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PROPERTIES AND REGULATION OF C-1-FRUCTOSE-1,6-DIPHOSPHATASE FROM SPINACH CHLOROPLASTS

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SUMMARY

Chloroplast fructose diphosphatase (EC 3.1.3.11) was purified according to the procedures of Racker and Schroeder [1] and Buchanan et al. [2] and the properties compared. Neither preparation contained fructose diphosphatase from the cytoplasm. The preparations had similar molecular weights, pH optima, affinities for fructose diphosphate and Mg^{2+} and were similarly activated by EDTA, dithiothreitol and cystamine.

Mg^{2+}, fructose diphosphate and dithiothreitol all activate chloroplast fructose diphosphatase more so at suboptimal pH values. The combined effects of these substances under estimated physiological conditions in the chloroplast stroma in the light and in darkness were consistent with almost full activity of the enzyme during illumination but no activity in the dark.

INTRODUCTION

Chloroplasts contain an alkaline C-1-fructose-1,6-diphosphatase for the specific hydrolysis of fructose 1,6-diphosphate to fructose 6-phosphate during operation of the Calvin cycle of photosynthetic CO_2 fixation in the stroma. The purification and properties of this enzyme from spinach chloroplasts have now been reported from several laboratories [1–5]. The possibility that this fructose diphosphatase has a regulatory function in photosynthesis is currently receiving considerable attention; the high negative free energy change involved in the hydrolysis of Fru-1,6-P_2 is a criterion for such a role [6]. Studies have shown that the enzyme can be activated by reduced ferredoxin or dithiothreitol [2] and the activity increased by relatively high levels of Mg^{2+}, especially at suboptimal pH [5, 7].

Recent investigations have shown that the Mg^{2+} concentration [8–10] and the pH [11] in the chloroplast stroma both rise markedly upon illumination and decrease again in the dark. Similar changes in the level of Fru-1,6-P_2 have also been recorded (Latzko, E. and Gibbs, M., unpublished). With this information, it seemed

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important to compare the kinetic properties of the chloroplast fructose diphosphatase under conditions approximating those believed to exist in the stroma. Two separate preparations of fructose diphosphatase, purified according to two different published procedures [1, 2] were compared and used for this investigation.

MATERIALS AND METHODS

Spinach was purchased from the local market. All biochemicals and auxillary enzymes were purchased from Boehringer, Germany. Dithiothreitol and cystamine were obtained from Sigma, U.S.A.

Chloroplast fructose diphosphatase was purified either according to Racker and Schroeder [1] or by the procedure of Buchanan et al. [2]. The specific activity of the former preparation varied considerably in fractions A–D [1] between 20 and 100 units per mg protein; that of the latter preparation was 22 units per mg protein.

Enzyme activity was determined in a coupled enzyme assay containing, in a final volume of 1 ml: 100 μmol Tris · HCl buffer, pH 8.8; 10 μmol $MgCl_2$; 2 μmol EDTA; 0.2 μmol NADP; 3 units phosphohexose isomerase and 1.5 units glucose-6-phosphate dehydrogenase and enzyme preparation containing approx. 0.05 units fructose diphosphatase.

The reaction was started by the addition of 0.6 μmol Fru-1,6-P_2 and the change in absorbance at 366 nm due to the reduction of NADP was followed with an Eppendorf photometer and recorder. After a lag period of less than 30 s, the enzyme activity remained constant for at least 5 min.

Protein was determined according to Lowry et al. [12].

RESULTS

Not all fructose diphosphatase in green leaves is located in the chloroplast; significant activity is also present in the cytoplasm [13] but this enzyme differs from its chloroplast counterpart in being fully active at pH 7.5 and sensitive to inhibition by AMP [13]. The data in Table I confirm that the enzymes purified from leaf homog-

TABLE I

EVIDENCE THAT THE PURIFIED FRUCTOSE DIPHOSPHATASES [1, 2] CONTAIN ENZYME ONLY FROM THE CHLOROPLAST

Enzyme activity was determined as described under Methods, except the pH of the reaction mixture was either 7.5 or 8.8. Soluble chloroplast extracts were obtained by breaking previously isolated chloroplasts [14] and collecting the supernatant after centrifugation at 10 000 × g for 10 min.

Source of enzyme	Activity (μmol/mg protein/min)	
	pH 7.5	pH 8.8
Whole leaf homogenate	0.14	0.49
Soluble extract from isolated chloroplasts	0.02	0.30
Purified preparations:		
According to Racker and Schroeder [1]	0.1	97
According to Buchanan et al. [2]	0.01	24

TABLE II

PROPERTIES OF CHLOROPLAST FRUCTOSE DIPHOSPHATASE PURIFIED FROM SPINACH LEAVES BY TWO DIFFERENT PROCEDURES

Enzyme activity was determined as described under Methods, except for the determination of pH optima, the buffer concentration was 150 mM, and the K_m for Mg^{2+} was estimated in the absence of EDTA. The activations by dithiothreitol and cystamine were measured at pH 7.9. Molecular weights were estimated on a Sephadex G-200 column, 28 cm × 60 cm, calibrated and evaluated as described by Andrews [16].

Property	Purification procedure	
	Racker and Schroeder [1]	Buchanan et al. [2]
Spec. act. (from Table I)	97	24
pH optimum	8.5	8.5
K_m Mg^{2+}	5 mM	4.5 mM
K_m Fru-1,6-P_2	0.30 mM	0.35 mM
Activation by 2 mM EDTA	+42 %	+50 %
Activation by 10 mM cystamine	7-fold	5-fold
Activation by 10 mM dithiothreitol	16-fold	15-fold
Molecular weight	161 000	147 000

enates using either of the methods from Racker and Schroeder [1] or Buchanan et al. [2] contain none of the cytoplasmic fructose diphosphatase; neither of these preparations, nor the soluble extract from isolated chloroplasts, showed activity when assayed at pH 7.5, although the activity at this pH in a whole leaf extract was almost 30 % of that at pH 8.8. Since the cytoplasmic enzyme is equally active at both pH values [13], this latter result is reasonably similar to the earlier observation [15] that about 80 % of the leaf fructose diphosphatase activity is located in the chloroplast.

The two purified preparations were found to be quite similar in most properties examined (Table II). The only marked difference was in the specific activity which was up to four times greater in the enzyme prepared according to Racker and Schroeder [1] compared to the preparation based on the method of Buchanan et al. [2]. Purified preparations studied by Preiss and Greenberg [3] and El-Badry [5] showed similar wide variation in the specific activity. The values for other properties of the enzyme shown in Table II are in general agreement with those previously reported [1–5].

Preiss et al. [7] and El-Badry [5] have shown that the activity of chloroplast fructose diphosphatase at suboptimal pH values is considerably enhanced by relatively high concentrations (20–40 mM) of Mg^{2+}. Previous studies from this laboratory confirmed this observation and showed in addition that the enzyme activity responded similarly to increased levels of Fru-1,6-P_2 [17]. As expected, the affinity for Fru-1,6-P_2 was reduced at lower pH values [7, 17]. The observation that chloroplast fructose diphosphatase is activated by reduced ferredoxin or dithiothreitol [2] prompted an extension of these studies. The effect of 10 mM dithiothreitol on the Fru-1,6-P_2 saturation curve of chloroplast fructose diphosphatase at pH values 7.9 and 8.8 is shown in Fig. 1. Activation by dithiothreitol was most marked at pH 7.9 although even at pH 8.8 substantial activation occurred at lower substrate concentrations. Associated with this activation was an apparent decrease in the sigmoidicity of the saturation

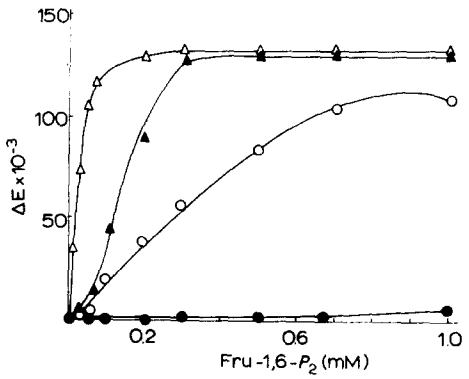

Fig. 1. Effect of pH and dithiothreitol on the Fru-1,6-P_2 saturation curve of chloroplast fructose diphosphatase purified according to Racker and Schroeder [1]. Enzyme activity was determined as described under Methods, except the pH and the concentration of Fru-1,6-P_2 were varied and dithiothreitol was added as shown. ●–●, pH 7.9; ▲–▲, pH 8.8; ○–○, pH 7.9 + 10 mM dithiothreitol; △–△, pH 8.8 + 10 mM dithiothreitol.

curve. Recent studies by El-Badry [5] also demonstrated a dithiothreitol activation which was more pronounced at lower pH values [5].

When the effect of 10 mM dithiothreitol on the Mg^{2+} saturation curve was examined at the same pH values, activation by dithiothreitol was again observed most noticeably at the lower pH and Mg^{2+} levels (Fig. 2). However, the effect was not as prominent as with low Fru-1,6-P_2 levels (Fig. 1). Finally, pH curves were determined at two different Fru-1,6-P_2 concentrations in the presence and absence of 10 mM dithiothreitol and the results (Fig. 3) again showed the dithiothreitol activation to be more effective at lower Fru-1,6-P_2 levels. Values similar to those in Figs 1–3 were obtained when fructose diphosphatase purified according to Buchanan et al. [2] was used.

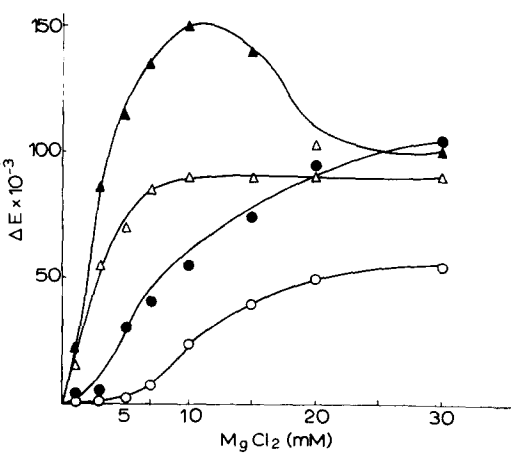

Fig. 2. Effect of pH and dithiothreitol on the Mg^{2+} saturation curve of chloroplast fructose diphosphatase purified according to Racker and Schroeder [1]. Enzyme activity was determined as described under Methods, except the pH and the concentration of Mg^{2+} were varied, EDTA was omitted and dithiothreitol was added as shown. ○–○, pH 7.9; △–△, pH 8.8; ●–●, pH 7.9 + 10 mM dithiothreitol; ▲–▲, pH 8.8 + 10 mM dithiothreitol.

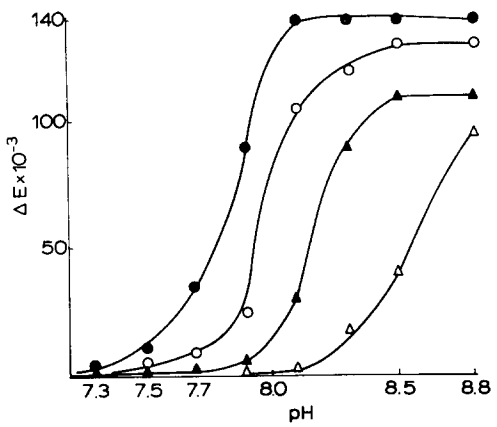

Fig. 3. Effect of pH and dithiothreitol at two different Fru-1,6-P_2 concentrations on the activity of chloroplast fructose diphosphatase purified according to Buchanan et al. [2]. Enzyme activity was determined as described under Methods except the concentration of Fru-1,6-P_2 was changed, the pH varied as shown and the buffer concentration doubled. △–△, 0.15 mM Fru-1,6-P_2; ○–○, 2 mM Fru-1,6-P_2; ▲–▲, 0.15 mM Fru-1,6-P_2 + 10 mM dithiothreitol; ●–●, 1 mM Fru-1,6-P_2 + 10 mM dithiothreitol.

These results, taken together, suggested that the changes believed to occur in the chloroplast stroma upon illumination were just those which favoured high fructose diphosphatase activity as shown in Figs 1–3: increased pH, elevated levels of Mg^{2+} and Fru-1,6-P_2, and the presence of a strong reductant. The fructose diphos-

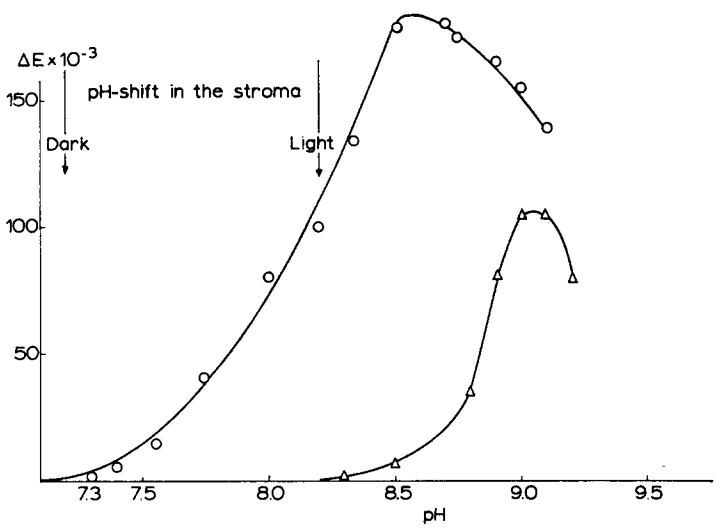

Fig. 4. Activity of chloroplast fructose diphosphatase under conditions approximating those believed to exist in the stroma in light (○–○) and in darkness (△–△). The pH shift in the stroma, calculated by Heldt et al. [11] to occur on transfer between light and darkness, is shown. Chloroplast fructose diphosphatase was purified according to Buchanan et al. [2] and assayed as described under Methods except the buffer concentration was doubled, EDTA was omitted and the following changes were made: ○–○, 1 mM Fru-1,6-P_2, 10 mM $MgCl_2$, 10 mM dithiothreitol; △–△, 0.2 mM Fru-1,6-P_2, 3 mM $MgCl_2$, no dithiothreitol. The pH was varied as shown.

phatase activity observed when these four variables were changed simultaneously between values approximating dark and light conditions is shown in Fig. 4, and superimposed on these results is the change estimated by Heldt et al. [11] to occur in the stroma during light-dark transitions. It is clear that the enzyme would be totally inactive in the dark but almost fully active in the light assuming the activity of the enzyme in vivo responds similarly to the purified enzyme used here.

DISCUSSION

Procedures for the purification [1–4] and crystallisation [5] of chloroplast fructose diphosphatase are now well established, but differences in the properties of these preparations leave some questions unanswered. For example, specific activities of homogeneous preparations range between 21.4 [5] and 118 [1]. Molecular weight estimations vary from 110 000 [4] to 195 000 [3]; in the present experiments values around 150 000 were obtained. There is considerable evidence that the enzyme dissociates into two halves [2, 4] as has been observed for the liver [18] and *Candida utilis* [19] fructose diphosphatases. The liver enzyme binds four molecules of Fru-1,6-P_2 per mol of enzyme [20] and appears to contain four subunits [21]. It would be interesting to know if the chloroplast enzyme is also composed of four subunits; the sigmoid saturation curves (Figs 1 and 2) would be consistent with this proposal.

Chloroplast fructose diphosphatase is unusual in that it can be activated by sulphydryl reagents such as dithiothreitol [2, 5], (Table II) and also by disulphides, e.g. cystamine [2], (Table II). An explanation of this is not clear but it is tempting to speculate that there exist rather unstable disulphide bonds necessary for molecular integrity (indeed the enzyme has a large content of cystine [2]) while reduced sulphydryl groups in the vicinity of the active site are essential for activity.

The kinetic studies reported in this paper provide strong evidence that, in the chloroplast stroma, Fru-1,6-P_2 is hydrolysed only during periods of illumination. Heldt et al. [11] have shown that the pH in the chloroplast stroma increases by 0.6 units upon illumination as a result of movement of protons into the thylakoids. There is now strong evidence that the exchange cation for this proton movement is mainly Mg^{2+} which moves from thylakoids into the stroma [9, 10]; the resultant increase in the concentration of Mg^{2+} has been estimated as 10 mM [8] and this increase is suspected to be an important facet of the light-activation of ribulose diphosphate carboxylase [22]. Both alkalisation of the stroma and increase in the concentration of Mg^{2+} could activate fructose disphosphatase in vivo (Figs 2 and 3). In addition, this activation may be amplified by a rise in Fru-1,6-P_2 generated by the Calvin cycle; the Fru-1,6-P_2 levels in isolated chloroplasts was previously estimated to increase from 0.01 mM to 0.2 mM after 5 min of light (Latzko, E. and Gibbs, M. unpublished). Finally, activation by a physiological reductant produced in the light could further ensure enzyme activation; this reductant may well be reduced ferredoxin which, as Buchanan et al. [2] have shown, activates the enzyme, although El-Badry [5] was unable to demonstrate significant activation by reduced ferredoxin and indeed obtained evidence that the mechanism of activation by reduced ferredoxin differs from that of dithiothreitol.

The observations from Heldt et al. [11] indicate that, even in the light, the pH in the chloroplast stroma would not exceed 8.0 provided the pH of the cytoplasm was

less than 7.6. This gives extra significance to the regulatory properties of fructose diphosphatase reported here since it is precisely at pH values less than 8.0 that, Fru-1,6-P_2, magnesium ions, dithiothreitol (Figs 1–3) and reduced ferredoxin [2] are more effective in activating the enzyme. An in vitro demonstration of this is clearly evident in Fig. 4. It will probably not be too long before in vivo evidence for changes in the fructose diphosphatase activity, dependent on changes in the stroma pH and/or Mg^{2+} content, are forthcoming. There has long been evidence for light induced activation of fructose diphosphatase in *Chlorella* [23] and similar data for isolated chloroplasts will be eagerly awaited.

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