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STUDIES ON THE REGULATION OF CHLOROPLAST FRUCTOSE-1,6-BISPHOSPHATASE

ACTIVATION BY FRUCTOSE 1,6-BISPHOSPHATE

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Summary

Chloroplast fructose-1,6-bisphosphatase (D-fructose 1,6-bisphosphate 1-phosphohydrolase, EC 3.1.3.11) isolated from spinach leaves, was activated by preincubation with fructose 1,6-bisphosphate. The rate of activation was slower than the rate of catalysis, and dependent upon the temperature and the concentration of fructose 1,6-bisphosphate. The addition of other sugar diphosphates, sugar monophosphates or intermediates of the reductive pentose phosphate cycle neither replaced fructose 1,6-bisphosphate nor modified the activation process. Upon activation with the effector the enzyme was less sensitive to trypsin digestion and insensitive to mercurials. The activity of chloroplast fructose-1,6-bisphosphatase, preincubated with fructose 1,6-bisphosphate, returned to its basal activity after the concentration of the effector was lowered in the preincubation mixture. The results provide evidence that fructose-1,6-bisphosphatase resembles other regulatory enzymes involved in photosynthetic CO_2 assimilation in its activation by chloroplast metabolites.

Introduction

Previous studies have provided evidence that chloroplast fructose-1,6-bisphosphatase (D-fructose 1,6-bisphosphate 1-phosphohydrolase, EC 3.1.3.11) is active in the light but almost inactive in darkness [1—5]. The ferredoxin-

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Abbreviations: Fru-1,6-P_2, D-fructose 1,6-bisphosphate; Fru-6-*P*, D-fructose 6-phosphate.

thioredoxin system constitutes one mechanism by which light regulates activity of this enzyme; in the regulation system thioredoxin, reduced photochemically via ferredoxin and ferredoxin-thioredoxin reductase, converts fructose-1,6-bisphosphatase to a form that displays activity at low (physiological) concentrations of Mg^{2+} [6—8]. Moreover, the rate of modification of the enzyme by reduced thioredoxin is slower that the rate of catalysis [6,7], Frieden has named this type of enzymes as hysteretic enzymes [9]. Another feature of chloroplast fructose-1,6-bisphosphatase is that it displays activity at high concentrations of Mg^{2+}, in the absence of any modifier [10,11]. Unlike activation mediated by reduced thioredoxin, the activation induced by high concentrations of Mg^{2+} is a fast and freely reversible process [7]. Earlier studies on the regulation of enzymes of the reductive pentose phosphate cycle, namely ribulose-1,5-bisphosphate carboxylase (3-phospho-D-glycerate carboxylase (dimerizing), EC 4.1.1.39) [12,13] and NADP-glyceraldehyde-3-*P* dehydrogenase [14—17], showed that both of these enzymes display hysteretic activation by chloroplast metabolites, that is, the rate of modification by an effector is slower than the rate of catalysis.

Although chloroplast fructose-1,6-bisphosphatase has been studied in some detail [7,10,11,18—24], an effect of intermediates of the Benson-Calvin cycle on enzyme activity has not been reported. Therefore, we decided to examine the effect of chloroplast metabolites on the activity of the enzyme.

During routine assays of fructose-1,6-bisphosphatase, that were carried out in the absence of modifiers and at low concentrations of Mg^{2+}, we consistently observed a lag phase in the reaction progress curve [7,25]. The data reported herein suggest that this lag phase is due to a substrate-induced hysteretic conversion of chloroplast fructose-1,6-bisphosphatase from an inactive to an active form during the catalytic phase of the reaction.

Materials and Methods

Reagents. Chemicals were obtained from the following sources: Tris from Serva Feinbiochemica (Heidelberg) and EDTA from Aldrich Chemical Co. (Milwaukee, WI). Glyceraldehyde-3-*P* and dihydroxyacetone phosphate were prepared from the monobarium salt of DL-glyceraldehyde-3-*P* diethylacetal and the dicyclohexylamine salt of dihydroxyacetone phosphate dimethylketal, respectively, according to the instructions provided by the supplier (Sigma Chemical Co., St. Louis, MO).

All other biochemicals and auxiliary enzymes were supplied by Sigma and were used without further purification.

Enzyme purification. Spinach leaves were purchased on the local market and kept frozen (−15°C) until used. Chloroplast fructose-1,6-bisphosphatase was purified from frozen spinach leaves by using a modification of the procedure described in an earlier report [7,24].

Assay of chloroplast fructose-1,6-bisphosphatase. The studies of enzyme activation by an effector were carried out using a two-stage assay [7,17]. Chloroplast fructose-1,6-bisphosphatase was preincubated in 0.1 ml of 0.1 M Tris-HCl buffer (pH 7.9), either in the presence or in the absence of an effector. After the preincubation period the enzyme was injected into the mixture that

was used to assay fructose-1,6-bisphosphatase activity (see below). Enzyme activity was assayed spectrophotometrically at 23°C by following the formation of Fru-6-*P* with a coupled enzyme assay. The assay mixture contained, in a final volume of 0.9 ml, 2 units of glucose-6-*P* dehydrogenase, 5 units of phosphoglucose isomerase and the following compounds: 100 μmol, Tris-HCl buffer (pH 7.9), 1 μmol, $MgSO_4$; 3 μmol, sodium Fru-1,6-P_2; and 1 μmol, NADP (sodium salt). NADPH formation was followed by measuring the change in absorbance at 340 nm with a Gilford 2000 spectrophotometer.

In activation experiments, where chloroplast fructose-1,6-bisphosphatase was preincubated with different concentrations of Fru-1,6-P_2, a variable amount of the sugar diphosphate was injected with the enzyme into the assay mixture. Therefore, in order to keep constant the concentration of substrate (3 mM) during the measurement of velocity, an amount of Fru-1,6-P_2 equal to that injected with the enzyme, was subtracted from the assay mixture. Such a procedure assured that any observed change in enzyme activity (catalysis) reflected changes that occurred during the preincubation [7,17].

Results

Kinetics of Fru-1,6-P_2 mediated activation of chloroplast fructose-1,6-bisphosphatase

In the hydrolysis of Fru-1,6-P_2 to Fru-6-*P*, catalyzed by chloroplast fructose-1,6-bisphosphatase, a distinct lag phase was observed when the activity was followed spectrophotometrically (Fig. 1). A similar time progress curve was obtained when the reaction was started by the addition of enzyme that was

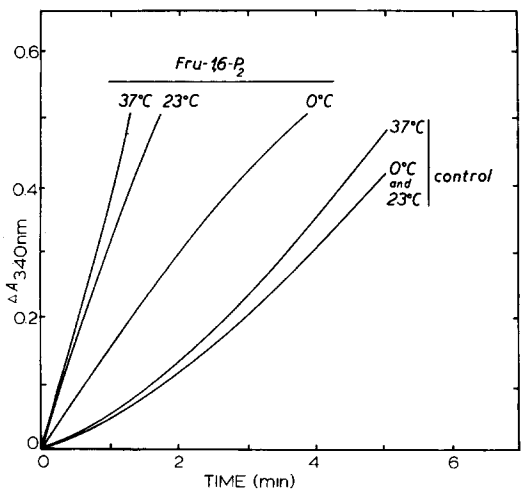

Fig. 1. Effect of preincubation with Fru-1,6-P_2 at different temperatures on the activity of chloroplast fructose-1,6-bisphosphatase. The enzyme (15 μg) was incubated in 0.1 ml of a solution containing 10 μmol of Tris-HCl buffer (pH 7.9) and, as indicated, 1.2 μmol of Fru-1,6-P_2. The preincubation was carried out at 0°C, 23 or 37°C. After 2 min the mixture was injected into a 1 cm light-path cuvette of 1.5 ml capacity, in a final volume of 0.9 ml. For the enzyme preincubated with Fru-1,6-P_2, the reaction mixture contained 1.8 μmol of Fru-1,6-P_2 instead of the 3 μmol that were used for the control enzyme. The change in absorbance at 340 nm was followed spectrophotometrically at 23°C.

either untreated or was previously preincubated in buffer. The lag period of 30—40 s was independent of the concentration of auxiliary enzymes and NADP, but was dependent on the concentration of either Mg^{2+} or Fru-1,6-P_2. The preincubation of the enzyme at various temperatures did not appreciably change the lag phase and the final (linear) velocity that was measured at a constant temperature (23°C). However, when chloroplast fructose-1,6-bisphosphatase was preincubated with 12 mM Fru-1,6-P_2 for 2 min prior to the initiation of the catalytic reaction, the lag phase was completely eliminated. Furthermore, the final (linear) rate of the activated enzyme (assayed at 23°C) was dependent on the temperature of preincubation. A 3-fold and a 4-fold increase were attained after preincubating the enzyme with Fru-1,6-P_2 at 23°C and 37°C, respectively; only a small effect was observed when activation was carried out at 0°C.

Neither Fru-6-*P* nor P_i formation were detected during the preincubation, because Mg^{2+}, a requirement for the enzyme activity, was omitted during preincubation and was present in the assay mixture. Moreover, in parallel experiments, we observed that neither Mg^{2+} nor NADP replaced Fru-1,6-P_2 in the activation of the enzyme. The Fru-1,6-P_2 mediated activation of chloroplast fructose-1,6-bisphosphatase is a readily reversible process because the enzyme deactivated rapidly once the Fru-1,6-P_2 concentration was lowered from 12 mM to 0.5 mM during the preincubation period. Other sugar diphosphates (glucose-1,6-bisphosphate, ribulose-1,5-bisphosphate, sedoheptulose-1,7-bisphosphate), sugar monophosphates (ribulose-5-*P*, ribose-5-*P*, fructose-1-*P*, Fru-6-*P*, glucose-6-*P*) or metabolic intermediates of the reductive pentose phosphate cycle (glyceraldehyde-3-*P*, dihydroxyacetone-*P*, 3-phosphoglyceric acid, ATP, NADPH, P_i) did not replace Fru-1,6-P_2 in the activation process. Time course studies showed that the Fru-1,6-P_2 induced activation of chloroplast fructose-1,6-bisphosphatase was relatively slow. After 15 s preincubation of the enzyme with the effector the initial velocity was about 70% of the maximum that was attained after a preincubation of 30 s. However the final (linear) velocity of the non-preincubated enzyme was 80% of the maximum activity obtained after 30 s preincubation.

Fig. 2 shows the response of fructose1,6-bisphosphatase to varying concentrations of Fru-1,6-P_2 during preincubation. The catalytic activity of the enzyme displayed a sigmoidal dependence on the concentration of this effector during preincubation ($A_{0.5}$ = 7 mM). Concentrations of Fru-1,6-P_2 lower than 2 mM had little effect on chloroplast fructose-1,6-bisphosphatase activity, whereas the enzyme activity was greatly enhanced at higher concentrations of this sugar diphosphate. The presence of the above mentioned chloroplast metabolites during preincubation did not alter the effect of Fru-1,6-P_2 on the enzyme. The activity of Fru-1,6-P_2 activated enzyme showed a hyperbolic dependence on Fru-1,6-P_2, when the concentration of this compound was varied during the measurement of activity ($S_{0.5}$ = 0.3 mM).

In order to characterize the activation process further, we studied the change in enzyme activity caused by varying the concentration of Mg^{2+} during the catalytic phase of the reaction (Fig. 3). In agreement with previously reported studies [18,20,21], the enzyme that had been preincubated in the absence of Fru-1,6-P_2, displayed sigmoid saturation curves (Hill coefficient: 2.9). The

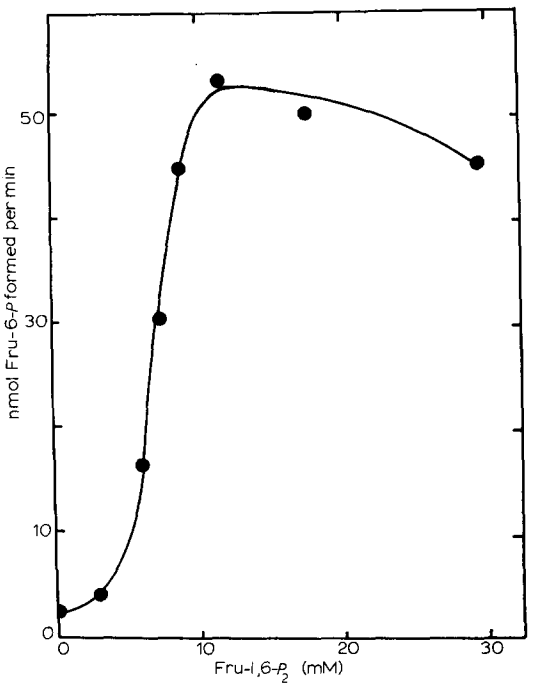

Fig. 2. Activation of chloroplast fructose-1,6-bisphosphatase by preincubation with varying concentrations of Fru-1,6-P_2. The enzyme (15 μg) was incubated for 2 min in 0.1 ml of 0.1 M Tris-HCl buffer (pH 7.9) with varying concentrations of Fru-1,6-P_2 as indicated. Following preincubation, the enzyme was injected into the assay mixture and Fru-6-*P* formation was followed spectrophotometrically. An amount of Fru-1,6-P_2 equal to that injected with the enzyme, was previously subtracted from the assay mixture, in order to keep the concentration of Fru-1,6-P_2 constant (3 mM) during the measurement of activity.

non-activated enzyme had a feeble activity at concentrations of Mg^{2+} lower than 1.5 mM, whereas maximum activity was attained between 10 and 20 mM ($A_{0.5}$ = 3.3 mM; V = 88 nmol Fru-6-*P* formed per min). Chloroplast fructose-1,6-bisphosphatase preincubated for 2 min with 12 mM Fru-1,6-P_2 displayed hyperbolic saturation curves (Hill coefficient = 1.1) and showed an appreciable activity at low concentrations of Mg^{2+}. Moreover, the activated enzyme possessed higher activity than its non-activated counterpart at any concentration of Mg^{2+} tested ($A_{0.5}$ = 2.0 mM; V = 118 nmol Fru-6-*P* formed per min).

Structural modification of chloroplast fructose-1,6-bisphosphatase following activation by Fru-1,6-P_2

Although the above mentioned results suggest that chloroplast fructose-1,6-bisphosphatase undergoes a structural modification upon incubation with Fru-1,6-P_2, we did not observe an appreciable change in the molecular weight of the enzyme in gel filtration experiments conducted in the presence and in the absence of Fru-1,6-P_2.

Therefore, we turned to another technique to detect structural changes, viz. the sensitivity of activated and non-activated fructose-1,6-bisphosphate to trypsin digestion. In this experiment chloroplast fructose-1,6-bisphosphatase was

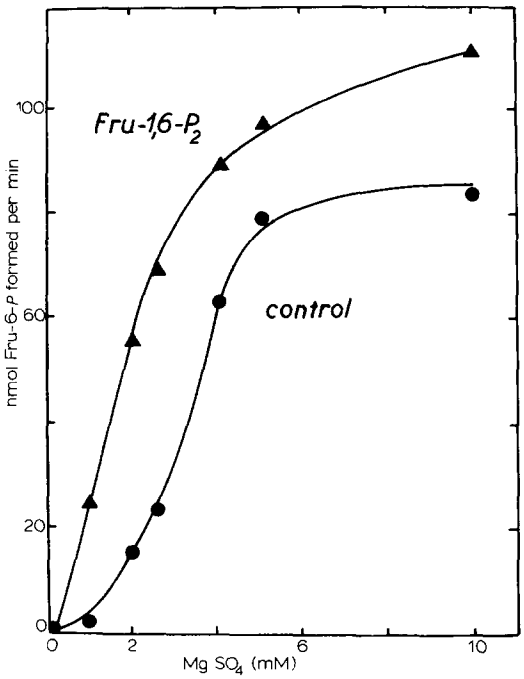

Fig. 3. Effect of Mg^{2+} concentration on the activity of chloroplast fructose-1,6-bisphosphatase preincubated in the presence and in the absence of Fru-1,6-P_2. The enzyme (7.5 μg) was incubated for 2 min at 23° C, in 0.1 ml of a solution containing 10 μmol of Tris-HCl buffer (pH 7.9) and, as indicated, 1.2 μmol of Fru-1,6-P_2. After preincubation the enzyme solution was injected into the assay mixture, containing variable concentrations of Mg^{2+}. The concentration of Fru-1,6-P_2 in the reaction mixture was arranged as described in Fig. 1.

TABLE I

EFFECT OF PREINCUBATION OF CHLOROPLAST FRUCTOSE-1,6-bisPHOSPHATE WITH FRU-1,6-P_2 ON TRYPSIN DIGESTION

The enzyme (30 μg) was incubated at 23°C in 0.06 ml of a solution containing 5 μmol Tris-HCl buffer (pH 7.9) and, as indicated, 1.2 μmol Fru-1,6-P_2. After 2 min, 20 μl of trypsin (1 mg/ml) were added and the proteolysis was carried out as indicated for either 10 or 15 min. 20 μl of soybean trypsin inhibitor (5 mg/ml) were added and enzyme was assayed.

Preincubation conditions	Relative fructose-1,6-bisphosphatase activity
Fructose-1,6-bisphosphatase, 12 min	100 *
Fructose-1,6-bisphosphatase, 2 min and trypsin, 10 min	8.6
Fructose-1,6-bisphosphatase, 2 min and trypsin, 15 min	3.8
Fructose-1,6-bisphosphatase plus Fru-1,6-P_2 12 min	100 **
Fructose-1,6-bisphosphatase plus Fru-1,6-P_2 2 min and trypsin, 10 min	55.2
Fructose-1,6-bisphosphatase plus Fru-1,6-P_2 2 min and trypsin, 15 min	23.3

* Actual value 33.8 nmol Fru-6-*P* formed per min.

** Actual value 93.2 nmol Fru-6-*P* formed per min.

TABLE II

EFFECT OF PRIOR TREATMENT OF CHLOROPLAST FRUCTOSE-1,6-bisPHOSPHATASE WITH FRU-1,6-P_2 ON ITS INHIBITION BY $HgCl_2$

The enzyme (15 μg) was activated for 2 min in 0.1 ml of a solution containing 50 mM Tris-HCl buffer (pH 7.9) and, as indicated, 12 mM Fru-1,6-P_2. 10 μl of 5 (nmol) $HgCl_2$ were added and the incubation was continued for 10 min. The mixture was then injected into the assay solution and activity was measured.

Preincubation conditions	Fructose-1,6-bisphosphatase activity (nmol Fru-6-*P* formed per min)
Fructose-1,6-bisphosphatase, 2 min	11.3
Fructose-1,6-bisphosphatase plus Fru-1,6-P_2, 2 min	55.5
Fructose-1,6-bisphosphatase plus Fru-1,6-P_2, 2 min and $HgCl_2$, 10 min	53.0

subjected to: (a) activation by preincubation for 2 min with a saturating concentration of Fru-1,6-P_2; (b) preincubation for a variable time with trypsin and (c) assay of enzyme activity by injecting the preincubated enzyme into a cuvette containing the reaction mixture plus trypsin inhibitor. As shown in Table I, trypsin treatment greatly lowered the activity of the non-activated fructose-1,6-bisphosphatase; only 9% of the original activity remained after 10 min of proteolysis. By contrast, the activity of the enzyme that had been previously activated with Fru-1,6-P_2 diminished only to about 45% under the same conditions. Another insight into the Fru-1,6-P_2 mediated activation of chloroplast fructose-1,6-bisphosphatase was obtained with the sulfhydryl reagent, $HgCl_2$. The enzyme obtained from the routine purification procedure was reported to have 16 -SH groups per mol of enzyme [23]. The importance of these residues to enzyme activity was revealed recently by their differential reactivity with reagents specific for -SH groups (Wolosiuk, R.A., unpublished data). The results depicted in Tables II and III demonstrate that the combined effects of Fru-1,6-Fru-1,6-P_2 and $HgCl_2$ on the final fructose-1,6-bisphosphatase activity depended on the order of addition of reactants to the enzyme. After preincubating chloroplast fructose-1,6-bisphosphatase with Fru-1,6-P_2 to generate

TABLE III

EFFECT OF PRIOR TREATMENT OF CHLOROPLAST FRUCTOSE-1,6-bisPHOSPHATASE WITH $HgCl_2$ ON ITS ACTIVATION BY FRU-1,6-P_2

The enzyme (45 μg) was incubated for 2 min in 0.1 ml of a solution containing 0.05 M Tris-HCl buffer (pH 7.9) and, as indicated, 5 nmol of $HgCl_2$. 20 μl of 60 mM Fru-1,6-P_2 were added and the incubation was prolonged for 10 min. The mixture was then injected into the assay solution and activity was followed spectrophotometrically.

Preincubation conditions	Fructose-1,6-bisphosphatase activity (nmol Fru-6-*P* formed per min)
Fructose-1,6-bisphosphatase, 2 min	34.6
Fructose-1,6-bisphosphatase plus $HgCl_2$, 2 min	0
Fructose-1,6-bisphosphatase plus $HgCl_2$, 2 min and Fru-1,6-P_2, 10 min	4.8

the activated form, a 10 min incubation with 50 μM $HgCl_2$ did not significantly alter the activity of the activated enzyme (Table II). However, then the same procedure was repeated, except that chloroplast fructose-1,6-bisphosphatase was first treated for 2 min with $HgCl_2$, followed by a 10 min preincubation with Fru-1,6-P_2, only 15% of the original (non-activated) activity was recovered (Table III).

Discussion

Earlier studies on chloroplast fructose-1,6-bisphosphatase have established that the reaction progress curve, measured in the absence of an enzyme modifier, displays a characteristic lag phase that is more pronounced at limiting concentrations of Mg^{2+} [7,20,25]. Preincubation of the enzyme with reduced thioredoxin shortened the lag phase and increased total enzyme activity [7]. The data presented in this paper show that a preincubation of chloroplast fructose-1,6-bisphosphatase with a chloroplast metabolite, Fru-1,6-P_2, led to similar effects i.e. the disappearance of the lag phase and an increase in the final (linear) velocity. Under the experimental conditions used, which closely resembled those found in the stroma of illuminated chloroplasts [26,27] the activation of chloroplast fructose-1,6-bisphosphatase by Fru-1,6-P_2 was a reversible, temperature dependent and relatively slow process. Therefore, the present findings support the earlier expressed view [7] that the phosphatase reaction consists of two events: (a) a modification phase in which the enzyme is converted from a less active to a more active state and (b) a catalytic phase in which the activated enzyme catalyzes the conversion of substrates to products. The rate of the modification phase is slower than the catalysis. Chloroplast fructose-1,6-bisphosphatase may thus be classified as a hysteretic enzyme according to the terminology suggested by Frieden [9].

The two-stage assay allows us to study the effect that the concentration of Fru-1,6-P_2 has on the activation and the catalytic step of fructose-1,6-bisphosphatase. Chloroplast fructose-1,6-bisphosphatase responds in a sigmoidal fashion when the concentration of Fru-1,6-P_2 is varied in the preincubation, whereas the Fru-1,6-P_2 activated enzyme displays a hyperbolic curve when the concentration of Fru-1,6-P_2 is varied during the measurement of velocity [cf; 11,20,21]. The concentration of Fru-1,6-P_2 in the stroma of illuminated chloroplasts [20,28], 0.4 mM, is lower than the observed $A_{0.5}$ in the activation of chloroplast fructose-1,6-bisphosphatase. Recent experiments in our laboratory [29] indicate that reduced thioredoxin-f decreases the $A_{0.5}$ for Fru-1,6-P_2. These results suggest that several mechanisms operate in conjuction in controlling the activity of chloroplast fructose-1,6-bisphosphatase during photosynthetic CO_2 assimilation.

Previous studies have shown that chloroplast fructose-1,6-bisphosphatase is almost inactive at low concentrations of Mg^{2+} (0.5—1 mM) whereas it displays activity at higher concentrations (10—20 mM) [10,11,18—21]. The enhancement of enzyme activity with Fru-1,6-P_2, as was shown previously with reduced thioredoxin [6,7,18,24], is more pronounced at low concentrations of Mg^{2+}, which apparently exist in the stroma of chloroplasts in the light [27]. Therefore, both the effector, Fru-1,6-P_2, and the ferredoxin-thioredoxin system

could provide mechanisms necessary for the activation of chloroplast fructose-1,6-bisphosphatase in the light.

Activation by Fru-1,6-P_2 does not appear to involve a large change in the molecular weight of chloroplast fructose-1,6-bisphosphatase. However, following activation by this effector, the enzyme is less sensitive both to proteolytic attack by trypsin and to inhibition by the -SH reagent, $HgCl_2$. These results suggest that Fru-1,6-P_2 induces a change in the conformation of the enzyme, without disturbing the quaternary structure.

The present evidence for a hysteretic activation of chloroplast fructose-1,6-bisphosphatase by its substrate adds a new feature to our understanding of the regulation of this key chloroplast enzyme. It is noteworthy that other chloroplast enzymes of the Benson-Calvin cycle, viz. NADP-glyceraldehyde-3-*P* dehydrogenase and ribulose-1,5-bisphosphate carboxylase respond in a similar manner to selected effectors [12—17]. Of these three enzymes fructose-1,6-bisphosphatase and NADP-glyceraldehyde-3-*P* dehydrogenase can be activated by the ferredoxin-thioredoxin system as well as by intermediates of the reductive pentose phosphate cycle [6,9,14—18,30]. More data are necessary to establish the relative importance of the effectors vs. the thioredoxin-linked mechanisms in the regulation of these enzymes during photosynthetic CO_2 assimilation.

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