BBA 66295

CHLOROPLAST AND CYTOPLASMIC ENZYMES

II. PEA LEAF TRIOSE PHOSPHATE ISOMERASES

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(Received November 6th, 1970)

SUMMARY

1. The predominant forms of pea *(Pisum sativum)* leaf chloroplast and cytoplasmic triose phosphate isomerases (D-glyceraldehyde 3-phosphate ketol-isomerase, EC 5.3.1.1), separated by isoelectric focusing, have been characterized.

2. With glyceraldehyde-3-*P* as substrate the two isoenzymes have broad, but not identical pH activity curves; the pH optimum for the chloroplast enzyme is 7.8, for the cytoplasmic enzyme, 7.1. The K_m for D-glyceraldehyde-3-*P* is 0.42 mM for the chloroplast, and 0.20 mM for the cytoplasmic enzyme. For dihydroxyacetone-*P* the K_m's are 1.1 and 0.39 mM, respectively.

3. Both forms of the enzyme are inhibited competitively (with glyceraldehyde-3-*P* as substrate) by several common metabolites. The number and nature of these compounds suggests that metabolic intermediates exert a cumulative effect on the activity of the triose-*P* isomerases, acting concertedly to modulate the activity of these enzymes in the green leaf.

INTRODUCTION

Triose phosphate isomerase (D-glyceraldehyde 3-phosphate ketol-isomerase, EC 5.3.1.1) catalyzes the interconversion of D-glyceraldehyde-3-*P* and dihydroxyacetone-*P*. The enzyme has been isolated from mammalian and avian tissues and from yeast[1-4] and purified from pea seeds[5] but there have been no studies of the enzyme from green leaves. The chloroplast isomerase has been separated from the cytoplasmic enzyme by isoelectric focusing[6].

The equilibrium for the triose-*P* isomerase reaction lies far to the right in favor of dihydroxyacetone-*P*; at 38° K is 22 (see ref. 7). The activity of this enzyme is extraordinarily high in whole leaf extracts[8] and in broken-chloroplast preparations[9]. In animals although the levels of the enzyme are quite high in tissue extracts, the

Abbreviations: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; Bicine, *N,N*-bis(2-hydroxyethyl)glycine.

triose phosphates are not in equilibrium[7]. The asymmetric labeling pattern observed in hexose in short-term photosynthesis experiments by GIBBS AND KANDLER[10] suggests that the triose-*P* isomerase system in green plants is likewise in disequilibrium.

The purposes of the present study were (1) to characterize the triose-*P* isomerases of leaf tissue and to determine whether there are differences in the biochemical properties of the chloroplast and cytoplasmic isoenzymes related to function within the cell, and (2) to find an explanation for the incongruity between the high levels of triose-*P* isomerase activity in extracts and the apparent disequilibrium in the system in the chloroplast.

Chloroplast and cytoplasmic triose-*P* isomerases were found to have different pH optima, Michaelis constants, and inhibitor constants. It would appear that although the proteins are very closely related they are neither structurally nor functionally identical.

The activity of both isomerases is affected by a number of common metabolites. These compounds probably exert a cumulative inhibitory effect on the activity of the enzymes *in vivo* thus lowering the effective concentration of the isomerases and leading to disequilibrium in the triose-*P* system in the green leaf.

MATERIALS AND METHODS

Growth conditions

Leaves from 10–14-day-old pea plants (*Pisum sativum*, var. Little Marvel) grown in a mixture of vermiculite and soil under 16 h artificial light, 8 h dark, were used in these experiments.

Isoelectric focusing

Chloroplast and cytoplasmic extracts, prepared as described previously[6], and containing 25 mg protein, were subjected to electrophoresis for 1.5 days in 3–6 ampholyte at 450 V, or 4–6 ampholyte at 500 V, at 10° in a 440-ml LKB isoelectric focusing column. Ampholyte and electrode solutions were made up with 5 mM mercaptoethanol. The cathode solution (made with 0.5 M 2-aminoethanol) was at the bottom of the column. The anode solution was 0.15 M H_3PO_4. Protein was estimated by the biuret method as described previously[6]. Fractions (36 drops, 2.8 ml) were collected and analyzed for isomerase activity. The pH of the individual fractions was measured at 0° with a Radiometer pH meter 26.

Enzyme assays

Triose-*P* isomerase activity was routinely assayed with glyceraldehyde-3-*P* as substrate as described by GIBBS AND TURNER[11]. The reaction mixture contained 1.4 μmoles DL-glyceraldehyde-3-*P*, 0.1 μmole NADH, 50 μmoles *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) potassium salt, pH 7.5, excess α-glycerol-*P* dehydrogenase (EC 1.1.1.8) and enzyme in a total volume of 1.0 ml. In the reverse reaction, with dihydroxyacetone-*P* as substrate, the assay method of BURTON AND WALEY[12] was used. The reaction mixture contained 2 μmoles dihydroxyacetone-*P*, 8.5 μmoles Na_3AsO_4, 1.2 μmoles NAD^+, 50 μmoles HEPES (potassium salt), pH 7.5, excess glyceraldehyde-3-*P* dehydrogenase (EC 1.2.1.12) and enzyme

in a total volume of 1.0 ml. The dehydrogenases were passed through a Sephadex G-25 column (2.5 × 30 cm, 100–300 μm) in 10 mM pH 7.5 HEPES (potassium salt), 5 mM mercaptoethanol buffer to remove $(NH_4)_2SO_4$. All reactions were run at 25°. Change in absorbance at 340 nm was followed on a Gilford 2400 recording spectrophotometer using the expanded scale. Under these conditions the reaction rate appeared to be linear and initial velocity was considered to be the change in A per unit time.

Determination of kinetic constants

Six substrate levels, varied at even reciprocal intervals between 40 μM and 1 mM (for D-isomer) were used when glyceraldehyde-3-*P* was substrate. With dihydroxyacetone-*P* as substrate the six concentrations were varied at reciprocal intervals between 0.2 and 2 mM. Values and standard error for K_m and K_i were estimated as described previously[13].

Reagents

DL-glyceraldehyde-3-*P*, dihydroxyacetone-*P* dimethylketal (di-monocyclohexylamine salt), NADH (Na^+), NAD^+, 2-(*N*-morpholino)ethane sulfonic acid (MES), HEPES, and *N*,*N*-bis(2-hydroxyethyl)glycine (Bicine), α-glycerol-*P* dehydrogenase and glyceraldehyde-3-*P* dehydrogenase (ammonium sulfate suspensions), ATP, ADP (Na^+), AMP (Na^+), fructose-1-*P* (Na^+), fructose-6-*P* (K^+), fructose-1,6-P_2 (Na^+), *P*-enolpyruvate (K^+), oxalacetic acid, ribose-5-*P* (Na^+), ribulose-1,5-P_2 (Na^+) and D-3-*P*-glycerate (Na^+) were obtained from Sigma, metal-free sucrose from Mann, sodium sulfate (suprapur) from Merck, Darmstadt, Sephadex from Pharmacia, and ampholine from LKB. The cyclohexylammonium salts of *P*-glycolic acid and 1-hydroxy-3-iodo-2-propanone phosphate diethylketal were the gift of Dr. F. C. Hartman. All other reagents were the highest quality commercially available. Dihydroxyacetone-*P* and iodoacetol-*P* were prepared from the ketals by acid hydrolysis. Free acids were neutralized with KOH. Pea seeds were obtained from Vaughan's Seed Company, Downers Grove, Ill.

RESULTS

One peak of triose-*P* isomerase activity is found in cytoplasmic extracts (Fig. 1). The apparent isoelectric point is 4.8. Two, and occasionally three peaks of activity are found in chloroplast extracts. The major chloroplast peak, pI', 4.75, is clearly separate from the cytoplasmic peak. The two minor, more cationic chloroplast peaks may represent variants of the chloroplast isoenzyme, partially denatured chloroplast enzyme or cytoplasmic contamination of the chloroplast extract. These pI' values differ somewhat from those reported previously, probably due to the use of mercaptoethanol in the present experiments. Also, in these experiments the fraction size was smaller.

Apparently the pea leaf isomerases form stable aggregate forms. Several peaks of activity were found when the isomerases were subjected to gel filtration or to centrifugation in sucrose density gradients. In this respect the plant isoenzymes resemble the bacterial isomerase (F. C. HARTMAN, personal communication).

Although the pH activity curves are very similar, the chloroplast and cyto-

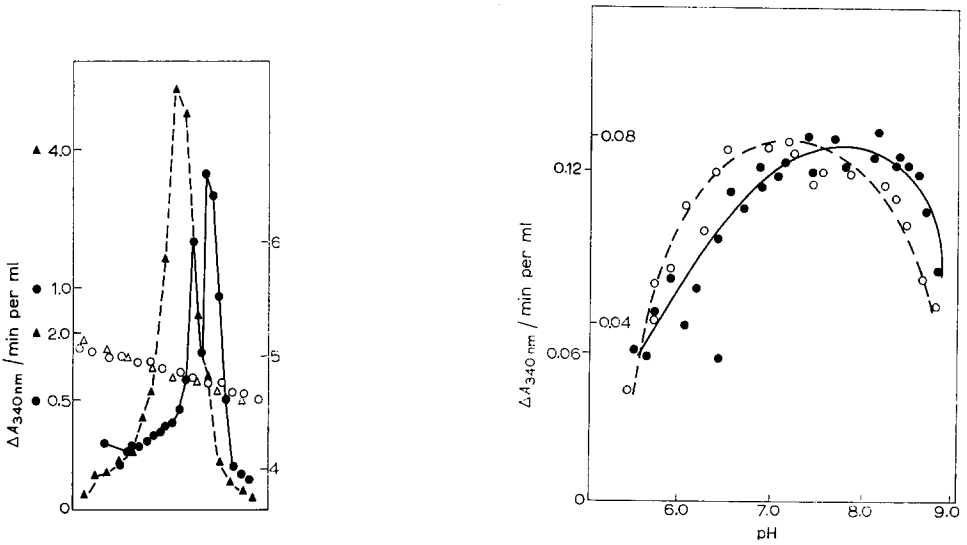

Fig. 1. Isoelectric focusing patterns of pea leaf triose-*P* isomerases. Chloroplast extract (circles) containing 25 mg protein was subjected to electrophoresis in 4–6 ampholyte and cytoplasmic extract (triangles) containing 20 mg protein was electrophoresed in 3–6 ampholyte. Activity is given by the filled symbols, pH of the fractions by the open symbols. Data were plotted for best fit to pH values. No units are given on the abscissa. The units used (drops) were consistent within each run but not between two runs. The major chloroplast peak is clearly separate from the cytoplasmic peak. This experiment was repeated 3 times with cytoplasmic extract and 7 times with chloroplast extract with consistent results.

Fig. 2. pH dependence of chloroplast (●, outside ordinate) and cytoplasmic (○, inside ordinate) triose-*P* isomerase catalyzed conversion of glyceraldehyde-3-*P* to dihydroxyacetone-*P*. Assay conditions as described under MATERIALS AND METHODS except that the pH and type of buffer (potassium salts of MES, HEPES or Bicine) was varied. pH was determined after activity was measured. The experiment was repeated 4 additional times with chloroplast and 3 with cytoplasmic isomerase, with consistent results. Clearly the pH activity curves for the two isomerases are not identical.

plasmic isomerases clearly do not have identical pH dependence. With glyceraldehyde-3-*P* as substrate there is a broad pH activity curve for either enzyme, but the curves are not superimposable (Fig. 2). The pH optimum for the chloroplast enzyme is 7.8, and for the cytoplasmic enzyme, 7.1. The pH dependence curves for rabbit

TABLE I

PROPERTIES OF PEA LEAF TRIOSE-*P* ISOMERASES

For details see text. Number of determinations in parentheses.

	Chloroplast	*Cytoplasmic*
p*I'*	4.75 (7)	4.8 (3)
pH optimum		
Glyceraldehyde-3-*P*	7.8 (5)	7.1 (3)
K_m (mM)		
D-Glyceraldehyde-3-*P*	0.421 ± 0.006 (24)	0.195 ± 0.003 (22)
Dihydroxyacetone-*P*	1.1 (5)	0.39 (7)

muscle and liver[3], yeast[3], and pea seed[5] isomerases are similar. With dihydroxyacetone-*P* as substrate and in the presence of arsenate the enzyme-catalyzed reaction is independent of pH between 6.3 and 8.8.

The K_m for D-glyceraldehyde-3-*P* is 0.42 mM for the chloroplast enzyme and 0.20 mM for the cytoplasmic isomerase (Table I). It is assumed that the pea leaf enzymes are specific for the D-form of glyceraldehyde-3-*P*. The K_m's for dihydroxyacetone-*P* are 1.1 mM for the chloroplast enzyme and 0.39 mM for the cytoplasmic enzyme. These values were obtained by substituting the K_p (observed K_m) values for dihydroxyacetone-*P* in the presence of arsenate and the K_i values for arsenate (when glyceraldehyde-3-*P* is substrate) into the formula for the competitive inhibitor constant and solving for K_m. It was assumed that arsenate is competitive, and that the inhibitor constants are the same for both substrates. These values are similar to the Michaelis constants for glyceraldehyde-3-*P* for triose-*P* isomerase from pea seeds[5] and algae[14] and, for both substrates, for the enzyme from avian[15] and mammalian sources[15,3] and yeast[3].

The equilibrium constant for the reaction calculated from the Haldane relationship for the cytoplasmic enzyme is 24 at 25°, which is in good agreement with the equilibrium constant, 22 at 38°, obtained by VEECH *et al.*[7], by analysis of equilibrium mixtures of the triose phosphates.

A number of different compounds inhibit the isomerases (Table II). At 10 mM concentrations glycolate, glyoxylate, NH_4Cl, KCl, EDTA, pyruvate, succinate, malate, glutamate, acetate, fumarate and α-ketoglutarate give 12% or less inhibition. At 0.7 mM ribulose-5-*P* gives 7% inhibition.

Like the pea leaf isoenzymes, the pea seed isomerase[5] and all other triose-*P*

TABLE II

INHIBITOR CONSTANTS FOR PEA LEAF TRIOSE PHOSPHATE ISOMERASES

Mean K_i's were estimated as described under MATERIALS AND METHODS. All of these compounds are competitive with glyceraldehyde-3-*P*. Number of determinations in parentheses.

Inhibitor	*Mean K_i (mM)*	
	Chloroplast	*Cytoplasmic*
$MgCl_2$	14 ± 2 (4)	5.8 ± 0.5 (4)
$MnCl_2$	3.9 ± 0.2 (6)	3.3 ± 0.1 (5)
Arsenate	12.0 ± 0.6 (2)	2.9 ± 0.1 (6)
Sulfate	9.2 ± 0.5 (2)	4.1 ± 0.2 (7)
ATP	6.6 ± 0.7 (2)	1.51 ± 0.05 (3)
ADP	5.1 ± 0.4 (3)	1.68 ± 0.06 (4)
AMP	4.5 ± 0.2 (4)	2.34 ± 0.09 (3)
Potassium phosphate	6.3 ± 0.7 (4)	1.40 ± 0.03 (4)
Sodium pyrophosphate	1.24 ± 0.07 (5)	0.59 ± 0.01 (2)
Fructose-1-*P*	19 ± 4 (2)	4 ± 0.2 (4)
Fructose-6-*P*	3.3 ± 0.1 (5)	2.65 ± 0.09 (5)
Fructose-1,6-P_2	2.3 ± 0.1 (3)	1.00 ± 0.06 (4)
P-Enolpyruvate	1.3 ± 0.1 (3)	0.66 ± 0.04 (5)
Citrate	8.7 ± 0.8 (4)	3.3 ± 0.1 (5)
Oxaloacetate	19 ± 1 (2)	3.1 ± 0.2 (2)
Ribose-5-*P*	does not inhibit (6)	3.6 ± 0.1 (3)
Ribulose-1,5-P_2	0.56 ± 0.05 (5)	0.24 ± 0.01 (4)
D-3-*P*-Glycerate	2.9 ±0.1 (5)	3.5 ± 0.2 (6)
2-*P*-Glycolate	15.2 ± 0.9 μM (5)	4.1 ± 0.2 μM (5)

isomerases[12,3,15] which have been tested are inhibited by phosphate, sulfate and arsenate. The yeast and muscle enzymes are not inhibited by fructose-6-*P*, fructose-1,6-P_2, or 3-*P*-glycerate and are less sensitive to inhibition by *P*-enolpyruvate than are the pea leaf enzymes[3]. The K_i for *P*-glycolate for these isoenzymes is of the same order as for the chicken and rabbit muscle enzymes[15]. The pea leaf enzymes seem to be somewhat more sensitive to inhibition by metabolic intermediates than are the animal and yeast isomerases.

The active site inhibitor 1-hydroxy-3-iodo-2-propanone phosphate[16,17] completely and instantaneously inhibits both pea leaf isomerases at concentrations as low as 10 μM. All triose-*P* isomerases including the bacterial, yeast, animal[18], and the chloroplast and cytoplasmic plant enzymes, are inhibited by this analog of dihydroxyacetone-*P*.

DISCUSSION

The cytoplasmic and chloroplast triose-*P* isomerases differ not only with respect to isoelectric point but also with respect to pH optima, Michaelis constants, and inhibitor constants (Table I). Whatever the physical and chemical reasons for these differences it is clear that within the leaf the chloroplast enzyme is not identical with the cytoplasmic enzyme.

The activity of the plant triose-*P* isomerases may by regulated by the concerted action of a number of metabolites. Fourteen metabolic intermediates were found which inhibit both isoenzymes. Other phosphorylated intermediates which were not tested must also inhibit the isomerases. Only one compound, *P*-glycolate, is a potent inhibitor. Labeled *P*-glycolate is a product of short-term photosynthesis in $^{14}CO_2$ (see ref. 19). Since μmolar concentrations of *P*-glycolate would completely inhibit triose-*P* isomerase it seems likely that the concentrations *in vivo* are rather low or that *P*-glycolate is compartmentalized away from the enzyme. *P*-Glycolate may be important in the regulation of triose-*P* isomerase activity in the chloroplast. Ribulose-1,5-P_2, a key intermediate in the reductive pentose phosphate cycle, is a somewhat less effective inhibitor. If the levels of this compound *in vivo* approproximate K_m for ribulose-1,5-P_2 carboxylase (EC 4.1.1.39) the isomerase will be mildly inhibited. Likewise the other intermediates found to affect the activity of the isomerases are probably mildly inhibitory *in vivo*. Each, by itself, would not be expected to compete effectively with substrate. The number of compounds affecting the isomerase, however, is significantly large. Acting together these compounds will inhibit the isomerases and the result will be disequilibrium between the triose phosphates. Changes in the flow of metabolism will result in altered steady state levels of the inhibitory metabolites, changes in triose-*P* isomerase activity and changes in the steady state levels of the triose phosphates.

Despite high levels of triose-*P* isomerase in animal tissues the triose phosphates are not in equilibrium in the livers of rats[7]. VEECH *et al.*[7], have concluded that in this system disequilibrium is due to inhibition by inorganic phosphate and to low physiological concentrations of glyceraldehyde-3-*P*.

Disequilibrium in the triose phosphate system in green plants was first observed by GIBBS AND KANDLER[10] who found that during short term photosynthesis in $^{14}CO_2$ specific activity of C-4 of hexose is higher than that of C-3. These workers suggested

that the pool of dihydroxyacetone-*P* in the chloroplast was being diluted with unlabeled dihydroxyacetone-*P*. Concerted inhibition of the chloroplast isomerase by photosynthetic intermediates however would result in slow equilibration of label between glyceraldehyde-3-*P* and dihydroxyacetone-*P*, and asymmetric labeling of hexose during short-term photosynthesis.

Disequilibrium may serve to maintain sufficient levels of glyceraldehyde-3-*P* for the aldolase catalyzed condensation of the triose phosphates to proceed. Although the equilibrium constant for the isomerase reaction is 22 the K_m for glyceraldehyde-3-*P* and dihydroxyacetone-*P* for mammalian fructose-1,6-P_2 aldolases (EC 4.1.2.7) are practically equal[20]. The same may be true for the plant aldolases. When the pentose shunt and the reductive pentose phosphate cycle are not operational, levels of phosphorylated intermediates (isomerase inhibitors) will be lower, the isomerase reaction will approach equilibrium, and the levels of glyceraldehyde-3-*P* will fall. This will force the aldolase reaction in the direction of cleavage of fructose-1,6-P_2. Inhibition of triose-*P* isomerase may well be a mechanism for the control of the direction of the aldolase reaction.

Chloroplast and cytoplasmic triose-*P* isomerases differ slightly with respect to isoelectric point, pH optima and K_m. Both enzymes are affected by a remarkable number of metabolites. Clearly the chloroplast and cytoplasmic triose-*P* isomerases are not identical, but extremely similar enzymes.

ACKNOWLEDGMENTS

I thank F. C. Hartman, Oak Ridge National Laboratory, for generous gifts of iodoacetol-*P* and *P*-glycolate, Mark Bresnik and Ephraim Casper for technical assistance, Ronald Golland for reprogramming the computer program of HANSON *et al.*[21], for the IBM 360, and James McCorkle and staff for growing the pea plants. This research was supported by grants from the University of Illinois, Chicago Circle, Research Board and from the National Science Foundation, GB 8626.

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