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ISOLATION AND CHARACTERIZATION OF LIGHT- AND DITHIOTHREITOL-MODULATABLE GLUCOSE-6-PHOSPHATE DEHYDROGENASE FROM PEA CHLOROPLASTS

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Glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate: NADP oxidoreductase, EC 1.1.1.49) has been purified to electrophoretic homogeneity from pea chloroplasts. The enzyme, which has a Stokes radius of 52 Å, is a tetramer made up of four 56000 Da monomers. The pH optimum is around 8.2. The enzyme is absolutely specific for NADP. The apparent K_m(NADP) is 2.4 ± 0.1 μM. NADPH inhibition of the enzyme is competitive with respect to NADP (mean K_i, 18 ± 5 μM) and is mixed ($K_p > K_m$, $V_{max} > V_p$) with respect to glucose 6-phosphate (mean crossover point, 0.5 ± 0.1 mM). The apparent K_m(glucose 6-phosphate) is 0.37 ± 0.01 mM. The purified enzyme is inactivated in the light in the presence of dilute stroma and washed thylakoids, and by dithiothreitol. Enzyme which has been partially inactivated by treatment with dithiothreitol can be further inactivated in the light in the presence of dilute stroma and washed thylakoids and reactivated in the dark, but only to the extent of the reverse of light inactivation. Dithiothreitol-inactivated enzyme is not reactivated further by addition of crude stroma or oxidized thioredoxin. Dithiothreitol-dependent inactivation of the enzyme follows pseudo-first-order kinetics and shows rate saturation. The enzyme which has been partially inactivated by treatment with dithiothreitol does not differ from the untreated control with respect to thermal and tryptic inactivation. However, enzyme which has been partially light inactivated shows different thermal and tryptic inactivation patterns as compared to the dark control. These observations suggest that the changes in the enzyme brought about by light modulation are not necessarily identical with those brought about by dithiothreitol inactivation.

Introduction

Light modulation provides one of the modes of enzyme regulation in photosynthetic systems. Several enzymes of the Calvin cycle are known to be activated whereas some enzymes of the oxidative pentose phosphate shunt and glycolysis are inactivated after illumination of whole leaves or intact or broken chloroplasts [1,2]. The effect of light is mimicked in vitro by strong disulfide reductants such as dithiothreitol. It is thought that light modulation of enzymes involves the flow of electrons through Photosystem I to the membrane-bound Light Effect Mediator (LEM), and soluble stromal factor, Protein Modulase, and finally to the enzyme molecule [1]. However, it is not actually known whether light modulation of the enzyme is reductive or is due to intramolecular disulfide-sulfhydryl rearrangements. An alternative scheme involving soluble ferredoxin, the enzyme ferredoxin-thioredoxin reductase and thioredoxin has been proposed by Buchanan and

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co-workers [1,2]. In the dark the effect of light is reversed (dark modulation). Dark reversal of light modulation is apparently mediated by oxidized thioredoxin [1].

Glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate : NADP oxidoreductase, EC 1.1.1.49), an enzyme of the oxidative pentose phosphate shunt, is one of the light-inactivated enzymes in pea (*Pisum sativum*) chloroplast and cytosol [1]. Both the chloroplastic and cytosolic glucose-6-phosphate dehydrogenases, which appear to be isoenzymes [3], are light inactivated when the plant is illuminated [1]. A part of the chloroplastic enzyme in pea leaves is bound to the thylakoid membrane and is released upon illumination [4].

Glucose-6-phosphate dehydrogenase has been purified from animal and microbial sources and extensively studied [5], but very little work has been reported on the enzyme from higher plants. It has been partially purified from sweet potato [6], black gram [7], spinach [8] and tobacco tissue culture cells [9]. In the present paper we report the isolation and characterization of pea chloroplast glucose-6-phosphate dehydrogenase which is light and dark modulatable.

Apparently, conformational changes in protein structure accompany light/dark modulation of enzyme activity [1]. The most metabolically significant change is probably in maximal velocity, but shifts in pH optima have been observed and in one case there is a change in apparent affinity for substrate after illumination [1]. We have examined the effect of light and dithiothreitol inactivation on the susceptibility of the enzyme to proteolytic and thermal inactivation. The results suggest that the dithiothreitol-inactivated enzyme is conformationally distinct from the light-inactivated enzyme.

Materials and Methods

Preparation of chloroplasts. Pea (*P. sativum* L. cv. Little Marvel) plants were grown for 12–15 days in vermiculite in a greenhouse. Chloroplasts were prepared from 8 kg of shoots as described in Ref. 10. The chloroplasts were lysed by suspension in 10 vol. of 50 mM Hepes (K^+), 1 mM EDTA, 2 mM $MgCl_2$, 10 mM KCl, pH 7.4 (Hepes-KCl buffer). A crude stromal fraction was separated from the thylakoids by centrifugation at 27000 × *g* for 20 min. The thylakoids were washed twice with Hepes-KCl buffer.

Assays. Glucose-6-phosphate dehydrogenase was assayed spectrophotometrically using a Cary 219 recording spectrophotometer connected to a thermostatically controlled constant temperature bath (25°C). The usual assay mixture contained 89 mM Tris-HCl buffer, pH 7.8, 0.05 mM NADP, 2.5 mM glucose 6-phosphate, 0.5 mM Hepes, 0.01 mM EDTA, 0.02 mM $MgCl_2$ and 0.1 mM KCl (final volume, 1 ml). The reaction was started by the addition of 10 μl of appropriately diluted enzyme. The initial rate of reaction was obtained from the linear increase in A_{340} recorded for a period of 5 min and the enzyme activity was estimated using the extinction coefficient of NADPH at 340 nm. An enzyme unit is defined as the amount of enzyme required to reduce 1 μmol of NADP to NADPH per min under the test conditions.

Protein was estimated by the method of Lowry et al. [11]. Chlorophyll was estimated according to the method of Strain et al. [12].

Purification of enzyme. Glucose-6-phosphate dehydrogenase was purified from pea chloroplasts. Hepes-KCl buffer was used throughout. All operations were carried out at 0–4°C, unless stated otherwise. The purification of the enzyme is summarized below and in Table I.

(1) Crude extract: The chloroplasts were lysed by resuspension in 10 vol. of Hepes-KCl buffer and homogenized in a glass tissue grinder. The supernatant fraction was separated from the particulate by centrifugation at 27000 × *g* for 15 min (200 ml).

(2) First $(NH_2)_2SO_4$ fractionation: The pH of the extract was adjusted to 7.4 with cold 1.7 M acetic acid, when necessary, and solid $(NH_4)_2SO_4$ was gradually added to 45% saturation. The pH of the solution was intermittently adjusted to 7.4 with cold 0.5 M NH_4OH. The supernatant fraction was discarded after centrifugation at 27000 × *g* for 20 min. The pellet was resuspended in 5 ml Hepes-KCl buffer, stirred for 30 min, and recentrifuged. The clear supernatant solution was then diluted for the next step (14 ml).

(3) Acid fractionation: NADP was added to the

supernatant solution to a final concentration of 0.1 mM and the pH was adjusted to 5.0 with cold 1.7 M acetic acid. The precipitate was discarded after centrifugation (5 min at 27000 × *g*) and the supernatant fraction was immediately brought back to pH 7.4 with cold 0.5 M NH_4OH solution (14.2 ml).

The addition of NADP at this step protected the enzyme. It did not interfere in the further purification steps, but it did increase the overall recovery.

(4) Poly(ethylene glycol) fractionation: The above solution was diluted to a protein concentration of about 5 mg/ml with Hepes-KCl buffer, solid poly(ethylene glycol) 6000 (1 g/10 ml extract) was added, and the solution was stirred vigorously for 2–3 h and then centrifuged (27000 × *g*, 20 min). The clear supernatant solution (4 ml) was used for the next step.

(5) Sepharose 6B gel filtration: The above sample was applied to a Sepharose 6B column (2.5 × 40 cm) which had been equilibrated with Hepes-KCl buffer and 2-ml fractions were collected. The active enzyme fractions were pooled (31 ml).

(6) Second $(NH_4)_2SO_4$ fractionation: The pooled fractions from the Sepharose column were brought to 35% saturation by gradual addition of solid $(NH_4)_2SO_4$. The pH was held constant at 7.4 by addition of cold 0.5 M NH_4OH solution. The precipitate was collected after centrifugation at 27000 × *g* for 20 min and suspended in Hepes-KCl buffer.

The enzyme at this stage appeared to be electrophoretically homogeneous (see Fig. 1) and was used for further studies after dialysis against Hepes-KCl buffer or after gel filtration over Sephadex G-25. The enzyme could be frozen at −20°C for 3–4 days without any detectable loss of activity.

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis at pH 8.3 in the presence and absence of SDS was carried out by the method of Laemmli [13]. Electrophoresis in the absence of SDS, with duplicate protein samples, was run at 7°C on 10% acrylamide gels. After completion, the gel slab was cut into two strips. One piece was stained for protein in 0.1% Coomassie blue (in 7% acetic acid) and destained with 7% acetic acid in 10% ethanol and the other piece was stained for enzyme activity. The activity staining solution was 50 mM Tris-HCl buffer, pH 7.8, 50 μM NADP, 2.5 mM glucose 6-phosphate, which contained 0.2 mg phenazine methosulfate and 0.1 mg nitroblue tetrazolium (total volume, 10 ml). The gel slice was incubated in the solution for 30 min in the dark at room temperature and then fixed with 5% acetic acid. Gels were scanned with a Zeineh soft laser scanning densitometer, Biomed Instruments, Inc., Chicago. SDS gel electrophoresis was conducted at room temperature (about 22°C) on 7.5% acrylamide gels. The gels were stained for protein with Coomassie blue as described above. Standards used in subunit molecular mass determination were bovine serum albumin, pyruvic kinase, ovalbumin, trypsin and ribonuclease.

Analytical gel filtration. The Stokes radius of glucose-6-phosphate dehydrogenase was estimated by gel filtration through a Sephadex G-200 column (2.5 × 41 cm) equilibrated with Hepes-KCl buffer (containing 100 mM KCl). The column was calibrated with proteins of known Stokes radii, namely ribonuclease, ovalbumin, bovine serum albumin, lactic dehydrogenase, pyruvic kinase and urease. In each case 3–5 mg protein were applied to the column and the peak elution volume was noted. Void volume was obtained from the elution of thyroglobulin. Stokes radius and frictional coefficient were estimated according to the method of Siegel and Monty [14].

Light and dark modulation assay. Hepes-KCl buffer was used in all of the light modulation assays. The assay mixture contained washed thylakoid membranes (20–40 μg chlorophyll), crude stromal extract (0.1–0.2 mg protein) and appropriately diluted enzyme in a total volume of 0.2 ml. The mixture was illuminated ($59 \cdot 10^3$ lx; two General Electric 120-V, 150-W floodlamps) in a controlled temperature bath (25°C). At timed intervals aliquots were withdrawn and added directly to the test solution which was kept ready for this purpose. The increase in absorbance with time was recorded as usual.

For dark modulation experiments, the enzyme/thylakoid/crude stromal extract mixture (as above) was illuminated for the desired period of time, then the light was turned off. Aliquots were withdrawn at timed intervals and the activity estimation was made as usual.

Isolation of partially light-inactivated enzyme. 0.85 ml purified enzyme was incubated with 0.05 ml crude stromal extract (10–15 mg/ml) and 0.1 ml washed thylakoids (2–4 mg/ml chlorophyll) in the light ($59 \cdot 10^3$ lx; two General Electric 120-V, 150-W floodlamps) at 25°C. After partial inactivation the light was turned off and the mixture was diluted 5-fold with Hepes-KCl buffer. The thylakoids were separated by centrifugation at $27\,000 \times g$ for 25 min and the supernatant solution was used for comparative studies. The control sample was given similar treatment in the dark.

Isolation of enzyme partially inactivated by dithiothreitol. Purified enzyme in Hepes-KCl buffer was incubated with dithiothreitol in the cold until the residual activity was approx. 50%. The above mixture was immediately applied to a Sephadex G-25 column (15 × 1 cm) which had been equilibrated with Hepes-KCl buffer and eluted with the same buffer. The protein fractions were pooled (total volume 0.5 ml). The control was given similar treatment in the absence of dithiothreitol. The enzyme passed through the column in about 3 min. All activity applied to the columns was recovered.

Substrate specificity. Substrate specificity was studied in the usual assay system. Various sugar substrates (at 5 mM) were substituted for glucose 6-phosphate. NAD was tested in an assay system which contained 0.5 mM NAD instead of 0.05 mM NADP. in each case the reaction was started by the addition of 10 μl appropriately diluted enzyme.

Steady-state kinetics and product inhibition. NADP and glucose 6-phosphate solutions were prepared, and if necessary neutralized, within 6 h of use. Concentrations of the solutions of NADP and glucose 6-phosphate were estimated enzymatically using commercially prepared glucose-6-phosphate dehydrogenase from yeast.

The K_m value for each substrate was estimated by varying the substrate concentrations at high and constant concentration of the other substrate in the usual assay system. For estimation of the K_m for NADP and of the K_i for NADPH against NADP, the NADP levels were 2, 4, 6, 10 and 20 μM and glucose 6-phosphate was 3.2 mM. Glucose 6-phosphate concentrations used were 0.267, 0.444, 0.667, 1.60 and 3.20 mM and NADP was 34 μM for estimation of K_m or crossover points. The reaction was started by addition of 10 μl appropriately diluted enzyme. Product inhibition studies were carried out in the presence of NADPH. Each substrate was varied at constant concentration of the other substrate in the absence and presence of NADPH. The data were analyzed with the aid of a program for hyperbolic saturation [15] on an IBM 4341 Computer at the University of Illinois, Chicago, Computer Center. Mean K_m, K_i and crossover point values and standard error were estimated according to the method of Anderson and Fuller [16,17]. Mean K_m values are from six independent experiments. The mean value for K_i(NADPH) (varied substrate, NADP) is from three experiments. The mean value for the crossover point (glucose 6-phosphate varied substrate) is from two independent experiments.

Chemicals. Sepharose 6B, Sephadex G-200 and Sephadex G-25 were from Pharmacia, Piscataway, NJ. Ammonium sulfate (enzyme grade) was from Schwarz/Mann, Orangeburg, NY. Other biochemicals and poly(ethylene glycol) 6000 were purchased from Sigma, St. Louis, MO. Pea seeds were obtained from Northrup and King, Chicago, IL. Crude thioredoxin of undefined specificity was prepared in this laboratory by Dr. A.H. Mohamed according to Ref. 10.

Results and Discussion

Purification and characterization of enzyme

A summary of the purification procedure is given in Table I. The enzyme has been purified 280-fold with an overall recovery of about 8%. The most highly purified preparations give a single protein band on native gel electrophoresis, which also stains for glucose-6-phosphate dehydrogenase activity (Fig. 1). The specific activity of the purified enzyme (3–4 U/mg protein) is markedly lower than that of the enzyme purified from other eucaryotic sources [18–21].

The enzyme preparation contained a significant amount of bound nucleotide and thus there was a peak in the absorption spectrum at 260 nm, but no indication of other chromophores (data not shown). Removal of nucleotides by chromatography over DEAE-cellulose or through activated charcoal resulted in a drastic loss of activity and thus pre-

TABLE I

SUMMARY OF PURIFICATION OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE FROM PEA CHLOROPLASTS (FROM 8 kg OF PEA LEAVES)

Steps	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification (-fold)	Recovery (%)
(1) Crude extract	24.0	1900	0.013	–	–
(2) $(NH_4)_2SO_4$ (I)	22.4	354	0.063	4.9	93
(3) Acid fractionation	11.6	78.1	0.15	12	48
(4) Poly(ethylene glycol) 6000 fractionation	6.4	3.8	1.7	131	27
(5) Sepharose 6B gel filtration	3.1	1.3	2.4	185	13
(6) $(NH_4)_2SO_4$ (II)	1.8	0.5	3.6	277	8

paration of apoenzyme was impossible. Some other glucose-6-phosphate dehydrogenases behave similarly [5].

The Stokes radius of the enzyme, estimated by analytical gel filtration, is 52 Å (data not shown). Identical results were obtained in three different experiments. The subunit molecular mass estimated by SDS gel electrophoresis is 56000 Da. Values obtained from three experiments with three different preparations were 56000, 56000 and 58000 Da (data not shown). Apparently, the enzyme is a 224000 Da tetramer, most likely a homotetramer. The subunit molecular masses of microbial glucose-6-phosphate dehydrogenases generally lie between 50000 and 60000 Da and, of mammalian enzymes, between 58000 and 67000 Da [5]. The subunit molecular masses of only two plant enzymes have been reported. Both were purified from tobacco tissue culture cells [9]. One has a monomeric mass of about 88000 Da. The other has a subunit mass of approx. 56000 Da. The pea leaf enzyme, then, appears to be similar to most other glucose-6-phosphate dehydrogenases in subunit molecular mass. The frictional ratio (f/f_0) of the chloroplast enzyme, estimated from the Stokes radius and subunit molecular mass data, is 1.3. The frictional coefficient of the enzyme from other sources varies from 1.17 for the brewer's yeast enzyme to 1.48 for the bovine adrenal monomer [5].

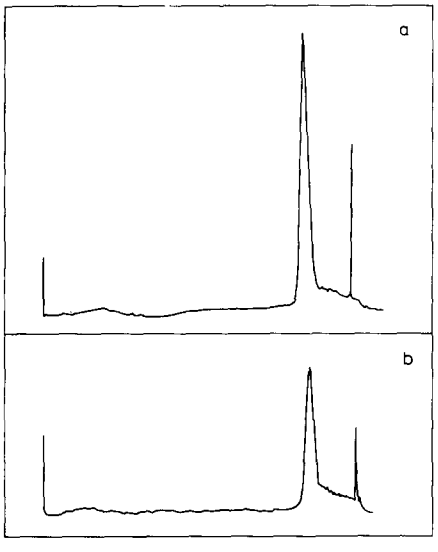

Fig. 1. Densitometric scans of purified glucose-6-phosphate dehydrogenase on native polyacrylamide gel electrophoresis. Protein samples (100 μg each) were subjected to electrophoresis. One slice was stained for protein with Coomassie blue and scanned at 670 nm (a), and the other piece was stained for enzyme activity and scanned at 405 nm (b).

Effect of pH

The pH optimum is at 8.2 (data not shown). The pH dependency is similar to that reported for the crude stromal enzyme [4], but the optimum is slightly lower, possibly because of the difference in buffer anions.

Substrate specificity

Substrate specificity was tested in the usual assay system as described in Materials and Methods. It is noteworthy that 0.5 mM NAD cannot replace 0.05 mM NADP (data not shown). All of the plant glucose-6-phosphate dehydrogenases studied so far have been shown to be absolutely NADP specific [6–9]. Some of the animal and microbial enzymes are specific for NADP and/or

NAD [5]. Likewise, 5 mM glucose, glucose 1-phosphate, glucosamine 6-phosphate and ribose 5-phosphate do not substitute for 2.5 mM glucose 6-phosphate (data not shown). However, 5 mM glucose 6-sulfate serves inefficiently (11% activity) as a substrate. It appears that changes in the substituent on C-6 of the glucose moiety can be tolerated by the pea chloroplast enzyme. Changes at C-1 or C-2 or a decrease in the size of the ring are not possible. Glucose 1-phosphate does not serve as a substrate for the enzyme from any source, but all of the other compounds which were examined do serve as substrates for some glucose-6-phosphate dehydrogenases [5]. This is the first report on sugar substrate specificity for a plant glucose-6-phosphate dehydrogenase.

Steady-state kinetics and product inhibition

The substrate K_m values for the pea leaf chloroplast enzyme are listed in Table II. Reported K_m(NADP) values for the enzyme from other sources vary from a low value of 1.7 μM for the enzyme from rat adipose tissue to 360 μM for the *Pseudomonas fluorescens* enzyme [5]. Similarly, the K_m values for glucose 6-phosphate vary from a low value of 12 μM for the human platelet enzyme to a high value of 5 mM for the enzyme from *Pseudomonas multivorans* and the housefly [5]. Among the plant enzymes the K_m value for NADP varies from 14 μM (black gram enzyme) [7] to 60 μM (tobacco tissue culture cell enzyme) [9]. The reported K_m values for glucose 6-phosphate range from 18 μM for the enzyme from hazel cotyledons [22] to 220 μM for the tobacco tissue culture cells enzyme [9]. The enzyme from sweet potato [6] and black gram [7] shows sigmoidal dependence on glucose 6-phosphate concentration. The pea chloroplast enzyme has the lowest K_m for NADP reported for a plant glucose-6-phosphate dehydrogenase. However, the K_m value for glucose 6-phosphate is in the range of that of the other plant enzymes.

NADPH is a competitive inhibitor with respect to NADP and gives mixed-type inhibition ($K_p > K_m$, $V_{max} > V_p$) with respect to glucose-6-phosphate (Table II). The K_i value obtained when NADP is the varied substrate, 18 $\pm$ 5 μM, is lower than that reported by Lendzian [23] for the spinach stromal enzyme, 70 μM, and higher than that reported by Muto and Uritani [24] for the sweet potato enzyme, 10 μM. The level of NADPH in the spinach chloroplast is about 500 μM [25]. Probably a considerable amount of the nucleotide is protein bound in vivo. If this is so, then control of the activity of the enzyme by reduction charge [26–28] seems likely. K_i(NADPH) values reported for the enzyme from other sources range from 0.07 μM (*Saccharomyces carlsbergensis*) to 150 μM (rat adipose tissue) [5].

Effect of dithiothreitol

Dithiothreitol inactivated the enzyme. The rate of inactivation was a function of dithiothreitol concentration (Fig. 2) and was increased in the presence of crude thioredoxin (data not shown).

TABLE II

K_m AND K_i VALUES OF PEA CHLOROPLAST GLUCOSE-6-PHOSPHATE DEHYDROGENASE

K_m(NADP)	2.4 $\pm$0.1 μM
K_m(glucose 6-phosphate)	0.37$\pm$0.01 mM
K_i(NADPH) (NADP varied)	18 $\pm$5 μM
Mixed Inhibition (NADPH) Crossover point (glucose 6-phosphate varied) ($K_p > K_m$, $V_{max} > V_p$)	0.5 $\pm$0.1 mM

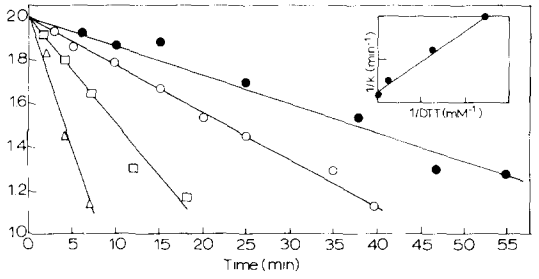

Fig. 2. Inactivation of glucose-6-phosphate dehydrogenase in the presence of dithiothreitol (DTT) in Hepes-KCl buffer at 25°C. The diluted enzyme (144 μg; spec. act. 3.4 U/mg protein) was incubated with different concentrations of dithiothreitol in a total volume of 0.2 ml. At different time intervals 10-μl aliquots were withdrawn and tested for enzyme activity. Dithiothreitol concentration was 50 (△), 5 (□), 1 (○), and 0.5 (●) mM. Inset shows double-reciprocal plot of rate constants for inactivation. Similar results were obtained in three different experiments. Under the same conditions less than 1% of activity is lost even after 8 h of incubation in the absence of dithiothreitol. Ordinate: log% residual activity.

The disappearance of enzyme activity with time in the presence of dithiothreitol followed pseudo-first-order kinetics and showed rate saturation (inset in Fig. 2). $K_{inactivation}$ is about 2.5 mM. Apparently, inactivation involves an initial reversible formation of an enzyme-dithiothreitol complex prior to covalent modification.

In contrast to the pea chloroplast enzyme, glucose-6-phosphate dehydrogenase from human erythrocytes is not sensitive to dithiothreitol [5]. It is interesting to note that the activity of the erythrocyte enzyme is scarcely affected by air oxidation even though 70% of the sulfhydryl groups of the enzyme are oxidized.

Thioredoxin is known to enhance the dithiothreitol-dependent inactivation of glucose-6-phosphate dehydrogenase in a partially purified system [10]. Thioredoxin also enhances the inactivation of native purified enzyme in the presence of dithiothreitol.

Light and dark modulation

Incubation of the purified native enzyme with crude stromal extract and washed thylakoids in the light resulted in a rapid decrease in enzyme activity (Fig. 3). If the same system, after illumination, was kept in the dark prior to estimation of activity, there was a time-dependent recovery of enzyme activity (dark modulation) (Fig. 3). The extent of dark reversal of light inactivation was essentially quantitative.

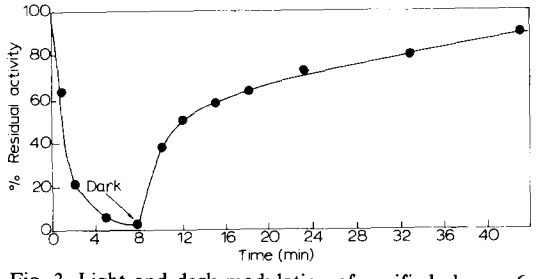

Fig. 3. Light and dark modulation of purified glucose-6-phosphate dehydrogenase (9.0 μg; spec. act. 3.2 U/mg protein) in the presence of crude stromal extract (0.2 mg protein) and washed thylakoids (30 μg chlorophyll) (total volume, 0.2 ml). At different time intervals 10-μl samples were withdrawn and added to the assay mixture for activity estimation. At this dilution, enzyme activity carried over from the stromal extract was not detectable, hence the observed light inactivation represents inactivation of the purified enzyme. Similar results were obtained in four different experiments.

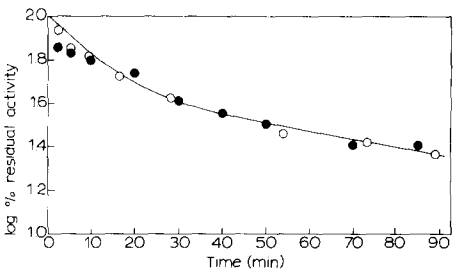

Fig. 4. Thermal inactivation of partially dithiothreitol-inactivated (●) and untreated control (○) pea chloroplast glucose-6-phosphate dehydrogenase at 43°C. The enzyme (0.22 mg; spec. act. 3.1 U/mg protein) was incubated in 20 mM dithiothreitol (final volume, 1.0 ml) for 15 min at 0°C (residual activity 46%) and then passed through a Sephadex G-25 column. Control was given similar treatment in the absence of dithiothreitol. Samples (0.5 ml) of the gel filtered enzyme were maintained at 43°C in a constant temperature bath. At different time intervals 20-μl aliquots were withdrawn and tested for enzyme activity. Similar results were obtained in three different experiments.

The enzyme which had been partially dithiothreitol inactivated was not reactivated when incubated with crude stromal extract or crude thioredoxin in the dark after removal of the reductant by gel filtration (Table III, rows 3 and 4). However, the enzyme which had been partially inactivated with dithiothreitol was further in-

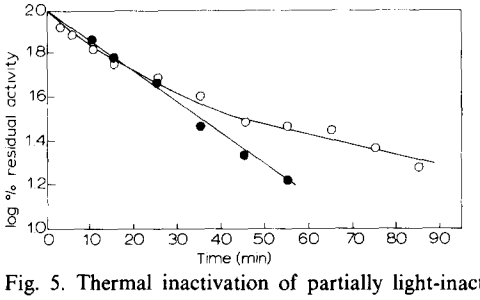

Fig. 5. Thermal inactivation of partially light-inactivated (●) and dark control (○) pea chloroplast glucose-6-phosphate dehydrogenase at 43°C. Enzyme (0.85 ml, 0.32 mg; spec. act. 3.5 U/mg protein) was incubated with crude stromal extract (0.05 ml, 0.58 mg protein) and washed thylakoids (0.1 ml, 0.17 mg chlorophyll) in the light at 25°C. After 15 min (residual activity 52%) the above mixture was diluted 5-fold with cold Hepes-KCl buffer and centrifuged. The control sample was given similar treatment in the dark. Aliquots (0.5 ml) of the supernatant solution from this treatment were maintained at 43°C in a constant temperature bath. At different time intervals small aliquots (0.02 ml in the case of control and 0.05 ml in the case of light-treated enzyme) were withdrawn and tested for enzyme activity. Similar results were obtained in three different experiments.

TABLE III

LIGHT AND DARK MODULATION OF UNTREATED AND OF PARTIALLY INACTIVATED (DITHIOTHREITOL-TREATED) GLUCOSE-6-PHOSPHATE DEHYDROGENASE FROM PEA CHLOROPLASTS

Enzyme, with the addition of stroma, thylakoids, and/or thioredoxin where indicated, was incubated in the dark and then, where noted, was exposed to light, and/or reincubated in the dark for additional time. The sequence was dark (20 min), or light (10 min), dark (25 min) in Expt. I and dark (10 min), or light (10 min), dark (30 min) in Expt. II. For Expt. I the treated enzyme (1.0 ml, 96 μg) was incubated in 1.8 mM dithiothreitol (total volume, 1.1 ml) for 15 min after which the dithiothreitol was removed by gel filtration. For Expt. II the treated enzyme was 150 μg protein in 1.0 ml for dithiothreitol treatment. Controls were also subjected to gel filtration. In Expt. I, runs 1 and 2, 0.1 ml untreated or treated (partially dithiothreitol-inactivated) enzyme was incubated with 10 μl stroma (0.145 mg protein) and 10 μl thylakoids (0.035 mg chlorophyll). At the times indicated 10-μl samples were withdrawn in the light and dark and activity was estimated. In runs 3 and 4, 0.1 ml treated enzyme was incubated with 10 μl stroma (0.145 mg protein) or 0.05 ml thioredoxin (2.72 μg protein) in the dark. At times indicated 10-μl samples were withdrawn and tested for enzyme activity. In Expt. II, runs 1 and 2, the stromal protein added was 0.12 mg and thylakoid chlorophyll was 0.012 mg. In run 3 stromal protein was 0.67 mg (which accounts for the extra activity). In run 4 thioredoxin was 2.72 μg. Activity is expressed as total enzyme units in the incubation mixture.

	Activity			
	Dark (0 min)	Dark (20 min)	Light (10 min)	Dark (25 min)
Expt. I				
(1) Untreated enzyme + stroma + thylakoids	0.102	0.102	0	0.102
(2) Treated enzyme + stroma + thylakoids	0.048	0.048	0	0.048
(3) Treated enzyme + stroma	0.055	0.055		
(4) Treated enzyme + thioredoxin	0.042	0.042		
Expt. II	(0 min)	(10 min)	(10 min)	(30 min)
(1) Untreated enzyme + stroma + thylakoids	0.144	0.144	0	0.123
(2) Treated enzyme + stroma + thylakoids	0.050	0.050	0	0.050
(3) Treated enzyme + stroma	0.153	0.153		
(4) Treated enzyme + thioredoxin	0.062	0.048		

activated in the light, and could be quantitatively reactivated to its partially inactivated state in the dark modulation system (Table III, row 2).

Thermal inactivation

Incubation of purified enzyme at elevated temperature results in a time-dependent loss of activity (Figs. 5 and 6). Thermal inactivation progresses in two distinct phases, a fast followed by a slow phase. The time-dependent thermal inactivation of the partially dithiothreitol-inactivated enzyme at 43°C followed a pattern similar to that of the untreated control (Fig. 4). But the thermal inactivation of the enzyme which had been partially light inactivated showed a single-phase first-order kinetic pattern (Fig. 5). Apparently, light inactiva-

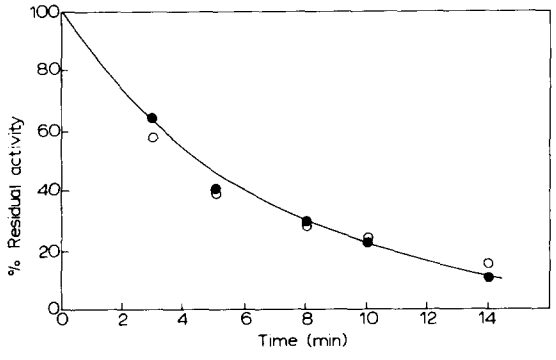

Fig. 6. Proteolytic inactivation of partially dithiothreitol-inactivated (●) and untreated control (○) pea chloroplast glucose-6-phosphate dehydrogenase at 25°C. Dithiothreitol-inactivated and untreated control were prepared as described in Fig. 4. Initial specific activity of the enzyme was 3.1 U/mg protein. The gel filtered enzyme (0.48 ml) was incubated with trypsin (0.02 ml, 20 μg) at 25°C. At different time intervals small aliquots (0.02 ml in the case of control and 0.05 ml in the case of treated enzyme) were withdrawn and tested for enzyme activity in an assay system containing 1 mg/ml soybean trypsin inhibitor. Similar results were obtained in two different experiments.

tion of glucose-6-phosphate dehydrogenase converts the enzyme into an alternative conformation which has a thermal stability different from that of the dark form. Dithiothreitol treatment does not bring about such a change in conformation.

Proteolytic inactivation

Light modulation has been shown to cause a change in proteolytic susceptibility of the enzyme in crude extracts [29]. The time-dependent loss of purified enzyme activity in the presence of trypsin is shown in Fig. 6. The inactivation pattern of partially dithiothreitol-inactivated enzyme in the presence of trypsin is similar to that of the untreated control under similar conditions. Trypsin inactivation of partially light-inactivated and dark control glucose-6-phosphate dehydrogenase is shown in Fig. 7. Note that the light-inactivated enzyme is more stable against proteolysis than is the enzyme in the dark control, unlike the dithiothreitol-inactivated enzyme. In contrast to these results, Schürmann and Wolosiuk [30] and Pradel et al. [31] have found that dithiothreitol treatment apparently causes a change in conformation of spinach chloroplastic fructose-bisphosphatase.

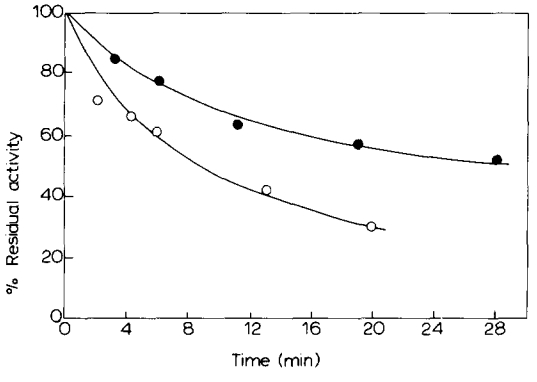

Fig. 7. Proteolytic inactivation of partially light-inactivated (●) and dark control (○) pea chloroplast glucose-6-phosphate dehydrogenase at 25°C. Light-inactivated and dark control enzymes were prepared as described in the legend to Fig. 5. Initial specific activity of the enzyme was 3.5 U/mg protein. Samples (0.48 ml) were incubated with trypsin (0.02 ml, 20 μg). At different time intervals small aliquots (0.02 ml in the case of control and 0.05 ml in the case of light-inactivated enzymes) were withdrawn and tested for enzyme activity in an assay system containing 1 mg/ml soybean trypsin inhibitor. Similar results were obtained in two different experiments. In related experiments with chymotrypsin no differences in the rate of inactivation of the light and dark forms of the enzyme were found (data not shown).

Concluding Remarks

We describe here the purification and characterization of a light- and dithiothreitol-modulatable glucose-6-phosphate dehydrogenase from pea chloroplasts. The isolation of a homogeneous preparation of this enzyme was reported earlier from this laboratory [32] but that preparation was not light modulatable, although it was inactivated in the presence of dithiothreitol. The unusual behavior of the enzyme probably resulted from modification of the enzyme during the course of purification. The present method allows the isolation of native enzyme which is both light and dithiothreitol modulatable.

Peak chloroplast glucose-6-phosphate dehydrogenase has a Stokes radius of 52 Å. Since the subunit molecular mass is about 56000 Da, the enzyme is probably a 224000 Da homotetramer.

The chloroplast enzyme is absolutely specific for NADP. No enzyme activity could be detected when NAD was substituted for NADP. There is a little activity when glucose 6-phosphate is replaced by glucose 6-sulfate in the assay medium. Other sugar analogues cannot serve as substrate. It appears that even a minor change in substrate structure is rarely tolerated by pea chloroplast glucose-6-phosphate dehydrogenase. Not all glucose-6-phosphate dehydrogenases show such specificity [5].

The chloroplast enzyme is not unusual in its kinetic constants, sensitivity to NADPH, or pH optimum.

These observations suggest that although pea chloroplast glucose-6-phosphate dehydrogenase differs from mammalian and microbial glucose-6-phosphate dehydrogenases with respect to its unique sensitivity to light and dithiothreitol modulation, the other physicochemical properties of the enzyme are similar to those reported for most glucose-6-phosphate dehydrogenases.

Light inactivation of purified glucose-6-phosphate dehydrogenase is presumably mediated through the Light Effect Mediator (LEM) and Protein Modulase components of the thylakoid membrane and stroma, respectively [1]. Dark re-

activation of the light-inactivated enzyme has been shown to be mediated by stromal thioredoxin [1]. Enzyme which has been partially inactivated by dithiothreitol treatment is further inactivated, and reactivated, in the light/dark modulation systems as is native enzyme. However, the recovery of enzyme activity after dark modulation is quantitative only with respect to the activity remaining after dithiothreitol inactivation, not to the original activity before dithiothreitol treatment. These results show that the inactivation caused by dithiothreitol treatment is not reversed by dark modulation. Further, there is no reversal of dithiothreitol inactivation by crude stromal extract or by partially purified oxidized thioredoxin.

The dithiothreitol-dependent inactivation follows pseudo-first-order kinetics and shows rate saturation with respect to dithiothreitol concentration. The enhanced rate of inactivation in the presence of crude thioredoxin suggests that thioredoxin functions as a catalyst to facilitate reduction of disulfide groups on the native enzyme molecule.

Thermal inactivation of purified glucose-6-phosphate dehydrogenase shows biphasic kinetics. Half of the enzyme activity is destroyed more rapidly than the remaining half. It may be that there are equimolar quantities of two forms of the enzyme, possibly light and dark forms, having similar physical properties. Alternatively, the inactivation pattern could be due to structural arrangements of subunits in the tetramer as has been proposed for the plant glyceraldehyde-3-phosphate dehydrogenases [33,34].

The thermal inactivation pattern of the partially dithiothreitol-inactivated enzyme is similar to that of the untreated control. Moreover, there is no difference in proteolytic inactivation of partially dithiothreitol-inactivated and untreated control enzyme in the presence of trypsin. The similarity in the inactivation patterns for thermal denaturation and for proteolytic degradation suggests that the enzyme wich has been partially inactivated by dithiothreitol contains a mixture of fully active and less active enzyme species. Thermal inactivation of partially light-inactivated glucose-6-phosphate dehydrogenase follows monophasic first-order kinetics while in the dark control the kinetics of thermal inactivation are biphasic. The change in kinetic pattern from biphasic to monophasic first order due to partial light inactivation suggests the transformation of one form of the enzyme to another form. Apparently, the two forms have different thermal stabilities and kinetic patterns. Further, there is a difference in the proteolytic susceptibility of the partially light-inactivated and dark forms. Since the partially light-inactivated enzyme used in these experiments can be further inactivated, it seems likely that there are at least three distinct forms of the enzyme, i.e., dark active. light-partially active and light inactive.

These observations suggest that the dithiothreitol-dependent modification of glucose-6-phosphate dehydrogenase is not necessarily the same as that mediated by light.

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