Purification and properties of a new glutathione-dependent thiol:disulphide oxidoreductase from rat liver

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A new GSSG-dependent thiol:disulphide oxidoreductase was extensively purified from rat liver cytosol. The enzymic protein shows molecular weight 40 000 as determined by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, and 43 000 as determined by thin-layer gel filtration on Bio-Gel P-100. The pI is 8.1. This enzyme converts rat liver xanthine dehydrogenase into an oxidase, in the presence of oxidized glutathione. Other disulphide compounds are either inactive or far less active than oxidized glutathione in the enzymic oxidation of rat liver xanthine dehydrogenase. The enzyme also catalyses the reduction of the disulphide bond of ricin and acts as a thioltransferase and as a GSH:insulin transhydrogenase. The enzymic activity was measured in various organs of newborn and adult rats.

Xanthine dehydrogenase (EC 1.2.1.37) from rat liver can be converted into an oxidase (EC 1.2.3.2) by treatment with proteolytic enzymes or by other treatments such as storage at -20°C and pre-incubation with either thiol reagents or whole liver homogenate (Stirpe & Della Corte, 1969). The effect of all these treatments except proteolysis can be reversed by thiols (Della Corte & Stirpe, 1972). This conversion of rat liver xanthine dehydrogenase can also be performed, in the presence of GSSG, by an enzyme of the thiol:disulphide-interchanging type, partially purified from rat liver supernatant (Battelli, 1980).

The present paper describes the purification procedure of this enzyme, which we propose to call GSSG-dependent thiol:disulphide oxidoreductase. The molecular weight and the isoelectric point have been determined. Disulphide-compound specificity and other enzymic activities have been examined.

This enzymic activity is widely distributed in the tissues of newborn and adult rats.

Materials and methods

Materials

Sephadex G-25 and Sephacryl S-200 (superfine grade) were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden; DEAE-cellulose and CM-cellulose were from Whatman, Maidstone, Kent, U.K. Bio-Rad Laboratories (Richmond, CA,

U.S.A.) supplied Bio-Gel P-100, acrylamide, *NN'*-methylenebisacrylamide, ammonium persulphate, Coomassie Blue and *NNN'N'*-tetramethylethylenediamine. Ampholines were supplied by LKB-Producter A.B., Stockholm-Bromma, Sweden. Polyvinylpyrrolidone, cystamine and L-cystine were obtained from Fluka, Buchs, Switzerland. NAD^{+} was purchased from Boehringer, Mannheim, Germany; lactose was from Merck, Darmstadt, Germany; SDS was from Baker Chemicals, Deventer, Holland. Other chemicals were supplied by Sigma Chemical Co., St. Louis, MO, U.S.A.

L-[^{14}C]Leucine (sp. radioactivity 300 Ci/mol) was from The Radiochemical Centre, Amersham, Bucks., U.K.

Ricin was prepared as described by Nicolson & Blaustein (1972) and Nicolson *et al.* (1974).

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The purification procedure was essentially as previously described (Battelli, 1980) with some modification (see Scheme 1).

Enzyme assay

Activity was tested by incubating suitable amounts of the enzymic preparations at 37°C for 20 min in 35 mM-Tris/HCl buffer, pH 8.1, containing 0.1 mM-EDTA, 0.15 mM-2-mercaptoethanol, 0.5 mM-GSSG and $170\text{ }\mu\text{g}$ of xanthine dehydrogenase/ml, partially purified from rat liver as described by Battelli (1980).

Abbreviations used: GSH, reduced glutathione; GSSG, oxidized glutathione; SDS, sodium dodecyl sulphate.

Rat liver

Homogenize in 3 vol. of 0.25 M-sucrose: centrifuge, discard pellet:
adjust supernatant to pH 5.5 with 0.2 M-acetic acid: centrifuge,
discard pellet.

Post-microsomal supernatant

Add $(\text{NH}_4)_2\text{SO}_4$ to 70% saturation, centrifuge, resuspend pellet in
20 mM-Tris/HCl buffer, pH 8.5: desalt through Sephadex G-25: elute
from DEAE-cellulose at pH 8.5.

DEAE-cellulose eluate

Add $(\text{NH}_4)_2\text{SO}_4$ to 70% saturation, centrifuge, resuspend pellet in
20 mM-potassium phosphate buffer, pH 6.1: desalt through Sepha-
dex G-25: elute from CM-cellulose at pH 6.1 with linear gradient
of 0.1–0.3 M-NaCl.

CM-cellulose eluate

Dialyse against solid polyvinylpyrrolidone up to 50 times
concentration: dialyse against 20 mM-sodium phosphate buffer,
pH 7.2: centrifuge, discard pellet.

Polyvinylpyrrolidone concentrate

Elute from Sephacryl S-200 at pH 7.2.

Sephacryl S-200 eluate: purified enzyme

All the centrifugations are at 19000 g for 20 min. All the operations were
carried out at 4°C.

Scheme 1. Summary of procedure for purification of the enzyme

Xanthine dehydrogenase and oxidase activities were assayed by measuring the formation of uric acid from the change in the A_{292} in the presence of NAD^+ (total activity) and the formation of NADH from the change in the A_{340} (dehydrogenase activity) (Stirpe & Della Corte, 1969).

One unit of GSSG-dependent thiol:disulphide oxidoreductase is defined as the amount of enzyme when:

$$\% \text{ D form in the absence of the enzyme} - \% \text{ D form in its presence} = 50$$

where % D form is given by the ratio $\text{NADH formed/uric acid formed}$.

Other enzymic activities

Purified GSSG-dependent thiol:disulphide oxidoreductase was tested for other enzymic activities as described in the respective references: thiol oxidase (EC 1.8.3.2) by the reaction with 5,5'-dithiobis-(2-nitrobenzoate) (Janolino & Swaisgood, 1975); protein disulphide-isomerase (EC 5.3.4.1) with reduced ribonuclease (Pery & Fougereau, 1968); thioltransferase with cysteine (Mannervik & Axelson, 1975); GSSG reductase (EC 1.6.4.2) with NADPH (Carlberg & Mannervik, 1975); and GSH:insulin transhydrogenase (EC 1.8.4.2) with insulin (Ibbetson & Freedman, 1976).

The ability of the enzyme to reduce the disulphide bond of ricin, the toxin from *Ricinus communis*, was tested by determining the inhibitory effect on protein synthesis of ricin preincubated with the oxido-

reductase. It is known that the inhibitory effect of ricin on cell-free protein synthesis is enhanced upon reduction of the toxin (Olsnes & Pihl, 1973, 1977). Protein synthesis was determined as described by Stirpe *et al.* (1980) with a lysate of rabbit reticulocytes prepared as described by Allen & Schweet (1962).

Physical-chemical analysis

Molecular weight was determined by SDS/polyacrylamide-gel electrophoresis (Weber & Osborn, 1969) with bovine serum albumin (mol.wt. 68 000), soya-bean trypsin inhibitor (mol.wt. 21 500) and the two chains of ricin (mol.wts. 32 000 and 34 000) as standards, and by thin-layer gel filtration on Bio-Gel P-100 layer (0.2 mm thick) equilibrated with 200 mM-NaCl in 50 mM-Tris/HCl buffer, pH 8.1 (Morris, 1964), with cytochrome c (mol.wt. 13 500), soya-bean trypsin inhibitor and bovine serum albumin as standards.

Isoelectric point was determined as described by Dale & Latner (1968), but on a 5% polyacrylamide gel, polymerized by $\text{NNN}'\text{N}'$ -tetramethylethylenediamine and ammonium persulphate. Gels were either cut for pH determination or stained with 0.1% Coomassie Blue in 20% (w/v) trichloroacetic acid and destained with 40% (v/v) methanol at 50°C.

Carbohydrate content was measured by the phenol/ H_2SO_4 reaction (Dubois *et al.*, 1956) with lactose as a standard.

Protein concentrations were determined by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard.

Tissue distribution

Tissues from 1–2 days newborn and from 200 g Wistar rats were homogenized with 3 vol. of 20 mM-Tris/HCl buffer, pH 8.5, mixed with an equal volume of DEAE-cellulose in the same buffer and centrifuged at 105 000 g for 60 min.

For each tissue, different amounts of the 105 000 g supernatant were tested and the enzymic content was calculated from the linear part of the curve.

Results

The results of the purification procedure are summarized in Table 1. The chromatography on DEAE-cellulose retained most of the rat liver supernatant proteins, including xanthine dehydrogenase.

After polyvinylpyrrolidone concentration, dialysis against a low-ionic-strength buffer was introduced,

Table 1. *Purification of GSSG-dependent thiol:disulphide oxidoreductase*

Assay conditions and units are described in the Materials and methods section. The converting activity could not be measured before the DEAE-cellulose step because of the interfering presence of endogenous xanthine dehydrogenase in the rat liver supernatant. At each purification step the activity was proportional to the added amount of the enzymic preparation.

Step	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)
DEAE-cellulose	110	802	5940	7.4
CM-cellulose	70	139	4060	29
Polyvinyl-pyrrolidone	2.5	2.62	600	229
Sephacryl S-200	18	1.29	480	372

Table 2. *Tissue distribution of the enzymic activity*
For details see the text.

Tissue	Adult rats (units/g of tissue)	Newborn rats (units/g of tissue)
Liver	43.20	33.14
Spleen	32.86	23.20
Skeletal muscle	18.52	6.14
Brain	14.72	13.86
Skin	14.40	12.66
Kidney	13.66	17.14
Lung	12.92	15.76
Heart	9.60	14.80

Table 3. *Enzymic activities of GSSG-dependent thiol:disulphide oxidoreductase*

Assay methods are described in the text. A unit of either thioltransferase or GSH:insulin transhydrogenase activity corresponds to 1 μ mol of NADPH formed/min at 25°C. Thiol oxidase activity was tested with 12 μ g of purified enzyme/ml for 5 h at 35°C. Protein disulphide-isomerase activity was tested with 12.5 μ g of purified enzyme/ml for 7 h at 37°C. GSSG reductase activity was tested with 1.7 μ g of purified enzyme/ml for 1 h at 25°C.

Enzyme	Activity (units/mg of protein)
Xanthine dehydrogenase converting activity	200.00
Thiol oxidase	Not detectable
Protein disulphide-isomerase	Not detectable
Thioltransferase	0.84
GSSG reductase	Not detectable
GSH:insulin transhydrogenase	1.32

Table 4. *Enzymic and non-enzymic conversion of xanthine dehydrogenase in the presence of various disulphide compounds*

Assay conditions and units are described in the Materials and methods section.

Disulphide compound	Concn. (mM)	D form (%)	
		Enzyme absent	Enzyme present
None		71	64
GSSG	0.5	62	2
Cystine	0.1	37	35
	0.4	8	12
Cystamine	0.005	43	40
	0.02	17	17
Dipyridyl disulphide	0.05	53	40
	0.2	5	0
Oxidized thiocetic acid	10	33	33
	15	28	27
Penicillamine	1.25	32	21
disulphide	2.5	12	5
Homocystine	1.25	33	16
	2.5	11	6

Fig. 1. *Elution profile of proteins and of the enzymic activity from a Sephacryl S-200 column*

Void volume (V_0) = 30 ml. —, Protein concentration; ----, enzymic activity. For details see the text.

because under these conditions the enzyme was retarded by Sephacryl S-200 and could be separated from the other proteins (Fig. 1). A 20 μ g sample of the enzymic protein, purified by this procedure, gave a unique band on SDS/polyacrylamide-gel electrophoresis.

Table 5. Summary of thiol:disulphide-interchanging enzymes

Enzymes	Enzymic activities	Animal tissue	Intracellular location	Mol.wt.	pI	Sugar content	Best substrate	Inhibitors	References
GSH:protein-disulphide oxidoreductase (EC 1.8.4.2)	GSH:insulin trans-hydrogenase (protein disulphide-isomerase)	Rat and bovine liver	Membrane-associated	60 000 (Hillson & Freedman, 1980)	4.1	12%	GSH	Cu ²⁺	Varadani (1972), Ansoerge <i>et al.</i> (1973), Morin <i>et al.</i> (1978)
Protein disulphide-isomerase (EC 5.3.4.1)	Protein disulphide-isomerase (GSH:insulin transhydrogenase)	Bovine liver	Microsomal fraction	42 000 (Hillson & Freedman, 1980)	4.65		Dithio-threitol		De Lorenzo <i>et al.</i> (1966)
GSSG reductase (EC 1.6.4.2)	GSSG reductase	Rat liver	Cytosol	125 000			NADPH and GSSG	Cu ²⁺	Mize & Langdon (1962), Carlborg & Mannervik (1975)
GSH:disulphide oxidoreductase	Protein disulphide-isomerase	Pig pituitary	Cytosol	170 000	7 and 4.4-5		GSH	Cu ²⁺ , N-ethylmaleimide	Lorenson & Jacobs (1981)
Thiol oxidase (EC 1.8.3.2)	Thiol oxidase (protein disulphide-isomerase)	Bovine milk		89 000		11%		EDTA	Janolino & Swaisgood (1975)
Thiol oxidase (EC 1.8.3.2)	Thiol oxidase (protein disulphide isomerase)	Rat skin	Cytosol	66 000	4.65		Dithio-threitol and dithio-erythritol		Takamori <i>et al.</i> (1980)
Thioredoxin	Disulphide reductase (GSH:insulin transhydrogenase)	Bovine liver	Cytosol	12 000			NADPH	Cu ²⁺	Engström <i>et al.</i> (1974)
Thioltransferase	Thioltransferase (GSH:insulin trans-hydrogenase; protein disulphide-isomerase)	Rat liver	Cytosol	11 000	9.5	8.6%	GSH		Axelsson <i>et al.</i> (1978)
GSSG-dependent thiol:disulphide oxidoreductase	Thiol:disulphide oxidoreductase (ricin disulphide reductase; thioltransferase; GSH:insulin transhydrogenase)	Rat liver	Cytosol	40 000-43 000	8.1	Not detectable	GSSG	Insensitive to Cu ²⁺ and to N-ethylmaleimide	Present experiments

Fig. 2. Effect of the enzyme on the inhibition of protein synthesis by ricin

Ricin was preincubated at 37°C for 60 min in 20 mM-sodium phosphate buffer, pH 7.2 (0.2 ml final volume), in the presence of either 10 mM-GSH (■) or 65 µg of a semi-purified preparation of the enzyme (before Sephacryl S-200 chromatography) (▲) or both (●).

With the purified enzyme the activity was linearly related to the concentration.

The chromatographic behaviour of the oxidoreductase suggested a basic nature of the enzymic protein; this was confirmed by isoelectric focusing, which showed pI 8.1. The fact that the enzyme was retarded in the Sephacryl S-200 chromatography was not due to a carbohydrate component of the enzymic molecule, since no sugar was detectable by the method used.

Similar values of molecular weight were obtained by SDS/polyacrylamide-gel electrophoresis (40 000) and by thin-layer gel filtration on Bio-Gel P-100 (43 000).

The tissue distribution of the enzyme is shown in Table 2. The oxidoreductase was detectable in all organs tested, in both newborn and adult rats. The liver showed the highest amount of activity. Except in kidney, lung and heart, the enzyme amount was higher in adult tissues than in the newborn.

The wide distribution of the oxidoreductase suggested the possibility that this enzyme could be involved in other thiol:disulphide-interchanging reactions. The enzyme showed activity as thiol-transferase, GSH:insulin transhydrogenase (Table 3) and ricin-disulphide-bond reductase (Fig. 2), whereas no thiol oxidase, protein disulphide-isomerase and GSSG reductase activities were detectable (Table 3).

The remarkable selectivity of the oxidoreductase for GSSG in the reaction with xanthine dehydrogenase is shown in Table 4.

GSSG-dependent thiol:disulphide oxidoreductase was unaffected by treatment at 37°C for 30 min either with 1 mM-CuSO₄ or with 1 mM-*N*-ethyl-maleimide.

Discussion

We have purified from rat liver an enzyme that catalyses the oxidation of xanthine dehydrogenase thiol groups essential for the dehydrogenase activity. The reaction is dependent on the presence of GSSG and converts the xanthine dehydrogenase into an oxidase.

On the basis of the properties shown in Table 5, the enzyme is clearly distinct from other thiol:disulphide-interchanging enzymes, and we can put forward three hypotheses on its possible biological role.

(1) It is not known how much of the xanthine dehydrogenase has *in vivo* a dehydrogenase (D) or an oxidase (O) activity. The GSSG-dependent thiol:disulphide oxidoreductase could convert *in vivo* the xanthine dehydrogenase from its D into the O form. Such a regulatory role has been suggested for milk thiol oxidase (Clare *et al.*, 1981), which catalyses the conversion from the D into the O form of milk xanthine oxidase, pretreated with dithiothreitol.

(2) Although the enzyme converts the xanthine dehydrogenase in the direction type D → type O only, it can act also in the opposite direction in the reduction of the disulphide bond of insulin and ricin. These observations suggest that the enzyme could separate polypeptide chains joined by disulphide bonds, as proposed previously for other enzymes (Varandani, 1972; Holmgren, 1979; Axelsson & Mannervik, 1980).

(3) The wide tissue distribution, together with its preference for glutathione, may suggest another wider biological role for the enzyme presented in this study, i.e. the maintenance of the 'GSH status' of the cell, the significance of which has been well documented by Kosower & Kosower (1978).

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