Purification of NADPH-dependent dehydroascorbate reductase from rat liver and its identification with 3α-hydroxysteroid dehydrogenase

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Rat liver cytosol has been found to reduce dehydroascorbic acid (DHAA) to ascorbic acid in the presence of NADPH. The enzyme responsible for such activity has been purified by ammonium sulphate fractionation, DEAE-Sepharose, Sephadex G-100 SF and Reactive Red column chromatography, with an overall recovery of 27%. SDS/PAGE of the purified enzyme showed one single protein band with an M_r of 37500. A similar value (36800) was found by gel filtration on a Sephadex G-100 SF column. The results indicate that the enzyme is a homogeneous monomer. The K_m for DHAA was 4.6 mM and the $V_{max.}$ was 1.55 units/mg of protein; for NADPH K_m and $V_{max.}$ were 4.3 μM and 1.10 units/mg of protein respectively. The optimum pH was around 6.2. Several typical substrates and inhibitors of the aldo–keto reductase superfamily have been tested. The strong inhibition of DHAA reductase effected by steroidal and non-steroidal anti-inflammatory drugs, together with the ability to reduce 5α-androstane-3,17-dione strongly, suggest the possibility that DHAA reductase corresponds to 3α-hydroxysteroid dehydrogenase. Microsequence analysis performed on the electro-transferred enzyme band shows that the N-terminus is blocked. Internal primary structure data were obtained from CNBr-derived fragments and definitely proved the identity of NADPH-dependent DHAA reductase with 3α-hydroxysteroid dehydrogenase.

INTRODUCTION

Ascorbic acid (AA) plays an important role in a variety of biological functions by participating in physiological reactions such as the production of collagen and catecholamine (reviewed in [1]), by sparing other antioxidants such as α-tocopherol, through its recycling [2,3] and by scavenging toxic free-radical products derived from carcinogens, oxidative metabolism, radiation and phagocytic infiltration [4–8].

All these reactions of AA utilize its property of being easily oxidized. The first oxidation (one-electron transfer) produces the ascorbate free radical (AFR); the disproportionation of pairs of AFRs not immediately reduced, or direct two-electron transfer, produce the fully oxidized dehydroascorbic acid (DHAA).

Along with synthesis *de novo* and dietary supply of AA, it is advantageous economically for the cell to recycle the oxidized forms by reducing them back to AA. With regard to AFR, several authors have reported the presence of a NADH-dependent AFR reductase activity in cellular membranes [9–11]. By contrast, such an activity is absent in the cytosol. As far as the fully oxidized form is concerned, the central role of GSH in reducing DHAA back to AA has been documented for a long time [12–15]. Recently, Wells et al. [16] have reported that purified glutaredoxin, a cytosolic enzyme, and protein disulphide isomerase, a microsomal enzyme, exhibit GSH-dependent DHAA reductase activity. Furthermore, a novel GSH-dependent enzyme of rat liver cytosol has been purified to homogeneity and characterized by us [17].

The possibility that multiple enzymatic pathways for DHAA reduction exist has been considered by Choi and Rose [18]. In rat colon mucosa, in fact, they described a DHAA reductase activity which requires NADPH rather than GSH as a hydrogen donor.

In view of this suggestion, we looked for such an activity in rat liver cytosol. The present study reports the existence of NADPH-dependent DHAA reductase activity also in liver cytosol; the purification and properties of the enzyme are described. Characterization studies carried out on the purified enzyme clearly show the identity of the NADPH-dependent DHAA reductase with 3α-hydroxysteroid dehydrogenase, an enzyme abundantly represented in rat liver cytosol which displays a number of functions [19–22].

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. Following ether anaesthesia of the animal, 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, M_r cut-off 6000–8000, Serva Feinbiochemica, Heidelberg, Germany) against two changes of a total 340 vol. of the homogenization buffer. The contents of the dialysis bag was centrifuged for 10 min at 1500 *g* to remove particles.

Enzyme purification

Unless otherwise stated, all steps were performed at 0–4 °C.

Liver cytosol, prepared from five rats by the procedure described above, was diluted 1.5-fold with buffer A and used for the following step.

Abbreviations used: AA, ascorbic acid; DHAA, dehydroascorbic acid; AFR, ascorbate free radical; PIR, Protein Identification Resource; PVDF, polyvinylidene difluoride.

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Step 1: ammonium sulphate fractionation

Powdered ammonium sulphate (46.6 g) was slowly added at 0 °C to the cytosol (162 ml) to give 50% 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 (175 ml), 34.0 g of powdered ammonium sulphate were added resulting in 80% saturation and the mixture was stirred and centrifuged again. The pellet was redissolved in 35 ml of 10 mM potassium phosphate buffer, pH 7.8 (buffer B), 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 used for the next step.

Step 2: DEAE-Sepharose chromatography

The protein solution obtained at step 1 was applied to a DEAE-Sepharose CL 6B (Sigma, St. Louis, MO, U.S.A.) column (2.5 cm × 9 cm) previously equilibrated with buffer B. After rinsing with 250 ml of the same buffer, the column was eluted with a 400 ml NaCl gradient set up as follows: chamber 1, stirred and connected to the column, containing 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 to a flow rate of 90 ml/h. The same flow rate was used for delivering the mixture to the column. Eluate fractions of volume 10 ml were collected and assayed for DHAA reductase activity. Fractions with activity were pooled and concentrated to 1.5 ml by ultrafiltration (Centriprep 10; Amicon, Beverly, MA, U.S.A.).

Step 3: gel-filtration chromatography

The sample obtained at 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 the first 125 ml, eluate fractions of volume 2.5 ml were collected. Fractions showing DHAA reductase activity were pooled, concentrated to 2.5 ml by ultrafiltration (Centricon 10, Amicon), and used for the next step.

Step 4: Reactive Red 120 chromatography

The sample obtained at step 3 was applied to a Reactive Red 120 3000-CL (Sigma) column (1.5 cm × 2 cm) equilibrated with buffer A. After rinsing with 20 ml of the same buffer, 2.5 ml fractions were collected with buffer A plus 1.5 M NaCl, pH 7.8, with gravity flow. Before testing, the fractions were passed through Sephadex G-25 columns (PD-10 columns; Pharmacia LKB Biotechnology, Uppsala, Sweden) equilibrated with buffer A in order to remove NaCl. Fractions containing DHAA reductase activity were pooled and stored at 0–4 °C. This protein was used as the final enzyme preparation for the experiments described below.

Enzyme assay conditions

Reaction mixtures consisted of: buffer A, enzyme solutions at various steps of purification, 200 μM NADPH, 6 mM DHAA, to give a final pH of 6.9. DHAA was prepared immediately before the assay by exposing a solution of AA in water to liquid bromine; excess bromine was removed by bubbling the solution with N_2 [13,23]. Control experiments were performed by using standard DHAA (Aldrich, Milwaukee, WI, U.S.A.). NADPH solution was freshly prepared in 10 mM potassium phosphate buffer, pH 8.0, to minimize the non-specific acid destruction. Reactions were initiated by the addition of substrate (DHAA) and the decrease in absorbance at 340 nm, associated with the oxidation of NADPH, was monitored at 30 °C. A molar absorption coefficient of 6220 litre·mol^{-1}·cm^{-1} was used. No oxidation of NADPH was observed in the presence of DHAA alone. The direct assay, i.e. the monitoring of the change in absorbance at 265 nm associated with the formation of AA [15], could not be used, due to the high absorbance of NADPH at that wavelength. However, identification of the reaction product (AA) was performed by h.p.l.c. [24] in many instances (see, for example, Figure 1). One unit of DHAA reductase activity was defined as the quantity of enzyme catalysing either the oxidation of 1 μmol of NADPH/min or the formation of 1 μmol of AA/min under the conditions of the assay.

SDS/PAGE

SDS/PAGE on slab gel was performed by the method of Laemmli [25] with 5% acrylamide for the stacking gel and 15% acrylamide for the separating gel. Samples were denaturated by boiling for 3 min in 0.1% (s/v) SDS in the presence of 5% (v/v) 2-mercaptoethanol. The gels were stained with Coomassie Brilliant Blue.

Determination of M_r

M_r of the native enzyme was estimated by gel filtration on a Sephadex G-100 SF column (same procedure as in step 3 of Enzyme purification section). The column was calibrated with BSA (M_r 66000), ovalbumin (M_r 45000), carbonic anhydrase (M_r 29000) and cytochrome *c* (M_r 12400). The M_r of the denatured enzyme was determined by SDS/PAGE as specified above.

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.) according to Hunkapiller [26]. The separation and identification of the phenylthiohydantoin amino acids was performed by an on-line phenylthiohydantoin analyser model 120A equipped with a control/data module 900A (Applied Biosystems). Internal amino acids sequences were obtained after protein cleavage with CNBr as described by Fontana and Gross [27]. The fragments were then separated by SDS/PAGE. The 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 [28]. After the run, the protein pattern was transferred to polyvinylidene difluoride (PVDF) membranes [29]. The blotting procedure was performed at 250 mA for 1 h using a Mini Trans-Blot Electrophoretic Transfer Cell apparatus (Bio-Rad Laboratories, Milano, Italy). The PVDF membranes were stained with 0.1% Coomassie Brilliant Blue R250 in 50% methanol, destained for 5 min in 50% methanol/10% acetic acid and air dried. The main protein bands resulting from the cleavage with CNBr were cut and submitted to microsequence analysis.

The primary structure obtained was compared with those contained in the PIR (Protein Identification Resource; Max Planck Institute, Munchen, Germany) data base of protein sequences.

RESULTS AND DISCUSSION

A NADPH-dependent DHAA reductase activity was observed to be present in rat liver cytosol. As shown in Figure 1, the DHAA reduction was evaluated both by NADPH oxidation and by AA formation. The two methods gave virtually the same results; a molar ratio of 1.2 ± 0.1 (NADPH consumed/AA formed) was calculated. To evaluate DHAA reduction activity, NADPH oxidation only has been used in all subsequent experiments.

The following studies have been dedicated to the purification of the enzyme responsible for this activity. Table 1 summarizes the results of a typical purification procedure, involving ammonium sulphate fractionation, followed by DEAE-Sepharose, Sephadex G-100 SF and Reactive Red chromatographies. The enzyme was obtained with an overall activity recovery of 27% and 125-fold purification.

The enzyme recovered after the last chromatographic step appeared pure on the basis of SDS/PAGE (Figure 2), which showed one single protein band with a M_r of 37500 ± 500 (mean $\pm$ S.D. of three determinations). On gel-filtration on Sephadex G-100 SF, the native enzyme showed a M_r of 36800 ± 4200 (mean $\pm$ S.D. of three determinations). The enzyme should therefore by considered as an homogeneous monomer.

The K_m for DHAA, calculated in the presence of 250 μM NADPH at pH 6.8, was 4.6 ± 0.8 mM, with a $V_{max.}$ of 1.55 ± 0.05 units/mg of protein (mean $\pm$ S.D. of three determinations); the K_m and $V_{max.}$ values for NADPH, calculated in the presence of 12 mM DHAA, were 4.3 ± 0.6 μM and 1.10 ± 0.07 units/mg of protein respectively (mean $\pm$ S.D. of three determinations).

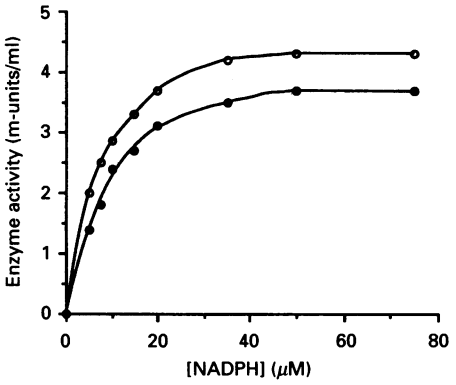

Figure 1 NADPH-dependent DHAA reductase activity in rat liver cytosol

DHAA (1 mM) was incubated in 100 mM potassium phosphate buffer, pH 7.2, in the presence of dialysed liver cytosol (4 mg of protein/ml) with increasing concentrations of NADPH. The enzymatic activity was evaluated either by measuring NADPH oxidation (○) or ascorbic acid formation (●) (see the Materials and methods section for details). No DHAA reduction was observed in the absence of dialysed cytosol.

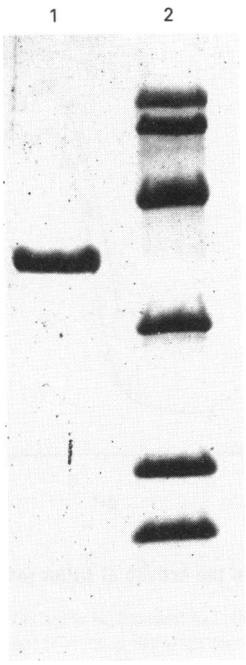

Figure 2 SDS/PAGE of purified DHAA reductase

Lane 1, 4 μg of purified enzyme; lane 2, M_r standards (top to bottom: 77000, 62000, 42700, 30000, 17000, 12300) (see the Materials and methods section for details).

Figure 3 shows the pH dependence of the enzyme activity. Optimum pH was around 6.2. The exposure of the purified enzyme to 56 °C for 5 and 10 min resulted in an activity decrease of 44 and 92% respectively. NADH, up to 350 μM, proved to be a very poor co-factor for the reduction of DHAA, accounting for 9% of the reduction compared with the same concentration of NADPH. Isodehydroascorbic acid was reduced 2-fold more effectively than DHAA (results not shown).

The data reported above strongly suggest that this enzyme is a member of the aldo–keto reductase superfamily. In fact this enzyme shares typical features with this superfamily, such as monomeric structure with relatively low M_r (30000–40000), dependence on NADPH as cofactor and localization in the cytoplasm. In addition three vicinal carbonyl groups are present in the DHAA molecule, and this might make it a suitable

Table 1 Purification of NADPH-dependent DHAA reductase from rat liver cytosol

Purification step	Total protein (mg)	Total activity*	Specific activity†	Purification factor	Yield (%)
Cytosol	1710	6177	3.6	1	100
$(NH_4)_2SO_4$ (50–80% satd.)	765	4694	6.1	1.7	76
DEAE-Sepharose CL-6B	24	3622	151	42	59
Sephadex G-100 SF	5.2	1889	363	101	31
Reactive Red 120	3.7	1666	450	125	27

* Determined as (m-units/ml) × total volume.
† Determined as m-units/mg of protein.

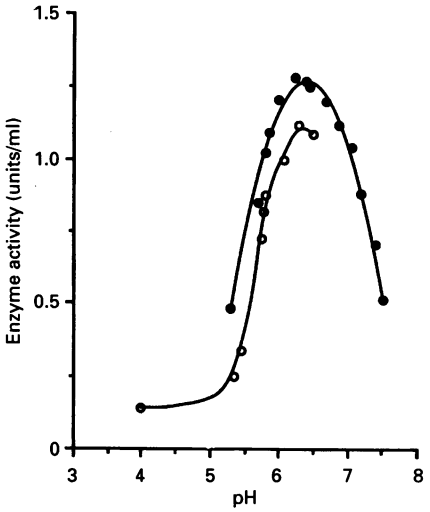

Figure 3 Effect of pH on the activity of DHAA reductase

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

Table 2 Substrate specificity of DHAA reductase

Enzyme activity was measured in the presence of purified enzyme and 200 μM NADPH in 100 mM potassium phosphate buffer, to give a final pH of 6.8 at 30 °C. Relative values of enzyme activity are percentages of that obtained with 6 mM DHAA. Substrates which were not sufficiently soluble in buffer were dissolved in methanol (control velocities were determined in the presence of appropriate quantities of the solvent). N.D., not detectable.

Substrate	Concentration	Relative enzyme activity (%)
Hydrindantin	62.5 μM	97
2,6-Dichlorophenol-indophenol	100 μM	20
D-Glucose	200 mM	0
Mannitol	22.5 mM	0
D-Glucuronate	50 mM	0
Chloral hydrate	6 mM	128
Phenylglyoxal	0.5 mM	144
	1 mM	225
DL-Glyceraldehyde	20 mM	37
	40 mM	64
4-Nitrobenzaldehyde	50 μM	182
4-Nitroacetophenone	2 mM	48
9,10-Phenanthrenequinone	5 μM	390
	10 μM	405
1,4-Benzoquinone	15 μM	158
	125 μM	281
Menadione	50 μM	N.D.
5α-Androstane-3,17-dione	6.25 μM	137

substrate for an enzyme of this class. In order to verify the hypothesis that DHAA reductase is a member of the aldo–keto reductase superfamily, several typical substrates have been tested (Table 2). Quinones such as 9,10-phenanthrenequinone and 1,4-benzoquinone as well as 4-nitrobenzaldehyde and 5α-androstane-3,17-dione were the best substrates. D-Glucuronate, the prototype substrate of aldehyde reductase [30], was not reduced; DL-glyceraldehyde, a typical substrate of aldose reductase [31], was reduced only at high concentration; glucose and mannitol were not reduced. With 9,10-phenanthrenequinone, 4-nitrobenzaldehyde and 5α-androstane-3,17-dione as substrates, NADH (200 μM) also proved to be a good cofactors accounting for 83%, 29% and 125%, respectively, compared with the same concentration of NADPH.

Table 3 Effects of inhibitors of DHAA reductase

Purified enzyme was incubated with the inhibitor at the indicated concentration for 2 min at 30 °C prior to initiating the enzyme reaction by the addition of 200 μM NADPH and 6 mM DHAA. Results are presented as the percentage relative DHAA reductase activity when compared with that in a control reaction in which the inhibitor was omitted. Inhibitors which were not sufficiently soluble in buffer were dissolved in methanol (control velocities were determined in the presence of appropriate quantities of the solvent).

Inhibitor	Concentration	Relative enzyme activity (%)
None	–	100
AA	6.0 mM	67
$NADP^+$	0.1 mM	51
Phenobarbital	1.0 mM	91
Pyrazole	10 mM	91
Dicoumarol	0.1 mM	51
1,10-Phenanthroline	1.0 mM	66
Quercetin	5 μM	71
	20 μM	45
	40 μM	5
Rutin	10 μM	46
Indomethacin	1.25 μM	46
	2.50 μM	33
	5.0 μM	17
6α-Methylprednisolone	5 μM	10.0
	10 μM	7.2
Betamethasone	5 μM	21
	10 μM	16

Table 3 lists a number of compounds which were tested as inhibitors of DHAA reductase. The enzyme is inhibited both by the end product, AA, and by the oxidized form of the cofactor, i.e. $NADP^+$. Since the enzyme is not significantly inhibited by pyrazole, it is clearly distinct from alcohol dehydrogenase. It can also be distinguished from aldehyde and aldose reductase, due to the lack of inhibition by phenobarbital [32]. This is in accordance with the fact that the enzyme does not reduce D-glucuronate and only moderately reduces DL-glyceraldehyde. Dicoumarol, which strongly inhibits DT-diaphorase at nM concentrations [33], inhibited DHAA reductase by 50% at concentrations of 100 μM. The best inhibitors were indomethacin, a non-steriodal anti-inflammatory agent, 6α-methylprednisolone and betamethasone, two steroidal anti-inflammatory agents, and quercetin and rutin, two flavonoid compounds.

As far as aldo–keto reductases are concerned, such a strong inhibition by anti-inflammatory drugs has been observed only in the case of rat liver 3α-hydroxysteroid dehydrogenase [19,34]. 5α-Androstane-3,17-dione, one of the typical substrates of the last enzyme, is also reduced by DHAA reductase (see Table 2). Similarly to 3α-hydroxysteroid dehydrogenase, DHAA reductase is also able to oxidize androsterone (50 μM) in the presence of both NAD^+ and $NADP^+$ (2.3 mM), with specific activities of 391 and 506 m-units/mg of protein.

Since these data suggest the possibility that DHAA reductase corresponds to 3α-hydroxysteroid dehydrogenase, micro-sequence analysis was performed on the protein in order to verify such a hypothesis. Like 3α-hydroxysteroid dehydrogenase [35], the N-terminus of the whole protein was blocked. In order to obtain any internal primary-structure information, we cleaved

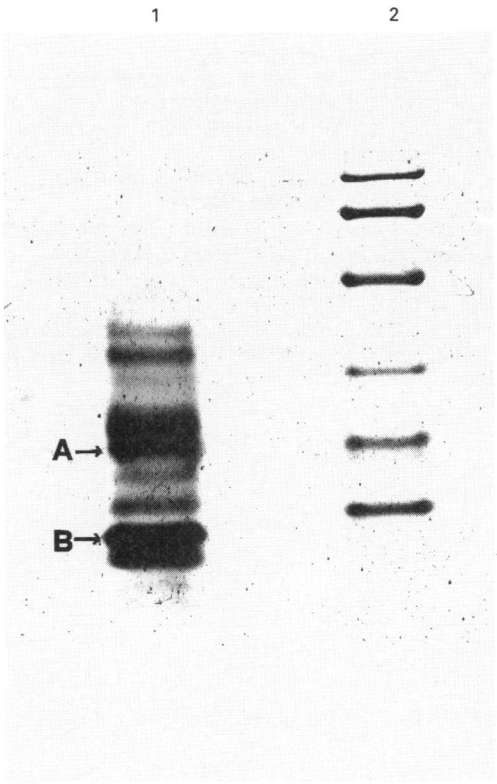

Figure 4 SDS/PAGE of cleaved DHAA reductase

Lane 1, CNBr-derived fragments; lane 2, M_r standards (top to bottom: 92500, 66000, 45000, 31000, 21500, 14400).

Peptide A1

Ala-Leu-Gln-Pro-Gly-Asp-Ile-Phe-Phe-Pro-Arg-Asp-Glu-His-Gly
121

Peptide A2

Glu-Lys-Cys-Lys-Asp-Ala-Gly-Leu-Ala-Lys-Ser-Ile-Gly-Val-Ser
152

Peptide A3

Leu-Asp-Tyr-Cys-Lys-Ser-Lys-Asp-Ile-Ile-Leu-Val-Ser-Tyr-Cys
203

Peptide B

Asp-Ser-Ile-Ser-Leu-Arg-Val-Ala-Leu-Asn-Asp-Gly-Asn-Phe-Ile
2

Figure 5 N-terminal primary structure of some peptides obtained by fragmentation of DHAA reductase with CNBr

Microsequence analysis was carried out on the peptides indicated in Figure 4. The position of the first amino acid residue in the whole sequence of 3α-hydroxysteroid dehydrogenase is also indicated.

the protein at methionine bonds with CNBr. Fragmentation of the protein led to the production of a series of polypeptides, well resolved in the Tricine electrophoresis gel (Figure 4). The proteolytic fragments corresponding to the main protein electrophoretic bands were subjected to microsequence analysis to determine their N-terminal primary structures. A first stretch of amino acid residues was obtained from Edman degradation of band B (Figure 4). The sequence obtained was used for a databank search (PIR, Protein Identification Resource): the primary structure was found to correspond to the N-terminal sequence of 3α-hydroxysteroid dehydrogenase [35], following the removal of the first modified methionine. Further confirmation of the identification was gained by sequencing band A, which was composed of three polypeptides (Figure 5), again easily identified as corresponding to internal sequences of 3α-hydroxysteroid dehydrogenase.

3α-Hydroxysteroid dehydrogenase is known to catalyse the NAD(P)$^+$-linked oxidoreduction of a wide variety of substrates, and, besides its activity as a hydroxysteroid dehydrogenase, it also displays a quinone reductase activity [19], an aromatic alcohol dehydrogenase activity [19], a dihydrodiol dehydrogenase activity [19–21], and a hydroxyprostaglandin dehydrogenase activity [22]. Our data attribute a new function to this versatile enzyme, i.e. the capacity to reduce DHAA to AA, thus regenerating the functional form of vitamin C. The enzyme therefore constitutes a further pathway for the reduction of DHAA, in addition to glutaredoxin [16] and to the GSH-dependent DHAA reductase previously purified by us [17].

The presence of three enzymatic pathways that catalyse the same reaction emphasizes the importance of AA in the cellular metabolism. The plurality of forms takes on added meaning when one considers physiology and pathology. Under physiological conditions, the basal level of DHAA could be effectively decreased by GSH-dependent DHAA reductase, due to the low K_m of this enzyme for DHAA. In many pathological conditions, where an oxidative stress occurs, intracellular GSH concentration falls and the DHAA level can increase significantly. In this situation the activity of NADPH-dependent DHAA reductase can be set into motion, due to its very high affinity for NADPH (K_m below 5 μM), in spite of the low affinity for DHAA (in terms of the calculated K_m).

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