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H_2O_2 DESTRUCTION BY ASCORBATE-DEPENDENT SYSTEMS FROM CHLOROPLASTS

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Summary

Washed lamellae from isolated spinach chloroplasts exhibited peroxidative activity with 3,3′-diaminobenzidine or ascorbate as electron donors. By heat treatment or by incubation of the chloroplasts with pronase a heat-labile enzymic activity (system A) and a heat-stable non-enzymic peroxidative activity (system B) could be differentiated.

System A is membrane-bound, reacts with 3,3′-diaminobenzidine and with ascorbate as electron donors, shows a sharp pH optimum between 7.5 and 8.0 with both substrates and is inhibited competitively by cyanide.

The heat-stable factor can be extracted from the chloroplast lamellae by heat treatment, reacts only with ascorbate as electron donor, shows increasing activity with higher pH values but no optimum and is not inhibited by cyanide.

Both peroxidative systems in connection with a relatively high concentration of ascorbate in chloroplasts should represent an important tool for the detoxification of H_2O_2 which is produced in these organelles by photosynthetic O_2 reduction.

Introduction

It has now been well established that photosynthesizing algae [1—4] and isolated chloroplasts [5] produce hydrogen peroxide by monovalent [6—9] and probably also by divalent [10] reduction of oxygen.

H_2O_2, however, inhibits photosynthesis by 50% at concentrations as low as

Abbreviation: Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid.

10^{-5} M [11]. Chloroplasts may not contain catalase, although some activity of this enzyme is usually found with preparations of isolated plastids. It was suggested [12] that this activity is derived from catalase, which was originally located in peroxisomes and had been liberated from them upon homogenization of the leaves (see Refs. 12—14). In addition, catalase inhibition by ascorbate was demonstrated [15] and, since this compound at concentrations up to 50 mM was found in chloroplasts [12,16], the importance of catalase, if present in chloroplasts, in the destruction of H_2O_2 in chloroplasts should only be very small.

However, chloroplasts contain a variety of substances which could react with hydrogen peroxide or its precursor O_2^- [12]. For instance, glycollate can be formed from dihydroxyethylthiaminepyrophosphate (see Ref. 17), dehydroascorbate originates from ascorbate, and GSSG from GSH [12]. All these reactions should proceed by a 2-electron oxidation; however, until now, no peroxidase has yet been demonstrated in chloroplasts [18].

In this paper, we wish to describe two ascorbate-specific peroxidative activities of isolated chloroplast lamellae, an enzymic and a non-enzymic one.

Methods

Preparation of chloroplast lamellae

Chloroplast lamellae were prepared from purified intact chloroplasts. The latter were isolated from 50 g washed spinach leaves by the following procedure: The deribbed leaves were ground in 100 ml pyrophosphate buffer for 5 s at 0°C [19]. The resulting mix was squeezed through eight layers of gauze and then centrifuged for 1 min at 2600 × *g*. The pellet was suspended in 2 ml buffer [20], containing 330 mM sorbitol, 2 mM $NaNO_3$, 2 mM EDTA, 1 mM $MnCl_2$, 1 mM $MgCl_2$, 0.5 mM K_2HPO_4, 50 mM Hepes, 20 mM NaCl. Sodium ascorbate was omitted. Whole cells and cell debris were spun down at 100 × *g* for 1 min and intact chloroplasts were separated from the supernatant by centrifugation at 750 × *g* for 1 min.

The intact plastids were then ruptured by the addition of 3 ml sorbitol- and ascorbate-free Hepes buffer. The lamellae were isolated by centrifugation at 4100 × *g* for 1 min, washed in 3 ml and finally suspended in 0.3 ml of the same medium. The supernatant was used as the aqueous chloroplast extract. Chlorophyll was measured according to the method of Arnon [21].

Peroxidase assay

Two assays of the peroxidative activity were used:

(i) Potentiometric determination of H_2O_2 consumption. 1.7 ml 0.1 M Tris-HCl buffer (pH 8.0), 0.05 ml 0.34 M sodium ascorbate and 0.05 ml chloroplast suspension were put into the cell of a Gilson oxygraph and flushed with N_2 until an oxygen concentration of approx. 10 μM was recorded. The peroxidative reaction was started by injection of 0.05 ml 8.16 mM H_2O_2. A small and sudden increase in the oxygen concentration was usually observed upon the addition of the H_2O_2; this was due to oxygen dissolved in the H_2O_2. No significant further increase of the oxygen concentration in the incubation mixture then occurred. The reaction was terminated after 15 s to 3 min by adding an

excess of catalase (EC 1.11.1.6). The resulting sharp increase in oxygen concentration was taken as a measure of unreacted H_2O_2. The consumption of H_2O_2 by the peroxidative reaction was calculated from the difference between added and unreacted H_2O_2. Instead of ascorbate, 2.8 mM 3,3'-diaminobenzidine was also used as an electron donor in this assay.

(ii) Continuous determination of the oxidation product. When 3,3'-diaminobenzidine was used as an electron donor, the peroxidative reaction could be followed directly by measuring the absorbance of the oxidation product dehydro-3,3'-diaminobenzidine and its condensation product, respectively. Since the photometrically-obtained results were usually scattered due to the effect of the chloroplast particles, each figure represents an average value of at least four single measurements. The reaction mixture contained in a total volume of 3 ml: 0.1 M Tris-HCl buffer (pH 7.6), 2.8 mM 3,3'-diaminobenzidine and various amounts of chloroplast lamellae, ranging from 15—75 μg chlorophyll. The reaction was initiated by the addition of 0.05 ml 8.16 mM H_2O_2. To avoid photochemical oxidation of the electron donor, the assay was performed in the dark.

Identification of the reaction products

(i) Didehydroascorbate. In order to avoid considerable didehydroascorbate degradation, the reaction was performed at pH 6.7, instead of pH 8.0. After termination of the reaction, the incubation mixture was lyophilized. The residue was dissolved in 0.1 ml H_2O and separated by thin-layer chromatography on silicagel DC 60 F_{254} (Merck) using ethanol/10% acetic acid (9 : 1, v/v) and acetic acid/acetone/methanol/benzene (5 : 5 : 20 : 70, v/v). Dehydroascorbate was detected by its violet fluorescence after spraying the chromatograms with 1,2-phenylenediamine reagent [22]. The substance cochromatographed with authentical dehydroascorbate (Ferak). Besides dehydroascorbate, unreacted ascorbate, as well as some unknown spots, were detected near the origin.

In order to quantify the amount of dehydroascorbate, [U-^{14}C]ascorbate (New England Nuclear; 0.0455 Ci/g) was used in the peroxidase assay. After thin-layer chromatography of the incubation mixture the radioactive spots were located on the chromatograms with a Betacamera and the radioactivity was determined with a methane flow-counter.

(ii) Dehydrodiaminobenzidine and its condensation products. After removal of the chloroplast lamellae by centrifugation the absorption spectrum of the oxidation product in the supernatant coincided well with that exhibited by the oxidation product of 3,3'-diaminobenzidine by means of horseradish peroxidase (Fig. 2). An absorption maximum was found between 465 and 480 nm. When the chloroplast membranes were not separated from the supernatant, as was the case with the continuous assay, the absorption maximum was shifted towards longer wavelengths. Therefore, in the continuous assay the increase in absorbance at 517 nm was recorded.

Results

1. Evidence for peroxidative activity in chloroplast lamellae

Demonstration of peroxidative activity with the classical electron donors,

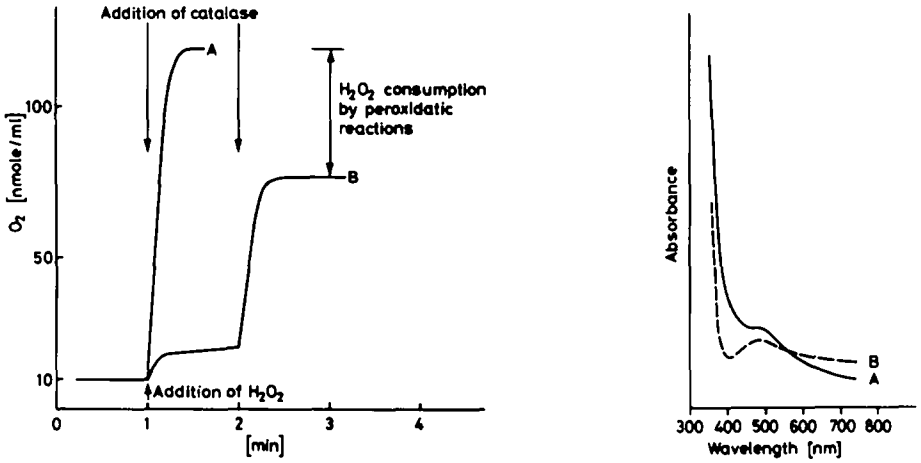

Fig. 1. Oxygen trace of the potentiometric determination of peroxidative activities with a Clark electrode. The assay medium contained 1.7 ml 0.1 M Tris-HCl buffer (pH 8.0), 50 µl 0.34 M ascorbic acid and 50 µl chloroplast lamellae (1.5 µg chlorophyll/µl). The reaction was started by addition of 50 µl 8.16 mM H_2O_2 and stopped by an excess of catalase. The sharp increase after the addition of H_2O_2 in trace B was due to oxygen dissolved in the H_2O_2 solution. A further negligible increase was probably due to a small activity of catalase still associated with lamellae or to a buffer effect. The consumption of H_2O_2 was calculated from the difference in oxygen release between the reacted (B) and the unreacted (A) sample. 1 nmol O_2 is equivalent to 2 nmol H_2O_2.

Fig. 2. Absorption spectra of the reaction products of 3,3'-diaminobenzidine with H_2O_2 catalyzed by horseradish peroxidase (EC 1.11.1.7) (A) or by chloroplast lamellae (B). The assay mixture contained in a total volume of 3 ml 0.1 M Tris-HCl buffer (pH 7.6) with 2.8 mM 3,3'-diaminobenzidine and 50 µl chloroplast suspension (2.3 µg chlorophyll/µl) or 10 µl horseradish peroxidase (0.5 units/µl). The reactions were started by addition of 50 µl 8.16 mM H_2O_2. After 5 min, the samples were centrifuged and the absorption spectra of the supernatants were measured.

guaiacol [23] or benzidine [24], was unsuccessful. However, use of the more electronegative electron donor, 3,3'-diaminobenzidine, resulted in the formation of the brown condensation product of dehydrodiaminobenzidine. The formation rates of this substance were found to depend linearly on the amount of chloroplast lamellae in the incubation mixture. Since the reaction could be strongly inhibited by the addition of ascorbate, this compound apparently functioned also as an electron donor. This was shown in a system consisting of chloroplast lamellae, ascorbate and H_2O_2, where the oxygen concentration could be followed continuously with a Clark electrode (Fig. 1). H_2O_2 consumption was calculated from the difference in oxygen liberation between an unreacted and the reacted sample upon addition of catalase. Since the H_2O_2 decomposition by catalase would yield a continuously increasing oxygen concentration in the reaction mixture, the nearly constant oxygen level shown by the oxygen trace in Fig. 1 excludes H_2O_2 consumption by catalatic action. Thus, ascorbate-dependent H_2O_2 consumption must be due to peroxidative activity of the chloroplast membranes.

2. Differentiation of an enzymic and a non-enzymic peroxidative activity

To examine the nature of the peroxidative activity, this activity was determined after treatment of the chloroplast lamellae at various temperatures. Pre-

TABLE I

INFLUENCE OF HEAT TREATMENT ON THE PEROXIDATIVE ACTIVITIES OF SPINACH CHLOROPLAST LAMELLAE

The assay was performed as described in the legends to Fig. 3 (with 3,3′-diaminobenzidine as electron donor) and Fig. 1 (with ascorbate as electron donor). The membrane suspension was preincubated at different temperatures as shown below. Heat labile activity was calculated from the difference between the total activity and the heat stable activity. chl, chlorophyll.

Preincubation time (min)	Preincubation temperature (°C)	H_2O_2 consumed (μmol/mg chl per h)		Diaminobenzidine oxidized (μmol/mg chl per h)
		Total ascorbate-dependent activity	Heat-labile activity	3,3′-Diaminobenzidine-dependent activity
0	—	551	336	12
15	40	215	0	0
15	60	341	0	0
15	80	361	0	0
30	100	325	0	0

treatment of the membranes at temperatures higher than 35°C resulted in a considerable, but never complete, loss of activity with ascorbate as electron donor. With 3,3′-diaminobenzidine, no activity could be detected after heat treatment (Table I).

The data of Table I show one peroxidative activity with 3,3′-diaminobenzidine as electron donor which proved to be heat-labile. However, with ascorbate two activities could be separated, a heat-labile (system A) and a heat-stable one (system B). The latter usually increased with higher preincubation temperatures and could be solubilized completely upon heating of the chloroplasts for 20 min at 80°C (Table I).

A further differentiation between both systems was achieved by inhibitor studies. System A was inhibited in a competitive manner by CN^-, whereas system B was not. An enzymic nature of system A was indicated not only by its heat inactivation but also by its inactivation by pronase. The portion of heat-labile peroxidase activity corresponded well with the pronase-sensitive part of the total activity (Table II).

TABLE II

EVIDENCE FOR THE ENZYMIC CHARACTER OF PEROXIDATIVE SYSTEM A IN SPINACH CHLOROPLAST LAMELLAE BY INCUBATION WITH PRONASE

The membrane suspension was preincubated with purchased pronase (15 U/120 μg chlorophyll) for 40 min at 30°C (pH 7.5). The peroxidative activity was tested as described in the legend to Fig. 1. Pronase itself exhibited some peroxidative activity, which was substracted as background.

	H_2O_2 consumed (μmol/mg chlorophyll per h)
Total peroxidative activity	565
Heat-labile activity	350
Heat-stable activity	215
Pronase-labile activity	338
Heat-labile/pronase-labile activity	1.03

TABLE III

EVIDENCE FOR DIDEHYDROASCORBATE AS PRODUCT OF ASCORBATE-DEPENDENT PEROXIDATIVE SYSTEMS OF SPINACH CHLOROPLASTS

The assay was performed as described in the legend to Fig. 1, except a variation in the assay medium was made. It consisted of 1.65 ml 0.05 M Hepes buffer (pH 6.7) with 2 mM EDTA, 50 µl chloroplast suspension (2.1 µg chl/µl), 50 µl 0.068 M [U-^{14}C]ascorbic acid and 100 µl 8.16 mM H_2O_2 in a total volume of 1.86 ml. The lyophilized supernatant was separated by thin-layer chromatography. The radioactive substances were cochromatographed with authentical ascorbate and dehydroascorbate and the radioactivity was measured with a methane flow counter.

	Experiment	H_2O_2 consumed (µmol/mg chlorophyll per h)	Didehydroascorbate produced (µmol/mg chlorophyll per h)
Total activity	a	1456	1740
	b	1286	1032
Heat-stable activity		58	162
Heat-labile activity	a	1398	1578
	b	1230	870

3. Characterization of the peroxidative systems

Identification of the reaction products. When ascorbate was used as substrate, didehydroascorbate could be expected as the reaction product. Since this compound is rather unstable in the presence of H_2O_2, and particularly at an alkaline pH, the peroxidative reaction was performed at pH 6.7 and [U-^{14}C]-ascorbate was used. After a reaction period of 15 s, the chloroplast lamellae were removed by centrifugation and the lyophilized supernatant was separated by thin-layer chromatography. The ^{14}C-labelled spots cochromatographed with dehydroascorbate and ascorbate and their radioactivity was measured with a methane flow counter.

Table III shows the stoichiometry of H_2O_2 and ascorbate consumption and dehydroascorbate formation. Although some decay of dehydroascorbate was obviously inevitable, the data of Table III suggest a 1 : 1 stoichiometry between the consumption of H_2O_2 and ascorbate on the one hand and the production of dehydroascorbate on the other.

When 3,3′-diaminobenzidine was used as electron donor the brown condensation product of dehydrodiaminobenzidine was formed, which was identified by its absorption maximum at 465—480 nm (Fig. 2). Since this product is also formed by nascent oxygen from catalatic H_2O_2 decomposition [25,26], an assay without 3,3′-diaminobenzidine was performed in the oxygraph cell. No oxygen production was observed during the reaction period. Therefore, at least at pH 7.6, the oxidation of 3,3′-diaminobenzidine by H_2O_2 must have occurred by a peroxidative mechanism.

pH optima. The peroxidative reaction with 3,3′-diaminobenzidine as substrate exhibited a sharp pH optimum between 7.5 and 8.0 (Fig. 3). With ascorbate as electron donor, a pH curve was obtained which showed the features of two superimposed curves (Fig. 3). This became evident when the pH dependence of system B was determined separately: a more or less linear increase of the activity with rising pH values, but no optimum, was observed. Since the pH

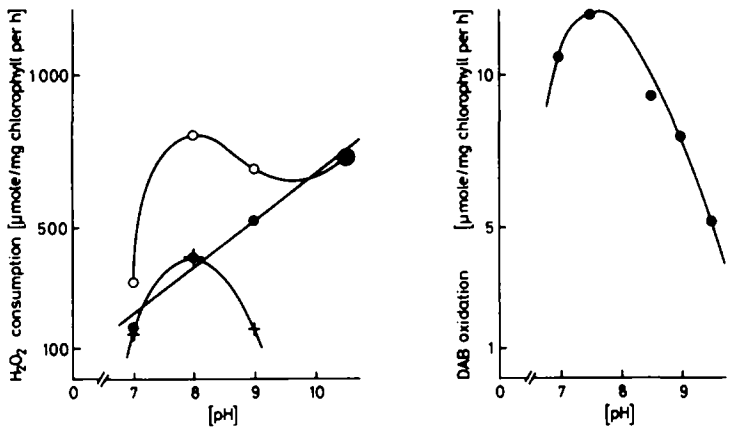

Fig. 3. pH dependence of the peroxidative activities in chloroplast lamellae. With ascorbate as electron donor the assay was performed as described in the legend to Fig. 1. The pH optimum of the heat labile activity (+————+) was calculated from the difference between the pH curve of the total (○————○) and that of the heat stable (●————●) peroxidative activity which could be measured directly. With 2.8 mM 3,3′-diaminobenzidine as electron donor the peroxidative activity was measured in an assay medium containing buffer, chloroplast lamellae equivalent to 15—60 µg chlorophyll and 50 µl 8.16 mM H_2O_2 in a total volume of 3 ml. The absorbance of the oxidation product dehydro-3,3′-diaminobenzidine was recorded at 517 nm. Buffer systems: 0.1 M potassium phosphate buffer (pH 5—8), 0.1 M Tris-HCl buffer (pH 7.2—9), 0.1 M 2-amino-2-methyl-1,3-propandiol buffer (pH 8.6—10.6).

dependence of system A could not be determined directly, the difference between the pH curves obtained with both systems and that of system B should reveal the pH characteristic of the enzymic system. This curve coincided with the pH optimum found with 3,3′-diaminobenzidine as substrate (Fig. 3).

Substrate specificity. True peroxidase activity could only be observed with ascorbate, which is known to be present in chloroplasts, and with the artificial electron donor 3,3′-diaminobenzidine (Table IV). The activity in the latter system was small as compared to that with ascorbate. With glutathione, a small activity was sometimes observed. This activity, however, never did show the characteristics of true catalysis such as substrate saturation, K_m or K_i.

TABLE IV

SUBSTRATE SPECIFICITY OF THE PEROXIDATIVE ACTIVITIES OF SPINACH CHLOROPLASTS

The assay was performed as described in the legend to Fig. 1. Various concentrations of each possible electron donor were used. The reaction with GSH showed no substrate saturation curve.

Substrate	Maximum concentration in the assay (mM)	Maximum rates (µmol H_2O_2 consumed/mg chlorophyll per h)	
		System A	System B
Ascorbate	10	1400	500
3,3′-Diaminobenzidine	2.8	15	0
GSH	15	(10)	
Dithioerythritol	10	0	
NADH	5	0	
NADPH	5	0	

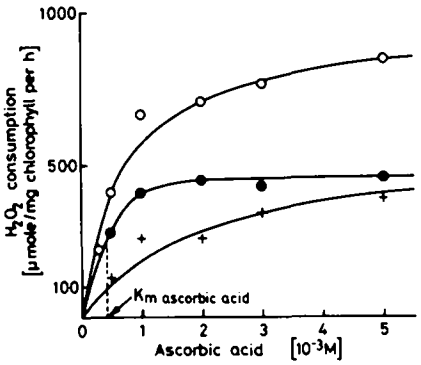

Fig. 4. Determination of K_m(ascorbate) for the heat labile peroxidative activity in chloroplast lamellae. The assay was performed as described in the legend to Fig. 1. The substrate saturation curves for the total activity (○———○) and the heat stable activity (+———+) were determined directly. The Michaelis-Menten plot for the heat labile activity (●———●) was calculated from the difference between both curves. The $K_{m(ascorbate)}$ for the heat-labile peroxidative activity is $0.4 \cdot 10^{-3}$ M.

Kinetic data. With saturating concentrations of ascorbate or 3,3′-diaminobenzidine the $K_{m(H_2O_2)}$ was $3 \cdot 10^{-5}$ M and with saturating concentrations of H_2O_2 the $K_{m(3,3'\text{-diaminobenzidine})}$ was $2.5 \cdot 10^{-3}$ M. Under these conditions the $K_{m(ascorbate)}$ could be determined directly for system A + B and for system B, respectively. When both substrate curves were plotted in a Michaelis-Menten diagram, the difference between both curves revealed a third saturation curve (Fig. 4), which was ascribed to system A. From these curves, a $K_{m(ascorbate)}$ for system A was calculated at $0.4 \cdot 10^{-3}$ M and for system B at $1.4 \cdot 10^{-3}$ M. The $K_{m(ascorbate)}$ of system A corresponded well with the $K_{i(ascorbate)}$ in the 3,3′-diaminobenzidine assay ($0.5 \cdot 10^{-3}$ M).

CN inhibited system A and the reaction with 3,3′-diaminobenzidine in a competitive manner with respect to H_2O_2. The $K_{i(CN^-)}$ was found to be $2.4 \cdot 10^{-5}$ M. System B was not inhibited by CN^-.

Discussion

It is important, during experiments on the detoxification of H_2O_2 in the chloroplast using low potential electron donors (such as ascorbate or 3,3′-diaminobenzidine), that photochemical oxidation of the electron donors by the action of photosystems should be avoided; for this reason, the experiments reported here were performed in the dark. The lability of system A upon heat or pronase treatment and the kinetic data indicate that it has an enzymic activity. Because the reaction with 3,3′-diaminobenzidine showed identical behaviour as system A with respect to heat or pronase treatment, pH optimum, $K_{m(H_2O_2)}$ and inhibition with CN^-, it is concluded that both systems are identical, i.e. that system A can use ascorbate, as well as 3,3′-diaminobenzidine, as electron donors. The idea is corroborated by the correspondence of $K_{m(ascorbate)}$ and $K_{i(ascorbate)}$ in the assay with 3,3′-diaminobenzidine.

Up to now, attempts to solubilize system A have been unsuccessful. There-

for, no substantiated suggestions can be made as to the involvement of a heavy-metal ion in the peroxidative reaction. The strong competitive inhibition of the reaction with H_2O_2 by CN^-, however, could easily be explained if the enzyme contains such a metal ion, as in other peroxidases.

Because of its relatively high affinities for H_2O_2 and ascorbate, system A should be useful to protect chloroplasts against damage by H_2O_2.

Contrarily, system B, although acting as a catalyst, appears to be a substance other than a protein, because of its heat stability and its resistance to pronase. Considerable activity of this system was always found in an aqueous chloroplast extract. By its heat extractability and stability, this system resembles the 'oxygen-reducing factor' described by Elstner and Heupel [27], catalyzing the production of O_2^- plus H_2O_2 from O_2 plus reduced ferredoxin or reduced ferredoxin:NADP reductase (EC 1.6.7.1); the reaction is competitively inhibited by ascorbate. However, in contrast to the activity of system B, that of the 'oxygen-reducing factor' can be precipitated by ethanol.

System B appears to have lower activity than system A for ascorbate and hence its function in detoxification of H_2O_2 is not quite obvious. However, rates as high as 500—1400 μmol H_2O_2 consumed by system A per mg chlorophyll per h were only observed with spinach leaves grown and harvested in spring. In winter or summer spinach, or in old leaves, the activity of system A was low and usually less than that of system B. Under these conditions, the non-enzymic system could be of considerable importance for the destruction of H_2O_2 by chloroplasts. It should be noted that both system A and system B activities were also found in pea chloroplasts.

Based on their findings of reduced glutathione at a concentration of 3.5 mM in the chloroplast stroma, a glutathione reductase from chloroplasts and a considerable non-enzymic reduction power of reduced glutathione with respect to didehydroascorbate, Foyer and Halliwell [28] proposed a cycle for the continuous removal of toxic species of reduced oxygen in the chloroplast. The ascorbate-specific peroxidative reactions described here fit well into this sequence of reactions. In photosynthesizing chloroplasts, the pH of the stroma is about 8 [29] and thus the enzymic peroxidase should work maximally at rates up to 1400 μmol/mg chlorophyll per h which is up to 20 times that of the glutathione reductase of chloroplasts. The activity of system B at pH 8 would be approx. 200—600 μmol/mg chlorophyll per h. Thus the rates of ascorbate consumption could be about 5—25 times that of ascorbate regeneration by the 'Foyer and Halliwell cycle'. These high peroxidative activities together with the large stock of ascorbate inside the chloroplast warrant a permanent and efficient detoxification of H_2O_2 by this organelle.

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