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A DUAL EFFECT OF Ca^{2+} ON CHLOROPLAST FRUCTOSE-1,6-BISPHOSPHATASE

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SUMMARY

Chloroplast fructose-1,6-bisphosphatase, isolated from spinach leaves, was activated by preincubation with Ca^{2+} (or Mn^{2+}), fructose-1,6-bisphosphate and dithiothreitol-reduced thioredoxin-f. Upon activation, the enzyme displayed high activity when measured at low concentrations of both fructose-1,6-bisphosphate and Mg^{2+}. On the contrary, the activity of chloroplast fructose-1,6-bisphosphatase was inhibited by Ca^{2+}. These results suggest that Ca^{2+} (or Mn^{2+}) is potentially important in the regulation of the chloroplast fructose-1,6-bisphosphatase reaction (activation and catalysis).

INTRODUCTION

In cyanobacteria, algae and the chloroplasts of higher plants, Fru-P_2ase catalyzes the hydrolysis of Fru-1,6-P_2 to Fru-6-P and P_i during the operation of the reductive pentose phosphate cycle of photosynthetic CO_2 assimilation (1-4). Investigations over the last fifteen years provided evidence that the activity of chloroplast Fru-P_2ase is regulated by light (5-8).

The changes which occur upon illumination both in pH and in the concentration of Mg^{2+} in the stroma of chloroplasts (9-14)

ABBREVIATIONS

Fru-1,6-P_2	:	D-fructose-1,6-bisphosphate
Fru-P_2ase	:	fructose-1,6-bisphosphatase (EC 3.1.3.11)
Fru-6-P	:	D-fructose-6-phosphate
DTT	:	dithiothreitol
EGTA	:	ethylene glycol bis-(β-amino-ethyl ether) N,N,N',N' tetraacetic acid
TRIS	:	Tris-(hydroxymethyl) amino-methane
P_i	:	inorganic phosphate

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have been correlated with the high activity of purified chloroplast Fru-P_2ase observed at high concentrations of Mg^{2+} and alkaline pH (1,3,15-18). In addition, other mechanisms have been proposed to account for the activation of chloroplast Fru-P_2ase; the activity of the enzyme is enhanced by the ferredoxin-thioredoxin system, effectors, and a recently found iron-sulfur protein (ferralterin) (19-23).

We have recently shown that reduced thioredoxin-f interacts with Fru-1,6-P_2 in the activation of chloroplast Fru-P_2ase (24). However, in our earlier studies on the regulation of chloroplast Fru-P_2ase the concentrations of Fru-1,6-P_2 used were higher than the reported values for the stromal concentration in illuminated chloroplast (0,4 mM) (24,25). We now report that either Ca^{2+} or Mn^{2+} enhance the activation of chloroplast Fru-P_2ase mediated by Fru-1,6-P_2 and reduced thioredoxin. The activated enzyme displays high activity at low concentrations of Fru-1,6-P_2 (0.4 mM).

MATERIALS AND METHODS

Isolation of chloroplast thioredoxins and Fru-P_2ase

Published procedures were used for the isolation of chloroplast Fru-P_2ase and thioredoxin-f (26,27).

Protein was estimated by the method of Lowry et al with bovine serum albumin as standard (28).

Determination of chloroplast Fru-P_2ase activity

Chloroplast Fru-P_2ase was routinely assayed by the two-stage assay devised earlier(20,29). The enzyme was preincubated at 23°C in 0.1 ml of a solution containing 3 µg of chloroplast thioredoxin-f and the following (in µmol): TRIS-HCl buffer (pH 7.9), 10; DTT, 0.25; Fru-1,6-P_2 , 0.04; and $CaCl_2$, 0.01. After preincubation the mixture was injected into 0.9 ml of the reaction mixture for the determination of Fru-P_2ase activity containing 2 units yeast glucose-6-P dehydrogenase and 5 units yeast phosphoglucose isomerase and the following (in µmol): TRIS-HCl buffer (pH 7.9), 50; $MgSO_4$, 1; sodium Fru-1,6-P_2 , 0.36; EGTA,0.02; and NADP, 1. The change in absorbance at 340 nm was followed at 23°C with a Gilford 2000 spectrophotometer.

Reagents

Biochemicals were purchased from Sigma Chemical Co.(St.Louis, MO). Fru-1,6-P_2 solutions were treated batchwise with DOWEX-50

(H^+ form) and Chelex-100. Chemicals were of analytical reagent grade.

RESULTS AND DISCUSSION

Effect of Ca^{2+} on the activation of chloroplast Fru-P_2ase

In the course of studies aimed at diminishing the concentration of Fru-1,6-P_2 , we found that the addition of Ca^{2+} to the preincubation medium, greatly enhanced the activity of the enzyme measured at 0.4 mM Fru-1,6-P_2 . As shown in Table I, the maximum activity was obtained after preincubating chloroplast Fru-P_2ase with Fru-1,6-P_2 and DTT-reduced thioredoxin, in the presence of 100 µM Ca^{2+} . In this experiment, both Fru-1,6-P_2 and DTT-reduced thioredoxin-f were lowered to concentrations that did not modify chloroplast Fru-P_2ase (24). Mn^{2+}, at low concentrations (10 µM),replaced Ca^{2+} in the activation of chloroplast Fru-P_2ase.

TABLE I

Activation of chloroplast Fru-P_2ase by Ca^{2+} , Fru-1,6-P_2 and DTT-reduced thioredoxin-f.

Preincubation conditions	Fru-P_2ase activity (umol F-6-P formed . min^{-1} . mg $prot^{-1}$)
Thioredoxin-f	0
Fru-1,6-P_2	0
Ca^{2+}	0
Thioredoxin-f plus Fru-1,6-P_2	3.8
Thioredoxin-f plus Ca^{2+}	0
Fru-1,6-P_2 plus Ca^{2+}	0
Thioredoxin-f plus Fru-1,6-P_2 and Ca^{2+}	73.2

Chloroplast Fru-P_2ase (0.5 µg) was preincubated for 10 min at 23° C in 0.1ml solution containing 10 µmol TRIS-HCl buffer (pH 7.9), 0.25 µmol DTT, and, as indicated, 3 µg of thioredoxin-f, 0.04 µmol Fru-1,6-P_2 , and 0.01 µmol $CaCl_2$. After preincubation Fru-P_2ase activity was assayed as described under Materials and Methods.

TABLE II

Effect of EGTA on the activation of chloroplast Fru-P_2ase by Ca^{2+}, Fru-1,6-P_2 and DTT-reduced thioredoxin-f.

Preincubation conditions	Fru-P_2ase activity (μmol F-6-P formed . min^{-1} . mg $prot^{-1}$)
Complete, 10 min	44
Complete plus EGTA, 10 min	0
Complete 10 min, plus EGTA, 10 min	41.5

Chloroplast Fru-P_2ase (2.5 μg) was preincubated at 23°C in 0.1ml of a solution containing 3 ug chloroplast thioredoxin-f and the following (in μmol): TRIS-HCl buffer (pH 7.9), 10; DTT, 0.25; Fru-1,6-P_2, 0.04; $CaCl_2$, 0.01 and, as indicated, EGTA, 0.02. Following preincubation the enzyme was injected into the assay mixture and Fru-6-P formation was followed spectrophotometrically as described in Materials and Methods.

To see whether the presence of Ca^{2+} is required after the enzyme was activated, chloroplast Fru-P_2ase was (i) preincubated for 10 min with Ca^{2+}, Fru-1,6-P_2 and DTT-reduced thioredoxin-f, (ii) preincubated for additional 10 min with EGTA, and (iii) injected into the mixture used for the assay of Fru-P_2ase activity. Table II shows that EGTA did not decrease the rate of catalysis of the activated chloroplast Fru-P_2ase, whereas when EGTA was added at the beginning of the preincubation the enzyme had no activity.

In addition, parallel experiments revealed that the activated chloroplast Fru-P_2ase subjected to gel filtration, to remove both Ca^{2+} and Fru-1,6-P_2, was eluted as an active enzyme (data not shown). On the basis of these results, it would seem that Ca^{2+} partakes only in the process of activation of chloroplast Fru-P_2ase by Fru-1,6-P_2 and DTT-reduced thioredoxin-f.

Fig.1 shows the response of chloroplast Fru-P_2ase activity to varying concentrations of Ca^{2+} in the preincubation. Although the

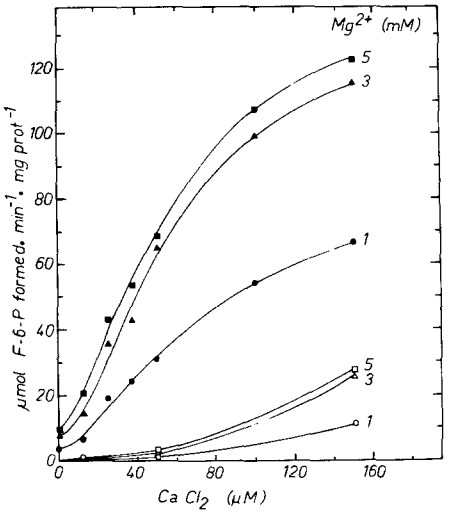

Figure 1. Effect of Ca^{2+} on the activation of chloroplast Fru-P_2ase. Chloroplast Fru-P_2ase (0.5 to 2.5 ug) was preincubated as indicated, with varying concentrations of Ca^{2+} ,Fru-1,6-P_2 and DTT, in the presence (● , ▲ , ■) and absence (○ , △ , □) of thioredoxin-f, as described under Materials and Methods. Following activation, Fru-6-P formation was assayed, as indicated, at three different concentrations of Mg^{2+} , according to Materials and Methods.

enzyme activity varied with the concentration of Mg^{2+} in the catalysis, the apparent $A_{0.5}$ for Ca^{2+} was not modified (55 µM). It should be pointed out that in the absence of thioredoxin, chloroplast Fru-P_2ase was activated by Fru-1,6-P_2 and Ca^{2+} . However, the rate of catalysis measured was one fifth of that obtained when the enzyme was preincubated with the complete system.

Effect of Ca^{2+} on the activity of chloroplast Fru-P_2ase

While attempting to maximize the activation of chloroplast Fru-P_2ase , we observed, in agreement with other investigators (30), that Ca^{2+} is an inhibitor of Fru-P_2ase activity. Fig.2 shows that the activity of the activated chloroplast Fru-P_2ase , decreased when the concentration of total Ca^{2+} increased in catalysis. The inhibition of Fru-P_2ase activity by Ca^{2+} depended on the concentration of Mg^{2+} used during the measurement of velocity; the apparent $I_{0.5}$ for Ca^{2+} were 7 µM, 26 µM, 40 µM when the activity was assayed at 1 mM, 3 mM and 5 mM Mg^{2+} respectively. In

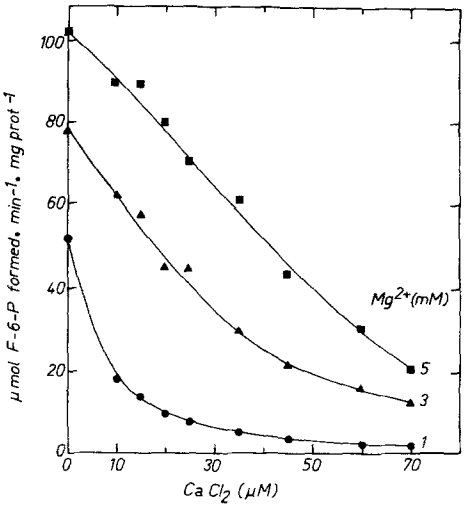

Figure 2. Effect of Ca^{2+} on the activity of chloroplast Fru-P_2ase. Chloroplast Fru-P_2ase (2.5 ug) was preincubated with Ca^{2+}, Fru-1,6-P_2 and DTT-reduced thioredoxin-f as described under Materials and Methods. After activation, Fru-P_2ase activity was assayed, as indicated, at three different concentrations of Mg^{2+} and varying concentrations of Ca^{2+} as outlined in Materials and Methods.

this particular experiment, EGTA was ommitted from the mixture for assaying Fru-P_2ase activity, except in those conditions where EGTA chelated the residual Ca^{2+} carried with the enzyme (maximum activity).

These results show that the inhibition produced by Ca^{2+} on the activated chloroplast Fru-P_2ase was more pronounced when the activity was measured at low concentrations of Mg^{2+}. Apparently, the activity of the activated Fru-P_2ase would be hindered by the Ca^{2+} carried with the preincubation mixture. We therefore decided to analyze the dependence on the concentration of Mg^{2+} of chloroplast Fru-P_2ase activity, assayed both in the presence and in the absence of EGTA. As shown in Fig.3, the activated Fru-P_2ase measured in the presence of 20 μM EGTA, was more active at any concentration of Mg^{2+}, than the enzyme assayed in the absence of EGTA. Moreover, the Mg^{2+} dependence of the activated enzyme was lower when the assay was carried out in the presence of EGTA ($A_{0.5}$ = 0.7 mM vs. $A_{0.5}$ = 2 mM).

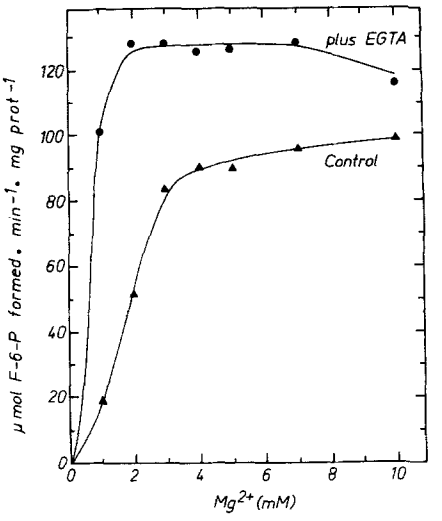

Figure 3. Effect of Mg^{2+} concentration on the activity of chloroplast Fru-P_2ase assayed in the presence and in the absence of EGTA. The enzyme (0.5 ug) was preincubated as described in section 2. Following preincubation the enzyme solution was injected into the assay mixture containing variable concentrations of Mg^{2+} , and as indicated, 20 uM EGTA. The rate of catalysis was followed according to Materials and Methods.

In parallel experiments we observed that the activity of the Fru-P_2ase subjected to gel filtration after activation was slightly modified by EGTA. These results suggest that EGTA exerts its effect on the activity of the activated chloroplast Fru-P_2ase by chelating the Ca^{2+} carried with the enzyme when the preincubation mixture is injected into the solution for assaying Fru-P_2ase activity.

CONCLUSIONS

Previous work (20,22,24) provided evidence that the Fru-P_2ase reaction consists of (i) a modification phase in which the enzyme is converted from an inactive to an active form and (ii) a catalytic phase in which the active enzyme hydrolyses Fru-1,6-P_2 to Fru-6-P and P_i . The present data show that the effect of Ca^{2+} on the modification phase is opposite to its action on catalysis. Ca^{2+} enhances the activation of chloroplast Fru-P_2ase mediated by Fru-1,6-P_2 and DTT-reduced thioredoxin-f (24); on the contrary,

it inhibits the catalytic activity. The dual effect of Ca^{2+} on chloroplast Fru-P_2ase is further stressed by Mg^{2+} ; the apparent $A_{0.5}$ for Ca^{2+} is not modified by Mg^{2+} , whereas the apparent $I_{0.5}$ for Ca^{2+} depends on the concentration of Mg^{2+} .

In addition the present data show that chloroplast Fru-P_2ase activated with Ca^{2+} , Fru-1,6-P_2 and DTT-reduced thioredoxin-f displays high activity at low (physiological) concentrations of both Fru-1,6-P_2 and Mg^{2+} (11-15,25).

Until now, the evidence available points to Ca^{2+} as an inhibitor of Fru-P_2ase (15,30). Moreover, no free Ca^{2+} would be present within the chloroplasts (12). Therefore, it remains to be investigated whether Ca^{2+} and Mn^{2+} (which can replace Mg^{2+} as a cofactor in catalysis(1)) are involved, in vivo, in the light mediated regulation of chloroplast Fru-P_2ase .

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