Plant Physiol. (1980) 66, 815-817
0032-0889/80/66/0815/03/$00.50/0

Inhibition by Catalase of Dark-mediated Glucose-6-Phosphate Dehydrogenase Activation in Pea Chloroplasts[1]

Received for publication January 25, 1980 and in revised form June 13, 1980

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ABSTRACT

Dark activation of light-inactivated glucose-6-phosphate dehydrogenase was inhibited by catalase in a broken pea chloroplast system. Partially purified glucose-6-phosphate dehydrogenase from pea leaf chloroplasts can be inactivated *in vitro* by dithiothreitol and thioredoxin and reactivated by H_2O_2. The *in vitro* activation by H_2O_2 was not enhanced by horseradish peroxidase, and dark activation in the broken chloroplast system was only slightly inhibited by NaCN. These results indicate that the dark activation of glucose-6-phosphate dehydrogenase may involve oxidation by H_2O_2 of SH groups on the enzyme which were reduced in the light by the light effect mediator system.

Light modulates the activity of several chloroplastic enzymes by a process which couples electron flow through the photochemical apparatus to the reduction of a disulfide bond or a thiol-disulfide exchange on the modulated enzyme. Electrons are transferred from PSI to the enzyme via the thylakoid-bound light-effect mediator and a thioredoxin-like stromal factor (3, 5). When a broken pea leaf chloroplast preparation is illuminated or treated with DTT, the light-activatable enzymes associated with the reductive pentose pathway are activated and glucose-6-P dehydrogenase is inactivated. In darkness, the light effects are reversed, with the light-activated enzymes becoming inactivated and glucose-6-P dehydrogenase becoming activated (1).

Since the light effect is a reductive process dependent upon either photosynthetic electron flow or an artificial reducing agent, such as DTT, it seems reasonable to suggest that the dark effect may involve an oxidation of the light-modulated enzyme, possibly the conversion of two sulfhydryl groups to a disulfide. This view seems to be supported by experiments showing that thioredoxin-activated fructose 1,6-bisphosphatase can be inactivated by the oxidizing agents dehydroascorbate, tetrathionite, and oxidized glutathione (18) and that light or DTT activation of malate dehydrogenase is reversed by diamide, a thiol oxidant (4).

If the dark reversal of the light effect *in vivo* is, in fact, due to oxidation of light-reduced enzyme sulfhydryl groups, the identity of the naturally occurring oxidant is still in question. The demonstration of H_2O_2 synthesis and accumulation within the intact chloroplast (7, 9) and the capability of H_2O_2 to oxidize sulfhydryl groups (14, 15) makes this compound a likely candidate for such an oxidant. This possibility is also indicated by the work of Kaiser (13), which demonstrated that H_2O_2 inhibits the Calvin cycle but increases the formation of products of the oxidative pentose phosphate pathway in isolated chloroplasts.

The purpose of the experiments presented here was to determine if H_2O_2 might be involved in the dark activation of pea leaf chloroplast glucose-6-P dehydrogenase which has previously been inactivated by light.

MATERIALS AND METHODS

Plant Material. Pea (*Pisum sativum* L., var. Little Marvel) plants were grown in Vermiculite in a greenhouse and were harvested when they were between 9 and 11 days old. Whole shoots were washed in distilled H_2O and used in the preparation of isolated chloroplasts.

Chloroplast Preparation. Chloroplasts were prepared as described previously (1) and were washed once in 0.33 M sorbitol, 50 mM Hepes, 2 mM $MgCl_2$, 2 mM EDTA (pH 7.2) and then were broken by osmotic lysis in 50 mM Hepes, 10 mM KCl, 2 mM $MgCl_2$, 1 mM EDTA (pH 7.2). This preparation was used in the light-modulation experiments.

Enzyme Assays. Glucose-6-P dehydrogenase (EC 1.1.1.49) activity was assayed as described previously (6). Change in A_{340} was followed at room temperature (about 20 C) using a Cary 219 or Gilford 2400 recording spectrophotometer. In the experiments with partially purified glucose-6-P dehydrogenase, the change in fluorescence of NADPH was followed using an Eppendorf photometer 1100 adapted for fluorimetry. Activity determinations and experiments were run in triplicate.

Light-Dark Modulation. Glucose-6-P dehydrogenase in the broken chloroplast preparation was inactivated in a constant temperature bath of 25 C as described previously (2) except that the light source was two General Electric 150-w, 120-v reflector flood lamps. Light intensity was 5,500 ft-c. After approximately 15 min in the light, the chloroplast preparation was transferred to darkness and samples withdrawn after 0, 15, 30, 60, and 90 s in the dark. Modulation was stopped by dilution of the sample in 10 volumes ice-cold distilled, deionized H_2O, which then was centrifuged at 12,000*g* for 10 min, and the supernatant was taken for enzyme assay. In some experiments, catalase (EC 1.11.1.6) or NaCN was added to the chloroplast preparation. The degree of dark activation varies from one chloroplast preparation to the next and was, therefore, not identical in all experiments. However, comparisons were made only between results obtained from the same preparation.

Experiments with Partially Purified Glucose-6-P Dehydrogenase. Pea leaf chloroplasts were prepared essentially as described above, except that the lysed chloroplasts were centrifuged and the stromal fraction was applied to a column of Sephacryl S-200. The eluant was assayed for glucose-6-P dehydrogenase and thioredoxin

[1] This research was supported by National Research Foundation Grant PGM 77-08355 (to L. E. A.).

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activities, and the active fractions were taken for experimental use. Thioredoxin activity is defined as the ability to enhance the rate of inactivation of chloroplast glucose-6-P dehydrogenase by DTT (5). Inactivation of the glucose-6-P dehydrogenase was accomplished by incubation of 10 μl enzyme fraction with 10 μl thioredoxin fraction and 5 μl 50 mM DTT for 20 min. This then was added to 0.85 ml 0.1 M Tris-HCl (pH 7.8) containing various concentrations of H_2O_2, or H_2O_2 plus peroxidase (EC 1.11.1.7), incubated for 10 min, and then assayed for glucose-6-P dehydrogenase activity to determine the degree of reactivation of the enzyme.

Chemicals. Biochemicals, including bovine liver catalase and horseradish peroxidase, were obtained from Sigma. H_2O_2 was from Fisher. Analytical reagent grade chemicals were used throughout. Pea seeds were obtained from Northrup and King, Chicago.

RESULTS AND DISCUSSION

Experiments were run to assess the involvement of H_2O_2 in the dark reversal of light-mediated glucose-6-P dehydrogenase inactivation. Figure 1 shows the effect of inclusion of approximately 7,500 units of catalase (added at the beginning of the light period)/ml of broken chloroplast preparation. Activation of the enzyme during the first 90 s after transfer from light to darkness was strongly suppressed in the presence of catalase, as compared to controls. The same results were obtained with controls containing either no catalase or boiled catalase. Dark activation of glucose-6-P dehydrogenase, therefore, appears to be dependent upon the availability of H_2O_2 generated during the light reactions. These results are compatible with the proposal of Kaiser (13) that H_2O_2 may affect the activity of chloroplast enzymes by oxidation of light-generated SH groups on the enzyme molecule.

It is possible to inactivate purified pea leaf chloroplast glucose-6-P dehydrogenase *in vitro* using DTT and thioredoxin in place of light-generated reductants. An enzyme preparation which had been inactivated in this way was incubated with H_2O_2 and the activity then was reassayed. The results in Table I show that the enzyme is indeed reactivated in the presence of H_2O_2. Reactivation in this system is apparently nonenzymic and was not enhanced by addition of 35 units of horseradish peroxidase/ml reaction mixture. It cannot be determined from these results whether the H_2O_2 is interacting directly with reduced groups on the enzyme molecule or is bringing about a reverse electron flow through thioredoxin.

Although reactivation of the purified glucose-6-P dehydrogenase by H_2O_2 was not increased in the presence of horseradish peroxidase, this does not exclude the possibility of an enzyme-mediated oxidation occurring in the chloroplast system. Figure 2 shows the results of a dark-activation experiment in which 5 mM NaCN was included in the chloroplast preparation. NaCN was added at the end of the light period but before transfer to darkness, so as not to interfere with light inactivation of the enzyme. NaCN had no influence on the activity of the purified glucose-6-P dehydrogenase (data not shown), so any effects in the broken chloroplast system were due to interference with the dark-activation process. Five mM NaCN should strongly inhibit reactions catalyzed by a typical heme-containing peroxidase, but Figure 2 indicates only slight interference with dark activation of glucose-6-P dehydrogenase. This suggests that the predominant mechanism of the H_2O_2 effect may be nonenzymic oxidation of enzyme SH groups or else oxidation via some other type of enzyme. The fact that CN does seem to cause a slight inhibition indicates some type of peroxidase might be involved, although perhaps only to a minor extent. The participation of a thylakoid-bound peroxidase might explain previous results indicating that the dark-mediation

Table I. *Activation of Partially Purified Glucose-6-P Dehydrogenase by Hydrogen Peroxide*

Partially purified glucose-6-P dehydrogenase was inactivated by incubation with DTT and thioredoxin for 20 min. The inactivated enzyme then was incubated for 10 min with H_2O_2 or H_2O_2 + 35 units horseradish peroxidase (HRP), and the activity was assayed to determine the degree of reactivation.

H_2O_2 Concn.	Glucose-6-P Dehydrogenase Specific Activity	Control
mM	*nmol NADPH/mg protein·min*	%
0	40.0	100
0.5	48.8	122
1	57.2	143
5	110.0	275
10	124.8	312
1 + HRP	54.0	135

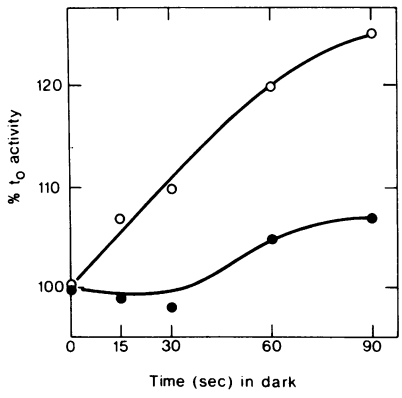

FIG. 1. Time course for dark activation of glucose-6-P dehydrogenase with (●) and without (○) added catalase (7,500 units) in pea leaf chloroplasts. Activity at time zero was 1.81 nmol NADPH formed/mg Chl·min. The concentration of Chl in the chloroplast preparation was 1.47 mg/ml. Light inactivation and dark activation procedures are described under "Materials and Methods." Similar results were obtained in experiments using controls either without catalase or with boiled catalase.

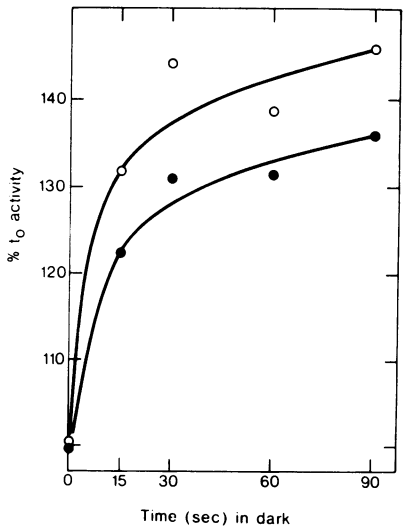

FIG. 2. Time course for dark activation of glucose-6-P dehydrogenase with (●) and without (○) 5 mM NaCN in pea leaf chloroplasts. Activity at time zero was 4.52 nmol NADPH formed/mg Chl·min. The concentration of Chl in the chloroplast preparation was 0.74 mg/ml. Light inactivation and dark activation procedures are described under "Materials and Methods."

system is partially dependent upon the presence of the membrane fraction of the chloroplasts (L. E. Anderson, unpublished). The degree of inhibition of dark activation by CN may underestimate the role of a peroxidase since CN also would interfere with H_2O_2 removal by catalase or ascorbate peroxidation systems (10), thereby allowing the H_2O_2 to accumulate to higher levels.

Chloroplastic H_2O_2 production results from electron transport through both PSI and PSII (8) and apparently occurs in both broken and intact chloroplasts (7, 9, 16). Despite the presence of various removal systems, H_2O_2 seems to accumulate to a certain level, although the actual steady-state level of H_2O_2 inside the chloroplast or in other parts of the cell is uncertain. Estimates have run from 14 μM in chloroplasts (9) to millimolar or higher concentrations in nonphotosynthetic tissue (17).

The inhibition by catalase of dark activation of glucose-6-P dehydrogenase could be compatible with the scheme proposed by Wolosiuk and Buchanan (19), in which H_2O_2 converts glutathione from the reduced to the oxidized state and the oxidized glutathione, in turn, oxidizes the light modulated enzyme in the dark. However, the data of Halliwell and Foyer (11), which indicates that chloroplasts contain a high ratio of reduced to oxidized glutathione regardless of light conditions, and the results presented here showing activation of partially purified glucose-6-P dehydrogenase by H_2O_2 alone would seem to favor a direct oxidation of the enzyme by H_2O_2. However, the possibility of an oxidized intermediate (such as thioredoxin) functioning *in vivo* cannot be ruled out.

These results may offer at least a partial explanation for the inhibition of photosynthesis by H_2O_2, which has been reported by Kaiser (12) and Robinson *et al.* (16) and are consistent with the following hypothesis of light/dark modulation. Although H_2O_2 is being produced in the light and accumulates to some unknown steady-state level, the flux of electrons through the light-effect mediator is adequate to maintain the modulatable enzymes in the reduced state. In darkness, or in the light with added H_2O_2, this cannot be maintained and the enzymes shift to the oxidized form. Inactivation of the light activated enzymes and activation of glucose-6-P dehydrogenase by this mechanism are in good agreement with Kaiser's (13) finding that H_2O_2 inhibits carbon incorporation into Calvin cycle intermediates but stimulates 6-phosphogluconate formation.

Acknowledgments—The authors thank Dr. Anthony Ashton for providing the partially purified glucose-6-P dehydrogenase and thioredoxin and Lawrence Sykora and staff for raising the pea plants.

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