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Inhibition of photosynthesis in isolated spinach chloroplasts by added fructose-1,6-diphosphatase

Small amounts of juice from spinach leaves added to isolated spinach chloroplasts photosynthesizing with $H^{14}CO_3^-$ strongly affect the rate of fixation[1]. From 90% inhibition to 20% stimulation is observed, depending on the amount of added juice and on the ratio of added PP_i to added juice. Gel filtration of the juice produces two inhibitory fractions. Fraction A contains compounds of high molecular weight, including a complex of a large molecule with a small inhibitory molecule. Small amounts of Fraction A are inhibitory only in the presence of PP_i. Fraction B contains the small molecule without the large molecule, and its inhibition is much less affected by PP_i. A complex in Fraction A apparently dissociates to give a small molecule (Factor B) and a relatively inactive large molecule. Inactive retentate, obtained by dialysis of leaf juice, greatly increases the inhibitory effect of limited amounts of the Factor B in the presence of PP_i, added in optimal amounts.

We have now found that purified fructose diphosphatase (D-fructose-1,6-diphosphate 1-phosphohydrolase, EC 3.1.3.11) from spinach chloroplasts duplicates the behavior of inactive retentate just described, in nearly all respects.

Spinach juice was isolated and clarified as described previously[1]. Factor B was partially purified by treatment of the clarified juice as follows: 1.4 l of clarified juice, from 4 kg spinach leaves, was ultrafiltered (through Amicon "DIAFLO" UM-10). The ultrafiltrate was concentrated by vacuum freeze-drying and dissolved in 140 ml water. This solution was boiled for 4 min. To the combined supernatant solution *plus* washings, methanol was added to 80%. The 10 g white precipitate was dissolved in 96 ml of water and gel-filtrated in 16 ml portions, through Sephadex G-10 in a column (55 cm × 3.4 cm). Factor B, identified by absorption in ultraviolet at 255 nm, was found in the fractions 330–360 ml. After vacuum freeze-drying, the 1.5 g of white powder was dissolved in 50 ml water and precipitated again by addition of methanol to 80%, giving 0.7 g.

Fructose diphosphatase was purified as follows: Spinach chloroplasts, isolated as before, were sonicated at 0° for 30 sec (Biosonik, Model BPI, Bronwill Scientific Co., Rochester, N.Y.) and then centrifuged 2 h at 36000 × *g*. From the supernatant solution, the precipitate from 33 to 70% acetone was taken (after 3 h at −14°), redissolved in buffer (50 mM Tris, pH 7.4, 2 mM glutathione, 0.2 mM EDTA, 1 mM $MgCl_2$), dialyzed against water for 4 h and buffer for 8 h twice, then centrifuged at 36000 × *g* for 10 min. The supernatant solution was passed through a DEAE column. The eluate was fractionated with $(NH_4)_2SO_4$, the 40 to 90% precipitate being taken, dissolved, and dialyzed as before, and the retentate was gel-filtered with Sephadex G-200 (always with the same buffer). Eluent fractions were assayed either by P_i released[2,3] or by NADPH produced ($A_{340\ nm}$) in the presence of fructose diphosphate, glucose phosphate isomerase (EC 5.3.1.9), glucose-6-phosphate dehydrogenase (EC 1.1.1.1.49) and NADP.

Spinach chloroplasts were isolated and assayed for rate of photosynthesis in the presence of various additions as described before[1,4].

The effects on photosynthesis rate in isolated spinach chloroplasts of additions of fructose diphosphatase and of Factor B, separately and together, are given in

TABLE I

PHOTOSYNTHESIS RATES IN ISOLATED SPINACH CHLOROPLASTS WITH ADDED FRUCTOSE DIPHOSPHATASE AND FACTOR B

Isolated spinach chloroplasts photosynthesized for 10 min with $H^{14}CO_3^-$ (see text). Additions were made prior to start of 5 min preillumination. Control was in complete Soln. C (*cf.* ref. 1) (which contains 5 mM PP_i). Weight of "Factor B" is given as weight of the impure powder, prepared as described in text. Enzyme was dissolved in Soln. C *minus* PP_i and ascorbate and added in 80 μl per flask. Total volume of each flask was 0.5 ml, and each flask contained chloroplasts with 46 μg chlorophyll.

Additions	*Rate* (μ*moles* ^{14}C/*mg chlorophyll per h*)	*Inhibition* (%)
Control	104	—
+240 μg fructose diphosphatase	83	20
+150 μg Factor B	78	25
+240 μg fructose diphosphatase +150 μg Factor B	3	97

Table I. As was seen previously in the study with Factor B *plus* retentate from dialysis of juice[1], enzyme and Factor B exhibit a strong synergistic inhibitory effect on the rate of photosynthesis.

The effect of added PP_i on the inhibition by a combination of Factor B and of diphosphatase is shown in Fig. 1. The effects are similar to those seen upon variation of PP_i concentration with added spinach juice[1]. With smaller amounts of enzyme and Factor B, only small inhibition is seen without added PP_i. As the concentration of added PP_i increases for a given amount of enzyme and Factor B, a maximum inhibition is reached. A further threefold increase in PP_i concentration reverses the inhibition.

Thus, added fructose diphosphatase causes the principal effects on photosynthesis rates in isolated chloroplasts seen previously with spinach juice retentate. With either retentate or purified enzyme, the dependence of effects on Factor B and added PP_i is the same. However, we could not yet demonstrate the formation of a complex between Factor B and purified enzyme. Possibly the complex seen with gel filtration

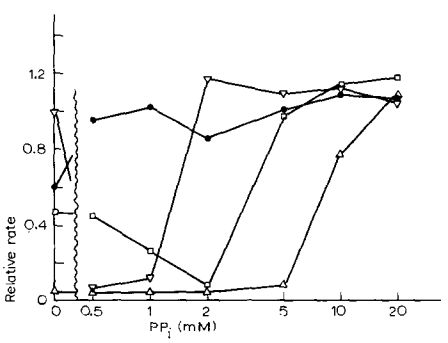

Fig. 1. Dependence of photosynthetic CO_2 fixation rate of added fructose diphosphatase, Factor B, and PP_i. Control (complete Soln. C)[1]: 149 μmoles CO_2 fixed per mg chlorophyll per h. 46 μg chlorophyll per flask, 40 μg fructose diphosphatase per flask (except in control). Amount of Factor B (prepared as in text) μg per flask: ●, 0; ▽, 25; □, 50; △, 125.

of juice (Factor A) was, in fact, a complex between Factor B and some protein other than diphosphatase. Fraction A would thus include that complex *plus* fructose diphosphatase.

A full interpretation of these effects must await the results of kinetic experiments on the behaviors of all intermediate compounds in the photosynthetic carbon reducing cycle. Earlier studies showed that Fru-1,6-P_2 as well as several other compounds move rapidly between the isolated, photosynthesizing chloroplasts and the suspending medium[5]. In contrast, Fru-6-P was well retained in the chloroplasts. The ratio of the diphosphate found in the medium to diphosphate retained in the chloroplasts was found to be 35, while the corresponding ratio for the monophosphate was nearly 100-fold less. We suggest that added fructose diphosphatase converts Fru-1,6-P_2 in the medium to Fru-6-P. This conversion would lower the concentration of Fru-1,6-P_2 both inside and outside the chloroplasts and, at the same time, would convert it to a metabolite which may not readily reenter the chloroplasts.

Fru-1,6-P_2 stimulated CO_2 fixation by isolated spinach chloroplasts which were capable only of very low fixation rates[6]. With isolated chloroplasts fixing CO_2 at high rates, Fru-1,6-P_2 had a stimulatory effect, whereas other carbon cycle intermediate compounds and cofactors tested were ineffective or inhibitory[7]. This effect was seen only after 10 min photosynthesis, when the rate of fixation declines. Thus, the level of Fru-1,6-P_2 seems to be important in the control of photosynthesis rate.

PP_i at higher concentrations inhibits fructose diphosphatase[8]. Thus, we can understand that PP_i in high levels reverses the inhibition of fixation due to this enzyme. There remains to be explained the stimulation of inhibition by Factor B and PP_i in lower concentration in the presence of the enzyme, and also the general stimulation of fixation rates by PP_i alone[4,7].

One possibility, suggested earlier[1], is that PP_i and Factor B both enter the chloroplasts where they might affect the activity of internal fructose diphosphatase. However, unpublished experiments show no effect of Factor B in moderate amounts on diphosphatase activity. Also, PP_i added externally does not appear to be metabolized by the chloroplasts (except after hydrolysis)[4].

We are now inclined to attribute effects of PP_i to an alteration of the rate of transport through the chloroplast outer membrane of Fru-1,6-P_2 and other intermediate compounds. Factor B may have a similar role. Thus, PP_i and Factor B might regulate *in vivo* the flow of carbon from the photosynthetic carbon cycle to the biosynthetic reactions in the cytoplasm.

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