Effect of hydrogen peroxide on spinach (*Spinacia oleracea*) chloroplast fructose biphosphatase

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Thiol-treated spinach (*Spinacia oleracea*) chloroplast fructose biphosphatase (EC 3.1.3.11) is severely inhibited by H_2O_2 , whereas the freshly purified enzyme is little affected. Dithiothreitol reverses inhibition by H_2O_2 , indicating that essential thiol groups are oxidized during H_2O_2 inactivation. A new role for the dithiol and thioredoxin systems that are operative in illuminated chloroplasts is proposed.

H_2O_2 is formed in the light by spinach (*Spinacia oleracea*) chloroplasts (Forti & Gerola, 1977), probably due to transfer of reducing equivalents generated by photosynthetic electron transport to O_2 to form the toxic superoxide radical ($O_2^{\cdot -}$) (Asada *et al.*, 1974; Egneus *et al.*, 1975). Superoxide is then converted into H_2O_2 by superoxide dismutase, high activities of which are present in chloroplasts (Jackson *et al.*, 1978). H_2O_2 slowly inactivates chloroplast superoxide dismutase (Asada *et al.*, 1975) and rapidly inhibits CO_2 fixation by type-A chloroplasts at the level of fructose biphosphatase and sedoheptulose biphosphatase (Egneus *et al.*, 1975; Kaiser, 1976; Forti & Gerola, 1977; Heldt *et al.*, 1978). Clearly, H_2O_2 must be removed from chloroplasts in order to allow the Calvin cycle to operate, yet these organelles contain no catalase (for a review, see Halliwell, 1978). However, Foyer & Halliwell (1976) proposed that NADPH, reduced glutathione and ascorbate act via glutathione reductase (Halliwell & Foyer, 1978) and ascorbate peroxidase (Grodén & Beck, 1979; Kelly & Latzko, 1979) to remove H_2O_2 .

Charles & Halliwell (1980a) proposed, on the basis of kinetic evidence, that a reduced form of the enzyme fructose biphosphatase, obtained by treatment with thiol compounds, is the form that actually functions in the Calvin cycle. If this is so, it follows from the results of Heldt *et al.* (1978) that thiol-treated fructose biphosphatase should be susceptible to inactivation by H_2O_2 . The present paper reports the effects of H_2O_2 on thiol-treated fructose biphosphatase under physiological conditions.

Materials and methods

Materials

All reagents were purchased from Sigma Chemical Co., Poole, Dorset, U.K., except for H_2O_2 , which

was purchased from BDH Chemicals Ltd., Poole, Dorset, U.K. Spinach (*Spinacia oleracea*) was purchased locally.

Incubation of fructose biphosphatase with H_2O_2 and subsequent assay of enzyme activity

Incubation mixtures contained, in a total volume of 1 ml, 100 mM-Tris/HCl buffer, pH 8.0, 0.5 mM-EDTA, 10 mM-MgCl₂ and about 0.1 unit (μ mol/min) of fructose biphosphatase. The incubation was initiated by addition of H_2O_2 at a final concentration of 0–2.5 mM and enzyme activity determined at various times as follows. Reaction mixtures contained, in a total volume of 2 ml, 100 mM-Tris/HCl buffer, pH 8.0, 0.5 mM-EDTA, 10 mM-MgCl₂, 0.3 mM-NADP⁺, 3 units (μ mol/min) of glucose phosphate isomerase (EC 5.3.1.9), 1 unit (μ mol/min) of glucose 6-phosphate dehydrogenase (EC 1.1.1.49) and 100 μ M- or 3 mM-fructose 1,6-bisphosphate. The reaction was started by the addition of 200 μ l of the above incubation mixture [about 0.02 unit (μ mol/min) of fructose biphosphatase] and monitored by the ΔA_{340} due to reduction of NADP⁺. Dithiothreitol or H_2O_2 at the concentrations used did not affect the coupled enzyme assay. The amount of enzyme required to hydrolyse 1 μ mol of fructose 1,6-bisphosphate/min at 25°C (with 1 mM-fructose 1,6-bisphosphate) was defined as 1 unit.

Removal of dithiothreitol from thiol-treated fructose biphosphatase

Before incubation of thiol-treated fructose biphosphatase with H_2O_2 , the dithiothreitol used for thiol treatment was removed by filtration of the enzyme preparation through a column (24 cm $\times$ 0.8 cm²) of Sephadex G-25. Fractions (500 μ l) were collected and the active fractions, completely free of dithiothreitol, were pooled. The pooled fractions

constituted the thiol-treated fructose bisphosphatase preparations used in the present experiments.

Purification and thiol treatment of spinach chloroplast fructose bisphosphatase

The procedures used were as described previously (Charles & Halliwell, 1980a).

Results

Charles & Halliwell (1980a) showed that freshly purified spinach chloroplast fructose bisphosphatase requires non-physiological concentrations of substrate (3 mM) to achieve its maximum rate, whereas the thiol-treated form shows the same maximal activity at the concentrations of substrate present in the chloroplast stroma (100 μ M; Heldt *et al.*, 1980).

Freshly purified fructose bisphosphatase was found to be virtually unaffected by incubation for 5 min with H_2O_2 at concentrations as high as 2.5 mM when assayed at saturating (3 mM) fructose 1,6-bisphosphate (Fig. 1). In contrast, 0.3 mM- H_2O_2 inhibited the thiol-treated enzyme, assayed at

Fig. 1. Effect of H_2O_2 on the activity of fructose bisphosphatase as a function of $[H_2O_2]$

Fructose bisphosphatase was incubated with various amounts of H_2O_2 for 5 min and enzyme activity was subsequently determined as described in the Materials and methods section, the concentration of fructose 1,6-bisphosphate being 100 μ M or 3 mM as shown. ●, Thiol-treated fructose bisphosphatase assayed with 100 μ M-fructose 1,6-bisphosphate; ■, freshly purified fructose bisphosphatase assayed with 3 mM-fructose 1,6-bisphosphate (the freshly purified enzyme shows virtually no activity when assayed at 100 μ M-substrate). The specific activity of both enzyme preparations, corresponding to 0% inhibition, was 154 units/mg of protein.

Fig. 2. Effect of H_2O_2 on the activity of fructose bisphosphatase as a function of time

Fructose bisphosphatase was incubated with 0.3 mM- H_2O_2 and enzyme activity was determined at various times as described in the Materials and methods section, the concentration of fructose 1,6-bisphosphate being 100 μ M or 3 mM as shown. ●, Thiol-treated fructose bisphosphatase assayed with 100 μ M-fructose 1,6-bisphosphate; ■, freshly purified fructose bisphosphatase assayed with 3 mM-fructose 1,6-bisphosphate. The specific activity of both enzyme preparations, corresponding to 0% inhibition, was 154 units/mg of protein.

Fig. 3. Effect of dithiothreitol on the activity of thiol-treated fructose bisphosphatase after treatment with H_2O_2

Thiol-treated fructose bisphosphatase was incubated with 0.3 mM- H_2O_2 as described in the Materials and methods section. The arrows indicate the time of addition of 50 mM-dithiothreitol to the enzyme. Enzyme activity was subsequently determined at various times, the concentration of fructose 1,6-bisphosphate being (100 μ M) (●) or 3 mM (■). The specific activity of the enzyme preparation corresponding to 0% inhibition was 154 units/mg of protein.

100 μ M-substrate concentration, by 70% after 5 min, and 1 mM- H_2O_2 inhibited almost completely. Heldt *et al.* (1978) reported a 76% inhibition of CO_2 fixation in type-A spinach chloroplasts by treatment with 0.3 mM- H_2O_2 for 5 min.

Virtually complete inhibition of the thiol-treated enzyme (assayed at 100 μ M-substrate) was also seen after 15 min incubation with 0.3 mM- H_2O_2 (Fig. 2), whereas the freshly purified enzyme was inhibited by only 7% (assayed at 3 mM-substrate).

Activity could be substantially (70%) restored to the thiol-treated enzyme after inhibition by H_2O_2 by raising the substrate concentration used in the assay to 3 mM, which suggests that the major effect of H_2O_2 is to convert the thiol-treated form into the 'low-affinity' freshly purified form of the enzyme. To check this, a sample of thiol-treated enzyme was treated with 0.3 mM- H_2O_2 for various times and then incubated for 15 min with 50 mM-dithiothreitol. Fig. 3 shows that approx. 50% of the activity, as assayed at 100 μ M-substrate concentration, could be restored by the thiol treatment. The same enzyme assayed at 3 mM-substrate concentration lost much less activity, and the lost activity was not restored by thiol treatment (Fig. 3).

Discussion

Since Heldt *et al.* (1978) have demonstrated that fructose biphosphatase is a principal point of inhibition of the Calvin cycle by H_2O_2 , we investigated the effects of H_2O_2 on the two available forms of the enzyme, freshly purified and thiol-treated fructose biphosphatase. Figs. 1 and 2 show that thiol-treated fructose biphosphatase is severely inhibited by H_2O_2 , whereas freshly purified fructose biphosphatase is little affected. This observation is in good agreement with previous kinetic data (Charles & Halliwell, 1980a), which suggested that thiol-treated fructose biphosphatase is well suited to a role *in vivo*. Inhibition of thiol-treated fructose biphosphatase by H_2O_2 is reversible by assay at high substrate concentrations or by dithiothreitol treatment (Fig. 3), indicating that the effect of H_2O_2 is an oxidation of essential thiol groups, which causes a reversion of the enzyme to a form whose affinity for substrate and cofactor is too low and whose pH optimum is too high (pH 8.8 as opposed to pH 8.0 for the thiol-treated enzyme) to allow it to operate under physiological conditions (Charles & Halliwell, 1980a).

As discussed in the introduction, H_2O_2 might be removed by an ascorbate/glutathione system in chloroplasts, but it seems that this process is not always completely efficient, since CO_2 fixation by intact chloroplasts is often stimulated by the addition of catalase (e.g. Egneus *et al.*, 1975; Heldt

et al., 1978). For the Calvin cycle to operate at significant rates, fructose biphosphatase and sedoheptulose biphosphatase must be maintained in the reduced (thiol-treated) forms throughout periods of illumination. This can be achieved *in vivo* by the light-dependent generation of membrane-bound dithiol groups and of the dithiol form of the protein thioredoxin, whose action is mimicked *in vitro* by dithiothreitol (Anderson & Avron, 1976; Buchanan *et al.*, 1979). Although it has been suggested that the thiol systems play a part in controlling the rate of the Calvin cycle at the fructose biphosphatase step, this seems unlikely, because the rates of reversion of the reduced enzyme to the oxidized form in the dark are very slow, both *in vivo* and *in vitro*, and conversion of the oxidized form back into the reduced form by these systems is also slow (for a review, see Halliwell, 1978). It seems more likely to us that the dithiol systems exist *in vivo* to prevent inhibition of the reduced forms of fructose biphosphatase and sedoheptulose biphosphatase by any light-generated H_2O_2 that escapes the action of the ascorbate/glutathione system, and that fine control of the enzyme activity *in vivo* is achieved by changes in stromal pH and cation concentrations (Kelly *et al.*, 1976; Heldt *et al.*, 1978; Charles & Halliwell, 1980a,b).

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