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Glutathione and ascorbic acid in spinach (*Spinacia oleracea*) chloroplasts

The effect of hydrogen peroxide and of Paraquat

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The stroma of spinach chloroplasts contains ascorbic acid and glutathione at millimolar concentrations. [Reduced glutathione]/[oxidized glutathione] and [ascorbate]/[dehydroascorbate] ratios are high under both light and dark conditions and no evidence for a role of oxidized glutathione or dehydroascorbate in the dark-deactivation of fructose bisphosphatase could be obtained. Addition of H_2O_2 to chloroplasts in the dark decreases the above ratios, an effect that is reversed on illumination. Addition of Paraquat to illuminated chloroplasts caused a rapid oxidation of reduced glutathione and ascorbate, and apparent loss of dehydroascorbate. Paraquat rapidly inactivated fructose bisphosphatase activity, as assayed under physiological conditions.

Ascorbic acid and glutathione are present in the stroma of spinach (*Spinacia oleracea*) chloroplasts (for review see Halliwell, 1981). Since chloroplasts produce H_2O_2 upon illumination (Nakano & Asada, 1981) and yet contain little, if any, catalase or 'non-specific' peroxidase activity, Foyer & Halliwell (1976) suggested that the H_2O_2 was disposed of by an 'ascorbate-glutathione cycle' as follows:

$$\text{Ascorbate} + H_2O_2 \rightarrow \text{dehydroascorbate} + 2H_2O \quad (1)$$

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

$$\text{GSSG} + \text{NADPH} + H^+ \rightarrow 2\text{GSH} + \text{NADP}^+ \quad (3)$$

An ascorbate peroxidase enzyme catalysing reaction (1) (Groden & Beck, 1979) and a glutathione reductase (reaction 3; Halliwell & Foyer, 1978) enzyme are present in spinach chloroplasts. Reaction (2) appears to be non-enzymic in spinach chloroplasts (Foyer & Halliwell, 1976, 1977), although a dehydroascorbate reductase may be present in pea chloroplasts (Jablonski & Anderson, 1981). However, no direct evidence for a role of GSH or As in removing H_2O_2 in intact chloroplasts has yet been reported.

Upon illumination of chloroplasts the activity of the Calvin-cycle enzyme fructose bisphosphatase, as assayed at physiological concentrations of substrate and Mg^{2+}, increases (Leegood & Walker, 1980*a*; Laing *et al.*, 1981), probably owing to reductive conversion of the enzyme into a form with greater affinity for its substrate (Charles & Halliwell, 1981*a*). In the dark, enzyme activity decreases again. Wolosiuk & Buchanan (1977) found that fructose bisphosphatase could be deactivated *in vitro* by GSSG or DAs and suggested that they were formed for this purpose in darkened chloroplasts. However, Halliwell & Foyer (1978) discovered that the [GSH]/[GSSG] ratio in spinach chloroplasts was high under both light and dark conditions. Since they made the latter measurement after 10 min of darkness, this does not rule out the possibility that a 'burst' of GSSG or DAs formation immediately after switching off the light could deactivate fructose bisphosphatase (Buchanan, 1980).

In the present paper we report the concentrations of As, DAs, GSH and GSSG in type A spinach chloroplasts in both light and dark, and the effect of H_2O_2 and of the herbicide PQ upon the amounts present. The effect of H_2O_2 on the activity of fructose bisphosphatase in isolated spinach chloroplasts has been reported previously (Charles & Halliwell, 1981*a*). The present paper also reports the effect of PQ on this enzyme. PQ is reduced by Photosystem I and generates H_2O_2 and the superoxide radical, $O_2^{-\bullet}$, within the chloroplast (Dodge, 1975; Halliwell, 1981).

Abbreviations used: GSH, reduced glutathione; GSSG, oxidized glutathione; As, ascorbate; DAs, dehydroascorbate; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent); PQ, Paraquat.

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Materials and methods

Reagents

Fresh spinach leaves were obtained from New Covent Garden Market, London, S.W.8, U.K.

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Glutathione reductase was from Boehringer, 2-vinylpyridine from Aldrich and all other reagents from Sigma Chemical Corp., including PQ (Methyl Viologen).

Chloroplast isolation

Type A chloroplasts (Hall, 1972) were isolated from washed mature spinach leaves as described by Charles & Halliwell (1981*a*), except that Mg^{2+}, Mn^{2+} and EDTA were omitted from the preparation and resuspension medium. Their percentage intactness was determined by the ferricyanide method (Lilley *et al.*, 1975). Chlorophyll was measured by the method of Arnon (1949). Spinach chloroplast fructose bisphosphatase was purified, reduced and assayed as described by Charles & Halliwell (1980*a*). The enzyme assay contained 100 μM substrate and 5 mM-$MgCl_2$.

Determination of As and DAs

A modification of the method of Okamura (1980) was used. The assay is based on the reduction of Fe^{3+} to Fe^{2+} by As in acidic solution. The Fe^{2+} then forms complexes with bipyridyl, giving a pink colour that absorbs at 525 nm. Total ascorbate (As + DAs) is determined through a reduction of DAs to As by dithiothreitol. For the concentrations quoted, half a sample of chloroplast preparation was assayed for total ascorbate content, and the other half was assayed for As only. DAs concentrations were then deduced from the difference.

A sample (400 μl; 250–350 μg of chlorophyll) of the chloroplast preparation was added to 200 μl of 10% (w/v) trichloroacetic acid. After vortex-mixing it was allowed to stand in ice for 5 min. NaOH (10 μl, 5 M) was added, mixed and the mixture centrifuged for 2 min in a Microfuge. To a 200 μl sample of the supernatant was added 200 μl of 150 mM-NaH_2PO_4 buffer, pH 7.4, and 200 μl of water. To another 200 μl of supernatant sample was added 200 μl of buffer, 100 μl of 10 mM-dithiothreitol and, after thorough mixing and being left at room temperature for 15 min, 100 μl of 0.5% (w/v) *N*-ethylmaleimide. Both samples were vortex-mixed and incubated at room temperature for >30 s. To each was then added 400 μl of 10% (w/v) trichloroacetic acid, 400 μl of 44% (v/v) H_3PO_4, 400 μl of 4% (w/v) bipyridyl in 70% (v/v) ethanol and 200 μl of 3% (w/v) $FeCl_3$. After vortex-mixing, samples were incubated at 37°C for 60 min and the A_{525} was recorded. A standard curve in the range 0–40 nmol of As or DAs was used for calibration.

Determination of GSH and GSSG

The method used employed the specificity of glutathione reductase and was a modification of that of Griffith (1980). A portion (50 μl; about 25 μg of chlorophyll) of chloroplast suspension was quickly mixed with 250 μl of resuspension medium (330 mM-betaine/50 mM-Hepes/KOH, pH 7.6) and 150 μl of 10% (w/v) sulphosalicylic acid. After centrifugation, 150 μl of the supernatant was added to 700 μl of 0.3 mM-NADPH, 100 μl of 6 mM-DTNB, 50 μl of glutathione reductase (10 units/ml), all reagents being prepared in 125 mM-NaH_2PO_4 buffer, containing 6.3 mM-EDTA, at pH 7.5. Reaction was followed as ΔA_{412} and the total glutathione content was calculated from a standard curve.

To measure GSSG, 150 μl (about 75 μg of chlorophyll) of chloroplast suspension was added to 75 μl of sulphosalicylic acid. After mixing and centrifugation, to 180 μl of the supernatant was added 12 μl of 2-vinylpyridine followed by 20 μl of 50% (v/v) triethanolamine, the latter being placed on the side of the tube above the level of the liquid. The solution was vortex-mixed for 30 s and left at 25°C for 25 min. A portion (150 μl) of the resultant solution was assayed as above. Calibration curves were carried out using GSSG samples treated exactly as above, since triethanolamine slightly alters the rate of colour development.

Results

Type A chloroplasts (Hall, 1972) were prepared from different batches of leaves and analysed for their contents of ascorbate and glutathione by sensitive assay methods. Table 1 summarizes the results obtained. GSH, GSSG, As or DAs added to the chloroplasts in known amounts could be quantitatively detected in subsequent assay. The [GSH]/[GSSG] and [As]/[Das] ratios were always high and did not change under light or dark conditions. There was no evidence for a burst of GSSG or DAs formation on switching off the light (Fig. 1 shows a typical example). The intactness of the chloroplast preparations used was 70% or more, as determined by the ferricyanide method.

Table 1. *Ascorbate and glutathione concentrations in illuminated spinach chloroplasts*

Values are given as means ± S.D. for *n* chloroplast preparations from different batches of leaves. Concentrations are calculated by assuming an internal chloroplast volume of 23 μl per mg of chlorophyll. Several chloroplast preparations contained no detectable DAs. The chloroplast intactness was 70% or more as measured by the ferricyanide method.

Substrate measured	Concentration (mM)
As ($n = 40$)	12.9 ± 2.8
DAs ($n = 38$)	1.12 ± 1.2
Total ascorbate ($n = 39$)	13.5 ± 1.9
GSH ($n = 42$)	4.2 ± 1.7
GSSG ($n = 41$)	0.3 ± 0.17
Total glutathione ($n = 40$)	4.5 ± 1.6

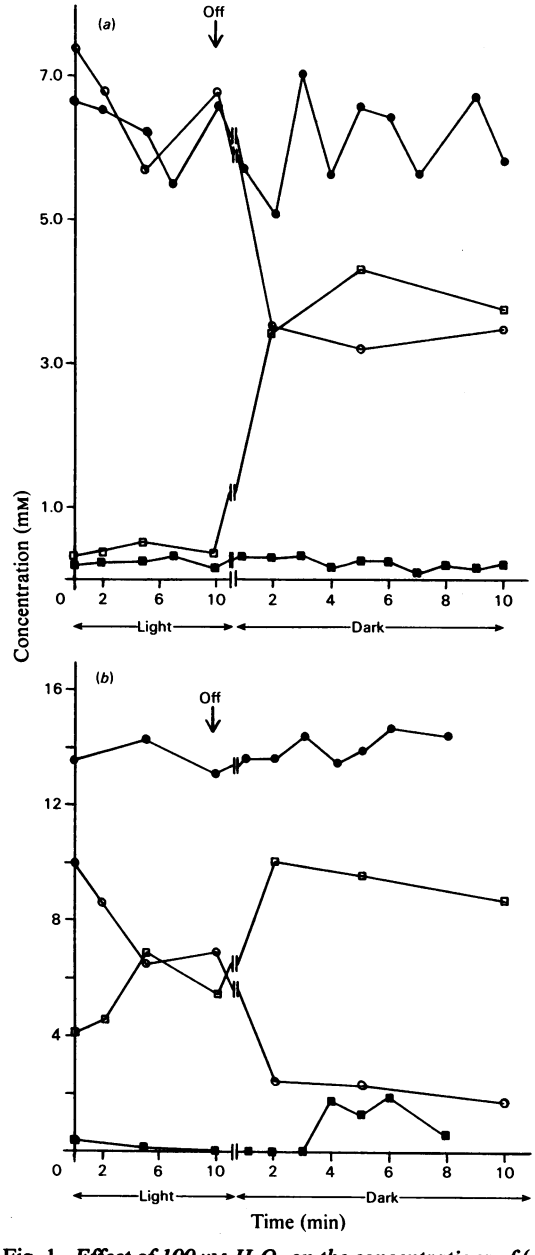

Fig. 1. *Effect of 100 μM-H_2O_2 on the concentrations of (a) GSH and GSSG and (b) As and DAs in illuminated and darkened type A chloroplasts*

(*a*) The light was switched on at time zero and off as indicated by the arrow. Where indicated, H_2O_2 at a final concentration of 100 μM was added at time zero. Portions were removed at the times indicated and assayed for GSH and GSSG, as described in the Materials and methods section. Symbols: ●, GSH; ■, GSSG; ○, GSH, +H_2O_2; □, GSSG, +H_2O_2. (*b*) Conditions were as described for (*a*). Portions were removed at the times indicated and assayed for As and DAs as described in the Materials and methods section. Symbols: ●, As; ■, DAs; ○, As, +H_2O_2; □, DAs, +H_2O_2. Similar results were obtained in ten experiments using chloroplasts from different batches of leaves.

H_2O_2 is a fairly non-polar molecule and crosses the chloroplast envelope readily (Egneus *et al.*, 1975). The effect of addition of H_2O_2 at a final concentration of 100 μM to illuminated type A chloroplast preparations is shown in Figs. 1(*a*) and 1(*b*). There was a slow oxidation of part of the As present to DAs but little change in the GSH and GSSG levels. On turning off the light, however, the [As]/[DAs] ratio decreased markedly within 2 min and GSH was equally quickly converted into GSSG (Figs. 1*a* and 1*b*). Under both light and dark conditions, a fall in the concentration of total ascorbate on addition of H_2O_2 was recorded. A high [GSH]/[GSSG] ratio rapidly re-appeared upon illumination of H_2O_2-treated dark-adapted chloroplasts (Table 2).

Addition of PQ to illuminated type A chloroplasts caused a rapid loss of GSH and transient formation of GSSG (Fig. 2*a*). However, despite a massive loss of As on paraquat addition, there was no significant increase in DAs detected (Fig. 2*b*). The loss of total ascorbate may be an indication of immediate degradation of DAs formed from oxidation of As. GSSG is also quickly lost. Addition of PQ to darkened chloroplasts produced much slower, although significant, changes (Table 3). PQ also caused a rapid inactivation of fructose bisphosphatase activity, as assayed at physiological concentrations of substrate and Mg^{2+} (Fig. 3).

Physiological concentrations of GSSG (0.3 mM; see Table 1) and DAs (1 mM; see Table 1) had no effect on the activity of fructose bisphosphatase,

Table 2. *Effect of illumination on the concentrations of GSH and GSSG in H_2O_2-dark-adapted type A chloroplasts*

A final concentration of 100 μM-H_2O_2 was added to isolated type A chloroplasts in the dark. After 2 min, portions were removed for the assay of GSH and GSSG as described in the Materials and methods section. At the same time, the light was switched on and further portions were removed after 2 min and 5 min of illumination and assayed for GSH and GSSG. Similar results were obtained in three different experiments using chloroplasts from different batches of leaves.

Time (min)	[GSSG] (mM)	[GSH] (mM)
Before addition	0.12	4.21
After addition		
2 (in the dark)	2.82	1.40
2 (illuminated)	0.47	3.52
5 (illuminated)	0.32	3.83

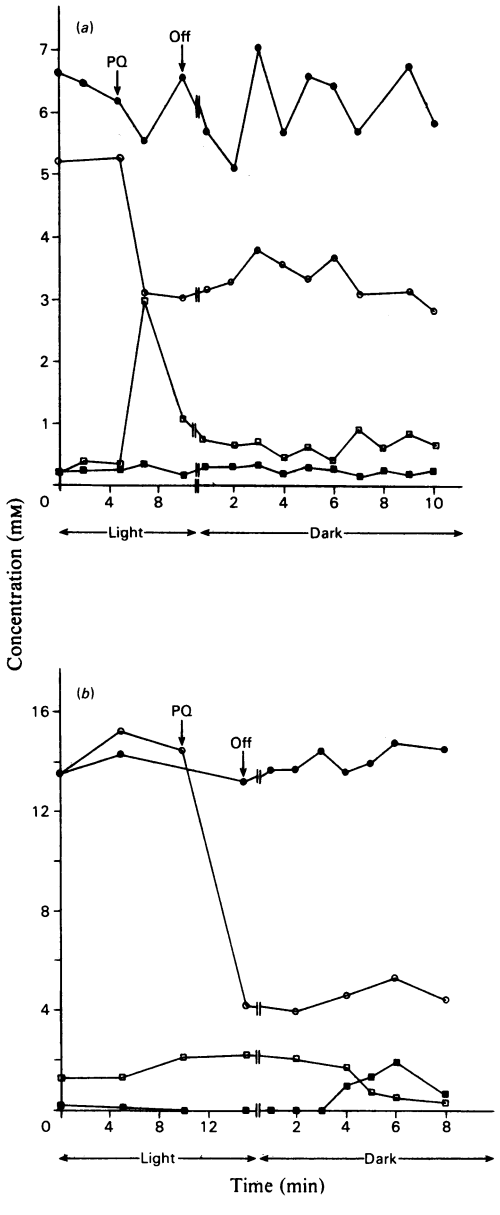

Fig. 2. *Effect of 100 μM-Paraquat on the concentrations of (a) GSH and GSSG and (b) As and DAs in illuminated and darkened type A chloroplasts*

(*a*) The light was switched on at time zero and off as indicated by the arrow. Where indicated Paraquat at a final concentration of 100 μM was added. Portions were removed at the times indicated and assayed for GSH and GSSG as described in the Materials and methods section. Symbols: ●, GSH; ■, GSSG; ○, GSH, +PQ; □, GSSG, +PQ. (*b*) Conditions were as described for (*a*). Portions were removed at the times indicated and assayed for As and DAs as described in the Materials and methods section. Symbols: ●, As; ■, DAs; ○, As, +PQ; □, DAs, +PQ. Similar results were obtained in eight different experiments using chloroplasts from different batches of leaves.

Table 3. *Effect of 100 μM-Paraquat on ascorbate and glutathione levels in type A chloroplasts maintained in the dark*

A final concentration of 100 μM-Paraquat was added to isolated type A chloroplasts maintained in the dark. Portions were removed at the times indicated and assayed for GSH, GSSG, As and DAs as described in the Materials and methods section. Similar results were obtained in eight different experiments using chloroplasts from different batches of leaves.

Time after Paraquat addition (min)	[DAs] (mM)	[As] (mM)	[GSSG] (mM)	[GSH] (mM)
0	3.23	7.47	0.10	3.26
2	3.05	7.83	0.12	3.90
5	0.57	7.66	0.32	3.07
10	1.42	6.97	1.24	1.98

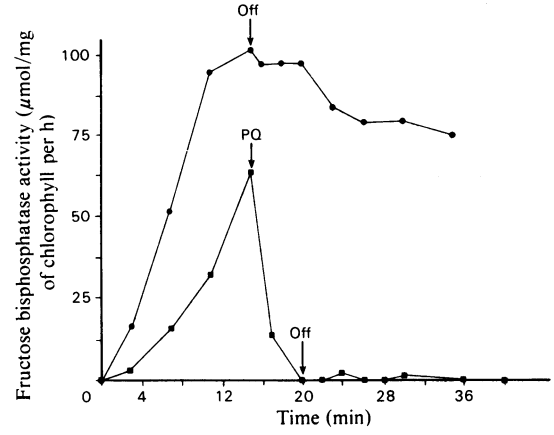

Fig. 3. *Effect of 100 μM-Paraquat on the physiological activity of fructose bisphosphatase in isolated type A chloroplasts*

Illumination was commenced at time zero to generate the physiological (reduced) form of fructose bisphosphatase. Where indicated, Paraquat at a final concentration of 100 μM was added and the light turned off as indicated by the arrows. Portions were removed at the times indicated and assayed for enzyme activity as described in the Materials and methods section. Similar results were obtained in five different experiments using chloroplasts from different batches of leaves.

either purified or in the reduced form (results not shown).

Discussion

The [GSH]/[GSSG] and [As]/[DAs] ratios are maintained high in chloroplasts under both light and dark conditions – there is no sign of a burst of GSSG or DAs formation when the light is turned off

(Figs. 1 and 2). Deactivation of enzymes such as fructose bisphosphatase must therefore be achieved by other mechanisms, as already suggested (Charles & Halliwell, 1980*a*,*b*, 1981*a*,*b*; Leegood & Walker, 1980*a*,*b*; Soulie *et al.*, 1981), especially as physiological concentrations of GSSG and DAs have no effect on the purified reduced form of fructose bisphosphatase.

The oxidation of As on addition of H_2O_2 to chloroplasts in the light or dark is consistent with the operation of an ascorbate peroxidase enzyme (Groden & Beck, 1979), which must therefore be catalytically active under both conditions. Perhaps the net amount of As oxidized in the light is limited by the recycling of DAs at the expense of GSH, which is in turn recycled by glutathione reductase. Consistent with this, addition of H_2O_2 in the dark produced a greater oxidation of As accompanied by a fall in the [GSH]/[GSSG] ratio (Fig. 1); presumably the rate of the glutathione reductase reaction is limited by the supply of NADPH in the dark. Further evidence for this suggestion comes from the observation that illumination of H_2O_2-treated dark-adapted chloroplasts caused a marked rise in the [GSH]/[GSSG] ratio (Table 2).

Addition of PQ to illuminated chloroplasts causes a rapid uptake of O_2 and inhibition of CO_2 fixation (Dodge, 1975), accompanied by the formation of H_2O_2 and $O_2^{-\bullet}$. Removal of electrons from the Photosystem I acceptor by PQ also prevents generation of NADPH and so glutathione reductase cannot operate. There is a rapid oxidation of both GSH and As (Fig. 2) and a loss of the physiologically-active form of fructose bisphosphatase (Fig. 3), presumably due to the action of H_2O_2 (Charles & Halliwell, 1980*c*, 1981*a*) and to the fact that electrons will no longer be available to reduce thioredoxin and activate the enzyme (Leegood & Walker, 1980*a*,*b*). Once formed, the DAs disappears rapidly. In the absence of GSH to recycle it, DAs might be degraded into oxalic acid and L-threonic acids (Habermann *et al.*, 1968). GSSG is also lost. In illuminated chloroplasts the addition of PQ obviously imposes a great 'oxidative stress'. PQ had a much slower action on As and GSH in the dark, consistent with the light-dependence of its toxic effect on plants (Dodge, 1975). Nevertheless it can probably be reduced slowly in the dark by a 'back flow' of electrons from NADPH to the electron transport chain (e.g. Leegood & Walker, 1981) or even by glutathione reductase itself (Richmond & Halliwell, 1982) and thus give rise to some formation of H_2O_2 and $O_2^{-\bullet}$.

In summary all these results are consistent with the operation of an ascorbate/glutathione cycle (Foyer & Halliwell, 1976) in the intact chloroplast and support the conclusion that GSSG and DAs are not involved *in vivo* in dark deactivation of fructose bisphosphatase.

Note added in proof (Received 6 January 1983)

Foyer *et al.* (1983) have independently performed experiments that support the conclusions reached in the present paper.

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