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The Fructose 1,6-Diphosphatase of *Dictyostelium discoideum**

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ABSTRACT: Fructose 1,6-diphosphatase from the slime mold *Dictyostelium discoideum* was found to have an alkaline pH optimum (9.0–9.5). Little or no activity occurred at physiological pH unless EDTA was added. In the presence of 0.2 mM EDTA an additional peak of activity, equal to that at pH 9.5, appeared at pH 7.5–8.0. EDTA stimulated activity at each pH tested (7.5, 8.5, and 9.5). Stimulation of activity was greatest at pH 7.5 and progressively decreased at high pH. The reaction was completely dependent upon the presence of Mg^{2+} or Mn^{2+}. At optimum concentrations the reaction rate with Mg^{2+} was about three times higher than with Mn^{2+}. In addition to EDTA, mercaptoethanol, cysteine, dithiothreitol, KCN, and histidine stimulated the reaction rate at pH 7.5. Adenosine monophosphate, deoxyadenosine monophosphate, 3′,5′-cyclic adenosine monosphosphate, adenosine diphosphate and adenosine triphosphate did not stimulate nor inhibit enzyme activity at pH 7.5 in the presence of 0.2 mM EDTA. Fructose 1,6-diphosphate was slightly inhibitory above 0.2 mM. The low activity of the enzyme as well as the lack of regulation of its activity by 3′,5′-cyclic adenosine monophosphate suggests that it may not be important in gluconeogenesis.

Fructose 1,6-diphosphatase (D-fructose 1,6-diphosphate 1-phosphohydrolase, EC 3.1.3.11) is an enzyme which has been extensively studied by many workers (see reviews by Atkinson, 1966; Wood, 1966). The FDPase from many organisms has an alkaline pH optimum and essentially no activity at physiological pH. Many compounds, such as EDTA and some sulfhydryl reagents, can activate this enzyme at the latter pH. Depending upon the source of the enzyme, activity at physiological pH can be inhibited by AMP, FDP, by both compounds or by neither (Scala *et al.*, 1968). Evidence obtained *in vivo* and *in vitro* in mammalian systems indicates that FDPase is important in the regulation of gluconeogenesis (Krebs *et al.*, 1964; Scrutton and Utter, 1968).

FDPase has been reported in cell free extracts of *Dictyostelium discoideum* by Cleland and Coe (1968). The activity of the enzyme when compared to that found in other organisms was unusually low and did not change during differentiation. A recent study of the phosphofructokinase of *D. discoideum* (Baumann and Wright, 1968) showed that this enzyme had an unusual regulatory pattern, inconsistent with its role as a key enzyme in metabolic regulation of glycolysis and gluconeogenesis. The low level of FDPase found by Cleland and Coe (1968) as well as evidence discussed by Baumann and Wright (1968) suggested that during differentiation of *D. discoideum* the hexose units of glycogen (already present in amoeba) are used as precursors for the synthesis of polysaccharides and trehalose which accumulate during fruiting body formation. In such a system gluconeogenesis from amino acids would not be of great importance, as is also

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suggested from the data of Pannbacker and Wright (1967). The regulatory pattern of the FDPase from *D. discoideum* was investigated with the expectation of gaining further insight into the metabolism of this organism during differentiation.

Materials and Methods

Materials. Glucose 1-phosphate, glucose 6-phosphate, fructose 1-phosphate, fructose 6-phosphate, nucleotides, NADP, NADH, phosphoenolpyruvate, pyruvate, mercaptoethanol, dithiothreitol, cysteine, histidine, and the enzymes used in the assays were obtained from Calbiochem. FDP (98–100% pure), imidazole, glycine, triethanolamine, and streptomycin sulfate were obtained from Sigma. All other chemicals were obtained from Fisher Scientific Co. Unless otherwise stated, sodium salts of acids were used.

Assays. FDPase activity was measured at 25° by two methods, a spectrophotometric assay and a P_i assay. The spectrophotometric assay consisted of coupling F-6-P formation with excess phosphoglucose isomerase and G-6-P dehydrogenase and measuring the rate of NADP reduction (Racker and Schroeder, 1958; Rosen, 1966). The reaction mixture (1 ml) consisted of 0.2 mM FDP, 1.5 mM $MgCl_2$, 0.3 mM NADP, and either 40 mM triethanolamine buffer (pH 7.5) or 40 mM glycine buffer (pH 9.5). In some experiments 0.2 mM EDTA was added. The assay was completed within 10 min. Unless otherwise stated the spectrophotometric assay was used in all experiments. A unit of enzyme was defined as the amount catalyzing the formation of 1 μmole of F-6-P/min, at pH 9.5 and 25°. The P_i assay consisted of measuring the P_i released from FDP. The reaction mixture (1 ml) was the same as used for the spectrophotometric assay except for the use of 0.5 mM FDP and the omission of NADP and the assay enzymes. The reaction was terminated in 15 min by the addition of 1 ml of cold 10% (w/v) trichloroacetic acid. Precipitated protein was removed by centrifugation and the supernatant was assayed for P_i by the method of Fiske and SubbaRow (1925). Zero-time controls were always included to correct for the P_i present initially. In experiments designed to test the stoichiometry of the reaction FDP was measured by coupling to aldolase, triose phosphate isomerase, and α-glycerophosphate dehydrogenase (Racker, 1947). Protein was assayed by the method of Lowry *et al.* (1951).

Enzyme Preparation. D. discoideum was grown as previously described (Ward and Wright, 1965). Cells were harvested either at the stage of late aggregation or early culmination. Unless otherwise stated, cells from the early culmination stage were used as the source of enzyme. The harvesting, storage of cells, and initial purification steps were identical with those described by Baumann and Wright (1968). Table I summarizes the enzyme purification. Fraction A is the crude preparation from which nucleic acids were removed by streptomycin sulfate to give fraction B. Fraction C consists of the protein precipitated between 50 and 60% $(NH_4)_2SO_4$ saturation. After centrifugation this precipitate was resuspended in 50 mM Tris-HCl (pH 8.0) and immediately used in assays. No significant NADPH oxidase activity was detected in this fraction. Upon storage for 72 hr at 4° the enzyme lost about 40% of its activity. Due to the extremely low activity of the FDPase in crude extracts and the reasonable stoichiometry (see below) obtained with fraction C, further purification was not attempted. The strain of *Escherichia coli* used for growth of the myxamoebae did not contain FDPase detectable either at pH 7.5 or 9.5 with or without 0.2 mM EDTA. Hence, the FDPase activity observed in crude extracts was not the result of contamination by the *E. coli* enzyme.

Results

Stoichiometry of the FDPase reaction was determined as shown in Table II. The reaction mixture contained 1.5 mM $MgCl_2$ and 40 mM TEA buffer (pH 7.5) with 0.2 mM EDTA or 40 mM glycine buffer (pH 9.5) with no added EDTA. After a 15-min incubation an equal amount of cold 0.7 M $HClO_4$ was added. The mixture was brought to pH 7.0 with 5 *N* KOH and centrifuged. An aliquot of the supernatant solution was assayed for P_i by the method of Fiske and SubbaRow (1925). $MgCl_2$ and dithiothreitol were added to a final concentration of 5 and 1 mM, respectively. To different aliquots appropriate purine nucleotides and enzymes were added in order to measure FDP and F-6-P as described in Methods. Mixtures without enzyme, as well as the reaction mixture with enzyme but no substrate, were incubated and assayed as described.

Substrate Specificity. No hydrolysis of G-1-P, G-6-P, F-1-P, or F-6-P was detected with the P_i assay when these compounds were tested at 0.5 mM concentrations at either pH 7.5 with 0.2 mM EDTA or at pH 9.5.

Effect of pH and EDTA. As is seen in Figure 1, in TEA–glycine buffer the enzyme exhibited a pH optimum between 9.0 and 9.5 and very little activity at pH 7.5

TABLE I: Enzyme Purification.[a]

Fraction	Vol (ml)	Protein (mg/ml)	Sp Act.[b]	Recov (%)	Purifn Factor
A	100	39	0.0036	100	1
B	98	32.6	0.0041	93	1.2
C	9.5	25.7	0.0202	35	5.6

[a] All assays performed at pH 9.5 as described in Methods. [b] Units/mg of protein.

TABLE II: Stoichiometry of the Fructose 1,6-Diphosphatase Reaction.[a]

Reaction Component	Initial Concn (μmoles/ml)	Final Concn (μmoles/ml)	Net Changes (μmoles)
Fructose 1-phosphate	0.58[b]	0.13	−0.45
	(0.63)[c]	(0.27)	(−0.36)
Fructose 6-phosphate	0.00	0.40	+0.40
	(0.00)	(0.32)	(+0.32)
P_i	0.09	0.59	+0.50
	(0.12)	(0.53)	(+0.41)

[a] The reaction was terminated in 15 min and the substrate and products were assayed as described in Methods. [b] Reaction carried out in 40 mM glycine buffer (pH 9.5). [c] Reaction carried out in 40 mM TEA (pH 7.5) in the presence of 0.2 mM EDTA (in parentheses).

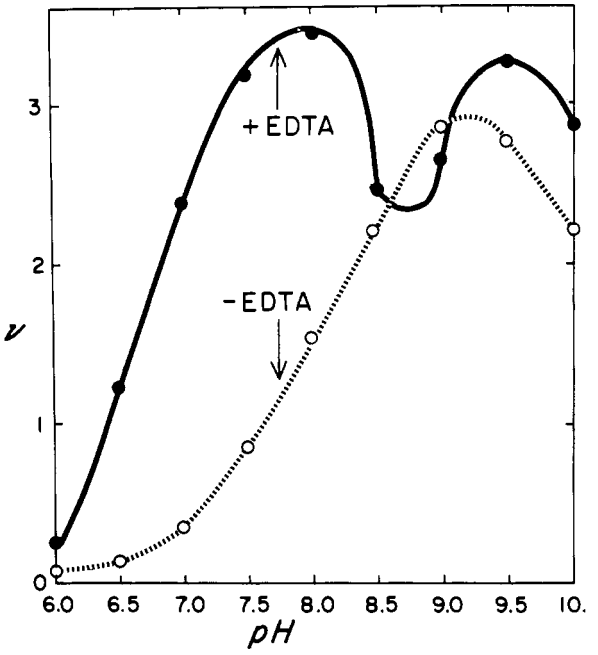

FIGURE 1: Effect of pH on enzyme activity with 1.5 mM $MgCl_2$. (○) no added EDTA; (●) with 0.2 mM EDTA. Assayed in a mixture of 40 mM triethanolamine and 40 mM glycine buffers as described in Methods. v = mμmoles/min.

Addition of 0.2 mM EDTA resulted in two pH optima, one at 7.5–8.0, and the other at about 9.5; different enzyme preparations differed slightly in the relative height of the two peaks. A greater variability in different preparations was observed in the extent of the dip at pH 8.5–9.0. Enzyme preparations from cells at the stage of late aggregation did not differ significantly from enzyme prepared from the stage of early culmination in either the pH optima or the degree of activation by EDTA. The effect of EDTA concentration on activation of FDPase was tested in TEA buffer (pH 7.5), Tris buffer (pH 8.5), and glycine buffer (pH 9.5), all at 40 mM concentration (Figure 2). Activation occurred at each pH but was greatest at 7.5. The concentration optimum for EDTA at this pH was about 0.2 mM. Increase in EDTA in all cases resulted in activation and then inhibition. As the pH was raised this change occurred at lower EDTA concentrations and the degree of inhibition was greater. The relative activity at pH 7.5 with 0.2 mM EDTA and at pH 9.5 without EDTA varied according to the buffer. In an equimolar mixture of TEA and glycine buffer the activity at both pH values was about the same. However, when TEA buffer was used alone at pH 7.5, the activity was always lower compared with the activity in glycine buffer alone, at pH 9.5. Variation of activity in different buffers has been reported for the FDPase from other organisms (McGilvery, 1964).

The effect of various compounds on enzyme activity was tested at pH 7.5. Table III gives the results of those compounds which showed activation. The following compounds tested at the same concentration as those in Table III showed no effect: AMP, 3′,5′-AMP, dAMP, P_i, PEP, imidazole, and succinate. FDP tested at 0.5 and 1 mM exhibited no activation. ADP, ATP, and PP_i were tested at 1 and 2 mM with 1.5 mM $MgCl_2$ plus a concentration of $MgCl_2$ equivalent to the tested compound. No activation was observed. A control was included with the highest concentration of the tested compound and 0.2 mM EDTA. In all cases the activity with 0.2 mM EDTA and the tested compound was the same as that expected from EDTA activation alone. Recently Pogell *et al.* (1968) have demonstrated activation of rabbit muscle FDPase at pH 7.5 by phosphofructokinase. A preparation of phosphofructokinase from *D. discoideum* was prepared by $(NH_4)_2SO_4$ precipitation (Baumann and Wright, 1968). Using this fraction, no activation of the FDPase was observed at pH 7.5, in the range of 0.05–5 mg of protein/ml.

Attempts were made to demonstrate inhibition of enzyme activity by AMP, 3′,5′-AMP, dAMP, PEP, P_i, ATP, ADP, and PP_i in the presence of 0.2 mM EDTA at pH 7.5. To avoid effects due to chelation, ATP, ADP, and PP_i were tested with 1.5 mM $MgCl_2$ plus an amount of $MgCl_2$ equivalent to the concentration

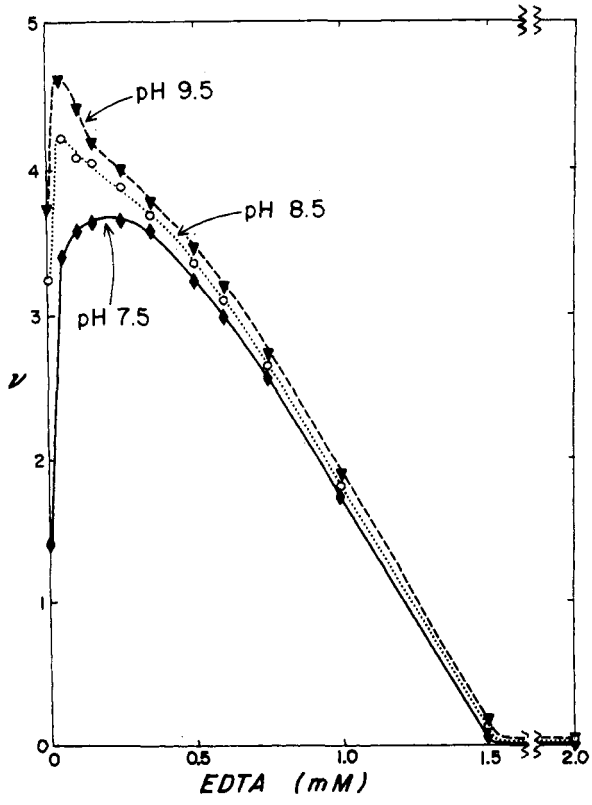

FIGURE 2: Effect of EDTA on enzyme activity with 1.5 mM $MgCl_2$. Assayed in (♦) 40 mM triethanolamine (pH 7.5), (○) 40 mM Tris buffer (pH 8.5), and (▼) 40 mM glycine buffer (pH 9.5), as described in Methods. v = mμmoles/min.

TABLE III: Compounds Which Activate Fructose 1,6-Diphosphatase at pH 7.5.[a]

Compd Added	Concn (mM)	Rel Act.[b]
None		23
EDTA	0.2	100
Mercapto-ethanol	1	47
	10	71
Cysteine	1	71
	10	62
Dithiothreitol	1	77
	10	80
KCN	1	80
	10	97
Histidine	1	65
	10	94

[a] Assayed in 0.04 M triethanolamine buffer (pH 7.5) as described in Methods. [b] Activity relative to that obtained in the presence of 0.2 mM EDTA (taken to be 100).

of the tested compound. When tested at 0.5, 1.0, 1.5, and 2.0 mM all of these compounds exhibited less than 10% inhibition.

The effect of Mg^{2+} and Mn^{2+} was tested at pH 7.5 with 0.2 mM EDTA and at pH 9.5 without the latter compound. As can be seen from Figure 3, the reaction is completely dependent upon the presence of one or the other divalent cation. The minimal concentration of $MgCl_2$ giving the highest activity was about 1.5 mM at both pH values. The optimum concentration for Mn^{2+} at pH 9.5 was about 0.1 mM. At pH 7.5, in the presence of 0.2 mM EDTA, the optimum concentration was about 0.03 mM. The highest activity with Mg^{2+} was usually two to three times higher than with Mn^{2+}. Unlike Mg^{2+}, Mn^{2+} did not show any inhibition in the 1.0–5.0 mM concentration range.

The effect of FDP concentration of enzyme activity was tested at pH 7.5 in the presence of 0.2 mM EDTA. Figure 4 shows a Lineweaver and Burk (1934) plot of such an experiment. A typical substrate inhibition curve was observed. FDP was inhibitory at concentrations above 0.2 mM. At 1.0 mM FDP enzyme activity was 10% lower than at 0.2 mM FDP. The K_m for FDP in the presence of 1.5 mM $MgCl_2$ was about 6×10^{-5} M. Determination of K_m in two other experiments gave values which fell within 20% of the value obtained in Figure 4.

Discussion

The FDPase from *D. discoideum* resembles in its EDTA activation and pH optima the FDPase from *Candida utilis* (Rosen *et al.*, 1965) and *Polysphondylium*

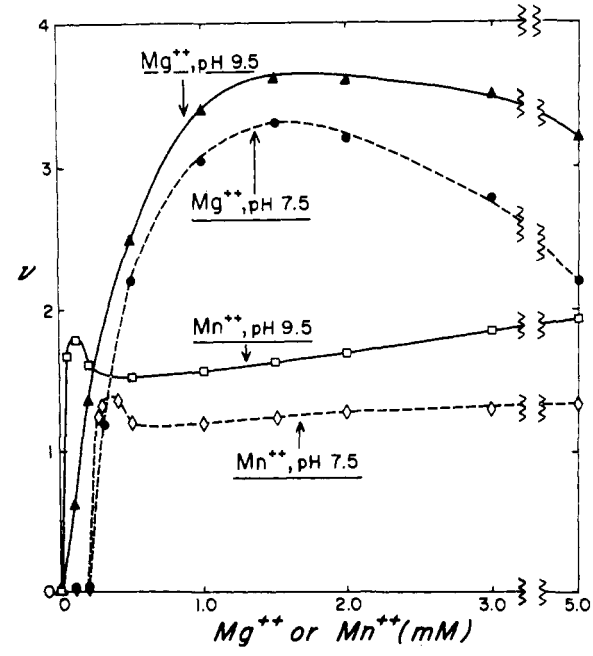

FIGURE 3: Effect of Mg^{2+} and Mn^{2+} on enzyme activity. Assayed in (●, ◇) 40 mM triethanolamine buffer (pH 7.5) with 0.2 mM EDTA, and (▲, □) 40 mM glycine buffer (pH 9.5) with no added EDTA, as described in Methods. v = mμmoles/min.

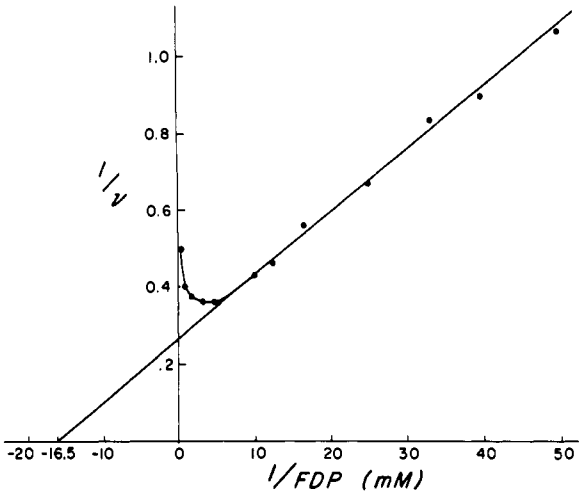

FIGURE 4: Lineweaver and Burk plot of the effect of fructose 1-diphosphate concentration on enzyme activity. Assayed in 40 mM triethanolamine buffer (pH 7.5) with 0.2 mM EDTA as described in Methods. v = mμmoles/min.

pallidum (Rosen, 1966). Like the enzyme from the latter organism, the FDPase of *D. discoideum* was not inhibited by AMP or dAMP and only slightly inhibited by FDP. Enzymes from all three organisms required Mg^{2+} or Mn^{2+} for activity. The maximal activity with Mg^{2+} was higher than with Mn^{2+}. Optimal activity with Mn^{2+} is reached at a lower concentration than with Mg^{2+}.

Since the activity of *D. discoideum* FDPase is unaffected by AMP, ADP, or ATP, there may be no direct relationship between enzyme activity and the energy state of the cells. This as well as the unusually low FDPase activity found in *D. discoideum* suggests that gluconeogenesis may not be important during the developmental cycle of this organism. Further evidence supporting this conclusion has been discussed by Baumann and Wright (1968).

In *D. discoideum*, FDPase may be important for the cycling of triose or hexose units through the pentose phosphate cycle. Such cycling would result in NADPH formation necessary for biosynthetic reactions which may occur during differentiation. Evidence for an active pentose phosphate cycle in this organism has been obtained by Wright *et al.* (1964).

The natural activiator for FDPase at physiological pH is unknown. Cleland and Coe (1968) have reported a lack of stimulation of FDPase activity by EDTA at pH 7.5 in crude extracts of *D. discoideum*. We have confirmed this finding. Absence of EDTA stimulation of enzyme activity in the crude extract suggests the presence of an inhibitor, which appears to be removed during purification. If this is the case the activity of this enzyme in the cell would be even lower than that observed in extracts assayed at pH 9.5, a possibility consistent with a minor role for this enzyme in development of the slime mold.

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