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The Presence of Glutathione and Glutathione Reductase in Chloroplasts: A Proposed Role in Ascorbic Acid Metabolism

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Abstract. Both glutathione and an NADPH-dependent glutathione reductase are present in spinach (*Spinacia oleracea* L.) chloroplasts. It is proposed that glutathione functions to stabilise enzymes of the Calvin cycle, and it may also act to keep ascorbic acid in chloroplasts in the reduced form.

Key words: Glutathione – Chloroplasts – Calcin cycle – Ascorbic acid – *Spinacia.*

Introduction

The simple tripeptide glutathione (GSH) is found in almost all living cells and takes part in numerous biochemical reactions, as well as helping to stabilise certain enzymes by preventing oxidation of their thiol groups (Jocelyn, 1972). The oxidised form of glutathione (GSSG) may be converted back to the reduced form (GSH) by the activity of the enzyme glutathione reductase, which catalyses the reaction given in Equation (1).

$$GSSG + NADPH + H^+ \rightarrow 2GSH + NADP^+. \quad (1)$$

Glutathione reductase is present in many animal and plant tissues (Jocelyn, 1972), including spinach leaves (Anderson et al., 1953). The subcellular location of this enzyme has been extensively investigated in animal tissues, and it appears to be mainly located in the cytosol (Jocelyn, 1972). However, the location of this enzyme in plant tissue does not appear to have been studied by modern techniques, although Hendley and Conn (1953) did not detect significant quantities of the enzyme in broken spinach chloroplast preparations.

In the present paper, evidence is presented to show that spinach chloroplasts do contain GSH and glutathione reductase, and possible functions of this enzyme are discussed.

Abbreviations: GSH = tripeptide glutathione; GSH = reduced form of glutathione; GSSG = oxidised form of glutathione

Many plant tissues contain dehydroascorbate reductase (Mapson, 1958), which catalyses the reaction shown in Equation (2)

$$\text{dehydroascorbate} + 2GSH \rightarrow GSSG + \text{ascorbate}. \quad (2)$$

The relation of this enzyme to glutathione reductase is also discussed in the present paper.

Materials and Methods

Materials

Dehydroascorbic acid (>95% pure) was purchased from Koch-Light Ltd., Colnbrook, Bucks, U.K. Other reagents were of the highest purity available from BDH Chemicals Ltd., London, U.K. or from the Sigma Chemical Corp., Kingston-upon-Thames, Surrey, U.K. Spinach leaves were obtained from New Covent Garden Market, London, U.K. *Codium fragile,* from Bembridge, Isle of Wight, was kept in aerated sea-water at greenhouse temperatures (10–20° C).

Enzyme Assays

$NADP^+$-glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.9) was assayed by the fall in absorbance at 340 nm as NADPH was oxidised (Bradbeer, 1969), glycollate oxidase (EC 1.1.3.1) by the formation of glyoxylate phenylhydrazone (Feierabend and Beevers, 1972), citrate synthetase (EC 4.1.3.7) by the formation of coenzyme A (Barbareschi et al., 1972) and cytochrome c oxidase (EC 1.9.3.1.) by the fall in absorbance at 550 nm as reduced cytochrome c was oxidised (Tolbert et al., 1969). Glutathione reductase (EC 1.6.4.2) was assayed by the fall in absorbance at 340 nm; reaction mixtures contained, in a total volume of 3 ml: enzyme, EDTA (3 μmol), GSSG (10 mg), NADPH (0.3 mg) and Tris buffer (260 μmol) adjusted to pH 7.5 with HCl. Corrections were made for any NADPH oxidation in the absence of GSSG.

Dehydroascorbate reductase (EC 1.8.5.1) was assayed by a modification of the method of Yamaguchi and Joslyn (1951). Dehydroascorbate (2 μmol), GSH (5 μmol), enzyme and KH_2PO_4 buffer (35 μmol) adjusted to pH 6.3 with KOH were incubated in a total volume of 1.00 ml at 25° C for 5 min. Then 0.1 ml of 10% (w/v) metaphosphoric acid was added. The solution was centrifuged and ascorbic acid in the supernatant measured by titration against dichlorophenol-indophenol. Controls without enzyme and without GSH were carried out to correct for any non-enzymic reduction and

any ascorbate present in the fraction being assayed. Dehydroascorbate was dissolved in N_2-saturated buffer immediately before use.

Each of the above assays was only used in a range in which the rate of reaction was proportional to the amount of extract added. During fractionations, recoveries of the activity present initially in the homogenate were always calculated: results of experiments with recoveries not in the range 90–110% were usually discarded.

Unless otherwise stated, all homogenisations and fractionations were performed at 0–4° C and the homogenate squeezed through a double layer of muslin before use. Chlorophyll was assayed by the method used by Arnon (1949).

Chloroplast Isolation

Spinach: Intact [type A in the classification of Hall (1972)] spinach chloroplasts were prepared as described by Reeves and Hall (1973), except that Hepes-KOH (50 mM) at pH 7.5 was the buffer used in the homogenisation medium, the concentration of bovine serum albumin was raised to 1% (w/v) and dithiothreitol (1 mM) replaced ascorbate. The chloroplast pellets were resuspended gently in homogenisation medium. Unless otherwise stated, the chloroplasts were diluted 10× with distilled water and allowed to stand for 30 s to rupture the envelopes (Walker, 1971) immediately before assay of enzyme activities.

Where indicated, the chloroplast preparations were washed by gentle resuspension in homogenising medium (40 ml), followed by centrifugation as described by Reeves and Hall (1973). *Codium* chloroplasts were prepared by a modification of the method of Schönfeld et al. (1973). Algal fronds (100 g) were homogenised for 15 s at 0–4° C at full speed in an Ato-Mix (MSE Ltd., Crawley, Sussex, UK) in 100 ml of medium (0.8 M sorbitol, 1% (w/v) bovine serum albumin, 0.1 M Tris-HCl buffer, pH 7.5). The homogenate was squeezed through 2 layers of muslin and centrifuged at 4,000 *g* for 90 s. The pellet was resuspended in homogenising medium and centrifuged again. This procedure was repeated. Finally, the twice-washed chloroplast preparation was resuspended in homogenising medium.

Nicotiania. Chloroplasts from leaves of *N. excelsior* were prepared by the method used for spinach chloroplasts (see above).

Results

Subcellular Localisation of Enzymes

As expected (Mapson, 1958), both glutathione reductase and dehydroascorbate reductase were detected in homogenates of spinach (*Spinacia oleracea* L.) leaves: activities of both enzymes were usually in the range of 1–2 μmol of product ($NADP^+$ or ascorbic acid respectively) formed/min/mg chlorophyll. When leaf homogenates in 0.5 M sucrose-50 mM Tris-HCl pH 7.5 were centrifuged at 12,000 *g* for 30 min, over 90% of both enzymes was recovered in the supernatant fraction. Also, centrifugation of leaf homogenates at 100,000 *g* for 1 h to sediment microsomes (Halliwell, 1974a) did not increase the amount of these two enzymes pelleted.

These results suggest that glutathione reductase and dehydroascorbate reductase are either non-particulate, or are released from some other organelle during homogenisation and centrifugation. The most likely candidate is chloroplasts, which lose their envelopes and stromal contents during homogenisation and prolonged centrifugation (Walker, 1971; Halliwell, 1974a). To test this hypothesis, "intact" chloroplasts were prepared by rapid centrifugation: these type A (Hall, 1972) chloroplasts retain their stromal enzymes, such as $NADP^+$-glyceraldehyde-3-phosphate dehydrogenase (Latzko and Gibbs, 1968). Table 1 shows that the distribution of glutathione reductase in such experiments was very similar to that of $NADP^+$-glyceraldehyde-3-phosphate dehydrogenase, whereas little dehydroascorbate reductase was found in the chloroplast fraction.

Table 1. Localisation of enzymes in intact spinach chloroplasts

A–F refer to 6 experiments on different batches of leaves. Intact chloroplasts were prepared, as described in the Materials and Methods section. Enzyme assays were carried out on homogenate, pellet and supernatant fractions and the activity in the chloroplast fraction expressed as a % of the activity present in the homogenate. Recoveries of enzyme activities in pellet and supernatant fractions were 90–110% of the activity of the homogenate. Although the chloroplasts pelleted were up to 80% intact, as assayed by ferricyanide-dependent O_2 uptake (Lilley et al., 1975), they only represented a small proportion of the total chloroplasts in the homogenate, and so the percentage of enzymes pelleted was small

Expt.	% of the enzyme activity present in the homogenate that was recovered in the chloroplast pellet		
	$NADP^+$-glyceraldehyde-3-phosphate dehydrogenase	glutathione reductase	dehydroascorbate reductase
A	25	16	4
B	22	19	0
C	27	21	0
D	20	18	6
E	11	9	–
F	16	13	0

These results suggest that some glutathione reductase is present in intact chloroplasts. However, since intact chloroplast fractions also contain peroxisomes and mitochondria (e.g. Halliwell, 1973), it is important to check that the activity present is not due to contamination by these organelles. Table 2 shows the results of an experiment designed to check this. The supernatant obtained after pelleting intact chloroplasts was further centrifuged at 6,000 *g* for 15 min, a procedure which sediments most peroxisomes and mitochondria (Halliwell, 1974a). Table 2 shows that the 6,000 *g* pellet, rich in peroxisomes (glycollate oxidase marker – Tolbert et al., 1969) and mitochondria (cytochrome oxidase marker), contained no glutathione reductase, and so this enzyme cannot be asso-

ciated with these organelles. The 6,000 *g* pellet also contained a large amount of chloroplasts (chlorophyll marker), but these were "broken", i.e. had lost stromal enzymes, as shown by the low activities of $NADP^+$-glyceraldehyde-3-phosphate dehydrogenase (Latzko and Gibbs, 1968). Again, the distribution of glutathione reductase was similar to that of $NADP^+$-glyceraldehyde-3-phosphate dehydrogenase.

Table 2. Localisation of enzymes in spinach leaf homogenates
Intact chloroplasts were prepared as described in the Materials and Methods section. The supernatant from the rapid centrifugation was further centrifuged at 6,000 *g* for 15 min at 0–4° C. All fractions were assayed for the enzymes stated below: glycollate oxidase is a marker for peroxisomes, cytochrome oxidase for mitochondria, chlorophyll for total chloroplasts and $NADP^+$-glyceraldehyde-3-phosphate dehydrogenase ($NADP^+$-GPDH) for intact chloroplasts. Similar results were obtained when citrate synthetase was used instead of cytochrome oxidase as a marker for mitochondria

	Fraction				Chlorophyll recovered (%)
	glycollate oxidase	cytochrome oxidase	glutathione reductase	$NADP^+$-GPDH	
Homogenate	100	100	100	100	100
"Intact chloroplast" pellet	7	15	17	25	50
6,000 *g* pellet	26	62	0	1	45
6,000 *g* supernatant	67	11	85	66	5
(% recovery in supernatant plus pellet fractions of the activity present in the homogenate)	(100)	(88)	(102)	(93)	(100)

The possibility of contamination of the intact chloroplast fraction by cytosolic (soluble) enzymes must also be considered. However, washing the chloroplast fraction (see Materials and Methods) did not decrease the ratio of glutathione reductase to $NADP^+$-glyceraldehyde-3-phosphate dehydrogenase. Further, if the glutathione reductase present was due to cytosolic contamination, then it would be *outside* the chloroplasts and freely accessible to its substrates. Table 3 shows, however, that little activity of glutathione reductase could be detected if the chloroplasts were allowed to remain intact during the assay by including 0.33 M sorbitol and 0.3% (w/v) bovine serum albumin in the reaction medium. Considerable activity was detected if the chloroplasts were broken by osmotic shock (see Materials and Methods) before assay. The "latency factor" (ratio of enzyme activity in intact to that in broken chloroplasts) was the same for both glutathione reductase and $NADP^+$-glyceraldehyde-3-phosphate dehydrogenase. We conclude that spinach chloroplasts contain glutathione reductase as a stromal enzyme. We have also detected glutathione reductase in chloroplast fractions isolated from leaves of tobacco (*N. excelsior*) and fronds of the alga *Codium fragile*. Table 3 shows that the measurable glutathione reductase activity of *Codium* chloroplasts was greatly increased after the extremely tough

Table 3. Evidence for enzyme latency in spinach and *Codium* chloroplast fractions

Chloroplasts were prepared, as described in the Materials and Methods section. Assays of spinach chloroplasts for $NADP^+$-glyceraldehyde-3-phosphate dehydrogenase and glutathione reductase were carried out using reaction media (see Materials and Methods) to which sorbitol and bovine serum albumin had been added (final concentrations 0.33 M and 0.3% (w/v) respectively). Chloroplasts were either assayed directly or broken by osmotic shock (see Materials and Methods) and then assayed

Codium chloroplasts were assayed in normal reaction mixtures (i.e. without added sorbitol or albumin), either directly or after sonication for 15 s at 0–4° C, using a PG100 MSE Ultrasonic disintegrator, model 150W, set on medium power, with a 1/8th inch microprobe and a solution volume of 5 ml. $NADP^+$-glyceraldehyde-3-phosphate dehydrogenase was very unstable in sonicated chloroplast fractions and required immediate assay

Chloroplast source		$NADP^+$-glyceraldehyde-3-phosphate dehydrogenase ($NADP^+$-GPDH) (μmol NADPH oxidised/min/mg chl)	Glutathione reductase (GR) (μmol NADPH oxidised/min/mg chl)	Latency factor (activity in intact chloroplasts/activity in broken chloroplasts)	
				$NADP^+$-GPDH	GR
Spinach	(a) Intact	0.33	0.06		
	(b) Disrupted before assay	4.87	0.84	0.068	0.071
Codium	(a) Intact	0.149	0.180		
	(b) Sonicated before assay	0.456	0.370	0.327	0.486

envelopes characteristic of these chloroplasts (Trench et al., 1973) had been ruptured by sonication; similar results were obtained with $NADP^+$-glyceraldehyde-3-phosphate dehydrogenase.

Measurement of the Glutathione Content of Chloroplasts

The presence of glutathione reductase in spinach chloroplast fractions suggests that it may play a role in chloroplast metabolism. Freshly isolated, washed, spinach chloroplast fractions were therefore examined for the presence of GSH and GSSG, using the sensitive and specific enzyme assay method described by Tietze (1969). Little, if any, GSSG was detected. However, GSH was always present in chloroplasts. Three separate preparations from different batches of leaves contained 81, 63 and 79 nmol of GSH/mg chlorophyll. If we assume the osmotic volume of spinach chloroplasts to be 21 µl/mg chlorophyll (D.O. Hall – personal communication), it may be calculated that the average internal concentration of GSH in these three chloroplast fractions was 3.5 mM.

Relation of Ascorbic Acid Metabolism to Glutathione Metabolism

High concentrations of ascorbic acid are present in spinach chloroplasts (Gerhardt, 1964; Walker, 1971). Ascorbic acid in chloroplasts can react with the superoxide radical, $O_2^{\cdot -}$, helping to protect them against this cytotoxic species (for a review see Halliwell, 1974b). Also, evidence is accumulating to suggest that illuminated chloroplasts produce H_2O_2 in vivo (Jennings and Forti, 1974; Egneus et al., 1975). Since chloroplasts seem to contain little, if any, catalase (Tolbert, 1971), the well-known reaction of ascorbate with H_2O_2 may also play an important protective role.

Reaction of ascorbate with H_2O_2 or $O_2^{\cdot -}$ – produces dehydroascorbate. Although little, if any, dehydroascorbate reductase was detected in intact chloroplasts (Table 1), Mapson (1958) showed that GSH could non-enzymically reduce dehydroascorbate at pH values above 7. Table 4 shows that the rate of this reaction at mM concentrations of GSH, such as are found in chloroplasts, (see above) is quite fast. Since the chloroplast stroma becomes alkaline during photosynthesis (Pick et al., 1973; Heldt et al., 1973) it seems likely that any dehydroascorbate produced would be rapidly reduced by GSH, and the GSSG so obtained in turn reduced by NADPH in the presence of glutathione reductase.

Table 4. Non-enzymic reduction of dehydroascorbate by GSH

Reaction mixtures contained, in a total volume of 2.00 ml, the following reagents at the final concentrations stated: KH_2PO_4-Na_2HPO_4 buffer of the stated pH (50 mM), dehydroascorbate (1.5 mM) and GSH (1.5 mM). They were incubated at 25° C for 5 min. Metaphosphoric acid (0.1 ml) was then added and ascorbate estimated as described in the Materials and Methods section. No ascorbate was formed in the absence of GSH at any pH value.

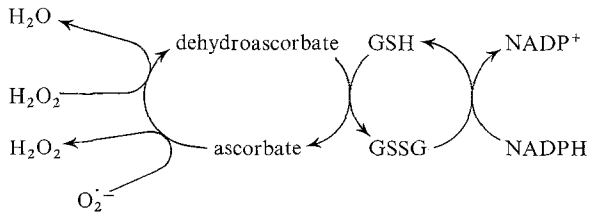
pH	µmol ascorbate produced/5 min	% of the dehydroascorbate present that was reduced to ascorbate by GSH/5 min
6.0	0	0
6.5	0.21	7
7.0	0.27	9
7.5	0.44	15
8.0	1.02	34
8.5	1.37	46

Discussion

The results presented in this paper show that chloroplasts contain GSH and glutathione reductase, which appears to be a stromal enzyme, present in intact but not in broken chloroplasts. This explains the observation of Hendley and Conn (1953), discussed in the Introduction. The equilibrium position of all glutathione reductases so far studied greatly favours reduction of GSSG (Jocelyn, 1972), which would account for the fact that the glutathione present in chloroplasts is mainly in the reduced form. The NADPH required for the action of glutathione reductase would be obtained from the electron transport chain in the light; in the dark, NADPH could be provided by the action of glucose-6-phosphate dehydrogenase (Schnarrenberger et al., 1972), which is activated in the dark (Anderson, 1975).

What is the function of the GSH-glutathione reductase system in chloroplasts? Many enzymes of the Calvin cycle require low molecular weight thiols for activity, and GSH is frequently used to stabilise these enzymes in vitro (e.g. Bradbeer, 1969). For example, Buchanan et al. (1972) were unable to demonstrate light activation of chloroplast fructose diphosphatase by reduced ferredoxin and a "protein factor" unless GSH was present.

Table 4 shows that GSH will non-enzymically reduce dehydroascorbate back to ascorbate at pH values greater than 7, which are known to be obtained in the stroma of illuminated spinach chloroplasts. Hence a cycle of reactions (see below) might operate to help remove chloroplast H_2O_2 and $O_2^{\cdot -}$. The significance of this cycle is under investigation.



In the absence of dehydroascorbate reductase in chloroplasts (Table 1), we would not expect this cycle to operate at pH values below, say, 7.5.

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