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Light-Dependent Reduction of Dehydroascorbate by Ruptured Pea Chloroplasts[1]

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ABSTRACT

Glutathione dehydrogenase (EC 1.8.5.1) was partially purified from pea shoots. The pH optimum was 7.6. The K_m values for GSH and dehydroascorbate were 4.4 and 0.44 millimolar, respectively. The enzyme was inhibited by iodoacetate and $CuSO_4$ but not significantly by $ZnCl_2$ or NaN_3. Part of the total enzyme activity was associated with isolated chloroplasts.

Illuminated ruptured chloroplasts, in the presence of 50 micromolar NADP(H) and substrate concentrations of GSH or GSSG, catalyzed (dehydroascorbate plus glutathione)-dependent O_2 evolution with the concomitant reduction of dehydroascorbate to ascorbate. Oxidation of ascorbate by ascorbate oxidase activity associated with the chloroplasts was relatively insignificant. $ZnCl_2$ inhibited (dehydroascorbate plus glutathione)-dependent O_2 evolution but not ascorbate formation. The reaction was attributed to light-dependent reduction of GSSG (involving glutathione reductase) coupled to the reduction of dehydroascorbate (involving glutathione dehydrogenase). Light-dependent reduction of GSSG appears to be the rate-limiting step in this reaction sequence at physiological concentrations of GSH.

Chloroplasts reduce O_2 in the light to H_2O_2 (2, 4). Foyer and Halliwell (5) have proposed a mechanism for the reduction of H_2O_2 in chloroplasts involving ascorbate as the reductant. Their model also postulates that the DHA[3] produced in this reaction is successively reduced by GSH and NADPH, the latter involving GSSG reductase (EC 1.6.4.2) according to the following reaction sequence:

$$2\ H_2O + 2\ NADP \rightarrow 2\ NADPH + 2\ H^+ + O_2 \quad (I)$$

$$2\ NADPH + 2\ H^+ + 2\ GSSG \rightarrow 4\ GSH + 2\ NADP \quad (II)$$

$$4\ GSH + 2\ DHA \rightarrow 2\ ascorbate + 2\ GSSG \quad (III)$$

$$2\ H_2O + 2\ DHA \rightarrow 2\ ascorbate + O_2 \quad (IV)$$

Reaction III proceeds nonenzymically, especially at alkaline pH. The enzyme GSH dehydrogenase (GSH:DHA oxidoreductase, EC 1.8.5.1) which catalyzes reaction III has been described in a number of plants (12) and partially purified from peas (16) and spinach (6). Foyer and Halliwell (5) were unable to detect GSH dehydrogenase activity in spinach chloroplasts and concluded that the enzyme was localized predominately in the cystosol. They proposed that DHA was reduced nonenzymically by GSH in chloroplasts, especially as light-induced alkalization of chloroplast stroma (10) provides favorable conditions for the nonenzymic reaction.

We reported previously (11) that illuminated ruptured chloroplasts catalyze the reduction of GSSG with the concomitant evolution of O_2. The properties of these reactions were consistent with the operation of light-coupled GSSG reductase (reactions I and II). Taken in conjunction with the proposal of Foyer and Halliwell (5), this suggests that the reduction of DHA, and ultimately H_2O_2, could be light coupled via GSSG reductase. This mechanism predicts that in the presence of catalytic amounts of GSH or GSSG, chloroplasts will exhibit DHA-dependent O_2 evolution with the concomitant reduction of DHA to ascorbate. Previously, we reported that ruptured chloroplasts did not support DHA-dependent O_2 evolution in the presence of 40 μM GSSG, 50 μM NADPH, and 1 mM DHA (11). In this paper we report that illuminated ruptured chloroplasts in the presence of 50 μM NADPH, 0.94 mM DHA, and ≥0.15 mM GSH or ≥0.1 mM GSSG exhibit characteristics consistent with the operation of reactions I to IV and that reaction III involves a chloroplast GSH dehydrogenase.

MATERIALS AND METHODS

Chemicals. DHA was obtained from Koch-Light, Bucks., England; the purity as estimated by oxidation of GSH in the presence of purified GSH dehydrogenase was 94%. Cyt *c* (horse heart), ascorbate oxidase (lyophilizate), and GSSG were obtained from Boehringer, Mannheim, Germany. GSH was obtained from Sigma and contained 0.06 mol GSSG/mol GSH by assay with glutathione reductase. Acidic substrates were adjusted to pH 7.0 with KOH.

Plant Material and Chloroplasts. Pea seedlings (*Pisum sativum* cv Massey Gem) were raised as described previously (11). Chloroplasts were prepared by a modification of the method used by Anderson and House (1). Pea shoots (100 g) were blended for 4 s in an icy slush of extracting medium (250 ml) containing 0.33 M D-glucose, 50 mM Na_2HPO_4, 50 mM KH_2PO_4, 0.2 mM $MgCl_2$, 0.1% (w/v) NaCl, 0.1% (w/v) BSA adjusted to pH 6.5 with HCl. After squeezing through muslin, chloroplasts were recovered by centrifugation (2500*g* for 30 s). The surface of the pellet was rinsed (without resuspending) with medium containing 0.33 M D-glucose, 0.1% (w/v) BSA, and 0.04 volume of incubating medium. The pellet was resuspended in medium containing 0.33 M D-sorbitol adjusted to pH 7.6 with Tris (washing medium) and, after centrifuging at 2,500*g* for 20 s, the supernatant solution was aspirated. In some experiments this procedure was repeated four more times, but unless otherwise stated, the chloroplasts were washed once. The washed chloroplasts were resuspended in 1 to 2 ml of incubating medium containing 0.33 M D-sorbitol, 50 mM Hepes, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM Na_2EDTA, and 0.1% (w/v) BSA adjusted to pH 7.6 with KOH. Sonicated chloroplasts were prepared as described previously (11). Osmotically shocked chloro-

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[3] Abbreviations: DHA, dehydroascorbate; GAP, glyceraldehyde 3-phosphate.

plasts were prepared by diluting intact chloroplasts (1 volume) in 6 volumes H_2O. After 5 min, the concentration of the medium was adjusted to that of incubating medium. Chloroplasts were completely shocked by this procedure as determined by the intactness test (11).

O_2 Evolution and Reduction of DHA. Reactions were performed in incubating medium at 25 C in white light. O_2 evolution was measured polarographically (3) in a pair of model DW oxygen electrodes supplied by Hansatech, Norfolk, England. Standard reaction mixtures (1 ml) for the determination of (DHA plus GSH)-dependent O_2 evolution by ruptured chloroplasts contained 200 μg Chl/ml, 50 μM NADPH, 2 mM GSH, and the reaction was initiated by addition of DHA to a final concentration of 0.94 mM.

Reduction of DHA was followed by treating samples (0.2 ml) with 1 ml of 1.67% (w/v) metaphosphoric acid and analyzing the supernatant solution for ascorbate by titration with 2,6-dichlorophenol indophenol. Standard reaction mixtures for (DHA plus GSSG)-dependent O_2 evolution by sonicated chloroplasts contained 200 μg Chl/ml, 0.2 mM GSSG, and 50 μM NADPH. Following cessation of GSSG-dependent O_2 evolution (11), denoting reduction of GSSG to GSH, (GSSG plus DHA)-dependent O_2 evolution was initiated by the addition of DHA to a final concentration of 0.94 mM. GSSG-dependent O_2 evolution by ruptured chloroplasts was determined as described previously (11).

Partial Purification of GSH Dehydrogenase. GSH dehydrogenase was purified essentially as described by Foyer and Halliwell (6). Pea leaf tissue was extracted in 0.1 M K-phosphate (pH 6.3) (2.5 ml/g fresh weight of tissue). The extract was squeezed through muslin and centrifuged at 15,000*g* for 30 min. The supernatant solution (referred to as crude extract) was treated with solid $(NH_4)_2SO_4$ (0.291 g/ml; about 50% saturated). After removing insoluble material by centrifugation (10,000*g* for 15 min), more $(NH_4)_2SO_4$ (0.194 g/ml) was added. The pellet was dissolved in 10 mM K-phosphate (pH 6.3) and subjected to gel filtration through Sephadex G-100 equilibrated with the same buffer. Active fractions were pooled and stored at −20 C.

Enzyme Assays. GSH dehydrogenase was assayed by two methods. Method 1 involved GSH-dependent consumption of O_2 in the presence of DHA and ascorbate oxidase. Reaction mixtures (1 ml) contained 100 mM K-phosphate (pH 6.3), 2 units of ascorbate oxidase, 1.88 mM DHA, 50 mg BSA, and enzyme extract. Following preincubation for 5 min at 25 C, the reactions were initiated by the addition of GSH to a final concentration of 4 mM and O_2 consumption monitored polarographically. Enzyme activity, corrected for controls lacking GSH and enzyme extract, is expressed as μmol O_2 consumed/min (GSH dehydrogenase units). Method 2 involved GSH-dependent reduction of DHA. Reaction mixtures (1 ml) contained 50 mM K-phosphate (pH 6.3), 1.88 mM DHA, and enzyme extract. Following preincubation at 25 C for 5 min, the reactions were initiated by the addition of GSH to a final concentration of 4 mM. After 4 min, the reactions were terminated with 0.15 ml of 10% (w/v) metaphosphoric acid and the supernatant solution (1 ml) analyzed for ascorbate as described above. The rate of ascorbate production in the absence of enzyme gradually decreased during the incubation period but the enzyme-dependent rate was constant. All results are the means of duplicate determinations corrected for controls lacking GSH and enzyme extract and are expressed as GSH-dependent formation of ascorbate in μmol/min. Because of the stoichiometry relating O_2 consumption (method 1) to the formation of ascorbate (method 2), the rates for method 2 are twice those for method 1.

Catalase activity was assayed polarographically. Reaction mixtures (1 ml) containing 50 mM K-phosphate (pH 7.0) and enzyme extract were preincubated for 5 min at 25 C and the reactions initiated by addition of H_2O_2 to a final concentration of 10 mM. Catalase activity is expressed as μmol O_2 evolved/min. GSSG reductase (11), NADP-specific GAP dehydrogenase (9), and Cyt *c* oxidase (14) were assayed at 25 C. Reduced Cyt *c* was determined spectrophotometrically at pH 7.4 (13). The ascorbate oxidase activity of fractions associated with the purification of GSH dehydrogenase was assayed as in (15) (method 1). Ascorbate oxidase activity of sonicated chloroplast preparations was determined polarographically by ascorbate-dependent O_2 consumption at 25 C in the dark (method 2). Reactions were conducted in chloroplast-incubating medium containing 200 μg Chl/ml and initiated by addition of L-ascorbate to a final concentration of 2.8 mM. Activity is expressed as μmol O_2 consumed/min (units).

Subcellular Fractionation of Pea Leaf Tissue. In the following schedule, incubating medium and extracting medium refer to the media described for the preparation of chloroplasts. Pea shoots were blended in extracting medium; the suspension obtained after squeezing through muslin is designated crude unfractionated homogenate (fraction I, see Table I). Chloroplasts were isolated by centrifugation and washed three times as per the standard procedure (fraction II), but the initial 2,500*g* supernatant solution (fraction VI) and the combined chloroplast washings (fraction V) were retained. The 2,500*g* supernatant solution (fraction VI) was subjected to further centrifugation at 10,000*g* for 15 min to yield the 10,000*g* supernatant solution (fraction VII) and the 10,000*g* pellet which was resuspended in incubating medium (fraction VIII). After washing, the chloroplasts (fraction II) were sonicated and the supernatant solution (stroma, fraction III) was recovered by centrifugation at 20,000*g* for 15 min; the pellet (grana) was resuspended in incubating medium (fraction IV). All fractions were sonicated and assayed for GSH dehydrogenase activity by method 1 in 100 mM K-phosphate (pH 7.6).

Chloroplast intactness, Chl, protein, and GSH were determined as described previously (11).

RESULTS

Properties of GSH Dehydrogenase. Crude extracts of pea leaf tissue catalyzed GSH-dependent consumption of O_2 in the presence of DHA and exogenous ascorbate oxidase. The specific activity was constant at protein concentrations up to 0.3 mg/ml and the mean rate at pH 6.3 was 0.089 μmol O_2/min·mg protein. Crude extracts also contained ascorbate oxidase activity amounting to about 70% of the GSH dehydrogenase activity as determined by O_2 consumption. Purification of GSH dehydrogenase enhanced the specific activity 112-fold and essentially removed all of the ascorbate oxidase activity; the residual ascorbate oxidase activity accounted for the oxidation of less than 0.1% of the ascorbate produced by purified GSH dehydrogenase in a 5-min assay.

Some properties of purified GSH dehydrogenase were examined by method 2. The enzyme was relatively stable at −20 C (15% decrease after 6 days), but at 0 C the activity decreased by 25% after 24 h and at 90 C for 5 min, activity was abolished. The pH optimum in K-phosphate and Tris-HCl buffers was 7.6. The nonenzymic rate of reduction of DHA increased with pH up to 7.8 but decreased at higher values. The ratio of the nonenzymic to enzymic rate increased above pH 7.8. At alkaline pH, aerobic oxidation of exogenous ascorbate (0.5 mM) in the absence of GSH and enzyme was negligible (less than 1% for the duration of GSH dehydrogenase assays).

The apparent K_m values, calculated from double reciprocal plots, were 0.44 mM for DHA and 4.4 mM for GSH in the presence of 12.5 mM GSH and 1.88 mM DHA, respectively. DHA concentrations ≥1 mM inhibited enzyme activity (e.g. 18% inhibition at 1.88 mM). L-Cysteine, 2-mercaptoethanol, and thioglycollate (each 4 mM) did not substitute for GSH in the enzyme catalyzed reaction, although these compounds did promote nonenzymic reduction of DHA. $ZnCl_2$ and NaN_3 (both at 0.1 and 1 mM) did not significantly inhibit enzyme activity. $CuSO_4$ (0.1 mM) and iodoacetate (0.1 and 1 mM) inhibited activity by 44%, 85%, and 99%, respectively.

Subcellular Distribution of GSH Dehydrogenase Activity in Pea

Table I. *Subcellular Distribution of GSH Dehydrogenase in Pea Leaf Tissue*

The reactions were prepared as described under "Materials and Methods." GSH dehydrogenase was determined by method 1 in 100 mM K-phosphate (pH 7.6). The intactness of the washed chloroplasts (fraction II) prior to sonication was 76%.

Fraction	GSH Dehydrogenase		
	units	*units/mg Chl*	*units/mg protein*
I Crude unfractionated homogenate	5.46	0.369	0.030
II Washed chloroplasts	0.421[a]	0.225	0.038
III Stroma from II	0.415		0.086
IV Grana from II	0.006		0.001
V Chloroplast washings (three washes)	0.120	0.016	0.005
VI Low speed supernatant solution (2,500*g*)	4.33	0.889	0.035
VII 10,000*g* supernatant solution from VI	4.19		0.043
VIII 10,000*g* pellet from VI	0		0.000

[a] Value calculated by summation of fractions III and IV.

Shoots. The distribution of GSH dehydrogenase activity in subcellular fractions of pea shoots, prepared in iso-osmotic medium, is shown in Table I. The recoveries of GSH dehydrogenase activity, protein, and Chl in fractions III to VI relative to fraction I were 89%, 90%, and 97%, respectively. Most of the activity was associated with fraction VI, but the activity/mg protein of this fraction was similar to fraction I. The activity associated with fraction VI was not removed by centrifugation (fraction VIII). The activity/mg protein in chloroplast stroma (fraction III) was greater than all other fractions and approximately 3 times greater than fraction I.

The association of GSSG reductase, catalase, Cyt *c* oxidase, and NADP-specific GAP dehydrogenase with chloroplasts during a series of washes was examined (Table II). Relative to the activity of GSSG reductase, the activity of catalase and Cyt *c* oxidase decreased with each successive wash; after five washes the ratio decreased by 90 and 85%, respectively. The ratio of catalase to NADP-specific GAP dehydrogenase activity decreased by a similar amount (95%) but the ratio of GSH dehydrogenase to GAP dehydrogenase increased with the first wash and remained approximately constant during subsequent washes. The activity of GSH dehydrogenase in intact chloroplasts (washed five times) was enhanced approximately 100-fold when the chloroplasts were sonicated. These results suggest that part of the total GSH dehydrogenase activity of pea shoots is associated with the soluble component of chloroplasts.

Table II. *The Association of GSSG Reductase, GSH Dehydrogenase, and Other Enzymes with Chloroplasts during a Series of Washes*

Following each wash, chloroplast intactness was determined and a sample sonicated, centrifuged at 20,000*g* for 15 min, and the supernatant solution assayed for the enzymes shown. GSH dehydrogenase activity was determined by method 1.

No. washes	Experiment 1			
	Intactness	GSSG reductase	Catalase: GSSG reductase	Cyt *c* oxidase: GSSG reductase
	%	*μmol/min·mg Chl*	*ratio*	
0	65	0.409	147.5	48.9
1	68	0.354	41.1	25.7
2	79	0.390	10.6	8.87
3	78	0.349	7.56	7.45
4	68	0.361	7.23	6.54
5	72	0.344	4.16	5.20

No. washes	Experiment 2			
	Intactness	GAP dehydrogenase	Catalase:GAP dehydrogenase	GSH dehydrogenase: GAP dehydrogenase
	%	*μmol/min·mg Chl*	*ratio*	
0	74	5.04	9.36	0.011
1	67	6.69	3.58	0.020
2	86	8.42	2.33	0.027
3	87	12.18	1.19	0.021
4	85	9.50	1.13	0.023
5	94	10.89	1.08	0.024

(DHA plus Glutathione)-dependent O_2 Evolution by Ruptured Chloroplasts. Ruptured chloroplasts in the light evolved O_2 when supplied with 50 μM NADPH and 40 to 200 μM GSSG (Fig. 1A). After the evolution of approximately 0.4 mol O_2/mol GSSG supplied, O_2 evolution ceased. This is consistent with the stoichiometric reduction of GSSG to GSH by light-coupled GSSG reductase (11). Subsequent addition of 0.94 mM DHA caused resumption of O_2 evolution by chloroplasts previously supplied with 0.1 and 0.2 mM GSSG (equivalent to 0.2 and 0.4 mM GSH at the conclusion of GSSG-dependent O_2 evolution) but not at lower concentrations of GSSG (Fig. 1A). Figure 1B shows that 0.2 to 0.4 mM GSH could replace 0.1 and 0.2 mM GSSG as the source of glutathione, concentrations less than 0.15 mM GSH did not support DHA-dependent O_2 evolution. These data indicate that the concentration of GSSG used in our previous study (40 μM) was too low to detect (DHA plus glutathione)-dependent O_2 evolution (11).

Some properties of (DHA plus glutathione)-dependent O_2 evolution were examined and compared with GSSG-dependent O_2 evolution (without DHA). The mean initial rate of (DHA plus GSH)-dependent O_2 evolution was 11.6 μmol/mg Chl·h (SD 2.6) and for GSSG-dependent O_2 evolution 11.3 μmol/mg Chl·h (SD 3.2). Analogous rates of O_2 evolution for osmotically shocked chloroplasts were 44 and 43% less for (DHA plus GSH)- and GSSG-dependent O_2 evolution. Neither reaction proceeded in the dark or in the presence of 2 μM DCMU. Substituting NADP for NADPH did not affect the rate of the reactions but in the absence of NADP(H) the rate decreased by 85%. The rate of O_2 evolution for both reactions was independent of NADPH concentration from 30 to 100 μM. NADH (50 μM) did not substitute for NADP(H) in either reaction. Sonicated chloroplasts did not catalyze O_2 evolution in the presence of DHA when GSH was replaced with L-cysteine, 2-mercaptoethanol, thioglycollate, or DDT (each 2 mM).

Ruptured chloroplasts, preincubated in the dark for 4 min with 0.2 mM $ZnCl_2$, an inhibitor of GSSG reductase (8), did not catalyze GSSG-dependent O_2 evolution in the light but if GSSG was

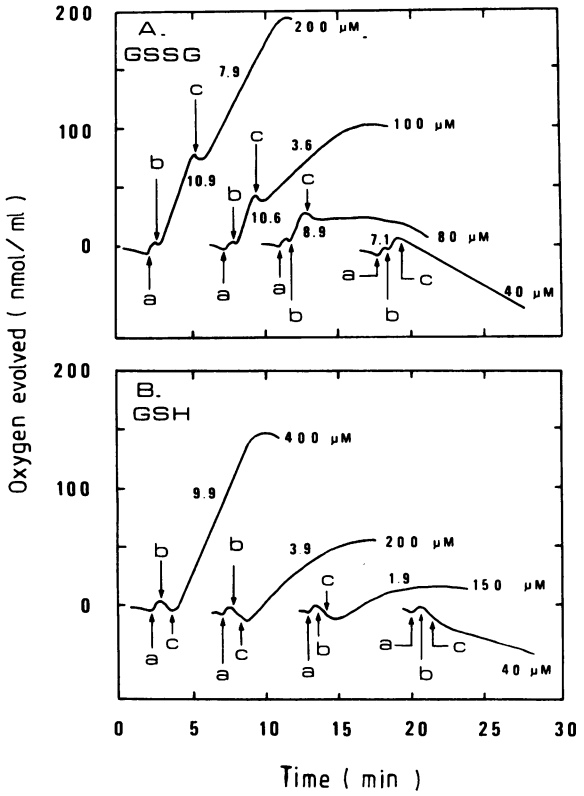

FIG. 1. Effect of concentration of GSSG (A) and GSH (B) on O_2 evolution by sonicated chloroplasts in the presence of 50 μM NADPH and 0.94 mM DHA. All reaction mixtures initially contained 50 μM NADPH and sonicated chloroplasts (200 μg Chl/ml) in the dark. Reaction mixtures were illuminated at the times indicated by (a) and GSSG or GSH (concentration specified for each experiment) was added at time (b). DHA (0.94 mM) was added at time (c). Values beside the curves represent the rate of O_2 evolution in μmol/mg Chl·h. Chloroplast intactness prior to sonication, 80%.

present during the preincubation GSSG-dependent O_2 evolution in the light was unaffected. $ZnCl_2$ (0.2 mM) had no effect on the uncoupled rate of $Fe(CN)_6^{3-}$-dependent O_2 evolution of sonicated or osmotically shocked chloroplasts (200 μg Chl/ml). This implies that the inhibition of GSSG-dependent O_2 evolution by $ZnCl_2$ was not due to inhibition of reaction I. Sonicated chloroplasts preincubated in the dark with 0.2 mM $ZnCl_2$, 2 mM GSH, and 50 μM NADPH, did not catalyze (DHA plus GSH)-dependent O_2 evolution in the light. Addition of DHA (0.94 mM) during the preincubation did not alleviate the inhibition. Conversely, the addition of $ZnCl_2$ (0.2 mM) during (DHA plus GSH)-dependent O_2 evolution had no significant effect on O_2 evolution. These data are consistent with the operation of light-coupled GSSG reductase (reactions I and II) in (DHA plus glutathione)-dependent O_2 evolution and DHA as the eventual electron acceptor.

Relation Between (DHA plus Glutathione)-dependent O_2 Evolution and Reduction of DHA to Ascorbate. Illuminated sonicated chloroplasts in the presence of 2 mM GSH catalyzed the quantitative reduction of 1.4 mM DHA to ascorbate (Fig. 2A). This reaction was associated with the evolution of O_2 although O_2 evolution continued for a further 7 min after cessation of DHA reduction. During the period of rapid reduction of DHA, the concentration of GSH decreased, but as reduction of DHA approached completion, the concentration of GSH returned to the initial concentration (Fig. 2A). In the absence of chloroplasts the rate of reduction of DHA was decreased by 92% (Fig. 2B). These data are consistent with the reduction of DHA by GSH in a reaction catalyzed by chloroplast GSH dehydrogenase followed

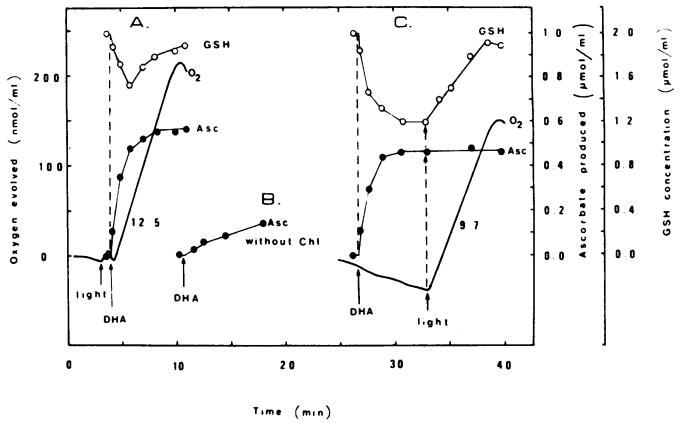

FIG. 2. Effect of continuous light (A) and dark pretreatment prior to illumination (C) on the reduction of DHA to ascorbate (●), the concentration of GSH (○) and O_2 evolution (——) by sonicated chloroplasts in the presence of GSH and DHA. The nonenzymic reduction of DHA by GSH is also shown (B). Incubation mixtures A to C initially contained 50 μM $NADPH_2$ and 2 mM GSH in the dark; A and C also contained sonicated chloroplasts (200 μg Chl/ml). Reactions were initiated with 1.4 mM DHA and illuminated as shown. Values beside the continuous curves denote the rate of O_2 evolution in μmol/ml Chl·h. Chloroplast intactness prior to sonication, 78%.

by subsequent reduction of the GSSG produced in this reaction by light-coupled GSSG reductase (Fig. 2C). In the dark, light-coupled reduction of GSSG is eliminated thereby permitting an estimate of the GSH oxidized and DHA reduced. Under these conditions, 1.74 mol GSH were oxidized/mol ascorbate formed (see reaction III). In a subsequent light period the concentration of GSH was restored to 96% of the original concentration in a reaction associated with the evolution of 0.25 mol O_2/mol of GSH regenerated in good agreement with reactions I and II. After cessation of O_2 evolution in the light phase the ratios of O_2 evolved to ascorbate formed for the experiments shown in Figure 2, A and C, were 0.43 and 0.41 (see reaction IV). In other experiments similar to those shown in Figure 2C, 0.2 mM $ZnCl_2$ did not inhibit the reduction of DHA by GSH during the dark phase but, in the subsequent light phase, O_2 evolution and regeneration of GSH from GSSG (formed during the reduction of DHA) were inhibited completely.

After correcting for the appropriate stoichiometries, the results in Figure 2, A and C, demonstrate that the rate of GSH oxidation by GSH dehydrogenase is 4.6 times greater than the rate of formation of GSH by GSSG reductase. This would account for the transitory net consumption of GSH in the experiment shown in Figure 2A. The GSSG reductase of peas is inhibited by GSH (11). Figure 3 shows that the relative activities of GSSG reductase and GSH dehydrogenase of chloroplast extract are dependent on the concentration of GSH. At the GSH concentrations reported in chloroplasts (3.5 mM, see ref. 5), the dehydrogenase was approximately 6 times more active than the reductase.

Ascorbate Oxidase Activity in Relation to Reduction of DHA and (DHA plus Glutathione)-dependent O_2 Evolution. Ascorbate oxidase activity was associated with chloroplasts subjected to a single wash. The mean rate of activity as determined by ascorbate-dependent O_2 consumption in the dark (method 2) was 1.82 μmol/mg Chl·h (SD 1.23). The activity decreased when chloroplasts were washed prior to sonication in much the same manner as described for catalase and Cyt *c* oxidase (Table II); the ascorbate oxidase activity of chloroplasts washed five times was 0.53 μmol/mg Chl·h (71% decrease). Conversely the rate of (DHA plus GSH)-dependent O_2 evolution was unaltered by the washing procedure.

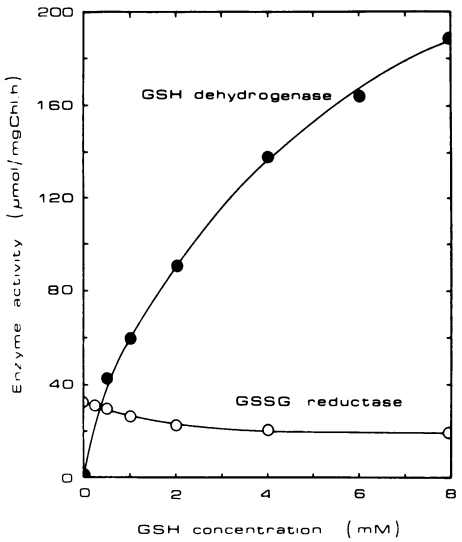

FIG. 3. Effect of GSH concentration on the activities of GSH dehydrogenase (●) and GSSG reductase (○) of chloroplast extract. GSH dehydrogenase activity was determined by method 1 except that incubations were conducted in chloroplast incubating medium containing 0.94 mM DHA; activity is expressed as μmol GSH oxidized/mg Chl·h. GSSG reductase activity was determined as in (11) except that incubations were conducted in chloroplast incubating medium containing 0.4 mM GSSG and 0.1 mM NADPH; activity is expressed as μmol GSH produced/mg Chl·h.

DISCUSSION

The properties of the pea leaf GSH dehydrogenase are in good agreement with those described for the enzyme in spinach (6), although the pH optimum we report is somewhat higher than reported previously for the pea enzyme (16). The higher specific activity of the enzyme in washed chloroplasts and chloroplast extracts relative to the other subcellular fractions (Table I) suggests that at least some of the enzyme is associated with pea chloroplasts. The large increase in activity following sonication of washed chloroplasts, implying that exogenous GSH and/or DHA are inaccessible to the enzyme in intact chloroplasts and the retention of GSH dehydrogenase and NADP-specific GAP dehydrogenase (but not catalase and Cyt *c* oxidase) activities during repeated washing of chloroplasts (Table II) support this conclusion. In this respect our results on the subcellular distribution of GSH dehydrogenase activity in peas differ from spinach (5).

The properties of (DHA plus glutathione)-dependent O_2 evolution catalyzed by ruptured pea chloroplasts (Fig. 1) are consistent with the operation of light-dependent GSSG reductase activity coupled to GSH dehydrogenase as shown in reactions I to IV. This is exemplified by the specific requirement for GSH/GSSG and NADP/NADPH in addition to DHA and light. Either the oxidized or reduced forms of glutathione or NADP support DHA-dependent O_2 evolution inasmuch as these are subject to turnover in reactions I to III, but other thiols and disulfides would not substitute for GSH/GSSG and NADH would not replace NADPH. The quantitative reduction of DHA to ascorbate (Fig. 2) during (DHA plus GSH)-dependent O_2 evolution demonstrates that DHA is the eventual electron acceptor. In the presence of substrate amounts of GSH, reduction of DHA to ascorbate is independent of light (reaction III, Fig. 2C); the evolution of O_2 in a subsequent light period is associated with the light-dependent reduction of GSSG formed in reaction III (Fig. 2C).

The demonstration of (DHA plus GSSG)-dependent O_2 evolution we report here is inconsistent with our previously unsuccessful experiments in which 40 μM GSSG was used (11). Figure 1A shows that a concentration of at least 0.1 mM GSSG was required for DHA-dependent O_2 evolution. This is consistent with the relatively low affinity of pea leaf GSH dehydrogenase for GSH (K_m 4.4 mM). We reported previously that the GSSG reductase of ruptured pea chloroplasts as determined by GSSG-dependent O_2 evolution was not inhibited by 0.2 mM $ZnCl_2$ (11). In these experiments chloroplasts were preincubated with $ZnCl_2$ in the dark in the presence of 0.2 mM GSSG, conditions which prevent inhibition of GSSG reductase by $ZnCl_2$ (8). When ruptured chloroplasts were preincubated in the dark with 0.2 mM $ZnCl_2$ prior to addition of GSSG, GSSG-dependent O_2 evolution was inhibited completely. The inhibition of (DHA plus GSH)-dependent O_2 evolution by 0.2 mM $ZnCl_2$ is consistent with the proposed role of GSSG reductase in light-coupled reduction of DHA.

Some of the molar ratios determined by experiment are in good agreement with the stoichiometries described by equations I to IV. They include ascorbate produced:GSH consumed (reaction III) during the dark phase of the experiment shown in Figure 2C, the O_2 evolved to GSH formed during the subsequent light period (reactions I and II) and the O_2 evolved to ascorbate produced during the experiment. However, the ratio of O_2 evolved to DHA supplied was typically 50% of the stoichiometry predicted by reaction IV (Fig. 2A). This could, in part, be due to the instability of DHA at alkaline pH (16) but it also raises the question whether oxidation of ascorbate by ascorbate oxidase could be coupled to reactions I and IV, effectively making O_2 the eventual electron acceptor. However, chloroplasts washed once prior to sonication oxidize ascorbate at a rate of 7% of the production of ascorbate by the same chloroplasts supplied with DHA and GSH.

Whereas both ascorbate and GSH occur in chloroplasts (5, 7), our results imply that DHA could act as a physiological oxidant of GSH in chloroplasts in a reaction catalyzed by GSH dehydrogenase. Oxidation of GSH by DHA also proceeds nonenzymically but the enzyme-catalyzed reaction appears to be quantitatively more important. From the experiment shown in Figure 2C, the nonenzymic and chloroplast-dependent rates were 0.011 and 0.155 μmol ascorbate/min·ml of reaction mixture. The volume of intact chloroplasts containing 200 μg Chl (the amount per ml of reaction mixture) was estimated from the data in (5) at 4.2 μl. Little is known about the concentrations of ascorbate-DHA and the operation of reactions I to IV in intact chloroplasts, but, assuming that the enzyme-dependent rate of intact chloroplasts proceeds at the rate we report for sonicated chloroplasts, then the nonenzymic rate within the volume of intact chloroplasts would amount to only 0.03% of the enzymic rate. Foyer and Halliwell (5) have proposed that light-induced alkalization of chloroplast stroma (10) provides more favorable conditions for the nonenzymic reaction. When corrections are made to the data described above with respect to pH, the nonenzymic rate at pH 8 still only represents 0.06% of the enzymic rate. We conclude therefore that GSH dehydrogenase activity affords the most likely mechanism for reduction of DHA in pea chloroplasts.

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