Thioltransferase activity of bovine lens glutathione S-transferase

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A Mu-class glutathione S-transferase purified to electrophoretic homogeneity from bovine lens displayed thioltransferase activity, catalysing the transthiolation reaction between GSH and hydroxyethyl disulphide. The thiol-transfer reaction is composed of two steps, the formation of GSSG occurring through the generation of an intermediate mixed disulphide between GSH and the target disulphide. Unlike glutaredoxin, which is only able to

catalyse the second step of the transthiolation process, glutathione S-transferase catalyses both steps of the reaction. Data are presented showing that bovine lens glutathione S-transferase and rat liver glutaredoxin, which was used as a thioltransferase enzyme model, can operate in synergy to catalyse the GSH-dependent reduction of hydroxyethyl disulphide.

INTRODUCTION

S-Thiolation of proteins is one of the most relevant reactions occurring in cells undergoing oxidative insult and the lens is a tissue in which this process occurs to a significant extent [1–3]. Both glutathione and cysteine residues have been shown to be linked to lens proteins through mixed disulphide bonds, either as a consequence of oxidative insult or as a result of aging [4]. Lens proteins have been shown to undergo a massive thiolation process *in vitro* in isolated cultured lens subjected to either hyperbaric oxygen or H₂O₂ treatment [5–7]. The oxidation of lens protein -SH has also been demonstrated *in vivo* in cataractous lenses and has been observed to increase with age in the normal human lens nucleus [4,8,9]. Enzymes have been shown to be subjected to S-thiolation [10–14] and the possibility that their concerted modification could operate as a metabolic process to counteract oxidative stress has been considered [15]. Indeed, whether protein thiolation is the unavoidable consequence of disulphide accumulation or is a programmed plan of cell defence is still an open issue. Besides its potential ability to act as a metabolic antioxidant system, protein thiolation has been proposed as an intracellular redox buffer which can partially reduce disulphides generated by the antioxidant-scavenging actions of thiols [15]. Moreover the thiolated proteins and eventually those products deriving from their inter- or intra-molecular transthiolation rearrangement can often be recovered in their native form when reducing conditions are restored. This feature offers a unique advantage to the cell over the irreversible modification that occurs in the side chain of amino acids of proteins subjected to direct action of radical oxygen species [16–21].

Despite the fact that transthiolation reactions can in many cases rapidly proceed without enzyme assistance, cells possess enzymes that can catalyse the reduction of protein disulphide or protein mixed disulphide bonds. Whereas protein disulphide isomerase (EC 5.3.4.1) is generally considered to act as a chaperone-like protein, inducing cysteine residues to form proper disulphide bonds [22], glutaredoxin (Grx) (also named thioltransferase) is considered to be the catalyst of reducing processes since it allows the recovery of reduced thiol residues from disulphide bonds [23–26]. The transthiolation reaction consists of two well-defined steps. The first step determines the partial reduction of the disulphide to form a mixed disulphide with

GSH. In the second step the intermediate mixed disulphide is further reduced by GSH, and glutathione disulphide is produced:

Although Grx mRNA has been isolated from lens tissue [27], the enzyme is not very abundant [27–30], and attempts at purification have ended with an apparently non-homogeneous protein preparation [27]. Nevertheless, thioltransferase activity is readily detected in lens extracts. In this paper we show that most of the thioltransferase activity detectable in the lens is not that of Grx but is the result of a side activity of the abundant glutathione S-transferase (GST) (EC 2.5.1.18) [31,32]. Data are provided to substantiate our hypothesis that lens GST and Grx may act together in the GSH-dependent reduction of disulphide bonds.

EXPERIMENTAL

Materials

Sephadex G-75, Sephadex G-100 SF, Sephadex G-50, Mono P HR 5/20, Superdex HR 10/30, Pharmalyte 8.0–10.5, Polybuffer 96, Ampholine PAG plates and isoelectric focusing calibration kit were purchased from Pharmacia Biotech (Uppsala, Sweden). Penthyl agarose, GSH, 1-chloro-2,4-dinitrobenzene (CDNB), glutathione reductase (EC 1.6.4.2, 190 units/mg), bovine liver GST (10 units/mg), NADPH, dithiothreitol (DTT), hydroxyethyl disulphide (HEDS), Coomassie Blue R250, gel-filtration molecular-mass markers and amino acid sequence reagents were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). DE-52, CM-52 and GF/F discs were obtained from Whatman (Maidstone, Kent, U.K.). Biophoresis horizontal electrophoresis cell, Bio-Gel HTP, acrylamide, *N,N'*-bisacrylamide, *N,N,N',N'*-tetramethylethylenediamine and SDS/PAGE molecular-mass markers were purchased from Bio-Rad (Hercules, CA, U.S.A.). Dialysis membranes and Centriprep 10 were from Amicon (Beverly, MA, U.S.A.). Dialysis tubes were from Medicell International Ltd. (London, U.K.). Cellulose nitrate filters were obtained from Sartorius (Gottingen, Germany). All inorganic chemicals were of reagent grade from BDH (Poole, Dorset, U.K.). Calf eyes were obtained from a local slaughterhouse soon after slaughter; the lenses were removed and frozen at –20 °C until needed.

Abbreviations used: ROS, radical oxygen species; Grx, glutaredoxin; GST, glutathione S-transferase; DTT, dL-dithiothreitol; CDNB, 1-chloro-2,4-dinitrobenzene; HEDS, hydroxyethyl disulphide; CE, capillary electrophoresis; 2-ME, 2-mercaptoethanol; 2-ME-SG, S-glutathionyl-2-mercaptoethanol.

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Fused silica capillaries for capillary electrophoresis (50 cm $\times$ 50 μ m internal diameter, 360 μ m optical path length) were supplied by Polymicro Technologies (Phoenix, AZ, U.S.A.) and assembled in the Beckman cartridge format.

Purification of GST as a thioltransferase enzyme

After the incision of the capsule, 300 frozen bovine lenses (approx. 600 g) were suspended in 1.5 litres of 10 mM sodium phosphate/HCl, pH 7.0 (buffer A), containing 5 mM DTT and stirred in an ice-cold bath for 1 h. The suspension was centrifuged at 22000 *g* at 4 °C for 40 min and the supernatant, referred to as crude extract, was applied to a DE-52 column (3.5 cm $\times$ 30 cm) pre-equilibrated with buffer A supplemented with 2 mM DTT. The column was washed with 1.5 litres of the same buffer. The enzyme was not retained by the resin and the eluate was subjected to $(\text{NH}_4)_2\text{SO}_4$ fractionation. The protein fraction precipitating between 65 and 80% salt saturation was collected as a pellet, resuspended in 10 mM potassium phosphate/HCl, pH 7.0 (buffer B), and extensively dialysed against buffer B supplemented with $(\text{NH}_4)_2\text{SO}_4$ to 30% salt saturation. It was then applied to a penthyl agarose column (1.4 cm $\times$ 5.5 cm). The enzyme was not retained by the resin and the eluate, after dialysis against 10 mM potassium phosphate/HCl, pH 6.0, was concentrated and then applied to a CM-52 column (1.4 cm $\times$ 6 cm). Again, the enzyme was not retained by the resin and the eluate was directly applied to a Bio-Gel HTP column (1.4 cm $\times$ 9 cm) pre-equilibrated in buffer B supplemented with 50 mM NaCl. The column was washed with the same buffer until the A_{280} of the eluate reached baseline values, and then a linear gradient from 50 to 200 mM NaCl in buffer B was applied. The enzyme activity was eluted at 160 mM NaCl. Active fractions were pooled, concentrated and applied to a Sephadex G-75 column (2.8 cm $\times$ 90 cm) pre-equilibrated and eluted with buffer B containing 100 mM NaCl. Active fractions were pooled, concentrated and subjected to a second chromatographic step on Sephadex G-75. Active fractions were pooled, dialysed against 25 mM Tris/acetic acid, pH 8.8, and applied to Mono P HR 5/20, used in an FPLC system. A linear pH gradient between 8.5 and 7.5 was generated by fluxing the column with pH 7.5 elution buffer (0.11 ml of Pharmalyte 8.0–10.5, 9.5 ml Polybuffer 96, water to 100 ml, brought to pH 7.5 with acetic acid). Active fractions were pooled, concentrated and applied to a Superdex 200 HR 10/30 column, used in an FPLC system. The column was pre-equilibrated and eluted with buffer B containing 150 mM NaCl. Fractions containing enzyme activity were pooled, dialysed against buffer B, concentrated to 0.4 mg/ml protein with a specific activity of 74.3 m-units/mg and stored at –20 °C. Attempts to include in the purification procedure a glutathione–agarose affinity chromatography step failed, since the enzyme became inactivated after adsorption to the resin.

Partial purification of rat liver Grx

Male Sprague–Dawley rat livers were suspended in 33% (w/v) 100 mM potassium phosphate/HCl, pH 7.2 (buffer C), and centrifuged for 15 min at 22000 *g* at 4 °C. The supernatant was centrifuged at 100000 *g* at 4 °C for 60 min. The 100000 *g* supernatant was dialysed extensively in a 6000–8000 Da cut-off dialysis tube against buffer C and concentrated with Centriprep 10. A 1 ml volume of the sample was then loaded on a Sephadex G-100 SF column (2.5 cm $\times$ 50 cm) and the column was eluted with buffer C. Active fractions were pooled, concentrated to a final volume of 2 ml, and applied to a Sephadex G-50 column (2.8 cm $\times$ 90 cm). The column was eluted with buffer B containing 150 mM NaCl, and active fractions were pooled and concentrated to 0.85 mg/ml protein with a specific activity of 270 m-units/mg.

Measurement of enzyme activities

GST activity was measured at 25 °C by following the increase in A_{340} which parallels the conjugation of GSH to CDNB [33]. One unit of GST activity is defined as the amount of enzyme that leads to the formation of 1 μ mol of S-2,4-dinitrophenylglutathione/min. An absorption coefficient at 340 nm of 9.6 mM^{–1}·cm^{–1} for S-2,4-dinitrophenylglutathione was used.

Thioltransferase activity was measured at 30 °C as previously described by using HEDS as disulphide substrate [34]. One unit of thioltransferase activity is defined as the amount of enzyme that leads to the oxidation of 1 μ mol of NADPH/min. The change in absorption coefficient at 340 nm associated with NADPH oxidation was 6.22 mM^{–1}·cm^{–1}.

Determination of the components of the transthiolation reaction

A capillary electrophoresis (CE) method was optimized in order to detect products and intermediates of the transthiolation reaction between HEDS and GSH. A Beckman P/ACE system 2100, controlled by Beckman System Gold software, was used. The analyses were performed at a constant voltage of 30 kV with the cathode positioned at the outlet, using 100 mM Tris/borate buffer, pH 8.5, filtered through a 0.22 μ m cellulose nitrate filter, as electrolyte. The temperature of the capillary during electrophoresis was kept constant at 25 °C. Between runs the capillary was washed with 100 mM NaOH and water, before reconditioning with the electrolyte. Under these conditions, whereas HEDS and 2-mercaptoethanol (2-ME) were electroeluted at the same time as the migration front, GSH, GSSG and S-glutathionyl-2-mercaptoethanol (2-ME-SG) appeared to be resolved as single peaks with retention times of 6.2 min, 6.9 min and 5.4 min respectively. Peak areas were proportional to the concentration of different thiol/disulphide compounds from 5 to 100 μ M (M. Dal Monte, unpublished work). When incubation mixtures were analysed for components of the transthiolation reaction, 20 μ l aliquots were supplemented with 1 μ l of 1 M HCl, centrifuged for 3 min on a Beckman Microfuge E, and analysed by CE.

Synthesis of 2-ME-SG

GSH (4 mM) and HEDS (30 mM) were incubated in buffer B for 2 h at 20 °C, and the transthiolation reaction was followed by CE. At the end of the incubation, the mixture was concentrated to a clear powder residue which was resuspended in 1 ml of water and applied to a DE-52 column (2.1 cm $\times$ 20 cm) pre-equilibrated with water. The column was washed with water until the A_{254} of the eluate reached the baseline value. The mixed disulphide was eluted from the column by water acidified to pH 2 with HCl. Mixed-disulphide-containing fractions were pooled and neutralized with NaOH. CE analysis of the synthesized mixed disulphide revealed the occurrence of a single peak with a retention time of 5.4 min. The same analysis after treating the compound with 5 mM DTT at 25 °C for 30 min revealed the disappearance of the 5.4 min peak and the generation of two new peaks with retention times of 3.6 and 6.2 min, associated with 2-ME and GSH respectively.

Molecular-mass determination

The apparent molecular mass of GST subunits was determined by SDS/PAGE as described by Laemmli [35]. The following standards were used for calibration: BSA (66 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa). The apparent molecular mass of the native enzyme was determined by gel filtration on a Superdex 200 HR 10/30 column, equilibrated

and eluted in buffer B supplemented with 150 mM NaCl. The proteins used for calibration were IgG (160 kDa), BSA (66 kDa), β -lactoglobulin (35 kDa) and cytochrome *c* (12.4 kDa).

Isoelectric focusing

Isoelectric focusing of purified GST was performed at 4 °C on a Biophoresis horizontal electrophoresis apparatus as previously reported [36]. The following standards were used for calibration: amyloglucosidase (pI 3.50); Methyl Red (pI 3.75); soya-bean trypsin inhibitor (pI 4.53); β -lactoglobulin A (pI 5.20); bovine carbonic anhydrase B (pI 5.85); human carbonic anhydrase B (pI 6.55); horse myoglobin, acidic (pI 6.85); horse myoglobin, basic (pI 7.35); lentil lectin, acidic (pI 8.15); lentil lectin, middle (pI 8.45); lentil lectin, basic (pI 8.65); trypsinogen (pI 9.30).

Amino acid sequencing of GST

The partial N-terminal amino acid sequence was determined by direct Edman degradation in a Milligen 6600 protein sequencer. The N-terminal sequence obtained was compared with those stored in the Swiss Protein database using a PC gene program.

Other methods

Protein concentration was determined by the Coomassie Blue-binding assay [37], using BSA as the standard.

RESULTS

Purification of a thioltransferase activity from bovine lens and its identification as GST

The purification of a protein displaying thioltransferase activity was performed by a variety of classical methods (Table 1). The activity and protein determination at different steps indicated the effectiveness of the isolation procedure adopted, which ended with a purification factor of approx. 190 and an overall yield of 0.28 % of the initial activity. The final enzyme preparation with a specific thioltransferase activity of 74.3 m-units/mg appeared to be homogeneous by SDS/PAGE analysis and displayed a single protein band with a molecular mass of 25 kDa.

Besides the thioltransferase activity, Table 1 also shows the results of measurements at different purification steps of GST activity of the enzyme preparations. Gel filtration of the purified enzyme on a Superdex 200 column calibrated with molecular-mass standard proteins gave rise to a symmetrical elution peak with a retention time compatible with an apparent molecular

Figure 1 Effect of GST on the transthiolation process between GSH and HEDS

The time course of formation/consumption at 25 °C of 2-ME-SG (○, ●), GSSG (△, ▲) and GSH (□, ■) in the transthiolation process between 0.2 mM GSH and 0.2 mM HEDS in either the absence (open symbols) or presence (closed symbols) of 0.7 μ M GST is reported.

Figure 2 GSH-dependent reduction of 2-ME-SG catalysed by GST

The time course of formation/consumption at 25 °C of GSSG (△, ▲), 2-ME-SG (○, ●) and GSH (□, ■) in the transthiolation process between 0.5 mM GSH and 0.09 mM 2-ME-SG in either the absence (open symbols) or presence (closed symbols) of 1.6 μ M GST is reported.

Table 1 Purification of thioltransferase activity

Thioltransferase and GST activities were measured as reported in the Experimental section. The GST activity is reported in parentheses.

Step	Activity (m-units)	Specific activity (m-units/mg)	Yield (%)	Purification factor
Crude extract	92 (3680)	0.4 (16)	100	1
DE-52	47	0.13	51	0.3
(NH ₄) ₂ SO ₄	35	7.8	38	19.5
Penthyl agarose	17.5	11.7	19	29
CM-52	14.7	13.7	16	34
Bio-Gel HTP	11.9	15.4	13	38.5
Sephadex G-75 (I)	10.2	33.7	11.1	84
Sephadex G-75 (II)	6.7 (280)	43.2 (1805)	7.3	108
Mono P	0.7	48	0.76	120
Superdex 200	0.27 (11)	74.3 (3027)	0.28	186

mass of 45 kDa. The isoelectric point of the enzyme, analysed by isoelectrofocusing, was 7.1. The N-terminal amino acid sequence analysis of the polypeptide chain (PMILGYWDIRGLAH-ATRL) revealed that the purified protein was a Mu-class GST [38–46].

Mechanism of action of GST as a thioltransferase-acting enzyme

Reagents, products and intermediate species of the transthiolation process were analysed by the CE technique (see the Experimental section). Figure 1 shows the time course of GSH consumption and 2-ME-SG and GSSG formation during the transthiolation reaction catalysed by 0.7 μ M GST. GST caused

Figure 3 Comparative action of GST and Grx on the transthiolation process between GSH and HEDS

The time course of the formation at 25 °C of 2-ME-SG (A) and GSSG (B) in the transthiolation process between 0.2 mM GSH and 0.2 mM HEDS is reported. The incubations were performed in either the absence of enzymes (○) or the presence of 0.7 μ M rat liver Grx (▲), 0.7 μ M GST (●) or a mixture of both enzymes each present at a final concentration of 0.7 μ M (△). The inset shows the relative initial rate of reaction, as evaluated from the time curve data in (B). Bar *a* refers to the non-enzymic reaction; bars *b*, *c* and *d* refer to the reactions catalysed by Grx, GST and Grx + GST respectively.

a significant increase in the rate of synthesis of the mixed disulphide intermediate. Although to a lesser extent, the second step of the transthiolation process was also enhanced by the enzyme. This is shown in Figure 2, in which the GSH-dependent reduction of 2-ME-SG was followed in the presence of 16 μ M GST.

A comparison of the catalytic action of GST and Grx is shown in Figure 3, in which the time course of 2-ME-SG (Figure 3A) and GSSG (Figure 3B) formation in the presence of these enzymes is reported. The rate of the formation and the steady-state level of the intermediate mixed disulphide, the synthesis of which is catalysed by GST, appeared to be reduced both in the Grx-catalysed reaction and when Grx was present together with GST. On the other hand, both GST and Grx enhanced the rate of GSSG formation (Figure 3B). In this case, when both enzymes were present in the reaction mixture, a synergistic effect on the initial rate of the overall reaction (i.e. GSSG formation) was observed (Figure 3B inset).

Kinetic characterization of GST as a thiol-transfer-catalysing enzyme

The kinetic parameters K_m and k_{cat} for the GSH-dependent reduction of HEDS were determined by double-reciprocal plot analysis (results not shown) of the initial reaction rates of the overall transthiolation reaction. A k_{cat} of 0.019 ± 0.002 s⁻¹ and a K_m of 265 ± 80 μ M and 470 ± 90 μ M for GSH and HEDS respectively were measured. When the initial rates for mixed disulphide formation as a function of substrate concentration were analysed in double-reciprocal plots (results not shown) a k_{cat} of 0.28 ± 0.13 s⁻¹ and a K_m of 50 ± 6 μ M and 1 ± 0.4 mM for GSH and HEDS respectively were measured.

The effect of HEDS on the nucleophilic attack of GSH on CDNB catalysed by the enzyme appears to be a competitive-type inhibition with respect to CDNB and a mixed-type (uncompetitive) inhibition with respect to GSH (Figure 4).

DISCUSSION

The isolation from bovine lens of a protein displaying thiol-transferase activity resulted in the purification of a GST. The

Figure 4 Inhibitory action of HEDS on GST activity

The effectiveness of HEDS as an inhibitor of the GST-catalysed formation of glutathione-CDNB adduct is analysed by double-reciprocal plots at different concentrations of CDNB and GSH. (A) Inhibition of HEDS towards CDNB, in the presence of 1 mM GSH; HEDS was used at the following concentrations: 0 (●), 0.5 (△), 0.75 (○) and 1 (▲) mM. (B) Inhibition of HEDS towards GSH, in the presence of 1 mM CDNB; HEDS was used at the following concentrations: 0 (●), 1 (△), 2 (○) and 4 (▲) mM. In each reaction mixture the GST concentration was 40 nM.

high purification factor and rather low purification yield, which are shown in Table 1, came from a purification strategy intended to discriminate as much as possible, with a variety of purification steps, the enzyme under purification from other contaminants present. The identical ratio between thioltransferase and GST specific activities observed at different purification steps, including crude extract and pure enzyme preparation, indicate that the two activities co-purify. The enzyme is a dimer of 25 kDa subunits, displaying a specific activity of 3 units/mg as GST and 74 m-units/mg as thioltransferase acting on HEDS as substrate. The N-terminal amino acid analysis allows the classification of the lens enzyme as a Mu-class GST.

The ability of lens GST to act as a thioltransferase by catalysing the GSH-dependent reduction of HEDS is not a common feature of GST enzymes. A commercial preparation of purified bovine liver GST, for instance, which displays a specific activity of 10 units/mg, is devoid of any thioltransferase activity (results not shown). Nevertheless, the mechanism of action of GST acting as a thiol-transfer catalyst appears to involve the same sites as those used by the enzyme to transfer GSH on a variety of electrophilic molecule centres other than disulphides. This is strongly suggested by the inhibitory action exerted on GST-catalysed glutathionyl-CDNB adduct formation by HEDS, which acts as a competitive or mixed-type inhibitor with respect to CDNB or GSH respectively (Figure 4). Thus the specific thioltransferase activity of lens GST is its ability to recognize the disulphide bond of HEDS as a substrate-reactive centre for a GSH-substitution reaction, a typical feature of GST enzymes. In this context, it is not surprising that GST mainly activates the first step of the transthiolation process. As shown in Figure 1, a 35-fold increase in the initial rate of formation of the intermediate mixed disulphide with respect to the uncatalysed reaction was observed when $0.7 \mu\text{M}$ GST was present in the reaction mixture. Indeed, kinetic analysis of the first step of the transthiolation reaction revealed, for both GSH and HEDS, Michaelis-type behaviour with a K_m of $50 \pm 6 \mu\text{M}$ and $1 \pm 0.5 \text{ mM}$ for GSH and HEDS respectively. The k_{cat} value of $0.28 \pm 0.15 \text{ s}^{-1}$ measured for this first reaction step as compared with the k_{cat} for the overall reduction of HEDS ($0.019 \pm 0.002 \text{ s}^{-1}$) indicates that the generation of GSSG is the limiting step of the process. Nevertheless, although less efficiently, GST also enhances the second step of the transthiolation process. This is clearly evident in Figure 2, in which, at a rather high enzyme concentration, a significant increase in the rate of the intermediate mixed disulphide reduction with respect to the uncatalysed process is observed.

The higher catalytic activity of GST in producing the mixed disulphide represents the major functional difference between this enzyme and Grx. The latter enzyme has been shown to catalyse the transthiolation process by acting essentially on the second step of the overall process, with the first step dependent only on the chemical reactivity of the reagents [47]. This feature is also evident in our study in which Grx isolated from rat liver was used as a comparative thioltransferase enzyme model. The comparison between lens GST and Grx in catalysing the reduction of HEDS reveals the unusual complementarity of action of these two enzymes. This is highlighted by the enhancement of the rate of the GSH-dependent disulphide reduction observed when both enzymes were present in the incubation mixture (Figure 3B inset). Under these conditions an apparent synergistic effect on the initial reaction rate was observed.

In conclusion, bovine lens GST can enhance the GSH-dependent reduction of disulphides, a feature that may be significant in organs or tissues, like the lens, in which Grx is not very abundant [27–30]. Moreover, the unique ability of GSTs to activate the first step of the transthiolation reaction, which is the

limiting step in the Grx-catalysed reaction, means that they have the potential, when present, also to activate the process in those tissues that possess Grx. This is especially useful in conditions (e.g. oxidative) in which decreased GSH levels may reduce the formation of the mixed disulphide intermediate, which is the true substrate for Grx. Whether the ability of lens GST to catalyse transthiolation is a common feature of Mu-class enzymes is under investigation. As it has the potential to enhance the cellular kinetic control of disulphide reduction, it should be considered as a member of the family of thiol/disulphide-interconverting enzymes.

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