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Plant Dihydroxyacetone Phosphate Reductases[1]

Purification, Characterization, and Localization

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ABSTRACT

A cytosolic form of dihydroxyacetone phosphate (DHAP) reductase was purified 200,000-fold from spinach (*Spinacia oleracea L.*) leaves to apparent electrophoretic homogeneity. The purification procedure included anion-exchange chromatography, gel filtration, hydrophobic chromatography, and dye-ligand chromatography on Green-A and Red-A agaroses. The enzyme, prepared in an overall yield of 14%, had a final specific activity of about 500 μmol of DHAP reduced min^{-1} mg^{-1} protein, a subunit molecular mass of 38 kD, and a native molecular mass of 75 kD. A chloroplastic isoform of DHAP reductase was separated from the cytosolic form by anion-exchange chromatography and partially purified 56,000-fold to a specific activity of 135 μmol min^{-1} mg^{-1} protein. Antibodies generated in rabbits against the cytosolic form did not cross-react with the chloroplastic isoform. The two reductases were specific for NADH and DHAP. Although they exhibited some dissimilarities, both isoforms were severely inhibited by higher molecular weight fatty acyl coenzyme A esters and phosphohydroxypyruvate and moderately inhibited by nucleotides. In contrast to previous reports, the partially purified chloroplastic enzyme was not stimulated by dithiothreitol or thioredoxin, nor was the purified cytosolic enzyme stimulated by fructose 2,6-bisphosphate. A third DHAP reductase isoform was isolated from spinach leaf peroxisomes that had been prepared by isopycnic sucrose density gradient centrifugation. The peroxisomal DHAP reductase was sensitive to antibodies raised against the cytosolic enzyme and had a slightly smaller subunit molecular weight than the cytosolic isoform.

All organisms need glycerol phosphate for the synthesis of glycerolipids, and some algae use glycerol as an osmoticum (26). NAD^+-dependent glycerol-3-phosphate dehydrogenase from muscle and liver has been purified and partially characterized (21). Isoforms of *sn*-glycerol-3-phosphate:NAD^+ oxidoreductase (EC 1.1.18) in higher plants and algae are referred to as DHAP[3] reductases because at physiological pH and substrate concentrations the enzymes are essentially inactive as dehydrogenases (10, 11). In plants and algae, there are cytosolic and chloroplast stromal forms of DHAP reductases (7, 10) that use DHAP from photosynthesis or glycolysis to form glycerol phosphate. So far, DHAP reductases have only been partially purified from spinach (*Spinacia oleracea* L.) leaves (24), castor bean endosperm (5), *Dunaliella tertiolecta* (8, 15), and *Chlamydomonas reinhardtii* (18). To study and compare different DHAP reductases from both higher plants and algae at the protein and gene levels, we have purified two forms of DHAP reductase from spinach leaves, one of them to apparent electrophoretic homogeneity, and have generated antibodies against this isoform.

MATERIALS AND METHODS

Plant Material and Chemicals

Spinach (*Spinacia oleracea* L.) was bought from local markets or grown in growth chambers in soil with 8 h of light (200 μEm^{-2} s^{-1}) and 16 h of dark at 22°C. DEAE-cellulose (DE52) was from Whatman; Sephacryl S-200 and phenyl Sepharose CL-4B were from Pharmacia; the dye-ligand agaroses (Matrex gel Red-A and Matrex gel Green-A) were from the Amicon Corporation. Unless otherwise indicated, chemicals were purchased from Sigma Chemical Company. DHAP, glyceraldehyde phosphate, and hydroxypyruvate phosphate were prepared by hydrolyzing the cyclohexylamine salts as directed by Sigma. Molecular standards for SDS-PAGE and hydroxylapatite (Bio-Gel HTP) were purchased from Bio-Rad. Rabbit muscle DHAP reductase and Percoll were from Sigma.

Enzyme Purification

pH adjustments of buffers were made at room temperature. Unless otherwise indicated, all purification steps were carried out at 4°C. All gravity values refer to the maximum radius of the rotors.

Homogenization and ammonium sulfate fractionation were done as described by Gee et al. (10). The protein, precipitated between 40 (243 g/L) and 80% (516 g/L) saturation with ammonium sulfate, was dialyzed for 16 h against two changes of 10 L of buffer A (25 mM Tris [pH 7.0], 10 mM 2-mercaptoethanol) and centrifuged at 40,000*g* for 10 min, and the supernatant fluid was applied to a DE52 column (2.6 × 80 cm) previously equilibrated with buffer A. After the column was washed with 1.2 L of buffer A, adsorbed protein was eluted with a 2-L linear gradient of 0 to 0.4 M NaCl in buffer A at a flow rate of 110 mL/h. Two peaks of DHAP

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[3] Abbreviations: DHAP, dihydroxyacetone phosphate; ACP, acyl carrier protein.

reductase activity were recovered from DEAE-cellulose (Fig. 1). Peak II was concentrated with 80% ammonium sulfate, dialyzed for 16 h against two changes of buffer A, and then chromatographed at a flow rate of 25 mL/h on a Sephacryl S-200 column (2.6 × 90 cm) equilibrated with buffer A. Four preparations, each from 1.5 to 2.0 kg of spinach leaves, were run successively, and fractions with activity were combined after this column step and frozen (−18°C) in 2 M glycerol until used for further purification. The preparation stored in this way lost little enzyme activity during a period of several weeks. All subsequent steps were with material from 7.25 kg of spinach leaves.

DHAP reductase from Sephacryl S-200 was adjusted to 0.1 M NaCl and loaded on a Green agarose column (2.5 × 12 cm) equilibrated with 0.2 M NaCl in buffer A. The column was washed with 100 mM NaCl in buffer A until the absorbance at 280 nm was below 0.02, and then bound protein was eluted with a linear salt gradient (total volume 1 L) from 0.1 to 1.5 M NaCl in buffer A at a flow rate of 50 mL/h. DHAP reductase-containing fractions were bound to a phenyl Sepharose column (1.5 × 20 cm) equilibrated with 0.5 M NaCl in buffer A (buffer B). Elution was done by washing the column with 500 mL of buffer A at a flow rate of 30 mL/h, and eluted DHAP reductase was rechromatographed on a second Green agarose column (1.5 × 12 cm) under conditions described above but scaled to the smaller size of the column. DHAP reductase from Green agarose was applied to a Red agarose column (0.7 × 10 cm) equilibrated with buffer B. The column was washed with 10 column volumes of buffer B, and then DHAP reductase was eluted with 30 mL of 2.5 mM NADH in buffer B at a flow rate of 15 mL/h. After dialysis for 16 h against two changes of 2 L of buffer A, DHAP reductase was bound to a second Red agarose column (0.7 × 5 cm) in buffer A. The column was subsequently washed with buffer A and buffer B, and DHAP reductase was eluted with 20 mL of 2.5 mM NADH in buffer B at a flow rate of 15 mL/h. The purified enzyme was concentrated and desalted in Centricon 30 microconcentrators (Amicon) and stored at −18°C in 2 M glycerol in buffer A. Stored this way, the enzyme lost 20% of its original activity in 1 month.

The purification procedure for DHAP reductase peak I from DE52 cellulose (Fig. 1) was similar to that used for peak II with the following modifications. The buffers did not contain 2-mercaptoethanol. The Green agarose columns were omitted. After Sephacryl S-200 DHAP reductase was bound to a hydroxylapatite column (2.5 × 16 cm) in 25 mM Tris, pH 7.0, it was eluted with a linear gradient from 0 to 0.1 M phosphate at pH 7.0. DHAP reductase was bound to phenyl Sepharose in 30% ammonium sulfate in 25 mM Tris, pH 7.0, and eluted with a linear gradient from 30 to 0% ammonium sulfate in Tris buffer. The first Red agarose column was equilibrated with 50 mM NaCl in Tris buffer.

Preparation of Subcellular Organelles

A crude chloroplast fraction was obtained using the method of Cerović and Plesničar (4), and chloroplasts were further purified on a Percoll gradient according to the method of Goyal et al. (13). Purified intact chloroplasts were broken in 25 mM Tris (pH 7.0), 10 mM 2-mercaptoethanol, and 5 mM $MgCl_2$, membranes removed by centrifugation at 120,000*g* for 1 h, and the stromal proteins subjected to chromatography on DE52 cellulose as described in the legend to Figure 4.

For the isolation of peroxisomes, spinach leaves were homogenized with a Waring blender at medium speed for 5 s in homogenization buffer consisting of 25 mM Tricine-NaOH (pH 7.5), 1 mM EDTA, 2 mM $MgCl_2$, 10 mM KCl, and 25% (w/w) sucrose. After centrifugation at 2,000*g* for 10 min, peroxisomes were collected by centrifugation at 10,000*g* for 15 min and further purified by density gradient centrifugation at 102,000*g* for 3 h on sucrose step gradients consisting of 5 mL of 60%, 5 mL of 50%, 15 mL of 48%, 10 mL of 45%, 3 mL of 40%, and 10 mL of 35% (w/w) sucrose in 25 mM Tricine-NaOH, pH 7.5. The purified peroxisomes were broken by osmotic shock with 25 mM Tris (pH 7.0), 10 mM 2-mercaptoethanol, and 5 mM $MgCl_2$, the membranes removed by centrifugation at 120,000*g* for 1 h, and the soluble proteins chromatographed on DE52.

Quantitative Determinations

DHAP reductase activity was measured spectrophotometrically at room temperature by monitoring the decrease of absorbance at 340 nm. A 1-mL assay mixture contained 0.2 mM NADH and 1 mM DHAP in either 17 mM each of Hepes/Tricine/Mes at pH 6.9 or 50 mM Mes at pH 6.1. Unless otherwise indicated, all assays were run at pH 6.9.

Marker enzyme assays to identify cellular components were as described previously (2). Protein was determined by the method of Bradford (3) using BSA as a standard. Chl was determined according to the method of Arnon (1).

Electrophoresis and Western Blotting

SDS-PAGE was on gradient gels from 7.5 to 15% acrylamide (20), and proteins on SDS gels were visualized by silver staining (16).

Native PAGE was done on minigels because of the instability of DHAP reductase during prolonged electrophoresis. Buffer and gel systems were the same as for SDS-PAGE, except that SDS was omitted. After electrophoresis, the gels were incubated in DHAP reductase assay mixture (see above) for 30 min at room temperature and then scanned spectrophotometrically at 340 nm.

For western blot analyses, proteins were transferred from gels onto nitrocellulose according to the method of Towbin et al. (25). Nonspecific reactions were blocked with 3% nonfat dry milk for 1 h at room temperature, and incubation with the antibody (1:1000 dilution) was for 3 h at room temperature. Immunoreactive products were detected using protein A or anti-rabbit immunoglobulin G coupled to alkaline phosphatase (14).

Antibody Production

For the generation of polyclonal antibodies, rabbits were injected with the purified cytosolic DHAP reductase in the lower leg near the popliteal lymph node. The initial injection consisted of 10 μg of protein in 20 mM sodium phosphate (pH 7.4) and 0.14 mM NaCl, emulsified in Freund's complete

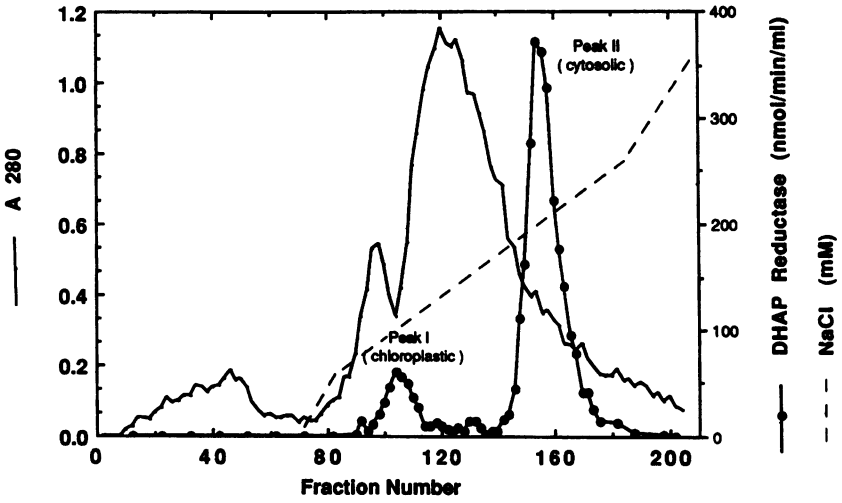

Figure 1. Chromatography of DHAP reductase from spinach leaf homogenates on DE52. A dialyzed 40 to 80% ammonium sulfate fraction was applied to the column. Elution was done with a salt gradient from 0 to 0.4 M NaCl in buffer A. Fractions 1 to 82 and fractions 183 to 205 were 22 mL each; fractions 83 to 182 were 11 mL each. The A_{280} (—●—) corresponds to a 1:40 dilution.

adjuvant. Subsequent injections at 4-week intervals were with 5 μg of protein in the same buffer emulsified in Freund's incomplete adjuvant.

Immunoprecipitations

Immunoprecipitations were done overnight at 4°C in 50 mM Tris (pH 7.0), 150 mM NaCl, and 1% (v/v) Triton X-100. Antiserum or preimmune serum (200 μL) was used in a total volume of 1 mL. The immunoprecipitated protein was removed by centrifugation in a microcentrifuge at full speed for 15 min. The supernatant fraction was then assayed for DHAP reductase activity.

RESULTS

Purification of DHAP Reductases

After homogenization of spinach leaves, ammonium sulfate fractionation of the crude extract, and chromatography on DE52, two fractions of DHAP reductase activity were resolved (Fig. 1). The minor peak (peak I), eluting at a low concentration of salt (0.1 M NaCl), was shown to be in the chloroplasts (see "Localization of Peaks I and II as Separated by DEAE Chromatography") and will, therefore, be referred to as chloroplastic DHAP reductase. The major peak (peak II) was apparently from the cytosol (see "Localization of Peaks I and II as Separated by DEAE Chromatography") and will, therefore, be referred to as cytosolic DHAP reductase. The subsequent purification of cytosolic DHAP reductase to apparent electrophoretic homogeneity, as summarized in Table I, afforded an almost 200,000-fold enrichment of the specific activity up to 500 μmol min^{-1} mg^{-1} of protein; this was obviously due to the low abundance of DHAP reductase protein, considering that, of 120 g of total soluble protein extracted from 7.25 kg of spinach leaves, only 50 μg of protein was in the purified cytosolic DHAP reductase fraction. By far the highest increase in specific activity was achieved by chromatography on Red agarose because of the high affinity of the enzyme for the dye, from which DHAP reductase could be removed only by a combination of a high concentration of salt (0.5 M) and NADH. However, none of the other purification steps could be omitted without loss of purity of the preparation. The progress of purification is demonstrated in Figure 2. The purified enzyme, obtained in

Table I. *Purification of Spinach Leaf Cytosolic DHAP Reductase*[a]

Purification Step	Total Activity	Total Protein[a]	Specific Activity	Recovery	Purification
	μmol min^{-1}	mg	μmol min^{-1} mg^{-1}	%	Fold
Soluble leaf homogenate	—[b]	116,800[a]	—	—	—
Dialyzed 40–80% $(NH_4)_2SO_4$ fraction	171	68,400	0.0025	100[b]	1[b]
DEAE-cellulose (DE52)	116	3,500	0.033	68	13
Sephacryl S-200	88	1,200	0.073	51	29
Green agarose (first)	75	133.1	0.563	44	225
Phenyl Sepharose	61	27.5	2.23	36	893
Green agarose (second)	56	13.4	4.18	33	1,670
Red agarose (first)	38	0.22	171.0	22	68,473
Red agarose (second)	24	0.048	495.0	14	198,167

[a] Based on preparation from 7250 g of spinach leaves. [b] Activity could not be detected in the crude homogenate. Recovery and fold purification, therefore, were based on the activity in the dialyzed 40 to 80% ammonium sulfate fraction.

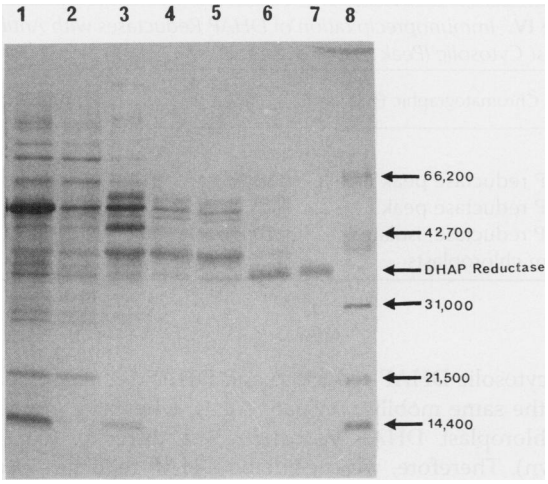

Figure 2. Evaluation of purification of cytosolic DHAP reductase by SDS-PAGE after each chromatographic column. Lane 1, Preparation (peak II) after DEAE-cellulose; lane 2, after Sephacryl S-200; lane 3, after first Green agarose; lane 4, after phenyl Sepharose; lane 5, after second Green agarose; lane 6, after first Red agarose; lane 7, after second Red agarose; lane 8, molecular mass standards (in daltons). Proteins were visualized by silver staining. A band at 38,000 was visible for the purified DHAP reductase.

an overall yield of 14%, was a single band of about 38 kD on SDS gels. Data obtained from velocity sedimentation of cytosolic DHAP reductase (data not shown) suggested a mol wt of the enzyme of 75,000, indicating that the holoenzyme is a homodimer. An overview of the partial purification of the chloroplast DHAP reductase is given in Table II. This enzyme had a lower apparent affinity for phenyl Sepharose and Red agarose than the cytosolic counterpart, resulting in a less pure preparation of the enzyme (see Fig. 3, lane 3).

Partial Characterization of DHAP Reductases

The purified DHAP reductases were not active with substrates other than DHAP, such as glyceraldehyde phosphate, phosphohydroxypyruvate, 3-phosphoglycerate, phosphoglycolate, hydroxypyruvate, pyruvate, and fructose 1-phosphate. The activity with NADPH at pH 6.9 was only 3% of the activity with NADH for both reductases. The pH optimum of the cytosolic DHAP reductase was 6.1, and the pH optimum of the chloroplastic DHAP reductase was 6.9 (data not shown). The effects of inhibitors on the two DHAP reductases are shown in Table III. Palmitoyl CoA inhibited both enzymes most severely. Similar inhibition was obtained with stearoyl CoA and oleoyl CoA. CoA, acetyl CoA, hexanoyl CoA, dodecanoyl CoA, and free fatty acids were not inhibitory. NaCl stimulated slightly up to concentrations of 0.25 M and inhibited at concentrations higher than 0.5 M.

Table III. *Inhibition of DHAP Reductase Activity*[a]

Inhibitor	Isoform	
	Cytosolic	Chloroplastic
	% *activity*	
None (control)	100	100
2.5 μM palmitoyl CoA	50	85
15 μM palmitoyl CoA	0	60
60 μM palmitoyl CoA	0	0
1 mM phosphohydroxypyruvate	10	40
10 mM ATP	20	50
10 mM CTP	60	80
0.1 M NaCl	150	116
0.25 M NaCl	122	113
2 M NaCl	14	13

[a] The assay mixture contained 1 mM DHAP and 0.2 mM NADH. The pH of the mixture was 6.1 for the cytosolic enzyme and 6.9 for the chloroplastic enzyme.

Antibodies raised in rabbits against the cytosolic DHAP reductase did not cross-react with the chloroplastic DHAP reductase on western blots, whereas a good reaction was obtained with the cytosolic DHAP reductase (Fig. 3). Furthermore, the enzymes had different mobility on native gels (data not shown). Preliminary data obtained by velocity sedimentation also suggest that the chloroplast DHAP reductase is a monomer of 38 kD, whereas the cytosolic DHAP reductase is a homodimer (see above).

Localization of Peaks I and II as Separated by DEAE Chromatography

When intact chloroplasts were isolated from spinach leaves as described in "Materials and Methods" and the stromal fraction was subjected to chromatography on DEAE-cellulose, almost all of the DHAP reductase activity applied to the

Table II. *Partial Purification of Spinach Leaf Chloroplastic DHAP Reductase*

Purification Step	Total Activity[a]	Total Protein[a]	Specific Activity	Recovery	Purification
	μmol min^{-1}	*mg*	*μmol min*$^{-1}$ *mg*$^{-1}$	%	*Fold*
Dialyzed 40–80% $(NH_4)_2SO_4$ fraction	199	84,604	0.002	100	1.0
DEAE-cellulose (DE52)	32	4,552	0.007	16	2.9
Sephacryl S-200	15	n.d.[b]	n.d.	7.4	n.d.
Hydroxylapatite	14	1,287	0.011	6.8	4.4
Phenyl Sepharose	10	429	0.024	5.2	10.0
Red Agarose (first)	5.8	n.d.	n.d.	2.9	n.d.
Red Agarose (second)	4.2	0.031	135.0	2.1	56,320

[a] Based on preparation from 7250 g of spinach leaves. [b] n.d., Not determined.

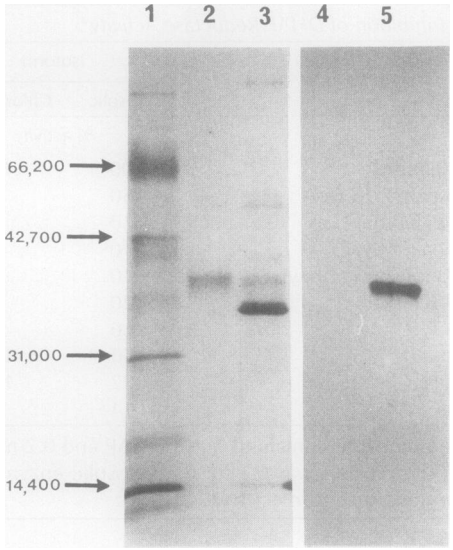

Figure 3. Western blot and silver stain analyses of DHAP reductases following SDS-PAGE. Lanes 1 to 3, Silver stain; lanes 4 and 5, western blot using antibodies raised against the purified cytosolic enzyme. Lane 1, Molecular mass standards (in daltons); lanes 2 and 5, purified cytosolic DHAP reductase; lanes 3 and 4, partially purified chloroplastic DHAP reductase.

column eluted in one peak at about 0.1 M NaCl (Fig. 4). This DHAP reductase was not specifically immunoprecipitated by antibodies against the purified DHAP reductase peak II, nor was the DHAP reductase peak I from DEAE chromatography of the crude leaf homogenate (Table IV). Furthermore, the DHAP reductase from the chloroplast stroma and DHAP reductase peak I had the same mobility on native gels (data not shown) and were both more active at pH 6.9 than at pH 6.1, whereas DHAP reductase peak II was more active at pH 6.1. For these reasons, DHAP reductase peak I was considered to be a chloroplastic DHAP reductase.

A cytosolic DHAP reductase previously isolated (10) was completely immunoprecipitated by antibodies generated against the purified DHAP reductase peak II. Furthermore,

Table IV. *Immunoprecipitation of DHAP Reductases with Antibody against Cytosolic (Peak II) DHAP Reductase*

DEAE Chromatographic Fraction	Water	Preimmune Serum	Antiserum
		% *activity*	
DHAP reductase peak II	100	81	01
DHAP reductase peak I	100	45	54
DHAP reductase, isolated from chloroplasts	100	55	55

this cytosolic DHAP reductase and DHAP reductase peak II had the same mobility on native gels, whereas the mobility of chloroplast DHAP reductase was different (data not shown). Therefore, we considered DHAP reductase peak II to be cytosolic.

Peroxisomal DHAP Reductase

Because rat and mouse liver peroxisomes contain NAD^+-linked glycerol phosphate dehydrogenase (9), it had to be shown that the DHAP reductase activity isolated from chloroplasts was not due to contamination by peroxisomes. For this reason, peroxisomes were isolated by differential centrifugation and isopycnic centrifugation of a 10,000*g* pellet on a sucrose step gradient. Following fractionation of the gradient, fractions were assayed for catalase and fumarase activity to determine the position of peroxisomes and mitochondria respectively (Fig. 5). Chl content was measured (1) to determine the location of chloroplasts and thylakoids in the gradient. Peroxisomal fractions with the highest catalase activity (fractions 3 to 6) had low fumarase activity and Chl content (Fig. 5). Thus, there was little contamination of the peroxisomal fraction by mitochondrial and thylakoid markers.

Throughout the gradient, no DHAP reductase activity was detectable, probably because of the presence of inhibitors. After the peroxisomes in fractions 3 to 6 from Figure 5 were broken by osmotic shock and soluble proteins were subjected to chromatography on DE52-cellulose, one peak of DHAP reductase activity was eluted from the column (Fig. 6). After

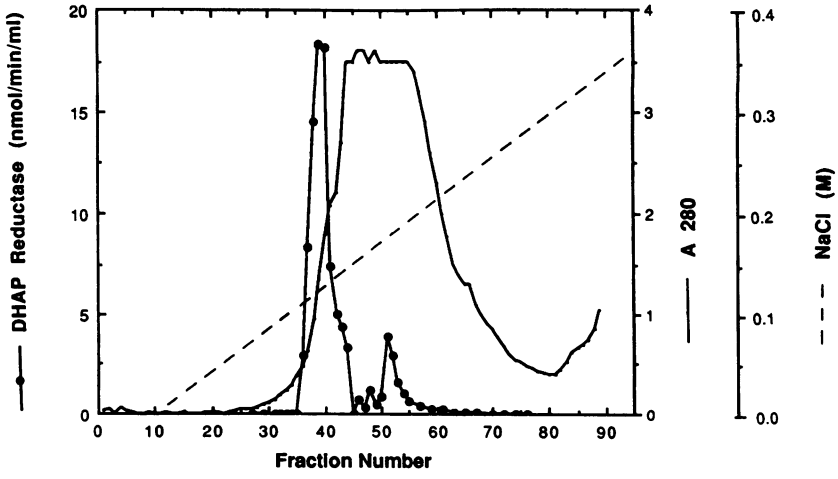

Figure 4. Chromatography of DHAP reductase isolated from intact chloroplasts on DEAE-cellulose. Chloroplast stroma prepared as described in "Materials and Methods" was applied to a DE52 column (1.5 × 20 cm) that had been equilibrated with 25 mM Tris (pH 7.0), 10 mM 2-mercaptoethanol. After the column was washed thoroughly with the same buffer, adsorbed proteins were eluted with a linear gradient of NaCl (0–0.4 M, 150 mL) in 25 mM Tris (pH 7.0), and 10 mM 2-mercaptoethanol.

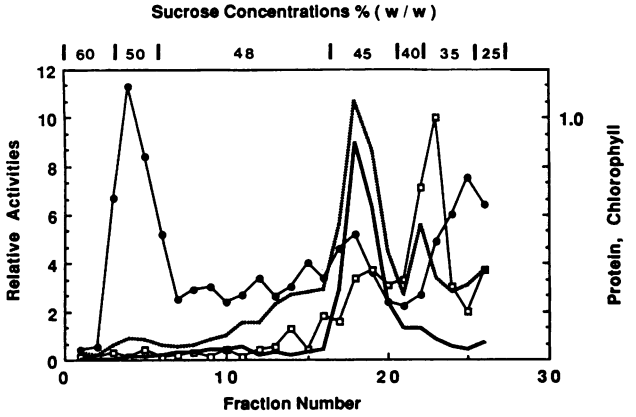

Figure 5. Separation by sucrose density gradient centrifugation of organelles contained in 10,000g pellet resuspended in 25% (w/w) sucrose. ●, Catalase; □, fumarase; ·····, protein (1 arbitrary unit = 10 mg/mL); ——, Chl (1 arbitrary unit = 2 mg/mL).

soluble proteins from mitochondrial fractions (fractions 22 and 23 from Fig. 5) had been chromatographed on DEAE-cellulose, DHAP reductase activity was insignificant (data not shown). Therefore, it appears that there is no DHAP reductase activity in mitochondria. The peroxisomal DHAP reductase was more active at pH 6.1 than at pH 6.9 and was immunoprecipitated to 80% when incubated with the antibody against the cytosolic DHAP reductase. Therefore, peroxisomal DHAP reductase was clearly different from chloroplastic DHAP reductase. Soluble proteins from peroxisomes have been chromatographed on Red agarose under the same conditions used for the cytosolic DHAP reductase, and eluted protein was analyzed by western blotting together with purified cytosolic DHAP reductase. As shown in Figure 7, peroxisomal DHAP reductase exhibited a slightly lower sub-

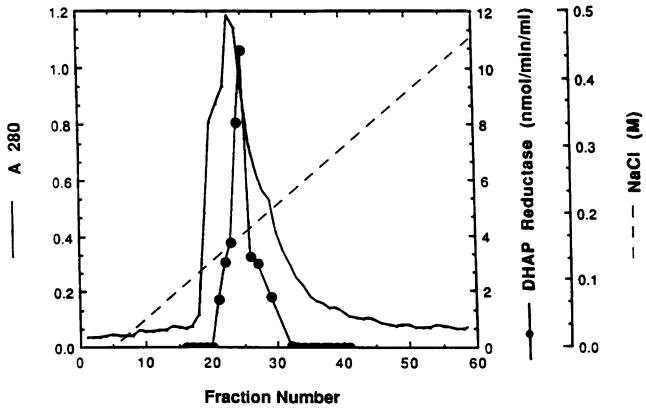

Figure 6. Chromatography of soluble proteins from peroxisomes on DEAE-cellulose. Soluble peroxisomal proteins, prepared as described in "Materials and Methods," were applied to a DE52 column (0.7 × 20 cm) equilibrated with 25 mM Tris (pH 7.0), 10 mM 2-mercaptoethanol. After the column was washed with the same buffer, adsorbed proteins were eluted with a linear gradient of 0 to 0.5 M NaCl (40 mL).

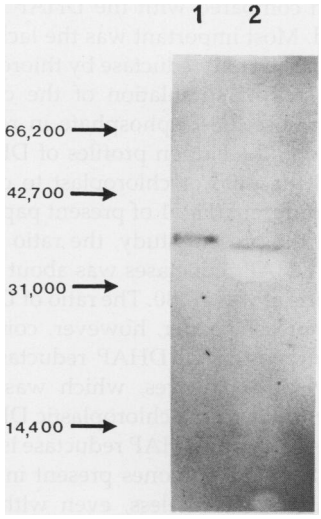

Figure 7. Comparison of peroxisomal DHAP reductase with purified cytosolic DHAP reductase by western blotting. Soluble proteins from peroxisomes were chromatographed on Red agarose. Proteins were eluted from the column with 2 mM NADH in 25 mM Tris (pH 7.0), 10 mM 2-mercaptoethanol, and 0.5 M NaCl and were subjected to SDS-PAGE and western blotting together with purified cytosolic DHAP reductase. Lane 1, Purified cytosolic DHAP reductase; lane 2, peroxisomal protein after Red agarose. Antibodies were raised against the purified cytosolic enzyme.

unit mol wt than the cytosolic DHAP reductase and, therefore, appeared to be a different isoform.

DISCUSSION

Results of the present study suggest the occurrence of spinach leaf DHAP reductase in three cell compartments: cytosol, chloroplasts, and peroxisomes.

Physiologically, DHAP reductase is involved in glycerolipid biosynthesis, catalyzing the reduction of DHAP to glycerol 3-phosphate in leaf cells. There are at least two pathways for the synthesis of glycerolipids, each of which produces different sets of glycerolipids (19). The end product of chloroplast fatty acid synthesis is acyl-ACP. Although chloroplast glycerol 3-phosphate acyl transferase in vitro uses both acyl CoA and acyl-ACP substrates, in vivo acyl-ACP is used rather than acyl-CoA (6). Unless glycerol 3-phosphate is imported from the cytosol, chloroplast DHAP reductase would prevent the use of acyl-CoA for chloroplast glycerolipid synthesis because the presence of acyl-CoA would inhibit the production of glycerol 3-phosphate from photosynthetic DHAP, making acyl-ACP the only substrate for the transferase. The second site of glycerolipid synthesis, the cytoplasmic side of the ER membrane, would more likely use glycerol 3-phosphate synthesized by the cytosolic DHAP reductase that uses DHAP from glycolysis or DHAP exported from chloroplasts.

Our previous investigation of DHAP reductase isoforms in leaves had also indicated the presence of a chloroplastic and a cytosolic DHAP reductase (8). However, the purified isoforms described in the present study had quite different

properties when compared with the DHAP reductases previously reported. Most important was the lack of stimulation of the chloroplastic DHAP reductase by thioredoxin and DTT as well as the lack of stimulation of the cytosolic DHAP reductase by fructose 2,6-bisphosphate in recent investigations. Furthermore, the elution profiles of DHAP reductases from DE52 and the ratio of chloroplast to cytosolic DHAP reductase were different (Fig. 1 of present paper versus figure 1 of ref. 10). In the present study, the ratio of the cytosolic to chloroplastic DHAP reductases was about 80:20, whereas in previous reports, it was 20:80. The ratio of DHAP reductase isoforms in the present paper, however, coincides with the ratio previously reported for DHAP reductases from senescent or market spinach leaves, which was approximately 80:20 for the cytosolic to the chloroplastic DHAP reductases (12). This suggests that the DHAP reductase isoforms isolated in the present study are the ones present in spinach leaves during senescence. Nevertheless, even with fresh spinach leaves from plants grown in a growth chamber, both crude and pure chloroplast preparations by the isolation procedures used in this paper were not stimulated by DTT or thioredoxin, nor was the cytosolic form stimulated by fructose-2,6-bisphosphate. We have not reconciled these differences. The length of time to prepare the homogenate and to perform the ammonium sulfate fractionation and dialysis steps to first reveal activity are variables that need further evaluation.

After a chloroplast stromal fraction had been chromatographed on DE52, a smaller peak of DHAP reductase activity eluted from the column at a higher salt concentration than the major chloroplast peak (Fig. 4). This indicated either the presence of a second chloroplastic isozyme or contamination of chloroplasts by other cell compartments. The discovery of DHAP reductase activity in the isolated leaf peroxisomal fraction seems to be the third isoform in spinach leaves. Previously, this isoform had not been detected in small peroxisomal preparations (10). In the present study, no DHAP reductase activity was detectable even in larger peroxisomal fractions from a sucrose gradient, until the soluble proteins from the broken peroxisomes had been chromatographed on DE52. This is similar to the failure to detect any of the isoforms in crude homogenates until inhibitors had been removed by partial purification (10). Thus, several reasons for not detecting the peroxisomal form of DHAP reductase are its low activity relative to the other two forms, its inhibition by acyl-CoAs or other unknown components in crude preparations or in peroxisomes, and its elution position on chromatography columns in a region of high endogenous NADH oxidation, which lowered the sensitivity of the assay. The peroxisomal DHAP reductase was clearly different from the chloroplastic form and appeared to have a smaller subunit mol wt than the cytosolic form as determined by SDS-PAGE. This is similar to the peroxisomal glycerol-3-phosphate dehydrogenases from rat and mouse liver, where the peroxisomal form had a higher electrophoretic mobility than the cytosolic counterpart (9). Insignificant DHAP reductase activity in plant mitochondrial fractions is probably due to peroxisomal contamination and is consistent with the situation in mitochondria from animal cells, where only a flavin adenine dinucleotide-dependent glycerol-3-phosphate dehydrogenase is present (21).

Both purified cytosolic and chloroplastic DHAP reductases were specific for DHAP and NADH, the activity with NADPH being only 3% of the activity with NADH. Less pure preparations when assayed at pH 6.0 had 50% as much activity with NADPH as with NADH, which might be attributed to the presence of acid phosphatases, which would convert NADPH to NADH (17).

Because of the low abundance of the isoforms of DHAP reductase, the purification of the cytosolic form was extremely high. The final specific activity of about 500 μmol min^{-1} mg^{-1} protein, however, fits approximately in the range for DHAP reductases from animal cells, such as those from rat brain (22) and from *Drosophila* (23), that had a specific activity of about 150 μmol min^{-1} mg^{-1}.

LITERATURE CITED

1. **Arnon DI** (1949) Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. Plant Physiol **24:** 1–15
2. **Bergmeyer HU, Gawehn K, Grassl M** (1974) Enzymes as biochemical reagents. *In* HU Bergmeyer, ed, Method of Enzymatic Analysis, Ed 2, Vol 1. Academic Press, Inc., New York, pp 438–439, 452–453
3. **Bradford MM** (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72:** 248–254
4. **Cerović ZG, Plesničar M** (1984) An improved procedure for the isolation of intact chloroplasts of high photosynthetic capacity. Biochem J **223:** 543–545
5. **Finlayson SA, Dennis DT** (1980) NAD^+-specific glycerol 3-phosphate dehydrogenase from developing castor bean endosperm. Arch Biochem Biophys **199:** 179–185
6. **Frentzen M, Heinz E, McKeon TA, Stumpf PK** (1983) Specificities and selectivities of glycerol-3-phosphate acyltransferase and monoacylglycerol-3-phosphate acyltransferase from pea and spinach chloroplasts. Eur J Biochem **129:** 629–636
7. **Gee R, Goyal A, Byerrum RU, Tolbert NE** (1989) Two isozymes of dihydroxyacetone phosphate reductase in *Dunaliella*. Plant Physiol **91:** 345–351
8. **Gee R, Goyal A, Gerber D, Byerrum RU, Tolbert NE** (1988) Isolation of dihydroxyacetone phosphate reductase from *Dunaliella* chloroplasts and comparison with isozymes from spinach leaves. Plant Physiol **88:** 896–903
9. **Gee R, Tolbert NE** (1982) Glycerol phosphate dehydrogenase in rat and mouse liver peroxisomes. Ann NY Acad Sci **368:** 417–419
10. **Gee RW, Byerrum RU, Gerber DW, Tolbert NE** (1988) Dihydroxyacetone phosphate reductase in plants. Plant Physiol **86:** 98–103
11. **Gee RW, Byerrum RU, Gerber DW, Tolbert NE** (1988) Differential inhibition and activation of two leaf dihydroxyacetone phosphate reductases. Plant Physiol **87:** 379–383
12. **Gee RW, Byerrum RU, Gerber DW, Tolbert NE** (1989) Changes in the activity of the chloroplastic and cytosolic dihydroxyacetone phosphate reductase during maturation of leaves. Plant Physiol **89:** 305–308
13. **Goyal A, Betsche T, Tolbert NE** (1988) Isolation of intact chloroplast from *Dunaliella tertiolecta*. Plant Physiol **88:** 543–546
14. **Harlow E, Lane D** (1988) Immunoblotting. *In* Antibodies: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, pp 471–510
15. **Haus M, Wegmann K** (1984) Glycerol-3-phosphate dehydrogenase (EC 1.1.1.8.) from *Dunaliella tertiolecta*. 1. Purification and kinetic properties. Physiol Plant **60:** 283–288
16. **Heukeshoven J, Dernick R** (1983) Simplified method for staining of proteins in polyacrylamide gels and the mechanism of silver staining. Electrophoresis **6:** 103–112
17. **Kleczkowski LA, Edwards EG** (1989) Identification of hydroxy-

pyruvate and glyoxylate reductases in maize leaves. Plant Physiol **91:** 278–286

18. **Klöck G, Kreuzberg K** (1989) Kinetic properties of a *sn*-glycerol-3-phosphate dehydrogenase purified from the unicellular alga *Chlamydomonas reinhardtii*. Biochim Biophys Acta **991:** 347–352
19. **Kunst L, Browse J, Somerville C** (1988) Altered regulation of lipid biosynthesis in a mutant of *Arabidopsis* deficient in chloroplast glycerol-3-phosphate acyltransferase activity. Proc Natl Acad Sci USA **85:** 4143–4147
20. **Laemmli UK** (1970) Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature **227:** 680–685
21. **Lin ECC** (1977) Glycerol utilization and its regulation in mammals. Annu Rev Biochem **446:** 765–795
22. **McGinnis JF, de Villis J** (1974) Purification and characterization of rat brain glycerol phosphate dehydrogenase. Biochim Biophys Acta **364:** 17–27
23. **Niesel DW, Bewley GC, Miller SG, Armstrong FB** (1980) Purification and structural analysis of the soluble *sn*-glycerol-3-phosphate dehydrogenase isozymes in *Drosophila melanogaster*. J Biol Chem **255:** 4073–4080
24. **Santora GT, Gee R, Tolbert NE** (1979) Isolation of a *sn*-glycerol-3-phosphate: NAD oxidoreductase from spinach leaves. Arch Biochem Biophys **196:** 403–411
25. **Towbin H, Staehelin T, Gordon J** (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA **76:** 4350–4354
26. **Wegmann K** (1971) Osmotic regulation of photosynthetic glycerol production in *Dunaliella*. Biochim Biophys Acta **2234:** 317–323