ENHANCEMENT OF CHLOROPLAST FRUCTOSE-1,6-BISPHOSPHATASE ACTIVITY BY FRUCTOSE-1,6-BISPHOSPHATE AND DITHIOTHREITOL-REDUCED THIOREDOXIN-f

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1. Introduction

Studies with cyanobacteria, algae and isolated chloroplasts have shown that light influences the activity of regulatory enzymes of the reductive pentose phosphate cycle of CO_2 assimilation [1–5]. Similar changes of the activity of these enzymes were obtained in vitro by one of the following mechanisms: the ferredoxin–thioredoxin system [6–10]; enzyme effectors [11–14]; changes in $[Mg^{2+}]$ and pH [15–18].

Chloroplast Fru-P_2ase, whose activity is controlled by light [1–4], catalyzes the hydrolysis of Fru-1,6-P_2 to Fru-6-P and P_i, in the presence of Mg^{2+} (eq. (1)):

$$\text{Fru-1,6-}P_2 + H_2O \longrightarrow \text{Fru-6-P} + P_i \qquad (1)$$

Earlier studies showed that the activity of a homogeneous preparation of chloroplast Fru-P_2ase is enhanced by:

(i) High $[Mg^{2+}]$, in a fast and freely reversible process [7,17,18];
(ii) Thioredoxin reduced either photochemically via ferredoxin and ferredoxin–thioredoxin reductase or chemically with DTT [6,7]. The rate of activation of the enzyme by the ferredoxin–thioredoxin system is slow relative to the rate of catalysis. Moreover, the deactivation of the active form of the enzyme requires the presence of an oxidant;
(iii) Fru-1,6-P_2, as an enzyme effector, in a relatively slow and freely reversible process [14].

Abbreviations: Fru-P_2ase, fructose-1,6-bisphosphatase (EC 3.1.3.11); Fru-1,6-P_2, D-fructose-1,6-bisphosphate; Fru-6-P, D-fructose-6-phosphate; DTT, dithiothreitol; TES, *N*-Tris-(hydroxymethyl)-methyl-2-aminoethane sulfonic acid; P_i, inorganic phosphate

The effect of either the ferredoxin–thioredoxin system or Fru-1,6-P_2 on chloroplast Fru-P_2ase was more prominent when the assay of enzyme activity was carried out at low $[Mg^{2+}]$. In an extension of our work on the regulation of chloroplast Fru-P_2ase by the enzyme effector and reduced thioredoxin, the question arises whether the activity of the phosphatase is affected by the presence of both modifiers. We have therefore designed experiments to study the effect of both reduced thioredoxin and Fru-1,6-P_2 on the activity of chloroplast Fru-P_2ase. We now report that limiting concentrations of both reduced thioredoxin-f and Fru-1,6-P_2, which by themselves have little effect on the activity of the enzyme, greatly enhanced the activity of chloroplast Fru-P_2ase. These results provide the first evidence for the earlier expressed view that reduced thioredoxin interacts with enzyme effectors in the activation of regulatory enzymes of the Benson–Calvin cycle [6,7,19].

2. Materials and methods

2.1. *Isolation of chloroplast thioredoxins and Fru-P_2ase*

Spinach leaves were obtained from the local market and kept frozen (−15°C) until used. Spinach chloroplast thioredoxin-f and Fru-P_2ase were purified as in [7,20,21].

2.2. *Assay of chloroplast Fru-P_2ase activity*

The activity of chloroplast Fru-P_2ase was measured by the two-stage assay devised earlier [7,13,14]. The enzyme was preincubated at 23°C for 5 min in 0.1 ml solution containing chloroplast thioredoxin-f, as indicated and [μmol]: TES–NaOH buffer (pH 7.9),

5; DTT, 0.5. After preincubation the mixture was injected into 0.9 ml assay solution (in 1 cm lightpath 1.5 ml cuvette) containing 2 units yeast glucose-6-phosphate dehydrogenase, 5 units yeast phosphoglucose isomerase and [μmol]: TES–NaOH buffer, (pH 7.9), 50; $MgSO_4$, 1; sodium Fru-1,6-P_2, 3; NADP, 1. The NADPH formation was measured by following ΔA_{340} with a Gilford 2000 spectrophotometer. As in [7,14] when Fru-1,6-P_2 was added to the preincubation mixture, an equal amount was subtracted from the solution that was used to assay Fru-P_2ase activity. Such a procedure assured that, after injecting the enzyme solution, Fru-1,6-P_2 was kept constant at 3 mM.

2.3. *Reagents*

Biochemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Chemicals were of analytical reagent grade.

3. Results and discussion

3.1. *Activation of chloroplast Fru-P_2ase by Fru-1,6-P_2 and DTT-reduced thioredoxin-f*

We have shown that the preincubation of chloroplast Fru-P_2ase, purified to homogeneity, with DTT-reduced thioredoxin-f increases the activity of the enzyme [6,7,21]. The rate of activation of the phosphatase by chemically reduced thioredoxin-f was slower than the rate of catalysis, and dependent upon the concentration of thioredoxin-f. However, we reported the enhancement of Fru-1,6-P_2 hydrolysis, after chloroplast Fru-P_2ase was preincubated with Fru-1,6-P_2 in absence of Mg^{2+} [14]. The enzyme activity depended on both [Fru-1,6-P_2] and the time of preincubation. Like the effect of reduced thioredoxin, the changes in activity induced by Fru-1,6-P_2 could be ascribed to a modification of Fru-P_2ase which takes place more slowly than did the catalytic step. The results depicted in table 1 confirmed that the activity of chloroplast Fru-P_2ase increases following preincubation of the enzyme with high concentrations of either DTT-reduced thioredoxin-f or Fru-1,6-P_2.

Table 1
Activation of spinach chloroplast Fru-P_2ase by dithiothreitol-reduced thioredoxin-f and Fru-1,6-P_2

Preincubation conditions	Fru-P_2ase activity (nmol Fru-6-P formed/min)
Control	2.4
Thioredoxin (80 μg)	2.8
DTT + thioredoxin (20 μg)	6.1
DTT + thioredoxin (80 μg)	16.0
Fru-1,6-P_2 (3 mM)	2.4
Fru-1,6-P_2 (12 mM)	7.2

Enzyme (10 μg) was incubated for 5 min in 0.1 ml solution containing 0.05 M TES–NaOH buffer (pH 7.9), 5 mM DTT and as indicated, chloroplast thioredoxin-f or Fru-1,6-P_2. The mixture was then injected into the assay solution and activity was followed spectrophotometrically at 23°C as in section 2

Table 2
Effect of limiting concentrations of dithiothreitol-reduced thioredoxin-*f* and Fru-1,6-P_2 on chloroplast Fru-P_2ase

Preincubation conditions	Fru-P_2ase activity (nmol Fru-6-P formed/min)
Control	3.3
Thioredoxin	3.3
DTT + thioredoxin	4.8
Fru-1,6-P_2	3.9
Fru-1,6-P_2 + DTT	2.1
Fru-1,6-P_2 + thioredoxin	5.1
Fru-1,6-P_2 + DTT and thioredoxin	21.7
Fru-1,6-P_2 + DTT and thioredoxin, $-Mg^{2+}$	0.3

Chloroplast Fru-P_2ase (10 μg) was preincubated for 5 min at 23°C in 0.1 ml solution containing 5 μmol TES–NaOH buffer (pH 7.9) and, as indicated, 20 μg thioredoxin-f 0.5 μmol DTT, and 0.3 μmol Fru-1,6-P_2. Following preincubation the mixture was injected into the assay solution and activity was measured as in section 2

To see whether the thioredoxin system interacts with the effector in the activation of chloroplast Fru-P_2ase, we assayed the rate of catalysis after preincubating the enzyme with concentrations of both DTT-reduced thioredoxin-f and Fru-1,6-P_2, that by themselves did not change appreciably the activity. As shown in table 2, low concentrations of either DTT-reduced thioredoxin-f or Fru-1,6-P_2 have little effect on the activity of chloroplast Fru-P_2ase. By contrast, the activity of Fru-P_2ase was enhanced 7-fold following preincubation in the presence of both modifiers. As with other regulatory enzymes of chloroplasts, the activation phase was appreciably slower than the catalytic phase [19]. Thus, to obtain maximal activity, the enzyme was preincubated with

Table 3
Effect of Fru-1,6-P_2 on chloroplast Fru-P_2ase previously activated by DTT-reduced thioredoxin-f

Treatment	Fru-P_2ase activity (nmol Fru-6-P formed/min)
Control	2.9
6 min, Thioredoxin	4.8
1 min, Fru-1,6-P_2	2.6
6 min, Thioredoxin + Fru-1,6-P_2	40.2
1 min, Thioredoxin + Fru-1,6-P_2	6.1
5 min, Thioredoxin, and 1 min, Fru-1,6-P_2	19.4

Except for the time and the indicated differences during preincubation, experimental conditions were as in table 2

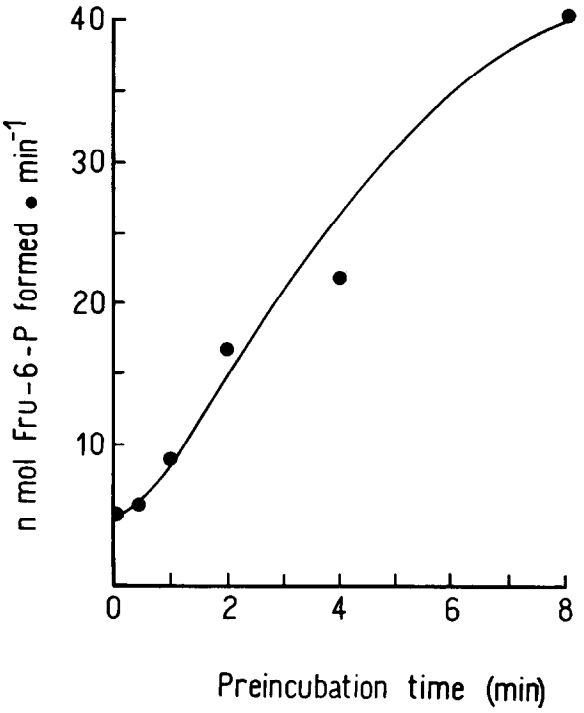

Fig.1. Effect of time of preincubation on the activation of chloroplast Fru-P_2ase by Fru-1,6-P_2 and DTT-reduced thioredoxin. Chloroplast Fru-P_2ase (10 μg) was preincubated at 23°C for the indicated times in 0.1 ml solution containing 20 μg chloroplast thioredoxin-f and [μmol]: TES–NaOH buffer (pH 7.9), 5; DTT, 0.5; Fru-1,6-P_2, 0.3. Following preincubation the enzyme was injected into the assay mixture and Fru-6-P formation was followed spectrophotometrically. Except for the time of preincubation and the amount of Fru-1,6-P_2 in the assay cuvette (2.7 μmol), experimental conditions were as in section 2.

both the effector and reduced thioredoxin. Figure 1 shows that the activity of Fru-P_2ase increased progressively as a function of the preincubation time. The preincubation time required for obtaining half maximum activity ($t_{0.5}$), when chloroplast Fru-P_2ase was activated by low concentrations of both modifiers, was 5 min, whereas when the enzyme was preincubated with high (saturating) concentrations of reduced thioredoxin-f, $t_{0.5}$ = 12 min and Fru-1,6-P_2, $t_{0.5}$ = 30 s [7,14].

In the activation of chloroplast Fru-P_2ase by low concentrations of both reduced thioredoxin-f and Fru-1,6-P_2 the question arises whether the presence of Fru-1,6-P_2 is required for the entire period of preincubation. Therefore, we examined the effect of Fru-1,6-P_2 added after the enzyme was treated for a certain period of time with reduced thioredoxin-f. In these experiments the enzyme was:

(i) Preincubated for 5 min with reduced thioredoxin-f;
(ii) Preincubated for an additional 1 min with Fru-1,6-P_2;
(iii) Injected into the reaction mixture and assayed for Fru-P_2ase activity.

As seen in table 3, the enzyme modified with DTT-reduced thioredoxin-f, responded rapidly to a short preincubation with Fru-1,6-P_2; a 3-fold enhancement was observed in enzyme activity. Nevertheless, this rate of catalysis was 50% of that obtained when the enzyme was treated for 6 min with both the sugar diphosphate and reduced thioredoxin. Therefore, we decided to carry out the experiments under conditions that produce maximal stimulation of enzyme activity; i.e., by preincubating chloroplast Fru-P_2ase with low concentrations of both Fru-1,6-P_2 and DTT-reduced thioredoxin-f.

3.2. *Kinetics of the activation of chloroplast Fru-P_2ase by DTT-reduced thioredoxin-f and Fru-1,6-P_2*

As reported [14], the response of chloroplast Fru-P_2ase to varying [Fru-1,6-P_2] during preincubation fits a sigmoidal curve (fig.2). The addition of 20 μg DTT-reduced thioredoxin-f (which by itself caused no change in activity) greatly enhanced the activity of the enzyme. Such an increase in the rate of catalysis was accompanied by a decrease in the sigmoidal shape of the curve. Thus, the $A_{0.5}$ for Fru-1,6-P_2 changed from 8 to 1 mM, when low concentrations of DTT-reduced thioredoxin-f were added to the preincubation. Moreover, the enzyme preincubated with reduced

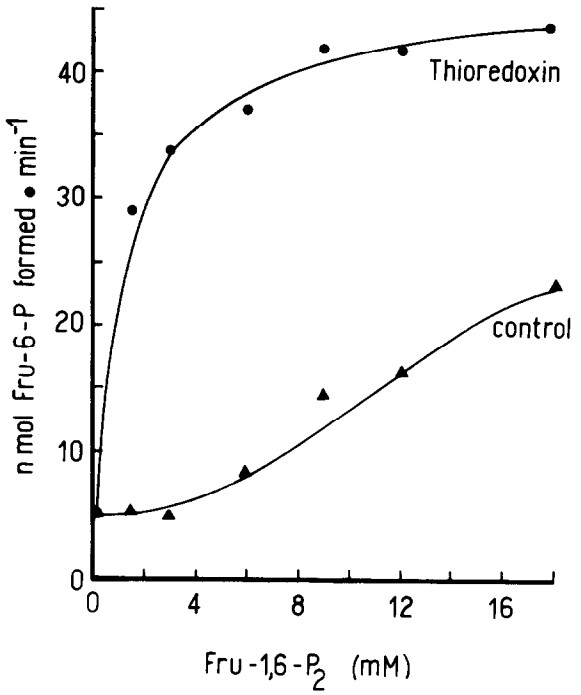

Fig.2. Activation of chloroplast Fru-P_2ase by preincubation with DTT-reduced thioredoxin-f and varying [Fru-1,6-P_2]. The enzyme (10 μg) was preincubated for 5 min with varying [Fru-1,6-P_2] as indicated, in 0.1 ml solution containing 5 μmol TES-NaOH buffer (pH 7.9) in the presence (•) or in the absence (▲) of 0.5 μmol DTT and 20 μg chloroplast thioredoxin-f. Enzyme activity was assayed spectrophotometrically at 23°C by following Fru-6-P formation. The concentration of all components was kept constant during the measurement of activity by: (a) subtracting from the assay mixture an amount of Fru-1,6-P_2; (b) adding to the control assay mixture an amount of DTT and thioredoxin-f equal to that injected with the enzyme.

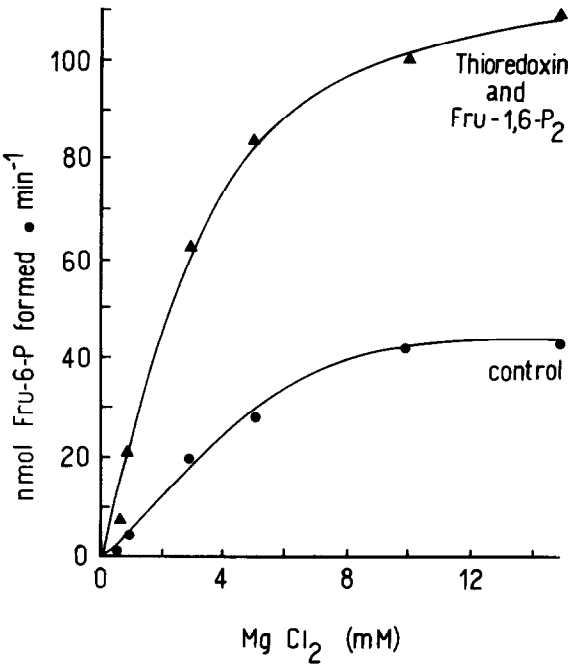

Fig.3. Effect of [Mg^{2+}] on the activity of chloroplast Fru-P_2ase preincubated with Fru-1,6-P_2 and DTT-reduced thioredoxin-f. Chloroplast Fru-P_2ase (10 μg) was preincubated for 5 min at 23°C in 0.1 ml solution containing 5 μmol TES–NaOH buffer (pH 7.9) and, as indicated, 0.3 μmol Fru-1,6-P_2, 0.5 μmol DTT and 20 μg chloroplast thioredoxin-f. Following preincubation the enzyme solution was injected into the assay mixture, containing variable [Mg^{2+}], and NADP reduction was followed spectrophotometrically. Except for the amount of Fru-1,6-P_2 in the assay mixture (2.7 μmol), experimental conditions were as in section 2.

thioredoxin possessed higher activity than its control counterpart at any [Fru-1,6-P_2].

One of the distinctive features of chloroplast Fru-P_2ase is its requirement of Mg^{2+} for enzyme activity. Earlier studies [17,18] have shown that the enzyme is almost inactive when the assay of activity is carried out at low [Mg^{2+}] (0.5–1 mM), but is active at high [Mg^{2+}] (10–20 mM). The preincubation of the chloroplast Fru-P_2ase with either reduced thioredoxin-f or Fru-1,6-P_2 renders an enzyme form that is active at low [Mg^{2+}], whereas the activity is enhanced 40–80% at high [Mg^{2+}].

The data in fig.3 show that the activity of chloroplast Fru-P_2ase, that had been preincubated in absence of any modifier, fitted a sigmoidal curve (Hill coefficient, 3.0; V_{max}, 42 nmol Fru-6-P formed/min). However, the response of the enzyme, preincubated for 5 min with both DTT-reduced thioredoxin-f and Fru-1,6-P_2, to varying [Mg^{2+}] in the assay medium, displayed a hyperbolic curve (Hill coefficient, 1.1; V_{max}, 120 nmol Fru-6-P formed/min).

4. Concluding remarks

Chloroplast Fru-P_2ase increases its catalytic rate following preincubation with low concentrations of both chemically-reduced thioredoxin-f and Fru-1,6-P_2. The enhancement of enzyme activity is more pronounced when the Fru-P_2ase assay is carried out at low [Mg^{2+}]. Like other regulatory enzymes of the carbon reduction cycle the rate of activation is slower relative to the rate of catalysis. Moreover, the increase

in the activity, observed when Fru-1,6-P_2 is added to chloroplast Fru-P_2ase, pre-treated with reduced thioredoxin, suggests a sequential effect of these modifiers on the enzyme during the activation.

The activity of key enzymes of the reductive pentose phosphate cycle is modified by the products of the light reactions of photosynthesis. The present results support the view that the ferredoxin–thioredoxin system would operate in conjunction with other light-mediated mechanisms in chloroplasts, in controlling the activity of regulatory enzymes of the Benson–Calvin cycle [6,19].

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