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Differential Inhibition and Activation of Two Leaf Dihydroxyacetone Phosphate Reductases[1]

ROLE OF FRUCTOSE 2,6-BISPHOSPHATE

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ABSTRACT

The chloroplastic and cytosolic forms of spinach (*Spinacia oleracea* cv Long Standing Bloomsdale) leaf NADH:dihydroxyacetone phosphate (DHAP) reductase were separated and partially purified. The chloroplastic form was stimulated by dithiothreitol, reduced thioredoxin, dihydrolipoic acid, 6-phosphogluconate, and phosphate; the cytosolic isozyme was stimulated by fructose 2,6-bisphosphate but not by reduced thioredoxin. End product components that severely inhibited both forms of the reductase included lipids and free fatty acids, membranes, and glycerol phosphate. In addition, two groups of inhibitory peptides were obtained from the fraction precipitated by 70 to 90% saturation with $(NH_4)_2SO_4$. Chromatography of this fraction on Sephadex G-50 revealed a peptide peak of about 5 kilodaltons which inhibited the chloroplastic DHAP reductase and a second peak containing peptides of about 2 kilodaltons which inhibited the cytosolic form of the enzyme. Regulation of the reduction of dihydroxyacetone phosphate from the C_3 photosynthetic carbon cycle or from glycolysis is a complex process involving activators such as thioredoxin or fructose 2,6-bisphosphate, peptide and lipid inhibitors, and intermediary metabolites. It is possible that fructose 2,6-bisphosphate increases lipid production by stimulating DHAP reductase for glycerol phosphate production as well as inhibiting fructose 1,6-bisphosphatase to stimulate glycolysis.

There are two forms of sn-3-phosphoglycerol dehydrogenase (EC 1.1.1.8) or NADH:DHAP[2] reductase in leaves (3, 6). In the leaf, the isozyme in the chloroplast is stimulated fivefold by reduced spinach (*Spinacia oleracea*) thioredoxin or twofold by DTT (3), whereas the cytoplasmic form is not affected by thioredoxin. The two forms in the leaf have been completely separated and partially purified by chromatography on DEAE cellulose as well as by gel filtration on Sephacryl S-200 (3). The chloroplast form represents 70 to 80% of the total DHAP reductase activity in normal leaves. Because the catalysis as a reductase is physiologically irreversible, other dehydrogenase reactions must also be involved for oxidation of glycerol phosphate (3, 4).

This paper describes inhibitors and activators of the two forms of NADH:DHAP reductase from leaves. Because these inhibitors are so effective, no activity had earlier been detectable in crude leaf homogenates or chloroplast preparations (3, 6). Enzyme activity could only be measured by first precipitating a protein fraction with 35 to 70% saturated $(NH_4)_2SO_4$ followed by dialysis. However, it has now been possible to detect the DHAP reductase in a crude homogenate after centrifugation at 110,000*g* for 1 hr and addition of BSA to remove most lipids and membranes and addition of DTT to stimulate the chloroplastic form.

In addition to NADH:DHAP reductase, thioredoxin regulates the activity of a number of chloroplast enzymes, and, therefore, stimulation of the DHAP reductase isozyme in the chloroplast by thioredoxin is consistent with previous findings with other chloroplast enzymes. An increase in the activity of DHAP reductase would lead to increased production of glycerol phosphate, which in turn would be used for lipid synthesis or might yield glycerol by dephosphorylation.

The present study indicates that Fru 2,6-P_2 stimulates the cytoplasmic DHAP reductase, which could also result in increased lipid synthesis or amounts of glycerol in the cytoplasm. Recent investigations (7–11) have indicated that Fru 2,6-P_2 also inhibits cytosolic Fru 1,6-P_2 phosphatase to block the formation of sucrose and promote glycolysis. Thus, regulation of the cytosolic DHAP reductase isozyme appears to be another cytoplasmic systems that is regulated by Fru 2,6-P_2.

MATERIALS AND METHODS

Materials. Spinach (*Spinacia oleracea* cv Long Standing Bloomsdale) was grown in the greenhouse in soil with Hoagland nutrients with 8 h light and 16 h dark. At night a tent was placed over the plants to shade them from other light sources.

DHAP was prepared from the dimethylketal dimonocyclohexylamine salt by treatment with Dowex 50-H+ as described by Sigma Chemical Co. The Sephacryl S-200 and Sephadexes were obtained from Pharmacia Corp., ultrapure $(NH_4)_2SO_4$ was from Swartz/Mann Inc., and DEAE cellulose was DE-52 purchased from Whatman Ltd. Phosphatidyl choline and linolenic acid were sonicated to form an emulsion (2), and dihydrolipoic acid was dissolved in ethanol. The sonicated detergents were made up in a 0.5% stock solution and then added to the assay mixtures after the addition of enzyme.

Fru 2,6-P_2 from Sigma Chemical Co. was dissolved in water. Sedo 1,7-P_2 from the same source was dissolved in water at a 10 mM concentration and then adjusted to pH 2 with HCl for 2 min to hydrolyze any contaminating Fru 2,6-P_2 before readjusting the pH to 7.

Enzyme. The protein in the 35 to 70% saturated $(NH_4)_2SO_4$ fraction was further purified as previously described (3). The chloroplastic and cytoplasmic forms of DHAP reductase were

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[2] Abbreviations: DHAP, dihydroxyacetone phosphate; Fru 2,6-P_2, fructose 2,6-bisphosphate; Fru 1,6-P_2, fructose 1,6-bisphosphate; Fru 6-P, fructose 6-phosphate, Sedo 1,7-P_2, sedoheptulose 1,7-bisphosphate.

completely separated and purified on Sephacryl S-200 without dialysis of the $(NH_4)_2SO_4$ fraction. If the $(NH_4)_2SO_4$ fraction were assayed, the enzyme had to be dialyzed against 10 mM Tris at pH 7.0, because $(NH_4)_2SO_4$ was inhibitory. Because Tris was also somewhat inhibitory, it was necessary that the final concentration in the enzyme assay be less than 1 mM Tris.

DHAP reductase activity was measured, as described previously (3), by the reduction of DHAP with NADH as monitored at 340 nm. A 1 ml assay mixture contained 500 μl of 200 mM Hepes at pH 6.9, 50 μl of 4.4 mM NADH, 100 μl of 10 mM DHAP, varying volumes of activators and inhibitors, and 200 μl of enzyme solution. As shown previously (3), the pH optimum of both isozymes was about 6.9 to 7.0.

Preparation of Inhibitors. The inhibitory peptides were isolated in two ways. The greatest amount of material was obtained by precipitating the peptides overnight at about 8°C, after increasing the 70% saturated $(NH_4)_2SO_4$ solution of the homogenate from which the reductases had been removed to 90% saturation with $(NH_4)_2SO_4$. Seventy percent saturation with $(NH_4)_2SO_4$ was accomplished by adding 532 g/L of original homogenate, and for 90% saturation an additional 152 g of $(NH_4)_2SO_4$/L was added. The precipitate was collected by centrifugation and resuspended to 10 mM Tris buffer at pH 7.0. The inhibitory peptides could also be obtained by concentrating by lyophilization the small mol wt fraction from the Sephacryl S-200 column used to separate the isoenzymes. The resuspended peptides from either procedure were then chromatographed on a column of Sephadex G-50 fine (2.5 × 90 cm) and eluted with water.

To study the effect of thylakoid membranes on DHAP reductase, the crude homogenate was prepared in 100 mM Tris at pH 7.0, 20 mM ascorbate, 10 mM mercaptoethanol, and 5 PVPP/100 g leaves. The homogenate was centrifuged at 20,000*g* for 1 h, and the resulting precipitate was resuspended in 10 mM Tris at pH 7.0 and 10 mM mercaptoethanol and called the thylakoid fraction. Due to severe inhibition by trace levels of lipids and membranes, the supernatant was further centrifuged for 1 h at 110,000*g* to remove other membranes (ER, microsomes) before one could detect enzyme activity.

RESULTS

Stimulation by Sulfhydryl Compounds or Fructose 2,6-Bisphosphate. The two isozymic forms of DHAP reductase were first precipitated by 35 to 70% saturated $(NH_4)_2SO_4$ and completely separated from each other on Sephacryl S-200, as reported previously (3, 6), so that differential regulation of their activity could be studied. The chloroplastic form was stimulated by DTT (Table I), but this DTT effect was less than from reduced thioredoxin from spinach leaves (3). In addition, dihydrolipoic acid stimulated the chloroplastic form (Table I), although long chain fatty acids without sulfhydryl groups were very inhibitory (next section). Other chloroplast enzymes that are stimulated by DTT and reduced thioredoxin are also stimulated by high concentrations of dihydrolipoic acid. Mercaptoethanol, glutathione, and cysteine did not stimulate either DHAP reductase form (3).

Fru 2,6-P_2 stimulated the cytosolic form of DHAP reductase but not the chloroplastic form (Fig. 1). A concentration of about 25 μM Fru 2,6-P_2 produced maximum stimulation of about 2.3-fold. Fru 1,6-P_2 had little or no stimulatory effect on DHAP reductase. Sedo 1,7-P_2 at 0.5 mM inhibited by 50% both the chloroplastic and cytosolic forms of DHAP reductase (Fig. 2). If the Sedo 1,7-P_2 solution had not been treated for 2 min at pH 2 to destroy contaminating Fru 2,6-P_2, some stimulation of the cytosol isozyme was observed.

Phosphate at concentrations around 5 mM stimulated the chloroplastic NADH:DHAP reductase and somewhat inhibited the cytosolic form (Fig. 2). At higher concentrations above 20 to 30 mM phosphate, both forms of DHAP reductase were inhibited. The stimulation of the cytosolic form by 25 μM Fru 2,6-P_2 (Fig. 1) was the same in either Hepes buffer or in a 5 mM phosphate buffer, but otherwise the effect from combinations of Fru 2,6-P_2 and phosphate has not yet been explored.

The chloroplastic form of DHAP reductase was slightly stimulated by about 5 μM 6 P-gluconate, whereas the cytosolic isozyme was inhibited (Fig. 2). ATP alone or ATP and Mg^{2+} at a 1:1 concentration ratio were slightly stimulatory to both isoenzymes of DHAP reductase. AMP had no effect on either enzyme at the concentrations examined. Phenylmethyl sulfonyl fluoride, an inhibitor of proteases, inhibited the cytosolic enzyme by 50% at concentrations over 0.5 mM but had little effect on the chloroplastic form. Glycerol and 3-P-glycerate at concentrations up to 10 mM did not inhibit the reductases, and 100 μM 3′,5′-cyclic AMP had no effect (data not shown). Although no correlation is attached to these inhibitory compounds at this time, there is a clear preferential inhibition of the two isozymes by different metabolites.

Inhibition by End Products. At over 25 μM, the product, glyc-

Table I. *Effect of Dithiothreitol and Dihydrolipoic Acid on the Cytoplasmic and Chloroplastic Forms of Dihydroxyacetone Phosphate Reductase*

Lipoic acid was dissolved in ethanol and added to the assay mixture. Equal amounts of ethanol were added to controls. Ethanol in these concentrations did not change the rate of the reaction from controls containing no ethanol. The data presented are averages of two experiments in which different enzyme preparations were used.

Concentration	DTT		Dihydrolipoic Acid	
	Chloroplast	Cytosol	Chloroplast	Cytosol
mM	*% enzyme activity*			
0	100	100	100	100
0.1			110	100
0.2			135	95
0.5	150	100	147	100
1.0	160	120	190	105
2.5	210	120		
5.0	215	120		

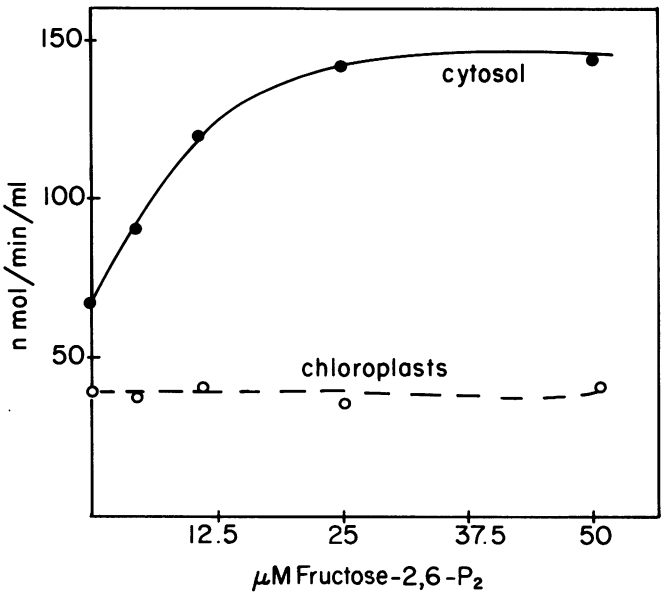

FIG. 1. Fru 2,6-P_2 stimulation of the cytosolic form of DHAP reductase.

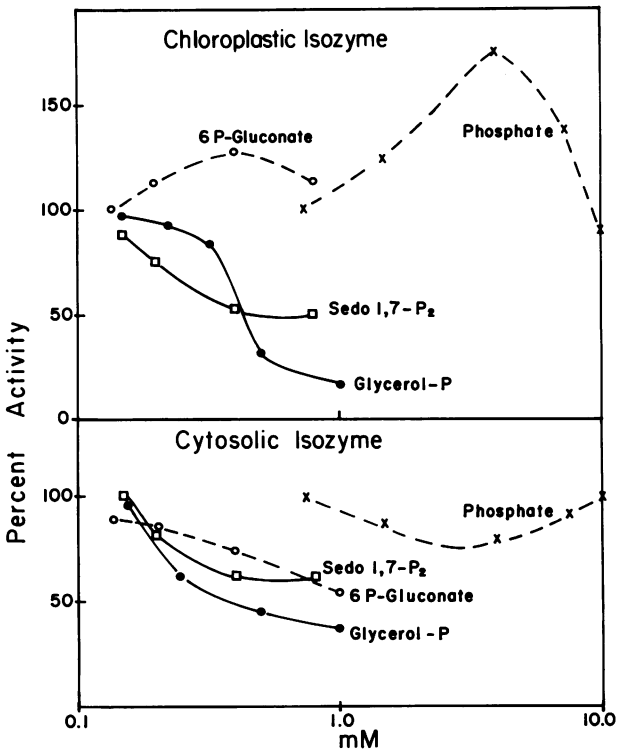

FIG. 2. Regulation of both the chloroplastic and cytosolic forms of DHAP reductase by metabolites.

erol phosphate from DHAP reduction, was a strong inhibitor of both the cytosolic and chloroplastic isoenzymes of NADH:DHAP reductase (Fig. 2). This result is suggestive of end product inhibition, but the inhibition can also be considered representative of inhibition by other phosphate esters of the C_3 cycle (see above). Since large amounts of glycerol phosphate are not known to accumulate in leaves, it may not play a significant role in regulating the reductase activity. Rather, glycerol-P is converted into lipids, hydrolyzed to glycerol (12), or oxidized back to DHAP (4). However, a combination of 6 P-gluconate, Sedo 1,7-P_2, glycerol-P, and perhaps other phosphate esters should severely restrict the chloroplastic DHAP reductase activity.

Both forms of NADH:DHAP reductase from leaves were strongly inhibited by low concentrations of lipids, free fatty acids, and detergents. Inhibition by a representative compound of each is presented in Table II. At over 10 μM, concentrations of any of these materials, the reductase activities are nearly completely inhibited. Inhibition by palmitic acid was similar to that by linolenic acid (Table II), but the detergent, deoxycholate, unlike Triton X-100, was not inhibitory at these low concentrations. Severe inhibition by lipids and membranes had prevented earlier detection and study of these enzymes and had forced us to initiate the enzyme purification before detecting measurable activity (3, 6). Thus, no activity can be detected in the leaf homogenates or chloroplast preparations even with the addition of DTT and/or BSA. During isolation of the enzyme, 0 to 35% saturation with $(NH_4)_2SO_4$ has been used to clarify the homogenate, after which the activity was precipitated by 35 to 70% saturation with $(NH_4)_2SO_4$, and the precipitate was dialyzed to remove the $(NH_4)_2SO_4$ and peptides both of which were also inhibitory.

No enzyme activity in the supernatant of the homogenate was detectable after centrifugation at 20,000 or 110,000g for an hour to remove membranes and bulk lipids (Table III). However, activity in these clarified homogenates could be detected provided that BSA was added to absorb residual fatty acids and/or DTT was added to stimulate the major chloroplastic form of the enzyme. Even the activity of the dialyzed enzyme preparation after fractionation by 35 to 70% saturated ammonium sulfate could be increased by BSA and, of course, by DTT.

To emphasize the severe inhibition of DHAP reductase by membrane fractions, the thylakoid fraction, obtained by centrifuging the crude homogenate at 20,000g for 1 h, was added back to an active enzyme preparation with both isozymes prepared by precipitation between 35 to 70% saturation with $(NH_4)_2SO_4$ and dialysis. Addition of the thylakoid fraction to the active enzyme preparation resulted in severe inhibition (Table IV). If the thylakoid preparation was boiled or membranes were removed by centrifugation there was no inhibition. Similar inhibitions were noted for activity in the supernatant fraction from the homogenate following centrifugation at 100,000g.

Inhibition by Peptides from Leaf Extracts. Dialysis of the 35 to 70% saturated $(NH_4)_2SO_4$ fraction was necessary to measure the NADH:DHAP reductase activity. The dialysate was concentrated by freeze drying and was chromatographed on Sephadex G-50 while monitoring for peptides at 280 nm (Fig. 3). Three fractions were obtained, and each inhibited the partially purified DHAP reductase activities. The third or last fraction to elute from Sephadex G-50 absorbed at 260 and 320 nm, as well as 280 nm, and contained small molecules, probably phenolics and salts. The third fraction was biuret negative for peptides and would have also contained $(NH)_2SO_4$ which inhibited both forms of the reductase. The other two peptide peaks, which were measured by 280 nm absorption and were biuret positive, were collected separately and concentrated by freeze drying. The first peak would have contained peptides of about 5 kD and peak II would have contained peptides of about 2 kD. The largest and most active amount of the two peptide fractions was obtained in a 70 to 90% saturated $(NH_4)_2SO_4$ precipitate from the original homogenate. Thus, DHAP reductase was isolated from the 35 to 70% saturated $(NH_4)_2SO_4$ fraction, and the inhibitors were primarily in the 70 to 90% saturated $(NH_4)_2SO_4$ fraction.

The first and second peptide fraction from the Sephadex G-50 chromatography of the 70 to 90% saturated $(NH_4)_2SO_4$ or from the dialysate of the reductase preparation differentially inhibited the two forms of the enzyme (Table V). For these analyses, the isozymes had been separated by chromatography on Sephacryl S-200 (3), and their activity was measured in the presence of increasing aliquots of the two separate and concen-

Table II. *Inhibition of the Isolated Chloroplastic and Cytoplasmic Forms of Spinach Leaf DHAP Reductase by Lipids*

Concentration	Linolenic Acid		Phosphatidyl Choline		Triton X-100		Octyl Glucose	
	Chloroplast	Cytosol	Chloroplast	Cytsol	Chloroplast	Cytosol	Chloroplast	Cytosol
μM				% enzyme activity				
0	100	100	100	100	100	100	100	100
1.5	63	58	74	75	73	95	86	96
3.0	40	43	42	43	70	69	41	61
6.0	15	33	34	20	50	52	15	26
15.0	0	0	0	8	36	10	0	20

Table III. *Activity of Dihydroxyhydroxyacetone Phosphate Reductases in Spinach Leaf Homogenates*

The 35 to 70% $(NH_4)_2SO_4$ fraction was dialyzed against 10 mM Tris at pH 7.0 and 10 mM mercaptoethanol overnight with three buffer changes. Based on the standard assay with 1 mM DTT, the total enzyme activity in the 35 to 70% saturated $(NH_4)_2SO_4$ fraction after dialysis was 10-fold that detectable in the 20,000 *g* supernatant.

Additions	Crude Homogenate	20,000*g* Supernatant	Dialyzed 35–70% Saturation $NH_4(SO_4)_2$ Fraction
		nmol/min/ml homogenate	
None	0	0	75
1 mM DTT	0	30	300
6 mg BSA/ml	0	57	100
0.6 mM dihydrolipoic acid	0	16	122

Table IV. *Inhibition of Partially Purified Dihydroxyacetone Phosphate Reductases by the Thylakoid Membrane Fraction from Homogenate*

After centrifuging the homogenate at 20,000*g* for 20 min, the thylakoid fraction was resuspended in a volume of 10 mM Tris at pH 7.0 and 10 mM mercaptoethanol equal to the volume of the original homogenate. The partially purified reductase was the dialyzed fraction after precipitation by 35 to 70% saturated $(NH_4)_2SO_4$. Assays were run in the presence of 1 mM DTT, which stimulated the chloroplast isozyme but not the cytosolic form in the preparation.

Thylakoid Fraction	Inhibition
μl	%
0	0
5	11
10	65
20	97
Boiled, 20	0
Supernatant, 20[a]	0

[a] The supernant refers to 20 μl of the supernatant from the thylakoid fraction after additional centrifugation at 140,000*g* for 1 h.

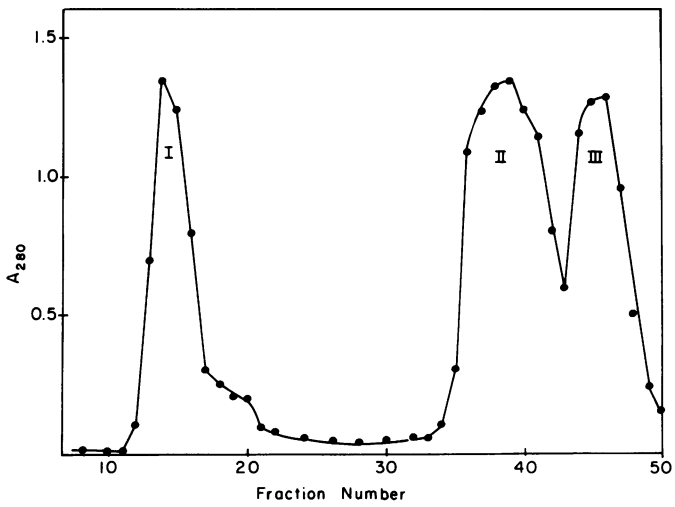

FIG. 3. Separation of peptide fractions which differentially inhibit the isozymes of DHAP reductase.

trated peptide fractions. Fraction I with the larger peptides inhibited the chloroplastic DHAP reductase preferentially. Fraction II consisting of the smallest peptides preferentially inhibited the cytosolic DHAP reductase (Table V). When solutions of either peptide fraction were placed in a boiling water bath for 10 min, all inhibitory activity was lost.

DISCUSSION

Three types of general regulation of the NADH:DHAP reductase from the chloroplast and from the cytosol of leaves have been explored. The first type involves activators, which include reduced thioredoxin and DTT for the chloroplastic isozyme and Fru 2,6-P_2 for the cytosolic form. This pattern is similar to the regulation of the two fructose bisphosphate phosphatases from leaves (1, 10, 11) and follows the general pattern of thioredoxin regulation of enzymes in the chloroplast and Fru 2,6-P_2 regulation of enzymes in the cytosol (Fig. 4). Stitt (11) has reviewed the proposed role of Fru 2,6-P_2 in the control of glycolysis by Fru 2,6-P_2 inhibition of a cytosolic Fru 1,6-P_2 phosphatase. In the light, Fru 2,6-P decreases in concentration resulting in a release from inhibition of the Fru 1,6-P_2 phosphatase activity. This increase in phosphatase activity causes a decrease in the rate of glycolysis and promotes sucrose synthesis. In the dark, Fru 2,6-P_2 increases in concentration (8, 9) resulting in inactivation of cytosolic Fru 1,6-P_2 phosphatase and an increase in the rate of glycolysis. Our data can be interpreted to indicate that reduced thioredoxin and Fru 2,6-P_2 also regulate the chloroplastic and cytosolic NADH:DHAP reductases, respectively. This type of regulation could control glycerol-P production for lipid synthesis in conjunction with the triose-P shuttle between the two compartments (Fig. 4). Fru 2,6-P_2 inhibits the cytosolic Fru 1,6-P_2 phosphatase and stimulates the cytosolic DHAP reductase resulting in increased glycerol-P production. In the light, the DHAP reductase in the chloroplast would be stimulated by reduced thioredoxin, and the reductase in the cytosol would be inhibited by decreased levels of Fru 2,6-P_2. Thus, the possibility of lipid synthesis in the chloroplast would be favored in the light. In the dark the chloroplastic DHAP reductase would decrease in activity in the absence of reduced thioredoxin, but the cytosolic DHAP reductase activity should be increased because of increased concentrations of Fru 2,6-P_2. Thus, it would appear that in the dark, lipid synthesis outside the chloroplast would be favored. Therefore, Fru 2,6-P_2 seems to have a regulatory role on two major metabolic pathways, namely glycolysis and lipid synthesis. In the light, chloroplast lipid and cytoplasmic sucrose should be stimulated by F2,6-P_2, and in the dark, cytosolic glycolysis and lipid synthesis should increase.

A second type of regulation involves the general but severe inhibition of both forms of DHAP reductase by fatty acids, lipids or detergents, and membranes. This inhibition complicates isolation and quantitation of DHAP reductase activities. We have measured from mature spinach leaves about 10 μmol of the chloroplastic DHAP reductase activity/h/mg Chl with DTT as an activator (3) and about 3 to 4 μmol without Fru 2,6-P_2 as an activator for the cytoplasmic form. If spinach leaf reduced thioredoxin were used, the chloroplastic isozyme activity would be about 20 μmol, and if Fru 2,6-P_2 had been present when the cytosolic enzyme was assayed, about 6 to 8 μmol of the cytosolic

Table V. *Differential Inhibition of the Two Isoenzymic Dihydroxyacetone Phosphate Reductases from Spinach Leaves by Small Peptide Fractions*

The 35 to 70% $(NH_4)_2SO_4$ fraction was dialized and the dialysate was concentrated by lyophylization. The concentrated dialysate was chromatographed on Sephadex G-50 and monitored at 280 nm. There were three peaks absorbing at 280. Differential inhibition by the first two peaks are presented here. The third peak which corresponded with small mol wt molecules inhibited both the chloroplast and cytosolic forms. The peptides precipitated by 70 to 90% $(NH_4)_2SO_4$ saturation were chromatographed on Sephadex G-50. The chromatagraph had three 280 nm absorbing peaks (Fig. 3). The results of the first two are presented here. The third also inhibited both enzyme activity.

Volume of Peptide Fraction	Dialysate from Reductase				From 70–90% $(NH_4)_2SO_4$ Precipitate			
	Peptides I		Peptides II		Peptides I		Peptides II	
	Chloroplast	Cytosol	Chloroplast	Cytosol	Chloroplast	Cytosol	Chloroplast	Cytosol
μl	% enzyme activity							
0	100	100	100	100	100	100	100	100
25	77	100	100	90	32	90	90	60
50	75	98	90	75	10	80	80	52
100	50	93	85	50	0	75	70	31
200	38	90	80	35	0	70	65	0

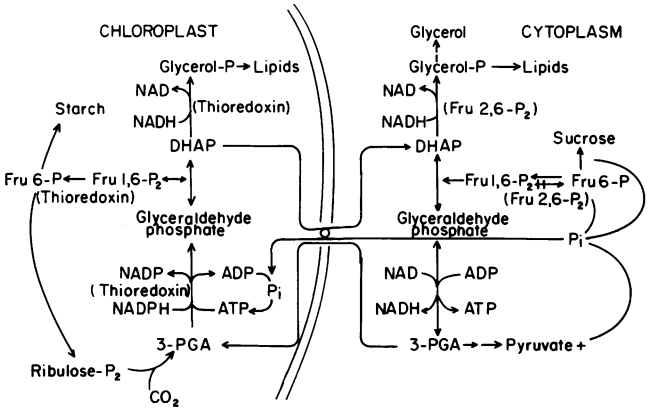

FIG. 4. Metabolic pathways for DHAP production and reduction in spinach leaves.

reductase would have been observed. Measurement of these activities may be underestimated and may vary greatly during isolation because of these effectors. As an example, no DHAP reductase activity can be detected until the lipids and membranes are largely removed, either by $(NH_4)_2SO_4$ fractionation or, as described herein, by prolonged centrifugation and the use of BSA to absorb fatty acids. A previous study has indicated that fatty acids strongly inhibit glycerol-3-phosphate dehydrogenase (assayed as a DHAP reductase) from rabbit muscle and liver (5), and it was suggested that fatty acids may have a regulatory role on the enzyme *in vivo*.

Both forms of DHAP reductase appear to be soluble (3). The cytosolic form is not in an organelle, and the chloroplastic form is in the stroma as judged from its rapid loss from broken chloroplasts. It is not known how these soluble activities function in the cell, which contains lipids and fatty acids which inhibit them. The structural integrity that is destroyed during leaf homogenation appears to release sufficient fatty acids and membrane fragments to inhibit the DHAP reductases completely.

A third type of regulation of DHAP reductase that has been explored in this paper is that by specific peptide inhibitors. A large (about 5 kD) and a smaller (~2 kD) fraction were separated from the leaf homogenate, first by precipitation with 70 to 90% saturated $(NH_4)_2SO_4$ and then chromatography on Sephadex G-50. The larger mol wt peptide fraction preferentially inhibited the chloroplast form of DHAP reductase, and the fraction of lesser mol wt inhibited the cytoplasm form. These peptides were also present in the enzyme protein fraction from $(NH_4)_2SO_4$ fractionation and, like the lipids, caused underestimation of the enzyme activity.

The complexities arising from three classes of regulating components for DHAP reductase means that characterization of each must precede a general understanding of the potential for glycerol-P synthesis. The synthesis of glycerol-P for lipids by leaf cells must be substantial, yet carefully controlled, or else most of the carbohydrate from photosynthesis, the triose phosphate shuttle, or glycolysis would be drained away by reduction of the key intermediate, DHAP. In fact, this does occur in some algae which produce primarily glycerol as the end product of photosynthesis. In higher plants, however, the transport of sucrose necessitates a complex, compartimentalized regulatory restriction on the DHAP reductase activities to force carbon flow to sucrose. Because Fru 2,6-P_2 inhibits the cytosolic Fru 1,6-P_2 phosphatase (7, 9) and stimulates the cytosolic DHAP reductase activity, Fru 2,6-P_2 appears to play a key regulatory role to stimulate in the dark both glycolysis and glycerol formation in the cytosol. Additional regulation by lipids and peptides may be necessary to restrict glycerol-P formation because of its direct competition with glycolysis.

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