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INHIBITION OF PHOTOSYNTHESIS IN ISOLATED SPINACH CHLOROPLASTS BY INORGANIC PHOSPHATE OR INORGANIC PYROPHOSPHATASE IN THE PRESENCE OF PYROPHOSPHATE AND MAGNESIUM IONS

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SUMMARY

Inhibition of photosynthesis in isolated spinach chloroplasts by P_i is decreased by the presence of PP_i and increased with increasing Mg^{2+} concentration. Previously reported regulation of this photosynthesis by protein factors from spinach leaves appears to be due mostly to pyrophosphate phosphohydrolase (EC 3.6.1.1) activity which converts PP_i to P_i and to the effects of PP_i and Mg^{2+} on this pyrophosphatase activity.

INTRODUCTION

Spinach leaf juice exerts a strong inhibitory effect on the photosynthetic fixation of CO_2 by isolated spinach chloroplasts in a medium containing PP_i [1]. One of the inhibitory factors was found to be Mg^{2+}, while another factor was a protein [2, 3]. In the presence of an appropriate amount of PP_i, a concentration of Mg^{2+} about equal to or slightly less than the PP_i concentration could stimulate the rate of CO_2 fixation, while addition of a small amount of the protein factor, in the absence of Mg^{2+}, could also stimulate the rate of CO_2 fixation. The presence of these amounts of Mg^{2+} and protein factor together could cause a very large (90 % or more) inhibition of the rate of CO_2 inhibition ("synergistic inhibition").

Other characteristics of this inhibitory effect are: (1) a requirement for a low level of PP_i; (2) the inhibition can be overcome by higher levels of PP_i, (3) either the protein factor or Mg^{2+} alone can inhibit when added in higher concentrations, and (4) inhibition by Mg^{2+} alone, protein factor alone, or a combination of the two is accompanied by increased movement of intermediate compounds of the reductive pentose phosphate cycle, especially dihydroxyacetone phosphate, out of the chloroplasts into the suspending medium [3].

A partially purified preparation of D-fructose-1,6-disphosphate 1-phosphohydrolase, EC 3.1.3.11 (hexosediphosphatase), was found to reproduce the activity of the protein factor, leading at first to a conclusion that the protein factor might be hexosediphosphatase [2]. However, heat treatment which denatured the protein

and completely destroyed the hexosediphosphatase activity released a factor which still exhibited inhibitory activity [3]. Moreover, the protein factor could be separated from hexosediphosphatase activity by gel filtration [4], and has a lower molecular weight than hexosediphosphatase.

A Mg^{2+}-dependent pyrophosphate phosphohydrolase, EC 3.6.1.1 (inorganic pyrophosphatase), was previously isolated from spinach chloroplasts by procedures which follow some of the same steps that are used in the purification of the protein factor [5]. The hexosediphosphatase from chloroplasts which gave the synergistic inhibitory activity reported earlier [2] was isolated by a procedure which also resembled that used in the isolation of inorganic pyrophosphatase [5]. The two enzymatic activities were separated by an ion-exchange chromatography on DEAE, but later work showed this to give incomplete separation. The protein factor (isolated either from previously isolated chloroplasts or from whole leaves) contains considerable pyrophosphatase activity, and significant amounts of P_i are released by the hydrolysis of PP_i under the conditions used in our studies. Photosynthesis by isolated chloroplasts is inhibited by such levels of P_i. The synergistic inhibitory activity we reported appears to be due to the stimulation of pyrophosphatase activity by Mg^{2+}, resulting in the release of P_i into the medium. PP_i reduces the inhibitory effect of P_i on chloroplast CO_2 fixation.

EXPERIMENTAL

Isolation of inhibition protein factor

A partially purified preparation of hexosediphosphatase was prepared from spinach leaves as previously reported [2], except that the spinach leaves were ground and used directly (no chloroplast isolation) and the $(NH_4)_2SO_4$ precipitation step was eliminated. Gel filtration on Sephadex G-150 (instead of G-200) resulted in separation of an hexosediphosphatase active fraction from a smaller molecular weight protein fraction, which was inhibitory to CO_2 fixation in isolated chloroplasts. The further purified hexosediphosphatase fraction did not cause such inhibition. The inhibitory protein factor has pyrophosphatase activity.

Chloroplast photosynthesis

Chloroplasts were prepared from fresh spinach as described earlier [6]. These chloroplasts were suspended in Solution C and placed in small serum-stoppered flasks (total suspension volume, 0.5 ml) on a shaker over an illuminated light table [6]. Varying amounts of protein factors, inorganic pyrophosphatase, or of P_i were added to flasks as indicated in the tables. After 5 min preillumination, 25 μl of solution of $NaH^{14}CO_3$ (0.15 M, 20 Ci/mole) was added and the chloroplasts were allowed to photosynthesize for 5 min before killing with 4.5 ml methanol.

For determination of acid-stable radioactive compounds formed, an aliquot portion of the killed chloroplast suspension was run onto filter paper, two drops of glacial acetic acid were added, and the wet area of filter paper was dried with a stream of N_2. After standing several hours, the radioactivity on the paper was measured with a pair of thin-window GM tubes. Chlorophyll content was determined, and $^{14}CO_2$ fixation was expressed as μmoles $^{14}CO_2$ fixed/mg of chlorophyll per h [6].

Assay for pyrophosphatase activity

Inorganic pyrophosphatase activity was assayed by measuring P_i released into the reaction mixture [7]. Yeast inorganic pyrophosphatase was purchased from Worthington Biochemical Corp.

Comparison of effect of protein factor and of P_i on chloroplast photosynthesis

In our usual assay of protein factor activity, the chloroplast preparation is in contact with the protein for a total of 10 min (5 min preillumination and 5 min with $H^{14}CO_3^-$) for measurement of fixation. The amounts of protein used in our experiments with protein factor contain sufficient inorganic pyrophosphatase activity to convert 5 mM PP_i to 10 mM P_i in the flask by the end of the 10-min period. In other experiments we added amounts of P_i sufficient to give concentrations ranging from 0.5–10.5 mM to the flasks at the beginning of the preillumination period.

RESULTS

Up to 93 % decrease in the rate of $^{14}CO_2$ uptake is seen with 10.5 mM P_i and no PP_i (Table I). These are the concentrations which would result from the complete conversion of 5 mM PP_i to P_i (the Solution C contains 0.5 mM P_i). Thus the severe inhibition reported to result from addition of protein factor plus Mg^{2+} can be accounted for if the additions result in substantial hydrolysis of PP_i. It is noteworthy that, at all levels of P_i, inhibition is greater with 4 mM Mg^{2+} than with 1 mM Mg^{2+}, and is less in the presence of PP_i than in the absence of PP_i. Thus there are some effects of Mg^{2+} and PP_i on the inhibition by P_i which do not require any exogenous pyrophosphatase activity.

The "synergistic inhibitory activity" seen upon addition of protein factor plus

TABLE I

EFFECTS OF P_i, PP_i AND Mg^{2+} ON RATE OF PHOTOSYNTHESIS BY ISOLATED SPINACH CHLOROPLASTS

Control rate (1.00 in table) was 170 μmoles of $^{14}CO_2$/mg chlorophyll per h.

Concentrations*		Relative Rates	
Mg^{2+} (mM)	P_i (mM)	$-PP_i$	$+5$ mM PP_i
1.0	0.5	0.82	1.00
1.0	1.5	0.73	0.95
1.0	2.5	0.63	0.87
1.0	5.5	0.35	0.63
1.0	10.5	0.065	0.31
4.0	0.5	0.47	0.96
4.0	1.5	0.32	0.89
4.0	2.5	0.21	0.73
4.0	5.5	0.079	0.49
4.0	10.5	0.027	0.18

* Addition of 0, 0.5, 1.0, 2.5 and 5.0 μmoles of P_i were made to the 0.5 ml of Solution C which already contains 0.5 mM P_i.

TABLE II

EFFECTS OF "PROTEIN FACTOR" AND OF YEAST PYROPHOSPHATASE ON RATE OF PHOTOSYNTHESIS BY ISOLATED SPINACH CHLOROPLASTS

Control rate (1.00 in table) was 125 μmoles $^{14}CO_2$/mg chlorophyll per h. Concentration of PP_i was 5.0 mM. The pyrophosphatase activity of the protein factor was 2.8 units/mg, while that of yeast pyrophosphatase was 600 units/mg (one unit = 1 μmole P_i released/min). Thus 240 μg of protein factor would release $0.24 \times 2.8 \times 10 = 6.7$ μmoles of P_i in 10 min (5 min preillumination plus 5 min with $H^{14}CO_3^-$) giving 13.4 mM P_i in the reaction mixture (volume 0.5 ml).

	Relative rates	
	1 mM Mg^{2+}	4 mM Mg^{2+}
No addition	1.00	0.76
Protein factor (240 μg)	0.83	0.14
Yeast pyrophosphatase (0.5 μg)	0.93	0.14

4 mM Mg^{2+} can be exactly duplicated by the addition of purified yeast pyrophosphatase (Table II). While the specific activity of pyrophosphatase in the protein factor is low, it is sufficient to convert all of the PP_i to P_i in the reaction flasks during 10 min. Little inhibition is seen with 1 mM Mg^{2+} and either protein factor or yeast pyrophosphatase.

DISCUSSION

The "synergistic" inhibition of CO_2 fixation in isolated spinach chloroplasts caused by addition of "protein factor" plus 4 mM Mg^{2+} in the presence of 5 mM PP_i is now seen to be the result of pyrophosphatase activity resulting in conversion of 5 mM PP_i to 10 mM P_i, which severely inhibits CO_2 fixation. The "synergistic" requirement for Mg^{2+} is not required for the P_i inhibition, since one of the roles of Mg^{2+} is to activate the pyrophosphatase [5]. There remains the observation that Mg^{2+} alone (with low levels of PP_i) can stimulate (at low levels) or inhibit (at higher levels) CO_2 fixation by isolated choroplasts. Also, Mg^{2+} alone increases the rate of movement of metabolites out of the chloroplasts, while increased PP_i (with low levels of Mg^{2+})has the opposite effects. These phenomena are perhaps due, in part, to endogenous pyrophosphatase and the resulting P_i released, and to the effects of Mg^{2+} in activating this enzyme. Excess PP_i could inactivate the enzyme by complexing Mg^{2+}.

That we failed to accept this rather simple explanation of the effects earlier was due in part to an early observed severe "synergistic" inhibition with only 1 mM PP_i plus 1.5 mM Mg^{2+} [3], which could only give a maximum of 2 mM P_i. This level of P_i does not cause severe inhibition. However, with spinach chloroplasts currently being isolated in this laboratory, severe synergistic inhibition with protein factor or yeast inorganic pyrophosphatase plus 1 mM PP_i and 1.5 mM Mg^{2+} is no longer observed, despite many experiments. Presumably this is yet another example of the variability of isolated spinach chloroplast preparations over the seasons and with differently grown spinach. It is possible that the impure factor used in earlier experiments contained factors other than pyrophosphatase which influence the rate of photosynthesis during the first 5 min. However, the severe inhibitory effect, de-

pendent on Mg^{2+} and PP_i concentrations, appears to be due for the most part to the inorganic pyrophosphatase as activated by Mg^{2+} in the presence of low levels of PP_i.

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