Dehydroascorbate and dehydroascorbate reductase are phantom indicators of oxidative stress in plants

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Abstract In many physiological studies dehydroascorbate (DHA) reductase is regarded as one of the chloroplast enzymes involved in the protection against oxidative stress. Here, evidence is presented that plant cells do not possess a specific DHA reductase. The DHA reductase activities measured in plant extracts are due to side reactions of proteins containing redox-active dicysteine sites. Native gel electrophoresis combined with specific activity staining revealed three different proteins with DHA reductase activity in leaf and chloroplast extracts. These proteins have been identified as thioredoxins and trypsin inhibitors (Kunitz type) by Western blot analysis. The essential regulatory functions of thioredoxins in chloroplast metabolism are strongly inhibited in the presence of as little as 50 μM DHA. Thus, the intracellular DHA concentration should be kept below 50 μM but not all proteins with DHA reductase activity are effective enough for this purpose. A specific DHA reductase is frequently demanded as part of the enzymatic equipment to avoid oxidative stress. We argue that this is not necessary because in chloroplasts DHA does not accumulate to any significant extent due to the high activities of monodehydroascorbate reductase and of reduced ferredoxin.

Key words: Thioredoxin; Dehydroascorbate reduction; Oxidative stress; *Spinacia oleracea*

1. Introduction

In chloroplasts molecular oxygen is reduced to superoxide at the reducing sides of the photosystems in higher plants. The superoxide radical anion is efficiently removed by superoxide dismutase, and the generated hydrogen peroxide is detoxified to water by ascorbate peroxidase (Fig. 1). It is well established that the synthesis rate of hydrogen peroxide is significantly increased in stress situations generated by air pollutants, high light, chilling etc. Asada and co-workers calculated that without recycling of ascorbate H_2O_2 would deplete the available ascorbate pool via ascorbate peroxidase within minutes [1]. This necessitates a reducing system to regenerate ascorbate. The primary product of ascorbate peroxidase is monodehydroascorbate (MDA), which is rapidly returned into the ascorbate pool either by NADPH-dependent MDA reductase or non-enzymatically by reduced ferredoxin (Fig. 1). If MDA radicals are not reduced they disproportionate to ascorbate and dehydroascorbate (DHA) with a rate constant of 10^5 M^{-1} s^{-1} [2]. In plant extracts a glutathione-dependent DHA reductase activity has been observed [3] which will recycle DHA to ascorbate (Fig. 1). An increase of DHA reductase activity and an accumulation of DHA have been frequently implied as biochemical indicators of oxidative stress in plant metabolism [4] but a characterization of DHA reductase has remained elusive because of rapid loss of enzyme activity. Here we report that plant cells do not possess and do not require a specific DHA reductase.

2. Materials and methods

All chemicals and reagents were of the highest purity available. Trypsin inhibitor from soybean (Kunitz type) was obtained from Boehringer. Antibodies raised against thioredoxin *m* and *f* from spinach chloroplasts were generous gifts from Dr. B. Buchanan, University of California, Berkeley, CA, USA. Samples of spinach thioredoxin *m* and *f* were supplied by Dr. P. Schürmann, University of Neuchâtel, Switzerland, which is gratefully acknowledged. Antibodies raised against trypsin inhibitor were produced in cooperation with Dr. Giorgio Tiscar, University of Bari, Italy. NADP-malate dehydrogenase and fructose-1,6-bisphosphatase were isolated from spinach leaves and assayed by published procedures [5,6]. Spinach leaves were purchased on local markets and lots of 100 g leaf material were extracted in 200 ml of potassium phosphate buffer (50 mM, pH 7.8) in a Waring blender. The homogenate was passed through four layers of miracloth and centrifuged at 13000 rpm for 20 min. Isolation of chloroplasts from spinach leaves followed the method described by Mourioux and Douce [7]. Activity staining of dehydroascorbate reduction following native gel electrophoresis on 1% agarose gels was performed by a method described by De Tullio et al. [8], which involves the reactions between the ascorbate formed, cyanoferrate(III), and ferric chloride. Immobilon P-membrane (Millipore) was used for Western blots and antisera were applied in 1:1000 dilution. Anti-rabbit IgG peroxidase conjugate and diaminobenzidine (from Sigma) were used to determine the first antibody.

3. Results and discussion

Recently we described the purification of an apparently novel protein with glutathione-dependent DHA reductase activity from spinach chloroplasts [9]. N-terminal sequence analysis of this protein revealed that it is highly homologous with trypsin inhibitors of the Kunitz type. Both the isolated protein from spinach chloroplasts and commercially available soybean trypsin inhibitor showed DHA reductase activity in the reduced state and acted as trypsin inhibitors in the oxidized state [9]. Their redox behavior must be due to the presence of conserved cysteine or cystine residues, respectively. Redox changes between a dithiol and disulfide also supply the reducing equivalents for the reduction of DHA to ascorbate. In the case of a trypsin inhibitor from potato two cysteines are arranged in a C P F C sequence, reminiscent of the redox-active sites found in thioredoxin (C G P C), glutaredoxin (C P Y C), and protein disulfide isomerase (C G P C) [2,9,10]. All the

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Abbreviations: DHA, dehydroascorbate; DHA reductase, dehydroascorbate reductase; MDA, monodehydroascorbate; MDA reductase, monodehydroascorbate reductase

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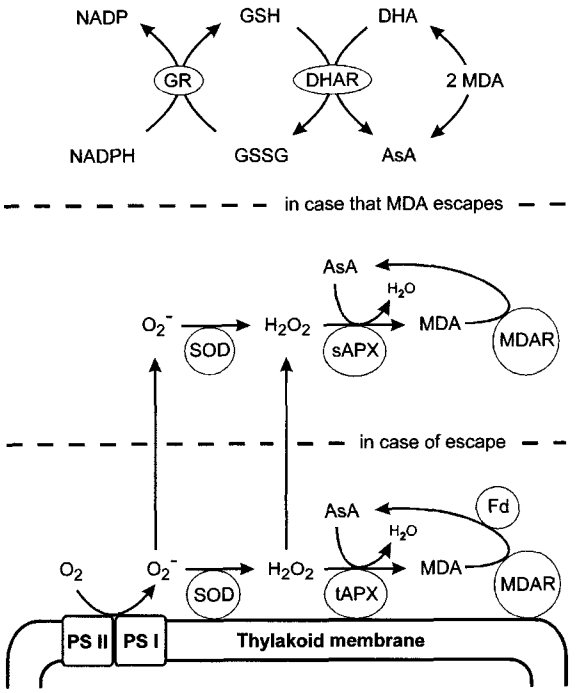

Fig. 1. A general view of the Mehler peroxidase process and ascorbate-glutathione cycle protecting the chloroplast metabolism against oxidative stress exerted by hydrogen peroxide (H_2O_2) and dehydroascorbate (DHA). From bottom to top: PS II, PS I: photosystems II and I of the thylakoid membrane; Fd: ferredoxin; O_2^-: superoxide; SOD: superoxide dismutase; tAPX: thylakoid-bound isoform of ascorbate peroxidase; MDA: monodehydroascorbate; MDAR: monodehydroascorbate reductase; AsA: ascorbic acid; sAPX: stromal isoform of ascorbate peroxidase; DHAR: dehydroascorbate reductase; GSH: reduced glutathione, GSSG: oxidized glutathione; GR: glutathione reductase.

latter proteins have been shown to possess DHA reductase activity. In mammalian cells proteins with DHA reductase activity have been described which were subsequently characterized as glutaredoxin [11]. In contrast, when we isolated glutaredoxin from green plant tissue it was observed that spinach glutaredoxin possesses no DHA reductase activity [12]. The structural differences between mammalian and plant glutaredoxins responsible for their different reactivity are currently under investigation. Recently, Kato et al. reported the isolation of a new DHA reductase from rice bran. The determined N-terminal amino acid sequence was not similar to the N-termini of other known DHA reducing proteins [13]. But it must be taken into account that proteins from storage tissue frequently possess extra amino acids at the N-termini which are not present in the corresponding proteins in tissues of developed plants [14]. Unfortunately, it was not investigated whether the rice bran protein with DHA reducing activity possesses additionally thioredoxin, glutaredoxin or protein disulfide isomerase activity [13]. Thus, further investigations are necessary to elucidate the real nature of this protein from rice bran.

3.1. *DHA as inhibitor of thioredoxin-dependent enzyme regulation*

Millimolar concentrations of DHA have been measured in the stromal compartment [15]. In view of these considerable concentrations and our knowledge that thioredoxins can be oxidized by DHA [9] we investigated whether DHA interferes with the thioredoxin-dependent redox regulation of CO_2 fixation, sulfate assimilation, ATP synthesis, etc. The thioredoxin system is vital for chloroplast metabolism because redox control of at least 12 different enzymes is achieved by the reductive cleavage of regulatory disulfide bridges in these target enzymes [16]. Indeed, as shown in Fig. 2, the thioredoxin-dependent activity of NADP-malate dehydrogenase is inhibited by 50% in presence of 50 μM DHA and reduced to less than 10% by 1 mM DHA. Thus, in the presence of the millimolar concentrations of DHA reported in stroma NADP-malate dehydrogenase would be completely inactive, resulting in serious consequences for the chloroplast metabolism because the enzyme is part of the defence system protecting plant cells against photoinhibition [17]. Another thioredoxin-dependent enzyme, glutamate synthase, is also inhibited by DHA (not shown), but DHA does not inhibit all thioredoxin-dependent stromal target enzymes with the same efficiency. The Calvin-Benson cycle enzyme fructose-1,6-bisphosphatase is not inhibited when the regulatory sites of the enzyme are protected in its protein complex with thioredoxin (Fig. 3) [6]. Here, each subunit of the tetrameric enzyme possesses a single regulatory disulfide bridge whereas in dimeric NADP-malate dehydrogenase each subunit contains two different regulatory disulfide bridges and only one of these sites is shielded by thioredoxin [18,5]. However, when DHA was added to reduced thioredoxin prior to fructose-1,6-bisphosphatase activation, thioredoxin-dependent activation of the target did not occur because of the oxidation of thioredoxin by DHA. Obviously, DHA must be regarded as a toxic substance which disturbs the various regulatory functions of the thioredoxin system in chloroplast metabolism. Consequently, chloroplasts should be equipped with a DHA reductase which keeps the DHA concentration at least below 50 μM. However, all proteins with DHA reductase activity isolated from chloroplasts possess apparent K_m values for DHA in the millimolar range [9,13]. The question arises which other mechanisms would prevent DHA accumulation.

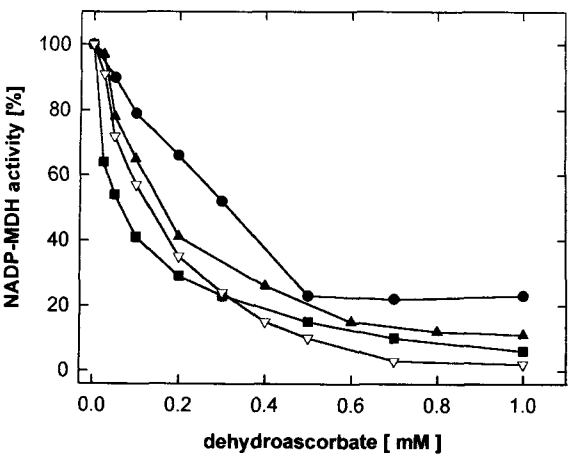

Fig. 2. Inhibtion of NADP-malate dehydrogenase from spinach chloroplasts by dehydroascorbate. NADP-MDH was activated either with dithiothreitol (●) or reduced thioredoxin *m* (▲) and *f* (▽) from spinach chloroplasts or with *Escherichia coli* thioredoxin (■). The activity after complete reductive activation was set at 100% and the inhibition was measured after addition of variable amounts of dehydroascorbate.

3.2. Screening for proteins with DHA reductase activity

It has been observed repeatedly that DHA reductase activity measured in crude extracts or in enriched fractions of spinach leaves is very unstable [3]. To ensure that DHA reductase was not being overlooked throughout a purification procedure as described [9,12] we analyzed DHA reductase activity patterns in cell-free protein extracts from isolated chloroplasts by native gel electrophoresis combined with activity staining for DHA reduction. In protein extracts of spinach chloroplasts we detected three different DHA reductase activities (Fig. 4). Western blot analysis revealed that two signals are due to thioredoxin *f* and thioredoxin *m* and that the third signal represents a Kunitz-type trypsin inhibitor. No additional DHA reductase species could be detected by this experimental procedure. Thus, it is concluded that no specific DHA reductase is present in spinach chloroplasts but all measurable DHA reductase activity is due to these different redox-active proteins. Lack of a specific detoxifying enzyme would be highly surprising if DHA concentrations were really in the millimolar range.

3.3. DHA accumulation is unlikely

The origin of DHA is the non-enzymatic disproportionation of MDA. Thus, the formation of DHA is determined by the half-life of MDA. Molecular oxygen is reduced to superoxide at the photosystems, thylakoid-bound superoxide dismutase and ascorbate peroxidase remove superoxide and hydrogen peroxide. The generated MDA is a substrate either of reduced ferredoxin or of thylakoid-bound MDA reductase. Thus, MDA will be preferentially removed at its formation site. In case MDA escapes, a stromal MDA reductase prevents an accumulation of MDA. Both MDA reductase and reduced ferredoxin remove MDA with rate constants of 10^7–10^8 M^{-1} s^{-1} [19] whereas the disproportionation rate of MDA is only 10^5 M^{-1} s^{-1} [2]. The enzyme equipment of the chloroplasts can, therefore, be regarded as very efficient and it is improbable that significant amounts of DHA will accumulate. This is supported by the fact that the measured MDA concentration in chloroplasts is about 10^{-8} M. It is, therefore, not a priori necessary to require a specific DHA reductase in chloroplasts. Nevertheless it is thinkable that the capacity of MDA scavenging is overcome under certain stress circumstances and that a DHA reductase would still be desirable. In these situations it must be taken into account that glutathione non-enzymatically reduces DHA to ascorbate at high rates, especially at alkaline pH which prevails in the light [20].

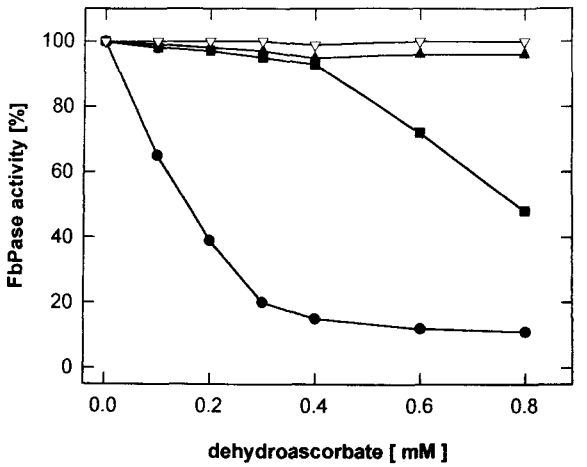

Fig. 3. Inhibtion of fructose-1,6-bisphosphatase from spinach chloroplasts by dehydroascorbate. FbPase was activated either with dithiothreitol (●) or reduced thioredoxin *m* (▲) and *f* (▽) from spinach chloroplasts or *Escherichia coli* thioredoxin (■). The activity after complete reductive activation was set at 100% and the inhibition was measured after addition of variable amounts of dehydroascorbate.

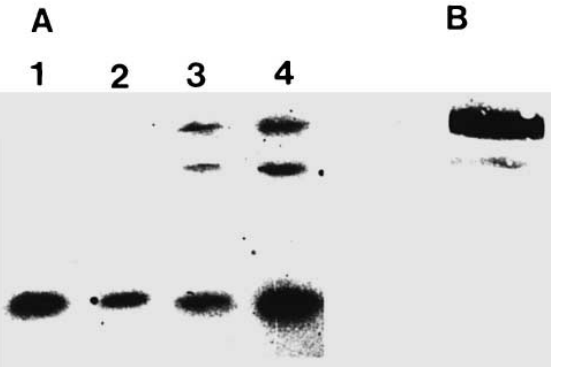

Fig. 4. A: Native gel electrophoresis in 1% agarose gels (200×160×4 mm) in combination with activity staining for glutathione-dependent dehydroascorbate reductase activity. Dehydroascorbate reducing proteins were indicated as dark blue signals. Lane 1: commercially available soybean trypsin inhibitor of the Kunitz type (50 μg in 200 μl); lane 2: protein with dehydroascorbate reductase activity isolated from spinach chloroplasts (50 μg in 200 μl); lane 3: spinach chloroplast protein extract (800 μg in 200 μl); lane 4: spinach leaf protein extract (940 μg in 200 μl). B: Western blot analysis of spinach leaf proteins with dehydroascorbate reductases after native gel electrophoresis as in A (lane 4), same scale. Antiserum against thioredoxin *f* from spinach chloroplasts was applied. The bands became enlarged during semi-dry blotting.

3.4. DHA reductase activity and DHA pool size do not indicate oxidative stress

It is important to consider that in biological solutions ascorbate is rapidly oxidized to DHA within minutes at physiological pH. Only under acidic conditions (pH 2) ascorbate is stable enough to be measured which explains why it cannot be quantified enzymatically [21,22]. In studies of oxidative stress in chloroplasts many steps are necessary to isolate the cell organelles and considerable time elapses. Thus, it is impossible by presently available methods to prepare samples without artifactual generation of DHA during the experimental procedure. This fact readily explains the high concentrations of DHA measured in chloroplast and plant extracts [21,22].

Increasing DHA concentrations and/or DHA reductase activity have been regarded as biochemical indicators of increasing oxidative stress in chloroplast metabolism [4]. As outlined in this report, both indicators are misleading and falsify the interpretations. Moreover, in leaf extracts no extra DHA reductase was detected outside the chloroplasts (Fig. 4, lane 4). We have to conclude that plant cells do not possess any specific dehydroascorbate reductase and that the activities measured in total leaf extracts are due to unrelated proteins present in chloroplasts. It cannot be excluded, however, that additional proteins with *un*specific DHA reductase activity exist outside the chloroplasts [3]. Unspecific DHA reductase activity associated with several unrelated proteins may explain why, unexpectedly, DHA reductase activity was not elevated in extracts of stress-treated plants [23]. To circumvent the

poor reproducibility of DHA reductase determination DHA pools have been analyzed. This approach also has difficulties as in some studies DHA was not detectable at all in plant leaf extracts [21]. These results support our opinion that DHA is not a constitutive metabolite occurring in considerable amounts in plant metabolism.

In conclusion, the regeneration of ascorbate is obviously not coupled to a glutathione-dependent DHA reductase (Fig. 1). The glutathione reductase present in leaves is not directly involved in the regeneration of ascorbate and is probably not of importance in avoiding oxidative stress as frequently discussed [4]. This offers an explanation why increased levels of glutathione reductase in transgenic plants do not confer increased tolerance against oxidative stress [24]. The above arguments should help to avoid future misconceptions about a stress defense in plant biology.

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