

Spinach leaf 6-phosphofructo-2-kinase

Isolation of a new enzyme form that undergoes ATP-dependent modification

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A novel form of 6-phosphofructo-2-kinase was partially purified from spinach (*Spinacia oleracea* L.) leaves. As isolated, the new enzyme form possessed little or no enzymatic activity. However, pretreatment with 2 mM Mg-ATP dramatically increased the activities of 6-phosphofructo-2-kinase and fructose-2,6-bisphosphatase. Activation only occurred in the presence of magnesium plus ATP; either alone was ineffective. The ATP activation was reversed by treatment with alkaline phosphatase and could be completely restored by subsequent incubation with Mg-ATP. Thus, protein phosphorylation appears to be the mechanism involved. This is the first evidence that higher plants contain an interconvertible form of the enzyme.

Regulation; P_i; ATP; Fructose-2,6-bisphosphatase; 6-Phosphofructo-2-kinase; (*Spinacia oleracea* L.)

1. INTRODUCTION

F26BP occurs widely in both plant and animal tissues, and is thought to play a key role in the regulation of carbohydrate metabolism. Consequently, the enzymes which synthesize and degrade F26BP (F6P,2K and F26BPase, respectively) must be highly regulated. In plants [1,2] and animals

[3,4] both enzymes are regulated by metabolites. In addition, the animal enzyme is subject to covalent modification which provides a mechanism to override metabolite control. In liver, a cAMP-dependent protein kinase phosphorylates the protein, and thereby inhibits the kinase and activates the phosphatase activity [3]. Preliminary attempts to phosphorylate the spinach leaf enzyme with cAMP-dependent protein kinase were unsuccessful [5]. However, in spinach leaves, activity of F6P,2K has been shown to increase during the day and decrease in the dark [6,7]. Changes in F6P,2K activity were most pronounced when assays contained limiting substrate concentrations, suggesting that some modification of the protein occurred which altered substrate affinities. The mechanism involved has not been identified.

During purification of spinach leaf F6P,2K, we attempted to modify the elution profile during Mono Q chromatography by inclusion of ATP in the gradient, as has been reported for protein kinase C from mouse brain [8]. We observed that elution of the 'native' enzyme was unaffected, but a new peak of enzyme activity was recovered in the

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Abbreviations: F26BP, fructose 2,6-bisphosphate; F6P,2K, 6-phosphofructo-2-kinase; F26BPase, fructose-2,6-bisphosphatase; F6P, fructose 6-phosphate; G6P, glucose 6-phosphate; PMSF, phenylmethylsulfonyl fluoride; BAD, benzamidine

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gradient. This paper documents the apparent existence of an inactive form of F6P,2K in spinach leaves which can be activated by pretreatment with ATP. These preliminary results suggest that plants contain a mechanism for the covalent modification of F6P,2K.

2. MATERIALS AND METHODS

Fresh spinach (*Spinacia oleracea* L.) leaves were obtained from local suppliers. The leaves were thoroughly washed, deveined, and frozen in liquid nitrogen.

2.1. Enzyme extraction and partial purification

Approx. 400 g frozen leaf tissue was combined with 800 ml grind buffer which contained 50 mM Tris-HCl (pH 8.0), 10% (v/v) glycerol, 0.1% (v/v) 2-mercaptoethanol and, where indicated, 5 mM each of PMSF and BAD. The tissue was thoroughly homogenized in a 5 l capacity Waring blender for 1 min at maximum line voltage. The resulting brei was filtered through 2 layers of cheesecloth and then centrifuged at $20000 \times g$ for 20 min. The supernatant was combined with solid ammonium sulfate (20% saturation), mixed for 20 min at 0–4°C, and then centrifuged as above. Additional ammonium sulfate was added to the supernatant to 40% saturation, and the above procedure repeated. The pellet was resuspended in 40 ml grind buffer and combined with 25 ml blue agarose resin (Bio-Rad), that had been equilibrated with grind buffer. The blue agarose suspension was gently mixed for 20 min at 0–4°C and then filtered under partial pressure. The resulting cake was resuspended in 40 ml grind buffer supplemented with 300 mM KCl, and the mixing/filtration procedure repeated. Elution was achieved by resuspending the blue agarose in 40 ml grind buffer containing 2 M KCl. The filtrate was collected in 8 ml aliquots which were stored at –80°C.

2.2. Mono Q chromatography

Approx. 8 ml blue agarose eluate was rapidly desalted and concentrated to 1 ml using Centricon-30 microconcentrators (Amicon, Danvers, MA). The 1 ml concentrate was brought to a final volume of 5 ml with 50 mM Tris-HCl (pH 8.0), 10% (v/v) ethylene glycol and 0.1% (v/v)

2-mercaptoethanol (buffer A). Final protein concentration was 2–3 mg/ml as determined according to Bradford [9]. The protein solution was chromatographed on a Mono Q column using a Pharmacia FPLC system (Pharmacia, Piscataway, NJ) at a flow rate of 1 ml/min. The column was equilibrated with buffer A and eluted with a 0–500 mM KCl linear gradient in the same buffer. The 1 ml fractions were collected and assayed immediately for activities. All steps were performed at 4°C.

2.3. Assay of F6P,2K and F26BPase activities

F6P,2K was assayed in a mixture of 25 mM Mops/50 mM Tris-HCl (pH 8.0), 50 μ l protein, 5 mM P_i , 5 mM F6P, 20 mM G6P, 2 mM ATP, 5 mM $MgCl_2$, 5 mM phosphocreatine, and 4 U/ml creatine phosphokinase in a final volume of 0.1 ml. Where indicated, pH was varied as specified. The reactions were terminated after 10 min by addition of an equal volume of 0.1 N NaOH.

F26BPase (low substrate assay) activity was determined by monitoring the disappearance of F26BP over a 10 min time course at 2.5 min intervals as in [10]. Reactions were initiated by addition of 0.5 μ M F26BP. The determination of high substrate (15 μ M) F26BPase activity was by modification of the continuous spectrophotometric assay reported by Cseke et al. [10]. Assay mixtures (1 ml) contained 50 mM Tris-HCl (pH 7.5), 100 μ l protein, 1 mM NAD and 2 units each of G6P dehydrogenase (from *Leuconostoc*) and phosphoglucose isomerase. The reaction was initiated by addition of 15 μ M F26BP and followed by the increase in absorbance at 340 nm.

Detection of F26BP (in the F6P,2K and low-substrate F26BPase assays) was the bioassay [11] based on stimulation of potato tuber pyrophosphate-D-fructose-6-phosphate-1-phosphotransferase.

Chemicals were obtained from Fisher (Fair Lawn, NJ); biochemicals and enzymes were purchased from Sigma (St. Louis, MO).

3. RESULTS AND DISCUSSION

3.1. Resolution of various forms of F6P,2K

Spinach leaf F6P,2K activity was concentrated and partially purified by ammonium sulfate frac-

tionation and blue agarose chromatography. The resulting sample was purified approx. 20-fold with an overall yield of 40%. Chromatography of the partially purified sample on Mono Q (FPLC) resolved a minor peak of activity followed by two major activity peaks (designated I–III in order of elution; fig.1A). Preincubation of the partially purified F6P,2K preparation with 2 mM Mg-ATP for 10 min at 25°C prior to Mono Q chromatography resulted in a substantial activation of total F6P,2K activity; either magnesium or ATP alone were ineffective (not shown). Subsequent chromatography of the Mg-ATP-activated sample on Mono Q revealed a dramatic activation of peak I activity (fig.1C); recovered activity of peaks II and III was unaffected by pretreatment with Mg-ATP. Considering total recovery of F6P,2K activity, the purification achieved during Mono Q chromatography was about 22-fold.

Inclusion of the protease inhibitors PMSF and BAD (5 mM each) in the initial extraction buffer, followed by partial purification as described

above, markedly affected recovery of F6P,2K activity during Mono Q chromatography. As shown in fig.1B, recovery of peak II activity was increased (relative to peak II activity in fig.1A) and peak III was not detected. Consequently, the peak III enzyme appears to be a proteolytically modified form of the native enzyme (peak II). Preincubation of the partially purified sample, prepared with protease inhibitors, with Mg-ATP again resulted in appearance of peak I activity (fig.1D). In both experiments (plus or minus protease inhibitors), F6P,2K activity essentially co-eluted with F26BPase activity, and activation of peak I activity by ATP pretreatment increased both kinase and phosphatase activities.

Overall, these results suggest that spinach leaves contain a novel form of F6P,2K. The new form is catalytically inactive as isolated but can be activated by pretreatment with ATP. Neither the appearance of this new form nor the ATP activation can be ascribed to protease action. The effect of ATP was persistent; activity of desalted peak I

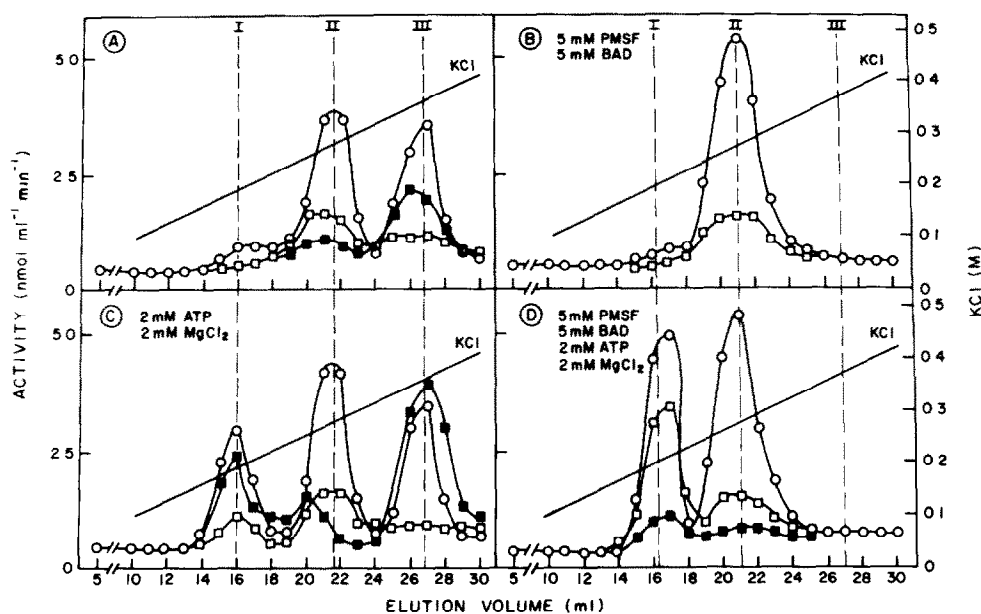


Fig.1. Resolution of three forms of spinach leaf F6P,2K by Mono Q chromatography. Spinach leaf extracts were prepared in the absence (A,C) and presence (B,D) of protease inhibitors (5 mM each of PMSF and BAD) and partially purified by ammonium sulfate fractionation and blue agarose chromatography as described in section 2. The partially purified samples were incubated at room temperature for 10 min in the absence (A,B) or presence of 2 mM Mg-ATP (C,D) prior to Mono Q chromatography. Fractions were assayed for F6P,2K activity (○) and F26BPase activity with low (0.5 μ M, □) or high (15 μ M, ■) F26BP as substrate.

recovered from Mono Q was stable for at least 1 week and after repeated freeze-thaw cycles. This strongly suggests that some covalent modification may be involved, since hysteretic behavior would be expected to reverse relatively rapidly after removal of ATP.

In an effort to determine whether appearance of peak I activity involved modification of peak II enzyme, a partially purified F6P,2K sample was chromatographed on Mono Q and the resolved fractions were individually incubated with ATP prior to assay. As shown in fig.2, almost all of the activity recovered was in peak II when fractions were preincubated at room temperature in the absence of ATP prior to assay. Preincubation of the fractions with ATP had no effect on peak II activity, but strongly activated F6P,2K activity in the region of peak I. As shown in the inset of fig.2, ATP-dependent activation of peak I enzyme required approx. 15 min to reach completion. The peak I enzyme appears to elute at the same salt concentration when in the active and inactive state. Thus, it is clear that peak I activity does not arise by modification of the peak II enzyme.

3.2. Kinetic characterization

Table 1 lists the K_m values for various forms of the enzyme. The apparent $K_m(\text{ATP})$ was about 0.5 mM for all three forms; however, differences in affinities for the other substrate, F6P, and the activator, P_i , were noted among the different forms. Relative to the native enzyme (peak II), the $K_m(\text{F6P})$ of peak I was increased about 2-fold and the $K_a(P_i)$ was increased 4-fold. The proteolytically modified form (peak III) also showed changes in kinetic parameters (i.e. reduced affinities) relative to the native enzyme form.

The pH dependence of enzyme activity also revealed differences among the forms. As shown in fig.3, native enzyme (peak II) had a fairly sharp pH optimum at pH 7.5. The peak I enzyme activity (after ATP activation) also exhibited a rather sharp pH profile but the pH optimum was shifted to slightly higher values (pH 7.8–8.0). Another important difference is that the enzymic activity of peak I enzyme approached zero at pH 7.0 whereas peak II enzyme had considerable activity at neutral pH. The pH dependence of the peak III enzyme was not determined. The differences in kinetic

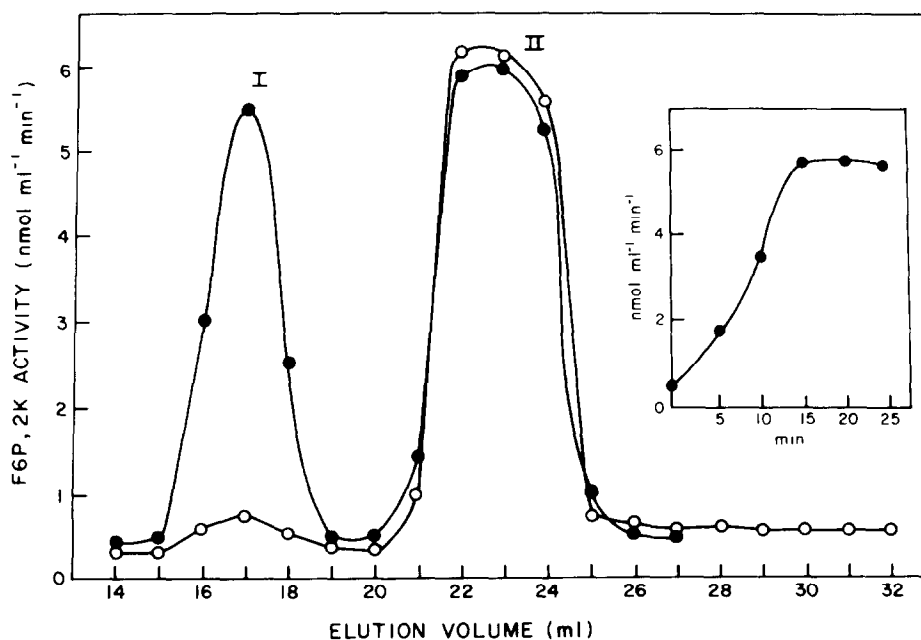


Fig.2. Partially purified spinach leaf F6P,2K (prepared with protease inhibitors) was chromatographed on Mono Q and the resolved fractions were assayed individually after preincubation at room temperature in the absence (○) and presence (●) of 2 mM Mg-ATP. (Inset) Fractions 16 and 17 were pooled, and the time course of activation by ATP determined at room temperature.

Table 1

Kinetic characterization of the three forms of spinach leaf F6P,2K resolved by Mono Q chromatography

Enzyme form	K_m (mM)		K_a (mM) P_i
	ATP	F6P	
I	0.5	1.0	2.0
II	0.5	0.5	0.5
III	0.5	0.8	1.5

Peak fractions from Mono Q were pooled, concentrated and desalted prior to assay as described in section 2. Values for forms I and II are means of 3 separate determinations; values for form III are from a single experiment

properties (table 1), pH dependence (fig.3) and elution from Mono Q (figs 1,2) strongly suggest that peak I enzyme, after activation by ATP, is not identical to the native enzyme (peak II).

3.3. Mechanism of ATP activation

One possible mechanism to explain the covalent modification of peak I F6P,2K by ATP is protein phosphorylation. If this is the mechanism, the ATP effect should be reversed by alkaline phosphatase, as has been demonstrated for the liver enzyme [12]. To test this possibility, active peak I enzyme was obtained by Mono Q chromatography as shown in fig.1D. The active enzyme was treated with alkaline phosphatase, and the two enzymes were then separated by gel filtration on a Superose 6B column. The peak I enzyme was almost completely inactivated by the phosphatase treatment (table 2, expt 2), but could be subsequently reactivated by treatment with Mg-ATP (table 2, expt 3). The action of the alkaline phosphatase was completely blocked by 75 mM KF (not shown). These results strongly suggest that the mechanism of ATP activation involves protein phosphorylation; the dephosphoenzyme is catalytically inactive and the phosphoenzyme is active.

3.4. Conclusions

The present results suggest that spinach leaves contain two distinct forms of F6P,2K: one that retains activity during purification and is unaffected

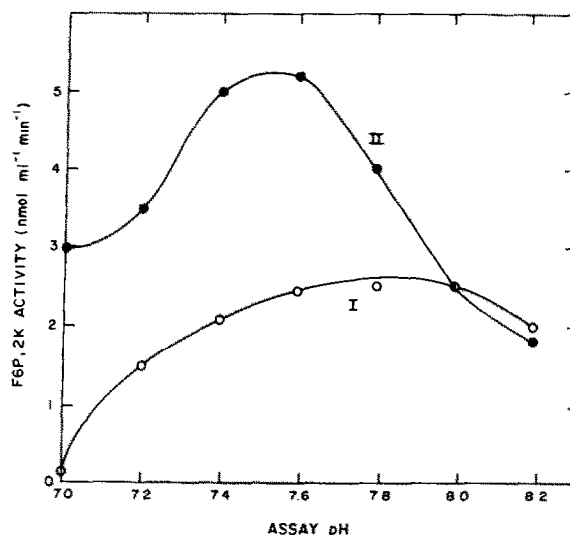


Fig.3. pH dependence of peak I and II F6P,2K activity. The forms of the enzyme were resolved by Mono Q chromatography as in fig.1D. Peak fractions were pooled, concentrated and desalted prior to assay.

by pretreatment with ATP (peak II), and one that is isolated during purification in a largely inactive state but can be activated in vitro by incubation with ATP (peak I). The nature of activation of peak I by ATP appears to involve protein phosphorylation based on the reversal of the ATP activation by alkaline phosphatase. If phosphorylation is the mechanism, then the protein kinase involved appears to co-elute with the peak I enzyme. As protein kinases tend to co-purify with their target enzymes [13] this appears to be a realistic possibility. Previous attempts to phosphorylate the plant F6P,2K involved purification of the active enzyme [5]. Failure to achieve phosphorylation in vitro may have resulted because the form sensitive to ATP modification (peak I) separates from the active peak II enzyme during purification. Because peak I enzyme is normally obtained in an inactive state, it may be easily 'lost' during purification.

Our preliminary results provide the first evidence that plants contain a mechanism for regulation of F6P,2K by covalent modification. It is tempting to speculate that this mechanism may be responsible for the diurnal changes in F6P,2K activity (associated with K_m changes) that have been documented to occur in spinach leaves [6,7].

Table 2

Inhibition of peak I F6P,2K activity by alkaline phosphatase and reactivation by Mg-ATP

Expt	Preincubation conditions	Recovered F6P,2K activity (nmol·ml ⁻¹ ·min ⁻¹)
1	control	16.0
2	alkaline phosphatase	< 1
3	expt 2 followed by Mg-ATP	15.5

Activated peak I enzyme was obtained by Mono Q chromatography. The peak I F6P,2K (50 µg/ml) was incubated without (expt 1) or with (expts 2,3) alkaline phosphatase (35 µg) at 25°C for 15 min. The 1 ml samples were then subjected to gel filtration on a Superose 6B column (125 ml), which separated the 2-kinase and alkaline phosphatase activities. In expt 3, the alkaline phosphatase-inactivated enzyme was reactivated by incubation with 2 mM Mg-ATP prior to assay. Commercially available alkaline phosphatase (type V, Sigma) was prepurified by Mono Q and Superose 6B chromatography before use in these experiments. In all cases, the Superose 6B column was equilibrated with 50 mM Tris-HCl (pH 7.5), 100 mM KCl and 10 mM DTT; the flow rate was 0.5 ml·min⁻¹

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