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The 58 kDa mouse selenoprotein is a BCNU-sensitive thioredoxin reductase

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Abstract The flavoprotein thioredoxin reductase [EC 1.6.4.5] ($NADPH+H^+$+thioredoxin-$S_2 \rightarrow NADP^+$+thioredoxin-$(SH)_2$) was isolated from mouse Ehrlich ascites tumour (EAT) cells. Like the counterpart from human placenta but unlike the known thioredoxin reductases from non-vertebrate organisms, the mouse enzyme was found to contain 1 equivalent of selenium per subunit of 58 kDa. The K_M values were 4.5 μM for NADPH, 480 μM for DTNB and 36 μM for *Escherichia coli* thioredoxin, the turnover number with DTNB being $\approx 40\ s^{-1}$. As mouse is a standard animal model in cancer and malaria research, thioredoxin reductase and glutathione reductase [EC 1.6.4.2] from EAT cells were compared with each other. While both enzymes in their 2-electron reduced form are targets of the cytostatic drug carmustine (BCNU), no immunologic cross-reactivity between the two mouse disulfide reductases was observed.

Key words: Chemotherapy; Malaria; Selenoenzyme purification; Thioredoxin reductase; Glutathione reductase; Mouse EAT cell

1. Introduction

Only very few mammalian selenoenzymes, such as glutathione peroxidase and thyroid hormone deiodinase, have so far been identified [1,2]. Human thioredoxin reductases, dimeric flavoenzymes with subunit M_r values of 55 kDa, were reported to contain one selenocysteine per subunit [3–5]. This suggested to us that the 58 kDa band described in mouse selenoprotein maps [6,7] might represent thioredoxin reductase. Thioredoxin reductases exhibit a rather broad substrate specificity [8–13]. Inter alia they are involved in deoxyribonucleotide synthesis [9], in cellular redox control [14] and in the detoxification of endogenous cytotoxins like *NK*-lysin [10]. It is furthermore noteworthy that thioredoxin reductase activity in all tumour cell lines studied so far is by an order of magnitude higher than in normal cells ([4] and references therein).

High molecular weight thioredoxin reductases are closely related to glutathione reductase [5,8,9,15,16]. Mouse glutathione reductase has been studied in detail as a marker antigen and as a target of cytostatic and antiparasitic agents such as carmustine [17,18]. A number of effects ascribed to glutathione reductase inhibition in vivo may in fact concern the newly discovered thioredoxin reductase. In the present study we therefore directly compared TrxR with GR.

2. Materials and methods

2.1. Materials and assay systems

EAT cells HD 34 [19] were a gift of Prof. C. Granzow, Heidelberg; *E. coli* thioredoxin was kindly provided by Prof. C.H. Williams Jr., Ann Arbor, MI, and carmustine (BCNU) by Prof. G. Eisenbrand, Kaiserslautern. Rabbit anti-(human placenta thioredoxin reductase)-serum (Eurogentec) and rabbit anti-(human glutathione reductase)-serum were used as described in [20]. Porcine horseradish peroxidase-coupled anti-(rabbit IgG)serum was from DAKO, Hamburg. All chemicals used were of the highest available purity. Protein was determined using the BioRad protein assay [21], with bovine serum albumin (Pierce) serving as a standard. Glutathione reductase was assayed as described in [25]. For thioredoxin reductase activity, the two assays described in [22] were used at 25°C. The DTNB reduction assay proved to be sufficiently specific for monitoring the purification process. Using this assay one enzyme unit is defined as the NADPH-dependent production of 2 μmol 2-nitro-5-thiobenzoate (ε_{412nm} 13.6 $mM^{-1}\ cm^{-1}$) per min. Bovine serum albumin in the assay buffer resulted in up to 25% increase in activity but was omitted in order to obtain standardized results. The experiments with the cytostatic agent carmustine were conducted as described in [5].

2.2. Isolation of thioredoxin reductase from EAT cells

Unless explicitly otherwise stated, all steps of enzyme purification were carried out at 0–4°C.

Eighteen milliliters of a frozen EAT cell pellet was thawed in the presence of 11 ml of extraction buffer at 38–40°C (10 μM FAD, 100 μM PMSF in TE buffer). Another 20 μl aliquot of 100 mM PMSF was added, and the suspension was stirred magnetically for 10 min at 4°C. The homogenate was adjusted to pH 8.3 with 5 M NH_4OH, and 6 ml of a −20°C cold mixture of chloroform/1-butanol (2:5, v:v) was added rapidly under vigorous stirring. Most hemeproteins and pyridine nucleotide-dependent oxidoreductases denature under these conditions [23] but GR and TrxR are recovered in high yields. The brownish mass was stirred for another 10 min, left for 1 h and then centrifuged for 30 min at 29 000 × *g*. The supernatant was set aside while the precipitate was resuspended in 7 ml of extraction buffer and centrifuged as above. The supernatants were combined, filtered through glass wool and adjusted to pH 8.3. This fraction contained 13 U TrxR.

Per 1 ml of the chloroform–butanol extract, 1 ml of cold acetone was slowly added under stirring. The resulting suspension was left for 1 h and then centrifuged (10 min, 26 000 × *g*). The pellet was taken up in a small volume of TE buffer, dialysed exhaustively against 2-fold diluted TE buffer and centrifuged (20 min, 29 000 × *g*). The supernatant was set aside, the precipitate was resuspended in 2 ml of TE buffer and again centrifuged.

The combined supernatants were applied to a 1 × 7 cm DEAE-52 cellulose column (Whatman), equilibrated with TE buffer and operated at room temperature. After washing the column with 2 vol. of TE buffer and 1 .vol of 50 mM NaCl in TE buffer, thioredoxin reductase activity was eluted with 90 mM NaCl in TE buffer. The pool was concentrated, washed with TE buffer in a Centricon 30 (Amicon) and applied to a 0.6 × 2.6 cm column of TE-equilibrated

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Abbreviations: BCNU, 1,3-bis-(2-chloroethyl)-1-nitrosourea; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); PMSF, phenylmethylsulfonylfluoride; TE buffer, 50 mM Tris-HCl, 1 mM EDTA, adjusted to pH 7.6 at room temperature; TrxR, thioredoxin reductase; GR, glutathione reductase

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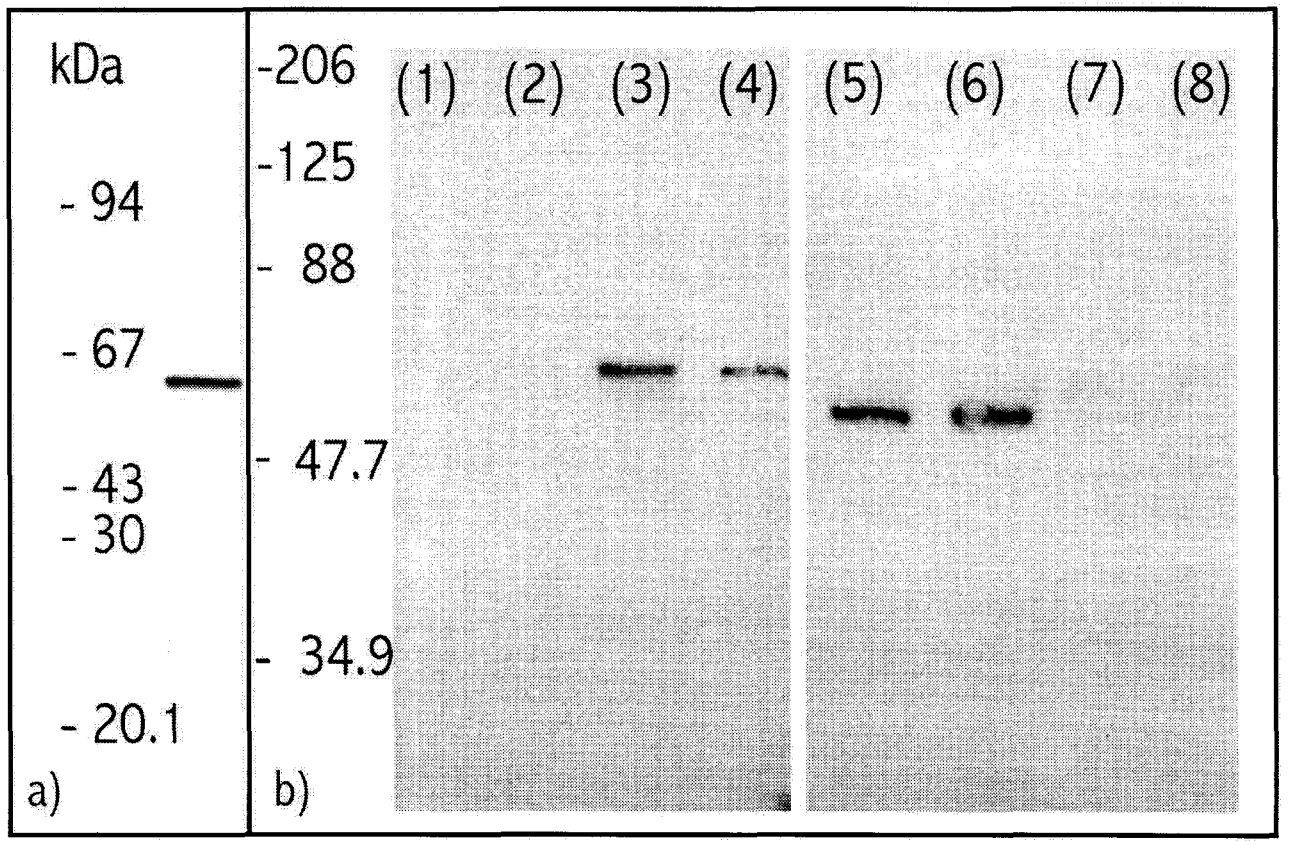

Fig. 1. SDS-PAGE and silver staining of 150 ng mouse thioredoxin reductase. The apparent subunit molecular mass of 58 kDa is probably by 5% too high (see text). Immunoblots demonstrating specific reactivity of human and mouse TrxR and GR with the cognate antisera. Lanes 1 and 5: 100 ng human glutathione reductase; lanes 2 and 6: 100 ng mouse glutathione reductase; lanes 3 and 7: 100 ng human thioredoxin reductase; lane 4 and 8: 100 ng mouse thioredoxin reductase. The proteins in lanes 1–4 where exposed to rabbit anti-(human thioredoxin reductase)serum and in lanes 5–8 to rabbit anti-(human glutathione reductase)serum.

2′,5′-ADP-Sepharose 4B (Pharmacia) which was subsequently rinsed with 2 vol. of the same buffer.

To remove non-specifically bound protein at high ionic strength the column was consecutively washed with 1 vol. 100 mM KCl in TE buffer, 0.7 vol. 200 mM KCl in TE buffer and 1 vol. 100 mM KCl in TE buffer. In order to prepare the column for specific elution steps the ionic strength was then decreased using 2 vol. 2-fold diluted TE buffer. Even though most pyridine nucleotide-dependent enzymes had been removed in the first purification step(s) (see above) some specifically binding protein remained. NAD(H) binding proteins were removed with 2 vol. 1.2 mM NADH in TE buffer and 2 vol. of TE buffer. To remove contaminating NADP(H) binding proteins the column was washed with 2 vol. 100 μM $NADP^+$ in TE buffer and finally with 1 vol. 500 μM $NADP^+$ in TE buffer. TrxR activity was eluted with 1.5 mM $NADP^+$. (The GR which was still on the column can be eluted and purified as a electrophoretically homogeneous protein according to [17].) The TrxR pool — which was now free of GR activity — was concentrated and washed with TE buffer in a Centricon 30. To remove residual contaminating proteins of 20–30 kDa (as judged by SDS-PAGE) the pool was reapplied to the regenerated 2′,5′-ADP–Sepharose column. The column was washed with 2 vol. 50 μM NADPH in TE buffer and 1 vol. 150 μM NADPH in TE buffer before TrxR activity was eluted with 500 μM NADPH in TE buffer. The pool was concentrated and washed in a Centricon 30. Purity was checked by 10% SDS-PAGE (BioRad) and silver staining (Quick-silver, Amersham).

Table 1
Comparison of human placenta thioredoxin reductase and mouse EAT thioredoxin reductase

Protein	Human TrxR	Mouse TrxR
Source	Placenta (0.2 U/g)	EAT cells (1–2 U/g)
Subunit M_r	55 kDa	55 kDa
Selenium content per mol subunit	0.93 mol	0.94 mol
Specific activity (DTNB reduction assay)	35 U/mg	37 U/mg
K_M for NADPH (DTNB reduction assay)	6.5 μM	4.5 μM
K_M for DTNB	400 μM	480 μM
K_M for *E. coli* thioredoxin	25 μM	36 μM
Reaction with anti-(human thioredoxin reductase)serum	yes	yes
Reaction with anti-(human glutathione reductase)serum	none	none
Effect of 1 mM carmustine		
in the absence of NADPH	no inhibition	
in the presence of 100 μM NADPH	inactivating modification	

The data for human TrxR apply for the enzyme isolated from placenta [5,22,33]. Isoenzymes have been reported for human lung adenocarcinoma cells [3] and human T-cells [4]. The kinetic data shown for the human and mouse EAT enzyme are comparable with rat liver TrxR [8].

The selenium contents of a 250 μl sample containing 550 nM TrxR subunits and of a 250 μl protein-free TE buffer as a control were determined by atomic absorption spectroscopy at Runnebaum and Fuchs Laboratories, Heidelberg.

3. Results and discussion

From 18 ml of packed Ehrlich ascites tumour cells 20 μg thioredoxin reductase with a specific activity of 37 U/mg was isolated. The enzyme was more than 98% pure by SDS-PAGE analysis. The purification procedure allows the simultaneous preparation of glutathione reductase. The specific activity of the purified GR (189 U/mg) compares well with the value reported previously [17]. As shown in Table 1 and Fig. 1, mouse thioredoxin reductase closely resembles its human counterpart in selenium content as well as in its enzymatic and immunologic properties. The subunit sizes of 58 kDa for mouse TrxR and 55 kDa for mouse GR, as judged from the SDS-PAGE analysis, are similar for the respective human enzymes (Fig. 1). Disulfide reductases tend to exhibit a 5% higher M_r by SDS-PAGE analysis than by amino-acid sequence analysis [4,5,15,17]. Therefore we used an M_r value of 55 kDa per FAD-containing TrxR subunit for stoichiometric calculations. With respect to the selenium content, thioredoxin reductase represents one and possibly the only function associated with the 55–60 kDa band described for mouse selenoprotein maps [6,7].

NADPH-reduced glutathione reductase can be irreversibly inactivated by carmustine (BCNU) whereas the enzyme in its oxidized state is not affected. The nature of this modification, a carbamoylation of the distal catalytic site-cysteine, has been studied in atomic detail for the human enzyme [24,25]. As shown in Table 1, reduced mouse thioredoxin reductase was, as its human equivalent [5,26], also irreversibly modified by BCNU at therapeutic concentrations. Consequently the possibility that thioredoxin reductase is the major target of nitrosourea drugs must be considered.

Despite the detailed pharmacological similarity of the two disulfide reductases, thioredoxin reductase does not react with anti-(human glutathione reductase)serum and vice versa (Fig. 1). This result indicates that differential immunolocalisation of the two disulfide reductases in the mouse brain [18] is possible when studying the effects of cytostatic or antimalarial drugs.

Like humans, mice are susceptible to cerebral malaria [27]. Thioredoxin reductase of *Plasmodium falciparum*, the causative agent of human tropical malaria, is *not* a selenoenzyme [16,28]. This difference between mammalian host and parasite enzyme might be exploited for the design of selective antiparasitic drugs [29]. Similar considerations apply to other pathogens like fungi and bacteria, whose thioredoxin reductases differ greatly from their mammalian counterparts (see [5,9,30,31] for references).

Our results support the notion of Holmgren and coworkers [32], that the selenium content is not a peculiarity of the human enzyme but a feature of various mammalian thioredoxin reductases.

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