Purification and characterization of glutathione-dependent dehydroascorbate reductase from rat liver

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GSH-dependent enzymic reduction of dehydroascorbic acid to ascorbic acid has been studied in rat liver cytosol. After gel filtration of cytosol on Sephadex G-100 SF, dehydroascorbate reductase activity was recovered in two distinct peaks, one corresponding to glutaredoxin (an enzyme already known for its dehydroascorbate reductase activity) and another, much larger one, corresponding to a novel enzyme different from glutaredoxin. The latter was purified to apparent homogeneity. The purification process involved $(NH_4)_2SO_4$ fractionation, followed by DEAE-Sepharose, Sephadex G-100 SF and Reactive Red chromatography. SDS/PAGE of the purified enzyme in either the presence or absence of 2-mercaptoethanol demonstrated a single protein band of M_r 31000. The M_r determined by both Sephadex G-100 SF chromatography and h.p.l.c. was found to be approx. 48000. H.p.l.c. of the denatured enzyme gave an M_r value identical with that obtained by SDS/PAGE (31000). The apparent K_m for dehydroascorbate was 245 μM and the $V_{max.}$ was 1.9 μmol/min per mg of protein; for GSH they were 2.8 mM and 4.5 μmol/min per mg of protein respectively. The optimal pH range was 7.5–8.0. Microsequence analysis of the electrotransferred enzyme band showed that the N-terminus is blocked. Data on internal primary structure were obtained from CNBr- and *N*-chlorosuccinimide-derived fragments. No significative sequence similarity was found to any of the protein sequences contained in the Protein Identification Resource database.

INTRODUCTION

There is increasing evidence suggesting that oxidative stress plays an important role in pathological conditions associated with toxic, inflammatory, ischaemic and radiation injury (reviewed in [1] and [2]). Protection against oxidative stress is afforded by several antioxidant systems, including enzymic (superoxide dismutase, catalase, GSH peroxidase and GSH transferases) and non-enzymic (vitamin E, β-carotene) systems. Of the water-soluble antioxidants, ascorbate is thought to act by both scavenging aqueous free radicals [3–7] and reducing tocopheroxyl radicals back to α-tocopherol [8,9].

After reaction with free radicals, ascorbate undergoes one-electron oxidation to semidehydroascorbic acid (ascorbyl radical). Dismutation or further oxidation converts semidehydroascorbic acid into dehydroascorbic acid (DHAA). Consistent with this, in conditions of prolonged oxidative stress, a decrease in the cellular level of ascorbate, with a concomitant increase in DHAA, has been observed *in vivo* [10,11]. DHAA is metabolized by the cell to diketogulonic acid, which is further catabolized and lost in the urine.

The antioxidant potential of ascorbate is believed to be restored by cell systems capable of reducing its oxidized forms. Such systems presumably maintain an effective steady-state concentration of the antioxidant in basal conditions as well as during oxidative stress, independently of *de novo* synthesis and dietary supply. Such ascorbate-regenerating systems play an additional role in maintaining cell integrity, as DHAA itself is known to have toxic effects, as shown in erythrocytes [12], pancreatic islets [13], cultured lens epithelial cells [14] and even in the intact animal [15,16].

With regard to the one-electron-oxidized form of ascorbate (ascorbyl radical), several authors have shown the existence of an NADH-dependent semidehydroascorbate reductase activity in cellular membranes [17–19]. In contrast, such an activity is absent from the cytosol [17–19]. The mechanism by which the two-electron-oxidized form of ascorbate, DHAA, is reduced is still debated. It has been suggested that GSH can reduce DHAA back to ascorbate [20–23]. Liver cells and erythrocytes have been reported to contain an enzyme capable of reducing DHAA to ascorbate using GSH as hydrogen donor [20]. Subsequently, a DHAA reductase activity has been described in crude extracts of various other cell types, including neutrophils, lymphocytes and cultured fibroblasts [21]. However, the existence of a DHAA reductase has been questioned. Several authors in fact have shown that GSH can reduce DHAA to ascorbate chemically, without the participation of any enzymic activity [22,23]. Wells et al. [24] reported that purified glutaredoxin and protein disulphide-isomerase (PDI) exhibit DHAA reductase activity. Moreover, the presence of a soluble DHAA-reducing activity associated with a protein with an M_r higher than glutaredoxin has also been suggested [25].

The present study reports the purification and properties of a novel cytosolic DHAA reductase activity which is independent of glutaredoxin.

MATERIALS AND METHODS

Cytosol preparation

Male Sprague–Dawley rats (200–250 g), maintained on a pellet diet (Nossan, Correzzana, Italy), were fed *ad libitum* until used. After ether anaesthesia, the liver was perfused through the portal vein with ice-cold saline. Liver homogenates (33%, w/v) were prepared in ice-cold 100 mM potassium phosphate buffer, pH 7.2 (buffer A) using a Potter–Elvehjem homogenizer. The homogenate was centrifuged at 20000 *g* for 15 min, and the supernatant was further centrifuged at 100000 *g* for 60 min. The cytosolic fraction was then dialysed overnight at 4 °C (Spectra/Por tubing,

Abbreviations used: DHAA, dehydroascorbic acid; PDI, protein disulphide-isomerase; HED, hydroxyethyl disulphide; NCS, *N*-chlorosuccinimide.
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M_r cut-off 6000–8000; Serva Feinbiochemica, Heidelberg, Germany) against two changes of a total of 340 vol. of the homogenization buffer. The contents of the dialysis bag were centrifuged for 10 min at 1500 *g* to remove particles.

Gel filtration of cytosol

Dialysed cytosol was concentrated threefold by ultrafiltration (Centriprep 10; Amicon, Beverly, MA, U.S.A.). All DHAA reductase activity was recovered in the concentrate. Then 1 ml of sample was loaded on to a Sephadex G-100 SF (Sigma Chemical Co., St. Louis, MO, U.S.A.) column (2.5 cm × 50 cm) equilibrated with buffer A at 4 °C. Proteins were eluted with the same buffer at a flow rate of 0.25 ml/min. After discarding 100 ml, eluate fractions of 2 ml were collected. DHAA reductase and thioltransferase activities were measured in all fractions.

The Sephadex G-100 SF column was calibrated with BSA (M_r 66000), ovalbumin (M_r 45000), carbonic anhydrase (M_r 29000) and cytochrome *c* (M_r 12400).

Enzyme purification

Unless otherwise stated, all steps were performed at 0–4 °C. Liver cytosol, prepared from 13 rats following the procedure above, was diluted 1.5-fold with buffer A and used as follows.

Step 1: $(NH_4)_2SO_4$ fractionation

Powdered $(NH_4)_2SO_4$ (104.3 g) was slowly added at 0 °C to cytosol (320 ml) to give 55% saturation. Sufficient 1 M NH_4OH was added to keep the pH constant. After being stirred for 30 min, the mixture was centrifuged at 18000 *g* for 30 min. To the supernatant (357 ml), 57.4 g of powdered $(NH_4)_2SO_4$ was added resulting in 75% saturation and the mixture was stirred and centrifuged again. The pellet was redissolved in 30 ml of buffer A and dialysed overnight at 4 °C against 4 litres of the same buffer. The dialysed protein solution was then centrifuged at 1500 *g* for 10 min and the resulting supernatant was concentrated to 5 ml by ultrafiltration (Centriprep 30; Amicon). The proteins were then transferred to 10 mM potassium phosphate buffer, pH 7.8 (buffer B) by two passages through Sephadex G-25 M columns (PD-10 columns; Pharmacia LKB Biotechnology, Uppsala, Sweden) equilibrated with buffer B.

Step 2: DEAE-Sepharose chromatography

The protein solution obtained from step 1 was applied to a DEAE-Sepharose CL 6B (Sigma) column (2.5 cm × 9 cm) previously equilibrated with buffer B. After being rinsed with 250 ml of the same buffer, the column was eluted with a 450 ml NaCl gradient set up as follows: chamber 1, stirred and connected to the column, contained 500 ml of buffer B; chamber 2, containing buffer B plus 150 mM NaCl, was connected to chamber 1 with a peristaltic pump (Minipuls 2, Gilson, Villiers le Bel, France) adjusted at a flow rate of 90 ml/h. The same flow rate was used to deliver the mixture to the column. Eluate fractions of 10 ml were collected and assayed for DHAA reductase activity. Fractions with higher specific activity were pooled and concentrated to 1.5 ml by ultrafiltration (Centriprep 30).

Step 3: Gel-filtration chromatography

The sample obtained from step 2 was applied to a Sephadex G-100 SF column (2.5 cm × 50 cm) equilibrated with buffer A. Proteins were eluted with the same buffer at a flow rate of 0.25 ml/min. After discarding 100 ml, eluate fractions of 2 ml were collected. Fractions showing higher DHAA reductase activity were pooled and concentrated to 0.5 ml by ultrafiltration (Centricon 10; Amicon) and used for the next step.

Step 4: Reactive Red 120 chromatography

The sample obtained from step 3 was applied to a Reactive Red 120 3000-CL (Sigma) column (1.5 cm × 2 cm) equilibrated with buffer A containing 1.5 mM $MgCl_2$. DHAA reductase activity was recovered by eluting the column with 5 ml of the same buffer at gravity rate. Eluate fractions of 0.5 ml were collected. Fractions containing DHAA reductase activity were pooled and stored at 0–4 °C. This protein solution was used as the final enzyme preparation for the experiments described below.

Enzyme assay conditions

The GSH-dependent DHAA reductase activity was determined by the direct spectrophotometric assay of Stahl et al. [23] monitoring the change in A_{265} associated with the formation of ascorbate. A molar absorption coefficient of 14800 was used. When not otherwise described, the test was performed in 100 mM potassium phosphate buffer, pH 7.2, in the presence of 0.25 mM DHAA and 1 mM GSH. Chemical reduction brought about by GSH alone was subtracted. The reaction was linear at least up to 2 min at 30 °C. DHAA was prepared immediately before the assay by exposing water solutions of ascorbic acid to liquid bromine; excess bromine was removed by bubbling the solution with N_2 [21,25]. Control experiments were performed by using standard DHAA (Aldrich Chemical Co., Milwaukee, WI, U.S.A.).

Glutaredoxin thiol-transferase activity was evaluated by measuring the reduction of hydroxyethyl disulphide (HED) in the presence of GSH as described by Holmgren [26].

PDI activity was assayed by measuring transhydrogenase activity as described by Lambert and Freedman [27]. As a positive control an enriched fraction was used, obtained by homogenizing rat liver in Triton X-100, followed by heat-treatment and $(NH_4)_2SO_4$ fractionation, i.e. the first three steps of the purification procedure reported by Hillson et al. [28].

Protein concentration was determined by measuring A_{280} (BSA as standard).

SDS/PAGE

SDS/PAGE on slab gel was performed by the method of Laemmli [29] with 5% acrylamide for the stacking gel and 15% acrylamide for the separating gel. Samples were denatured by boiling for 3 min in 0.1% (w/v) SDS in either the presence or absence of 5% (v/v) 2-mercaptoethanol. The gels were stained with Coomassie Brilliant Blue.

Determination of M_r

The M_r of native enzyme was estimated by both Sephadex G-100 SF chromatography (same procedure as in step 3 of the enzyme purification) and h.p.l.c. on an ultraspherogel SEC 2000 column (7.5 cm × 300 mm; Beckman, San Ramon, CA, U.S.A.). With h.p.l.c., proteins were eluted by buffer A at a flow rate of 1 ml/min and detected at 280 nm. M_r standards were the same as those used for calibration of the Sephadex G-100 SF column (see above). The M_r of denatured enzyme was determined by both SDS/PAGE as specified above and h.p.l.c. In the latter case, enzyme and standards were denatured as described by L'Italien [30] by treatment with 8 M urea, followed by reduction and alkylation of thiol groups. Denaturated proteins were eluted by buffer A containing 8 M urea at a flow rate of 0.5 ml/min.

Sequence analysis

The N-terminal amino acid sequence was determined by submitting the samples to automatic Edman degradation on a protein sequencer model 470A (Applied Biosystems, Foster City, CA, U.S.A.) as described by Hunkapiller [31]. Separation and identification of the phenylthiohydantoin-amino acids was performed by using an on-line Applied Biosystems analyser model 120A equipped with a control/data module 900A. Internal amino acid sequences were obtained after protein cleavage with CNBr and *N*-chlorosuccinimide (NCS) as described by Fontana and Gross [32]. The fragments were then separated by SDS/PAGE. Electrophoresis was carried out in a discontinuous polyacrylamide system using a 4% (w/v) stacking gel on top of a 10% (w/v) resolving gel as described by Schagger and von Jagow [33]. After the run, the protein pattern was transferred to poly(vinylidene difluoride) membranes [34]. The blotting procedure was performed at 250 mA for 1 h using a Mini Trans-Blot Electrophoretic Transfer Cell apparatus (Bio-Rad Laboratories S.r.l., Milano, Italy). The poly(vinylidene difluoride) membranes were stained with 0.1% Commassie Brilliant Blue R250 in 50% methanol, destained for 5 min with 50% methanol/10% acetic acid and air-dried. The main protein bands resulting from the cleavages with CNBr and NCS were cut out and submitted to microsequence analysis.

The primary structure obtained was compared with those contained in the Protein Identification Resource database of protein sequences (Max Planck Institute, München, Germany).

RESULTS AND DISCUSSION

All results given are means ± S.D. of three determinations. Figure 1 shows reduction of 100 μM DHAA by GSH in the presence or absence of dialysed cytosol. Total DHAA reduction (cytosol plus GSH) is clearly higher than chemical reduction (GSH alone) at all the GSH concentrations tested. The insert to Figure 1 shows the net cytosol-catalysed reduction with typical saturation kinetics. This confirms the presence in the cytosol of an enzyme activity able to reduce DHAA in the presence of GSH.

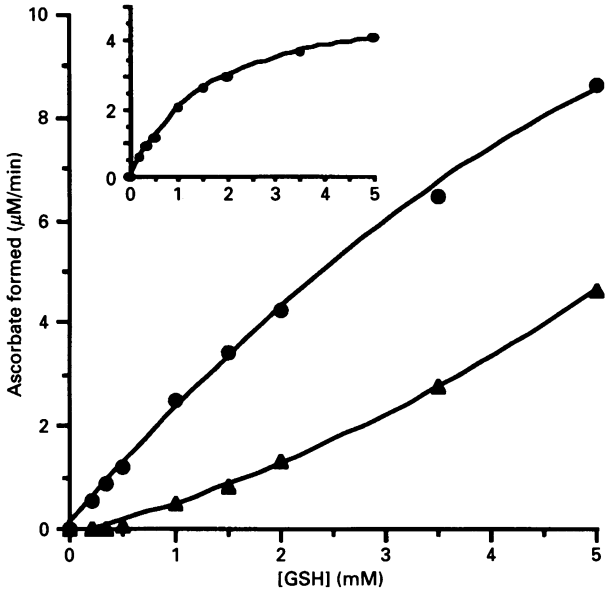

Figure 1 GSH-dependent DHAA reduction

DHAA (100 μM) was incubated in 100 mM potassium phosphate buffer, pH 6.8, in the presence (●) or absence (▲) of dialysed liver cytosol (4 mg of protein/ml), with increasing concentrations of GSH (see the Materials and methods section for details). The inset shows net cytosol-catalysed DHAA reduction, calculated as the difference in activity in the two conditions.

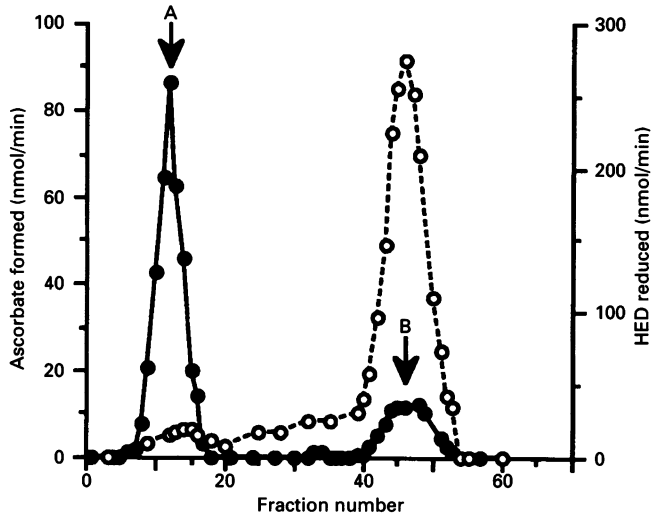

Figure 2 Gel filtration of dialysed rat liver cytosol on Sephadex G-100 SF

Dialysed cytosol was fractionated as detailed in the Materials and methods section. Fractions were assayed for DHAA reductase activity (●) and thioltransferase activity, measured by the reduction of HED (○) (see the Materials and methods section for details).

Table 1 Purification of GSH-dependent DHAA from rat liver cytosol

Purification step	Total protein (mg)	Total activity*	Specific activity†	Purification (fold)	Yield (%)
Cytosol	9699.0	12879	1.3	1.0	100
$(NH_4)_2SO_4$ (55–75% satn.)	2173.3	7636	3.5	2.7	59
DEAE-Sepharose CL-6B	20.3	3265	160.8	123.7	25
Sephadex G-100 SF	2.4	1256	519.2	399.4	10
Reactive Red 120	0.85	649	764.5	588.1	5

* Determined as ascorbate formed [(nmol/min per min) × total volume].

† Determined as ascorbate formed (nmol/min per mg of protein).

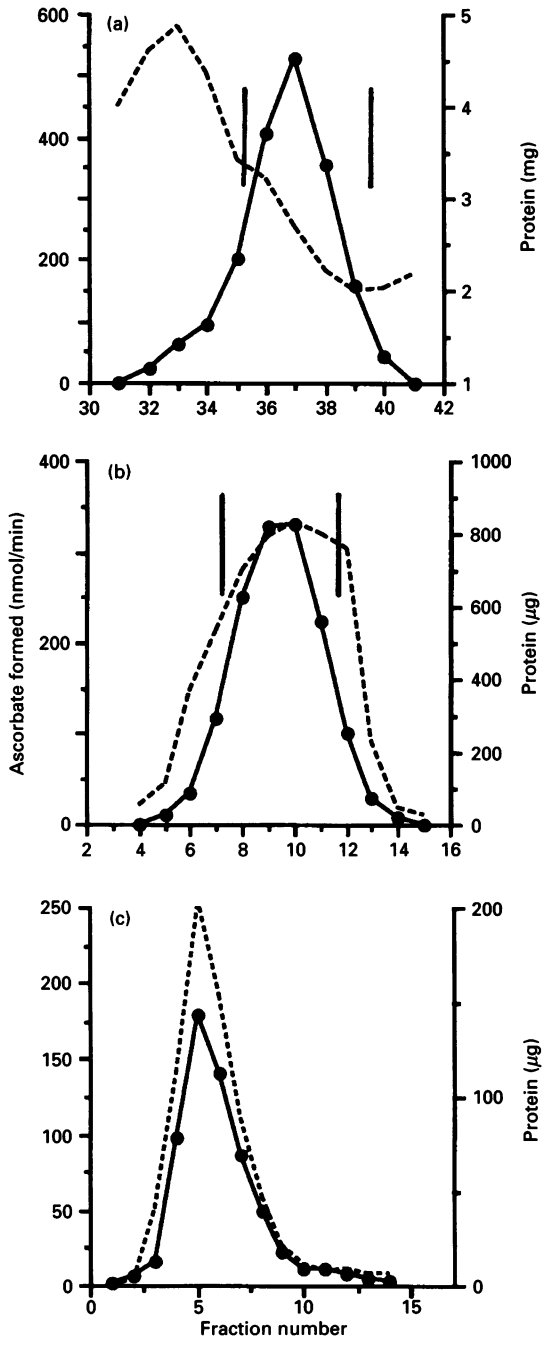

Figure 3 Chromatographic steps for purification of DHAA reductase

Fractions were assayed for protein concentration (----) and DHAA reductase activity (●) (see the Materials and methods section for details). (**a**) DEAE-Sepharose CL 6B chromatography. The protein solution obtained from $(NH_4)_2SO_4$ fractionation was loaded on to a column of DEAE-Sepharose CL 6B (2.5 cm × 9 cm) and eluted as described in the Materials and methods section. Fractions between the two vertical lines were used for the next step of the purification procedure. (**b**) Sephadex G-100 SF chromatography. Fractions from the DEAE-Sepharose CL 6B chromatography were pooled and chromatographed on a Sephadex G-100 SF column (2.5 cm × 50 cm) as described in the Materials and methods section. Fractions between the two vertical lines were used for the next step of the purification procedure. (**c**) Reactive Red 120 chromatography. Fractions from the Sephadex G-100 SF chromatography were pooled and chromatographed on a Reactive Red 120 column (1.5 cm × 2 cm) as described in the Materials and methods section.

Dialysed cytosol was subjected to gel filtration in order to verify whether this DHAA-reducing activity could be recovered

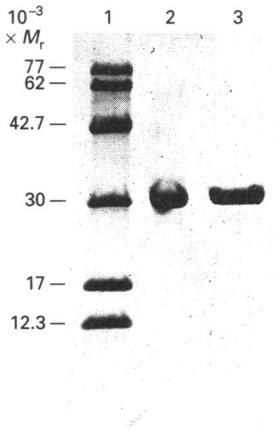

Figure 4 SDS/PAGE of purified DHAA reductase

Lane 1, M_r standards; lane 2, 4 μg of purified enzyme in the absence of 2-mercaptoethanol; lane 3, 4 μg of purified enzyme in the presence of 5% (v/v) 2-mercaptoethanol (see the Materials and methods section for details).

in one or more well-defined fractions. Figure 2 shows the results of a typical experiment. GSH-dependent DHAA reductase activity was recovered in two distinct peaks (A and B). The smaller one (peak B) was eluted with a volume corresponding to an M_r slightly lower than 12000. Peak B was co-eluted with the typical thioltransferase activity of glutaredoxin (i.e. reduction of HED) (Figure 2). This result suggests that part of the cytosolic GSH-dependent DHAA reduction is carried out by glutaredoxin, in accordance with the report of Wells et al. [24]. However, a larger peak (peak A) of GSH-dependent activity was eluted with a volume corresponding to an M_r of approx. 48000. Only a small amount of thioltransferase activity was found in this fraction.

Another enzyme with DHAA reductase activity is PDI [24]. However, it is a membrane-bound enzyme on the endoluminal side of microsomes [35] removable by Triton treatment and having an M_r of 107000 on Sephadex G-200 [36]. In any case, no PDI activity, tested as insulin transhydrogenase activity, was found in the fractions corresponding to peak A (results not shown). A preparation of solubilized PDI was used as a positive control for PDI activity (see the Materials and methods section). The activity of this preparation was 4.6 ± 0.7 nmol/min per mg of protein. Therefore the DHAA reductase activity of peak A should be ascribed to a novel enzyme different from either glutaredoxin or PDI.

Further treatments were carried out to purify the DHAA reductase corresponding to peak A. The enzyme was purified about 600-fold with respect to cytosol by the procedure described in the Materials and methods section with a yield of about 5%. A representative purification is summarized in Table 1. Figures 3(a) and 3(b) show the elution profile of both enzyme activity and protein from DEAE-Sepharose and Sephadex G-100 SF columns respectively. On the basis of SDS/PAGE only a few contaminants were present in the enzyme preparation after Sephadex chromatography (results not shown). These contaminants were removed by elution through the Reactive Red column; a typical elution profile is shown in Figure 3(c). The DHAA reductase recovered after this step was apparently pure, as judged by SDS/PAGE (Figure 4): a single protein band was obtained migrating with an M_r of 31000 ± 1000 in the presence and absence of 2-mercaptoethanol, which indicates the absence of interchain disulphide bridges.

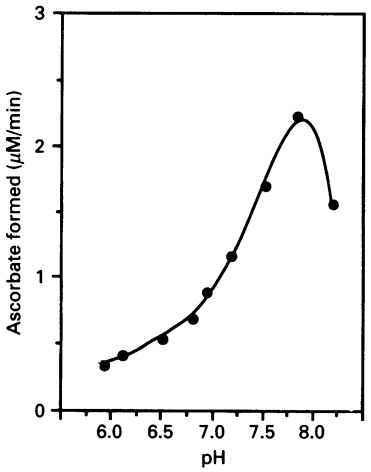

Figure 5 Effect of pH on the activity of DHAA reductase

DHAA reductase activity was measured at various pH values in 100 mM potassium phosphate buffer (see the Materials and methods section for details).

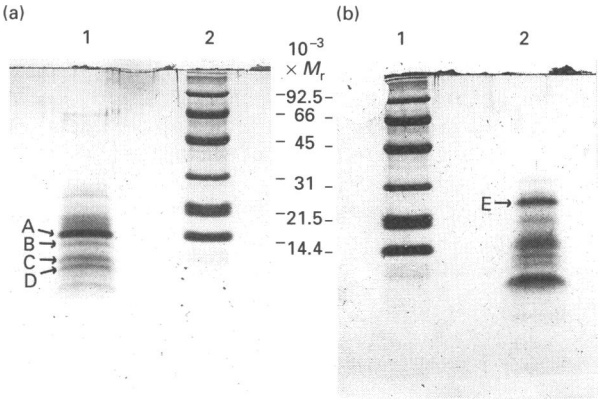

Figure 6 SDS/PAGE of cleaved DHAA reductase

(**a**) Lane 1, CNBr-derived fragments; lane 2, M_r standards. (**b**) Lane 1, M_r standards; lane 2, NCS-derived fragments. The protein bands indicated with letters were subjected to microsequence analysis.

CNBr peptide A

1	5	10	15	20
Val Leu Lys Ala Asn Gly Ile His Xxx Glu Ile Ile Asn Ile Asn Leu Leu Asn Xxx Pro				

CNBr peptide B

1	5	10
Gln Asp Tyr Ala Ile Gly Pro Arg Phe Gln		

CNBr peptide C

1	5	10
Ile Glu Asp Leu Val Ala Ile Glu Xxx Glu		
Pro		Val
		Asn

CNBr peptide D

1	5
Ser Xxx Glu Leu Asn Ala Asp	
Val	Gly

NCS peptide E

1	5
Ser Phe Glu Glu Asn Asn Phe Gly	

Figure 7 N-Terminal primary structure of some peptides obtained by fragmentation of DHAA reductase with CNBr or NCS

Microsequence analysis was carried out on the peptides indicated in Figure 6.

The purified enzyme was subjected to gel filtration on Sephadex G-100 SF. By comparison with standards ranging in M_r from 66000 to 12400 an M_r value of 48900 ± 1000 was calculated. A similar value (48500 ± 650) was obtained by h.p.l.c., whereas h.p.l.c. of the denatured enzyme gave a single peak with an M_r value of 31300 ± 3100 which was identical with that obtained by SDS/PAGE. Thus, however determined, the M_r value of the native enzyme was always higher than that of its denatured form. Such a discrepancy could be ascribed to the protein having an asymmetric shape and/or to interactions between charged protein groups and the gel matrix.

Figure 5 shows the pH-dependence of the enzyme activity. The optimal pH range was 7.5–8.0.

The K_m for GSH, calculated in the presence of 1.5 mM DHAA (at pH 7.2), was 2.8 ± 0.6 mM with a $V_{max.}$ of 4.5 ± 1.3 μmol/min per mg of protein. The apparent K_m for DHAA, calculated in the presence of 3 mM GSH, was 245 ± 62 μM with a $V_{max.}$ of 1.9 ± 0.1 μmol/min per mg of protein. Although not completely saturating for GSH, this concentration kept the rates of spontaneous reaction between GSH and DHAA low compared with the catalysed reaction.

As for the partially purified enzyme (peak A in Figure 2), the purified enzyme was devoid of either thiotransferase or insulin transhydrogenase activity (results not shown).

Exposure of the purified enzyme to 50 or 75°C for 5 min decreased its activity by 50 and 90% respectively.

The enzyme was capable of reducing isodehydroascorbate as effectively as DHAA (results not shown). The ability of other thiol compounds to substitute for GSH was also tested. Cysteine and acetylcysteine proved to be very poor substrates (8 and 4% of the GSH-driven activity respectively); no enzymic DHAA reduction at all was detected with lipoate, CoA, dithiothreitol or 2-mercaptoethanol. Thus DHAA reductase seems to have a quite specific requirement for GSH as hydrogen donor.

Microsequence analysis conducted on the whole protein gave no results, indicating that its N-terminus was blocked. In order to obtain information on the internal primary structure, we cleaved the protein at methionine and tryptophan. Fragmentation of the protein with CNBr and NCS led to the production of a series of polypeptides that were well resolved by SDS/PAGE (Figure 6). The proteolytic fragments corresponding to the main protein electrophoretic bands were submitted to microsequence analysis to determine their N-terminal primary structure. Sequence data were obtained by analysis of bands from both the cleavage procedures (Figure 7) and were compared with the known primary structures contained in the Protein Identification Resource databank. No sequence similarity was found to any of the proteins in the database, utilizing pentapeptide stretches with the Protein Identification Resource 'scan' option. Thus our results indicated that the protein has not one of the known primary structures.

In conclusion, our data demonstrate the presence in the rat liver cytosol of a DHAA reductase that is different from glutaredoxin. This novel enzyme accounts for most of the DHAA

reduction carried out by liver cytosol, at least under our experimental conditions (see Figure 2). The lack of similarity to other previously sequenced proteins means that it must be structurally different from the two enzymes already known to carry out GSH-dependent DHAA reduction (i.e. glutaredoxin and PDI). We do not know at present whether DHAA reduction is the only property of this protein. Further studies are required to obtain a better understanding of this new enzyme.

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