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IMPROVED IN VITRO LIGHT ACTIVATION AND ASSAY SYSTEMS FOR TWO SPINACH CHLOROPLAST ENZYMES

PETER SCHÜRMANN[a] and JEAN-PIERRE JACQUOT[b]

[a]*Laboratoire de Physiologie végétale et Biochimie, Université de Neuchâtel, Chantemerle 20, CH-2000 Neuchâtel (Switzerland), and* [b]*Laboratoire de Biologie végétale, E.R.A. au C.N.R.S. No. 799, Université de Nancy I, Case Officielle No. 140, F-54037 Nancy Cedex (France)*

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Summary

An improved system for the in vitro light activation of the chloroplast enzymes fructose-1,6-bisphosphatase and NADP-dependent malate dehydrogenase is described. Through the presence of the monothiol β-mercaptoethanol in the reaction mixtures the activated forms of the enzymes can be stabilized and their activity determined spectrophotometrically.

Chloroplast enzymes, like fructose-1,6-bisphosphatase and NADP-dependent malate dehydrogenase, are in vivo activated by light. This activation has been shown in vitro to depend on photochemically reduced ferredoxin, ferredoxin-thioredoxin reductase, a novel enzyme, and enzyme specific thioredoxins as well as the respective enzymes [1—4].

While comparing different plant thioredoxin fractions for their ability to activate spinach chloroplast fructose-1,6-bisphosphatase and NADP-dependent malate dehydrogenase we found it desirable to set up a light activating system comparable for both enzymes, and to be able to separate experimentally activation phase from reaction phase. In this report we describe such a system.

Light activation of enzymes. Pure chloroplast fructose-1,6-bisphosphatase or purified chloroplast NADP-dependent malate dehydrogenase are activated under anaerobic conditions in the presence of saturating amounts of reduced spinach ferredoxin, pure ferredoxin-thioredoxin reductase and pure, enzyme specific thioredoxin. Ferredoxin is reduced in the light by twice washed, heated thylakoid membranes using ascorbate/dichloro-

phenolindophenol as electron donor. The activated forms of the enzymes, which seem to be very labile, are well enough stabilized by the presence of β-mercaptoethanol in the reaction mixture that an aliquot of the activation mixture can be rapidly transferred to a spectrophotometer cuvette for the assay of the enzyme activity. The composition of the activation mixture for the two enzymes studied is identical with exception for the thioredoxins which are specific for their respective enzymes. The optimal pH for the activation of both enzymes has been found to be between 7.8 and 8.0. With time the activation follows saturation kinetics and is, under the conditions used in these studies, complete after 13—15 min. Completely activated fructose-1,6-bisphosphatase showed a specific activity of 33 units/ mg enzyme and NADP-dependent malate dehydrogenase 10 units/mg under the experimental conditions described.

Table I presents evidence that in the system used the activation depends on light, the presence of thylakoid membranes, ferredoxin, ferredoxin-thioredoxin reductase, thioredoxin and the enzyme tested. The omission of one of these components abolishes the activation.

Table II shows the enhancing effect of the presence of β-mercaptoethanol in the activation mixture. On its own β-mercaptoethanol does not induce any activity of the enzymes as can be seen from the dark controls. However, its omission from the mixtures exposed to light greatly reduces the amount of enzyme activity measured after activation. Table II also shows that under

TABLE I

REQUIREMENTS FOR PHOTOCHEMICAL ACTIVATION OF CHLOROPLAST ENZYMES

The complete activation mixture of 100 µl contained 10 µmol Tris-HCl buffer (pH 7.9), 1 µmol sodium ascorbate, 0.01 µmol 2,6-dichlorophenol indophenol, 1.4 µmol β-mercaptoethanol, 8 µg spinach ferredoxin, 4.2 µg pure ferredoxin-thioredoxin reductase, chloroplast thylakoids (5 µg chlorophyll) washed twice and heated for 5 min at 55° C to destroy their oxygen-evolving capacity, and depending on the enzyme to be activated 4.2 µg pure thioredoxin$_f$ and 4.25 µg pure fructose-1,6-bisphosphatase or 3.9 µg pure thioredoxin$_m$ and 18 µg purified malate dehydrogenase. Activation was carried out in conical centrifuge tubes stoppered with serum caps. The activation mixtures were gassed with N_2 for 3 min, then illuminated in a constant temperature water bath at 25° C with 18 000 lux. After 5 min activation 20 µl of the activation mixture were rapidly injected into the spectrophotometer cuvette containing the complete reaction mixture for the assay of fructose-1,6-bisphosphatase or malate dehydrogenase and the absorbance change at 340 nm was followed in a spectrophotometer at 25° C. The fructose-1,6-bisphosphatase reaction mixture contained in 0.98 ml (in a 1-cm cuvette of 1 ml capacity) 0.7 units glucose-6-phosphate dehydrogenase. 1.75 units phosphoglucose isomerase, 100 µmol Tris-HCl buffer (pH 7.9), 1.5 µmol $MgSO_4$, 0.1 µmol EDTA, 2.4 µmol fructose-1,6-bisphosphate, 1 µmol NADP, and 14 µmol β-mercaptoethanol. The malate dehydrogenase reaction mixture contained in 0.98 ml 100 µmol Tris-HCl buffer (pH 7.9), 1.7 µmol oxaloacetate, 0.5 µmol EDTA, 0.2 µmol $NADPH_2$ and 14 µmol β-mercaptoethanol. Activities are expressed as nmol/min per total activated enzyme.

Activation conditions	Enzyme activities	
	Fructose-1,6-bisphosphatase	Malate dehydrogenase
Dark, complete	0	1
Light, complete	116	56
Light, minus thylakoid membrane	0	2
Light, minus ferredoxin	0	2
Light, minus ferredoxin-thioredoxin reductase	0	2
Light, minus thioredoxin	0	2
Light, minus respective enzyme	0	1

TABLE II

EFFECTS OF β-MERCAPTOETHANOL AND NITROGEN ON ACTIVATED ENZYMES

The mixture contained 36 μg malate dehydrogenase. Otherwise assay conditions were the same as given in Table I. The 100% enzyme activities were expressed as nmol/min per total activated enzyme: 116 for fructose-1,6-bisphosphatase and 63 for malate dehydrogenase. MET, β-mercaptoethanol.

Activation conditions	Assay conditions	Enzyme activities (%)	
		Fructose-1,6-bisphosphatase	Malate dehydrogenase
Dark, complete	+ MET	0	2
	— MET	0	2
Light, complete	+ MET	100	100
	— MET	65	56
Light, minus MET	+ MET	41	59
	— MET	13	51
Light, minus nitrogen	+ MET	0	3
	— MET	0	3

the experimental conditions used the enzymes can only be activated under anaerobic conditions. The presence of oxygen completely inhibits the activation.

Spectrophotometric assay of fructose-1,6-bisphosphatase. We have optimized the coupled spectrophotometric assay [5] for the test of the light-activated fructose-1,6-bisphosphatase. Addition of β-mercaptoethanol to the assay mixture stabilizes the activated enzyme form in the spectrophotometer cuvette and allows the kinetic assay without continuous illumination. So far the light activated fructose-1,6-bisphosphatase has always been tested by measuring P_i released during an extended reaction period under continuous illumination [1,4]. The improved assay system separates activation phase from reaction phase and allows the continuous monitoring of the enzyme reaction. Omission of β-mercaptoethanol from the assay mixture reduces the detectable fructose-1,6-bisphosphatase activity to 65% of the control, omission from activation and assay mixture reducing it even more, i.e. to 13% (Table II). The fructose-1,6-bisphosphatase reaction, measured as NADP reduction in the coupled assay, proceeds after an initial lag phase of 30—60 s under all conditions linear with time.

Spectrophotometric assay of NADP-dependent malate dehydrogenase. Activated malate dehydrogenase has been measured by following spectrophotometrically the oxaloacetate-dependent $NADPH_2$ oxidation [6].

Similar to the fructose-1,6-bisphosphatase, addition of β-mercaptoethanol to the assay mixture apparently stabilizes the activated enzyme and approximately doubles the reaction rates (Table II). Under all conditions the reaction rates start linearly with time without any detectable lag phase.

In summary, these results provide evidence that the in vitro light activated forms of chloroplast fructose-1,6-bisphosphatase and NADP-dependent malate dehydrogenase are stabilized by the presence of β-mercaptoethanol in the reaction media. This enabled us to separate activation phase from reaction phase and to use identical activation conditions for

both enzymes. Furthermore, the use of an optical test for fructose-1,6-bisphosphatase considerably improves the sensitivity and accuracy of the assay. This method should help in the study of ferrodoxin-thioredoxin reductase, light-activated fructose-1,6-bisphosphatase and NADP-dependent malate dehydrogenase.

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