation at 19200*g* for

Abbreviation used: GSH, reduced glutathione.

60min. The cytosol fraction was finally adjusted to pH7.5 with ice-cold 0.1M-NaOH, and any floating lipid material was eliminated by filtration through gauze.

The cytosol fraction did not exhibit any detectable NADPH oxidation in the assay systems for thioredoxin reductase or thioltransferase when a disulphide substrate was lacking. Thus no extraneous NADPH oxidase or GSH oxidase activity contributed to the NADPH consumption measured.

Methods

Antibody inhibition studies

A 20% (w/v) cytosol fraction from rat liver was incubated with either anti-thioredoxin antibodies or anti-(glutathione reductase) antibodies. Antibodies were added to the cytosol fraction until maximal inhibition was obtained. Less than 4% of glutathione reductase activity remained after addition of 0.1vol. of the immunoglobulin G fraction of anti-(glutathione reductase) serum to 1vol. of the cytosol fraction. No detectable activity of thioredoxin remained after addition of 0.5vol. of the corresponding immunoglobulin G fraction. The incubation time to reach this inhibition was 30min. A 200μl portion of the treated cytosol fraction was then added to an assay system of 1ml volume containing (final concentrations): 0.5mM-GSH, 0.1mM-NADPH, 1mM-EDTA and 0.13M-sodium phosphate buffer, pH7.5. Finally, different low-M_r disulphides and thiosulphate esters were added to a concentration of 3mM in the assay system. For insulin and partially digested bovine serum albumin the final concentration was 0.5mg/ml, and for trypsin the concentration was 5mg/ml. The disulphide reduction was measured spectrophotometrically at 340nm by monitoring the oxidation of NADPH. The anti-(glutathione reductase) antibodies did not inhibit either the thioredoxin system or the thioltransferase. The anti-thioredoxin antibodies did not inhibit thioltransferase, but had a limited inhibitory effect (<30% inhibition) on glutathione reductase. This lack of complete specificity does not affect the results qualitatively, but may lead to a slight over-estimation of the contribution of the thioredoxin system to some of the disulphide-reducing activities of the cytosol.

Reconstitution studies

In these experiments on the disulphide-reducing systems, pure proteins were added in ratios proportional to their estimated average cellular concentrations. The percentage of the physiological concentrations varied from 2% to 50% for the different proteins. The estimated physiological concentrations of the different proteins were: thioredoxin, 10μM; thioredoxin reductase, 2μM (cf. Luthman & Holmgren, 1982); thioltransferase, 0.4unit/ml (in the present study determined with cystamine as disulphide substrate; Axelsson *et al.*, 1978); glutathione reductase, 6units/ml (cf. Carlberg *et al.*, 1981). Cystamine (3mM) and insulin (0.5mg/ml) were assayed as disulphide substrates in the reconstituted system in 0.1M-sodium phosphate buffer, pH7.5, containing 1mM-EDTA, 0.1mM-NADPH and 0.5mM-GSH (when thioltransferase was present). The disulphide reduction was monitored spectrophotometrically at 340nm.

Results

Inhibition of disulphide-reducing systems with antibodies

The two disulphide-reducing systems are referred to below as the 'thioltransferase' system and the 'thioredoxin' system. Inhibitory antibodies for both systems were available in the form of anti-(glutathione reductase) antibodies (Carlberg *et al.*, 1981) and anti-thioredoxin antibodies (Holmgren & Luthman, 1978). The antibodies were used in a cytosol fraction of rat liver containing both the thioltransferase system and the thioredoxin system. Both purified thioredoxin and GSH were added to compensate for the dilution in the assay. In addition, NADPH was added to 0.1mM concentration. The antibody-treated test systems were preincubated for 30min before assay of disulphide reduction, measured spectrophotometrically at 340nm as oxidation of NADPH, and were compared with an untreated control. The activity remaining after treatment with antibodies to one of the disulphide-reducing systems was expected to be due to the other system, and vice versa. Table 1 shows the results of a series of such experiments. For the low-M_r disulphides cystine, cystamine, homocystine and the mixed disulphide of cysteine and GSH, as well as for the thiosulphate esters *S*-sulphocysteine and *S*-sulphoglutathione, the activity remaining after treatment with anti-thioredoxin antibodies ranged between 67 and 83% of the control activity. The complementary experiments with anti-(glutathione reductase) antibodies gave less than 20% activity remaining. Thus both series of measurements suggest that these substrates are primarily reduced by the thioltransferase system.

Insulin reduction was less strongly affected by anti-(glutathione reductase) antibodies than by anti-thioredoxin antibodies: 70% and 31% remaining activity respectively were obtained in the two cases. Thus the thioredoxin system appears to be more important with this substrate. On the other hand, trypsin appeared to be predominantly reduced by the thioltransferase system: no activity remained after treatment with anti-(glutathione reductase) antibodies (Table 1). Native bovine serum albumin is

Table 1. *Relative contributions of the thioltransferase- and thioredoxin-dependent reducing activities in rat liver cytosol*

For experimental details see the text. The values are the differences between control (100%) and remaining activity after treatment with antibodies to glutathione reductase (thioltransferase system) or antibodies to thioredoxin (thioredoxin system). The sum of the percentages for a given substrate will exceed 100% if the two systems compete under less-than-first-order conditions. Abbreviations: N.D., not determined.

	Contribution to reducing activity (%)	
Substrate	Thioltransferase system	Thioredoxin system
Cystamine	83	26
Mixed disulphide of cysteine and GSH	94	17
Cystine	N.D.	18
Homocystine	N.D.	19
S-Sulphocysteine	81	11
S-Sulphoglutathione	92	33
Insulin	31	70
Trypsin	100	14
Partially proteolysed serum albumin	29	19

not reduced by any of the two enzyme systems, but albumin pretreated with proteinases, to effect limited proteolysis, has scissile disulphide bonds (Axelsson & Mannervik, 1980). The activity with digested albumin remained at 70–80% of the untreated control for samples treated with either of the two antibody preparations. This finding suggests that both systems are about equally important and that one can 'take over' when the other is eliminated. It is also possible that the two systems react with different disulphide-containing fragments of the proteolysed albumin. Alternatively, a third, as yet unidentified, disulphide-reducing system might be present in the cytosol fraction. Support for this interpretation was obtained from the finding that simultaneous use of antibodies against glutathione reductase and thioredoxin did not eliminate more than about 80–90% of the disulphide-reducing activity with insulin, trypsin or cystamine as substrate. Another possibility would be that thioredoxin reductase could reduce disulphides directly without the aid of thioredoxin or with thioltransferase as a cofactor, but this explanation does not appear to be plausible in view of the high substrate-specificity of the enzyme.

Mammalian thioredoxin contains thiol groups that are prone to oxidation, which leads to inactivation of catalytic activity. Such oxidation can be reversed by treatment with 2mM-dithiothreitol (Luthman & Holmgren, 1982). In order to exclude the possibility

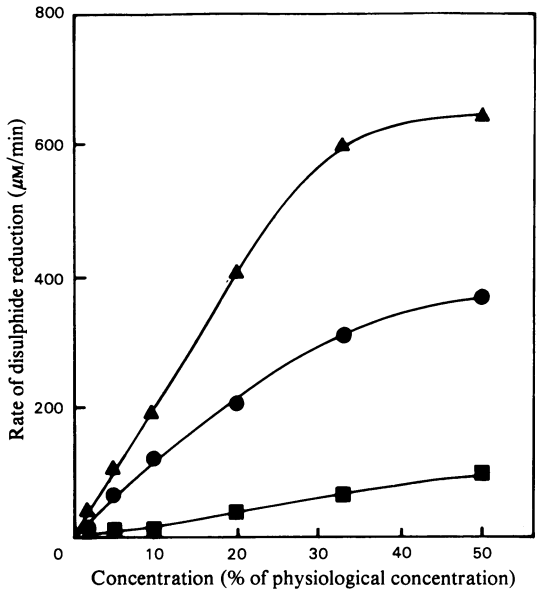

Fig. 1. *Reduction of cystamine by the thioltransferase system and the thioredoxin system*

The thioltransferase (●) and thioredoxin (■) systems were assayed separately or in combination (▲). The concentrations of the protein components of the systems were varied in ratios corresponding to the estimated mean cellular concentrations. For further details see the Methods section. The activities are expressed per ml of incubation mixture, and the concentrations of the proteins of the disulphide-reducing systems as percentage of the cellular concentrations.

that oxidative inactivation of the thioredoxin system would favour the relative contribution of the thioltransferase system, the cytosol fraction was pretreated with dithiothreitol. The percentage of activity remaining after inhibition with the two kinds of antibodies (cf. Table 1) did not change significantly on pretreatment with dithiothreitol. Thus the results in Table 1 do not appear to be biased as a result of inactivation of the thioredoxin system.

Reconstitution of disulphide-reducing systems from purified components

The crude cytosol fraction from rat liver might contain components in addition to the thioltransferase and thioredoxin systems that could affect disulphide reduction. Therefore reconstitution of the disulphide-reducing systems from purified components was made. The average cellular concentrations of the thioltransferase, glutathione reductase, thioredoxin and thioredoxin reductase were calculated, and the proteins were added to the assay system in proportion to their occurrence in the cell. Thus

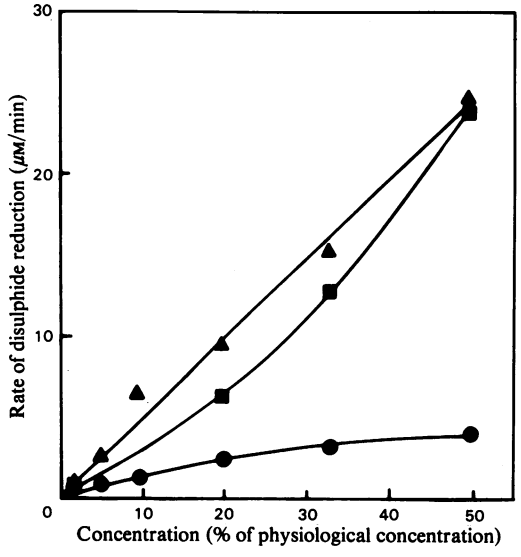

Fig. 2. *Reduction of insulin by the thioltransferase system* (●) *and the thioredoxin system* (■) *or the combination of the two systems* (▲)
For further details see the Methods section and the legend to Fig. 1.

protein concentrations ranging between 2 and 50% of the average values for both disulphide-reducing systems were tested with cystamine (Fig. 1), as a representative of low-M_r disulphides, and with insulin (Fig. 2), representing polypeptide substrates. Different protein concentrations were used to make possible extrapolation to the assumed concentrations *in vivo*.

In the experiments with cystamine as disulphide substrate it was found that the thioltransferase system was 3.5–10 times as active as the thioredoxin system in the protein concentration range studied (Fig. 1). This ratio is higher than that obtained in the inhibition experiments with antibodies (Table 1). The difference might be explained by the lack of GSH, which may protect the thioredoxin system, when the reconstituted thioredoxin system was tested by itself. This explanation of partial inactivation of the thioredoxin system in the absence of GSH is supported by the finding that the activity of the two systems combined was higher than the sum of the two separate activities (Fig. 1).

Insulin was reduced more efficiently by the thioredoxin system than by the thioltransferase system (Fig. 2). This finding is in agreement with the results of the inhibition experiments (Table 1). With this substrate the activities of the two systems were less than additive, suggesting approach to zero-order conditions with respect to insulin concentration.

Discussion

Both the thioltransferase system, consisting of cytosolic thioltransferase, GSH and glutathione reductase, and the thioredoxin system, consisting of thioredoxin and thioredoxin reductase, are capable of reducing low-M_r and protein disulphides at the expense of reducing equivalents from NADPH (Holmgren, 1979; Mannervik, 1980). The results of the present investigation show that the low-M_r substrates (including thiosulphate esters in addition to disulphides) are preferentially reduced by the thioltransferase system. With proteins or polypeptides, the relative importance of the systems depends on the nature of the disulphide substrate. For insulin reduction the thioredoxin system has the highest activity; for trypsin reduction the thioltransferase is more active. With proteolysed serum albumin both systems appears to be about equally efficient.

The data on the relative contributions of the two disulphide-reducing systems should be taken only as crude quantitative approximations of the conditions *in vivo*, because the conditions in the cell could not be reproduced exactly. The results nevertheless show clearly in a qualitative manner the relative importance of the two systems. It should also be noted that the systems act complementarily and that in most cases one can substitute for the other in disulphide reduction, if necessary.

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References

Axelsson, K. & Mannervik, B. (1980) *Acta Chem. Scand. Ser. B* **34**, 139–140
Axelsson, K., Eriksson, S. & Mannervik, B. (1978) *Biochemistry* **17**, 2978–2984
Carlberg, I. & Mannervik, B. (1975) *J. Biol. Chem.* **250**, 5475–5480
Carlberg, I., Altmejd, B. & Mannervik, B. (1981) *Biochim. Biophys. Acta* **677**, 146–152
Engström, N. E., Holmgren, A., Larsson, A. & Söderhäll, S. (1974) *J. Biol. Chem.* **249**, 205–210
Eriksson, B. & Eriksson, S. A. (1967) *Acta Chem. Scand.* **21**, 1304–1312
Eriksson, B. & Rundfelt, M. (1968) *Acta Chem. Scand.* **22**, 562–570
Holmgren, A. (1977) *J. Biol. Chem.* **252**, 4600–4606
Holmgren, A. (1979) *J. Biol. Chem.* **254**, 9113–9119
Holmgren, A. & Luthman, M. (1978) *Biochemistry* **17**, 4071–4077

Luthman, M. & Holmgren, A. (1982) *Biochemistry* **21**, 6628–6633

Mannervik, B. (1980) in *Enzymatic Basis of Detoxication* (Jakoby, W. B., ed.), vol. 2, pp. 229–244, Academic Press, New York

Mannervik, B. & Axelsson, K. (1978) in *Functions of Glutathione in Liver and Kidney* (Sies, H. & Wendel, A., eds.), pp. 148–153, Springer-Verlag, Berlin

Mannervik, B. & Eriksson, S. A. (1974) in *Glutathione* (Flohé, L., Benöhr, H. C., Sies, H., Waller, H. D. & Wendel, A., eds.), pp. 120–131, Georg Thieme, Stuttgart

Mannervik, B., Axelsson, K. & Larson, K. (1981) *Methods Enzymol.* **77**, 281–285

Segel, I. H. & Johnson, M. J. (1963) *Anal. Biochem.* **5**, 330–337