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Hydrogen Peroxide is Scavenged by Ascorbate-specific Peroxidase in Spinach Chloroplasts

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Intact spinach chloroplasts scavenge hydrogen peroxide with a peroxidase that uses a photoreductant as the electron donor, but the activity of ruptured chloroplasts is very low [Nakano and Asada (1980) *Plant & Cell Physiol.* 21: 1295]. Ruptured spinach chloroplasts recovered their ability to photoreduce hydrogen peroxide with the concomitant evolution of oxygen after the addition of glutathione and dehydroascorbate (DHA). In ruptured chloroplasts, DHA was photoreduced to ascorbate and oxygen was evolved in the process in the presence of glutathione. DHA reductase (EC 1.8.5.1) and a peroxidase whose electron donor is specific to L-ascorbate are localized in chloroplast stroma. These observations confirm that the electron donor for the scavenging of hydrogen peroxide in chloroplasts is L-ascorbate and that the L-ascorbate is regenerated from DHA by the system: photosystem I→ferredoxin→NADP→glutathione. A preliminary characterization of the chloroplast peroxidase is given.

Key words: Active oxygen — Ascorbate-specific peroxidase — Dehydroascorbate reductase — Hydrogen peroxide — Spinach chloroplast.

The univalent reduction of molecular oxygen that produces superoxide anion radicals (O_2^-) is unavoidable in chloroplasts, especially when the supply of carbon dioxide to the chloroplasts is repressed (Asada et al. 1977 and references cited in Nakano and Asada 1980). Recent experiments by Ziem-Hanck and Heber (1980) and by Steigner and Beck (1981) have indicated that the photoreduction of molecular oxygen is indispensable for the prevention of the over-reduction of electron carriers in the cyclic electron transport pathway, even in the presence of carbon dioxide. Hydrogen peroxide is produced in chloroplasts through the disproportionation of superoxide catalyzed by superoxide dismutase. It must be immediately scavenged because several enzymes in the CO_2-reduction cycle of the chloroplasts are very sensitive to hydrogen peroxide and the CO_2 fixation is inhibited (Kaiser 1976, 1979, Robinson et al. 1980).

In a previous paper (Nakano and Asada 1980), we demonstrated that intact spinach chloroplasts can scavenge hydrogen peroxide at a high rate with a peroxidase that uses a photoreductant as the electron donor. This means that the hydrogen peroxide produced in the chloroplasts is scavenged by the chloroplasts themselves and that diffusion and scavenging of the hydrogen peroxide by catalase in peroxisomes are very unlikely. Further, the physiological electron donor for the scavenging of hydrogen peroxide should be regenerated by the photochemical reactions in

Abbreviation: DHA, dehydroascorbate.

chloroplasts. The presence of glutathione reductase in chloroplasts (Foyer and Halliwell 1976, Schaedle and Bassham 1977, Jablonski and Anderson 1978) and the high concentrations of ascorbate (Gerhardt 1964) and glutathione (Foyer and Halliwell 1976) in the stroma, together with the presence of ascorbate-specific peroxidase (Groden and Beck 1979, Kelly and Latzko 1979) and DHA reductase (Foyer and Halliwell 1977) in leaf cells suggest the photosystem I → ferredoxin → NADP → glutathione → ascorbate system for the scavenging of hydrogen peroxide. This system was proposed by Foyer and Halliwell (1976).

We present here several lines of evidence which show that ascorbate is the electron donor for the scavenging of hydrogen peroxide in chloroplasts. The photoreductions of hydrogen peroxide and DHA that accompanied the evolution of oxygen were found in ruptured spinach chloroplasts only in the presence of GSH and DHA. We also confirmed that DHA reductase and a peroxidase whose electron donor is specific to ascorbate are located in the stroma of the chloroplasts. The intracellular distribution of the isozymes of peroxidase in spinach leaf cells and their specificities for electron donors indicate that the chloroplast peroxidase differs from the enzyme of spinach leaves isolated previously (Asada and Takahashi 1971).

Materials and Methods

Intact spinach chloroplasts, prepared as described previously (Nakano and Asada 1980), were used throughout the experiments. Crude intact chloroplasts were obtained by differential centrifugation and purified by Percoll-density centrifugation. The intactness of these chloroplasts was usually more than 90%, as estimated by the ferricyanide method (Heber and Santarius 1970). Ruptured chloroplasts were prepared from them by osmotic lysis prior use. All photoreactions were conducted at 25°C under light from a 300-watt to iodine lamp projector (400 kerg cm^{-2} sec^{-1}).

Hydrogen peroxide was determined fluorometrically, and oxygen evolution was measured with an oxygen electrode as described previously (Nakano and Asada 1980). The formation of ascorbate was followed by the increase in absorbance at 265 nm, based on an absorbance coefficient of 14 mM^{-1} cm^{-1}.

Ascorbate peroxidase was assayed from the decrease in absorbance at 290 nm (an absorbance coefficient of 2.8 mM^{-1} cm^{-1}) as ascorbate was oxidized. The reference wavelength of a Hitachi 356 dual-wavelength spectrophotometer was fixed at 310 nm. We used 290 nm in place of 265 nm because the absorbance of our assay mixture was too high at the absorption maximum of ascorbate. The reaction mixture for the peroxidase contained 50 mM potassium phosphate, pH 7.0, 0.5 mM ascorbate, 0.1 mM hydrogen peroxide and 0.1 mM EDTA in a total volume of 1 ml. The reaction was started by adding the enzyme or hydrogen peroxide, and the absorbance decrease was recorded 10 to 30 sec after this addition. Usually no correction for the oxidation of ascorbate in the absence of hydrogen peroxide was necessary, which shows the lack of or very low activity of ascorbate oxidase (EC 1.10.3.3) in spinach leaves. Correction was done for the low, non-enzymatic oxidation of ascorbate by hydrogen peroxide.

The assay mixture (1 ml) used to test the donor specificity of peroxidase contained 50 mM potassium phosphate, pH 7.0, 0.1 mM EDTA, 0.1 mM hydrogen perox-

ide and one of the following donors: 1 mM GSH, 10 μM cytochrome *c* (550 nm, 19 mM^{-1} cm^{-1}), 0.15 mM NAD(P)H (340 nm, 6.2 mM^{-1} cm^{-1}), 10 mM guaiacol (470 nm due to tetraguaiacol, 26.6 mM^{-1} cm^{-1}), 18 mM pyrogallol (430 nm due to purpurogallin, 2.47 mM^{-1} cm^{-1}) or 0.5 mM ascorbate (290 nm, 2.8 mM^{-1} cm^{-1}). The hydrogen peroxide-dependent oxidation of the donors was determined from the absorbance changes at the wavelengths and respective absorbance coefficients given above. When GSH was the donor, the reaction mixture contained 0.12 mM NADPH and glutathione reductase in order to determine GSSG by the decrease in the absorbance at 340 nm (Little et al. 1970).

The assay mixture for DHA reductase contained 50 mM potassium phosphate, pH 7.0, 2.5 mM GSH, 0.2 mM DHA and 0.1 mM EDTA in a final volume of 1 ml. Reaction rates were measured by the increase in absorbance at 265 nm 10 to 30 sec after adding the enzyme. Because GSSG has a low absorbance at 265 nm (0.18 mM^{-1} cm^{-1} at pH 7.0), the reaction rate was corrected by assuming the formation of 1 mol of GSSG for 1 mol of ascorbate formed, i.e., multiplying by a factor of 0.98. The reaction rate also was corrected for the non-enzymatic reduction of DHA by GSH (2.2 nmol ascorbate formed min^{-1} under our assay conditions). Cytochrome *c* oxidase was determined by monitoring oxygen uptake using the reaction mixture containing 50 mM HEPES, pH 7.6, 2 mM ascorbate, 20 μM horse heart cytochrome *c* and 0.2% Triton X-100.

Modified silica sol, Percoll, was obtained from Pharmacia. DHA was purchased from Koch-Light Co., Ltd. Horse heart cytochrome *c* (type III) and glutathione reductase were from Sigma. Horse radish peroxidase (grade II) was a product of Toyobo Co., Ltd.

Results

Hydrogen peroxide is photoscavenged by ruptured spinach chloroplasts in the presence of dehydroascorbate and glutathione

Unlike intact spinach chloroplasts, ruptured chloroplasts have very low photoscavenging activity for hydrogen peroxide; less than 10% that of intact ones (Nakano and Asada 1980). We now have found that ruptured chloroplasts recover the ability to photoreduce hydrogen peroxide when both GSH and DHA are added.

The addition of DHA and GSH to ruptured spinach chloroplasts caused oxygen evolution on illumination; i.e., under the present conditions DHA was a Hill oxidant. After oxygen evolution had ceased, addition of hydrogen peroxide induced oxygen evolution and the hydrogen peroxide disappeared at about one-third the rate it did in intact chloroplasts (Fig. 1). The photodecomposition of hydrogen peroxide and the accompanying evolution of oxygen were repeated if additional hydrogen peroxide was supplied. For each addition of hydrogen peroxide, the evolution of oxygen continued after the disappearance of hydrogen peroxide and ceased when about a half mol of oxygen had evolved if one mol of hydrogen peroxide had been added. This stoichiometry is expected when a photoreductant produced in the thylakoids supplies a reductant for hydrogen peroxide (Nakano and Asada 1980). These observations suggest that the evolution of oxygen after the disappearance of hydrogen peroxide is due to the photoreduction of DHA produced during the reduction of hydrogen peroxide. This is further supported by the following observa-

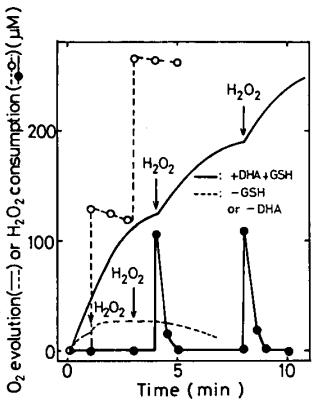

Fig. 1

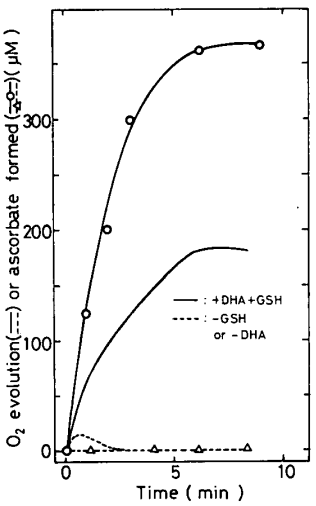

Fig. 2

Fig. 1 Photoscavenging of hydrogen peroxide by ruptured spinach chloroplasts in the presence of DHA and GSH. The complete mixture (1 ml) contained 50 mM HEPES buffer, pH 7.6, intact chloroplasts (227 μg of chlorophyll), 1.5 mM GSH and 0.4 mM DHA (solid lines). Either GSH or DHA was omitted from the complete mixture for the dotted-line curves. Where indicated, 120 μM H_2O_2 was added during illumination.

Fig. 2 Photoreduction of DHA and the accompanying evolution of oxygen by ruptured spinach chloroplasts in the presence of glutathione. The complete reaction mixture (1 ml) contained 50 mM HEPES buffer, pH 7.6, intact chloroplasts (72.8 μg of chlorophyll), 0.2 mM NADPH, 2.5 mM GSH and 0.4 mM DHA. The reaction was started by adding DHA to the illuminated mixture (solid lines). Where indicated either GSH or DHA was omitted, and the reaction was started on illumination (dotted lines). At intervals, 10 μl of the mixture was withdrawn with a microsyringe and placed in a cuvette containing 1 ml of 50 mM potassium phosphate, pH 7.6. The absorbance at 265 nm was determined immediately, and the amount of ascorbate formed was estimated.

tions: decomposition of hydrogen peroxide also was observed in the dark after chloroplasts had been illuminated in the presence of DHA and GSH, but without the evolution of oxygen (data not shown). In the dark, hydrogen peroxide may be reduced by the ascorbate formed from DHA in the light.

In the absence of DHA or GSH, a low rate of oxygen evolution was observed for about 30 sec just after the addition of hydrogen peroxide, but this quickly ceased. Under these conditions, the photodecomposition rate of hydrogen peroxide was very low, and no further evolution of oxygen took place when additional hydrogen peroxide was supplied (Fig. 1). The addition of 0.2 mM NADPH alone or with DHA and GSH had little or no effect on the photodecomposition of hydrogen peroxide and oxygen evolution when the chloroplast concentration was more than 200 μg of chlorophyll ml^{-1}. At 100 μg of chlorophyll ml^{-1}, the addition of 0.2 mM NADPH caused a 1.5-fold increase in both activities. The need for both GSH and DHA in the photoscavenging of hydrogen peroxide indicates that GSH does not function as a reductant of hydrogen peroxide and that there is no glutathione peroxidase in spinach chloroplasts, as shown later (Fig. 7).

Dehydroascorbate is photoreduced in ruptured and intact chloroplasts

The photoreduction of GSSG with the concomitant evolution of oxygen in pea

chloroplasts has been shown by Jablonski and Anderson (1978). We also confirmed the GSSG-dependent evolution of oxygen when ruptured spinach chloroplasts are illuminated in the presence of 0.2 mM NADPH and 1.5 mM GSSG. This GSSG-dependent evolution of oxygen took place only with ruptured chloroplasts; it was not detected with intact chloroplasts. This means that the envelope of the spinach chloroplast is not permeable to GSSG. This also provides evidence that glutathione reductase (EC 1.6.4.2) is localized in the chloroplast stroma, which agrees with the results of Foyer and Halliwell (1976), Schaedle and Bassham (1977) and Jablonski and Anderson (1978).

Ruptured pea chloroplasts showed no photoreduction of DHA, even in the presence of catalytic amounts of GSSG and NADPH (Jablonski and Anderson 1978). Ruptured spinach chloroplasts, however, could photoreduce DHA to ascorbate and evolve oxygen during the reduction in the presence of GSH (Fig. 1 and 2). The initial rate of ascorbate formation in Fig. 2 was about 100 μmol mg^{-1} of chlorophyll hr^{-1}. The stoichiometry of the evolution of one mol of oxygen for the photoreduction of two mol of DHA was obtained (Fig. 2); it suggests that DHA is reduced by GSH (reaction 3) and that GSH is regenerated by NADPH (reaction 2) which has been produced in the thylakoids by photoreduction with the concomitant evolution of oxygen (reaction 1).

$$2\,H_2O+2\,NADP^+ \longrightarrow 2\,NADPH+2\,H^++O_2 \quad (1)$$

$$2\,NADPH+2\,H^++2\,GSSG \longrightarrow 4\,GSH+2\,NADP^+ \quad (2)$$

$$4\,GSH+2\,DHA \longrightarrow 2\,GSSG+2\,Ascorbate \quad (3)$$

The summation of reactions (1), (2) and (3) gave the stoichiometry for the evolution of oxygen and for the formation of ascorbate. Under the conditions we used, DHA was reduced with exogenously added GSH in the dark, but there was no evolution of oxygen. The dark reduction of DHA, however, does not affect the overall stoichiometry shown in Fig. 2.

Photoreduction of DHA with ruptured chloroplasts took place only in the presence of GSH (Fig. 2), but intact spinach chloroplasts showed DHA-dependent evolution of oxygen even in the absence of GSH, although the rate at its maximum was half that of ruptured chloroplasts in the presence of GSH. Because of the slow permeation rate of DHA into intact chloroplasts (Fig. 5), the evolution of oxygen was low just after the addition of DHA to intact chloroplasts, but the rate increased gradually with time (Fig. 3B). When intact chloroplasts had been incubated first with DHA, oxygen evolution was high immediately after illumination (Fig. 3A). That GSH was not required for the photoreduction of DHA by intact chloroplasts is inferred from the high concentration of glutathione inside the chloroplasts for DHA reductase.

Dehydroascorbate reductase and ascorbate-peroxidase are localized in chloroplast stroma

The results we have repsented clearly show that in spinach chloroplasts hydrogen peroxide is reduced to water by a peroxidase that uses ascorbate as the electron donor, and that ascorbate is regenerated by the GSH produced from the NADPH photoreduced in the thylakoids. Therefore, the enzymes that participate in these processes should be present in chloroplasts. We have shown the localization of gluta-

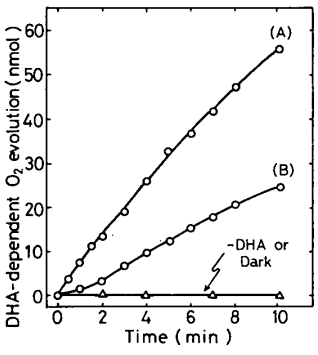

Fig. 3

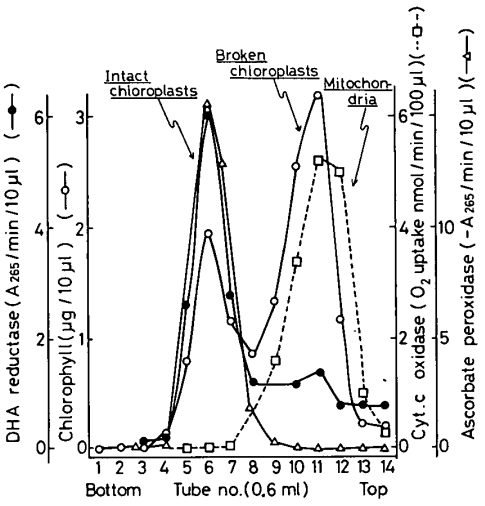

Fig. 4

Fig. 3 DHA-dependent O_2 evolution in intact spinach chloroplasts on illumination. The reaction mixture contained 330 mM sorbitol, 50 mM HEPES buffer, pH 7.6, and intact chloroplasts (24 μg of chlorophyll), in a total volume of 1 ml. After initial incubation of the mixture with 2 mM DHA in the dark for 5 min (A) or just after the addition of DHA (B), the reaction was started by illumination. Where indicated, DHA was omitted.

Fig. 4 Distribution of ascorbate peroxidase, DHA reductase, and cytochrome *c* oxidase from crude spinach chloroplasts after Percoll density centrifugation. Crude chloroplasts obtained by differential centrifugation were layered on a linear gradient of Percoll (10–90%, v/v) containing 50 mM HEPES buffer, pH 7.6, and 330 mM sorbitol, then they were centrifuged in a Sorvall ss-34 rotor at 6,500 rpm for 15 min. The enzymatic activities in each fraction were determined after lysis in the hypotonic assay mixture described in **Materials and Methods.**

thione reductase in chloroplasts and now present evidence for the localization of DHA reductase (EC 1.8.5.1) and a peroxidase that uses ascorbate as its electron donor in spinach chloroplasts.

Intact spinach chloroplast fraction obtained by differential centrifugation was purified further by Percoll density centrifugation. DHA reductase and ascorbate-peroxidase were found in intact chloroplasts, but there was no or only low activity in fractions containing broken chloroplasts and mitochondria (Fig. 4). Thus, both enzymes must be localized in the stroma of the chloroplast.

The enzymatic activities of DHA reductase and ascorbate-peroxidase were detectable only after Percoll-purified, intact chloroplasts had been ruptured hypotonically. The addition of substrates to the intact chloroplasts in the isotonic medium caused low or no oxidation of ascorbate (peroxidase) or formation of ascorbate (DHA reductase). These reactions were observed only when chloroplasts had been ruptured (Fig. 5). The low DHA reductase activity in intact chloroplasts indicates slow permeation of DHA and/or GSH as in the results shown in Fig. 3. The lack of or low ability of intact chloroplasts in isotonic medium to catalyze these reactions was due to the unavailability of substrates to the stroma enzymes, not to the effect of sorbitol. Under our assay conditions, 0.3 M sorbitol did not affect the enzymatic activities of ruptured chloroplasts. Because exogenous hydrogen peroxide is photodecomposed by intact chloroplasts (Nakano and Asada 1980), hydrogen peroxide

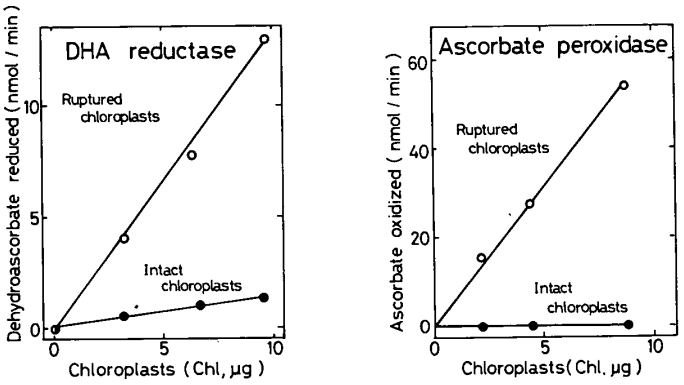

Fig. 5 Effect of lysis of intact spinach chloroplasts on ascorbate peroxidase and DHA reductase. The reaction mixture for ascorbate peroxidase (1 ml) contained 50 mM HEPES buffer, pH 7.6, 0.4 mM ascorbate and the Percoll-purified chloroplasts as indicated. The reaction was started by adding 0.1 mM H_2O_2. The mixture for DHA reductase contained 50 mM HEPES, pH 7.6, 0.5 mM GSH and the Percoll-purified chloroplasts as indicated. The reaction was started by adding 0.3 mM DHA. Under the above conditions, the chloroplasts were ruptured by osmotic lysis (ruptured chloroplasts). To maintain the intactness of chloroplasts, 330 mM sorbitol was added to the above mixture (intact chloroplasts).

should be able to pass across the envelope. Therefore, the results in Fig. 5 mean that ascorbate, DHA and GSH cannot cross the chloroplast envelope. Further, these observations rule out the binding or adsorption of ascorbate peroxidase and DHA reductase on the outside of the chloroplast envelope and confirm the localization of both enzymes inside the chloroplast.

Percoll-purified, intact chloroplasts were ruptured by osmotic shock in HEPES buffer; then the thylakoid and stroma fractions were obtained by centrifugation at 8,000 × *g* for 10 min. Ascorbate-peroxidase and DHA reductase were found exclusively in the stromal fraction; no activities were found in the thylakoid fraction (Fig. 6). Boiling of the stromal fraction for 10 min resulted in complete inactivation of both enzymes. Under our assay conditions, the rate of ascorbate oxidation by

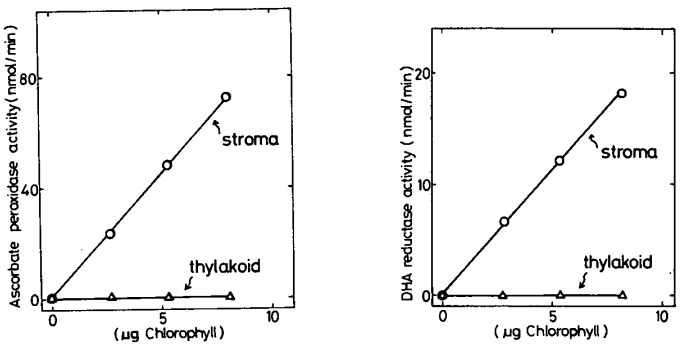

Fig. 6 Distribution of ascorbate peroxidase and DHA reductase in spinach chloroplasts. The Percoll-purified chloroplasts were ruptured osmotically in 50 mM HEPES buffer, pH 7.6, for 5 min at 5°C. Thylakoids and stroma were separated from the resulting mixture by centrifugation at 8,000 × *g* for 10 min. The thylakoid fraction (pellet) was washed by resuspension in the same buffer, then centrifuged as before. The enzymatic activities were determined as described in **Materials and Methods**. The amount of stroma is shown by the corresponding amount of thylakoid chlorophyll.

the chloroplast peroxidase was 200–400 μmol mg^{-1} of chlorophyll hr^{-1}. The rate of DHA reduction by DHA reductase was about 100 μmol mg^{-1} of chlorophyll hr^{-1} at its pH optimum.

Electron donor of chloroplast peroxidase is specific to ascorbate

Plant cells contain peroxidase in many isomeric forms, and the spinach leaf is no exception. Two isozymes of peroxidase from spinach leaves have been purified to the homogeneous state (Asada and Takahashi 1971). The polyacrylamide gel electrophoresis at pH 8.9 of the buffer extract of spinach leaves and visualization of the enzyme with 3-amino-9-ethyl carbazole as the electron donor (Shaw and Prasad 1970) indicated that at least five isozymes were present (data not shown). The stromal fraction from Percoll-purified chloroplasts, however, did not give a peroxidase band on the gel under the above conditions even when the stromal sample had an activity of 0.5 μmol of ascorbate min^{-1} for hydrogen peroxide-dependent oxidation.

The specificity of the electron donor for the chloroplast peroxidase was compared with that of the purified peroxidase A from spinach leaves (Asada and Takahashi 1971) and the buffer extract of the leaves. The chloroplast enzyme was highly specific to ascorbate; it did not catalyze the oxidation of guaiacol, horse heart cytochrome *c*, NADH$^+$, NADPH$^+$ and GSH (Fig. 7A). Because the chloroplast stroma showed a high oxidase activity for pyrogallol, we could not determine accurately the pyrogallol oxidation with the chloroplast peroxidase; its rate was below 5% that of ascorbate. The inability of the stroma to catalyze the oxidation of GSH with hydrogen peroxide agrees with the results in Fig. 1.

In contrast to the chloroplast peroxidase, purified spinach peroxidase A catalyzed the oxidation of guaiacol and pyrogallol at a high rate, but the oxidation

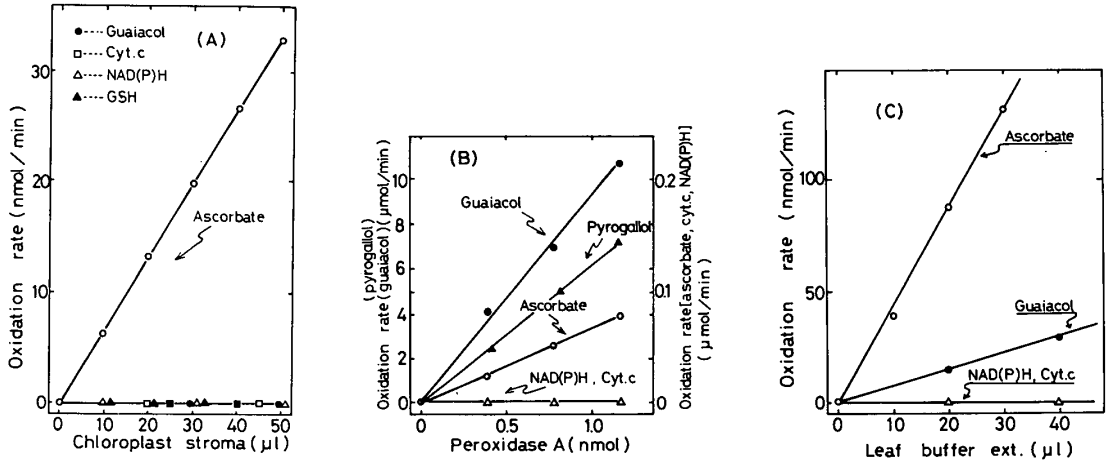

Fig. 7 Donor specificity of peroxidases in spinach chloroplast stroma (A) and leaf extract (C) and of spinach peroxidase A (B). Chloroplast stroma was prepared as described in Fig. 6. Purified spinach peroxidase A was prepared as previously reported (Asada and Takahashi 1971). The leaf buffer extract was prepared as follows: 50 g of spinach leaves was homogenized with 100 ml of 50 mM potassium phosphate, pH 7.0, then this mixture was filtered through gauze and centrifuged at 8,000 $\times g$ for 15 min. The supernatant (80 ml) was used as the extract. The oxidation of each donor was determined as described in **Materials and Methods**.

rate of ascorbate was only 0.8% that of guaiacol under our assay conditions. Horse radish peroxidase (an acidic isozyme, $A_{410}/A_{280}=3.4$) showed donor specificity similar to that of spinach peroxidase A; the oxidation rate of ascorbate was about 3% that of guaiacol. Thus, the chloroplast peroxidase differs from purified spinach peroxidase A and horse radish peroxidase in its high donor specificity for ascorbate. The buffer extract of spinach leaves catalyzed the oxidation of both guaiacol and ascorbate, but the oxidation of ascorbate was nearly 6-fold that of guaiacol (Fig. 7C). Therefore, most of the ascorbate oxidation catalyzed by the extract was accounted for by the chloroplast peroxidase; only a very minor part (0.14%) was catalyzed by guaiacol-oxidizable peroxidase, such as spinach peroxidase A.

Properties of dehydroascorbate reductase and peroxidase in the chloroplasts stroma

The apparent K_m for the substrates and the optimum pH of DHA reductase and the peroxidase were measured in the stromal fraction prepared from the Percoll-purified chloroplasts. The optimum pH values of DHA reductase and peroxidase were 8.3 and 7.0, respectively (Fig. 8). K_m values for the substrates of DHA reductase and the peroxidase were determined from double reciprocal plots of the reaction rates and the substrate concentrations. The substrate concentrations tested were up to 3.5 mM GSH and 0.4 mM DHA for DHA reductase and up to 0.5 mM ascorbate and 0.1 mM hydrogen peroxide for the peroxidase. The K_m values of DHA reductase were 70 μM for DHA when GSH was 3.5 mM and 1.4 mM for GSH when DHA was 300 μM at pH 8.3. These values are similar to those reported by Foyer and Halliwell (1977). Ascorbate peroxidase had a K_m of 0.3 mM for ascorbate when hydrogen peroxide was 100 μM and a value of 45 μM for hydrogen peroxide when ascorbate was 0.5 mM at pH 7.0. Thus, the K_m values of DHA reductase and ascorbate peroxidase as well as the concentrations of GSH (3.5 mM) and ascorbate (2.5 mM) in chloroplasts (Foyer and Halliwell 1976, Gerhardt 1964) confirm the participation of both enzymes in the scavenging of hydrogen peroxide in chloroplasts.

Under standard assay conditions, the chloroplast peroxidase was inhibited completely by 1 mM KCN or sodium azide, but was insensitive to 1 mM iodoacetate. The chloroplast enzyme catalyzed the oxidation of ascorbate by *t*-butylhydroperoxide with an apparent K_m of 0.45 mM. However, its rate with 2.0 mM *t*-butylhydroperoxide was about 5% that with 0.1 mM hydrogen peroxide.

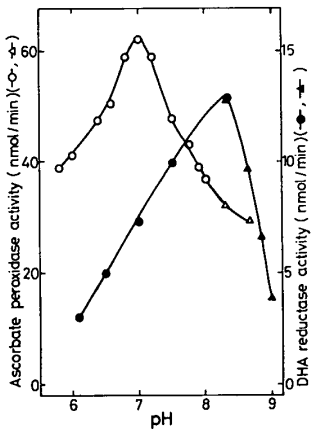

Fig. 8 pH Dependence of the ascorbate peroxidase and DHA reductase of spinach chloroplasts. Chloroplast stroma was prepared from intact chloroplasts as given in Fig. 6; stroma corresponding to 10 μg of chlorophyll of intact chloroplasts was used in each assay. Enzymatic activities were determined as described in **Materials and Methods**, except that 20 mM potassium phosphate, pH 6–8.5 (△, ▲) or 20 mM Tris-Cl$^-$, pH 8–9.5 (○, ●) was used.

Discussion

We showed previously that intact spinach chloroplasts scavenge hydrogen peroxide with a peroxidase that uses a photoreductant as the electron donor at a rate about 3-fold that of the bicarbonate-dependent evolution of oxygen. The photoscavenging activity of ruptured chloroplasts, however, was less than 10% that of intact ones (Nakano and Asada 1980). The recovery of the photoreduction of hydrogen peroxide and the concomitant evolution of oxygen in the ruptured chloroplasts when both GSH and DHA were added (Fig. 1) means that the photoreductant for the scavenging of hydrogen peroxide in spinach chloroplasts is ascorbate, not GSH. The absence of glutathione peroxidase in the chloroplast stroma (Fig. 7A) indicates that GSH is unable to reduce hydrogen peroxide.

The photoreduction of the DHA that accompanies the evolution of oxygen in ruptured and intact chloroplasts (Fig. 2 and 3), and the localization of DHA reductase and ascorbate-specific peroxidase in the stroma (Fig. 4 and 5) are evidence that spinach chloroplasts have a system that first reduces DHA and then hydrogen peroxide. Further evidence for the participation of GSH in the photoreduction of DHA and hydrogen peroxide is the inhibition of both reactions by a thiol reagent. The DHA-dependent evolution of oxygen and the reduction of hydrogen peroxide by illuminated intact chloroplasts were inhibited by 1 mM iodoacetate under the conditions given in Fig. 3 (A) and in a previous paper (Nakano and Asada 1980) (data not shown), although the chloroplast peroxidase was insensitive to the thiol reagent. The localization of glutathione reductase, ascorbate and glutathione in chloroplasts and our results provide conclusive evidence for the photogeneration of ascorbate via the system of photosystem I → ferredoxin → NADP → glutathione, as well as for the reduction of hydrogen peroxide by the ascorbate generated by this system (Fig. 9).

Our results show that the low photoscavenging rate of hydrogen peroxide in ruptured chloroplasts is due to the low concentrations of ascorbate and glutathione in the reaction mixtures. This is not surprising because the stromal components are diluted by osmotic lysis of intact chloroplasts. The concentrations of ascorbate and glutathione inside the chloroplast have been estimated to be 2.5 mM (Gerhardt 1964) and 3.5 mM (Foyer and Halliwell 1976). Under the conditions given in Fig. 1 (suspension of ruptured chloroplasts contained 214 μg of chlorophyll ml^{-1}), for example, ascorbate and glutathione were diluted to 24 and 33 μM, based on the assumption that the concentration of chlorophyll in intact chloroplasts is 25 mM (Nobel 1973). These concentrations are lower than the K_m values we found for the ascorbate of the chloroplast peroxidase (0.3 mM) and for the DHA (70 μM) and GSH (1.4 mM) of the DHA reductase. The GSH concentration in the ruptured chloroplasts is lower than the K_m value for GSSG of gutathione reductase (196 μM, Halliwell and Foyer 1978). NADPH had little effect on the hydrogen peroxide-photoscavenging system in ruptured chloroplasts at a high concentration of 200 μg chlorophyll ml^{-1}. This is inferred by the low K_m value for the $NADP^+$ of ferredoxin-NADP reductase (9.8 μM, Shin and Arnon 1965) and for the NADPH of glutathione reductase (2.8 μM, Halliwell and Foyer 1978). The concentration of pyridine nucleotides in intact chloroplasts has been shown to be about 1 mM (Krause and Heber 1976); therefore, under the conditions given in Fig. 1, their concentration in the medium is 9 μM.

Even when saturating concentrations of DHA and GSH were added, the photoscavenging activity of exogenous hydrogen peroxide by ruptured chloroplasts was about one-third that by intact ones. Incomplete recovery shows the importance of chloroplast integrity for the scavenging system of hydrogen peroxide, as well as the importance of the concentrations of glutathione and ascorbate. The rate of hydrogen peroxide scavenging by intact chloroplasts (200–500 μmol hydrogen peroxide mg^{-1} of chlorophyll hr^{-1}) was usually higher than the rate of ascorbate formation by DHA reductase in the stromal preparation (about 100 μmol ascorbate mg^{-1} of chlorophyll hr^{-1}). The reason for this is not yet clear, but photoactivation of DHA reductase or its activation by a stromal component cannot be excluded.

Chloroplast peroxidase was found in the stroma, but little or no activity was detectable in the thylakoids under our assay conditions (Fig. 6), which sharply contrasts with the results of Groden and Beck (1979). Soluble ascorbate-specific peroxidase also has been found in pea leaves (Kelly and Latzko 1979). Chloroplast peroxidase is characterized by its high specificity for ascorbate as the electron donor and differs from the other peroxidase isolated from spinach leaves (Asada and Takahashi 1971) and from horse radish peroxidase (Fig. 7). These are "typical" plant peroxidases which had high turnover for guaiacol, but the chloroplast peroxidase did not catalyze the oxidation of guaiacol. The "typical" peroxidases also catalyzed the oxidation of ascorbate, but the rate was only 1–3% that of the reaction with guaiacol (Fig. 7B). Catalysis of ascorbate oxidation has been shown for horse radish peroxidase (Maehly 1955) and for the cytochrome *c* peroxidase from yeast (Yonetani and Ray 1965), but the rates are very low in comparison with those for guaiacol and cytochrome *c*. Chloroplast peroxidase was inhibited by cyanide and azide. These poisons also inhibit the photoscavenging of hydrogen peroxide by intact chloroplasts (Nakano and Asada 1980); thus our results confirm the participation of chloroplast peroxidase in the scavenging of hydrogen peroxide.

Ascorbate-specific peroxidase has been detected in the catalase-lacking protozoa, *Euglena gracilis* (Shigeoka et al. 1980) and *Trypanosoma cruzi* (Boveris et al. 1980). The *Euglena* enzyme has been characterized as a hemoprotein. In preliminary results we found no ascorbate-specific peroxidase in a Cyanobacterium, *Plectonema boryanum*. The presence of the ascorbate-peroxidase in catalase-lacking chloroplasts from angiosperms is interesting in terms of the evolution of the hydrogen peroxide-scavenging system.

Foyer and Halliwell (1977) found DHA reductase in spinach leaves and partially purified it, but they did not show its localization in the chloroplasts. The absence of DHA reductase in the chloroplast caused them to suppose that the non-enzymatic reduction of DHA by GSH plays a role in the regeneration of ascorbate. But the localization of DHA reductase in the spinach chloroplast (Fig. 4) and the rate of non-enzymatic reduction indicate that most DHA is reduced by catalysis with the enzyme. We also found non-enzymatic reduction of DHA to ascorbate by GSH, the rate of which increased at a high pH as reported previously (Foyer and Halliwell 1977). In 50 mM phosphate buffer, the formation of ascrobate was 40, 25 and 3.5 nmol ml^{-1} min^{-1} at pH 8.3, 7.8 and 7.1, respectively, when the concentrations of GSH and DHA were 3.5 and 0.25 mM. These are the concentrations in the chloroplast (Gerhardt 1964, Foyer and Halliwell 1976) based on the assumption that 10% of the total ascorbate is in the oxidized form. If the

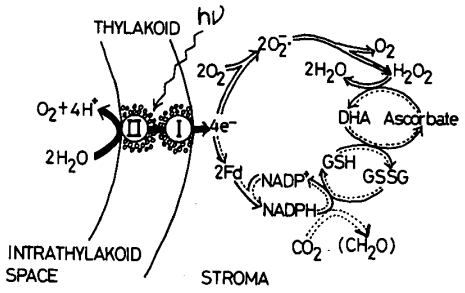

Fig. 9 Formation and scavenging of superoxide and hydrogen peroxide in chloroplasts. When molecular oxygen is reduced by two electrons from photosystem I, one molecule of hydrogen peroxide is produced (⟹). This hydrogen peroxide is reduced further to water by one molecule of ascorbate which has been generated by the (⟹) system with two electrons from photosystem I.

chlorophyll concentration in intact chloroplasts is 25 mM (Nobel 1973), then the DHA reductase activity in the stroma (about 100 μmol of ascorbate mg^{-1} of chlorophyll hr^{-1}) corresponds to 38 μmol of ascorbate ml^{-1} of chloroplasts min^{-1}. Thus, the non-enzymatic reduction of DHA by GSH is less than 0.1% of the enzymatic one, even at pH 8.3, and does not account for the regeneration of ascorbate in the chloroplast.

Fig. 9 shows the photoreduction of molecular oxygen in photosystem I which resulted in the formation of the superoxide anion radical (Asada et al. 1974) and its fate in chloroplasts. The O_2^- generated is converted to hydrogen peroxide and molecular oxygen by the superoxide dismutase present in chloroplasts at about 10 μM (Asada et al. 1973). O_2^- is reduced to hydrogen peroxide with ascorbate and GSH at the rates of 2.7×10^5 M^{-1} sec^{-1} (Nishikimi 1975) and of 6.7×10^5 M^{-1} sec^{-1} (Asada and Kanematsu 1976). O_2^- is also oxidized with oxidized plastocyanin and cytochrome *f* at the rate of about 10^6 M^{-1} sec^{-1} (Takahashi et al. 1980, Tanaka et al. 1978). However, when these reaction rates and concentrations are compared with those of the superoxide dismutase in the chloroplasts, it is clear that most of the O_2^- reacts with the superoxide dismutase (Asada et al. 1977).

The hydrogen peroxide produced is immediately reduced to water by ascorbate that is catalyzed by the ascorbate-specific peroxidase in the chloroplasts, and ascorbate is regenerated by the system we have proposed. When two electrons from photosystem I are transferred to molecular oxygen to produce two molecules of O_2^-, the transfer of two electrons from photosystem I to DHA is required to reduce hydrogen peroxide. Thus, in intact chloroplasts no apparent change in the oxygen concentration takes place even when O_2^- is produced in the absence of the physiological electron acceptor, CO_2 (Nakano and Asada 1980).

Even when only some electrons from photosystem I are transferred to molecular oxygen to produce O_2^-, e.g., 15 μmol of O_2^- mg^{-1} of chlorophyll hr^{-1}, the rate of hydrogen peroxide production in the chloroplasts is 10 μM sec^{-1} if we assume a chlorophyll concentration in them of 25 mM (Asada et al. 1977). On the other hand, CO_2 fixation in the chloroplasts is inhibited by about half with 10 μM hydrogen peroxide (Kaiser 1976, 1979). Thus, the scavenging of hydrogen peroxide in chloroplasts is indispensable if CO_2 fixation is to be maintained.

The production of superoxide anion radicals has been reported in mitochondria (Rich and Bonner 1978, Boveris et al. 1976) from plant and mammalian cells. NADH-dehydrogenase and ubiquinone-cytochrome *b* (Turrens and Boveris 1980) have been proposed as the sites of superoxide generation. The superoxide produced is disproportionated to hydrogen peroxide and molecular oxygen, as in chloroplasts because the superoxide dismutase is contained in mitochondria. The scavenging

system for hydrogen peroxide in mammalian mitochondria, however, differs from that proposed here for chloroplasts. Glutathione peroxidase has been suggested as the major scavenging enzyme of hydrogen peroxide in the mitochondria (Sies and Moss 1978, Nohl and Jordan 1980). What system participates in the removal of hydrogen peroxide in plant mitochondria remains to be determined.

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