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Chloroplast and Cytoplasmic Enzymes

IV. PEA LEAF FRUCTOSE 1,6-DIPHOSPHATE ALDOLASES[1, 2]

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ABSTRACT

Several peaks of aldolase activity are found in the isoelectric focusing pattern of pea (*Pisum sativum*) leaf chloroplast extracts. One peak, separated by 0.5 pH unit from the major chloroplast aldolase peak, is found when cytoplasmic extracts are focused. The chloroplast and cytoplasmic enzymes have a pH 7.4 optimum with fructose 1,6-diphosphate. The Michaelis constant for fructose-1,6-diphosphate is 19 μM for the chloroplast, 21 μM for the cytoplasmic enzyme, and for sedoheptulose 1,7-diphosphate, 8 μM for the chloroplast enzyme, 18 μM for the cytoplasmic enzyme. Both enzymes are inhibited by D-glyceraldehyde 3-phosphate and by ribulose 1,5-diphosphate. The similarity in the catalytic properties of the isoenzymes suggests that both enzymes have an amphibolic role in carbon metabolism in the green leaf.

In mammalian systems fructose-1,6-diP aldolases from different tissues have kinetic properties related to the function (synthetic or degradative) of the enzyme. Differences in the ratio V_{max} fructose-1,6-diP cleavage to V_{max} fructose-1,6-diP synthesis led Rutter and coworkers (15) to conclude that muscle aldolase is "more effective in cleaving fructose-1,6-diP whereas liver aldolase is relatively more effective in synthesis." Since in green plants chloroplast and cytoplasmic aldolases might also be expected to have opposing functions, it seemed of interest to compare the properties of the plant aldolases. Although spinach whole leaf (8, 14) and chloroplast (7) aldolases and a pea seed aldolase (10, 19, 20) had been studied prior to the present investigation, no higher plant aldolase known to be cytoplasmic had been characterized. The purpose of the experiments reported here was to characterize the pea leaf chloroplast and cytoplasmic aldolases and to compare the physical and kinetic properties of the isoenzymes.

The results indicate that the chloroplast and cytoplasmic aldolases are exceedingly similar enzymes, the pH optima and Michaelis constants for fructose-1,6-diP, fructose-1-P, and sedoheptulose-1,7-diP being almost identical. The membrane-bound form of the chloroplast enzyme resembles the soluble enzyme. The similarity in kinetic parameters suggests that, despite the compartmentalization of the plastid enzyme, the chloroplast and cytoplasmic aldolases have similar, not opposing, functions within the green leaf.

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[2] A preliminary report of these findings was presented at the II International Congress of Photosynthesis Research, Stresa, Italy, June, 1971 (4).

MATERIALS AND METHODS

Plant Material. Pea plants (*Pisum sativum*, var. Little Marvel) were grown as described previously (2).

Isoelectric Focusing. Chloroplast and cytoplasmic extracts, prepared as described previously (5) (except that the chloroplast extract was centrifuged 45 min at 500,000*g*), and containing 10 to 15 mg of protein, were subjected to electrophoresis for 1.5 days in pH 3 to 6 ampholytes at 450 v or pH 4 to 6 ampholytes at 500 v, in a 100-ml LKB isoelectric focusing column at 10 C. Ampholyte and electrode solutions were made up with 5 mM 2-mercaptoethanol. The anode solution (made with 0.5 N H_2SO_4) was at the bottom of the column. The cathode solution was 0.25 N NaOH. The density gradient was usually made with sucrose. Sorbitol was used in place of sucrose in experiments where aldolase activity was measured as fructose-1,6-diP formation. Fractions (9 drops, 0.7 ml) were collected and analyzed for enzyme activity. The pH of the individual fractions was measured at 0 C using a Radiometer pH meter 26.

Purification of Chloroplast and Cytoplasmic Aldolases. Chloroplast and cytoplasmic extracts were prepared as described previously (5) except that the procedure was scaled up for 1 kg of tissue, and the entire shoot of the plant was used. Chloroplasts were usually prepared beforehand and stored at −20 C. The method of Fluri *et al.* (8) for purification of spinach aldolase was followed, with some modifications. The chloroplast extract, in 10 mM potassium phosphate buffer, pH 7.5, was brought to pH 5.5 with 3.5 M acetic acid, and the supernatant fraction was made to 40% saturation with solid $(NH_4)_2SO_4$. Aldolase was extracted from the precipitate with 0.1 M tris-HCl, pH 7.5, which was 20% saturated with $(NH_4)_2SO_4$ and reprecipitated by addition of $(NH_4)_2SO_4$ to 55% saturation. The precipitated protein was dissolved in 2 to 4 ml of 13 mM potassium glycine, pH 7.5, 5 mM 2-mercaptoethanol, passed through a Sephadex G-25 column in the same buffer and further purified by isoelectric focusing in pH 4 to 6 ampholytes on a 440-ml column. Active fractions were passed through Sephadex G-25 in 10 mM potassium HEPES, 5 mM 2-mercaptoethanol to remove ampholyte and sucrose. Most of the chloroplast aldolase activity is lost after 2 to 3 weeks storage at −20 C.

The cytoplasmic extract was brought to pH 5 with 3.5 M acetic acid, centrifuged, and the supernatant solution was fractionated with solid $(NH_4)_2SO_4$. Aldolase activity was found in the fraction precipitating between 30 and 50% $(NH_4)_2SO_4$ saturation. The precipitated protein was dissolved in 25 ml of 10% $(NH_4)_2SO_4$ saturated, 0.1 M pH 7.4 tris-HCl, and reprecipitated

by the addition of $(NH_4)_2SO_4$ to 65% saturation. The precipitate was dissolved in 10 mM pH 7.4 potassium phosphate and was desalted by being passed through a Sephadex G-25 column in the same buffer. The excluded protein was adsorbed onto DEAE-cellulose (2.54 cm × 7 cm column) and eluted with a linear NaCl gradient (0–0.3 M NaCl in 10 mM potassium phosphate, pH 7.4). The active fractions were made to 65% saturation with $(NH_4)_2SO_4$ and subjected to gel filtration on a Sephadex G-200 column. The most highly purified fractions have a specific activity of 9 μmoles of triose-P formed per min per mg protein. The cytoplasmic enzyme is stable at −20 C for at least 6 months.

Enzyme Assays. Aldolase activity was routinely assayed as fructose-1,6-diP cleavage by the method of Wu and Racker (see ref. 5) using α-glycerol-P dehydrogenase and triose-P isomerase as coupling enzymes. The coupling enzymes were desalted by passage through Sephadex G-25 for studies of the kinetic properties of the aldolases. Triose-P isomerase was omitted when sedoheptulose-1,7-diP or fructose-1-P was used as substrate, since glyceraldehyde-3-P is not a product of aldol cleavage of these compounds. The hydrazine assay of Sibley and Lehninger (17) was used when *p*-hydroxymercuribenzoate was tested as an inhibitor. Aldolase activity was measured as fructose-1,6-diP synthesis by the colorimetric method of Roe as modified by Penhoet *et al.* (13).

Determination of Kinetic Constants. Six substrate levels, varied at even reciprocal intervals between 12 and 120 μM with fructose-1,6-diP as substrate, between 4 and 40 μM with sedoheptulose-1,7-diP, and between 11.5 and 115 mM with fructose-1-P, were used. Values and standard error for K*m* and K*i* were estimated as described previously (6).

Protein Determination. Protein was estimated by the biuret method as described previously (5) or, during the latter stages of enzyme purification, by the method of Warburg and Christian (see ref. 12).

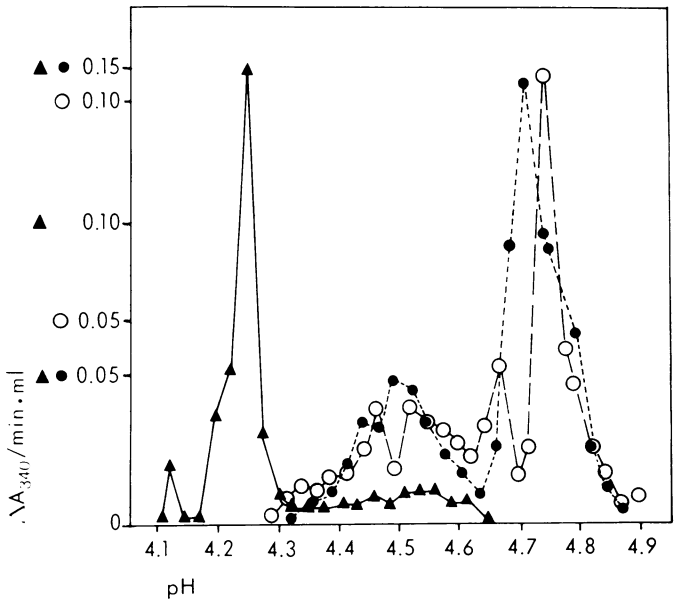

FIG. 1. Isoelectric focusing patterns of pea leaf fructose-1,6-diP aldolases. Soluble chloroplast (500,000*g* supernate, ○), particulate chloroplast (500,000*g* precipitate, ●) and cytoplasmic (▲) extracts were subjected to electrophoresis in pH 4 to 6, 4 to 6, and 3 to 6 ampholytes, respectively. pH values (abscissa) are the best straight-line fit to the actual values. Fraction size, as plotted, is therefore consistent within runs but not between runs. The chloroplast peaks are clearly separate from the cytoplasmic peak. This experiment was repeated 12 times with soluble chloroplast extracts, 4 times with particulate chloroplast fractions, and 5 times with cytoplasmic extracts with consistent results.

Reagents. DL-Glyceraldehyde-3-P, dihydroxyacetone-P dimethylketal (di-monocyclohexylamine salt), erythrose-4-P, DPNH, DPN, TPN, HEPES, tris, rabbit muscle aldolase, glyceraldehyde-3-P dehydrogenase, α-glycerol-P dehydrogenase, and α-glycerol-P dehydrogenase, triose-P isomerase mixture, ATP, ADP, AMP, fructose-1-P, fructose-1,6-diP, ribulose-1,5-diP, and sorbitol were obtained from Sigma Chemical, metal-free $(NH_4)_2SO_4$ and sucrose from Mann, Serva DEAE-cellulose and phosphocellulose from Gallard-Schlesinger, Sephadex from Pharmacia, and Ampholine from LKB. Dihydroxyacetone-P was prepared from the ketal by acid hydrolysis. Free acids were neutralized with KOH.

Sedoheptulose-1,7-diP was prepared from erythrose-4-P and dihydroxyacetone-P using rabbit muscle aldolase, by a modification of the procedure of Smyrniotis and Horecker (18). D-Glyceraldehyde-3-P was prepared from D-3-P-glyceric acid using P-glyceric acid kinase and glyceraldehyde-3-P dehydrogenase. L-Glyceraldehyde-3-P was prepared by converting the D-isomer from the commerically available racemic mixture to α-glycerol-P with triose-P isomerase and α-glycerol-P dehydrogenase.

All other reagents were the highest quality commercially available. Pea seeds were obtained from Atlee Burpee Company.

RESULTS

The apparent isoelectric point of the cytoplasmic aldolase is at pH 4.22 (Fig. 1). This experiment was repeated five times and maximum activity was found consistently at this pH. Additional minor peaks, which probably represent degraded pH 4.22 aldolase, were found in other experiments. Although the specific activity of the cytoplasmic enzyme is greater than that of the chloroplast enzyme in crude extract, during focusing the cytoplasmic enzyme loses much more activity.

When chloroplast aldolase is focused, four peaks of activity are found between pH 4.5 and 4.75 (Fig. 1). This experiment was repeated 12 times with crude chloroplast extracts or partially purified enzyme with similar results. Although the relative activity in the several peaks varies between experiments, most of the activity is invariably found at pH 4.75. The minor peaks represent either additional aldolase isoenzymes or degraded aldolase. Clearly the cytoplasmic enzyme is not identical with the chloroplast enzyme. There is no evidence for hybridization *in vivo* between chloroplast and cytoplasmic enzymes.

A portion of the chloroplast aldolase activity was associated with a green particulate fraction (500,000*g*, 30 min). The aldolase focusing pattern for this fraction was very similar to that of the soluble chloroplast enzyme (Fig. 1). Aldolase activity corresponded to chlorophyll as measured at 663 and 645 nm in 80% acetone. After focusing the aldolase in this fraction was still membrane-bound; after 40-fold dilution with 13 mM potassium glycine, pH 7, and 30 min centrifugation at 500,000*g* the activity was associated with the green precipitate. When particulate and soluble chloroplast fractions are treated with Triton X-100 and focused, the major peaks do not coincide exactly. The bound enzyme may be an additional aldolase isoenzyme.

Sedoheptulose-1,7-diP aldolase activity coincides with fructose-1,6-diP aldolase activity when chloroplast or cytoplasmic extracts are focused in pH 3 to 10 or pH 3 to 6 ampholytes. These results are consistent with the suggestion of Brooks and Criddle (7) that the chloroplast enzyme functions *in vivo* with

both of these substrates. It would appear that a single cytoplasmic aldolase, also, functions with both ketose phosphates.

No additional peaks of fructose-1,6-diP aldolase activity, measured as cleavage or as condensation, are found when extracts are focused in pH 3 to 10 or pH 3 to 6 ampholytes.

The chloroplast and cytoplasmic aldolases have broad, almost identical pH activity curves and the same optimum pH, 7.4, with fructose-1,6-diP as substrate (Fig. 2). With fructose-1-P the pH optimum is 7 for the chloroplast enzyme, about 6.5 for the cytoplasmic enzyme (Fig. 3). With sedoheptulose-1,7-diP the optimum pH is 7.8 for the cytoplasmic enzyme, 7.3 for the chloroplast enzyme (Fig. 4).

The pea leaf aldolases have essentially identical Michaelis constants for fructose-1,6-diP and similar Michaelis constants for sedoheptulose-1,7-diP (Table I). The extrapolated maximal velocity is 2-fold greater with fructose-1,6-diP than with sedoheptulose-1,7-diP. The apparent affinity for fructose-1-P is 500 times lower than for the diphosphate. These values are similar to those reported for the spinach enzymes (7, 8).

Ribulose-1,5-diP, an analog of the furanose form of fructose-1,6-diP (9), is both a poor substrate and a potent competitive inhibitor (Table I) of the pea leaf aldolases. Less than 5% of the activity obtained with fructose-1,6-diP is obtained with the pentulose diphosphate. The apparent affinities of the isoenzymes for ribulose-1,5-diP are about 10-fold lower than for fructose-1,6-diP (compare inhibitor constants and Michaelis constants, Table I). Ribulose-diP also affects the activity of the pea leaf triose-P and ribose-5-P isomerases (2, 3).

Michaelis constants for dihydroxyacetone-P and D-glyceraldehyde-3-P could not be estimated because D-glyceraldehyde-3-P is an inhibitor. In the presence of L-glyceraldehyde-3-P the isoenzymes are inactivated (Fig. 5). D-Glyceraldehyde-3-P inhibits the enzymes, but, since our aldolase preparations were contaminated with triose-P isomerase, it was not possible to study inactivation quantitatively with this stereoisomer. The rabbit muscle enzyme is inhibited by both isomers (11). Inhibition by D-erythrose-4-P was mixed (and varied between

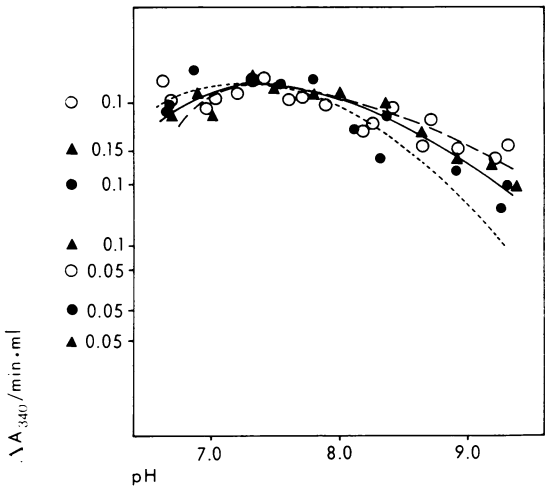

FIG. 2. pH dependence of soluble chloroplast (○, dashed line), particulate chloroplast (500,000*g* precipitate, ●, dotted line) and cytoplasmic (▲, solid line) aldolase catalyzed cleavage of fructose-1,6-diP. Each cuvet contained 100 μmoles of tris-HCl, 2.5 μmoles of fructose-1,6-diP, 0.1 μmole of DPNH, excess α-glycerol-P dehydrogenase, triose-P isomerase, and enzyme in a total volume of 1 ml. pH was determined after activity was measured. This experiment was done five times with chloroplast soluble, twice with chloroplast particulate, and four times with cytoplasmic aldolase with consistent results. The pH activity curves for the enzymes are very similar.

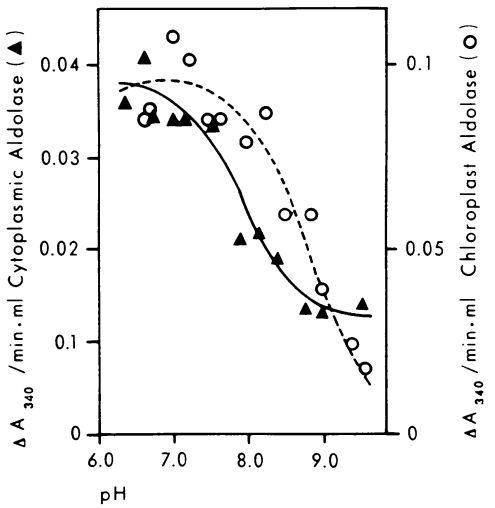

FIG. 3. pH dependence of chloroplast (○) and cytoplasmic (▲) aldolase catalyzed cleavage of fructose-1-P. Each cuvet contained 200 μmoles of tris-HCl, 400 μmoles of fructose-1-P, 0.1 μmole of DPNH, excess α-glycerol-P dehydrogenase, and enzyme in a total volume of 1 ml. pH was determined after activity was measured. The experiment was repeated twice with different preparations of enzyme in each case. The pH activity curves are similar but not identical.

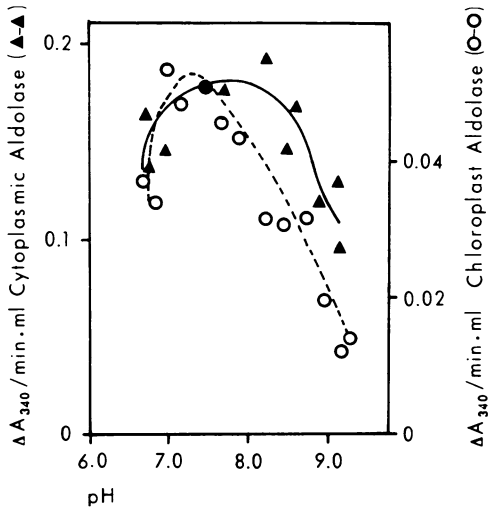

FIG. 4. pH dependence of chloroplast (○) and cytoplasmic (▲) aldolase catalyzed cleavage of sedoheptulose-1,7-diP. Each cuvet contained 100 μmoles of tris-HCl, 6 μmoles of sedoheptulose-1,7-diP, 0.1 μmole of DPNH, excess α-glycerol-P dehydrogenase and enzyme in a total volume of 1 ml with chloroplast enzyme. Less sedoheptulose-1,7-diP (1.3 μmoles) was used in this particular experiment with the cytoplasmic enzyme. Similar results were obtained with the higher concentration of heptulose-diP, and in another experiment with the chloroplast aldolase. pH was determined after activity was measured. The pH activity curves of the two aldolases are similar but not identical.

experiments), probably due to competition for and irreversible combination with the active site. Only high concentrations (6 mM) of this compound were inhibitory; the isoenzymes were not affected by 0.6 mM D-erythrose-4-P. The pea leaf aldolases are less sensitive to inhibition by L-glyceraldehyde-3-P and erythrose-4-P than the rabbit muscle aldolase (11) but more sensitive than the kidney and liver enzymes (1, 11).

ADP, 3-P-glyceric acid, citrate, and EDTA are competitive

Table I. *Properties of Fructose-1,6-diphosphate Aldolases*

Property	Cytoplasmic	Chloroplast	Chloroplast Membrane-bound	Spinach Whole Leaf (8)	Spinach Chloroplast (7)	Pea Seed (10)	Rabbit Muscle (15, 16)	Rabbit Liver (15, 16)
pI′	4.22	4.5–4.75	4.5–4.75					
pH optimum								
Fructose-1,6-diP	7.4	7.4	7.4	6–9	7.5	7.7–8.8	7–9	7.8
Fructose-1-P	6.5	7.0						
Sedoheptulose-1,7-diP	7.7	7.3						
K*m* (μM)								
Fructose-1,6-diP	21.2 ± 0.4	19.4 ± 0.5	24.0 ± 0.7	20	68	120	60	1
Fructose-1-P	8849 ± 587	7264 ± 851		10,000	3900	12,000	10,000	900
Sedoheptulose-1,7-diP	18 ± 2	7.8 ± 0.4			17			
V_{max} fructose-1,6-diP/V_{max} sedoheptulose-1,7-diP	2.3	2.15			1.7			
K*i* (mM)								
Ribulose-1,5-diP	0.18 ± 0.03	0.14 ± 0.01						
ADP	2.65 ± 0.09	2.00 ± 0.09						
3-P-Glyceric acid	2.0 ± 0.4	2.9 ± 0.6						
Citrate	2.7 ± 0.1	2.6 ± 0.1						

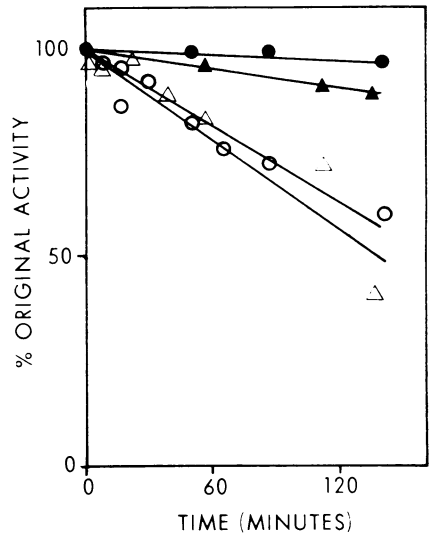

FIG. 5. Inactivation of pea leaf aldolases with L-glyceraldehyde-3-P. Chloroplast (circles) or cytoplasmic (triangles) aldolase was incubated with L-glyceraldehyde-3-P (0.4 mM, open symbols), at 25 C. Samples (100 μl) were removed at intervals, as indicated, and tested for aldolase activity using the standard assay. Controls (filled symbols) had water in place of L-glyceraldehyde-3-P. Both aldolases are slowly inactivated by this compound.

inhibitors of the pea leaf aldolases, but the inhibitor constants are large (2–3 mM) (Table I). ATP, AMP, P-enolpyruvate, pyruvate, ribose-5-P, glucose-6-P, sucrose, inorganic phosphate, inorganic sulfate, glutamate, succinate, fumarate, and L-malate have essentially no effect on the activity of either isoenzyme. Both enzymes are inhibited by *p*-hydroxymercuribenzoate; 80% inhibition occurs with 100 μM concentrations of this compound. Potassium ions and divalent metal ions have no effect on the activity of the enzymes.

It seemed possible that the activity of the particulate enzyme might be enhanced by light, since chlorophyll is associated with this fraction. We were not able to demonstrate any effect of white light on the activity of the membrane-bound enzyme at either high or low substrate concentrations. Apparently there is no latent or masked aldolase activity in the particulate fraction, since treatment of the fraction with Triton X-100, which solubilizes the enzyme, does not affect total aldolase activity.

Both chloroplast and cytoplasmic aldolases emerge from Sephadex G-200 slightly ahead of yeast alcohol dehydrogenase and therefore are about the same size as other class I aldolases (data not shown).

DISCUSSION

The relative proportion of reduced carbon recycling in the Calvin cycle and being converted into storage polysaccharides may be controlled at the level of aldolase. This enzyme is unique in catalyzing reactions on both sides of a metabolic branchpoint. It catalyzes the reaction of dihydroxyacetone-P with either glyceraldehyde-3-P to fructose-1,6-diP, or, with erythrose-4-P, to give sedoheptulose-1,7-diP. The fructose-diP is a precursor of storage polysaccharides and of erythrose-4-P, the alternative aldose-P substrate. The level of glyceraldehyde-3-P relative to dihydroxyacetone-P is controlled by triose-P isomerase, an enzyme which is negatively modulated by a number of metabolic intermediates (2). The activity of aldolase apparently is controlled directly by the relative levels of the aldose-P substrates and indirectly by the effect of modulators on the activity of triose-P isomerase.

We previously reported that pea leaf chloroplast and cytoplasmic triose-P isomerase, ribose-5-P isomerase, P-glyceric acid kinase, and fructose-1,6-diP aldolase have different isoelectric points (5). The properties of the isomerases have been compared. The triose-P isomerases differ slightly with respect to pH optima, Michaelis constants and inhibitor constants (2). The ribose-5-P isomerases have essentially identical kinetic properties (3). Like the triose-P isomerases and the ribose-5-P isomerases, the chloroplast and cytoplasmic fructose-1,6-diP aldolases are extraordinarily similar enzymes. There is little difference in pH optima, apparent affinity for substrates, and inhibition constants. There is no reason to believe that the chloroplast enzyme should be more effective for synthesis and the cytoplasmic enzyme more effective for cleavage. At the enzyme level there seems to be no advantage to the plant in having two exceedingly similar fructose-1,6-diP aldolases.

The similarity between the three pairs of chloroplast and cytoplasmic enzymes, aldolase, triose-P isomerase, and ribose-5-P isomerase, suggests that the chloroplast Calvin cycle en-

zymes and the analogous cytoplasmic enzymes do not have dissimilar functions within the green leaf. It seems possible that these very similar enzymes participate in both photosynthetic and dark metabolism and that in the green leaf *photosynthetic* carbon metabolism is not limited to the chloroplast and dark, catabolic metabolism is not entirely limited to the cytoplasm.

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