

Two Isoforms of Dihydroxyacetone Phosphate Reductase from the Chloroplasts of *Dunaliella tertiolecta*¹

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Three isoforms of dihydroxyacetone phosphate reductase in extracts from *Dunaliella tertiolecta* have been separated by a diethylaminoethyl cellulose column chromatography with a shallow NaCl gradient. The chloroplasts contained the two major isoforms, and the third, minor form was in the cytosol. The isoforms are unstable in the absence of glycerol and they are cold labile, but they may be partially reactivated at 35°C. The first chloroplast form to elute from the DEAE cellulose column was the major form when the cells were grown on high NaCl and it has been referred to as the form for glycerol production for osmoregulation or "osmoregulator form." The second form increased in specific activity when inorganic phosphate was increased in the growth media to stimulate growth, and it has been given the designation for the form for glyceride synthesis, "glyceride form." The osmoregulator form was stimulated by NaCl added to the enzyme assay, but not by reduced *Escherichia coli* thioredoxin. The glyceride form had properties similar to the enzyme in leaf chloroplast, such as inhibition by NaCl and by fatty acyl-coenzyme A derivatives and some stimulation by dithiothreitol, uridine diphosphate galactose, cytidine diphosphate dipalmitoyl diglyceride, and reduced *E. coli* thioredoxin. Thus, *Dunaliella* chloroplasts have a salt-stimulated osmoregulatory form of dihydroxyacetone phosphate reductase, which seems to have a role in glycerol production, and an isoform, which may be involved in glyceride synthesis and which has properties similar to the enzyme in chloroplasts of higher plants.

Leaves of higher plants contain both a chloroplastic and a cytoplasmic DHAP reductase form (Gee et al., 1988a, 1988b). The presence of DHAP reductase has also been reported in *Dunaliella tertiolecta* (Haus and Wegman, 1984; Marengo et al., 1985; Goyal et al., 1987) and *Dunaliella parva* (Gimmler and Lotter, 1982), and the enzyme was considered to be localized in the chloroplast (Brown et al., 1982; Gimmler and Lotter, 1982). More recently, we have described an active chloroplastic as well as a minor cytosolic isozyme of DHAP reductase in *Dunaliella* (Gee et al., 1989). The activity in the *Dunaliella* chloroplastic fraction was stimulated by thioredoxin, NaCl, and Mg²⁺. Because the minor form was absent in isolated chloroplasts or membrane fractions, it was designated as cytosolic. When *Dunaliella* is salt stressed or grown in salt medium, cells produce glycerol as an osmoticum (Wegmann, 1979; Avron, 1986; Goyal et al., 1986a, 1986b). The first enzyme for glycerol synthesis is a DHAP reductase

that reduces DHAP to glycerol-3-P. Glycerol-3-P is the precursor for both glycerol production and glyceride synthesis. NaCl inhibits the chloroplastic isoform of DHAP reductase in higher plants, but activates the algal chloroplastic isoform (Marengo et al., 1985; Gee et al., 1988c). Because of the different properties of the chloroplastic isoform in *Dunaliella* and in higher plants, we postulated that there may be different isoforms of DHAP reductase specifically involved in glycerol and glyceride synthesis.

In our previous work (Gee et al., 1989), the elution profile for the chloroplast DHAP reductase from DEAE cellulose chromatographic columns often was not symmetrical, indicating that there might be two chloroplastic forms. We have now been able to separate two isoforms from the *Dunaliella* chloroplast by modifying the DEAE chromatography and by varying the composition of the growth media to alter the ratio of the activities in the chloroplasts.

MATERIALS AND METHODS

Organism and Growth Conditions

Dunaliella tertiolecta (Commonwealth Scientific and Industrial Research Organization Marine Laboratory, Hobart, Australia) was maintained and cultured in a "*Dunaliella* medium" as described by Johnson et al. (1968) and modified by Goyal et al. (1987) or in a minimal medium (Sueoka, 1959). The rich, synthetic *Dunaliella* medium contained higher levels of phosphate (0.75 mM) than the minimal media, which contained 0.25 mM phosphate (Sueoka, 1959). In the present work, levels of NaCl and phosphate in the growth media have been varied. The algal cultures were grown with shaking in light (150 $\mu\text{E m}^{-2} \text{s}^{-1}$) on a 16:8 h light:dark cycle with about 3% CO₂ in air (v/v). After 3 or 4 d, while still in the log phase of growth, cells were harvested by centrifugation at 1000g for 5 min. Before harvesting, cultures were examined microscopically for bacterial or fungal contamination, and in some cases by plating on agar plates with a Glc yeast extract medium.

Isolation and Assay of the Enzyme

Dunaliella cells (about 2–4 mL packed cell volume) were broken in 30 to 40 mL of 100 mM Tris (pH 6.9), 20 mM ascorbate, and 5 mM DTT by two passes through a Yeda Press with 1500 p.s.i. of nitrogen. The homogenate was centrifuged at 40,000g for 30 min and the supernatant was

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Abbreviation: DHAP, dihydroxyacetone phosphate.

applied to a DEAE cellulose (DE 52) column (2.5 × 36 cm). The column was washed with 10 column volumes of 25 mM Tris (pH 6.9) and eluted with a linear gradient of 1 L of 0 to 0.4 M NaCl in 25 mM Tris at pH 6.9. If the enzyme preparation was to be stored, the active fractions were pooled and glycerol was added to a final concentration of 2 M and the enzyme was stored frozen at -18°C . To prove the presence of two major forms of DHAP reductase in the chloroplasts, intact chloroplasts were isolated from *Dunaliella* cells as previously described (Goyal et al., 1988) by density gradient centrifugation in Percoll, and the isolated chloroplasts were broken and chromatographed as described for whole cells. All steps of isolation were at 4°C .

The DHAP reductase activity was assayed at 25°C in the reductase direction at pH 6.9 expressed as $\mu\text{mol NADH oxidized h}^{-1} \text{mg}^{-1} \text{Chl}$ (Gee et al. 1988a). The glycerol-P dehydrogenase (oxidation direction) activity was assayed at pH 9.5 with 20 or 50 mM DL-glycerol phosphate and 2 mM NAD or NADP.

DHAP, as the dimethylketal dimonocyclohexylamine salt, was hydrolyzed with Dowex 50 H^{+} as described by Sigma. DEAE cellulose was DE-52 from Whatman Ltd. Thioredoxin was from *Escherichia coli* (CalBiochem). The reduction of thioredoxin by DTT before use and its preparation for the DHAP reductase assay has been described (Gee et al., 1988a; modification of Wolosiuk et al., 1980). The total Chl content was determined spectrophotometrically at 645 and 663 nm on an aliquot of cells, chloroplasts, or homogenate that had been extracted with 80% acetone (Arnon, 1949).

RESULTS

DEAE Cellulose Chromatography

Our previous isolation procedure for DHAP reductase isoforms from spinach leaves (Gee et al., 1988a) had involved

homogenization, concentration by ammonium sulfate fractionation, and chromatography on DEAE cellulose to remove inhibitors in the homogenate before significant activity could be detected. Ammonium sulfate also inhibits DHAP reductase. Because the activity of DHAP reductase in *Dunaliella* extracts was high, it was possible to omit the $(\text{NH}_4)_2\text{SO}_4$ step to concentrate the protein. Instead, the supernatant from *Dunaliella* cell homogenate could be applied directly to a DEAE cellulose column. Care had to be taken to keep the salt concentration in buffers low so that the activity would bind to the DEAE cellulose and not be eluted in the first fractions from the column. By using a shallow NaCl salt gradient (1 L of 0–0.4 M NaCl in 25 mM Tris, pH 6.9), three forms of DHAP reductase in *Dunaliella* extracts were separated as shown in Figure 1, A and B. In spite of earlier reports that only one chloroplastic DHAP reductase existed in *Dunaliella* (Brown et al., 1982; Gimmler and Lotter, 1982; Haus and Wegmann, 1984; Marengo et al., 1985), this type of elution profile with three activity peaks has now been observed with over 25 different cultures. The two major forms of DHAP reductase in *Dunaliella* were present in the intact isolated chloroplasts (data not shown; elution profiles were identical to data shown in Fig. 1, A and B). If cultures grown in high-salt media entered the stationary phase of growth, or growth was inhibited, only one peak of chloroplastic DHAP reductase was present (Fig. 1C) that exhibited properties of the osmoregulator form. In such cultures, the glyceride form is a minor component and its activity is masked by the osmoregulator form.

In designating these peaks, data will be presented that is consistent with the hypothesis that the first peak of activity is a chloroplastic isoform for glycerol production for osmoregulation, that the second peak is a different chloroplastic isoform that is similar to the form in the chloroplasts of

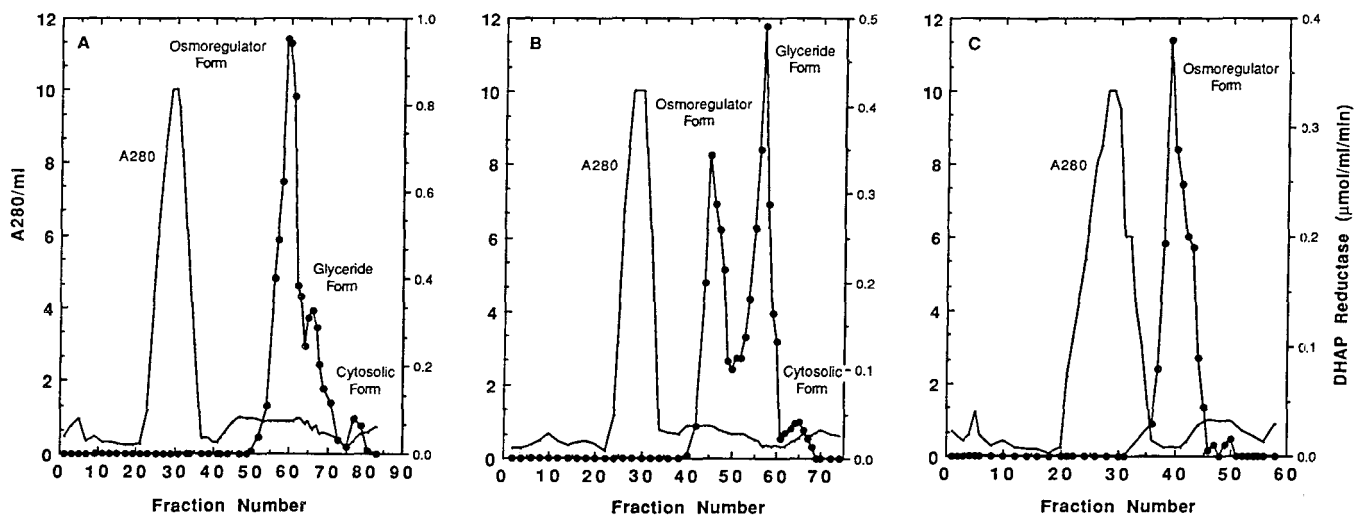


Figure 1. DEAE cellulose chromatographic separation of three DHAP reductase isoforms (●—●) from homogenates of *Dunaliella*. Each fraction contained 5 mL. The first peak is the osmoregulator form, the second peak is the glyceride form, and the third peak is the cytosolic form. The cells were grown in minimal medium containing 0.17 M NaCl and 0.25 mM phosphate (A), 2.5 mM phosphate (B), and 2.5 mM phosphate grown to stationary phase (C). Cells used for these preparations contained a total of about 10 mg Chl. Average activities found for the three peaks are given in Table I. Each part of the figure is from a different algal preparation with slight variations in column packaging, flow rates, and crude homogenate added to the column. Thus, the elution volumes for three isoforms shifted relative to each other. Their identifications were confirmed by at least three of the properties cited in Table III.

higher plants for glycerol-P production for formation of glycerides or lipids, and that the third smaller peak is a cytosolic isoform. Consequently, the first isoform of DHAP reductase eluted from the DEAE column will be called the chloroplastic "osmoregulator form," the second peak will be known as the chloroplastic "glyceride form," and the third peak will be called the "cytosolic form." It is recognized that for glycerol production by the osmoregulator form, glycerol-P is first produced and is then dephosphorylated by a specific glycerol-3-P phosphatase (Sussman and Avron, 1981).

The total DHAP reductase activity in the *Dunaliella* homogenate, as in leaf extracts (Gee et al., 1988c), increased after partial purification of the enzyme on the DEAE cellulose column (Table I). The increase in activity (Table I) after DEAE cellulose chromatography appears to be due primarily to an increase in the chloroplastic osmoregulator form of the DHAP reductase. The reason for this increase is not known, but it is unlikely to be due to NaCl removal since NaCl (up to 250 mM) actually stimulates the osmoregulator form of DHAP reductase. The *Dunaliella* cell pellet was resuspended and homogenized in 10 volumes of the extraction buffer, so that NaCl carried over in the homogenate did not exceed 150 mM. The activity as the glyceride form in leaf homogenates could not be measured until after DEAE cellulose chromatography, due to the removal of low mol wt inhibitors, such as fatty acids and other membrane components (Gee et al., 1988a, 1988b). However, the *Dunaliella* osmoregulator form was not inhibited by fatty acids and membrane components (next sections), so the nature of the inhibitors in the *Dunaliella* extract remains unknown. A more accurate value for total DHAP reductase activities is that from the fractions after DEAE cellulose chromatography, even though some inactivation from adverse temperature and passage of time may have occurred.

Effect of NaCl in Growth Media

The amount of NaCl in the minimal growth medium, which contains 0.25 mM phosphate, altered the ratio of activity of the two chloroplastic isoforms (Fig. 1, A and B; Table I). Increasing the NaCl concentration from 0.17 to 1.5 M while maintaining the phosphate level at 0.25 mM decreased the growth rate, as indicated by the decrease in total Chl content during 4 to 6 d of growth. The ratio of cell number to mg Chl varied only within 10%. The total DHAP

Table II. Effects of changing phosphate concentrations in *Dunaliella* growth medium on DHAP reductase activity in cells grown with 0.17 M NaCl

| Phosphate in media | mM | | | |
|---------------------------------------|---|------|-----|-----|
| | 0.025 | 0.25 | 2.5 | 25 |
| | $\mu\text{mol h}^{-1} \text{mg Chl}^{-1}$ | | | |
| Total DHAP reductase from DEAE column | 109 | 150 | 195 | 192 |
| Osmoregulator form, chloroplast | 72 | 108 | 96 | 90 |
| Glyceride form, chloroplast | 31 | 36 | 93 | 96 |
| Cytosolic form | 6 | 6 | 6 | 6 |
| | $\%$ of total activity | | | |
| Osmoregulator form, chloroplast | 66 | 72 | 50 | 47 |
| Glyceride form, chloroplast | 28 | 24 | 48 | 50 |

reductase per mg Chl, as measured in the crude homogenate, decreased only slightly with high salt and slower growth. However, the total DHAP reductase recovered from the DEAE column increased by 2.5-fold in the algae grown with 1.5 M NaCl. The significant increase in total activity was due to a severalfold increase in the osmoregulator form (Table I). The amount of increased activity with salt was the same on a Chl or a cell basis. Such an increase would be consistent with escalated glycerol production with increasing salinity (Wegmann 1979; Goyal et al., 1986b). With increasing salt concentration, the cytosolic activity remained low and constant, but as expected, the osmoregulator form in the chloroplast increased while the glyceride form was relatively unchanged (Table I).

Effect of Phosphate Concentration in Growth Medium

The *Dunaliella* growth medium contained 0.25 mM phosphate and 0.17 M NaCl; about 70% of the DHAP reductase activity was in the osmoregulator form and 25% was in the glyceride form (Fig. 1A; Table II). Upon increasing the phosphate concentration 10-fold (2.5 mM) or a 100-fold (25 mM), the percentage of the total DHAP reductase in the chloroplastic glyceride form increased from 25% to 50%, so that the amount of both chloroplastic forms became about the same at higher phosphate levels (Fig. 1B; Table II). Since the total DHAP reductase increased with increasing phosphate

Table I. Effect of changing NaCl in minimal growth medium (0.25 mM phosphate) on DHAP reductase activity

| NaCl in the media (M) | 0.17 | 0.5 | 1.0 | 1.5 |
|---|---|------|-----|-----|
| Total Chl (mg) in preparation | 10.8 | 11.2 | 8.0 | 4.2 |
| | $\mu\text{mol h}^{-1} \text{mg Chl}^{-1}$ | | | |
| Total DHAP reductase measured in crude homogenate | 110 | 88 | 83 | 73 |
| Total DHAP reductase from DEAE cellulose column | 86 | 125 | 136 | 227 |
| Osmoregulator form, chloroplast | 21 | 28 | 80 | 128 |
| Glyceride form, chloroplast | 57 | 90 | 48 | 91 |
| Cytosolic form | 8 | 7 | 8 | 8 |
| | $\%$ of total activity | | | |
| Osmoregulator form, chloroplast | 24 | 22 | 59 | 56 |
| Glyceride form, chloroplast | 66 | 72 | 35 | 45 |

concentration, the percentage of the total activity in the osmoregulator form decreased but the amount of activity remained about constant. This would be consistent with more rapid growth with adequate phosphate. Thus, increasing NaCl levels raised the amount of the osmoregulator form, whereas increasing growth by adding more phosphate added to the glyceride form. The cytosolic form did not change with either increased salt or phosphate levels. The amount of the three isoforms seems to be regulated independently of each other.

Stability

All three forms of the partially purified DHAP reductase were time and temperature labile and had to be stored frozen at -18°C in 2 M glycerol to retain activity for 1 month. The activity of the frozen preparations could be partially restored by warming at 35°C for 30 min or at room temperature ($25\text{--}30^{\circ}\text{C}$) for 3 h. After the first freeze-thaw cycle, activity could also be restored (up to 75%) by addition of 24 mM MgCl_2 , but if the enzyme was subjected to additional freeze-thaw cycles, 5 mM reduced DTT in addition to MgCl_2 was required. To achieve a linear enzyme assay with time, both DTT and 24 mM Mg^{2+} needed to be present. Each time one of the stored preparations was frozen and thawed, activity was lost, so that after four freeze-thaw cycles only 25% of the original activity could be recovered (data not shown). The enzyme could also be stored at 4°C in 2 M glycerol for 3 d. However, to recover most of the activity, heat activation was necessary. If preparations were stored frozen or at 4°C without glycerol, the activity was irreversibly lost and no reactivation occurred with warming, MgCl_2 , or DTT.

Substrate Specificity, pH Optimum, and K_m Values

DHAP reductase isoforms from leaves are highly specific for NADH (Gee et al., 1988c). It has been reported previously

that the activity in the chloroplasts of *Dunaliella* could use either NADH or NADPH without realizing that there were two isoforms present (Marengo et al., 1985; Gee et al., 1988c). However, as shown in Table III and Figure 2, both isoforms in the chloroplast can reduce DHAP with either NADH or NADPH. In this lack of pyridine nucleotide specificity, the *Dunaliella* chloroplastic isoforms for glycerol-P formation are different from the enzyme in spinach chloroplasts.

The pH optimum for both *Dunaliella* chloroplast reductase isoforms was about 6.9, and both showed a broad pH profile (Fig. 2). To measure any activity of either peak as a glycerol-3-P dehydrogenase, at least 20 mM DL-glycerol-P and 2 mM NAD^+ or NADP^+ at pH 9.5 was required (data not shown). The enzyme appears to be nearly irreversible under physiological conditions, as is also the case for isoforms from leaves.

Effect of NaCl on Activity of Isolated DHAP Reductase Forms from *Dunaliella*

The partially purified osmoregulator form from *Dunaliella* chloroplasts was stimulated at least 2-fold with 100 mM NaCl in the assay mixture. Since the buffers and other reagents in assay solutions also totaled about 100 mM, maximum activation occurred with about 200 to 250 mM salt, but higher salt concentrations became inhibitory. This NaCl stimulation of the osmoregulatory form was unique and was repeatedly observed in all enzyme preparations. The chloroplastic glyceride form from *Dunaliella* was inhibited by 100 mM NaCl (Table III), and, likewise, this isoform from leaf chloroplasts was severely inhibited by 25 mM NaCl (Gee et al., 1988c). The cytosolic form from *Dunaliella* or leaves was also inhibited by NaCl (Gee et al., 1989).

Differential Regulation of the Two Forms from the *Dunaliella* Chloroplast

Besides the data just presented on the difference between the two *Dunaliella* chloroplastic forms of DHAP reductase,

Table III. Properties of two *Dunaliella* chloroplastic DHAP reductases

| | Osmoregulator Form | Glyceride Form |
|--|---------------------------------------|--------------------------------|
| pH optimum | 6.8 to 7.0 | 6.8 to 7.0 |
| K_m (NADH), μM | 51 | 57 |
| K_m (NADPH), μM | 71 | 70 |
| K_m (DHAP), μM | 340 | 380 |
| Increased NaCl in media | Activity increased | Activity is lost |
| Increased phosphate in media | Stays the same | Activity increased |
| 100 mM NaCl in assay | Stimulated 2 to 3-fold | Inhibited 50% |
| Glutathione (100 μM) | No effect | No effect |
| DTT (1 mM) | Slight inhibition | Stimulated 3-fold |
| Reduced thioredoxin (20 μM) | Slight stimulation | Stimulated 4-fold |
| CoA derivatives (100 μM) | No effect | Inhibited |
| ATP (250 μM) | Inhibited | Inhibited |
| UDP-Gal (25 μM) | No effect | Activated 2-fold |
| CDP dipalmitoyl diglyceride (25 μM) | No effect | Activated 3-fold |
| Mg^{2+} (24 mM) | Activated approximately 3-fold | Activated approximately 3-fold |
| Ascorbate (1 mM) | Stimulated 2-fold | Stimulated 2-fold |
| Fru-2, 6-P ₂ (100 μM) | No effect | No effect |
| Fru-6-P (10–100 μM) | Stimulated 2-fold at 10 μM | Inhibited at 100 μM |
| Fru-1,6-P ₂ (1 μM to 1 mM) | Stimulated (20%) | |
| Sedoheptulose-1,7-P ₂ (25 μM) | No effect | Inhibited (20%) |
| Phosphate (1 mM) | Inhibited (50%) | Inhibited (50%) |

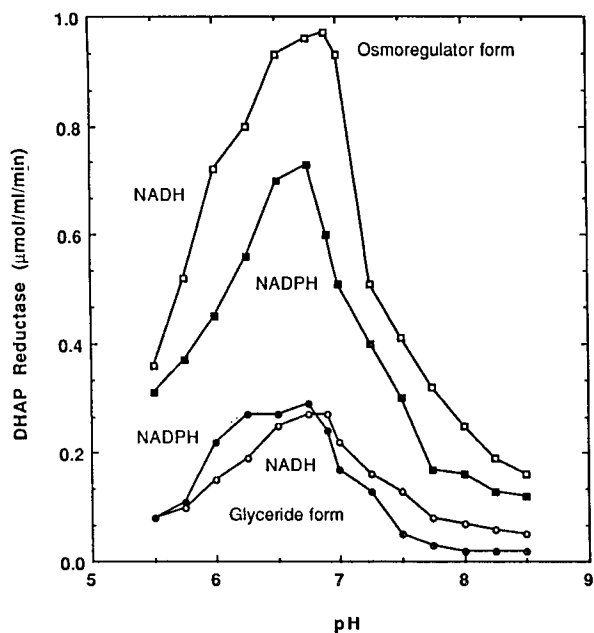


Figure 2. The pH optima and reduced pyridine nucleotide specificity for two chloroplastic isoforms of DHAP reductase from *Dunaliella*. The enzyme had been partially purified from a DEAE cellulose column (Fig. 1). Because the enzyme was from the late log phase, the glyceride form had less activity. The reaction in 1.0 mL has 33 mM Mes, 33 mM Hepes, 34 mM Tricine at the indicated pH values; DHAP was 1 mM and NADH or NADPH was 200 μ M.

the two forms were differentially regulated by inhibitors and activators of the glyceride form as previously described for leaf chloroplasts (Gee et al., 1988b). The glyceride form was inhibited by detergents, lipids, or long-chain acyl-CoA derivatives, regardless of whether it was isolated from chloroplasts, from leaves, or from *Dunaliella*. Oleoyl CoA, stearoyl CoA, and palmitoyl CoA inhibited the glyceride form, but did not inhibit the osmoregulator form (Fig. 3). The K_i for oleoyl CoA inhibition of the glyceride form was about 10 μ M. Likewise, the detergents deoxycholate and Triton X-100 inhibited the glyceride form, but not the osmoregulator form (Fig. 4). Chaps inhibited both forms. CDP-dipalmitoyl diglyceride at 1 μ M stimulated the glyceride form but became severely inhibitory at higher concentrations (Fig. 5). Micromolar concentrations of CDP-choline and UDP-Gal also stimulated the glyceride form (Fig. 5) but had no effect on the osmoregulator form (data not shown). Thus, in general the glyceride form for lipid synthesis was more sensitive to detergents and lipids than the osmoregulator form for glycerol synthesis. Both isoforms from the chloroplasts of *Dunaliella* were 50% inhibited by 1 mM Pi (Table III). Phosphoglycolate, ribulose-1,5-bisphosphate, and Fru-6-P inhibited the glyceride form, but not the osmoregulator form (Table III). In contrast, Fru-1,6-P₂ stimulated the osmoregulator form but did not affect the glyceride form (Table III). Reduced thioredoxin or DTT stimulated the glyceride form in the chloroplast, whereas these reducing agents have little stimulatory effect on the osmoregulator form.

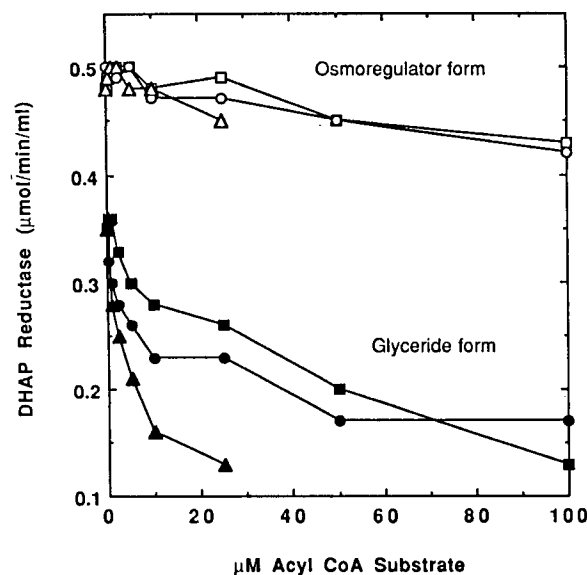


Figure 3. Effect of acyl-CoA substrates on two chloroplastic isoforms of DHAP reductase from *Dunaliella*. Osmoregulator form, open symbols; glyceride form, closed symbols. Palmitoyl CoA (○, ●); stearoyl CoA (□, ■); oleoyl CoA (▲, △).

DISCUSSION

The total activity of the DHAP reductases in *Dunaliella* greatly exceeds that in spinach leaves, in part because of high activity of the osmoregulator form for glycerol production. In addition, the DHAP reductase isoforms from *Dunaliella* had higher activity on a Chl basis than those from spinach leaves. V_{max} of the chloroplast DHAP reductase from spinach leaves

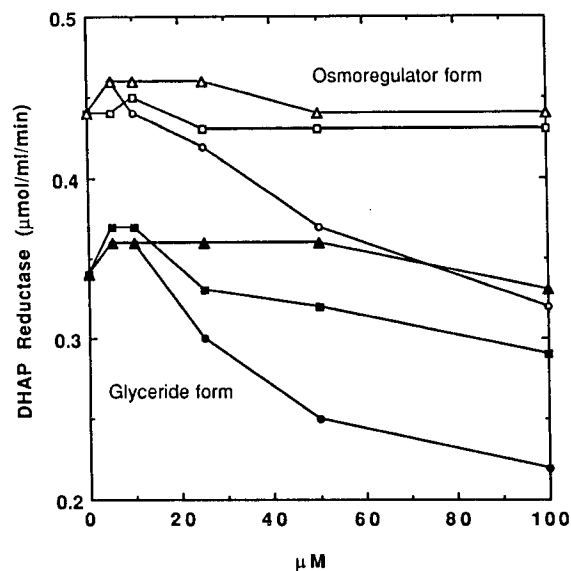


Figure 4. Effect of detergents on two chloroplastic isoforms of DHAP reductase from *Dunaliella*. Osmoregulator form, open symbols; glyceride form, closed symbols. Chaps (○, ●); Triton X-100 (□, ■); deoxycholate (▲, △).

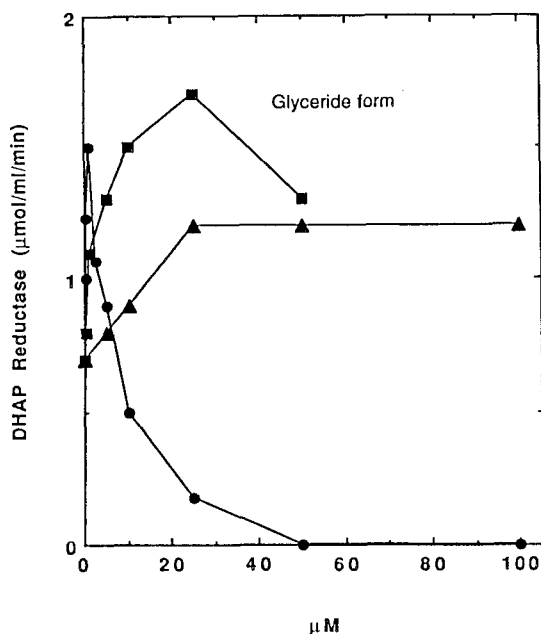


Figure 5. Effect of lipid precursors on chloroplastic glyceride isoform of DHAP reductase from *Dunaliella*. CDP-choline (●), CDP-dipalmitoyl diglyceride (■), UDP-Gal (▲).

was $13 \mu\text{mol h}^{-1} \text{mg}^{-1}$ Chl compared with 128 for the osmoregulator form and 100 for the glyceride form from *Dunaliella*. The spinach cytosol form had a V_{max} of $4 \mu\text{mol h}^{-1} \text{mg}^{-1}$ Chl, whereas V_{max} was 8 for this form in *Dunaliella*. Thus, starting with *Dunaliella* cells, detection of enzyme activity in crude homogenates was possible, whereas unknown inhibitors in the homogenates prevented measurements of the DHAP reductase isoforms from leaf tissues. The same inhibition of the glyceride isoform seems also to be occurring in *Dunaliella* extracts, but it was never recognized because the osmoregulator isoform was present in such large quantity, and it was not inhibited.

The chloroplast enzymes in *Dunaliella* had little preference for NADH or NADPH and may well use NADPH *in vivo* because of their chloroplast location. This pyridine nucleotide specificity for these algal reductases is different than that for the enzymes from higher plants, which are specific for NADH.

Previous investigations of the glycerol metabolism for osmoregulation in *Dunaliella* were based on DHAP reductase activity in the chloroplast (Brown et al., 1982; Gimmler and Lotter, 1982; Marengo et al., 1985; Goyal et al., 1987) for glycerol-P formation, coupled to a specific glycerol-P phosphatase (Sussman and Avron, 1981). These reports on DHAP reductase from *Dunaliella* spp. probably reflected only the osmoregulator form, because cultures were generally grown for more than 5 d or were harvested from the stationary phase, where only the osmoregulator form predominates (see Fig. 1C). Thus, previous investigators were not aware of the multiple isoforms of DHAP reductase in *Dunaliella*.

After reports that leaf or *Dunaliella* chloroplasts have a DHAP reductase glyceride isoform for glycerol-P formation

for lipid production (Gee et al., 1988a, 1988c), it was assumed that the glycerol-P pool in the *Dunaliella* chloroplasts would be used for either glycerol or for glyceride (lipid) synthesis. However, the presence in the *Dunaliella* chloroplasts of two distinctly different isoforms of DHAP reductase further suggests suborganelle enzymic localization of glycerol-P synthesis, one for glycerol production and one for the synthesis of lipids. We have no information on whether or how these two pathways might be functionally separated in the chloroplast. However, the properties of the DHAP reductase isoforms are well matched for the two uses of the glycerol-P produced (Table III). The osmoregulator form is stimulated by NaCl in contrast to the glyceride form, which is inhibited by NaCl. The glyceride form is tightly regulated by photosynthesis and lipid synthesis, being stimulated by growth-promoting factors such as phosphate in the growth medium, reduced thioredoxin from photosynthesis, or DTT, but inhibited by feedback regulators from lipid synthesis such as fatty acyl-CoA derivatives, lipids, and detergents. The glyceride form is also stimulated by lipid precursors such as UDP-Gal or CDP-dipalmitoyl diglyceride.

DHAP reduction by the osmoregulator form acts as if it might be linked directly with the glycerol-P phosphatase in *Dunaliella* cells. The chloroplastic osmoregulator DHAP reductase and glycerol-P phosphatase both are stimulated by NaCl and have a rather sharp pH optimum around 7.0 (Goyal, 1987). The free glycerol-P pool in this alga is about $200 \mu\text{M}$ (Goyal, 1987), whereas the apparent K_m of glycerol-P phosphatase for glycerol-P is about 2.7 mM (Sussman and Avron, 1981). Because of this unusually high K_m and lack of a substantial free glycerol phosphate pool *in vivo*, it is difficult to understand how substantially large rates of glycerol synthesis occur if DHAP reductase and glycerol-P phosphatase exist independently of each other in the chloroplast. It is reasonable to postulate that the osmoregulator form of DHAP reductase may be in a complex with or tightly associated with the glycerol-P phosphatase. This complex might result in a direct conversion of DHAP to glycerol. Mg^{2+} results in an increase in activity of all isoforms of DHAP reductase and a slight decrease in the apparent K_m for DHAP. This is also the case for glycerol-P phosphatase from *Dunaliella salina* (Sussman and Avron, 1981), where Mg^{2+} has been suggested to be complexed with the enzyme. Thus, it is possible that Mg^{2+} may be part of a DHAP reductase:glycerol-P phosphatase enzyme complex to support the high rates of glycerol formation observed in *Dunaliella*.

Because the two chloroplastic isoforms are regulated differently in *Dunaliella*, it seems reasonable to consider that salt stress (hyperosmotic stress) might inhibit the glyceride form and stimulate the osmoregulator form for glycerol production from DHAP. With only one form of DHAP reductase, it would be difficult to regulate two different functions. Depending on the extent of hyperosmotic stress, a large portion of DHAP can be derived from the products of starch breakdown (Avron, 1986; Goyal et al., 1986b; Goyal, 1987), whereas normally algae have a very low rate of starch breakdown in the light. A different isoform of DHAP reductase linked to glycerol production may be the way this alga copes with hyperosmotic stress.

Although the DHAP reductase isoform for glycerol-P for-

mation for glycerides is in the leaf and *Dunaliella* chloroplasts, the *Dunaliella* osmoregulator form of DHAP reductase and specific glycerol-P phosphatase for glycerol production have, so far, not been reported to be present in higher plants. Based on the very different properties of the two isoforms in *Dunaliella* chloroplasts, future work could be directed toward molecular engineering to put the *Dunaliella* osmoregulator form of DHAP reductase and glycerol-P phosphatase into plants to induce better osmoregulation with glycerol. Whereas previously, with only one known form of DHAP reductase in the chloroplast, it appeared difficult to alter glycerol-P production for glycerol osmoregulation without also altering lipid production, now with a different chloroplastic form of DHAP reductase for each function, it may be possible to regulate each end product differently.

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