Light-dependent Reduction of Oxidized Glutathione by Ruptured Chloroplasts

Received for publication July 29, 1977 and in revised form October 6, 1977

PETER P. JABLONSKI AND JOHN W. ANDERSON
Department of Botany, La Trobe University, Bundoora, Victoria 3083, Australia

ABSTRACT

Crude extracts of pea shoots (Pisum sativum) catalyzed oxidized glutathione (GSSG)-dependent oxidation of NADPH which was attributed to NADPH-specific glutathione reductase. The pH optimum was 8 and the K_m values for GSSG and NADPH were 23 μM and 4.9 μM, respectively. Reduced glutathione (GSH) inhibited the reaction. Crude extracts also catalyzed NADPH-dependent reduction of GSSG; the ratio of the rate of NADPH oxidized to GSH formed was 0.49. NADH and various substituted mono- and disulfides would not substitute for NADPH and GSH respectively. Per mg of chlorophyll, enzyme activity of isolated chloroplasts was 69% of the activity of crude extracts.

Illuminated sonicated pea chloroplasts, in the presence of catalytic amounts of NADPH, catalyzed GSSG-dependent O_2 evolution (mean of 10 determinations, 10.4 μmol per mg chlorophyll per hour, ± 1.4) with the concomitant production of GSH. The molar ratio of GSH produced to O_2 evolved was 3.8 and the highest ratios for O_2 evolved to GSSG added were 0.46 and 0.44. The K_m value for GSSG was 26 μM. GSH inhibited the reaction. The reaction was attributed to photosynthetically coupled glutathione reductase.

Ruptured chloroplasts, in the presence of catalytic amounts of GSSG and NADPH, did not catalyze sustained O_2 evolution in the presence of substrate amounts of hydrogen peroxide, dehydroascorbate, l-cystine, sulfite, or sulfate.

Glutathione reductase (glutathione oxidoreductase NADPH-specific, EC 1.6.4.2) has been described in a number of organisms (16). In animals the main function of glutathione reductase appears to be the reduction of GSSG which is formed during the reaction of H_2O_2 in a reaction catalyzed by glutathione peroxidase (15). Glutathione reductase has been reported in crude extracts from several plant species (10) and purified from germinating pea seeds (11). It has also been described in avocado mitochondria (18) and more recently in chloroplasts (6, 13).

Very little is known about the function of the enzyme(s) in plants. In mitochondria, GSH has been reported to reduce dehydroascorbate (18) but the significance of this reaction is not clear (10). It has been suggested that GSH serves a similar function in chloroplasts thereby providing an alternative mechanism to the reaction catalyzed by superoxide dismutase for the removal of free radicals of O_2 produced by the light reactions (6, 8). Another suggestion for the chloroplast enzyme is that GSH participates in the light-dependent regulation of the activity of various chloroplast enzymes by controlling the degree of oxidation of sulfhydryl groups of these enzymes (17). Part of this proposal is based on the requirement for light for the synthesis of NADPH and therefore the reduction of GSSG. Here, we report that ruptured (but not intact) pea chloroplasts, in the presence of catalytic amounts of NADPH, catalyze the reduction of GSSG to GSH in the light with the concomitant evolution of O_2. This system, which we attribute to photosynthetically coupled glutathione reductase activity, provides a means for testing some of the hypotheses concerning the physiological role of GSH and glutathione reductase in chloroplasts.

MATERIALS AND METHODS

Plant Material. Peas (Pisum sativum cv. Massey Gem) were soaked in water for 24 hr (1.6 mg/l of dry seeds) and raised in shallow trays of a potting mixture (coarse sand-vermiculite-peat moss, 1:1:1 by volume) in a growth cabinet at 20 C, 12-hr day. The seeds/seedlings were watered regularly with distilled H_2O for 8 days and thereafter with 10 mM KNO_3. The peas were harvested 12 to 15 days after imbibition.

Chloroplasts and O_2 Evolution. Chloroplasts were prepared as described previously (1). Oxygen evolution was measured at 25 C as previously reported (1) except that the O_2 electrodes (model DW) were supplied by Hansatech, Norfolk, England, and the reaction mixtures were illuminated by 100-w tungsten-halogen projector lamps. Chloroplast intactness was measured as before (1) except that the concentration of NH_4Cl was increased to 10 mM. Ruptured chloroplasts were prepared from intact chloroplasts either by sonicating for 1 min or on a Rapidis 600 sonicator (Ultrasonics Ltd., Yorks, England) or by diluting in 1 to 5 volumes of water. Independent measurements for intactness showed that sonicated chloroplasts were completely ruptured but osmotically shocked chloroplasts were still 7.6 to 26% intact. GSSG-dependent O_2 evolution was routinely measured at 25 C in 1 ml of incubating medium containing 50 μM NADPH, 200 μM GSSG, and ruptured chloroplasts (200 μg of Chl).

Preparation of Crude Extracts. Pea shoots were extracted in a pestle and mortar using 4 ml of 0.1 M Tris-HCl buffer (pH 7)/g fresh wt. The supernatant solution was recovered by centrifuging at 10,000g for 15 min. All operations were performed at 2 C.

Assay of Glutathione Reductase. Two methods were used.

1 This work was supported by a grant from the Australian Research Grants Committee.
2 Holder of a grant under the Tertiary Education Assistance Scheme.
3 Abbreviations: GSSG: oxidized glutathione; GSH: reduced glutathione; DTNB: 5,5'-dithiobis (2-nitrobenzoic acid).
4 Definition: the term "photosynthetically coupled" is used throughout this paper to refer to the generation of a reductant by photosystems I and II (e.g., NADPH) which is used as a substrate in the associated reactions.
Method 1 involved GSSG-dependent oxidation of NADPH. Reaction mixtures (3 ml) containing 1 μmol of GSSG, 0.4 μmol of Na₂EDTA, 400 μmol of K-phosphate buffer (pH 8) (pH 7 in some experiments) and 0.1 ml of diluted extract (up to 150 μg of protein) were preincubated at 25°C for 5 min. Reactions were initiated by addition of 0.1 μmol of NADPH and the rate of oxidation of NADPH monitored at 340 nm. Enzyme activity is expressed as the difference in the rate of oxidation of NADPH for incubation mixtures with and without GSSG in μmol min⁻¹. Method 2 involved the NADPH-dependent reduction of GSSG to GSH. Reaction mixtures (4 ml) containing 2 μmol of GSSG, 0.4 μmol of EDTA, 800 μmol of K-phosphate buffer (pH 8) and extract were preincubated at 25°C for 5 min. The reaction was initiated with 0.6 μmol of NADPH and samples (0.75 ml) were withdrawn at 1.5-min intervals and analyzed for GSH. Enzyme activity is expressed as the difference in the rate of GSH production for reaction mixtures with and without NADPH.

Subcellular Fractionation of Pea Leaf Tissue. Intact chloroplasts were prepared by the standard procedure (1) and the low-speed supernatant solution was retained. This fraction was subjected to further centrifugation at 20,000 g for 15 min; the precipitate is referred to as the 20,000 g pellet and the supernatant solution is designated cytosol. The chloroplasts were sonicated and insoluble material (grana) separated from chloroplast extract (stroma) by centrifugation at 20,000 g for 15 min. Both the grana and the 20,000 g pellet were resuspended in incubating medium (1). An independent crude extract was prepared from the same batch of tissue in buffer containing no osmoticum. This was used to measure total glutathione reductase in the tissue. Similarly, a further batch of tissue was used to determine total Chl.

Other Methods. GSH was measured by the method of Hafeman et al. (7) except that EDTA and NaCl were not incorporated into the metaphosphoric acid solution. Protein was precipitated with 2 volumes of trichloroacetic acid (15%, w/v), washed twice with acetone, solubilized in 2 m NaOH at 70°C for 1 hr, and measured by the method of Ellman (5) using crystalline BSA as standard. All other procedures were as described previously (1).

Chemicals. GSH was supplied by Sigma Chemical Co. It was found to be 96% pure using the DTNB extinction coefficient of Ellman (4). GSSG was obtained from Boeringer, G.m.b.H. Mannheim, Germany. It contained 1.92 μmol of GSH/g of GSSG as calculated from the DTNB reaction (4).

RESULTS

Properties of Glutathione Reductase in Crude Extracts. Crude extracts catalyzed GSSG-dependent oxidation of NADPH (method 1). The rate of the reaction was proportional to protein concentration up to 50 μg/ml of incubation mixture and varied from 0.062 to 0.129 μmol of NADPH oxidized mg of protein⁻¹ min⁻¹ (mean, 0.105; SD 0.023). Since 1 mg of soluble protein represents the amount extracted from approximately 200 mg fresh wt containing 0.31 mg of Chl, the mean rate was approximately equivalent to 20.3 μmol mg of Chl⁻¹ hr⁻¹. GSSG-dependent oxidation of NADPH was accompanied by the production of GSH (method 2). In separate experiments (detailed results not shown) the respective rates for given extracts by methods 1 and 2 were (in μmol of NADPH oxidized or μmol of GSH produced mg of protein⁻¹ min⁻¹): 0.065 and 0.15, 0.058 and 0.116, 0.062 and 0.115. The ratios of the rates of NADPH oxidized to GSH produced were 0.43, 0.50, and 0.54, respectively. Crude extracts did not catalyze significant GSSG-dependent oxidation of NADH (less than 1% of the rate of NADPH oxidation at 30 μM). The following substrates did not support oxidation of NADPH: 0.33 mM pyrrolycysteine, 0.33 mM L-cysteine, 0.33 mM L-djenkolate, 0.5 mM L-cystathionine.

The pH optimum for GSSG-dependent oxidation of NADPH in Tris-maleic acid-NaOH buffer was 8 (Fig. 1). The affinity of the reaction for GSSG and NADPH was high (Figs. 2A and 3A); the Kₐ values, calculated from double reciprocal plots, were 23 μM for GSSG and 4.9 μM for NADPH. GSSG-dependent oxidation of NADPH was not inhibited by 0.01 to 0.1 mM ZnCl₂ but 0.2 mM ZnCl₂ caused slight inhibition (4%). The reaction was inhibited by 2.5 to 10 mM GSH (Fig. 4) but not by 2.5 mM L-cysteine. Azide (0.1 mM) had no significant effect on enzyme activity (less than 0.7% inhibition).

Properties of Isolated Chloroplasts. The chloroplast preparations, prior to rupture, catalyzed the substrate-dependent O₂ evolution activities described previously (1) although the rates with some substrates were somewhat less. This is presumably due to differences in the variety of peas used. The intactness of each chloroplast preparation was measured prior to rupture; the mean value for 27 preparations was 68.3% (SD 16.3). The mean value for the uncoupled rate of O₂ evolution by osmotically shocked chloroplasts using ferricyanide as electron acceptor was 249 μmol mg of Chl⁻¹ hr⁻¹ (SD 65.3). Typical rates of O₂ evolution in μmol mg of Chl⁻¹ hr⁻¹ by intact chloroplasts for various physiological substrates were as follows: oxaloacetate, 9.3; P-glycerate, 19.3; aspartate plus α-ketoglutarate, 7.6; glutamine plus α-ketoglutarate, 3.7; nitrite, 9.7.

FIG. 1. Effect of pH on glutathione reductase activity in crude extracts. Enzyme activity was measured by method 1 in Tris-maleic acid-NaOH buffer and is expressed as μmol of NADPH oxidized min⁻¹ mg of protein⁻¹. Incubation mixtures contained 300 μmol of Tris, 300 μmol of maleic acid and NaOH as required to obtain the pH values specified.

FIG. 2. Effect of concentration of GSSG on (A) glutathione reductase activity of crude extracts (method 1) and (B) GSSG-dependent O₂ evolution by sonicated chloroplasts. Values shown for (B) were determined in the presence of 50 μM NADPH. Chloroplast intactness prior to sonication 87%.
Subcellular Distribution of Glutathione Reductase Activity in Pea Shoots. The distribution of enzyme activity, measured by method 2, is shown in Table I. The activity per mg of protein was greatest in the chloroplast extract (stroma). The total activity of chloroplasts/mg of Chl was 69% of that of the (total) activity in crude extracts (containing no osmotically) expressed per total Chl in the tissue. Chloroplast particular material (grana) and the 20,000g pellet contained negligible activity.

GSSG-dependent O₂ Evolution by Ruptured Chloroplasts. Illumination of sonicated chloroplasts in the presence of catalytic amounts of NADPH (50 nmol) initiated a slow rate of O₂ evolution which soon ceased. Subsequent addition of substrate amounts of GSSG (0.4 µmol) caused an immediate resumption of O₂ evolution (Fig. 5A) which continued at a constant rate for at least 3 to 5 min. GSSG-dependent O₂ evolution did not occur in the dark and was completely inhibited by 1.9 µM DCMU (Fig. 5B). Rates of GSSG-dependent O₂ evolution for sonicated chloroplasts varied from 7.3 to 12.2 µmol mg of Chl⁻¹ hr⁻¹ (mean, 10.4; SD 1.4). Sonicated chloroplasts exhibited GSSG-dependent O₂ evolution in the absence of NADPH but the rate was less than that observed in the presence of 50 µM NADPH (Fig. 3B) and gradually decreased during the incubation (e.g., after 10 min the rate was 35% of the initial rate without NADPH). Addition of NADPH (20-50 nmol) at any stage during the incubation caused an immediate enhancement in the rate of O₂ evolution which approximated that of incubation mixtures in which NADPH was added prior to GSSG. NADH (50 nmol) would not substitute for NADPH in this reaction.

Intact chloroplasts did not catalyze significant GSSG-dependent O₂ evolution (Fig. 6A) either in the presence or absence of NADPH (50 µM). Osmotically shocked chloroplasts exhibited GSSG-dependent activity in the presence (but not in the absence) of NADPH. The rates of O₂ evolution catalyzed by osmotically shocked chloroplasts were less than those for sonicated chloroplasts and were more variable (range, 1.9-10.2 µmol mg of Chl⁻¹ hr⁻¹; mean, 5.0; SD 2.6). This variability is attributed to the incomplete rupturing of osmotically shocked chloroplasts.

When ruptured chloroplasts were supplied with 200 nmol of GSSG in the presence of 50 nmol of NADPH, O₂ evolution commenced in the usual manner but ceased rather abruptly after the evolution of approximately 80 nmol. Evolution of O₂ was reinitiated by a further addition of 200 nmol of GSSG. This sequence could be repeated several times (Fig. 6B) but it was invariably found that both the amount of O₂ evolved and the rate of O₂ evolution decreased with each successive addition of GSSG. The molar ratio of O₂ evolved for each addition of GSSG was calculated for several experiments (Table II). In general, the highest molar ratios were obtained after the first addition of GSSG in those experiments having the fastest rates of O₂ evolution.

GSSG-dependent O₂ evolution was accompanied by the production of GSH. The formation of the two products was closely correlated although it was consistently noted that GSH production preceded O₂ evolution (Fig. 7). The sonicated...
The intact (200 chloroplasts contained 50 nmol of GSSG in the dark and intact or sonicated chloroplasts (200 µg of Chl). Treatments were made as shown. Values beside the curves represent the rate of O₂ evolution in µmol mg of Chl⁻¹ ml⁻¹. Amount of O₂ evolved for each successive addition of GSSG is also shown. Chloroplast intactness (A) 91%, (B) 73% (prior to sonication); volume, 1 ml.

chloroplasts used in the experiment described in Figure 7 catalyzed the production of 144 nmol of O₂ ml⁻¹ and 552 nmol of GSH ml⁻¹ when supplied with 305 nmol of GSSG ml⁻¹. This represents the production 3.8 mol of GSH per mol of O₂ evolved and 1.81 mol of GSH per mol of GSSG supplied. The maximum rates of GSH production and O₂ evolution were, respectively, 51 and 13.3 µmol mg of Chl⁻¹ hr⁻¹. Stroma, prepared from the same ruptured chloroplasts, catalyzed GSSG-dependent oxidation of NADPH (method 1) at a rate of 34 µmol mg of Chl⁻¹ hr⁻¹ when related to the Chl content of the chloroplasts from which the stroma was prepared.

The affinity of the GSSG-dependent O₂ evolution activity for GSSG was high (Fig. 2B); the K₅₀ value calculated from a double reciprocal plot was 26 µM. The abrupt cessation of O₂ evolution when limiting amounts of GSSG were supplied (Fig. 6B) was also indicative of a high affinity for GSSG. NAPDH concentrations of 20 to 50 µM were sufficient to promote maximum rate of O₂ evolution by sonicated chloroplasts (Fig. 3B).

Sonicated chloroplasts, in the presence of NADPH (50 nmol), did not catalyze O₂ evolution when GSSG was replaced with l-cystine, dL-homocystine, l-cysteine, L-djenkolate, L-cystathionine, or GSH (each 0.2 mm). Further, these compounds, at a concentration of 0.2 mm did not initiate any O₂ evolution by sonicated chloroplasts in the presence of catalytic amounts of NADPH (50 nmol) and GSSG (40 nmol) which could not be attributed to GSSG itself. Relatively high concentrations of GSH (2.5 to 10 mm) initiated O₂ evolution by sonicated chloroplasts (in the presence of catalytic amounts of NADPH) at rates of 3.9 to 7.9 µmol mg of Chl⁻¹ hr⁻¹. This reaction ceased after 0.5 to 3 min. Both the rate and the amount of O₂ evolved could be accounted for by the GSSG contamination of the GSH after allowing for the inhibitory effect of GSH (Fig. 4). In the presence of substrate amounts of GSSG, GSH (2.5–10 mm) inhibited O₂ evolution (Fig. 4). Azide (0.1 mm) inhibited GSSG-dependent O₂ evolution by 30%. NH₄Cl (0.2 to 6 mm) and ZnCl₂ (0.01 to 0.6 mm) had no significant effect upon GSSG-dependent O₂ evolution.

Studies of GSH Oxidation by Ruptured Chloroplasts. In theory, GSSG-dependent O₂ evolution by ruptured chloroplasts can be used to test hypotheses on the mechanism of reoxidation of GSH generated by glutathione reductase. In the presence of catalytic amounts of NADPH (50 µM) and GSSG (40 µM), addition of substrate amounts of a proposed physiological oxidizing agent should initiate and sustain O₂ evolution over and above that predicted from the catalytic amounts of GSSG supplied. The following substrates failed to fulfill this criterion; they also did not catalyze O₂ evolution in the absence of catalytic amounts of GSSG: dehydroascorbate (0.5 and 1 mm), H₂O₂ (0.1 to 0.3 mm) in the presence of azide (0.1 mm), sodium sulfate (0.1 and 1 mm), l-cysteine (0.1 mm). In the absence of catalytic amounts of GSSG, 0.1 and 1 mm sodium sulfite catalyzed O₂ evolution at rates of 1.7 and 2.4 µmol mg of Chl⁻¹ hr⁻¹, respectively, but this rapidly decreased and ceased entirely after 1 to 2 min.

5 Chloroplasts used to test this substrate were extracted in medium without isosacorbate (1).
**DISCUSSION**

Crude extracts of pea shoots catalyzed both GSSG-dependent oxidation of NADPH and NADPH-dependent reduction of GSSG. Since the mean ratio of NADPH oxidized to GSH produced was 0.49, NADH would not replace NADPH, and various substituted mono- and disulfides would not replace GSSG, we conclude that the activities we observed were catalyzed by NADPH-specific glutathione reductase. The properties of the reactions are in good agreement with those described for the glutathione reductase from peas (11) and wheat germ (3). Our results, however, differ somewhat from those reported for the enzyme from spinach chloroplasts (13) in being insensitive to ZnCl₂, have a distinct pH optimum (Fig. 1), and a lower $K_m$ value for GSSG (Fig. 2A). We conclude that the bulk of the activity is localized in chloroplasts and is recovered in the stroma following sonication (Table I). We cannot rule out the possibility that some activity is associated with other subcellular fractions though it is of interest that the chloroplasts from which the data in Table I were derived were 59% intact prior to sonication.

Inhibition of glutathione reductase by GSH (Fig. 4) does not appear to have been reported previously for the enzyme from plants although it has been described for the enzyme from human erythrocytes (14). Because of the high equilibrium constant for the reaction (11) the effect of GSH cannot be attributed to a simple effect on the back reaction. We suspect that GSH serves some role in regulating the activity of the enzyme. It is not clear, however, whether this would operate in intact chloroplasts since relatively high concentrations of GSH are required to inhibit the enzyme (Fig. 4) whereas the concentration in spinach chloroplasts has been estimated at 3.5 mm (6). Nevertheless, the inhibition of the pea enzyme by GSH might vary with the concentration of GSSG (14). In this event the GSH/GSSG ratio of chloroplasts might not only regulate the activity of chloroplast glutathione reductase but also other chloroplast enzymes (17).

Various lines of evidence suggest that the GSSG-dependent O₂ evolution catalyzed by ruptured chloroplasts was due to photosynthetically coupled glutathione reductase:

\[
2H_2O + 2NADP^+ \xrightarrow{hv} 2NADPH + 2H^+ + O_2 \quad (I)
\]

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

\[
2H_2O + 2GSSG \rightarrow 4GSH + O_2 \quad (III)
\]

Effects on either one of the two component reactions can be monitored by O₂ evolution. Reaction I required light and was sensitive to DCMU (Fig. 5B). Reaction II was inhibited by GSH (Fig. 4) and required substrate amounts of GSSG. It also required catalytic amounts of NADPH, the NADP so formed being recycled via reaction I. Evidence that NADP was recycled can be gauged from the observation that reaction mixtures containing as little as 10 nmol of NADP supported the production of 76 mol of O₂ before the experiment was terminated. The observed rates and molar ratios for O₂ evolved to GSSG added (Fig. 6B and Table II) and GSH produced (Fig. 7) approximate to the theoretical stoichiometry (reaction III). We presume that the small time difference between reduction of GSSG and O₂ evolution (Fig. 7) is due to the consumption of the initial catalytic amounts of NADPH in reaction II prior to the onset of cycling of NADP via reaction I. Our results are similar to those of Hendley and Conn (9) except that we did not add an exogenous source of glutathione reductase.

Most features of the GSSG-dependent O₂ evolution catalyzed by ruptured chloroplasts are similar to those catalyzed by the glutathione reductase of crude extracts. Azide (0.1 mm), however, caused 30% inhibition of the chloroplast system but had no significant effect on the activity of crude extracts (less than 0.7% inhibition). We attribute the effect of azide on the chloroplast system to an effect on reaction I (12). Foyer and Halliwell (6) have reported that sonicated, but not intact, chloroplasts catalyze GSSG-dependent oxidation of NADPH. This rules out the possibility that glutathione reductase is bound to the outside of the chloroplast membrane. Since intact chloroplasts also did not catalyze GSSG-dependent O₂ evolution (Fig. 6A), we infer that exogenous GSSG does not penetrate the chloroplast membrane or, if it does, it occurs at very slow rates. (The slow but increasing rate of O₂ evolution in Fig. 6A also occurs in the absence of GSSG.) Our results imply that the internal pool of GSH in chloroplasts, estimated at 3.5 mm (6), cycles between the oxidized and reduced states within the chloroplast itself and that the light reaction and possibly the GSH/GSSG ratio play an important part in this process. Sonication of chloroplasts catalyze high rates of GSSG-dependent O₂ evolution (mean 10.4 μmol mg of Chl⁻¹ hr⁻¹); this represents the production of 41.6 μmol of GSH mg of Chl⁻¹ hr⁻¹. These rates suggest that GSH serves an important role in some major reduction process(es) within the chloroplast. Our attempts to demonstrate sustained O₂ evolution by ruptured chloroplasts in the presence of catalytic amounts of NADPH and GSSG and substrate amounts of some proposed physiological oxidizing agents were unsuccessful. In particular, we were unable to detect any O₂ evolution in the presence of H₂O₂ (with 0.1 mm azide to inhibit catalase) or dehydroascorbate. These observations are inconsistent with the proposed roles of glutathione reductase and ascorbate suggested by Halliwell and Foyer (6, 8) for the removal of H₂O₂ and superoxide formed during illumination of chloroplasts (2).

**LITERATURE CITED**