N-terminal truncation affects the kinetics and structure of fructose-6-phosphate 2-kinase/fructose-2,6-bisphosphatase from *Arabidopsis thaliana*

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The enzyme fructose-6-phosphate 2-kinase (F6P,2K; 6-phosphofructo-2-kinase)/fructose-2,6-bisphosphatase (F26BPase) catalyses the formation and degradation of the regulatory metabolite fructose 2,6-bisphosphate. A cDNA encoding the bifunctional plant enzyme isolated from *Arabidopsis thaliana* (AtF2KP) was expressed in yeast, and the substrate affinities and allosteric properties of the affinity-purified enzyme were characterized. In addition to the known regulators 3-phosphoglycerate, dihydroxyacetone phosphate, fructose 6-phosphate and P_i , several metabolites were identified as important new effectors. PP_i , phosphoenolpyruvate and 2-phosphoglycerate strongly inhibited F6P,2K activity, whereas fructose 1,6-bisphosphate and 6-phosphogluconate inhibited F26BPase activity. Furthermore, pyruvate was an activator of F6P,2K and an inhibitor of F26BPase. Both kinase and phosphatase activities were rapidly inactivated by mild heat treatment (42 °C, 10 min),

but the presence of phosphate protected both enzyme activities from inactivation. In addition to the catalytic regions, the *Arabidopsis* enzyme comprises a 345-amino-acid N-terminus of unknown function. The role of this region was examined by the expression of a series of N-terminally truncated enzymes. The full-length and truncated enzymes were analysed by gel-filtration chromatography. The full-length enzyme was eluted as a homotetramer, whereas the truncated enzymes were eluted as monomers. Deletion of the N-terminus decreased the kinase/phosphatase activity ratio by 4-fold, and decreased the affinity for the substrate fructose 6-phosphate. The data show that the N-terminus is important both for subunit assembly and for defining the kinetic properties of the enzyme.

Key words: allosteric regulation, enzyme kinetics, fructose-2,6-bisphosphate, structure/function, terminal region.

INTRODUCTION

Fructose 2,6-bisphosphate (Fru-2,6- P_2) is a powerful regulator of carbohydrate metabolism in eukaryotic cells. The concentration of this metabolite controls the rate of interconversion between fructose 6-phosphate (Fru-6- P) and fructose 1,6-bisphosphate (Fru-1,6- P_2) in the cytosol. In animals and yeast Fru-2,6- P_2 serves as a regulator of glycolysis and gluconeogenesis [1]. In plants Fru-2,6- P_2 is involved primarily in the regulation of photosynthetic carbon partitioning [2]. Studies of transgenic plants have confirmed that a decrease in the level of Fru-2,6- P_2 in leaves increases the flux towards sucrose [3,4], whereas an increase in the level of Fru-2,6- P_2 increases the flux towards starch [5,6].

The synthesis and degradation of Fru-2,6- P_2 are catalysed by two specific enzyme activities, fructose-6-phosphate 2-kinase (6-phosphofructo-2-kinase; F6P,2K; EC 2.7.1.105) and fructose-2,6-bisphosphatase (F26BPase; EC 3.1.3.46). In animals these enzyme activities reside on a single bifunctional polypeptide. Several tissue-specific isoforms have been identified, and these isoforms are derived from different genes as well as from alternative splicing [7]. The animal enzymes are dimers of two identical subunits, each of which comprises the highly conserved catalytic region, as well as N- and C-terminal extensions which are more variable in sequence and size. The activities of some isoforms are regulated by the reversible phosphorylation of amino acid residues in the N- or C-terminus [7].

In plants, only limited information is available on the activities of F6P,2K and F26BPase. An enzyme with co-purifying F6P,2K and F26BPase activities has been obtained and partially character-

ized from spinach leaves [8]. The native form of this enzyme is a homotetramer. Recently cDNA and EST (expressed sequence tag) clones with homology to animal and yeast F6P,2K/F26BPases (known as F2KPs in plants) have been isolated from several plant species. The bifunctionality of the encoded enzyme has been confirmed by heterologous expression in *Escherichia coli* of the *Arabidopsis* (accession no. AF190739; AtF2KP) [9] and potato (AF073830) [10] F2KP cDNAs. In contrast with the animal enzymes, F2KP from *Arabidopsis* and potato is encoded by a single gene, as shown by Southern blot analysis [9,10]. A single F2KP could be detected in leaf tissue of several plant species by immunoblotting, suggesting that there is only one isoform in leaves [9]. However, the presence of isoforms specific for other tissues is still possible.

F2KPs differ from most animal F6P,2K/F26BPase isoforms in possessing long N-terminal extensions (345 amino acids in *Arabidopsis*). The catalytic region of the plant enzymes is highly conserved (88% identity between *Arabidopsis* and spinach) compared with the N-terminus (59% identity between *Arabidopsis* and spinach), which is composed of conserved motifs interrupted by highly variable sequences [9]. The N-terminus is not required for either of the two catalytic activities of F2KP [9,10]. However, it influences the ratio between the F6P,2K and F26BPase activities [9]. In the N-terminus of the F2KPs, several potential phosphorylation sites can be predicted [9]. Although reversible protein phosphorylation has been suggested as a regulatory mechanism in plants [11–13], regulatory phosphorylation sites are yet to be identified.

Abbreviations used: F6P,2K, fructose-6-phosphate 2-kinase (6-phosphofructo-2-kinase); F26BPase, fructose-2,6-bisphosphatase; F2KP, fructose-6-phosphate 2-kinase/fructose-2,6-bisphosphatase from plants; AtF2KP, F2KP from *Arabidopsis thaliana*; D_{1–318} (etc.), mutant protein lacking residues 1–318 (etc.) of full-length AtF2KP; PFP, PP_i-dependent 6-phosphofructo-1-kinase; Fru-6- P , fructose 6-phosphate; Fru-2,6- P_2 , fructose 2,6-bisphosphate; Fru-1,6- P_2 , fructose 1,6-bisphosphate; Glc-1,6- P_2 , glucose 1,6-bisphosphate; PEP, phosphoenolpyruvate; PGA, phosphoglycerate; DHAP, dihydroxyacetone phosphate.

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The kinetic properties of F2KP have been characterized in detail only for the spinach enzyme [8,14–17]. Both kinase and phosphatase activities are subject to allosteric regulation by metabolites, which are intermediates of photosynthetic carbohydrate metabolism. Thus the Fru-2,6- P_2 level becomes a signal which co-ordinates photosynthetic activity and the rates of biosynthesis of sucrose and starch. Further investigations have been hampered by difficulties in purifying this low-abundance and degradation-prone enzyme. The identification of cDNA clones encoding F2KP has allowed heterologous expression, which yields high amounts of the enzyme and facilitates purification. In previous studies we have used *E. coli* as a host for expression of F2KP, but have encountered problems in obtaining soluble and active enzyme. For the present study, yeast was chosen as the expression host.

In this paper we report the first heterologous expression of F2KP in yeast, and a detailed kinetic characterization of AtF2KP. The role of the N-terminal extension of this plant enzyme is investigated further, and it is shown to affect the kinetics, subunit assembly and stability of the enzyme.

MATERIALS AND METHODS

Expression constructs

For expression in yeast of AtF2KP (AF190739) [9], a series of fragments was amplified by PCR. The entire open reading frame was amplified using sense (5'-ATGGGGTTCAGGTGCATCG-3') and antisense (5'-TCCATGAGTTTGTAGCGT-3') primers. A number of N-terminal deletion mutants were made using the following sense primers: D₁₋₆₆ (i.e. mutant protein lacking residues 1–66 of full-length AtF2KP), 5'-ATGTCCGAGCTAAGCTTT-3'; D₁₋₁₂₅, 5'-ATGGTGATTGTTGAATTCGA-3'; D₁₋₁₇₉, 5'-ATGGAAGTTTGGAGCTCGAT-3'; D₁₋₂₄₉, 5'-ATGACGATCGTTGTTTCT-3'; D₁₋₃₁₈, (5'-ATGCTTGGACCTACTGCA-3' (underlined sequences indicate introduction of a new start codon). The amplification reactions were performed using *Pwo* DNA polymerase (Roche Molecular Biochemicals). To introduce a 3' A overhang, the PCR products were incubated for 10 min at 72 °C with 1 unit of *Taq* polymerase (Roche Molecular Biochemicals). The PCR products were cloned into pUni/V5-His-TOPO (Invitrogen) in-frame with the C-terminal 6 × His tag. The plasmids were recombined with pYES2.1E (Invitrogen) to yield the final expression constructs. The sequence of each plasmid was verified by restriction analysis and sequencing of the border between AtF2KP and pYES.1E using the T7 primer.

Yeast cultures

The yeast (*Saccharomyces cerevisiae*) strain used was INVSc1 (*MATa/MATα.hisΔ1/hisΔ1 leu2/leu2 trp1-289/trp1-289 ura3-52/ura3-52*; Invitrogen). Yeast cells were grown in 1 % yeast extract, 2 % (w/v) peptone and 2 % (w/v) glucose. For selection of transformants, yeast cells were grown on minimal medium without uracil [0.67 % (w/v) yeast nitrogen base without amino acids (Difco), 2 % (w/v) glucose and appropriate amino acids]. For induction of recombinant protein, yeast cells were grown in minimal medium containing 2 % (w/v) galactose at 30 °C for 20 h.

Purification of AtF2KP

Yeast cultures expressing the recombinant protein were pelleted and resuspended to a theoretical D_{600} of 50–100 in 20 mM sodium phosphate (pH 7.8)/500 mM NaCl (buffer A) containing 1 mM δ -aminocaproic acid, 0.5 mM benzamidine, 0.5 mM

EDTA, 0.125 mM phenanthroline, 1 mg·ml⁻¹ leupeptin, 1 mg·ml⁻¹ antipain, 1 mM PMSF and 0.1 % (v/v) β -mercaptoethanol. The cells were lysed by vortex-mixing for 5 × 30 s with an equal volume of acid-washed glass beads (425–600 μ m; Sigma). Cell lysates were cleared by centrifugation for 10 min at 20000 *g*. The supernatant from 50 ml of cell culture was applied to a 1 ml ProBond (Invitrogen) Ni²⁺-column equilibrated with buffer A, and the column was washed with 5 ml of buffer A, 5 ml of 20 mM sodium phosphate (pH 6.0)/500 mM NaCl (buffer B) and 2 ml of buffer B containing 50 mM imidazole. The recombinant protein was eluted with 2 ml of 350 mM imidazole in buffer B, and the buffer was exchanged using a Sephadex G-25 (Amersham Pharmacia Biotechnology) column equilibrated with 50 mM Mops/KOH, pH 6.0, 5 mM MgCl₂, 1 mM EDTA and 10 % (v/v) ethylene glycol (buffer C) before assaying for enzyme activity. All steps were performed at 4 °C.

Enzyme assays

The activities of F6P,2K and F26BPase were assayed by the detection of the formation or degradation of Fru-2,6- P_2 . One unit of activity was defined as the conversion of 1 μ mol of substrate per min. The assays were performed in buffer C at 25 °C. Unless indicated otherwise, F6P,2K activity was assayed in the presence of 10 mM Fru-6- P , 5 mM potassium phosphate and 2 mM ATP. F26BPase activity was assayed in the presence of 0.1 μ M Fru-2,6- P_2 . The amount of Fru-2,6- P_2 was quantified by measuring the activation of potato tuber PP_i-dependent 6-phosphofructo-1-kinase (PFK) [18].

Heat inactivation of F6P,2K

Samples of purified full-length AtF2KP and N-terminal mutant D₁₋₃₁₈ were incubated for 0, 2, 5, 10 or 15 min at 42 °C in buffer C in the presence or absence of 20 mM potassium phosphate. The samples were cooled on ice and assayed for F6P,2K and F26BPase activities.

Gel filtration

AtF2KP and D₁₋₃₁₈ were purified and 100 μ l of each preparation was applied to a Superose 6 HR 10/30 column (Amersham Pharmacia Biotechnology) equilibrated with buffer C containing 20 mM potassium phosphate. The column was eluted with 0.4 ml·min⁻¹ buffer C, and 0.4 ml fractions were collected and assayed for F6P,2K activity. Molecular mass standards (Amersham Pharmacia Biotechnology) were eluted under the same conditions.

Western blot analysis

Proteins were extracted from yeast cells by resuspending 10¹⁰ cells in 1 ml of 10 % (w/v) trichloroacetic acid and lysing the cells by vortex-mixing with an equal amount of acid-washed glass beads for 5 min. The extract was centrifuged for 20 min at 10000 *g* and the pellet was washed with 2 ml of cold acetone and centrifuged for 20 min at 10000 *g*. The pellet was redissolved in 500 μ l of SDS/PAGE sample buffer and heated to 100 °C for 5 min; the pH was adjusted with KOH until the colour changed from yellow to blue, and the mixture was centrifuged for 5 min at 10000 *g*. The supernatant was incubated for 10 min at 100 °C. Proteins were separated by SDS/PAGE, blotted and developed as described by Nielsen [19]. The antibody was raised against the C-terminus (amino acids 566–651) of AtF2KP [9]. Purified samples of AtF2KP and D₁₋₃₁₈ that had been used for kinetic analysis were mixed with SDS/PAGE sample buffer and the

immunoblots were performed as above. The amount of enzyme protein was quantified by density scanning, using known amounts of the antigen as standard.

RESULTS

Kinetic characterization of AtF2KP

A cDNA encoding AtF2KP was inserted into the yeast expression vector pYES2.1 under the control of a galactose-inducible promoter. Under the assay conditions used in this study, the native yeast F6P,2K and F26BPase activities were barely detectable in extracts of cells expressing a control plasmid. The 6×His-tagged protein from the supernatant of lysed induced cells was purified by affinity chromatography and used for characterization of kinetic properties. The F6P,2K activity of AtF2KP showed Michaelis–Menten kinetics for both substrates, Fru-6-*P* and ATP (Figures 1A and 1B). Hanes plots ($[S]/v$ versus $[S]$) were linear and Hill coefficients were close to unity (0.97 and 1.12 for Fru-6-*P* and ATP respectively). Hyperbolic fits gave estimated K_m values of 0.5 mM for Fru-6-*P* and 0.19 mM for ATP (Table 1). Deletion of the N-terminal 318 amino acids abolished the Michaelis–Menten kinetics of F6P,2K for Fru-6-*P* (Figure 1A). The Hill coefficient for these data was calculated as 0.7, suggesting weak negative co-operativity. The affinity for Fru-6-*P* was decreased by deletion of the N-terminus, and $S_{0.5}$ was estimated as 4.7 mM by approximating the data to a hyperbolic curve. The kinetics for ATP were barely affected (K_m 0.15 mM) (Figure 1B). The F26BPase activity also obeyed Michaelis–Menten kinetics (Figure 1C), and accordingly the Hill coefficients were close to unity (1.17 and 0.99 for AtF2KP and D_{1–318} respectively). Deletion of the N-terminus resulted in a small, non-significant increase in the K_m for Fru-2,6-*P*₂ from 28 to 44 nM (Table 1). Deleting the 318 N-terminal amino acids reduced the maximal activity of F6P,2K to 25% of that of AtF2KP, whereas the maximal F26BPase activity was reduced by only 30% (Table 1).

Effects of metabolites on enzyme activities

The F6P,2K and F26BPase activities of AtF2KP were measured in the presence of a concentration of 2 mM of a range of metabolites, including nucleotides, sugar nucleotides, phosphates, neutral sugars, sugar phosphates, phosphorylated C₃ compounds and intermediates of the citric acid cycle and the pentose phosphate pathway (Table 2). Of the nucleotides tested, only ADP affected F6P,2K, by reducing the activity to 50% of the control activity. F26BPase activity was slightly inhibited by ATP and ADP. As expected, P_i had opposite effects on the two activities: F6P,2K was activated (K_a 1.5 mM) and F26BPase was inhibited (K_i 0.5 mM) (Table 3). PP_i was a very strong inhibitor of F6P,2K, with a K_i of 0.13 mM, but did not influence F26BPase activity. Neutral sugars had no effect on F6P,2K activity, but inhibited F26BPase activity to 53–61% of the control activity. Sugar phosphates, except for glucose 1,6-bisphosphate (Glc-1,6-*P*₂) which was an inhibitor, had no effect on F6P,2K activity, but were powerful inhibitors of F26BPase. Of the sugar phosphates, Fru-6-*P* and Fru-1,6-*P*₂ were the strongest inhibitors of F26BPase, with K_i values of 0.1 mM and 0.08 mM respectively, whereas the K_i of Glc-1,6-*P*₂ was considerably higher (0.85 mM) (Table 3). Phosphorylated C₃ compounds, especially phosphoenolpyruvate (PEP), lowered both F6P,2K and F26BPase activities. The K_i values of C₃ compounds that inhibited F6P,2K activity ranged from 0.07 mM for PEP to 1.35 mM for dihydroxyacetone phosphate (DHAP) (Table 3). Pyruvate was a strong activator of F6P,2K, with a K_a of 1.4 mM, and an

Figure 1 Characterization of the substrate concentration dependence of AtF2KP and D_{1–318}

The F6P,2K and F26BPase activities of purified AtF2KP and D_{1–318} were measured at various substrate concentrations. Data are fitted to hyperbolic curves using Sigma Plot (SPSS Inc.). (A) F6P,2K activity of AtF2KP (●) and D_{1–318} (○) at increasing concentrations of Fru-6-*P* assayed in the presence of 2 mM ATP and 5 mM potassium phosphate; data are shown as percentages of the activity at 10 mM Fru-6-*P*. (B) F6P,2K activity of AtF2KP (●) and D_{1–318} (○) at increasing concentrations of ATP assayed in the presence of 5 mM potassium phosphate and 10 mM Fru-6-*P*; data are shown as percentages of the activity at 2 mM ATP. (C) F26BPase activity of AtF2KP (●) and D_{1–318} (○) at increasing concentrations of Fru-2,6-*P*₂; data are shown as percentages of the activity at 0.2–0.25 mM Fru-2,6-*P*₂. Values are means ± S.D. of experiments with three independent extracts.

inhibitor of F26BPase activity. Intermediates of the citric acid cycle also activated F6P,2K and inhibited F26BPase activity. The pentose phosphate pathway intermediate 6-phosphogluconate did not affect F6P,2K activity, but inhibited F26BPase to 23% of the control activity, with a K_i of 0.08 mM. The effects of metabolites on the activities of D_{1–318} were similar to those reported for full-length AtF2KP (results not shown).

Table 1 Apparent kinetic constants of F6P,2K and F26BPase activities of AtF2KP

K_m values were determined from the plots in Figures 1(A)–1(C) fitting the data to a hyperbolic curve (Sigma Plot). V_{max} was estimated from the measurement of activity of purified enzymes quantified by Western blot analysis.

Enzyme	F6P,2K			F26BPase	
	$K_m(\text{Fru-6-P})$ (mM)	$K_m(\text{ATP})$ (mM)	V_{max} (m-units/mg)	$K_m(\text{Fru-2,6-P}_2)$ (nM)	V_{max} (m-units/mg)
AtF2KP	0.5	0.19	180	28	270
D ₁₋₃₁₈	4.7*	0.15	44	44	170

* Data did not obey Michaelis–Menten kinetics; $S_{0.5}$ value was estimated by approximating the data to a hyperbolic curve.

Table 2 Inhibition or activation of F6P,2K and F26BPase activities of AtF2KP by metabolites

AtF2KP was purified, and F6P,2K and F26BPase activities were determined in the presence of 2 mM of a range of metabolites. The F6P,2K assay mixture contained 2 mM ATP, 5 mM potassium phosphate and 10 mM Fru-6-P. The F26BPase assay mixture contained 0.1 μ M Fru-2,6-P₂. Values are means $\pm$ S.D. of four assays.

Metabolite	Activity (% of control)		F6P,2K/F26BPase activity ratio
	F6P,2K	F26BPase	
Control	100 $\pm$ 7	100 $\pm$ 31	2.4
ATP	109 $\pm$ 14	66 $\pm$ 21	4.2
ADP	49 $\pm$ 4	58 $\pm$ 17	2.2
AMP	105 $\pm$ 10	73 $\pm$ 22	3.7
UDP	102 $\pm$ 2	71 $\pm$ 19	3.7
ADP-Glc	108 $\pm$ 10	77 $\pm$ 18	3.6
UDP-Glc	111 $\pm$ 6	84 $\pm$ 18	3.4
P _i	97 $\pm$ 8	32 $\pm$ 19	7.7
PP _i	4 $\pm$ 1	118 $\pm$ 30	0.1
Sucrose	98 $\pm$ 6	53 $\pm$ 13	4.1
Fructose	89 $\pm$ 9	61 $\pm$ 14	3.3
Glucose	98 $\pm$ 3	56 $\pm$ 11	3.9
Fru-6-P	92 $\pm$ 2	0 $\pm$ 22	*
Fru-1,6-P ₂	76 $\pm$ 3	8 $\pm$ 19	22.1
Glc-1-P	98 $\pm$ 6	48 $\pm$ 16	4.6
Glc-6-P	104 $\pm$ 10	34 $\pm$ 11	6.9
Glc-1,6-P ₂	58 $\pm$ 6	5 $\pm$ 10	24.4
DHAP	45 $\pm$ 14	40 $\pm$ 10	2.4
3-Phosphoglycerate	27 $\pm$ 2	54 $\pm$ 11	1.0
2-Phosphoglycerate	29 $\pm$ 3	51 $\pm$ 14	1.2
PEP	7 $\pm$ 1	36 $\pm$ 11	0.4
Pyruvate	218 $\pm$ 9	47 $\pm$ 11	9.6
Malate	142 $\pm$ 23	66 $\pm$ 16	4.5
Oxaloacetate	142 $\pm$ 32	62 $\pm$ 10	4.8
Citrate	146 $\pm$ 14	67 $\pm$ 14	4.5
Succinate	147 $\pm$ 14	53 $\pm$ 14	5.7
6-Phosphogluconate	100 $\pm$ 18	23 $\pm$ 18	9.1

* Value not calculated.

Table 3 Inhibition and activation constants of F6P,2K and F26BPase activities of AtF2KP

K_i and K_a were determined for the strongest inhibitors and activators. Values are means of two experiments.

Activity	Metabolite	K_i or K_a^* (mM)
F6P,2K	PP _i	0.13
	PEP	0.07
	3-PGA	0.35
	2-PGA	0.45
	DHAP	1.35
	P _i	1.5*
F26BPase	Pyruvate	1.4*
	P _i	0.5
	Fru-6-P	0.1
	Fru-1,6-P ₂	0.08
	Glu-1,6-P ₂	0.85
	6-Phosphogluconate	0.08

The stability of AtF2KP and D₁₋₃₁₈ was first studied by following the inactivation of F6P,2K when purified enzyme was incubated at 42 °C (Figure 3). The F6P,2K activity of both AtF2KP and D₁₋₃₁₈ decreased rapidly, and after 10 min almost no activity remained. Inclusion of 20 mM potassium phosphate efficiently protected the enzyme from heat inactivation (Figure 3), and 75 % of the F6P,2K activity of both AtF2KP and D₁₋₃₁₈ remained after 15 min of incubation under these conditions. The stability of both phosphatase and kinase activities was tested further at the standard assay temperature (25 °C) compared with that at 4 °C and 42 °C. Inactivation of both F6P,2K and F26BPase activities was negligible after a 10 min incubation at 25 °C, both in the presence and in the absence of phosphate (Table 4). The F26BPase activity was less sensitive than that of F6P,2K to incubation at 42 °C, and phosphate efficiently protected F26BPase against heat inactivation. Western blots showed that heat inactivation was not due to proteolytic degradation (results not shown).

Effects of N-terminal truncation on the structure and stability of AtF2KP

The subunit compositions of purified AtF2KP and D₁₋₃₁₈ were determined by gel filtration (Figure 2). AtF2KP was eluted as a 370 kDa protein. The molecular mass of the subunit predicted from the cDNA is 83 kDa, and AtF2KP was estimated to be a homotetramer. When using raw extracts of leaves from both *Arabidopsis* and spinach, F6P,2K activity was eluted at the same position (results not shown). Deletion mutant D₁₋₃₁₈ was eluted as 45 kDa protein, which corresponds to D₁₋₃₁₈ being a monomer, as the molecular mass predicted from the cDNA is 49 kDa. Elution of the enzyme activity as well defined peaks was, for both AtF2KP and D₁₋₃₁₈, strictly dependent on the presence of P_i in the buffer (results not shown).

N-terminal truncation series: effects on activities of F6P,2K and F26BPase

A series of N-terminally truncated AtF2KP proteins was prepared in order to investigate in more detail the function of this region. Protein with the first 66, 125, 179, 249 and 318 amino acids deleted from the N-terminus were expressed in yeast (Figure 4A). The activity ratio (F6P,2K/F26BPase) was halved, from 2.5 to 1.2, when the first 66 amino acids were deleted (Figure 4B). Deletion of 125, 179, 249 and 318 amino acids resulted in progressive further decreases in the activity ratio from 1.2 to 0.6. Overall the activity ratio was reduced 4-fold when the N-terminus was deleted completely.

Figure 2 Gel filtration of AtF2KP and D₁₋₃₁₈

The native molecular mass of AtF2KP (●) and D₁₋₃₁₈ (○) was determined by gel filtration. A 100 μ l sample of purified enzyme/protein standard was applied to a Superose 6 HR column equilibrated with buffer C containing 20 mM potassium phosphate. (A) Elution profile of F6P,2K activity; (B) elution of protein standards with defined molecular masses (ferritin, 440 kDa; catalase, 242 kDa; aldolase, 158 kDa; BSA, 67 kDa; ovalbumin, 43 kDa). K_{av} is the relative elution volume, calculated as $(V_e - V_0)/(V_1 - V_0)$, where V_e is the elution volume of the protein, V_0 is the elution volume of Blue Dextran, and V_1 is the column bed volume. mU, m-units.

Figure 3 Heat inactivation of AtF2KP and D₁₋₃₁₈ expressed in yeast

Purified samples of AtF2KP (●, ▼) and D₁₋₃₁₈ (○, ▽) were incubated at 42 °C, with (▼, ▽) or without (●, ○) 20 mM potassium phosphate, for the times indicated. The extracts were cooled on ice and assayed for F6P,2K activity. Values are means $\pm$ S.D. of experiments with three independent extracts.

DISCUSSION

F6P,2K/F26BPase is a key enzyme in the regulation of primary metabolism in eukaryotes. In most organisms Fru-2,6- P_2 regulates the rate of glycolysis and gluconeogenesis through allosteric regulation of 6-phosphofructo-1-kinase and fructose-1,6-bisphosphatase. Plants are autotrophic, and Fru-2,6- P_2 regulates

Table 4 Heat inactivation of F6P,2K and F26BPase activities of AtF2KP

AtF2KP was incubated at 4 °C, 25 °C or 42 °C for 10 min, in the absence or presence of 20 mM potassium phosphate. The extracts were cooled on ice and assayed for F6P,2K and F26BPase activities. Values are expressed as percentages of the activity at 4 °C, and are means $\pm$ S.D. of three independent extracts.

P _i concn during incubation (mM)	Incubation temperature (°C)	Activity (% of control)	
		F6P,2K	F26BPase
20	4	100 $\pm$ 10	100 $\pm$ 9
	25	77 $\pm$ 8	92 $\pm$ 17
	42	54 $\pm$ 9	89 $\pm$ 21
0	4	100 $\pm$ 8	100 $\pm$ 16
	25	76 $\pm$ 11	87 $\pm$ 15
	42	12 $\pm$ 6	33 $\pm$ 17

Figure 4 Deletion series of the N-terminus of AtF2KP

A series of N-terminal truncations of AtF2KP was made by deleting 66, 125, 179, 249 or 318 amino acids (D₁₋₆₆, D₁₋₁₂₅, D₁₋₁₇₉, D₁₋₂₄₉ and D₁₋₃₁₈ respectively). (A) Western blot analysis; (B) ratio between F6P,2K and F26BPase activities.

photosynthetic carbon metabolism [2]. Furthermore, plants are exceptional, since 6-phosphofructo-1-kinase is not affected by Fru-2,6- P_2 , whereas an alternative enzyme, PFP, is strongly activated by Fru-2,6- P_2 . Thus the plant enzyme, F2KP, faces a different challenge from the enzymes in heterotrophic eukaryotes, and studying F2KP may give valuable new information on the structure/function relationship of these unique enzymes.

Kinetic characterization of AtF2KP

For all three substrates, i.e. ATP, Fru-6- P and Fru-2,6- P_2 , the *Arabidopsis* enzyme (AtF2KP) followed Michaelis-Menten kinetics (Figure 1). The substrate affinities (Table 1) were comparable with those reported for the partially purified spinach enzyme [8,20]. Deletion of the 318-amino-acid N-terminus decreased the affinity of F6P,2K for Fru-6- P , whereas the affinity of F6P,2K for ATP and the affinity of F26BPase for Fru-2,6- P_2 were not significantly altered. Deletion of the N-terminus also decreased the V_{max} of F6P,2K more than that of F26BPase,

resulting in a decreased kinase/phosphatase ratio (Table 1 and Figure 4). This suggests that the N-terminus interacts primarily with the kinase region, modifying the Fru-6-*P* substrate binding site. Deletion studies of the liver [21] and testis [22] isoforms have also shown dramatic effects on F6P,2K activity, with decreases in both $V_{\max}$ and the affinity for Fru-6-*P*. The N-termini of the mammalian enzymes have no identity with the plant enzyme N-terminus, but the kinetics may indicate a functional similarity.

The effects of a range of metabolites on the activities of AtF2KP were tested. As expected, P_i activated F6P,2K and inhibited F26BPase (Tables 2 and 3). As a standard, all assays of F6P,2K activity included 5 mM P_i , whereas P_i was omitted from the F26BPase assays. The activity ratio between F6P,2K and F26BPase measured under standard conditions was 2.4, corresponding well with data for the native and *E. coli*-expressed enzymes [9]. In general, the kinase/phosphatase activity ratio increased in the presence of nucleotides, sugar nucleotides, neutral sugars, hexose phosphates and intermediates of the citric acid cycle and pentose phosphate pathways. In contrast F6P,2K activity was inhibited and the kinase/phosphatase ratio decreased in the presence of phosphorylated C_3 compounds and PP_i (Table 2).

The current model of allosteric regulation of F2KP is based primarily on characterization of the spinach enzyme. 3-Phosphoglycerate (3-PGA) is considered to be the most important inhibitor of F6P,2K activity, due to the similar K_i values of 3-PGA, 2-PGA and PEP [8] and the presence of these metabolites in a molar ratio of 10:1:3 [2]. For AtF2KP, PEP was by far the strongest inhibitor of F6P,2K (Tables 2 and 3). AtF2KP thereby resembles the maize enzyme [23]. For the maize enzyme the strong inhibition of kinase activity by PEP was suggested to be associated with C_4 photosynthetic metabolism, in which PEP is a key intermediate. Our data on the enzyme from *Arabidopsis* (C_3 metabolism) suggest that PEP is a more general inhibitor of F6P,2K activity in plants. Accumulation of PEP signals a sufficient supply of substrates in glycolysis, and inhibition of F6P,2K by PEP will decrease the level of Fru-2,6- P_2 and thereby promote sucrose biosynthesis in photosynthetic tissues. In contrast, pyruvate activates F6P,2K and inhibits F26BPase activity (Tables 2 and 3). A similar, though less pronounced, effect was observed for citric acid cycle intermediates. This has not been reported previously, and is in contrast with the properties of the animal isoforms of F6P,2K, which are inhibited by citrate [1]. The regulation of AtF2KP by pyruvate and PEP may sustain the anapleurotic role of PEP metabolism in plants [24] and secure a balance between PEP and pyruvate.

Of the hexose phosphates tested, Fru-6-*P*, Fru-1,6- P_2 and Glc-1,6- P_2 were the strongest inhibitors of F26BPase (Table 2). Inhibition by Fru-6-*P* is a general feature of F26BPases [1], and the K_i of 0.1 mM for the *Arabidopsis* enzyme is similar to the value for the spinach enzyme [8,14,15,20]. Glc-1,6- P_2 is known as a regulator of glycolytic metabolism [25]. However, this has not been substantiated in plants [26], and the inhibition of F26BPase by Glc-1,6- P_2 (Table 2) has no physiological relevance, since the K_i value is high (Table 3) compared with *in vivo* concentrations of Glc-1,6- P_2 [27]. The strong inhibition of F26BPase by Fru-1,6- P_2 (K_i 0.08) has not been reported previously for the bifunctional plant enzyme, and a monofunctional spinach F26BPase was only weakly inhibited ($K_i > 1$ mM) by Fru-1,6- P_2 [28]. The inhibition of F26BPase by Fru-1,6- P_2 will result in increased levels of Fru-2,6- P_2 and thereby inhibition of fructose-1,6-bisphosphatase, leading to further accumulation of Fru-1,6- P_2 . On the other hand, the plant enzyme PFP is activated by both Fru-2,6- P_2 and Fru-1,6- P_2 [29]. Accumulation of Fru-2,6- P_2 in response to increased levels of Fru-1,6- P_2 may therefore activate the reversible

formation of Fru-6-*P* and PP_i from Fru-1,6- P_2 and P_i . This could increase both the flux of carbon in the glycolytic direction and the formation of PP_i , which in plants may play a key role as a phosphate donor [30]. In accordance with this, we observed that PP_i is a strong inhibitor of F6P,2K. Accumulation of PP_i will result in decreased levels of Fru-2,6- P_2 and thereby deactivation of PFP and reduced formation of PP_i in cells with a net flux in the direction of Fru-6-*P* formation. The collective system may work as a ' PP_i -stat', as also outlined by Stitt [30]. Our data suggest that Fru-2,6- P_2 co-ordinates this system.

F26BPase was strongly inhibited (K_i 0.08 mM) by 6-phosphogluconate. This has not been reported previously for plant enzymes, but has been found for the rat islet [31] and liver [32] isoforms of F6P,2K/F26BPase.

The presence of neutral sugars at 2 mM slightly inhibited F26BPase activity. Since considerable amounts of sugars (50–100 mM) may accumulate in plant tissues, sugars may contribute to the inhibition of F26BPase, and thereby to the feedback inhibition of sucrose synthesis.

The diverse effects of metabolites on the activities of AtF2KP show that the level of Fru-2,6- P_2 in plant cells is a result of complex interactions between different metabolic pathways. The apparently conflicting regulatory mechanisms may exist because plants comprise both heterotrophic and autotrophic tissues. We have only discovered one gene encoding F2KP in *Arabidopsis*, and Western blots of *Arabidopsis* leaf tissues reveal a single band. Therefore one enzyme has to respond to quite diverse metabolic situations.

Structure and stability of AtF2KP

The native form of the AtF2KP enzyme is a homotetramer (Figure 2). This is in accordance with the data for the spinach enzyme [8], and shows that expression of AtF2KP in yeast yields a correctly folded and assembled holoenzyme. An additional bifunctional low-molecular-mass (L) isoform of the spinach enzyme was observed by Larondelle et al. [8]. The L-form was suggested to arise from proteolytic degradation of the H-form (high-molecular-mass isoform). Our data show that N-terminal truncation of AtF2KP results in disassembly of the enzyme subunits, yielding an L-form (Figure 2) which retains both kinase and phosphatase activity (Tables 1 and 4, Figure 4). Even deletion of only 126 amino acids of the N-terminus resulted in a shift of molecular mass, suggesting disassembly of subunits (results not shown).

Mammalian F6P,2K/F26BPases, which resemble the catalytic region of the plant enzyme, are homodimers [33]. The crystal structure of a modified 55 kDa rat testis F6P,2K/F26BPase [34] shows a head-to-head dimeric structure with close interactions between the two F6P,2K domains, which form an inter-monomeric 12-stranded β -sheet. Deletion of the N-terminus (24 and 30 amino acids) of the rat liver F6P,2K/F26BPase destabilized the dimeric structure of the enzymes [32]. Charged amino acids in the N-terminus are important for interactions with the catalytic domain and for stabilization of the tertiary structure [32,35]. A similar interaction may take place in AtF2KP, where the N-terminal region (amino acids 1–318) is very acidic (pI 4.7) and may interact with the catalytic regions, which are basic (pI 9.7). Our data show that the plant specific N-terminus is important for the quaternary structure of the plant enzyme and must contain domains that promote subunit assembly into a homotetramer.

To study further the effect of the N-terminus on stability, enzymes were subjected to a mild heat treatment. The enzyme activities proved to be extremely sensitive to incubation at 42 °C

(Figure 3, Table 4). The presence of the N-terminus did not influence the stability of the enzyme, which contrasts with the thermolability induced by N-terminal deletions of the rat testis enzyme [22]. On the other hand, both AtF2KP and the deletion mutant D₁₋₃₁₈ were protected from inactivation by the presence of P_i. The inactivation in absence of P_i was not due to proteolytic degradation (results not shown), suggesting that P_i stabilizes correct folding of the enzyme. At 25 °C, both activities were relatively stable, showing that the observed activation of the kinase by P_i is not overestimated due to enzyme stabilization during the assay. The presence of P_i was also required for the enzyme to elute as a well defined peak during gel filtration. The data show that P_i, in addition to serving as an allosteric regulator, has an important role in stabilizing the structure of AtF2KP.

Conclusions

In conclusion, a detailed analysis of the substrate affinities and allosteric properties of AtF2KP has identified PP_i, PEP, 2-PGA, Fru-1,6-P₂ and 6-phosphogluconate as important regulatory effectors, in addition to the more established regulators 3-PGA, DHAP, Fru-6-P and P_i. We have also shown that the N-terminus is important for subunit assembly and for defining the kinase/phosphatase ratio of the enzyme. This suggests that the formation of a tetrameric enzyme in plants is important to 'fine tune' the kinetic properties. However, the N-terminus has a relatively limited effect on the allosteric properties. Finally, we have shown that phosphate is strictly required for stability of both the F6P₂K and F26BPase activities of AtF2KP.

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