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HEXOSEDIPHOSPHATASE FROM SPINACH CHLOROPLASTS PURIFICATION, CRYSTALLIZATION AND SOME PROPERTIES

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SUMMARY

Hexosediphosphatase (D-fructose-1,6-bisphosphate 1-phosphohydrolase, EC 3.1.3.11) has been isolated, purified, and crystallized, from previously isolated spinach chloroplasts. The effects of various anions, cations, and sulfhydryl compounds were tested, and activation by Mg^{2+}, glycine, HCO_3^-, and sulfhydryl compounds is described. The purified enzyme is very specific for fructose 1,6-diphosphate and does not attack sedoheptulose-1,7-bisphosphate. The s_{20} value of the enzyme was 7.7, from which the molecular weight of the enzyme was estimated as 140 000.

INTRODUCTION

Hexosediphosphatase (D-fructose-1,6-bisphosphate 1-phosphohydrolase, EC 3.1.3.11) is an important enzyme in the regulation of gluconeogenesis in mammalian and microbial systems [1–4]. Hydrolysis of fructose 1,6-diphosphate (Fru-1,6-P_2) to fructose 6-phosphate is also an important reaction during the fixation of CO_2 in photosynthetic systems. The activity of hexosediphosphatase in photosynthetic *Chlorella* cells may be subject to regulation [5]. Morris [6] found that the activity of partially purified hexosediphosphatase from spinach chloroplasts is inhibited by $MgP_2O_7^{2-}$, $MgATP^{2-}$ and $MgADP^{2-}$. Hexosediphosphatase is a widely distributed enzyme in living systems. It has been isolated from many mammalian sources including rabbit liver [7], rabbit muscle [8–10], rabbit kidney [11], swine kidney [12, 13] and rat tissues [14]. The enzyme has also been isolated from a variety of microbial systems including *Dictyostelium discoideum* [15], *Candida utilis* [16, 17], *Polysphondylium pallidum* [18], *Pseudomonas saccharophila* [19], *Escherichia coli* [20]. and *Acinetobacter* sp. [21]. Hexosediphosphatase has also been isolated from some plant sources. These include castor bean endosperm and leaf [22, 23], navy bean [24] and wheat embryos [25]. Racker and Schroeder [26] isolated an alkaline hexosediphosphatase which they claimed could have little role in the reductive pentose phosphate cycle due to its

Abbreviation: Fru-1,6-P_2, fructose-1,6-diphosphate.

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apparent localization outside the chloroplast [26]. Smillie [27] has further investigated the localization of hexosediphosphatase and found that most of the enzymatic activity is localized in the chloroplast. This finding was confirmed by Latzko and Gibbs [28]. In this paper we report a method for the purification and crystallization of hexosediphosphatase from spinach chloroplasts. Some of the properties and characteristics of the enzyme are also reported.

MATERIALS AND METHODS

Chemicals

Fru-1,6-P_2, fructose 6-phosphate, ribulose 1,5-diphosphate, glucose 1-phosphate, glucose 6-phosphate, ribulose 5-phosphate, AMP, ADP, ATP, NADP, sorbitol, 2-*N*-morpholino ethane sulfonic acid, phosphoglucose isomerase and glucose-6-phosphate dehydrogenase were obtained either from CalBiochem or from Sigma Chemical Co.

Assay of enzymic activity

The enzyme hexosediphosphatase was assayed by the determination of NADPH produced, when the hexosediphosphatase reaction is coupled with the enzymatic reactions of phosphoglucose isomerase (EC 5.3.1.9) and glucose-6-phosphate dehydrogenase (EC 1.1.1.49). The reaction mixture (1.0 ml) contained 0.05 M Tris–HCl buffer (pH 8.7), 5 mM $MgCl_2$, 2 mM EDTA (dipotassium salt), 1 mM Fru-1,6-P_2; 0.2 mM $NADP^+$ (sodium salt), 5 μg phosphoglucose isomerase, 1.5 μg glucose-6-phosphate dehydrogenase, and hexosediphosphatase in the range of 0.01–0.02 unit. The temperature was 22 °C. One unit of enzyme activity is defined as the amount of enzyme activity catalyzing the hydrolysis of 1 μmole of Fru-1,6-P_2 to fructose 6-phosphate and P_i per min and specific activity is expressed as units per mg of protein.

For studying the substrate specificity of hexosediphosphatase, the hydrolysis of the sugar phosphates was assayed by the direct determination of P_i released in the enzymatic reaction by the procedure of Fiske and SubbaRow [29, 30]. The incubation mixture (1 ml) contained 0.05 M Tris–HCl buffer (pH 8.7), 5 mM $MgCl_2$, 2 mM EDTA, 1 mM of the sugar phosphate tested, and enzyme in an amount sufficient to release 0.05–0.1 mM of P_i in 10 min at 22 °C. The reaction was stopped by the addition of 2.5 M H_2SO_4 for the phosphate determination. The unit of enzyme activity was expressed in terms of μmoles of P_i formed under these conditions.

Determination of protein

Protein concentration was determined spectrophotometrically by measuring the absorption at 280 nm or by using the Lowry method of protein determination [31]. Bovine serum albumin was used as the standard in each of the two methods.

Disc gel electrophoresis

The method of Davis [32] was used to analyze for the homogeneity of the enzyme at the different stages of the purification procedure. Cross-linked polyacrylamide gel at pH 8.4 was used. Samples of 10–20 μl containing 50–100 μg of protein were used. All reagents used in the procedure were obtained from Canalco.

Determination of molecular weight

The sedimentation velocity method described by Chervenka [33] was used for the determination of s_{20} value of the enzyme. The run was performed using an An-D rotor at 59 780 rev./min.

Isolation and reduction of spinach ferredoxin

Spinach ferredoxin was isolated and purified by a procedure which involved acetone fractionation, according to San Pietro and Lang [34], followed by chromatography on DEAE-cellulose based on the method of Lovenberg et al. [35]. Its quantitative determination was based on the spectrum of the purified material. Ferredoxin was reduced either (a) in the presence of chloroplast particles and light [35], (b) by enzymatic catalyzed hydrogenation [36] or (c) chemically in presence of 0.15 mM solution of $Na_2S_2O_4$ under N_2, followed by dialysis against 0.05 M Tris–HCl buffer (pH 7.4). The reduction of ferredoxin was followed spectrophotometrically at 420 nm.

Isolation of the enzyme hexosediphosphatase

Hexosediphosphatase was purified by the following method:

Source of the enzyme. Field-grown, relatively young, spinach leaves were harvested and immediately stored over crushed ice in polyethylene bags in large ice chests. The leaves were deribbed and washed with ice-cold water and were dried between two sponges. The deribbed leaves were then chopped and divided into 50-g batches.

Isolation and sonication of chloroplasts. Chloroplasts were isolated by the method of Jensen and Bassham [37]. The yield of each 50-g leaf batch was suspended in 10 ml of basic buffer (0.05 M Tris–HCl buffer (pH 7.4), 0.002 M dithiothreitol, 0.0002 M EDTA, 0.001 M $MgCl_2$). The chloroplast suspension was sonicated for 30 s in batches of 50 ml, then it was centrifuged at 36 000 $\times g$ for 2 h. The supernatant was saved as the crude enzyme preparation (I).

Acetone fractionation. Acetone was added to the crude enzyme fraction to a concentration of 30%. The acetone had been pre-cooled at −14 °C and was added to the crude enzyme solution slowly while stirring at 4 °C. The enzyme in 30 % acetone was allowed to stand at 4 °C for 30 min, and the mixture was centrifuged at 13 200 $\times g$ for 4 min. The supernatant was collected and the acetone concentration in the supernatant was brought to 75%. The enzyme in 75% acetone was allowed to stand at −14 °C for 1–2 h. A copious precipitate formed and settled toward the bottom of the container. The upper layer of 75% acetone solution was decanted. The lower layer containing the precipitated enzyme was centrifuged for 1 min at 5000 $\times g$ and the pellets were collected. The precipitate was dissolved in the smallest possible volume of basic buffer and was dialyzed at 4 °C against water for 4 h, and against basic buffer twice for 8 h each time. The dialyzed mixture was centrifuged at 36 000 $\times g$ for 10 min, and the supernatant (II) was saved.

DEAE-cellulose column. The supernatant (II) was applied to DEAE-cellulose column, pre-equilibrated with 0.05 M Tris–HCl buffer (pH 7.4). The column was washed with 0.05 M Tris–HCl buffer (pH 7.4). Hexosediphosphatase did not stick to the DEAE-cellulose under these conditions and most of the activity passed down the column in the eluting buffer (III).

$(NH_4)_2SO_4$ fractionation. $(NH_4)_2SO_4$ powder was added slowly to the enzyme solution (III) while stirring. The pH of the mixture was maintained at about pH 7 using NH_4OH solution and pH paper as indicator. The precipitate formed at 45% $(NH_4)_2SO_4$ saturation was centrifuged at 13 200 $\times g$ for 10 min and was discarded. $(NH_4)_2SO_4$ powder was added to the supernatant to a concentration of 60% satn and the precipitate was collected by centrifugation at 13 200 $\times g$ for 10 min and was dissolved in basic buffer (IV).

Phosphocellulose column. Fraction (IV) was dialyzed against 0.05 M Tris–HCl buffer (pH 8.0) for 6 h. It was then applied to a cellulosephosphate column that had been pre-equilibrated with 0.05 M Tris–HCl buffer (pH 8.0). The column was washed with 0.05 M Tris–HCl buffer (pH 8.0), until no protein was coming out in the washing buffer. The column was then eluted with 1 mM Fru-1,6-P_2 in 0.05 M Tris–HCl buffer (pH 8.0). The fractions containing hexosediphosphatase were pooled and were dialyzed against a saturated solution of $(NH_4)_2SO_4$ preadjusted with NH_4OH to pH 8.0. The contents of the dialysis tubes were then centrifuged at 36 000 $\times g$ for 20 min and the pellets were dissolved in basic buffer (V).

Chromatography on Sephadex G-100 column. In most preparations Fraction V protein was homogeneous and no further purification was needed. However, in some preparations a trace of a contaminating protein was observed by polyacrylamide-gel electrophoresis. In these cases, Fraction V was chromatographed on a Sephadex G-100 column which was pre-equilibrated with Tris–HCl buffer (pH 8.0) and was eluted with the same buffer. The fractions containing hexosediphosphatase were pooled and were concentrated in the same manner as described for the protein eluted from the phosphocellulose column (VI). The specific activity of the homogeneous protein at this stage was 21.1 units per mg protein (Table I).

Crystallization of hexosediphosphatase. The concentration of protein in 1 ml of Fraction VI was adjusted to 27 mg/ml in a buffer containing 0.1 M Tris–HCl buffer (pH 8.0) and 10 mM $MgCl_2$. The solution was titrated with a cold (4 °C) saturated solution of $(NH_4)_2SO_4$ until a slight turbidity was observed. The solution was allowed to warm to room temperature where the turbidity disappeared. It was centrifuged at 36 000 $\times g$ for 10 min and the clear supernatant was cooled to cold

TABLE I

PURIFICATION OF SPINACH CHLOROPLAST HEXOSEDIPHOSPHATASE

Fraction	Protein concn (mg/ml)	Spec. act. (units/mg protein)	Yield (%)
I Sonicate supernatant	10	0.02	100
II Acetone fraction	21	0.68	60
III DEAE-cellulose column	7.5	3.3	49
IV 45–60% $(NH_4)_2SO_4$ fraction	20	8.2	73
V Phosphocellulose column	22	20.3	58
VI Sephadex G-100 column	21	21.1	52

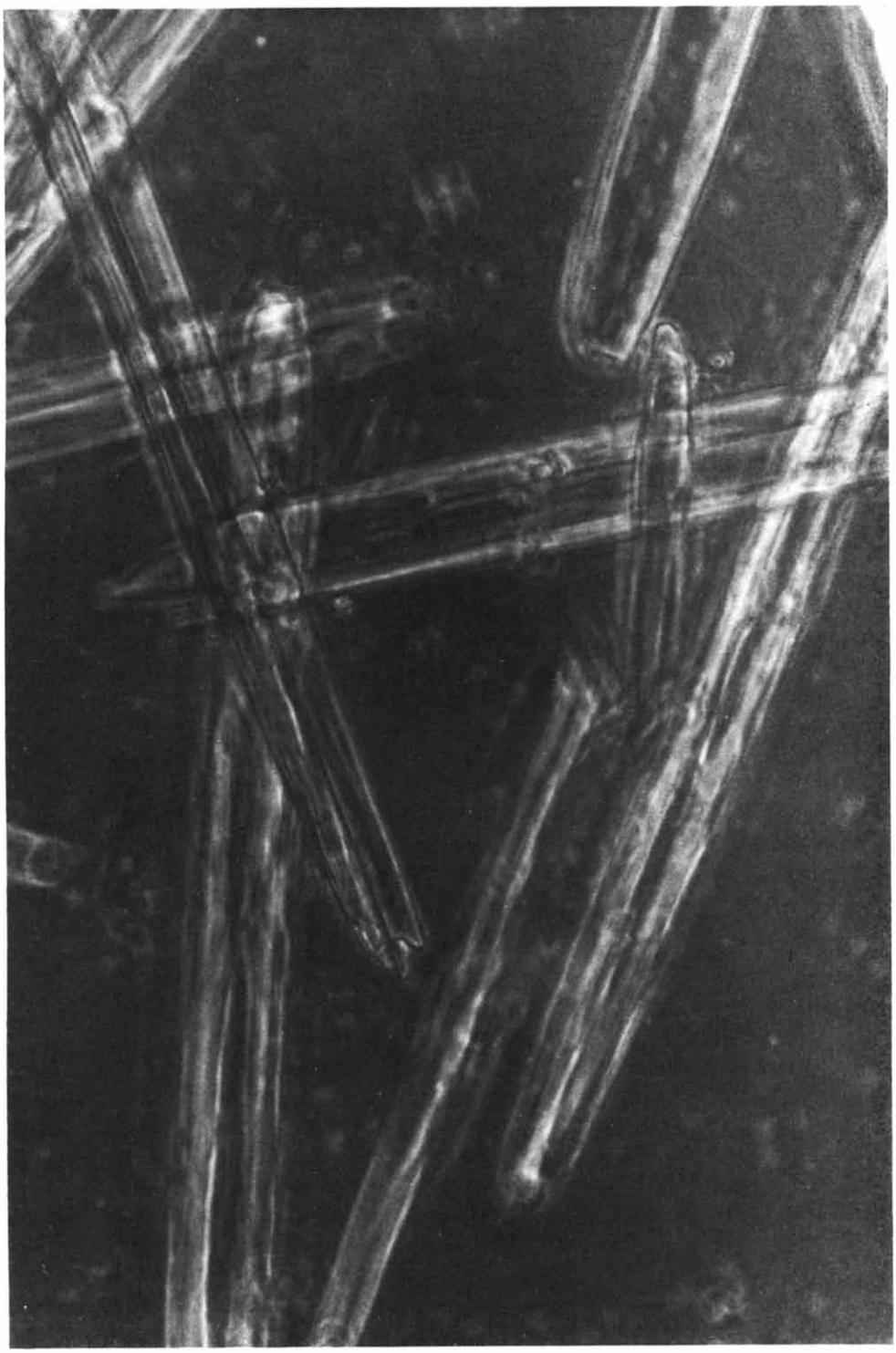


Plate 1. Crystals of hexosediphosphatase as seen by the phase-contrast microscope.

room temperature gradually by placing the tube containing the solution in a beaker containing water at room temperature and placing this assembly in the cold room (4 °C). The tube was then transferred to an ice bucket and was kept there on ice for a few days. The crystalline enzyme was obtained (VII). The enzyme in its crystalline state was not stable for over a month in crystallization suspension. However, redissolving the enzyme in basic buffer and storing the solution at 0 °C kept the enzyme activity stable for over 6 months.

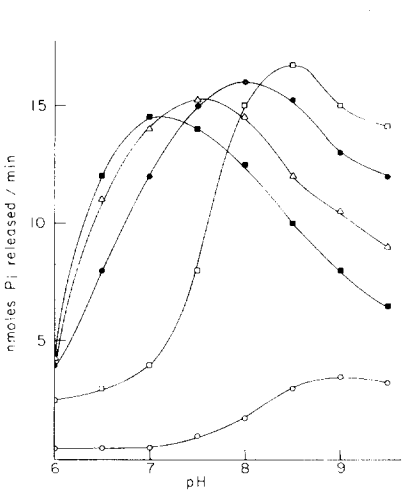
Homogeneity and molecular weight of hexosediphosphatase

Fractions V and VI were examined for their protein homogeneity using disk gel electrophoresis. Both fractions contained only one major band. In one run the phosphocellulose eluate (Fraction V) contained another minor band which was eliminated upon gel filtration on a Sephadex G-100 column.

Sedimentation rates data gave an s_{20} value of 7.7. Assuming a spherical protein with a partial specific volume of 0.725 cm, the molecular weight of the protein was estimated to be 140 000.

Effect of Mg^{2+} concentration on the pH optimum of hexosediphosphatase

In this study Mg^{2+} concentrations of 5–40 mM were used and the pH of the enzymatic reaction was varied from pH 6.0 to 9.5. At lower concentration of Mg^{2+} (1 and 5 mM) the pH optimum is around 8.5 (Fig. 1). As the Mg^{2+} concentration in the assay mixture increases, the pH optimum shifts towards the neutral pH and at 40 mM Mg^{2+} concentration the pH optimum is at pH 7. Our results are similar to those of Preiss et al. [38] obtained with partially purified hexosediphosphatase from spinach chloroplasts. These results suggest that the action of Mg^{2+} may partially be due to blocking some negative charges near the active site which renders the active site more accessible to the negatively charged substrate (Fru-1,6-P_2 or its Mg^{2+} salt). At the neutral pH the enzyme conformation is such that the negative charges are concentrated near the active site; thus more Mg^{2+} concentration is needed to neutralize these charges in order to make the active site more accessible to the negatively charged substrate. As the pH shifts to the alkaline pH, the enzyme conformation changes and consequently the negative charges become remote from the site; thus the Mg^{2+} concentration needed for maximum activity is much lower than that required at the neutral pH. Further evidence to support that the action of Mg^{2+} is on the enzyme was obtained by the failure of increasing concentration of anionic Fru-1,6-P_2 to inhibit the enzyme. McGilvery [39] calculated that a solution 1 mM in each of Fru-1,6-P_2 and $MgCl_2$ with sodium salts to bring the ionic strength to 0.077 (at 25 °C at pH 7), is expected to contain 19% as Mg–Fru-1,6-$P_2{}^{2-}$, and 3% Mg–H–Fru-1,6-$P_2{}^{-}$. If the pH was raised so as to completely ionize Fru-1,6-P_2, the concentration of the chelate would still only represent 26% of the total Fru-1,6-P_2. It is apparent from these calculations that the activity of hexosediphosphatase cannot be explained in terms of action of the Mg^{2+} on the substrate. Fig. 2 shows that Mg^{2+} has a direct effect on hexosediphosphatase. The sigmoidal shape of the curve of the dependence of activity of hexosediphosphatase on Mg^{2+} concentration shows that at both high and low pH (pH 7.0 and 8.7) the response of enzymic activity to Mg^{2+} concentration is sigmoidal at low Mg^{2+} concentrations. Such behavior may be interpreted as allosteric effect of Mg^{2+} on hexosediphosphatase [40].



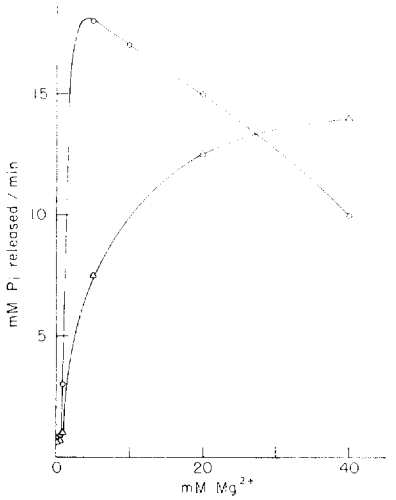


Fig. 1. Effect of Mg^{2+} concentration on the pH optimum of hexosediphosphatase. ○- - -○, 1 mM; □- - -□, 5 mM; ●- - -●, 10 mM; △- - -△, 20 mM; ■- - -■, 40 mM.

Fig. 2. Dependence of hexosediphosphatase activity on Mg^{2+} concentration. ○- - -○, pH 8.7; △- - -△, pH 7.0.

Effect of cations on hexosediphosphatase activity

Several other cations were tested for their possible activation of the enzyme. Mg^{2+} was by and large the most activating of all cations tested. Mn^{2+} partially replaced Mg^{2+} as the divalent cation required for the catalytic hydrolysis of Fru-1,6-P_2 by the enzyme. All other cations tested exhibited no activating effect on hexosediphosphatase (Table II). No activity for the hydrolysis of sedoheptulose 1,7-diphosphate, fructose 1-phosphate, or ribulose 1,5-diphosphate was induced by the presence of Zn^{2+}, Mn^{2+} or Ca^{2+}.

TABLE II

EFFECT OF CATIONS ON HEXOSEDIPHOSPHATASE ACTIVITY

Cation	Cation concn	nmoles of P_i released per ml* per min			
		5 mM	10 mM	20 mM	40 mM
Mg^{2+}		18	17	16.3	15.5
Mn^{2+}		8.6	8.5	7	5
Zn^{2+}		2.5	2.0	1.0	0.05
Fe^{3+}		0.5	0.5	0.2	0.05
Co^{2+}		1.5	1.4	0.5	0.2
Ni^{2+}		1.0	0.8	0.3	0.05
Au^{2+}		0.4	0.2	0.1	0.05
Cd^{2+}		1.0	0.5	0.5	0.08
Ca^{2+}		1.5	1.0	1.0	1.5

* Containing 1 μg of Fraction VI protein

Effect of anions on the activity of hexosediphosphatase

The effect of anions on the activity of hexosediphosphatase was carried out in assay mixtures 5 mM in Mg^{2+}. The sodium salt of the anions desired was used throughout this experiment. The concentrations of the anions were varied from 5 to 40 mM at pH 8.7. None of the anions tested (F^-, Cl^-, Br^-, I^-, SO_4^{2-} and HPO_4^{2-}) had any stimulating or inhibitory effect on the enzyme.

Activation of hexosediphosphatase by glycine

Glycine was found to activate hexosediphosphatase, at pH 8.7 and 5 mM Mg^{2+}, towards the hydrolysis of Fru-1,6-P_2 as shown in Fig. 3. The activity of the enzyme is increased by about 50% .The activation by glycine is not Mg^{2+} dependent. The glycine activation of the isolated enzyme raises the possibility of the regulation of hexosediphosphatase by glycine in vivo. The diversion of carbon from the carbon-reduction cycle to the synthesis of amino acids and proteins may be regulated at the hexosediphosphatase point [41]. It appears that a balance in favor of increased amount of soluble glycine results in the activation of hexosediphosphatase and an increase in the flow of fructose 6-phosphate to the synthesis of amino acids. The participation of glycine as a regulatory substance was further substantiated when it was found that glycine activates and enhances the CO_2 fixation by isolated chloroplasts in a parallel way to its activation to hexosediphosphatase (unpublished).

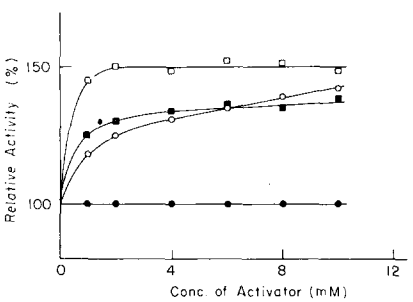


Fig. 3. Activation of hexosediphosphatase by glycine, HCO_3^-, and dithiothreitol, as a function of concentration of the activator. □- - -□, glycine; ■- - -■, HCO_3^-; ○- - -○, dithiothreitol; ●- - -●, control.

Activation of hexosediphosphatase by HCO_3^-

HCO_3^- which is the substrate for the enzyme ribulose-1,5-diphosphate carboxylase (EC 4.1.1.39) (which catalyzes the fixation of CO_2 using the five-carbon sugar diphosphate ribulose 1,5-diphosphate and resulting in the production of phosphoglyceric acid), the first enzymatic reaction in the carbon-reduction cycle was found to be an activator of spinach chloroplast hexosediphosphatase. This finding might suggest that CO_2 not only acts as the substrate for ribulosediphosphatase but also as the activator for some enzymes involved in the cycle.

Effect of nucleotides on hexosediphosphatase

$MgATP^{2-}$, $MgADP^{2-}$ and $MgP_2O_4^{2-}$ inhibited chloroplast hexosediphosphatase in a manner similar to that reported by Morris [6]. AMP, however, did not exert any inhibitory effect on the enzyme. Cyclic AMP was slightly activating.

Activation of hexosediphosphatase by ferredoxin and sulfhydryl reagents

Buchanan et al. [42, 43] claimed that reduced ferredoxin activates the enzyme hexosediphosphatase. Our results do not confirm this finding. The use of illuminated chloroplast to reduce ferredoxin was not suitable because of high background level of inorganic phosphate. Reduction catalyzed by partially purified hydrogenase led to interference by bacterial hexosediphosphatase. Reduction by $S_2O_4^{2-}$ proved to be the best method for our purposes. Reduced ferredoxin was introduced into the assay mixture just prior to the introduction of hexosediphosphatase. The reaction was allowed to proceed for 10 min, then was stopped by adding 0.1 ml of 50% trichloroacetic acid. The precipitate was centrifuged out, and the P_i produced in the enzymatic reaction was determined by the Fiske–SubbaRow method [29, 30]. Fig. 4 shows that Mg^{2+} concentration did not affect the relatively small amount of activation achieved in the presence of 100 μg of ferredoxin in a 1-ml assay mixture. Dithiothreitol followed a similar pattern of activation with the relative degree of activation at lower Mg^{2+} concentration (5–10 mM) about double that obtained at 40 mM Mg^{2+}. The activation of hexosediphosphatase by ferredoxin and dithiothreitol as a function of pH (Fig. 5) shows that the mechanism of activation of hexosediphosphatase by sulfhydryl compounds is different from that obtained by ferredoxin. At lower pH values (pH 6.5–7.5) the activation of the enzyme by sulfhydryl compounds was more than 100% of the activity in their absence while at higher pH the activation was much smaller. Ferredoxin, on the other hand, gave almost a constant small amount of activation at pH 6–9.5). Furthermore, other experiments showed that the effect of ferredoxin and dithiothreitol is additive which suggests that the mechanism of action of ferredoxin and sulfhydryl compounds on hexosediphosphatase is different.

Activation of hexosediphosphatase by $(NH_4)_2SO_4$

In the purification process activation of spinach chloroplast hexosediphosphatase was observed upon treatment with $(NH_4)_2SO_4$. This activation was unobtainable if $(NH_4)_2SO_4$ was used for fractionation before the use of acetone fractionation.

Specificity of chloroplast hexosediphosphatase

Chloroplast hexosediphosphatase is highly specific for Fru-1,6-P_2. The enzyme failed to attack sedoheptulose 1,7-diphosphate, ribulose 1,5-diphosphate, fructose

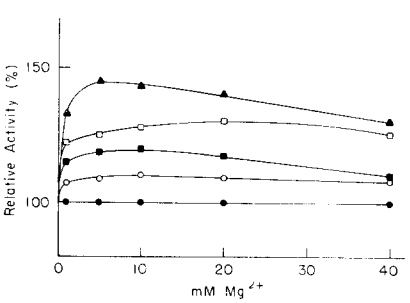


Fig. 4. Activation of hexosediphosphatase by glycine, HCO_3^-, dithiothreitol, and ferredoxin as a function of Mg^{2+} concentration. ▲- - -▲, 10^{-3} M glycine; □- - -□, 10^{-3} M HCO_3^-; ■- - -■, 10^{-3} M dithiothreitol; ○- - -○, 100 μg ferredoxin; ●- - -●, control.

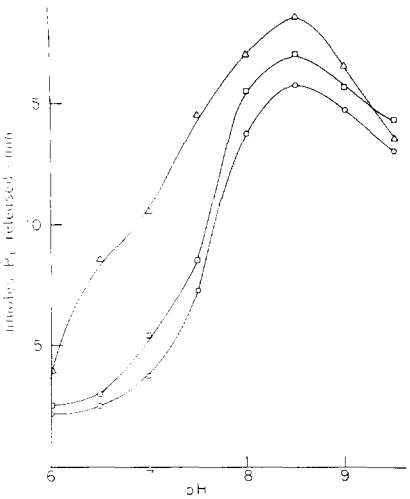


Fig. 5. Effect of pH on the activation of hexosediphosphatase by dithiothreitol and ferredoxin. ○- - -○, control (1 μg enzyme, no ferredoxin or dithiothreitol); □- - -□, 1 μg enzyme + 100 μg ferredoxin; △- - -△, 1 μg enzyme + 10^{-3} M dithiothreitol.

6-phosphate, fructose 1-phosphate, glucose 1-phosphate, glucose 6-phosphate, ribulose 5-phosphate, pyrophosphate, AMP, ADP or ATP. Changing the cation in the assay mixture from Mg^{2+} to Zn^{2+}, Mn^{2+} or Ca^{2+} and/or changing the pH to neutral pH (pH 7) did not induce any enzymic activity towards sedoheptulose 1,7-diphosphate, ribulose 1,5-diphosphate or fructose 1-phosphate.

DISCUSSION

The properties of the chloroplast hexosediphosphatase, including its pH optima shift with Mg^{2+} and its activation by glycine and by carbonate ion, are additional evidence that the chloroplast enzyme, like hexosediphosphatase from many other tissues, plays an important regulatory role in carbon metabolism in chloroplasts. This role was indicated earlier [5] by kinetic studies of *Chlorella pyrenoidosa* photosynthesizing in the presence of $^{14}CO_2$ and $^{32}P_i$, in which rapid changes in levels of Fru-1,6-P_2 and fructose 6-phosphate accompanied the light to dark and dark–light transition and the addition of several chemicals which produced general regulatory effects.

In view of the similar evidence from in vivo studies that the conversion of sedoheptulose 1,7-diphosphate to sedoheptulose 7-phosphate is also regulated [5], it is interesting that the purified hexosediphosphatase did not convert sedoheptulose 1,7-diphosphate to its monophosphate. Present evidence does not permit us to decide whether two separate enzymes are present in the chloroplasts for the hydrolysis of these two sugar diphosphates or the isolated enzyme has lost its capacity to convert sedoheptulose diphosphate, either through lability or because of lack of conditions necessary for activation of this function.

Kinetic studies of the properties of the enzyme isolated by the procedure in this report provide additional strong evidence for the allosteric properties of this

enzyme [43]. We have found no evidence for a large activation of the enzyme by reduced ferredoxin at any pH or Mg^{2+} concentration studied. However, Buchanan et al. [42, 43], who reported such activation by reduced ferredoxin, also reported a requirement for a protein factor of low molecular weight. Since we have not isolated such a factor, our experimental results are not necessarily in disagreement with those reports in which no specific activity of the enzyme was given.

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