The Regulation of Glucose-6-phosphate Dehydrogenase in Chloroplasts

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Glucose-6-phosphate dehydrogenase from intact pea chloroplasts is partially membrane bound and inactivated upon illumination. The inhibitory effect of light can be abolished by addition of methylviologen. Kinetic experiments with glucose-6-phosphate dehydrogenase reveal that, in the dark, the enzyme activity is strongly inhibited by the accumulation of NADPH. The inhibition of NADPH can be reversed by the addition of excess $NADP^+$. The non-Michaelis-Menten-type kinetics suggest that the enzyme is stringently regulated by the ratio of NADPH to $NADP^+$ plus NADPH, *i. e.*, the "reduction charge". These observations seem to indicate that in the light the inhibition of glucose-6-phosphate dehydrogenase is due to a high reduction charge, whereas in the dark the enzyme is controlled by the metabolic demand for reducing equivalents.

Introduction

Chloroplasts contain not only enzymes which catalyse the reactions of the Calvin cycle but also enzymes which are involved in the oxidative pentosephosphate cycle [1]. Hexosemonophosphates are used for the maintenance of the Calvin cycle, for starch synthesis, and for oxidation to 6-phosphogluconate, yielding NADPH and subsequently ribulose-5-phosphate plus carbon dioxide. It seems necessary that a stringent regulation of these reactions is maintained to control the carboxylation and decarboxylation reactions. The regulation of chloroplast key enzymes is exerted by light [2–7], the Mg^{2+} concentration, the pH shift in the stroma due to proton translocation [8, 9], and by the energy charge [10]. The glucose-6-phosphate dehydrogenase is also activated by Mg^{2+} and has a pH optimum similar to the Calvin cycle enzymes. In contrast to these enzymes it has been observed that the dehydrogenase is deactivated in light [11–13], suggesting that the oxidative pentosephosphate cycle is operating in the dark.

This investigation was undertaken to study the mechanism of the light inactivation of glucose-6-phosphate dehydrogenase.

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Abbreviations: G6P-glucose-6-phosphate; G6P-DH, glucose-6-phosphate dehydrogenase; MV, methylviologen.

Materials and Methods

Pea plants (*Pisum sativum*, var. Feltham first, Suttons Seeds, Reading, Engl.) were grown in vermiculite for 14 days with 12 hours light daily in the green house. The plants were kept for 14 hours in the dark before use. Chloroplasts were isolated from the plant material according to the method of Walker [14, 15] with the exceptions that ascorbate was omitted from the grinding medium, no Mn^{2+} was added to the resuspending medium and the Mg^{2+} concentration was 10 mM. After isolation, the chloroplasts were stored in ice and kept in darkness.

The G6P-DH assay was carried out in an Aminco spectrophotometer DW 2 UV/Vis operating in dual wavelenght mode with the measuring beam at the wavelenght of 334 nm and the reference beam at 450 nm. The reaction mixture contained: 50 mM Tricine-NaOH buffer pH 8.0, 10 mM $MgCl_2$, 1 mM G6P and 0.2 mM $NADP^+$, and chloroplasts (equivalent to 0.1 mg chlorophyll) in a final volume of 3 ml. Methylviologen was added as indicated at a concentration of 0.1 mM. The reaction was carried out at 23 °C. The light treatment of osmotically shocked chloroplasts was performed in a thermostated cuvette exposed to 50 000 lx white light.

The concentration of $NADP^+$ solution was estimated with purified G6P-DH (purified from yeast, Boehringer) as described above. Chlorophyll was measured according to Arnon [16].

Results

Intact chloroplasts were shocked in the assay medium immediately preceding the measurement of glucose-6-phosphate dehydrogenase. Table I shows

Table I. The influence of the substrates and light on the activity of glucose-6-phosphate dehydrogenase.

Additions	Initial rates	
	dark	light
—	100 *	65
G6P	98	74
$NADP^+$	102	30
G6P plus $NADP^+$	34	27

* The dark control is arbitrarly set to 100% and equal to a rate of 36 μmol NADPH formed·mg^{-1} chlorophyll·hour.

the rate of $NADP^+$ reduction as a function of the treatment of the chloroplasts prior to the assay. Samples were kept either in light or in darkness for 30 min. The dark control had an initial rate of 36 μmol·mg^{-1} chlorophyll·hour. Exposure of chloroplasts to 30 min of light, without the addition of substrates, caused a loss of one third of the original activity. The presence of G6P, during the light treatment, resulted in a minor deactivation of the enzyme activity, whereas the presence of $NADP^+$ alone showed a severe deactivation of the enzyme. In the presence of both $NADP^+$ and G6P, the rate was further decreased to 27% of the dark control. Neither G6P nor $NADP^+$, when present alone during the dark incubation, had any effect on the enzyme activity, however, the presence of both substrates together caused a 66% inactivation.

The influence of methylviologen, a mediator of pseudocyclic electron transport, on the glucose-6-phosphate dehydrogenase activity was studied. Table II summarizes the experiments which were

Table II. The influence of methylviologen in light on the activity of glucose-6-phosphate dehydrogenase.

Additions	Initial rates	
	without MV	with MV
—	65	103
G6P	74	100 *
$NADP^+$	30	100
G6P plus $NADP^+$	27	55

* 100% is equal to a rate of 36 μmol NADPH formed·mg^{-1} chlorophyll·hour.

carried out in light plus and minus methylviologen. In presence of 0.1 mM methylviologen, no deactivation of glucose-6-phosphate dehydrogenase occured either with G6P or with $NADP^+$. After the addition of both substrates and methylviologen to the chloroplast suspension the enzyme was allowed to react for 30 min in light. Afterwards the sample was removed to the dark, more $NADP^+$ added (G6P was in excess) and the initial reaction rate measured. In comparison to the dark control, a moderate loss of activity was observed but significantly less than the sample without methylviologen. Repetition of the experiments with methylviologen in presence of superoxide dismutase and/or catalase revealed that the addition of these enzymes had no influence on the "protective effect" of methylviologen. These data suggested that the superoxide radical was not involved in the above reaction (*cf.* ref. 17, 18). In agreement with the methylviologen experiments was the finding that the xanthin and xanthin oxidase could not reactivate the light caused loss of activity of glucose-6-phosphate dehydrogenase in the dark (data not shown).

Since the enzyme was deactivated in the dark as much as in the light by NADPH, the product of the reaction (see Table I), detailed studies of the kinetic of the enzyme reaction were carried out. The resuspended chloroplasts contained two forms of glucose-6-phosphate dehydrogenase, one which was membrane-bound (60 to 85% of the total activity) and one which appeared in the supernatant after shocking the chloroplasts and subsequent centrifugation (40 000 × *g*, 10 min).

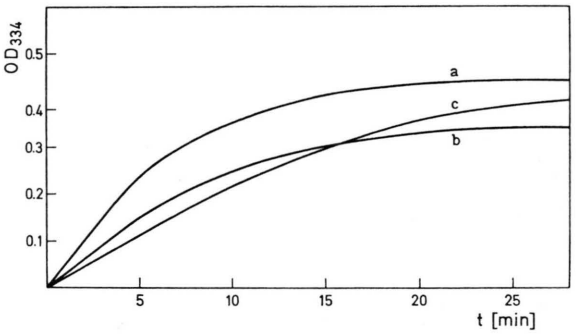

Fig. 1. The kinetics of the G6P-DH reactions with the shocked chloroplasts (trace a), the resuspended chloroplast lamellae (trace b) and the supernatant sample (trace c). The assay mixture is described in the Methods, except that the $NADP^+$ concentration was reduced to 0.075 mM. The trace were recorded at full scale (ΔOD_{334} of 0.5 was equal to 25 cm), the recording speed was 100 sec/inch.

Fig. 1 shows the kinetics of glucose-6-phosphate dehydrogenase measured immediately after the disruption of the chloroplasts by osmotic shock (trace a), of the resuspended lamellar membranes (trace b) and, of the supernatant (trace c). The reaction was recorded until termination of the reaction occured. After the addition of "external" glu-

cose-6-phosphate dehydrogenase (7 units) there was a further increase of $NADP^+$-reduction in the assay sample with the lamellar membranes. The same result was obtained by comparing the amount of NADPH produced with the added substrate concentration (20% of $NADP^+$ was not consumed during the test reaction). The horizontal line of the recorded traces showed that the NADPH was not reoxidized. The addition of 900 nmol of $NADP^+$ to the reaction mixture restored the activity of the enzyme and the same rate was observed as initially. The reaction rates were calculated from the different points of the curves and the concentration of NADPH estimated. Plotting *v*, the reaction velocity, *versus* the ratio of NADPH to $NADP^+$ plus NADPH, the reduction charge, reveals the dependence of the reaction rate on the $NADP^+$ concentration (Fig. 2). Curve a represents the assay with

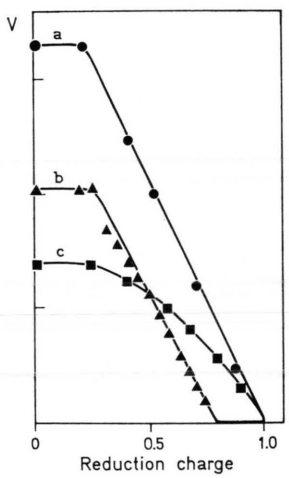

Fig. 2. The influence of the reduction charge on the reaction rate. The abscissa shows the reduction charge ranging from 0 to 1.0, the ordinate the reaction rate *v*, which were determined from the kinetic data of the experiments shown in Fig. 1.

whole chloroplasts shocked in the assay medium. Curve b, measured with the membrane-bound enzyme, showed non-Michaelis-Menten type kinetics as recognized from the sigmoidal shape of the curve. The reaction velocity was constant at reduction charge values from 0 to 0.25 whereas at lower values a sharp decline occured. At above 0.8 the reaction is completly inhibited. Trace c showed normal kinetics for the soluble enzyme.

All kinetic experiments contained Mg^{2+} in the assay medium. The omission of this ion drastically changes the activity of the glucose-6-phosphate dehydrogenase. The membrane-bound form was to 80%(±5%) inactivated whereas the activity of the soluble form was only reduced to 66%(±5%). Fig. 3 shows the influence of Mg^{2+} on the enzyme

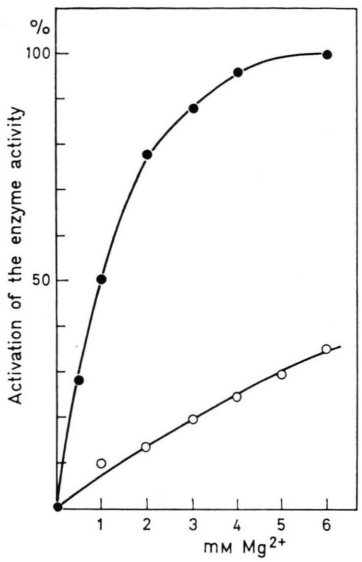

Fig. 3. The activation of G6P-DH by Mg^{2+}. The abscissa shows the Mg^{2+} concentration in the assay mixture, the ordinate the percentage of activation. The closed symbols represent the activation of the membrane-bound enzyme and the open symbols the activation of the soluble enzyme. The chloroplasts were shocked in Tricine-buffer without Mg^{2+} and the thylakoid lamellae were collected by centrifugation (40 000 × *g*, 10 min). The residue was resuspended in an equal amount of buffer and Mg^{2+} as added as indicated. The assay was carried out as described in the Methods and the initial reaction rates were determined.

activity. The chloroplasts were suspended in Tricine buffer with increasing amount of Mg^{2+} and the initial reaction rates were estimated. The ordinate shows the percentage of activation. The data were also used for the determination of the K_a values (Fig. 4). The K_a values were calculated from the Lineweaver-Burk plots and estimated as 1.2 mM Mg^{2+} for the activation of the membrane-bound enzyme and 16.7 mM for the soluble form.

The membrane-bound enzyme could be liberated by mild sonication. After 60 sec, 80% of the original activity of the membranes could be dedected in the supernatant.

Discussion

From observations made by different groups[4-6,9,13], it is known that sulfhydryl reducing agents have an effect on the activity of many chloroplast enzymes.

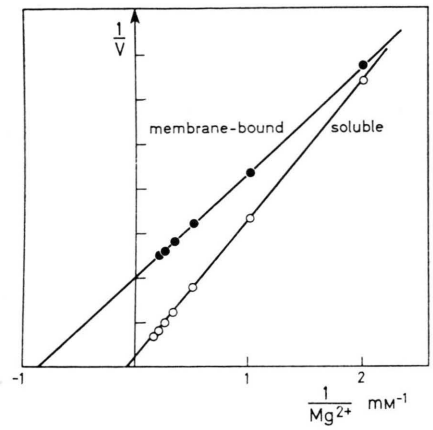

Fig. 4. The Lineweaver-Burk plots for the activation of G6P-DH by Mg^{2+}. The reciprocal values for the reaction rate v and the corresponding Mg^{2+} concentrations were determined from the data of the experiments shown in Fig. 3.

The results of Anderson [13] indicate that dithiothreitol inactivates the glucose-6-phosphate dehydrogenase. These observations might suggest that disulfide bridges are important for the function of this enzyme.

The inactivation of glucose-6-phosphate dehydrogenase by the action of light was investigated and and attempt was made to reactivate the enzyme by using reduced oxygen species [27], produced by illuminated chloroplasts in presence of methylviologen [17, 18] as oxidants. Indeed, the activity of glucose-6-phosphate dehydrogenase was preserved under these conditions (see Table II). The effect of methylviologen can be explained by the assumption the the reduced oxygen species had a protective influence on the enzyme molecules. Another explanation can be offered regarding the inhibition of electron flow to $NADP^+$ by methylviologen, and the assumption that NADPH is a negative allosteric effector. The first explanation can be ruled out by the results of the control experiments with superoxide dismutase and catalase: the protective effect of methylviologen was unaltered. Further kinetic studies verified the second explanation. Glucose-6-phosphate dehydrogenase activity is regulated by the level of NADPH. In illuminated chloroplasts the pool of $NADP^+$ is converted to NADPH and the enzyme activity would be shut off. In analogy to the term "energy charge", introduced by Atkinson [20] as a regulatory control for cell metabolism, the term "reduction charge", the ratio of NADPH to NADPH plus $NADP^+$ can be used for this control mechanism. Experiments by Heber [21, 22] have shown that, in the dark, chloroplast $NADP^+$ is only reduced from 5 to 15%. Upon illumination the percentage increases to more than 80%. If glucose-6-phosphate dehydrogenase is controlled by this ratio, it would be markedly inhibited in the light and, if the metabolic demand for reduction equivalents is low, it might also be inhibited in the dark. The regulation discussed above is limited to the membrane-bound enzyme. Mild sonication of the chloroplast lamellae causes liberation of the enzyme with concomitant changes of the regulatory functions. The differences between these two entities are also expressed by their different activation by Mg^{2+} ions.

Krause and Bassham [19] have already speculated about the role of NADPH as inhibitor of glucose-6-phosphate dehydrogenase and they could show that the addition of vitamin K_5 to *Chlorella* cells or isolated chloroplasts caused an immediate rise of 6-phosphogluconate.

The regulatory role of NADPH on glucose-6-phosphate dehydrogenase of erythrocytes, as studied by Luzzatto [23] and Bonsignore *et al.* [24], is in agreement with the results presented in this paper. Aside from the competition between $NADP^+$ and NADPH for an identical catalytic binding site, NADPH also acts as a heterotrophic allosteric inhibitor, bound to "a structural site" on the enzyme. Similar observations were reported by Blackkolb and Schlegel [25] for the glucose-6-phosphate dehydrogenase isolated from the chemiautotrophic bacterium *Hydrogenomonas* H 16. In this system, NADPH was also shown to be a competitive inhibitor and the sigmoidicity of the substrate saturation curve indicated the allosteric properties of the enzyme. This inhibitory effect of NADPH on the enzyme, isolated from blue green algae, was recently studied by Grossman and McGowan [28].

Chu and Bassham [26] reported that ribulosebisphosphate carboxylase was stimulated in presence of NADPH. This observation and our results with glucose-6-phosphate dehydrogenase might lead to a general view that, besides the change of Mg^{2+} concentration and pH-shift, the reduction charge has a main function as a metabolic control system in intact chloroplasts.

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Note added in proof: In support of our results, Lendzian and Bassham (Biochim. Biophys. Acta **396,** 260 – 275 [1975]) recently published similar results on the glucose-6-phosphate dehydrogenase of spinach chloroplasts and proposed a regulatory function of the ratio NADPH/$NADP^+$ on the enzyme activity in presence of ribulose-1,5-bisphosphate (RuDP).

In contrast to our results, which showed the influence of the reduction charge in the absence of RuDP on the membrane bound enzyme, these authors used a soluble chloroplast fraction in which they also observed a dependence of the inhibition on the RuDP level.

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