Isolation, Characterization, and Partial Purification of a Reduced Nicotinamide Adenine Dinucleotide Phosphate-dependent Dihydroxyacetone Reductase from the Halophilic Alga *Dunaliella parva*

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ABSTRACT

An NADP⁺-dependent dihydroxyacetone reductase, which catalyzes specifically the reduction of dihydroxyacetone to glycerol, has been isolated from the halophilic alga *Dunaliella parva*. The enzyme has been purified about 220-fold. It has a molecular weight of about 65,000 and is highly specific for NADPH. The pH optima for dihydroxyacetone reduction and for glycerol oxidation are 7.5 and 9.2, respectively. The enzyme has a very narrow substrate specificity and will not catalyze the reduction of glyceraldehyde or dihydroxyacetone phosphate. It is suggested that this enzyme functions physiologically as a dihydroxyacetone reductase in the path of glycerol synthesis and accumulation in *Dunaliella*.

It has long been known that the genus *Aerobacter* can use glycerol as its sole source of carbon and energy (15). Glycerol induces *Aerobacter* to form an NAD⁺-dependent glycerol dehydrogenase (EC 1.1.1.6) (16). The enzyme was partly purified from *Aerobacter aerogenes* (6, 7, 12, 13, 17) and *Escherichia coli* (1). NADP⁺ could not replace NAD⁺, and the best substrates were glycerol, 1,2 propanediol, or 2,3 butanediol. The enzyme was found to require NH_4^+, K^+, or Rb^+ for activity (13) and was inhibited by metal chelating agents (13, 17).

The halophilic alga *Dunaliella* produces glycerol as the major product of photosynthesis (2, 9, 20, 21). Free glycerol is produced and accumulated within the cells to a final concentration required to balance osmotically the external salt concentration (2). In a previous communication (3) we demonstrated the presence of a new enzyme, NADP⁺-dependent dihydroxyacetone reductase, in cell-free extracts of *Dunaliella parva*. The enzyme catalyzes the reaction: dihydroxyacetone + NADPH + $H^+ \rightleftharpoons$ glycerol + NADP⁺.

In this paper we report the isolation, partial purification, and characterization of this dihydroxyacetone reductase from *D. parva*. The characteristics of this enzyme suggest that it most probably functions in the synthesis and accumulation of free glycerol in this halophilic alga.

MATERIALS AND METHODS

All chemicals were of the highest quality commercially available. NADP⁺, NADPH, NAD⁺, and NADH were supplied by Sigma Chemical Company. Glycerol was obtained from Fisher Scientific Company. Dihydroxyacetone was obtained from Fluka.

Dunaliella parva, a unicellular green halophilic alga, was isolated from the Dead Sea, and was maintained by periodical transfers in artificial media or on agar slants (4, 10). The growth medium contained: 1.5 M NaCl, 24 mM $MgSO_4$, 20 mM $MgCl_2$, 10 mM $CaCl_2$, 4 mM $NaNO_3$, 1 mM KNO_3, 0.1 mM K_2HPO_4, 1.5 μM $FeCl_3$, 30 μM Na_2 EDTA, 185 μM H_3BO_3, 7.0 μM $MnCl_2$, 0.8 μM $ZnCl_2$ 0.02 μM $CoCl_2$, 0.2 μM $CuCl_2$, 20 mM tris-Cl, pH 7.4. Agar slants were prepared by the addition of 2% agar and 5 mM Na acetate to the above medium. Cultures were grown under illumination with fluorescent lamps (light intensity of about 200 ft-c). The cultures were gassed with a slow stream of air + 5% CO_2 and were slowly shaken. Temperature was maintained at 25 C. Strict sterility was not required because of the high salt content of the medium. Under these conditions the generation time was about 15 hr.

The activity of dihydroxyacetone reductase was followed in a Cary 16 recording spectrophotometer at 340 nm. The reaction was carried out in 1-cm light-path cuvettes at 25 C. The standard assay mixture for glycerol oxidation contained 30 mM Tricine-glycine, pH 9.2, 50 μM NADP⁺, 2.4 M glycerol, and dihydroxyacetone reductase in a total volume of 1 ml. The reaction was initiated by the addition of the NADP⁺ or the enzyme. The standard assay mixture for dihydroxyacetone reduction contained: 30 mM Tricine, pH 7.4, 100 μM NADPH, 5 mM dihydroxyacetone, and dihydroxyacetone reductase in a total volume of 1 ml. The reaction was started by the addition of the dihydroxyacetone. The initial slope was used to calculate the enzyme activity which was expressed as μmoles of NADP⁺ reduced or NADPH oxidized per min. One unit of enzyme activity is defined as the amount of enzyme which oxidizes 1 μmole NADPH/min at 25 C under the conditions specified above.

Protein concentration in the crude preparations was determined by absorbance readings at 280 nm and 260 nm (19). In purified preparations the Lowry method was employed (14). Bovine serum albumin (Sigma) was used as the protein standard.

RESULTS

Purification of Dihydroxyacetone Reductase. Table I summarizes the purification procedure. *D. parva* fragments were obtained by osmotic bursting of the algae (4). Logarithmic phase grown algae, harvested from 20 liters of algal culture

Table I. *Purification Procedure of Dihydroxyacetone Reductase from Dunaliella parva*

Fraction	Volume	Protein	Total Activity	Specific Activity	Purification	Yield
	ml	*mg/ml*	*units*	*units/mg protein*	*fold*	*%*
D. parva fragments	100	20	10	0.005	1	100
Cell-free extract	80	2.37	7.2	0.038	7	72
DEAE-cellulose	200	0.39	6.6	0.085	17	66
Ammonium sulfate (40–75%)	2.8	2.43	3.9	0.58	115	39
Sephadex G-200	20	0.105	1.7	0.80	160	17
Ammonium sulfate	0.6	1.6	1.1	1.11	220	11

containing 0.2 g of Chl, were washed twice by centrifugation with 1.5 M NaCl, 10 mM Tricine, pH 7.5, at room temperature. The pellet was diluted 30-fold with 5 mM Tricine, pH 7.5, at 4 C yielding a final NaCl concentration of about 50 mM. All following procedures were carried out at 4 C. Broken cells were removed by centrifugation and the cell-free extract contained the dihydroxyacetone reductase activity.

The cell-free extract was applied to a 2 × 20-cm column of DEAE-cellulose (Whatman, DE52), which had been equilibrated with 5 mM Tricine, pH 7.5. Dihydroxyacetone reductase was not adsorbed to the anion exchange cellulose and was eluted in the void volume with the above buffer.

Solid ammonium sulfate was slowly added with constant stirring to the total eluted volume to yield a 40% saturated solution (28.8 g to 100 ml). Stirring was continued for an additional 20 min and the precipitated protein was removed by centrifugation at 38,000g for 30 min (pellet discarded). To the supernatant an additional 25.5 g of solid ammonium sulfate was added per 100 ml of solution to obtain a 75% saturated solution. After about 12 hr the suspension was centrifuged and the resulting pellet dissolved in a minimal volume of 5 mM Tricine, pH 7.5.

The enzyme solution obtained from the previous step was applied to a column (2.5 × 45 cm) of Sephadex G-200 superfine (Pharmacia, Uppsala, Sweden) previously equilibrated with 5 mM Tricine, pH 7.5. A flow rate of 15 ml/hr was maintained and 2- to 4-ml fractions were collected. The elution profile of the enzyme from the Sephadex is presented in Figure 1. Enzyme fractions with high specific activity were pooled, and the protein was precipitated by the addition of solid ammonium sulfate to yield 95% saturation (68.4 g to 100 ml). This fraction was either used for determination of enzyme properties, or stored at −20 C. For ammonium sulfate removal, the final enzyme solution was applied to a small column of Sephadex G-25 (1 × 15 cm) and the enzyme was collected in the void volume.

ENZYME PROPERTIES

Stability. The enzyme preparations can be stored in 95% ammonium sulfate at −20 C for at least 2 months without loss of activity. However, only 50% of activity was retained when this preparation was thawed and frozen daily during that period.

Assay Requirement. From the data presented in Table II, it is clear that no components other than the substrates were required for activity. The reduction of dihydroxyacetone and the oxidation of glycerol by dihydroacetone reductase required NADPH or NADP$^+$, respectively. NADH or NAD$^+$ were inactive.

Effect of Enzyme Concentration. Under the standard assay conditions, the reaction rate was directly proportional to enzyme concentration over the range of 0 to 0.2 mg protein/ml. Higher concentrations were not assayed.

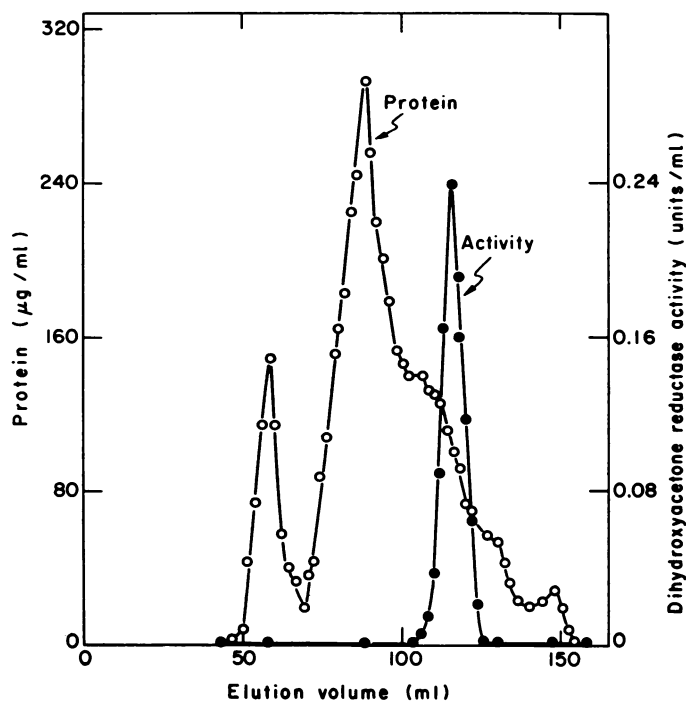*Determination of Molecular Weight.* Gel filtration of dihy-

FIG. 1. Elution profile of dihydroxyacetone reductase from Sephadex G-200 superfine. Elution volume measurement was started with the application of the enzyme solution to the column.

droxyacetone reductase on Sephadex G-200 column with other marker proteins, indicated that the dihydroxyacetone reductase has a mol wt of about 65,000.

pH Activity Curve. The dependence of enzymic activity on pH is shown in Figure 2. It is seen that dihydroxyacetone reduction proceeds best around pH 7.5, while the reverse reaction of glycerol oxidation has an optimum pH around 9.2.

Affinity for Substrates. The apparent K$_m$ values were calculated from Lineweaver-Burk plots. The K$_m$ values for glycerol and NADP$^+$, determined at pH 9.2, were 1.5 M and 15 μM, respectively. The K$_m$ values for dihydroxyacetone and NADPH, determined at pH 7.5, were 0.6 mM and 8 μM, respectively.

Equilibrium Constant. It is apparent that the pH at which the reaction is carried out will markedly affect the final equilibrium. Measurements were therefore made at different pH values in the range of 6.5 to 8.0. The reaction was carried out at 25 C and under the conditions specified under "Materials and Methods." From the absorption change at 340 nm, the amount of NADP$^+$ reduced was calculated and assumed to equal the concentration of dihydroxyacetone. The concentration of NADP$^+$ was obtained by subtracting the amount of NADPH formed from the amount of NADP$^+$ added. The

Table II. *Requirements for NADP+-dependent Dihydroxyacetone Reductase of Dunaliella parva*

The complete reaction mixture contained: for dihydroxyacetone reduction: 30 mM Tricine, pH 7.4; 100 μM NADPH or NADH; 5 mM dihydroxyacetone; and 8 μg dihydroxyacetone reductase in a total volume of 1 ml. For glycerol oxidation: 30 mM Tricine-glycine, pH 9.0; 50 μM NADP+ or NAD+; 2.4 M glycerol; and 8 μg dihydroxyacetone reductase in a total volume of 1 ml.

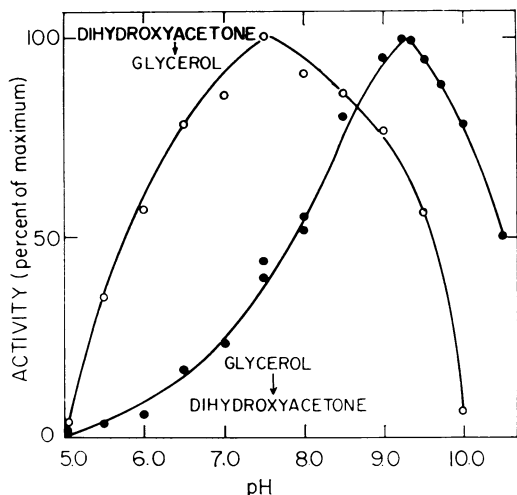| Components	Dihydroxyacetone Reduction	Components	Glycerol Oxidation
	μmoles NAD(P)H oxidized/mg protein·min		μmoles NAD(P) reduced/mg protein·min
Complete	1.05	Complete	2.1
System minus dihydroxyacetone	0.0	System minus glycerol	0.0
System minus NADPH	0.0	System minus NADP+	0.0
System minus NADPH + NADH	0.0	System minus NADP+ + NAD+	0.0
Boiled enzyme	0.0	Boiled enzyme	0.0

FIG. 2. Effect of pH on dihydroxyacetone reductase activity. The following buffers were used at 30 mM over the following pH ranges: Tricine, pH 5.0 to 8.0; Tricine-glycine, pH 7.5 to 9.2; and cyclohexylaminopropane sulfonic acid (Sigma) pH 9.0 to 10.5. 100% activity refers to 0.6 μmole NADP+ reduced or 1.2 μmoles NADPH oxidized/mg protein·min.

glycerol concentration (2.4 M) remained virtually constant. The equilibrium constant was calculated and found at all pH values tested around

$$K = \frac{(NADPH)(dihydroxyacetone)(H^+)}{(NADP^+)(glycerol)} = 5.1 \times 10^{-12}$$

It is seen that the equilibrium of the reaction catalyzed by dihydroxyacetone reductase is greatly in favor of glycerol formation around neutral pH.

Absorption Spectrum. The absorption spectrum of the most purified preparation had a single peak at 280 nm. The extinction at 280 nm was

$$E^{1\%}_{280\,nm} = 19.$$

Substrate Specificity. Dihydroxyacetone reductase was assayed with a number of different substrates. The K_m value for each of the substrates was determined beforehand. Table III shows that the highest activity was obtained with glycerol, with the next best substrate possessing only 26% of the activity with glycerol. The following alcohols had no detectable activity: sorbitol, mannitol, ethanol, lactate, and glycolate. The reverse reaction of NADPH oxidation was very poor (less than 5%), with respect to the activity with dihydroxyacetone, when assayed with the following substrates: glyceraldehyde, dihydroxyacetone phosphate, ribulose, sedoheptulose, and methylglyoxal.

Diaphorase Activity. As was previously shown, NADP+-dependent dehydrogenases retain diaphorase activity, reducing dichlorophenolindophenol by NADPH (8). Similarly, dihydroxyacetone reductase catalyzed the NADPH-dependent reduction of dichlorophenol indophenol with a reaction rate half that of dihydroxyacetone reduction.

Inhibitors and Activators. As illustrated in Table IV, SH groups seem essential for the activity of dihydroxyacetone reductase, as indicated by the inhibition of the enzyme by heavy metals and p-chloromercuribenzoate. Higher inhibitor concentrations were required for inhibition of the reverse reaction of glycerol oxidation, probably as a result of a protection by the very high glycerol concentration used. Heavy metal complex-forming agents such as EDTA, ethyleneglycol-bis(β-aminoethyl ether)-N,N'-tetraacetic acid, azide, and α,α-bipyridyl had no effect on the activity of dihydroxyacetone reductase. No

Table III. *Substrate Specificity of Dihydroxyacetone Reductase in the Oxidation of Alcohols*

The reaction mixture contained: 30 mM Tricine-glycine, pH 9.2; 50 μM NADP+; and 11 μg dihydroxyacetone reductase in a total volume of 1 ml. The activity with glycerol was 0.65 μmole NADP+ reduced/mg protein·min.

Alcohol	Apparent Km	Relative Activity
	M	
Glycerol	1.5	1.00
1,2-Propanediol	3.4	0.26
2,3-Butanediol	2.3	0.182
Ethanediol	3.5	0.145
Isoerythritol	1.2	0.091
Mesoerythritol	1.2	0.068

Table IV. *Effect of Inhibitors on the Activity of Dihydroxyacetone Reductase*

The reaction mixture contained: 30 mM Tricine, pH 7.4; 100 μM NADPH; 5 mM dihydroxyacetone; the indicated concentration of inhibitor and 10 μg dihydroxyacetone reductase in a total volume of 1 ml.

Inhibitor	Concentration	Inhibition
	mM	%
p-Chloromercuribenzoate	0.6	50
Cu^{2+}	1.0	50
Zn^{2+}	1.0	45
EDTA	1.0	0
α,α'-Dipyridyl	10	0
Hydroxylamine	5	0

inhibition was observed with monovalent ions such as sodium and lithium. The inhibitor of alcohol dehydrogenase, hydroxylamine (18), had no effect on the activity of dihydroxyacetone reductase.

Ammonium ions were found previously to activate the bacterial NAD+-dependent glycerol dehydrogenase in the presence of bicarbonate buffer in the reaction mixture (17). A similar ammonium activation of NADP+-dependent dihydroxyacetone reductase was observed with bicarbonate buffer at pH 9.2. However, this activation was not seen when bicarbonate was replaced by glycine or cyclohexylaminopropane sulfonic acid, or by increasing the concentrations of bicarbonate. It seems that the apparent activation effects observed with both of the bacterial and algal enzymes may be due to a decrease in the pH of the reaction mixture upon the addition of the acidic ammonium solution. The optimal activity of dihydroxyacetone reductase when bicarbonate served as the buffer was 8.9, somewhat lower than with other buffers (Fig. 2).

DISCUSSION

Two groups of enzymes have been previously shown to catalyze the formation of glycerol: (a) NAD+-dependent glycerol dehydrogenase from Aerobacter aerogenes (glycerol: NAD+ oxidoreductase EC 1.1.1.6) (6) which catalyzes the conversion of dihydroxyacetone to glycerol; (b) NADP+-dependent aldehyde reductases from various tissues of different mammalian species which catalyze the conversion of glyceraldehyde to glycerol (5, 11).

This report described the isolation and purification of a new enzyme from the halophilic alga D. parva which catalyzes the NADPH-dependent reduction of dihydroxyacetone to glycerol. This enzyme cannot be identified with any one of the above enzymes since NADH or glyceraldehyde could not replace NADPH or dihydroxyacetone. The thermodynamic equilibrium of the reaction catalyzed by this enzyme, and the low affinity for glycerol indicate that the formation of glycerol is strongly favored under physiological conditions.

So far dihydroxyacetone reductase was found only in the halophilic alga Dunaliella, which contains about 1 unit per 10^7 cells. It is most probably a cytoplasmic enzyme since after mild osmotic breakage most of the enzyme activity is excreted to the medium. The enzyme may play a major role in the osmotic regulation mechanism in Dunaliella. Recently we have shown that D. parva produces and accumulates large amounts of free glycerol within the cell which serves as the osmotic regulator of the cells (2). The above properties of dihydroxyacetone reductase indicate that this enzyme may catalyze the terminal step in the synthesis and accumulation of these high concentrations of intracellular glycerol in Dunaliella.

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