ENZYME REGULATION IN C_4 PHOTOSYNTHESIS

Role of Ca^{2+} in thioredoxin-linked activation of sedoheptulose bisphosphatase from corn leaves

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1. Introduction

Thioredoxin is now known to activate (or deactivate) a number of enzymes in different types of cells [1,2]. In regulating certain of these enzymes, thioredoxin appears to act alone, but with others, allosteric modifiers are involved. Members of the latter category include chloroplast fructose 1,6-bisphosphatase (Fru-P_2ase) and sedoheptulose 1,7-bisphosphatase (Sed-P_2ase), photosynthetic enzymes whose thioredoxin-linked activation is enhanced by their substrates – viz., fructose 1,6-bisphosphate (Fru-1,6-P_2) and sedoheptulose 1,7-bisphosphate (Sed-1,7-P_2) [3,4]. Recently, Hertig and Wolosiuk found that Ca^{2+} (or Mn^{2+}) is required in addition to Fru-1,6-P_2 for the thioredoxin-linked activation of Fru-P_2ase [5,6]. Interestingly, when present during catalysis, Ca^{2+} inhibited the enzyme.

In view of the similarity of its mode of regulation to that of chloroplast Fru-P_2ase, we deemed it worthwhile to study the effect of Ca^{2+} on a thioredoxin-linked substrate-specific Sed-P_2ase. Because of its greater stability and more extensive characterization, we selected the enzyme recently purified from leaves of corn [4], a classical C_4 plant, rather than a C_3 counterpart [7,8]. We now report that the corn Sed-P_2ase resembles Fru-P_2ase in that activation by chemically- or photochemically-reduced thioredoxin *f* (the chloroplast thioredoxin specific for the activation of Sed-P_2ase and Fru-P_2ase) requires Ca^{2+} (or Mn^{2+}) in addition to substrate (Sed-1,7-P_2). Moreover, as found with the chloroplast Fru-P_2ase, Sed-P_2ase was inhibited when Ca^{2+} was present during catalysis.

Abbreviations: Fru-P_2ase, fructose-1,6-bisphosphatase (EC 3.1.3.11); Fru-1,6-P_2, D-fructose-1,6-bisphosphate; Sed-P_2ase, sedoheptulose-1,7-bisphosphatase (EC 3.1.3.37); Sed-1,7-P_2, D-sedoheptulose-1,7-bisphosphate; DTT, dithiothreitol; EGTA, ethylene glycol bis-(β-aminoethyl ether) *N,N,N'N'* tetraacetic acid; Tris, Tris-(hydroxymethyl) amino-methane

Dedicated to Dr Luis F. Leloir on the occasion of his 75th birthday

Third paper of a series; first and second papers are identified in [4] and [19]

2. Materials and methods

2.1. *Reagents*

Biochemicals were obtained from Sigma Chemical Co. (St. Louis, MO). Solutions of Sed-1,7-P_2 were treated batchwise with Chelex-100. Other chemicals were obtained from commercial sources and were of the highest quality available.

2.2. *Analytical methods.*

Previously described methods were used for the determination of chlorophyll [9], protein [10], and P_i [11].

2.3. *Purification of the components of the ferredoxin–thioredoxin system and corn Sed-P_2ase*

Procedures previously devised were followed for the purification of ferredoxin [12], and thioredoxin *f* [13] from spinach leaves, and for ferredoxin–thioredoxin reductase from corn leaves [4]. The procedure in [4] was also used for purification of Sed-P_2ase from corn leaves.

2.4. *Preparation of chloroplast membranes*

Twice washed chloroplast membranes were pre-

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pared from chilled spinach leaves as described in [13, 14].

2.5. *Sed-P_2ase assay*

The activity of corn Sed-P_2ase was measured by the two-stage assay devised [15,16]. The P_i released from Sed-1,7-P_2 was determined after preincubation of the enzyme with Ca^{2+}, Sed-1,7-P_2, and thioredoxin *f* that was reduced either photochemically via the ferredoxin–thioredoxin system or chemically with DTT.

2.6. *Photochemical activation of Sed-P_2ase*

The reaction was carried out at 20°C in Warburg-Krippahl vessels containing (in the side-arm) 30 μg of corn Sed-P_2ase and (in the central compartment) 10 μg of spinach ferredoxin, 60 μg of spinach thioredoxin *f*, 7 μg of corn ferredoxin–thioredoxin reductase, twice-washed chloroplast membranes equivalent to 20 μg of chlorophyll, 40 μmol of Tris–HCl buffer (pH 8.4) and, as indicated (in μmol): $CaCl_2$, 0.08; and Sed-1,7-P_2, 0.2. Final volume, 0.42 ml. Vessels were first equilibrated with nitrogen gas for 6 min in the dark and were then illuminated for 5 min. The enzyme, then added from the side-arm, was activated by a 20 min illumination period. Light intensity, 20 000 lux. Following activation, an aliquot (0.05 ml) of the preincubation mixture was injected into the reaction mixture for assaying Sed-P_2ase activity.

2.7. *Chemical activation of Sed-P_2ase*

Corn Sed-P_2ase (6 μg) was preincubated for 20 min at 23°C in 0.1 ml of a solution containing 10 μmol of Tris–HCl buffer (pH 8.4) and, as indicated, 0.25 μmol of DTT, 0.05 μmol of Sed-1,7-P_2, 0.02 μmol of $CaCl_2$, 8 μg of spinach thioredoxin *f*. After preincubation, the mixture was injected in the reaction mixture for assaying Sed-P_2ase activity.

2.8. *Assay of Sed-P_2ase activity*

Sed-P_2ase activity was assayed at 23°C in a solution containing (in μmol): Tris–HCl buffer (pH 8.4), 100; $MgSO_4$, 10; Sed-1,7-P_2, 1.0; EGTA, 0.1. Final volume, 2.0 ml. After 4 min, the reaction was stopped by adding 2.0 ml of the reagent used for P_i analysis.

3. Results and discussion

During our earlier studies on the regulation of enzymes of photosynthetic CO_2 assimilation in chloroplasts, we devised an assay that consists of two stages, i.e., an activation stage followed by a reaction (catalytic) stage [15,16]. With this assay method, the enzyme is first activated by preincubation with a modifier (activation stage) and then is added to a mixture for measuring catalytic activity (reaction stage). Such a procedure makes it possible to study slow transitions in the conversion of inactive enzyme to a catalytically active form.

Table 1
Activation of corn Sed-P_2ase by Ca^{2+} Sed-1,7-P_2 and DTT-reduced thioredoxin *f*

Preincubation conditions	Sed-P_2ase activity (nmol P_i released)
Complete	23[a]
– DTT	3
– Sed-1,7-P_2	0
– Ca^{2+}	11
– thioredoxin *f*	13
Complete, + EGTA	1

[a] When Mn^{2+} (10 μM) replaced Ca^{2+} in the preincubation mixture, the corresponding Sed-P_2ase activity was 20 nmol P_i released

Corn Sed-P_2ase was preincubated for 20 min at 23°C in the chemical activation solution described under section 2, plus 0.1 μmol EGTA, as indicated. Following activation Sed-P_2ase activity was assayed as outlined in section 2

By applying the two-stage approach in the current study, we found that activation of corn leaf Sed-P_2ase requires Ca^{2+}. As shown in table 1, $CaCl_2$ (200 μM) doubled the activity of the enzyme when added to the preincubation mixture with chemically-(DTT) reduced thioredoxin *f* and substrate, Sed-1,7-P_2. The addition to the preincubation mixture of EGTA, a chelator with high affinity for Ca^{2+}, decreased the Sed-P_2ase activity to the basal (nonactivated) level (see also fig.1). As was earlier the case with Fru-P_2ase, a low (10 μM) concentration of Mn^{2+} could replace Ca^{2+} in the activation of Sed-P_2ase (table 1).

The activation of corn Sed-P_2ase was time-dependent. As shown in fig.1, activation of the enzyme by Ca^{2+}, Sed-1,7-P_2, and chemically-reduced thioredoxin *f* increased progressively with time up to 20 min. Thus, as reported in [4], corn Sed-P_2ase resembles other regulatory enzymes of the reductive pentose phosphate cycle in that it shows hysteretic behavior, i.e., its rate of activation is slow relative to its rate of catalysis [17].

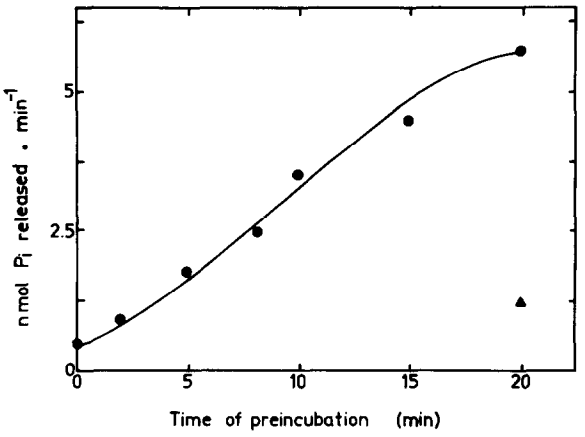

Fig.1. Effect of time of preincubation on the activation of corn Sed-P_2ase by Ca^{2+}, Sed-1,7-P_2 and DTT-reduced thioredoxin *f*. Corn Sed-P_2ase was preincubated in the presence (▲) or in the absence of EGTA (●). Except for time of preincubation, experimental conditions were as described in section 2.

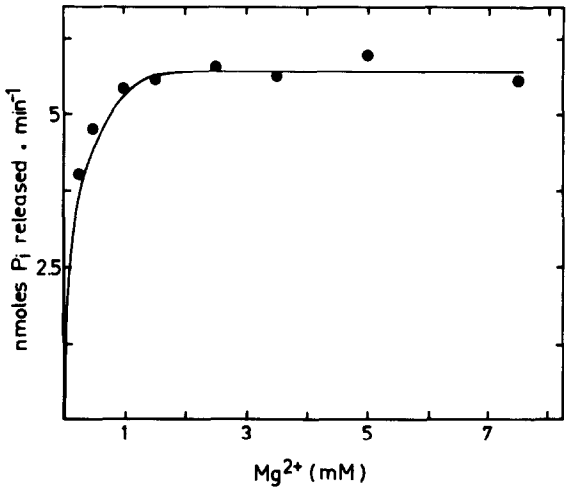

Fig.2. Effect of Mg^{2+} concentration on catalytic activity of corn Sed-P_2ase. Except for varying the Mg^{2+} concentration in the reaction mixture, conditions were as outlined in section 2.

In view of the finding that Ca^{2+} (or Mn^{2+}) enhanced the activation of Sed-P_2ase by chemically-reduced thioredoxin, the question arose as to whether a divalent metal was required when thioredoxin was reduced photochemically. An answer to this question was obtained in experiments based on our two-stage photochemical assay procedure devised earlier. Corn Sed-P_2ase was:

(i) Activated by preincubation in the light with Ca^{2+}, Sed-1,7-P_2, chloroplast membranes, and components of the ferredoxin–thioredoxin system (ferredoxin, thioredoxin, ferredoxin–thioredoxin reductase);

Table 2
Effect of Ca^{2+} and Sed-1,7-P_2 on the light activation of corn Sed-P_2ase via the ferredoxin–thioredoxin system

Preincubation conditions	Sed-P_2ase activity (nmol P_i released)
Light, complete	31.2
Light, – Ca^{2+}	22.8
Light, – Sed-1,7-P_2	11.6
Light, – Ca^{2+}, minus Sed-1,7-P_2	11.8
Dark, complete	13.2

Conditions and assay of enzyme activity were as described in section 2 for the photochemical activation of Sed-P_2ase. In the last treatment (Dark, complete) the vessels were kept in the dark throughout the preillumination and the reaction periods

(ii) Assayed in the dark for Sed-P_2ase activity.

As shown in table 2, maximal activation of the enzyme under these conditions was observed when Ca^{2+} was present during the activation stage. Significantly, a similar requirement of Ca^{2+} for activation was also found in independent experiments with the described [4] Fru-P_2ase from corn leaves (Wolosiuk, R. A., unpublished results).

Like its spinach counterpart [7], corn Sed-P_2ase requires a divalent cation for catalysis – Mg^{2+} or, less effectively, Mn^{2+}. When activated by chemically-reduced thioredoxin *f* and Sed-1,7-P_2 in the absence of Ca^{2+}, corn Sed-P_2ase required high (>10 mM) concentrations of Mg^{2+} ($A_{0.5}$ for Mg^{2+} was 5 mM). Addition of Ca^{2+} to the preincubation mixture significantly lowered the Mg^{2+} requirement. Thus, as shown in fig.2, when the preincubation mixture containing chemically reduced thioredoxin *f* and Sed-1,7-P_2 was supplemented with Ca^{2+}, Sed-P_2ase showed full activity at 1 mM Mg^{2+} ($A_{0.5}$ for Mg^{2+} was 0.3 mM). Activation of Sed-P_2ase in the presence of Ca^{2+} thus seems to increase the affinity of the enzyme for Mg^{2+} during catalysis.

In earlier studies with chloroplast Fru-P_2ase from spinach, it was found that Ca^{2+} has a dual effect on the ability of the enzyme to hydrolyze its substrate [5,6]. As described above, Ca^{2+} enhanced the activation of Fru-P_2ase by reduced thioredoxin *f* and its substrate, but when present during the reaction phase,

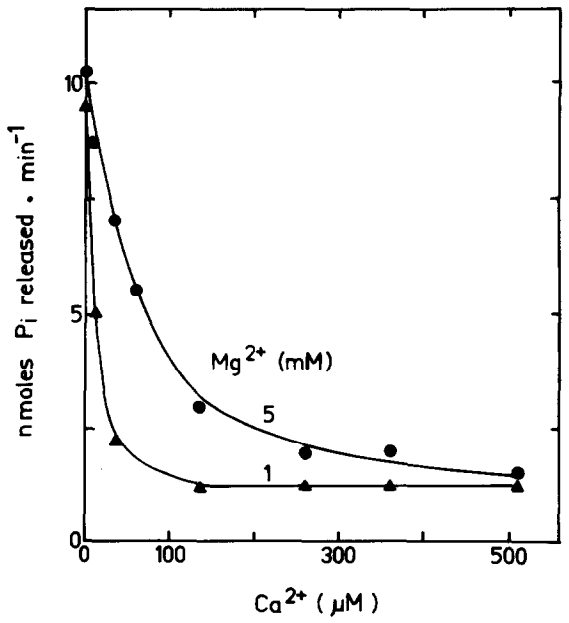

Fig.3. Effect of Ca^{2+} on the activity of corn Sed-P_2ase. Corn Sed-P_2ase was preincubated with Ca^{2+}, Sed-1,7-P_2, and DTT-reduced thioredoxin *f* as outlined in section 2. Following activation Sed-P_2ase activity was assayed, as indicated, at two different concentrations of Mg^{2+} and varying concentrations of Ca^{2+}, as described in section 2.

Ca^{2+} inhibited catalysis [18]. Because of its similarity to Fru-P_2ase, we considered it of interest to examine Sed-P_2ase in the latter capacity and found, in keeping with the earlier Fru-P_2ase results, an inhibitory effect of Ca^{2+} on catalysis. As shown in fig.3, Sed-P_2ase, which earlier had been activated by preincubation with Ca^{2+}, Sed-1,7-P_2, and chemically-reduced thioredoxin *f*, was inhibited when Ca^{2+} was added to the reaction mixture. Furthermore, similar to the spinach chloroplast Fru-P_2ase studied earlier, the Ca^{2+}-effected inhibition of catalysis depended on the Mg^{2+} concentration. The $I_{0.5}$ for Ca^{2+} dropped from 60 μM (at 5 mM Mg^{2+}) to 10 μM (at 1 mM Mg^{2+}) (cf. fig.3).

4. Concluding remarks

These results provide evidence that photosynthetic Sed-P_2ase purified from corn leaves resembles Fru-P_2ase in requiring both a divalent metal and its sugar phosphate substrate for activation by reduced thioredoxin *f*. Ca^{2+} (or Mn^{2+}) is required for the thioredoxin *f*-linked activation of both of these enzymes irrespective of whether the thioredoxin is reduced chemically by DTT or photochemically by chloroplasts supplemented with ferredoxin and ferredoxin–thioredoxin reductase. When activated in the presence of Ca^{2+}, corn leaf Sed-P_2ase becomes catalytically active at low concentrations of Mg^{2+} and Sed-1,7-P_2.

Like regulatory enzymes of the reductive pentose phosphate cycle in C_3 plants, the rate of activation of corn leaf Sed-P_2ase is slow relative to catalysis, thus confirming that this enzyme, like C_4 Fru-P_2ase, is a member of the hysteretic family of enzymes [4,6]. In view of current knowledge about Fru-P_2ase and Sed-P_2ase, it would appear that the ferredoxin–thioredoxin system could function jointly with a divalent cation (Ca^{2+} or Mn^{2+}) and effector substrates in the regulation of these enzymes in C_4 as well as C_3 photosynthesis. It is noteworthy that although the total concentration of Ca^{2+} in chloroplasts is apparently high [20,21], contrasting results were reported for the Ca^{2+} content of the stroma [21,22]. Thus, it remains to be seen whether Ca^{2+} is important in vivo in the activation of the fructose and sedoheptulose bisphosphatases or whether, as suggested in [23], Mg^{2+} is the cation active in this capacity. A related problem that remains unanswered is the relative photosynthetic contribution of the substrate-specific Sed-P_2ase studied here to the Sed-P_2ase activity of Fru-P_2ase that is unmasked either by dissociation [24] or by preincubation with Ca^{2+} [6].

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