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REGULATION OF THE ACTIVATION OF CHLOROPLAST FRUCTOSE-1,6-BISPHOSPHATASE: INHIBITION BY SPERMIDINE AND SPERMINE

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The activation of chloroplast fructose-1,6-bisphosphatase by fructose-1,6-bisphosphate, Ca^{2+}, DTT and chloroplast thioredoxin-f is prevented by either spermidine or spermine; on the contrary, other amino compounds do not replace polyamines in this reversible effect. On the other hand, neither spermidine nor spermine modify the catalysis of chloroplast fructose-1,6-bisphosphatase. The effect of spermidine, but not the effect of spermine, is reversed by increasing the concentration of Ca^{2+} in the activation; higher concentrations of fructose-1,6-bisphosphate or thioredoxin-f do not restore the control activity. The present results suggest that other regulatory mechanisms may control the activation of fructose-1,6-bisphosphatase in chloroplasts.

In cyanobacteria and chloroplasts of higher plants, the ferredoxin-thioredoxin system (1-2) (ferredoxin, ferredoxin-thioredoxin reductase and thioredoxin) has emerged as one mechanism by which light, through the photosynthetic electron transport system, modulates the activity of four enzymes of the Benson-Calvin cycle (3-6), one enzyme related to CO_2 fixation (7), two enzymes of the secondary plant metabolism (8-9), two enzymes of sulfate assimilation (10) and two enzymes involved in nitrogen metabolism (11). In vitro studies, carried out with those enzymes, revealed that the rate of conversion between two enzyme forms with different kinetic properties is slower than catalysis (enzyme hysteresis) (12). Reduced thioredoxin-f is the component of the ferredoxin-thioredoxin system that, both in C_3 and C_4 plants, partakes with other chloroplast components in the process of enzyme activation (13-15).

Recently we described that in the activation of chloroplast Fru-P_2ase, reduced thioredoxin-f acts synergistically with an effector (Fru-1,6-P_2 or

ABBREVIATIONS:

Fru-P_2ase, Fructose-1,6-bisphosphatase (EC. 3.1.3.11); Fru-1,6-P_2, D-Fructose-1,6-bisphosphate; DTT, dithiothreitol; Fru-6-P, D-Fructose-6-phosphate; Tris, Tris-(hydroxymethyl)-aminomethane.

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sedoheptulose-1,7-bisphosphate) and a bivalent cation (Ca^{2+} or Mn^{2+}) (16). On the contrary, once chloroplast Fru-P_2ase is active, thioredoxin-f does not modify the rate of Fru-1,6-P_2 hydrolysis. Since chloroplast Fru-P_2ase reaction may be regulated at two levels, in our studies on the light-mediated activation of enzymes, we explored the possibility that other chloroplast metabolites influence the activation, the catalysis or both. The present communication describes that the activation of chloroplast Fru-P_2ase by Fru-1,6-P_2, Ca^{2+}, DTT and thioredoxin-f is prevented by polyamines; on the other hand, these organic polycations do not influence the catalytic activity of the enzyme.

MATERIALS AND METHODS

All biochemicals and auxiliary enzymes were purchased from Sigma Chemical Co. (St. Louis, MO). Spinach leaves were obtained from the local market and kept frozen until used in the purification of chloroplast Fru-P_2ase (17) and thioredoxin-f (18). Enzyme activity was determined by following our two-stage assay for hysteretic enzymes. Chloroplast Fru-P_2ase (1.5 µg) was preincubated in plastic tubes at 23°C with 3.0 µg of chloroplast thioredoxin-f in a solution containing (in µmol): Tris-HCl buffer, pH 7.9, 10; DTT, 0.25; Fru-1,6-P_2, 0.04; and $CaCl_2$, 0.01. Final volume: 0.10 ml. After 15 min. the preincubation mixture was injected into the solution for Fru-P_2ase assay (16), and the rate of Fru-6-P formation was followed spectrophotometrically by a coupled enzyme assay (phosphohexose-isomerase and glucose-6-P dehydrogenase). Protein was assayed according to Lowry et al. (19).

RESULTS AND DISCUSSION

The two-stage assay used in previous studies revealed that the specific activity of chloroplast Fru-P_2ase is stimulated by preincubating the enzyme with Fru-1,6-P_2, Ca^{2+}, DTT, and thioredoxin-f (15). On the other hand, in the catalytic step, Fru-1,6-P_2 is obviously the substrate, Ca^{2+} acts as an inhibitor and neither DTT nor thioredoxin-f have any effect. In the course of studies on the regulation of the two processes that constitute the chloroplast Fru-P_2ase reaction, we found that certain polyamines inhibited the stimulation of enzyme activity. As shown in fig. 1, spermidine and spermine prevented the activation of chloroplast Fru-P_2ase by the concerted effect of Fru-1,6-P_2, Ca^{2+}, DTT, and thioredoxin-f, whereas putrescine was ineffective in this aspect. The concentrations of spermidine and spermine which decreased the specific activity of the enzyme to half of its maximal value were 3.8 mM and 0.7 mM, respectively. These

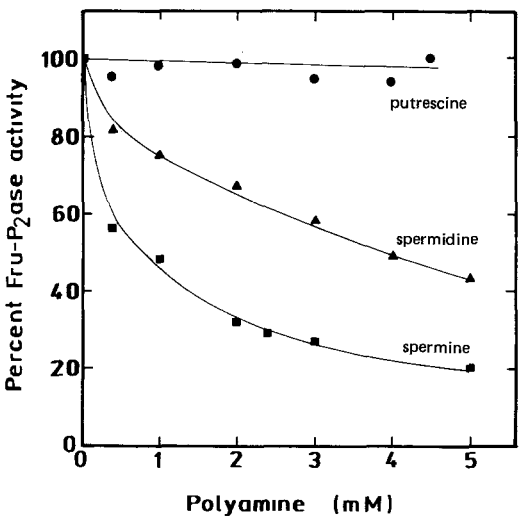

Fig. 1.- Effect of polyamines on the activation of chloroplast Fru-P_2ase. Chloroplast Fru-P_2ase (1.5 µg) was preincubated, as indicated, with varying concentrations of polyamines, as described under Materials and Methods. Following activation the rate of catalysis was assayed spectrophotometrically (16). The control activity, obtained in the absence of polyamines, was 57 µmol Fru-6-P-formed. min^{-1}. mg $prot^{-1}$.

results indicated that additional positive charges in these inhibitors enhance its interaction with the enzyme.

Polyamines are likely to function in the regulation of chloroplast enzymes by reversibly binding to a particular enzyme, and thus to modulate its activity. However, it is also known that as a result of charge neutralization, polyamines associate with macromolecules; in proteins, this interaction can lead to aggregation (20-22). To see whether the effect of polyamines is a reversible process or resulted from an irreversible inactivation of the enzyme, chloroplast Fru-P_2ase was (i) preincubated for 20 minutes with Fru-1,6-P_2, Ca^{2+}, DTT and thioredoxin-f in the presence of 5 mM spermine: (ii) transferred to a solution that contained all the previous components except the polyamine, and incubated for 15 minutes; and (iii) injected into the solution used for the assay of Fru-P_2ase activity. As shown in table I, the specific activity of chloroplast Fru-P_2ase was enhanced again once the concentration of spermine was decreased 50-fold by dilution. Similar results were obtained in parallel experiments in which spermidine was used instead of spermine. At the pH used in these studies (7.9), the inhibitory polyamines are totally protonated (23). On the basis that

TABLE I: Effect of prior treatment of chloroplast Fru-P_2ase with spermine on its activation by Fru-1,6-P_2, Ca^{2+}, DTT and thioredoxin-f.

Preincubation Conditions	Fru-P_2ase activity (μmol Fru-6-P formed . min^{-1} . mg $prot^{-1}$)
-Complete, 30 min	68
-Complete plus spermine, 20 min	17
-Complete plus spermine, 20 min and minus spermine, 15 min	51
-Complete plus spermine, 30 min	17

The enzyme (18 μg) was incubated at 23°C for 20 min in 0.05 ml of a solution containing 3 μg of chloroplast thioredoxin-f and the following (in μmol): Tris-HCl (pH 7.9) 5; DTT, 0.125; Fru-1,6-P_2, 0.02; $CaCl_2$, 0.005; and, as indicated, 0.25 μmol of spermine. An aliquot (0.01 ml) was injected into 0.5 ml of a solution of the same composition (except Fru-P_2ase) which contained, as indicated, 5 mM spermine. Following 15 min. incubation, 0.1 ml of the enzyme solution was injected into the mixture for assaying Fru-P_2ase activity (16).

the positively charged amino groups were the only functional components in spermidine and spermine which prevented the concerted activation of chloroplast Fru-P_2ase, we tested several other compounds. In this respect, ammonia, monoamines, aminoacids, and polyaminoacids (2 mM in the preincubation medium) were ineffective in preventing the synergistic stimulation of chloroplast Fru-P_2ase. On the contrary, two basic proteins, histone and protamine, completely inhibited the activity of Fru-P_2ase; however, in contrast to spermidine and spermine, the effect was not reversed by dilution.

In an attempt to ascertain whether the catalytic process of chloroplast Fru-P_2ase was modified when the enzyme was preincubated with Fru-1,6-P_2, Ca^{2+}, DTT, and thioredoxin-f in the presence of spermine, we determined the catalytic kinetic constants for both Mg^{2+} and Fru-1,6-P_2. As shown in fig. 2, the concentration of Mg^{2+} for half maximal Fru-P_2ase activity was not substantially changed; the $A_{0.5}$ for Mg^{2+} were 1.3 mM and 0.7 mM when the enzyme was activated in the presence and in absence of 0.5 mM spermine respectively. In parallel studies (not shown), we observed that the $S_{0.5}$ for Fru-1,6-P_2 of the spermine-treated chloroplast Fru-P_2ase remained constant with respect to the control enzyme. These results suggest, therefore, that the low specific activity of chloroplast Fru-P_2ase, following polyamine treatment, arose from lower amount of active enzyme rather than from a modified enzyme.

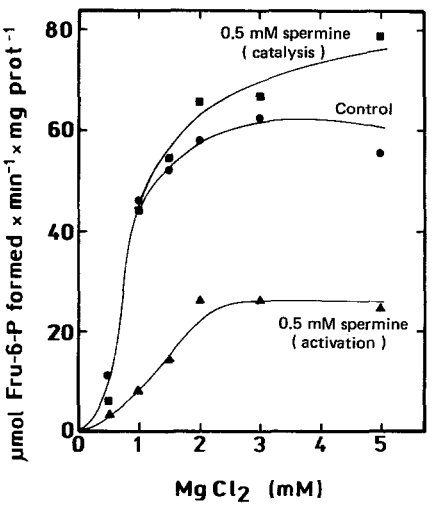

Fig. 2.- Effect of spermine on the activation and the activity of chloroplast Fru-P_2ase. The enzyme (1.5 µg) was preincubated in the presence (▲) or in absence (● ,■) of 0.5 mM spermine as outlined in Materials and Methods. After preincubation, the enzyme solution was injected into the assay mixture and the rate of catalysis was followed in the presence (■) or in absence (● ,▲) of 0.5 mM spermine.

Experiments designed to test the effect of polyamines on the catalysis showed that these organic polycations did not have any influence on this phase of chloroplast Fru-P_2ase. As depicted in fig. 2, when chloroplast Fru-P_2ase was activated by Fru-1,6-P_2, Ca^{2+}, DTT and thioredoxin-f, the presence of 0.5 mM spermine during the assay of Fru-1,6-P_2 hydrolysis did not change appreciably the activity of the enzyme. Although not shown, spermidine did not modify the catalytic phase of chloroplast Fru-P_2ase either.

The finding that the concerted activation of chloroplast Fru-P_2ase was prevented by either spermidine or spermine raised the question whether this effect could be reversed by the activating components. The results in Fig. 3 show that increasing the concentration of Ca^{2+} restored to control values the enzyme treated with spermidine. However, chloroplast Fru-P_2ase did not recover the specific activity of the control when preincubated in the presence of spermine. Moreover, as shown in fig. 4, high concentrations of either Fru-1,6-P_2 or thioredoxin-f did not abolish the effect of spermine. Whether polyamines as polycations compete with the bivalent cation at the activation site or bind directly to other anionic sites of chloroplast Fru-P_2ase causing a

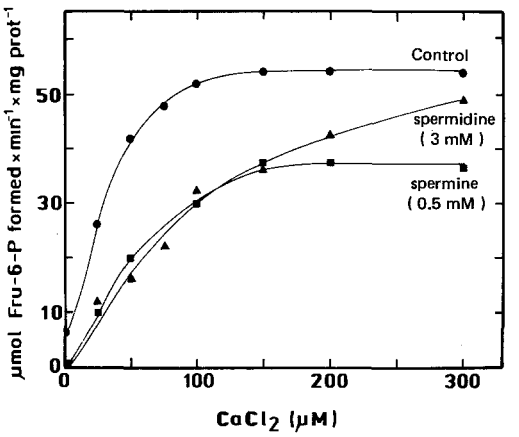

Fig. 3.- Effect of Ca^{2+} on the activation of chloroplast Fru-P_2ase. Chloroplast Fru-P_2ase (1.5 µg) was preincubated, as indicated, with varying concentrations of Ca^{2+} in the presence and absence of either spermidine or spermine, as described in Materials and Methods. Following preincubation, the enzyme solution was injected into the assay mixture and the rate of Fru-6-P formation was followed spectrophotometrically according to Materials and Methods.

conformational change remains to be established. In this regard, it is of interest that Ca^{2+} reversed totally the effect of spermidine, because both the bivalent cation and the polyamine are present in chloroplasts (24).

In chloroplasts, the formation of Fru-6-P from Fru-1,6-P_2 may be controlled at two levels: the light-mediated modulation of Fru-P_2ase and the catalysis. On

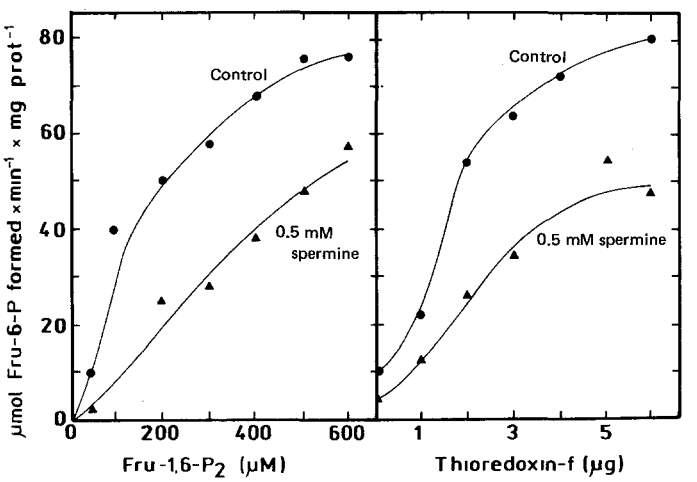

Fig. 4.- Effect of concentration of Fru-1,6-P_2 and chloroplast thioredoxin-f on the activation of chloroplast Fru-P_2ase. The enzyme (1.5 µg) was preincubated, as indicated, with varying concentrations of either Fru-1,6-P_2 or chloroplast thioredoxin-f, in the presence or in absence of 0.5 mM spermine according to Materials and Methods. After 15 min. preincubation, the enzyme solution was injected into the assay mixture and Fru-P_2ase activity was assayed as described in Materials and Methods.

the other hand, previous studies on the regulation of chloroplast Fru-P_2ase dealt with the activation of the enzyme and its reverse process, the deactivation (3, 17, 25). In this context, the posibility that each one of these processes may be in turn regulated was not considered. The above described results constitute an evidence that the concerted activation of chloroplast Fru-P_2ase may be prevented in a reversible manner by compounds which do not influence the catalytic step. Both spermidine and spermine directly affect the activation of chloroplast Fru-P_2ase mediated by an effector and a bivalent cation in the presence of reduced thioredoxin-f. It appears, therefore, that the amount of active enzyme in chloroplasts may be modulated by mechanisms other than the supply of the components of activation (reductants, effectors).

Spermidine, as well as an enzyme directly related to its synthesis (spermidine synthase), is present in cyanobacteria and chloroplasts of higher plants (24-26). More recently, it was shown that the incorporation of polyamines to chloroplasts is a light-dependent process (27). However, limited information is available on the physiological role of polyamines in chloroplasts; mainly, in senescence-related processes (28-29). Although more functions in chloroplasts may be modified by polyamines, the present results show that the function of an enzyme of the Benson-Calvin cycle is influenced by these organic polycations and they serve as a guide to the analysis of the role of polyamines in vivo.

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