FEBS Letters 352 (1994) 159–162

FEBS 14560

A novel dehydroascorbate reductase from spinach chloroplasts homologous to plant trypsin inhibitor

Susanne Trümper, Hartmut Follmann, Ingo Häberlein*

Biochemie, Fachbereich Biologie-Chemie der Universität Kassel, D-34109 Kassel, Germany

Received 21 July 1994; revised version received 18 August 1994

Abstract Dehydroascorbate reductase has been isolated from spinach chloroplasts and purified to apparent homogeneity. The N-terminal amino acid sequence of the enzyme is homologous to the Kunitz-type trypsin inhibitors from plant sources. It is shown that spinach DHA reductase and soybean trypsin inhibitor are both capable of reducing dehydroascorbate when in the reduced (thiol) form but acquire trypsin-inhibiting activity in the oxidized (disulfide) state. Reduced chloroplast thioredoxins also reduce dehydroascorbate.

Key words: Chloroplast enzyme; Dehydroascorbate reductase; Glutaredoxin; Spinach (*Spinacia oleracea*); Thioredoxin; Trypsin inhibitor

1. Introduction

Plant cells possess versatile antioxidant systems to protect organelles, membranes, and enzymes from the damaging action of toxic activated oxygen species [1,2]. In chloroplasts, H_2O_2 is produced in the Mehler reaction and also during superoxide dismutase-catalyzed disproportionation of the superoxide radical anion. H_2O_2 is then scavenged in a reaction sequence involving the redox pairs ascorbate/dehydroascorbate, reduced and oxidized glutathione, and NADPH/NADP. The first step of H_2O_2 degradation is performed by ascorbate peroxidase (EC 1.11.1.11) which reduces H_2O_2 to water. The primary product of ascorbate oxidation is monodehydroascorbate (MDA, also referred to as 'ascorbate free radical'). MDA is returned into the ascorbate pool by a NAD(P)H-dependent MDA reductase (EC 1.6.5.4). However, MDA readily disproportionates non-enzymatically to ascorbate and dehydroascorbate (DHA), necessitating an additional reducing system to regenerate ascorbate from DHA. DHA is reduced by glutathione, either non-enzymatically at a slow rate, or at much higher rates catalyzed by DHA reductase (EC 1.8.5.1) [3]. The oxidized glutathione formed will finally be reduced by glutathione reductase and NADPH as electron donor. Since NADPH is supplied by the photosystems II and I the reduction of H_2O_2 is a light-dependent process and coupled to the evolution of oxygen [4].

The capacity of this complex reaction sequence for efficient degradation of H_2O_2 in chloroplasts is part of the molecular mechanisms which determine the susceptibility of plants to different kinds of stress such as photoinhibition, high and low temperature, and drought [1,5,6]. Ascorbate is also involved in regenerating α-tocopherol from the α-chromanoxy radical, or in the synthesis of zeaxanthin in the xanthophyll cycle [1,7]. Obviously it plays a central role in the detoxification of activated oxygen and other antioxidant processes in chloroplasts.

All these functions depend on the continuous regeneration of ascorbate from its oxidized forms by MDA reductase and DHA reductase. Among the chloroplast enzymes involved in scavenging H_2O_2, only DHA reductase has remained poorly characterized which may in part be due to its low stability. Sometimes (e.g. in tomato plants [8]) the activity could not be found at all in crude extracts of isolated chloroplasts or leaves; the enzyme has been demonstrated in potato tuber, pea shoots, spinach leaves [3,9–12] but its state of purification is unsatisfactory. Other proteins such as glutaredoxin (thioltransferase) and protein disulfide isomerase (PDI) have also been shown to possess DHA reducing activity [13]. Here we describe the first complete purification of DHA reductase from spinach chloroplasts and its N-terminal amino acid sequence which, unexpectedly, relates the protein to Kunitz protease inhibitor.

2. Materials and methods

All chemicals and reagents were of highest purity available. Dehydroascorbate was obtained from Merck and glutathione from Pharma Waldhof. Soybean trypsin inhibitors were purchased from Sigma and Boehringer-Mannheim. Spinach thioredoxins were a gift from Dr. P. Schürmann, University of Neuchâtel. Glutaredoxin and thioredoxin from *E. coli* were prepared by published procedures.

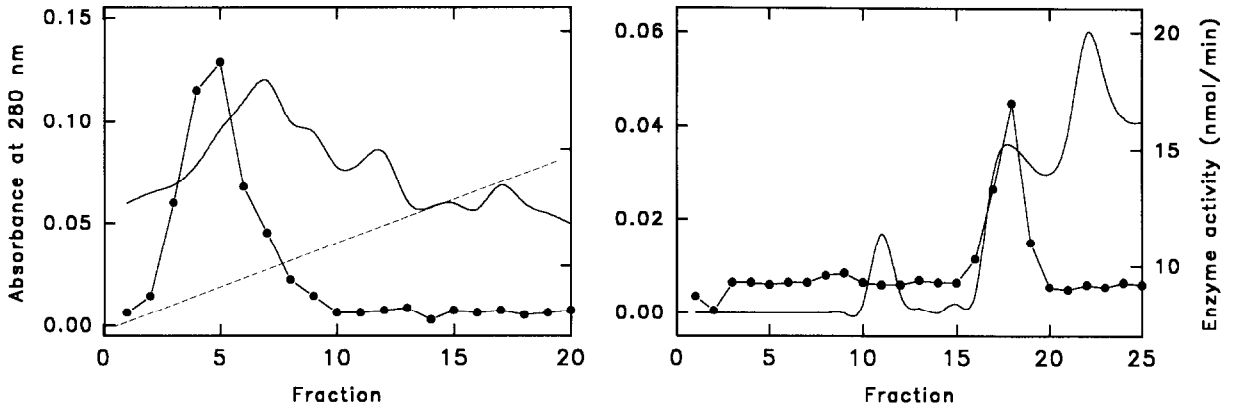
Isolation of intact chloroplasts from spinach leaves followed the method described by Mourioux and Douce [14]. The chloroplasts were washed three times. They were then incubated in a hypotonic, 25 mM K-phosphate (pH 7.0) buffer and heated to 80°C for 5 min. Heat-stable soluble proteins were separated from organelle debris and denatured proteins by centrifugation at 15,000 rpm for 15 min. The supernatant was applied onto a DEAE cellulose column (Whatman DE-52), and a salt gradient of 0–250 mM KCl in 2 × 100 ml was used to elute dehydroascorbate reductase (column size, 1.6 × 3 cm; flow rate, 30 ml/h). Fractions with DHA reductase activity were pooled and concentrated to a volume of 5 ml by Aquacide II. Gel filtration was performed on a Superdex 200 (Highload XK 16/60) column (Pharmacia) equilibrated with a 50 mM K-phosphate/100 mM NaCl buffer (pH 7.0). Fractions of 5 ml were collected at a flow rate of 1.5 ml/min. Fractions with dehydroascorbate reductase activity were pooled and again concentrated to 5 ml with Aquacide II. The protein samples were rechromatographed on the Superdex 200 column under identical conditions. Purity of the active fractions was analyzed by SDS-polyacrylamide gelelectrophoresis on 12% gels in a Tricine buffer system [15].

Dehydroascorbate reductase activity was assayed spectrophotometrically in 50 mM K-phosphate (pH 7.0) buffer containing 2 mM GSH, 1 mM dehydroascorbate, and 0.1 ml of enzyme sample in a total volume of 1 ml. The absorbance increase at 265 nm was observed for 2 min. Non-enzymatic reduction of dehydroascorbate by GSH was measured

*Corresponding author.

Abbreviations: DHA, dehydroascorbate; GSH, GSSG, reduced and oxidized glutathione; MDA, monodehydroascorbate.

SSDI 0014-5793(94)00947-3



1. Chromatography of dehydroascorbate reductase from spinach chloroplasts on DEAE cellulose (left) and Superdex 200 columns (right). Cf. ion 2 for experimental details. Drawn line, protein content (left scales); dashed line, KCl gradient (0–250 mM); ●, enzyme activity (right scales).

separate cell, and the increment over that blank activity was taken lehydroascorbate reductase activity. The absorbance coefficient of)rbate at 265 nm is $\varepsilon = 14{,}900\ M^{-1} \cdot cm^{-1}$.

lehydroascorbate reductase activity of trypsin inhibitors and thiore- in was observed after reduction with 5 mM dithiothreitol. 1.5 mg tein were dissolved in 0.25 ml of 50 mM K-phosphate buffer (pH and incubated with an equal volume of 10 mM dithiothreitol for h. A PD 10 column (Pharmacia) was then used to remove excess iothreitol. The protein-containing fractions (0.4 ml) were collected reas dithiothreitol eluted far behind the protein. Dehydroascorbate ıction was followed spectrophotometrically for 1 min in the absence ;SH. Trypsin inhibitor activity was determined in a standard trypsin ıy with *N*-benzoyl L-arginine-4-nitranilide as substrate [16].

or microsequencing, the purity of protein samples was verified by electrophoresis as described in [17]. Sequencing was done on an)lied Biosystems model 477A sequencer with HPLC identification)henylthiohydantoin amino acids.

Results

n view of the chloroplastic function of DHA reductase we)se isolated chloroplasts from spinach as a source for purifi- ion of the enzyme. Using modified assay conditions with ıydroascorbate and glutathione as substrates at pH 7 DHA uction could be readily detected in chloroplast extracts. The ivity remained in the heat-stable protein fraction under rk-up conditions common in the preparation of chloroplast)redoxins (see below). The enzyme was subjected to DEAE ulose ion exchange chromatography and to gel filtration on uperdex 200 column for purification (Fig. 1). DHA reduc- e eluted at a position corresponding to M_r = 38,000 Da from latter column. After rechromatography the material ap- ıred as a homogeneous protein of M_r = 20,000 Da on SDS polyacrylamide gel electrophoresis (Fig. 2) suggesting that the native enzyme is a dimer. The protocol leading to about 50-fold purification is summarized in Table 1. A purification scheme in which the heat denaturation step had been omitted did not reveal the presence of additional, potentially heat-sensitive enzyme fractions in chloroplasts.

Apparent K_m values for dehydroascorbate and GSH were determined with the pure enzyme. The data (1.0 mM and 7.0 mM, respectively, at pH 7.0 and 25°C) are in close agreement with those reported for partially purified DHA reductase preparations from potato and spinach [3,10]. The activity profile of spinach chloroplast DHA reductase had a maximum at pH 8.3. However, at that alkaline pH the enzyme was much less stable ($t_{1/2} \approx 30$ h at 4°C) than at pH 7 ($t_{1/2} > 1$ week), and at pH 7 it was active enough for all practical purposes. The enzyme was inhibited by iodoacetate and *N*-ethyl maleimide, indicating that SH groups are required for the reaction.

It was of interest to compare the new protein with the multiple forms of DHA reductase previously separated in an extract of whole spinach leaves [3]. The chloroplast enzyme clearly coincided with the first activity peak eluting from a DEAE Sephacel column (Fig. 1 in [3]) whereas all other DHA reducing fractions found in the leaf extract had no counterpart in the chloroplast. The various spinach DHA reductases also differed in their heat sensitivity, with the chloroplast protein being the only heat-stable fraction.

A sample of homogeneous protein was subjected to 2D-electrophoresis and then to microsequencing. Sequence alignment of the 17 N-terminal amino acids revealed, surprisingly, complete identity with the sequence of Kunitz trypsin inhibitor of soybean, and up to 50% homology to other plant protease

)le 1
ification of dehydroascorbate reductase from spinach leaf chloroplasts

	Total protein (mg)	Total activity ($nmol \cdot min^{-1}$)	Specific activity ($nmol \cdot min^{-1} \cdot mg^{-1}$)	Purification
oroplast lysate[a]	60	1600	26	1
ıt-stable supernatant	3.0	1440	480	18
AE cellulose eluate	2.1	1050	500	19
romatography on Superdex-200	0.09	100	1111	42
ne, rechromatography	0.02	30	1333	51

ıloroplast preparation starting from 500 g spinach leaves

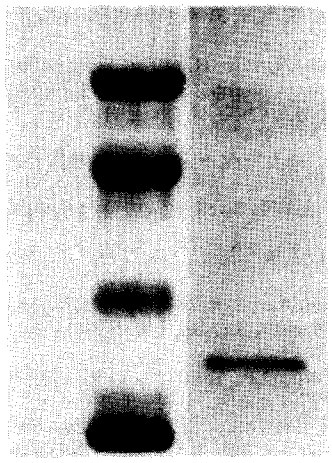


Fig. 2. SDS-PAGE of DHA reductase from spinach chloroplasts. Left lane: marker proteins. From top to bottom: bovine serum albumin, M_r = 66,000; ovalbumin, M_r = 45,000; chymotrypsinogen, M_r = 25,000; cytochrome *c*, M_r = 12,500. Right lane: purified enzyme.

inhibitors (Table 2). This unexpected observation prompted us to assay DHA reductase for trypsin inhibitor activity, and vice versa.

Plant protease inhibitors of the Kunitz type are proteins of M_r = 21,000 Da possessing two disulfide bonds [18]. Commercial preparations of soybean trypsin inhibitor showed no DHA reducing activity in standard assays. However, upon reduction by dithiothreitol the proteins served as DHA reductase but lost the trypsin inhibiting activity (Table 3). Dithiothreitol-reduced pancreatic ribonuclease was used as a control but showed no DHA reductase activity. We conclude that the reaction between reduced trypsin inhibitors and dehydroascorbate is a specific process. Conversely, the DHA reductase, isolated from spinach chloroplasts as a thiol protein, is not a trypsin inhibitor but it produced significant trypsin inhibition following oxidation by incubation with GSSG for 2 h at 6°C.

Glutaredoxin is another protein of entirely unrelated function (i.e. electron donor in ribonucleotide reduction) which exhibits DHA reductase activity [13] (Table 3). Glutaredoxin does not occur in the chloroplast but two thioredoxins, of comparable structure, are abundant chloroplast proteins. We therefore tested samples of reduced thioredoxin m and f from spinach chloroplasts and indeed observed reduction of dehydroascorbate by thioredoxin (Table 3). The values are low but are likely to be gross underestimates because specific electron donors (thioredoxin reductases) have not been included in these reaction mixtures and GSH does not reduce thioredoxins. Thus, DHA reduction could not proceed continuously in the assays.

4. Discussion

We have purified to homogeneity an active DHA reductase from spinach chloroplasts, thus substantiating that catalytic reduction of dehydroascorbate proceeds in the organelles [9,11,12]. The measured activity was similar to that of an earlier chloroplast preparation [12] but the enzyme has a lower specific activity than other DHA reductase fractions isolated from whole leaves [3].

Our present results indicate that the reduction of dehydroascorbate, essential for replenishing ascorbate pools, can be promoted by a number of different plant proteins. This is in accord with observations by others of multiple DHA reducing fractions, usually of unidentified nature, in leaves and seedlings [3,21]. In addition to proteins of 20–25 kDa molecular mass described previously [3,9,10] and in this work, plant thioredoxins (M_r = 12 kDa) have to be added as candidates for the catalysis of DHA reduction; this activity of thioredoxins went so far unrecognized [3,13].

The apparent identity of a DHA reductase and a trypsin inhibitor is extraordinary. Besides in their identical N-termini the two proteins coincide in molecular weight and heat stability. Whether the molecules exhibit sequence homology throughout,

Table 2
Sequence comparison between DHA reductase and Kunitz-type protease inhibitors

Protein	N-terminal (partial) sequence
Chloroplast DHA reductase (spinach)	D F V L D N E G N P L E N G G T Y ...
Trypsin inhibitor (soybean) [18]	D F V L D N E G N P L E N G G T Y ...
Trypsin inhibitor (silktree) [19]	K E L L D A D G D I L L N G G ? Y ...
Protease inhibitor pF4 (potato) [20]	... P V L D V A G K E L D S R L S Y ...

Table 3
Dehydroascorbate reductase and trypsin inhibitor activities of proteins

Protein	Redox and assay conditions	DHA reduction (μmol·min^{-1}·mg protein^{-1})	Trypsin inhibition (%)
Spinach chloroplast DHA reductase	reduced (native), GSH present	1.3	0
	oxidized by GSSG	0	30
Soybean trypsin inhibitor	oxidized (native)	0	100
	reduced, GSH present	0.25	0
Glutaredoxin (*E. coli*)	reduced, GSH present	7.4	–
Thioredoxin m (spinach chloroplast)[b]	reduced, no GSH	0.012	–
Thioredoxin f (spinach chloroplast)[b]	reduced, no GSH	0.032	–
Thioredoxin (*E. coli*)[b]	reduced, no GSH	0.024	–

[a] Product no. 190886 from Boehringer; product T 9003 from Sigma was less active.
[b] Chemically reduced by dithiothreitol, followed by removal of the reducing agent.

in particular in the cysteine regions, remains to be established in further studies. However, the above results already permit a chemically and physiologically sensible rationale in which the same protein functions as DHA reductase in the reduced (thiol) state in the chloroplast, a compartment characterized by reducing conditions, and in the oxidized (disulfide) form as protease inhibitor elsewhere. The partial, yet not quantitative mutual conversion between the two states in vitro (cf. Table 3) is no contradiction since chemical disulfide formation between cysteine residues and recovery of activity in proteins frequently remain imperfect in the absence of catalysts (e.g. protein disulfide isomerase).

The functional duality of one protein observed here represents a novel and, to our knowledge, unique case of protein differentiation.

Acknowledgements: This work has been supported by Sonderforschungsbereich 305, 'Ökophysiologie', and by Fonds der Chemischen Industrie. We wish to thank Dr. Claus Wasternack, Halle and Dr. A. Otto, Berlin-Buch for their generous help and expert advice.

References

[1] Foyer, C.H., Lelandais, M., Edwards, E.A. and Mullineaux, P.M. (1991) Curr. Topics Plant Physiol. 6, 131–144.
[2] Salin, M.L. (1987) Physiol. Plant. 72, 681–689.
[3] Hossain, M.A. and Asada, K. (1984) Plant Cell. Physiol. 25, 85–92.
[4] Asada, K. and Badger, M.R. (1984) Plant Cell. Physiol. 25, 1169–1179.
[5] Schöner, S. and Krause, H. (1990) Planta 180, 383–389.
[6] Smirnoff, N. and Colombé, S.V. (1988) J. Exp. Bot. 39, 1097–1108.
[7] Choudhury, N.K., Choe, H.T. and Huffaker, R.C. (1993) J. Plant Physiol. 141, 551–556.
[8] Walker, M.A. and McKersie, B.D. (1993) J. Plant Physiol. 141, 234–239.
[9] Foyer, C.H. and Halliwell, B. (1977) Phytochemistry 16, 1347–1350.
[10] Dipierro, S. and Borraccino, G. (1991) Phytochemistry 30, 427–429.
[11] Nakano, Y. and Asada, K. (1981) Plant Cell Physiol. 22, 867–880.
[12] Jablonski, P.P. and Anderson, J.W. (1981) Plant Physiol. 67, 1239–1244.
[13] Wells, W.W., Xu, D.P., Yanf, Y. and Rocque, P.A. (1990) J. Biol. Chem. 265, 15361–15364.
[14] Mourioux, G. and Douce, R. (1981) Plant Physiol. 67, 470–473.
[15] Schägger, H. and v. Jagow, G. (1987) Anal. Biochem. 166, 368–379.
[16] R.Geiger and H. Fritz (1984) in: H.U. Bergmeyer (Ed.) Methods of Enzymatic Analysis, 3rd. Edn. Vol. V., pp. 119–124, Weinheim, Verlag Chemie.
[17] O'Farrell, P.H. (1975) J. Biol. Chem. 250, 4007–4021.
[18] Kim, S., Hara, S., Hase, S. Ikenaka, T., Toda, H., Kitamura, K. and Kaizuma, N. (1985) J. Biochem. 98, 435–438.
[19] Odani, S., Ono, T. and Ikenaka, T. (1980) J. Biochem. 88, 297–301.
[20] Ishikawa, A., Ohta, S., Matsuoka, K., Hattori, T. and Nakamura, K. (1994) Plant Cell Physiol. 35, 303–312.
[21] Arrigoni, O. and De Tullio, M. (1994) J. Bioenerg. Biomembr., in press.