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ON THE MECHANISM OF THE LIGHT-INDUCED ACTIVATION OF THE NADP-DEPENDENT GLYCERALDEHYDE PHOSPHATE DEHYDROGENASE*

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SUMMARY

1. The effects of some metabolites on the activity of the NADP- and NAD-dependent glyceraldehyde phosphate dehydrogenase from spinach chloroplasts have been studied.

2. $NADP^+$ enhanced the activity of the NADP-dependent dehydrogenase while the NAD-dependent form was slightly inhibited. $NADP^+$ could not increase the activity of the NADP-dependent enzyme in addition to the activation by NADPH. The NAD-dependent dehydrogenase was not influenced by NAD^+ or NADH.

3. ATP was similarly effective in activating the NADP-dependent dehydrogenase as was found earlier for NADPH. The activation as a function of ATP concentration is described by a sigmoid curve indicating a cooperative mechanism. A combination of ATP and NADPH activated the NADP-dependent enzyme no more than did ATP or NADPH alone. The NAD-dependent form was slightly activated by ATP. ADP, phosphoglyceric acid, and glyceraldehyde phosphate did not modify the activity. AMP inhibited the NADP-dependent enzyme.

4. 50 mM $MgCl_2$ activated the NADP-dependent enzyme while higher concentrations had an inhibitory effect. The activation was about 1/10 that of NADPH. The activations by 50 mM $MgCl_2$ and NADPH were additive.

5. The results give further evidence for a cooperative mechanism of the activation. The known activation of the NADP-dependent enzyme *in vivo*, being closely connected with the light reactions of photosynthesis, is explained by an allosteric activation with NADPH and/or ATP as effectors. This is discussed in respect to the regulation of the reductive pentose phosphate cycle.

INTRODUCTION

There are two different mechanisms for the light-induced activation of the NADP-dependent glyceraldehyde phosphate dehydrogenase (D-glyceraldehyde-3-phosphate:NADP oxidoreductase (phosphorylating), EC 1.2.1.13): (1) an increase in the activity mediated directly or indirectly by phytochrome in etiolated plants[1-4]

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and (2) an activation in green leaves[5,6] which is reversible and dependent on the light reaction of photosynthesis[7]. This paper deals only with the latter process.

In a former paper[8] we have presented some evidence that the enzyme is activated by a cooperative mechanism with NADPH as effector. Until now a separation of the NADP- and the NAD-dependent glyceraldehyde phosphate dehydrogenase (D-glyceraldehyde-3-phosphate:NAD oxidoreductase (phosphorylating), EC 1.2.1.12) both being present in chloroplasts[9,10] did not succeed[11]. For this reason and on the basis of their own studies MELANDRI *et al.*[12] proposed the existence of only one glyceraldehyde phosphate dehydrogenase for higher plants which is active with both NADP and NAD.

Recently we have described the effect of NADPH on the NAD-dependent dehydrogenase[8]. Consequently we extended our work to the effects of other intermediates of photosynthesis on the activity of this enzyme. We also investigated the possibilities for a competitive mechanism according to MELANDRI *et al.*[12]. Our results present further evidence for a cooperative activation.

METHODS

Isolation of chloroplasts. Spinach was harvested from the field and stored in the dark at 4° (not longer than 2 h). Chloroplasts were isolated in a sucrose medium as described earlier[13,14]. The final chloroplast suspension contained 70–90% intact chloroplasts as tested with phase-contrast microscopy[15].

Incubation of chloroplast homogenate. Before incubation intact chloroplasts were destroyed by sonication (3 sec with Branson sonic power). The homogenate was diluted with 50 mM Tris buffer (final pH 7.6) to give a concentration of 150–200 μg chlorophyll per ml. The incubation mixture contained 3 mM dithiothreitol as reducing agent. The substances tested for their effects on the enzyme system were added to this solution, and controls were run separately. The homogenate was incubated at 22–23° in darkness. Enzyme activity was measured after 30–45 sec and then as indicated in the figures. For assay of activity 20–50 μl (corresponding to 3–10 μg chlorophyll) were removed from the incubation mixture which had an initial volume of 0.9 ml. The original enzyme activity was determined by graphic extrapolation of the control to zero time.

Preparation of leaf homogenate. 100 g washed spinach leaves were homogenized in a blender with 250 ml 50 mM Tris buffer (pH 8.0) containing 40 mM thioglycol, 5 mM sodium ascorbate, 3 mM cysteine, and 3 mM EDTA. Debris was removed by filtration through four layers of cloth.

Assay of enzymes. NADP- and NAD-dependent glyceraldehyde phosphate dehydrogenases were assayed in an optical test in the direction phosphoglyceric acid → glyceraldehyde phosphate according to HEBER *et al.*[9]. The test mixture in a final volume of 2.5 ml contained 125 μmoles Tris (pH 7.6), 3 μmoles ATP, 22 μmoles 3-phosphoglycerate, 0.4 μmole NADPH or NADH, respectively, 12.5 μmoles dithiothreitol, and 20 μmoles $MgSO_4$. The phosphoglycerate kinase (ATP:3-phospho-D-glycerate 1-phosphotransferase, EC 2.7.2.3) which is involved in this test system was not rate-determining (*cf.* ref. 8). Control measurements were run in the absence of substrate. Readings were made every 30 sec. The reaction was linear for 2 min.

Determination of chlorophyll. Prior to the incubation chlorophyll was estimated by extraction with 80% acetone (*cf.* ref. 16).

Reagents. Tris, acetone, ascorbate, cysteine hydrochloride, EDTA, $MgCl_2 \cdot 6\,H_2O$, and $MgSO_4$ were obtained from Merck, Darmstadt. ATP, ADP, AMP, NADPH, $NADP^+$, NADH, NAD^+, and glycerate 3-phosphate (tri-cyclohexyl ammonium salt) were from Boehringer, Mannheim; dithiothreitol from Calbiochem, Los Angeles; DL-glyceraldehyde 3-phosphate (free acid) from Sigma, St. Louis; and thioglycol from Schuchardt, München.

RESULTS

Effect of pyridine nucleotides

The effect of 3 mM $NADP^+$ on the NADP-dependent glyceraldehyde phosphate dehydrogenase is presented in Fig. 1a. After 20 min of incubation the activity is in-

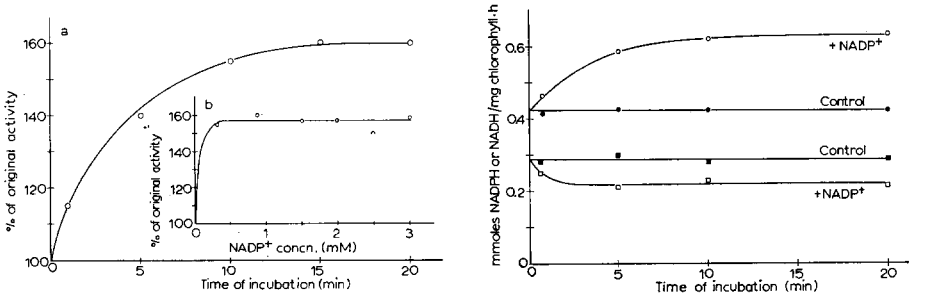

Fig. 1. a. Kinetics of activation of the NADP-dependent glyceraldehyde phosphate dehydrogenase by $NADP^+$. The chloroplast homogenate was incubated with 3 mM $NADP^+$ and the enzyme activity was assayed parallel to a control without $NADP^+$ (*cf.* METHODS). b. Activation of the NADP-dependent glyceraldehyde phosphate dehydrogenase as a function of $NADP^+$ concentration. Values represent the activation after 20-min incubation.

Fig. 2. Effect of $NADP^+$ on the NADP- and NAD-dependent glyceraldehyde phosphate dehydrogenase. 3 mM $NADP^+$ was added to one incubation mixture (*cf.* METHODS) out of which the NADP- and NAD-dependent glyceraldehyde phosphate dehydrogenases were assayed in parallel manner. ●—●, activity of the NADP-dependent enzyme without incubation with $NADP^+$; ○—○, activity of the NADP-dependent enzyme after incubation with 3 mM $NADP^+$; ■—■, activity of the NAD-dependent form in the absence of $NADP^+$; □—□, activity of the NAD-dependent dehydrogenase after incubation with 3 mM $NADP^+$.

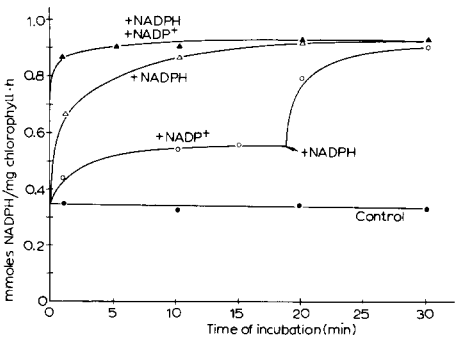

Fig. 3. Kinetics of activation of the NADP-dependent glyceraldehyde phosphate dehydrogenase by $NADP^+$ *plus* NADPH. ●—●, in the absence of additions; ○—○, at the start only $NADP^+$ (3 mM) was added, after 19 min of incubation addition of 3 mM NADPH; △—△, incubation. with 3 mM NADPH; ▲—▲, activation by 3 mM $NADP^+$ *plus* 3 mM NADPH.

creased by 60%. In comparison the same concentration of NADPH results in an activation of 200–450%[8]. The activation has already reached a plateau at 0.3 mM $NADP^+$ (Fig. 1b).

Parallel to the activation of the NADP-dependent dehydrogenase, the NAD-dependent glyceraldehyde phosphate dehydrogenase is inhibited (Fig. 2). Activation and inhibition have a ratio of about 5:1 as was found earlier by experiments *in vivo* with intact plants[7] and with isolated chloroplasts[14].

The activation by $NADP^+$ *plus* NADPH is the same as that of NADPH alone (Fig. 3). Addition of $NADP^+$ after 20-min incubation with NADPH does not lead to a further increase in activity. The same maximal activity is attained by the addition of NADPH to an incubation with $NADP^+$. Both effects are not additive.

The activity of the NAD-dependent dehydrogenase remained unchanged after incubation with 3 mM NADH or 3 mM NAD^+.

Influence of adenine nucleotides

6 mM ATP is similarly effective in activating the NADP-dependent enzyme (Fig. 4a), as was previously found for 3 mM NADPH[8]. Saturation of activation is

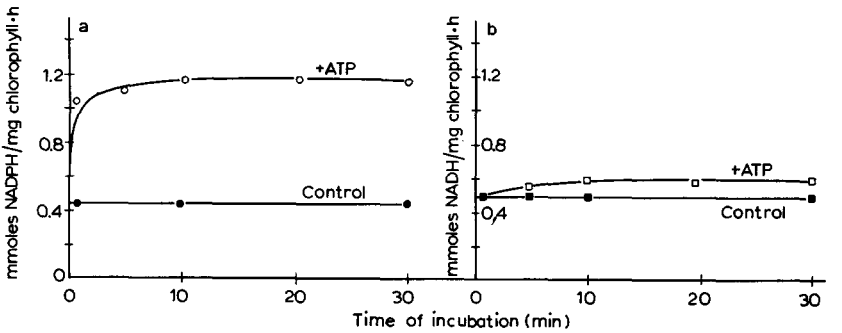

Fig. 4. Effect of incubation with 6 mM ATP on the NADP-dependent (a) and the NAD-dependent (b) glyceraldehyde phosphate dehydrogenase.

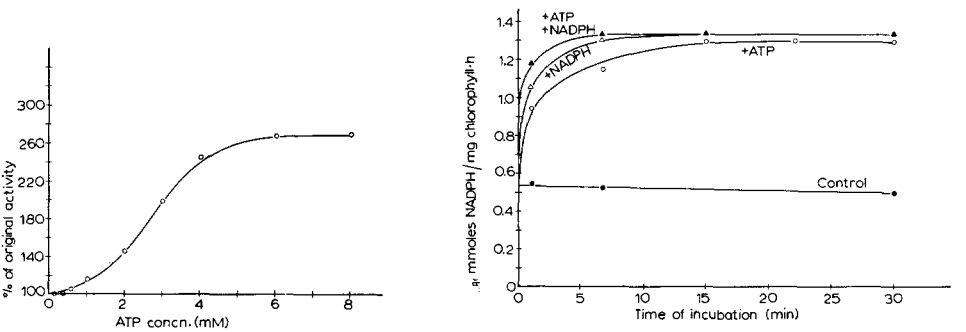

Fig. 5. Activation of the NADP-dependent glyceraldehyde phosphate dehydrogenase as a function of ATP concentration. Values were taken from a series of experiments after 20 min of incubation.

Fig. 6. Kinetics of activation of the NADP-dependent glyceraldehyde phosphate dehydrogenase by ATP *plus* NADPH. ●—●, without addition of effectors; ○—○, incubation with 8 mM ATP, after 19 min addition of 3 mM NADPH; △—△, incubation with 3 mM NADPH; ▲—▲, addition of 8 mM ATP *plus* 3 mM NADPH.

reached after 8 min incubation. The time of half-maximal activation varies from some seconds (Fig. 4a) to 1 min (Fig. 6). The NAD-dependent dehydrogenase is not inhibited but activated by about 16% by ATP (Fig. 4b).

The activation of the NADP-dependent enzyme as a function of ATP concentration does not follow Michaelis–Menten kinetics (Fig. 5). The curve has a sigmoid shape indicating a cooperative mechanism of the activation process. The activation attains a plateau with 6 mM ATP. For half-maximal activation the system needs 2.7 mM ATP. The enzyme cannot be activated in the test solution containing 1 mM ATP (*cf.* METHODS) as this ATP concentration is too low to result in a significant activation within a few minutes.

Regarding the final increase the combined effect of 8 mM ATP *plus* 3 mM NADPH is not greater than the activation by either ATP or NADPH alone (Fig. 6). Saturating concentrations of ATP activate as much as does NADPH. Addition of

TABLE I

EFFECTS OF SOME METABOLITES AND OF $MgCl_2$ ON THE ACTIVITY OF THE NADP- AND NAD-DEPENDENT GLYCERALDEHYDE PHOSPHATE DEHYDROGENASE

Activation and inhibition, expressed in percent of original activity, are indicated by "+" and "−", respectively.

Metabolite	*Concn. (mM)*	*Effect on glyceraldehyde phosphate dehydrogenase*	
		NADP-dependent	*NAD-dependent*
NADPH	3	+ ⩾ 200	—
$NADP^+$	3	+ 50	−23
NADH	3	—	0
NAD^+	3	—	0
ATP	8	+ ⩾ 200	+16
ADP	8	0	—
AMP	8	− 17	—
Phosphoglyceric acid	8	0	—
Glyceraldehyde phosphate	8	0	—
$MgCl_2$	50	+ 30	0

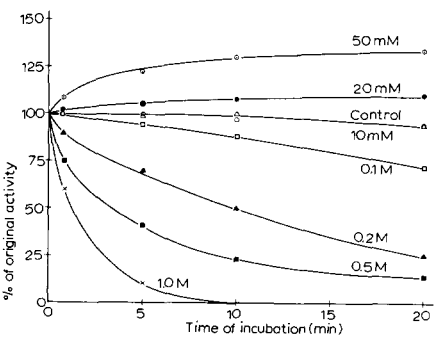

Fig. 7. Time curve of the effects of increasing $MgCl_2$ concentrations on the NADP-dependent glyceraldehyde phosphate dehydrogenase.

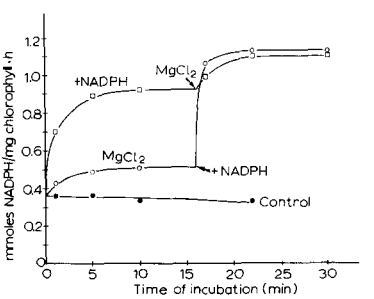

Fig. 8. Effects of $MgCl_2$ *plus* NADPH on the NADP-dependent glyceraldehyde phosphate dehydrogenase. ●—●, without additions; ○—○, incubation with 50 mM $MgCl_2$, after 15 min addition of 3 mM NADPH; □—□, incubation with 3 mM NADPH, after 15 min addition of 50 mM $MgCl_2$.

NADPH after 19 min of incubation with ATP does not further increase the activity. Addition of ATP does not further increase the activity.

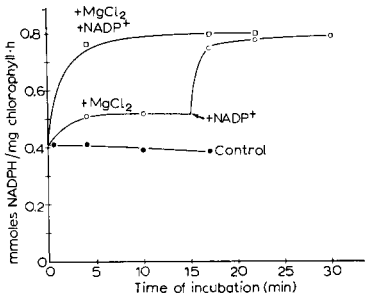

Fig. 9. Influence of $MgCl_2$ *plus* $NADP^+$ on the NADP-dependent glyceraldehyde phosphate dehydrogenase. ●—●, without additions; ○—○, incubation with 50 mM $MgCl_2$, after 15 min addition of 3 mM $NADP^+$; □—□, incubation with 3 mM $NADP^+$, after 15 min addition of 50 mM $MgCl_2$.

We could not find any influence of varied concentrations of ADP, 8 mM phosphoglyceric acid, and glyceraldehyde phosphate on the activity of the NADP-dependent dehydrogenase. 8 mM AMP decreases the activity after 6 min of incubation by 17% (see Table I).

Effect of $MgCl_2$

The effect of $MgCl_2$ depends largely on the concentration range in which it is used. The activity of the NADP-dependent dehydrogenase increases with increasing $MgCl_2$ concentrations in the range of 10–50 mM. The greatest activation was 30% after 20-min incubation (Fig. 7). When the $MgCl_2$ concentration is raised to a range between 0.1 and 1.0 M, the enzyme activity is increasingly inhibited.

50 mM $MgCl_2$ in addition to NADPH increases the activity above the plateau which is reached by NADPH alone (Fig. 8). This indicates that the effect of $MgCl_2$ is different from that of NADPH. A similar additive influence was found for the combination of $MgCl_2$ and $NADP^+$ (Fig. 9).

DISCUSSION

Activation in vivo

ATP increases the activity of the NADP-dependent glyceraldehyde phosphate dehydrogenase in the same way as we have found earlier for NADPH[8]. The kinetics of activation and the maximal enzyme activities are very similar for both effectors. The same maximal active state can be brought about by ATP as well as by NADPH and cannot be overcome by the presence of both nucleotides. Until now we could not decide whether ATP or NADPH or both nucleotides were the effectors *in vivo*.

In vivo $NADP^+$ cannot be an effector of the activation for the following reasons: The maximal increase of activity induced by $NADP^+$ is only 1/6 of the light-induced activation in intact spinach leaves[14]. Furthermore in photosynthesis, with which the activation *in vivo* is closely connected[7], $NADP^+$ is reduced to NADPH.

The activation by 50 mM $MgCl_2$ is about 1/10 that of NADPH and ATP *in vitro*

and of saturating light intensities *in vivo*. Therefore $MgCl_2$ can play only a minor role if any in the activation process. Ribulose diphosphate carboxylase is much more sensitive to an activation by Mg^{2+} (refs. 18–20) needing 10 mM Mg^{2+} for a 5-fold activation. In contrast to the effects of ATP, NADPH, and $NADP^+$, the influence of $MgCl_2$ is additive to the maximal state attained by NADPH. Perhaps the salt just provides the optimal ionic strength for the activation. We are about to clarify this question.

Mechanism of activation

The cooperative mechanism of the activation proposed in a previous paper[8] is confirmed. The effectors of the activation are produced in the light reaction of photosynthesis. So light has only an indirect influence on the activity.

The prerequisites of these comparative studies *in vivo* and *in vitro* are the slow activation and reversal of activation of the enzyme. The activity is maximal after about 10 min of exposure to the light *in vivo*[5,6] or incubation with effectors *in vitro*. *In vivo* about the same time is needed for reversal of activation[20]. This is why we can measure the activated form in a usual optical test.

The NADP- and the NAD-dependent dehydrogenases have very similar properties. We were not able to separate the enzymes by polyacrylamide-gel electrophoresis. Similar results were published by SCHULMAN AND GIBBS[11]. On the other hand our experiments do not support the hypothesis that the activation is brought about by a competition for the same binding sites of the coenzymes of an identical enzyme molecule. The activation by ATP, for example, cannot be explained by this assumption.

The activation of the NADP-dependent dehydrogenase by ATP *in vitro* is not accompanied by a decrease in the activity of the NAD-dependent form. This is not in agreement with our former model that both enzymes represent interconvertible forms. Indeed the effects of $NADP^+$ *in vitro* (Fig. 2) and of light *in vivo*[6,14] suggest an interconversion. Further data are necessary to clarify this disagreement.

Role of activation in CO_2 fixation

A maximal photosynthetic rate of 245 μmoles CO_2 fixed/mg chlorophyll·h was reported for spinach[21]. This rate represents the turnover number of the entire system of the reductive pentose phosphate cycle. The NADP-dependent glyceraldehyde phosphate dehydrogenase is generally accepted as a part of this system. In photosynthesis 2 moles of NADPH are needed for the reduction of 1 mole of CO_2. Therefore, for a photosynthetic activity of 245 μmoles CO_2/mg chlorophyll·h the theoretical minimal activity of the NADP-dependent dehydrogenase is 490 μmoles NADPH/mg chlorophyll·h. This, however, is in the range of highest enzyme activities found in darkened plants. With this activity, *i.e.* without activation, the NADP-dependent dehydrogenase would be a rate-limiting step in CO_2 fixation. We conclude that the activation of the NADP-dependent dehydrogenase is necessary in order to eliminate a barrier against maximal CO_2 fixation.

The activation *in vivo* is dependent on light intensity[22] which can be explained by an altered production of NADPH and ATP in the light reactions. With increasing assimilatory power the activity of the NADP-dependent dehydrogenase is raised so

that NADPH accelerates its own turnover and the enzyme activity should not determine the rate of CO_2 fixation.

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