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# Dihydroxyacetone Kinase Activity in *Dunaliella parva*[1]

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## ABSTRACT

**An enzyme catalyzing the phosphorylation of dihydroxyacetone has been identified in the halophilic alga, *Dunaliella parva*. Since glycerol and glyceraldehyde are not substrates, the enzyme is referred to as dihydroxyacetone kinase. Dihydroxyacetone kinase was purified 9-fold by ammonium sulfate fractionation followed by DEAE-cellulose chromatography.**

In algae, it has been demonstrated that osmoregulating mechanisms maintain water content at the appropriate level (2, 9, 12). Kauss (10, 11) has shown that *Ochromonas malhamensis* has a mechanism shuttling reversibly polyols between an osmotically active isofloridoside pool and an insoluble polysaccharide pool. The amount, per cell, of isofloridoside (a galactoside of glycerol) corresponds to the tonicity of the medium. In *Dunaliella parva* (4, 5, 15), glycerol has been demonstrated to be the osmoregulating solute. In other organisms, this role is apparently played by other solutes: cyclohexanetetrol in *Monochrysis lutheri* (6), sucrose in *Chlorella pyrenoidosa* (7, 8), and mannitol in *Platymonas subcordiformis* (13).

The mechanism of osmoregulation in *Dunaliella* is of particular interest because this alga grows in molar salt concentrations, and consequently, the concentration of the osmoregulating solute, glycerol, attains several molars. The enzymic activities involved in the pathway modulating glycerol concentration may, therefore, be expected to be unusually high. This was shown to be the case for dihydroxyacetone reductase by Ben-Amotz and Avron (5). The molecular simplicity of the osmoregulating solute, glycerol, as well as the expected high activities of the enzymes modulating its concentration make *Dunaliella* a choice model organism for studying the mechanism of osmoregulation.

The aim of the present study was to characterize further the enzymic pathway of the osmoregulating mechanism in *Dunaliella*.

## MATERIALS AND METHODS

All chemicals were of the highest purity commercially available. Dihydroxyacetone, glyceraldehyde, glyceraldehyde-3-phosphoric acid, rabbit glycerophosphate dehydrogenase, β-NADH, NADPH, DTT,[2] tris, MES, and HEPES were obtained from Sigma, DEAE-cellulose (DE11) from Whatman. *D. parva* was grown following Ben-Amotz and Avron (5), except for the $CuCl_2$ concentration which was 0.2 nM, and NaCl concentration which was either 0.8 M or 1.6 M.

Crude enzyme was prepared by centrifuging late log phase algal cell suspension for 15 min at 2,000*g*, at room temperature. The pellet was washed by suspending in a solution of NaCl of the same concentration as that employed during growth, followed by 10-min centrifugation at 6,000*g*. The pellet was cooled to 4 C, and the cells broken by resuspension in 9 volumes of ice-cold distilled $H_2O$. After standing 5 min, the suspension was centrifuged 15 min at 27,000*g*. The supernatant was used as the crude enzyme preparation.

The activity of dihydroxyacetone kinase was followed in a Cary 16 recording spectrophotometer at 340 nm. The reaction was carried out in 1-cm light path cuvettes at room temperature. The assay mixture contained 1.4 units of glycerophosphate dehydrogenase, and in final concentrations, 40 μM NADH, 600 μM ATP, 16 mM $MgCl_2$, 100 mM Tricine buffer (pH 7.5), and the enzyme fraction to be tested. Dihydroxyacetone to a final concentration of 1 mM was added to initiate the reaction. Final volume was 2 ml. The maximal rate attained within about 3 min was used to calculate enzyme activity.

For *Km* measurements, an Eppendorf 1100 fluorimeter connected to a recorder was used. Exciting light was through the 313 + 366 filter and a 420 nm cutoff filter protected the photomultiplier. This permitted lowering assay concentrations of NADH and enzyme by 1/10 while still observing steady reaction rates in the μM concentration range of the substrate dihydroxyacetone.

Protein concentration was determined by absorbance reading at 280 nm and 260 nm (14).

## RESULTS AND DISCUSSION

In *D. parva*, dihydroxyacetone reductase catalyzes the reversible reaction between glycerol and dihydroxyacetone. Under normal growth conditions (1.5 M NaCl), and assuming equal internal concentration of NADPH and $NADP^+$ and pH 7, glycerol concentration is 2.1 M and dihydroxyacetone 0.1 mM (5). Dihydroxyacetone is present in a sufficient concentration to be a component of either the path from polysaccharides to glycerol or from glycerol to polysaccharides.

If glycerol is synthesized from dihydroxyacetone, a likely precursor would be dihydroxyacetone-P. An enzyme-dephosphorylating dihydroxyacetone-P was, therefore, searched for, but evidence for significant activity could not be found.

An enzyme utilizing dihydroxyacetone was another possibility. A likely reaction was the phosphorylation of dihydroxyacetone leading to dihydroxyacetone-P, which could then be converted to polysaccharides by classical pathways. Indeed, bursting *D. parva* cells (by lowering the osmotic pressure) in the assay medium (3) showed the expected activity. Osmotically bursting *D. parva* cells followed by centrifugation yields in the supernatant dihydroxyacetone kinase activity indicating that the enzyme is soluble (Table I).

Two types of enzymes which are capable of phosphorylating dihydroxyacetone are known (1): glycerol kinase and triokinase. Glycerol kinase also phosphorylates glycerol, and the *Escherichia coli* enzyme also glyceraldehyde. Triokinase phosphorylates dihydroxyacetone and glyceraldehyde at similar rates.

The crude soluble extract from *Dunaliella* was assayed for its

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[2] Abbreviation: DTT: dithiothreitol.

ability to phosphorylate glycerol and glyceraldehyde. Table I indicates that no appreciable activity toward glyceraldehyde was observed (exp. 5). Furthermore, the dihydroxyacetone-phosphorylating activity of the crude extract was inhibited by glyceraldehyde (exp. 3). Had glyceraldehyde been phosphorylated, substantial activity should have been observed in the latter experiment in this assay, since the extract possessed high triose-P isomerase activity (exps. 6-8). High concentrations of glycerol did not competitively inhibit the phosphorylation of dihydroxyacetone (exp. 4), and furthermore, a direct phosphorylation assay using [$^{32}$P]ATP showed no significant glycerokinase or fructokinase activity (not shown). These properties are different from those of any known kinase and we, therefore, will refer to this enzyme as dihydroxyacetone kinase.

In order to check the relation between dihydroxyacetone kinase activity and the osmotic pressure of the growth medium, the algae were grown for several weeks at two different salt concentrations, 0.8 M and 1.6 M. The glycerol concentration per Chl is about 50% greater at 1.6 M NaCl than at 0.8 M NaCl (4). The dihydroxyacetone kinase activity of cells grown in either salt concentration was 54 $\mu$mol of dihydroxyacetone phosphorylated/min·g Chl. This activity is similar to that of dihydroxyacetone reductase (5).

Partial purification of the crude enzyme was obtained by fractionating the crude soluble extract containing 100 mM Tricine buffer, pH 7.5, 1 mM DTT, and 0.1 mM EDTA with ammonium sulfate. Most of the activity was recovered in the fraction precipitating between 18 and 36 g ammonium sulfate/100 ml. Further purification was obtained by fractionation of the ammonium sulfate fraction (after dialysis overnight against 10 mM Tricine buffer, pH 7.5, 1 mM DTT, and 0.1 mM EDTA) on a DEAE-cellulose column pre-equilibrated with dialysis buffer. Elution was with a linear gradient of 0 to 0.3 M NaCl. Dihydroxyacetone reductase activity peaked around 80 mM NaCl, and that of dihydroxyacetone kinase around 110 mM NaCl (Fig. 1). In the absence of DTT, dihydroxyacetone kinase slowly lost activity after the ammonium sulfate fractionation, and rapidly after the DEAE-cellulose chromatography (Table II). Purification was 3-fold after the ammonium sulfate fractionation and 9-fold after the DEAE-cellulose chromatography. Dihydroxyacetone kinase was very unstable after such a partial purification. Attempts at further purification resulted in a rapid loss of enzymic activity.

Some properties of crude dihydroxyacetone kinase were determined. The apparent *Km* toward dihydroxyacetone is of the order of 10 $\mu$M (Fig. 2). At high concentrations (above 200 $\mu$M), substrate inhibition is apparent. The apparent *Km* toward ATP, in the presence of an excess of $Mg^{2+}$ could not be determined because double reciprocal plots were curved downward in a manner suggesting negative cooperativity. The pH profile of dihydroxyacetone kinase shows a maximum of about pH 6.5 (Fig. 3). The requirement for 16 mM $Mg^{2+}$ in Tricine buffer, pH

Table 1: Substrate Specificity in Crude Soluble Extract

Reaction mixture contained glycerophosphate dehydrogenase, NADH, ATP, $MgCl_2$ and tricine pH 7.5, at the concentrations specified under Methods and, where indicated, 0.1 ml crude soluble extract, 1.25 mM dihydroxyacetone, 1.25 mM glyceraldehyde, 1 M glycerol and 0.75 mM glyceraldehyde-3-P. Cells were grown in 0.8 M NaCl. Soluble extract prepared from 10 liters of culture as described under Methods.

| Exp. No. | Additions | NADH oxidation nmoles/min |
|---|---|---|
| 1 | extract + dihydroxyacetone | 7.1 |
| 2 | extract | 1.2 |
| 3 | extract + dihydroxyacetone + glyceraldehyde | 2.9 |
| 4 | extract + dihydroxyacetone + glycerol | 8.0 |
| 5 | extract + glyceraldehyde | 1.2 |
| 6 | glyceraldehyde-3-P | 0.1 |
| 7 | extract + glyceraldehyde 3-P | 160 |
| 8 | extract + glyceraldehyde-3-P+glyceraldehyde | 102 |

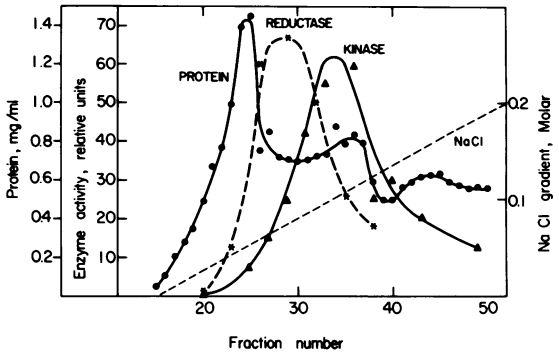


FIG. 1. DEAE-cellulose chromatography of *Dunaliella parva* crude extract. *Dunaliella parva* was grown in 0.8 M NaCl. Crude enzyme was prepared as described under "Materials and Methods" and was dialyzed against 10 mM Tricine buffer, pH 7.5. Specific activity of dihydroxyacetone kinase was 12 nmol NADH oxidized/min/mg protein. Bed volume of DEAE-cellulose column was 15 ml. It was loaded with 50 ml of dialyzed crude enzyme, washed with 20 ml of 10 mM Tricine buffer, pH 7.5, and eluted with 300 ml of NaCl gradient in the same buffer. Five-ml fractions were collected. Dihydroxyacetone kinase and protein were determined as described under "Materials and Methods." Dihydroxyacetone reductase activity was determined by measuring NADPH oxidation as described by Ben-Amotz and Avron (5). ▲——▲: dihydroxyacetone kinase; *- - -*: dihydroxyacetone reductase; ●——●: protein; -----: NaCl gradient.

Table II: Stabilization of Dihydroxyacetone Kinase

Crude soluble extract from Dunaliella parva containing 50 mM NaCl and 5mM tricine buffer pH 7.5 was passed through a DEAE-cellulose column. The dihydroxyacetone kinase activity was eluted by 5 mM tricine buffer, pH 7.5, containing 1 M NaCl. The active fraction showed 2.4 nmoles NADH oxidized /min/0.1 ml (100%). Aliquots of the active fraction were incubated with the indicated additions overnight at -20°C.

| Additions | Activity |
|---|---|
| | % of control |
| None | 9 |
| + DTT, 1 mM | 96 |
| + EDTA, 0.1 mM | 13 |
| + ATP, 1 mM | 1 |
| + DTT, 1 mM + EDTA, 0.1 mM + ATP, 1 mM | 112 |

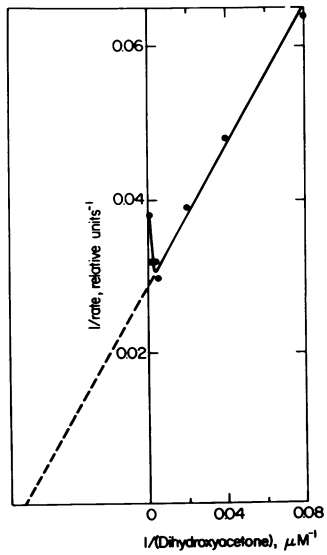


FIG. 2. Apparent *Km* of dihydroxyacetone kinase for dihydroxyacetone. Reaction rates were followed in a fluorimeter as described under "Materials and Methods." *Dunaliella parva* was grown in 0.8 M NaCl. Crude soluble enzyme and reaction mixtures were prepared as described under "Materials and Methods."

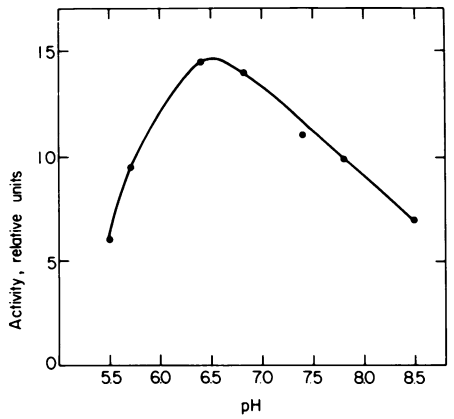


FIG. 3. pH profile of dihydroxyacetone kinase. Dihydroxyacetone kinase was purified by ammonium sulfate fractionation followed by DEAE-cellulose column chromatography. Reaction mixture contained, besides the usual components, 40 mM of each of the following buffers: tris, MES, HEPES. The reaction mixture was brought to the indicated pH values by adding HCl or NaOH. pH values were determined at the end of each reaction. In all cases, the rate-limiting step was the phosphorylation of dihydroxyacetone since adding more glycerophosphate dehydrogenase did not increase the reaction rate.

7.5, is lowered to 3 mM when the kinase is tested in MES buffer at pH 6.5.

## CONCLUSION

The presence of dihydroxyacetone reductase and dihydroxyacetone kinase in *D. parva* indicates a pathway by which glycerol may be transformed reversibly into dihydroxyacetone and therefrom irreversibly into dihydroxyacetone-P. In analogy with the osmoregulating mechanism which has been suggested to operate in *Ochromonas* (11), it seems reasonable to suggest that dihydroxyacetone-P is transformed by way of the usual glycolytic intermediates into a polysaccharide (a nonosmotic compound) when the level of the glycerol pool in *Dunaliella* is lowered during osmoregulation, and vice versa. The role of dihydroxyacetone kinase in *D. parva* would then be to catalyze the exit reaction from the osmoregulating glycerol pool.

It is interesting to note that the enzymic pathway of the osmoregulating pool seems to be catalyzed by enzymes which are unique. This may offer the organism a convenient means of controlling osmoregulation independent of other metabolic pathways.

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