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PROPERTIES OF SPINACH CHLOROPLAST FRUCTOSE-1,6-DIPHOSPHATASE

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Abstract—Photosynthetic fructose-1,6-diphosphatase (FDPase) fractions I and II, earlier purified from spinach leaves, show a similar amino acid composition, with the exception of a higher glutamic acid content in the latter. In both fractions glutamic and aspartic acids are the main amino acids. pH activity profiles of fractions I and II are similar, with optima at 8·65–8·70, both showing a high specificity for fructose-1,6-diphosphate. These two fractions are Mg^{2+}-dependent for activity, with an optimum Mg^{2+} concentration of 10 mM in standard conditions, which shifts to 5 mM when the Mg^{2+}/EDTA ratio is increased to 10; Mn^{2+} and Co^{2+} are slightly active. EDTA enhances FDPase activity slightly, with an optimum at 0·4–0·8 mM. Cysteine has no activating effect, and acts as an inhibitor above 10 mM. Both I and II have an optimum substrate concentration of 4 mM, and the substrate inhibits at concns above this value. Kinetic velocity curves are sigmoidal, with the concave zone located in the range of physiological substrate concns. (Hill coefficient 1·75 for both). This suggests a strong regulatory role of fructose-1,6-diphosphate. K_m values are $1{\cdot}4 \times 10^{-3}$ M (fraction I) and $1{\cdot}1 \times 10^{-3}$ M (fraction II). The highest activity rate occurs at 60°, in accordance with the high thermostability of both fractions; the activation energies are 14·3 kcal/mol (fraction I) and 13·0 kcal/mol (fraction II).

INTRODUCTION

In previous work[1] we found that spinach leaf photosynthetic fructose-1,6-diphosphatase (E.C. 3.1.3.11; FDPase) could be resolved into two active fractions I and II, with similar MWs and isoelectric points. Both are stable at acidic and neutral pH, but at pH 8·8 are split into similar subunits showing half the MW of native forms. So they can be considered as monomers of the original dimers I and II.

An enzymic diversity in connection with the existence of interconvertible monomeric and dimeric forms was earlier demonstrated in the *Rhodopseudomonas palustris* photosynthetic FDPase[2], and in the gluconeogenic one from rabbit liver[3] and from the mold *Candida utilis* [4]. However, no reports have been made concerning the existence of non interconvertible photosynthetic FDPases. Up to three FDPases occur in spinach[5], pea[6] and tapioca[7] leaves, with alkaline, acidic and neutral pH optimum, but only the former is the photosynthetic enzyme. In the present work we have compared the physico-chemical and kinetic properties of fractions I and II in order to determine the possible physiological significance of this heterogeneity.

RESULTS

The aminoacid composition of fractions I and II are shown in Table 1. The Buchanan's values[8] for the whole photosynthetic spinach FDPase are also included; these values, calcu-

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Table 1. Amino acid composition of FDPases from different sources*

Amino acid	Spinach leaves					Rabbit liver [10]
			Buchanan *et al* [8]			
	F I 92 000	F II 100 000	145 000	Correct to MW 92 000	Correct to MW 100 000	
Lysine	40	40	65	41	45	117
Histidine	10	7	12	8	8	14
Arginine	23	20	30	19	21	34
Aspartic acid	64	63	98	62	68	128
Threonine	26	27	28	18	19	70
Serine	49	48	78	49	54	79
Glutamic acid	67	79	216	136	149	81
Proline	29	28	43	27	30	52
Glycine	59	62	166	105	115	100
Alanine	45	40	64	40	44	106
Valine	46	47	67	42	46	104
Methionine	12	11	14	9	10	34
Isoleucine	43	48	57	35	39	70
Leucine	53	54	73	46	50	94
Tyrosine	25	32	36	23	24	52
Phenylalanine	21	16	25	16	17	40
Cystine (1/2)	not determined		210	132	145	20
Tryptophan	not determined		0	0	0	0

* Active fractions I and II were hydrolyzed with 6N HCl in sealed tubes under vacuum, for 24 hr at 100°. Results in number of residues per mol of enzyme.

lated on the assumption of a MW 145 000 by ultracentrifugation, have been also corrected to the values 92 000 (fraction I) and 100 000 (fraction II) which we have earlier determined [9]. The aminoacid composition of the gluconeogenic FDPase from rabbit liver is also shown.

The pH profiles for FDPase activity of fractions I and II are quite similar, with optima at 8·65–8·70 and a rapid decay in the acidic zone, to the extent that half maximal activity is at 8·4 and there is no activity below 8·2. Both fractions I and II show increasing activity with incubation temperature up to 60°, then decreasing quickly (Table 2). Activity also increases with substrate concentration, with a maximum around 4 mM; above this value a substrate inhibitory effect takes place. The added cysteine is quite ineffective, acting as an inhibitor above 10 mM. EDTA is not absolutely essential for activity but there is an enhacement up to a maximum at 0·4 and 0·8 mM EDTA concentration, respectively, for fractions I and II; above these values the activity goes down, and disappears at 3·2 mM (Table 3).

In the standard assay conditions a minimum

Table 2. Effect of incubating temperature on FDPase activity*

Incubating temperature (°C)	Activity (enzyme units)	
	Fraction I	Fraction II
4	0 021	0 022
16	0 047	0 059
28	0 109	0 128
37	0 152	0 170
45	0 234	0 240
60	0 263	0 267
75	0 044	0 029

* Active fractions I and II were assayed in the standard conditions, except that temp of incubation were as stated below.

Mg^{2+} concentration of 3 mM is required for activity with fractions I and II. There is a maximum at 10 mM Mg^{2+}, and an inactivating effect above this value (Fig. 1). If EDTA is omitted there is activity even at 1 mM Mg^{2+}, but then the increasing FDPase rate with Mg^{2+} concentration is slower. However, if the Mg^{2+}/EDTA ratio is constant in the value 10, activity at 1 mM Mg^{2+} is preserved, and the high rate of

Table 3. Effect of EDTA concentration on FDPase activity*

EDTA concentration (mM)	Activity (enzyme units) Fraction I	Fraction II
0·0	0·058	0·077
0·05	0 093	0 099
0 1	0 108	0 143
0 2	0 137	0·157
0·4	0 161	0·177
0·8	0·159	0 188
1·6	0·137	0·154
3·2	0 005	0·008
6·4	0 000	0 000
12·8	0 000	0 000

* active fractions I and II were assayed in the standard condition, except that EDTA concentrations were as stated below.

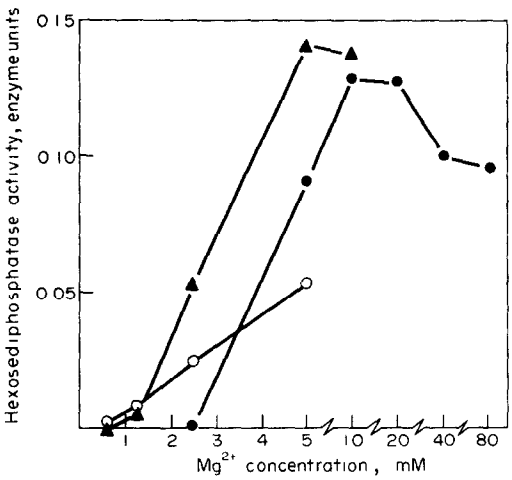

Fig. 1. Effect of Mg^{2+} concentration and Mg^{2+}/EDTA ratio on FDPase activity of fraction I. Assays performed in the standard conditions (●—●), and with the following exceptions: EDTA omitted (○—○); constant Mg^{2+}/EDTA ratio = 10(▲—▲)

FDPase enhancement with the increasing Mg^{2+} is again recovered. The optimum is now at 5 mM Mg^{2+} with both fractions.

Among the other divalent cations tested, only Mn^{2+} and Co^{2+} have shown some effect. Both were operative at below 2·5 mM concentration, at which Mg^{2+} is ineffective, but at higher concentration their activating capability was less (Table 4).

Fractions I and II are only active with fructose-1,6-diphosphate as substrate; other phosphates esters tested were ineffective. Figure 2 shows the plots of substrate concentration

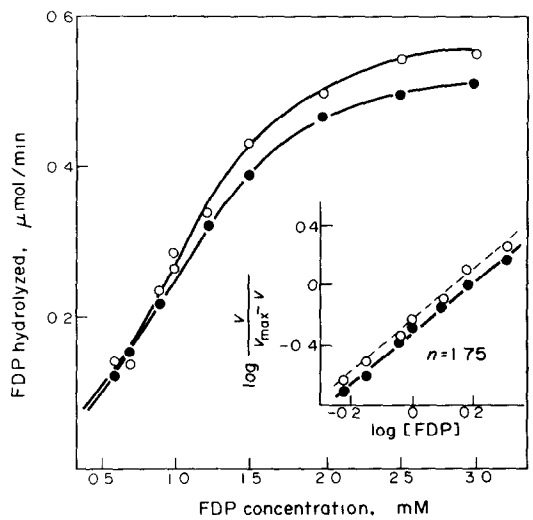

Fig 2 Velocity rate curves of FDPase active fractions I (●—●) and II (○—○) Inset is the Hill plots of fraction I (●—●) and II (○—○) n is the Hill coefficient. FDPase activities were determined in the reaction mixture 0 1 M Tris–HCl buffer pH 8·8, fructose-1,6-diphosphate at the indicated concentration, 5 mM $MgCl_2$, 1 6 mM EDTA, 5 mM cysteine and the enzyme preparation, in a final vol of 2 ml. After 30 min incubation at 28°, 1 ml of 5% trichloroacetic acid was added and the released Pi was determined

Table 4. Effect of Mg^{2+}, Mn^{2+}, and Co^{2+} on FDPase activation

Activator concentration (mM)	Activity (enzyme units) Mg^{2+} F.I	F II	Mn^{2+} F I	F II	Co^{2+} F I	F II
1·25						
2·5			0 033	0·040	0 025	0·025
5	0·096	0 107	0·017	0·023	0·026	0 036
10	0 132	0 174	0 010	0·013	0 021	0 025

* Active fractions I and II were assayed in the standard conditions, with the exception of Mg^{2+} and its sustitutes, tested at the concentrations stated below

versus reaction rate. A Hill coefficient $n = 1{\cdot}75$ was calculated from the Hill plots inset in the same figure. The K_m values for fractions I and II, were $1{\cdot}4 \times 10^{-3}$ and $1{\cdot}1 \times 10^{-3}$ respectively, from the reciprocal plots $1/v$ vs $1/S$. The Arrhenius plots of $1/T$ vs log v gave the values 14·3 and 13·0 kcal/mol as activation energies of fractions I and II.

Preincubation time at different temperatures for a 50% maintenance of activity clearly shows that at low temperatures (−18° and 4°) the enzyme is more stable in concentrated than in diluted solution, there is no difference at higher temperatures. The optimum for enzyme storage is 4°, at −18° there is a freezing inactivation. Fractions I and II do not show any difference in thermostability at similar concentrations.

DISCUSSION

The amino acid composition shows a similar pattern for fractions I and II with the exception of a higher glutamic acid content in the latter, in accordance with its higher elution volume from DEAE-cellulose column[1]. Glutamic and aspartic acids are the main amino acids in both fractions, and this could explain their low isoelectric points[1]. With the exception of a higher content in glycine and glutamic acid, a similar aminoacid composition has been found by Buchanan *et al.* (Table 1) for spinach photosynthetic FDPase, whereas the gluconeogenic enzyme is quite different, with a higher lysine content.

The high pH optimum we have found for FDPases I and II was considered to be photosynthetically nonphysiological by Racker and Schroeder[5]. However, Preiss *et al.* (11) found that Mg^{2+} at 40 mM shifts the pH optimum to more neutral zones. Although it is not clear whether the chloroplasts can reach that Mg^{2+} level, it is known that bean chloroplasts can accumulate up to 70% of the whole Mg^{2+} in the leaf[12]. Similar pH optima have been found in *Euglena*[13], *Phaseolus*[14] and the photosynthetic bacterium *Rhodospirillum rubrum*[15].

The high specificity of fractions I and II for fructose-1,6-diphosphate is in accordance with the results earlier found in other photosynthetic FDPases. On the contrary *Rhodopseudomonas* FDPase[16], as occurs with the gluconeogenic enzyme from different sources, is also active against sedoheptulose-1,7-diphosphate.

That photosynthetic FDPase requires a divalent metal activator is widely recognized, but whereas Mg^{2+} or Mn^{2+} is the physiological one is not clear. The former is more effective in castor-bean FDPase[17], with optimum at 5 mM, and the latter with the *Rhodopseudomonas* enzyme[16]. This is in agreement with our results. Ca^{2+}, Zn^{2+}, Fe^{2+}, Cu^{2+} and Co^{2+} are ineffective with FDPases from different sources[18].

The EDTA activating effect on gluconeogenic FDPase has always been considered to depend on some other mechanism than heavy metal chelation[19]. In spite of its great sensitivity to metal inhibition the same has been postulated for the photosynthetic enzyme[5, 13]. Nevertheless EDTA activation could not be demonstrated in *Phaseolus*[14] and castor-bean[17] FDPases. We have found a slight enhancement of activity by EDTA and the increased activity by Mg^{2+} is higher in the presence of EDTA.

Kinetic properties of fractions I and II deviated significantly from Michaelis–Menten kinetics, showing sigmoid velocity curves at the standard conditions tested (Fig. 2). However the Hill plots fit a straight line; the high Hill coefficient suggest that there is more than one binding-site for enzyme with the substrate, with a strong positive cooperation. The same occurs at neutral pH and low activator concentration with the *Rhodopseudomonas* enzyme, changing to hyperbolic at higher Mn^{2+} concentration[20]. This suggest a regulatory rol of Calvin cycle by fructose-1,6-diphosphate and Mg^{2+}, particularly considering that physiological fluctuations of the fructose-1,6-diphosphate in leaves[11] are located in the concave zone of the sigmoid. Apart from these low substrate concentrations, the K_m values are higher than that of 3×10^{-4} found by Racker and Schroeder[5] for spinach enzymes, and those of $7{\cdot}7 \times 10^{-4}$, $1{\cdot}6 \times 10^{-4}$ and 3×10^{-4} M in Phaseolus[14], castor-bean[17] and *Euglena*[13] enzymes, respectively, although none of these authors take account of the sigmoid kinetics.

The strong thermostability of both fractions I and II makes possible a maximum FDPase activity at 60°. App and Jagendorf[13] have

reported a similar situation for FDPase from *Euglena*; on the contrary, bacterial FDPases are not so stable[16]. The activation energies calculated for fractions I and II are in the range found for other phosphatases. Salmon liver gluconeogenic FDPase has an energy of activation of 115 kcal/mol; this high value could be explained considering the particular thermal habitat of salmon.

In conclusion, the similarity in kinetic properties of fractions I and II makes questionable the physiological role of these two photosynthetic FDPases and, from the point of view of origin, it is possible that one is formed from the other by endogenous proteolysis.

EXPERIMENTAL

Enzyme preparation. All the experiments were performed with pure fractions I and II isolated as described previously[1], lyophilized, and dissolved in buffer before use.

Enzymic activity and protein content determination. Enzyme activity was determined as described in Ref [1] Protein content was measured according to Ref. [21]

Aminoacid composition Samples were hydrolyzed with 6N HCl for 24 hr at 100° in sealed tubes under vac HCl was removed under vac and the aminoacid content determined in an autoanalyzer

Optimum activity conditions. The enzyme was assayed over the 4·0–10·4 with the following 50 mM buffer systems· acetate pH 4 0–5·2, maleate pH 5 2–6·8, Tris–HCl pH 7 0–8·8 and glycine–NaOH pH 8·8–10·4 In order to overcome the different nature and ionic strength of these buffer systems, the pH ranges of them were overlapped. Fructose-1,6-diphosphate, Mg^{2+}, EDTA and cysteine were assayed at the concn stated in Results, and Ca^{2+}, Mn^{2+}, Zn^{2+}, Sr^{2+}, Co^{2+}, Cd^{2+} and Cu^{2+} tested as Mg^{2+} substituted in the range 2·5–10 mM concn; all these activators were used as chloride Finally, the effect of temp on enzyme activity was studied by incubating between 4 and 75°

Substrate specificity and enzyme stability 14 physiological sugar-phosphate esters were assayed at 4 mM concn glucose-1-P, glucose-6-P, glucose-1,6-P, fructose-1-P, fructose-6-P, fructose-1,6-P, ribose-5-P, gluconate-6-P, ribose-1-P, ribulose-1,5-P, galactose-1-P, galactose-6-P, sedoheptulose-1,7-P glucuronate-1-P, and glycerol-2-P

Enzyme samples in dil (1–3 μg/ml) and conc (0·1–0·5 mg/ml) soln in 50 mM acetate buffer pH 5 5, were preincubated at different temps between −18° and 60° for periods from 30 min to 4 months, and their enzyme activities determined.

Kinetic assays. Reaction rates were determined at substrate concn between 0·6 and 3·0 mM, and the K_m values obtained from the plots of 1/v vs 1/S The activation energy was determined from plots of the log of initial reaction rates at 4, 16, 28, 37, 45 and 60° vs 1/T

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