N- and C-termini modulate the effects of pH and phosphorylation on hepatic 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase

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Liver and skeletal muscle isoforms of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (6PF2K/Fru-2,6-P₂ase) isoenzymes are products of alternatively spliced first exons of the same gene, with common kinase and bisphosphatase domains. The muscle-specific exon-1 encodes nine unique amino acids, that lack the cAMP-dependent protein kinase (PK-A) phosphorylation site, and differ in sequence from those encoded by the liver-specific exon-1 (32 amino acids), contributing to its much lower affinity for fructose 6-phosphate (Fru-6-P). PK-A phosphorylation of the liver isoform at Ser³² reduces the affinity of the kinase for Fru-6-P, and stimulates the bisphosphatase V_{max} . In the present study, we have defined the locus of interaction of the N-terminal residues with the N-terminal kinase and C-terminal domains by successive N- and C-terminal deletions. This study shows that: (1) residues Gly³-Glu⁶-Leu⁷ of the liver isoform are responsible for increasing the affinity of 6PF2K for Fru-6-P, maintaining the

inhibition of Fru-2,6-P₂ase activity, and mediating the effects of PK-A phosphorylation on the two activities; (2) the loss of Fru-6-P inhibition of the bisphosphatase and the enhancement of its V_{max} , rather than the inhibition of the kinase, may be responsible for the behaviour of the muscle isoform primarily as a bisphosphatase; (3) the composition of residues 24–32 of the liver form appears to confer the enhanced kinase catalytic rate of this form over that of the muscle isoform. It is concluded that specific regions of the N-terminus of liver and skeletal muscle 6PF2K/Fru-2,6-P₂ase have a role in adapting the two activities to work in the physiological range of pH and substrate concentrations found in each particular tissue.

Key words: enzyme kinetics, gluconeogenesis, glycolysis, hepatic glucose metabolism, structure/function.

INTRODUCTION

Hepatic 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (6PF2K/Fru-2,6-P₂ase) is a member of a family of bifunctional enzymes that catalyse the synthesis and hydrolysis of fructose 2,6-bisphosphate (Fru-2,6-P₂) [1–3]. This family includes distinct hepatic [4–6], skeletal muscle [7,8], heart [9,10], testes [11] and brain [12] isoforms, with widely different kinase/bisphosphatase activity ratios that, with the exception of the liver/muscle isoforms, are products of different genes [1,2]. The subunit of each homodimer is comprised of two distinct domains; an N-terminal kinase domain and a C-terminal bisphosphatase domain, which in the rat liver enzyme correspond to residues 1–250, and 251–470, respectively. There is a high degree of conservation only in the sequences of the catalytic and substrate binding regions of the kinase and bisphosphatase domains, with tissue- and species-specific divergence in the N- and C-terminal regions that usually contain protein kinase phosphorylation sites. For example the liver enzyme contains a cAMP-dependent protein kinase (PK-A) phosphorylation site at Ser³² that, when phosphorylated, results in the inhibition of the kinase and activation of the bisphosphatase [1]; phosphorylation of a similar residue at the C-terminal of the heart isoenzyme has the opposite effect on its activities, i.e. the activation of the kinase and inhibition of the bisphosphatase [1]. It has been postulated that these N- and C-terminal regions are the regulatory domains, responsible for the kinetic differences exhibited by the tissue-specific isoforms, and that phosphorylation contributes an ad-

ditional level of control [1]. Recently, the X-ray crystallographic analysis of a mutated testes 6PF2K/Fru-2,6-P₂ase defined the tertiary structure of both active sites [13]. However, the first 36 N-terminal residues of this isoform could not be visualized, and the model could, therefore, not delineate the role of N/C-terminal interactions in the regulation of the enzyme. The objects of the present work were to specifically define the locus of interaction of the Ser³² phosphorylation site with the N-terminal head of the liver isoform, and to delineate the effect of different areas of the first 32 amino acids on the catalytic rates of the kinase and bisphosphatase. In the process, we have kinetically characterized the bifunctional enzyme catalytic core, with respect to substrate (Fru-6-P) dependence or inhibition, and with respect to pH, in order to more fully investigate the mechanism by which specific regions of the initial portion of the N-terminus determine N- and C-terminal interactions.

EXPERIMENTAL

Materials

Restriction enzymes and bacteriophage T4 DNA ligase were from New England Biolabs and Boehringer Mannheim Chemicals. Phosphocellulose P11 was from Whatman Bio-Systems, Ltd. (Maidstone, Kent, U.K.). Blue-Sepharose was obtained from Pharmacia-LKB Biotechnology, Inc. Isopropyl β -D-thiogalactoside was from New Jersey Labscientific, Inc. (Livingston, NJ, U.S.A.). Fru-6-P, Fru-2,6-P₂, ATP and potato tuber pyrophosphate:fructose-6-phosphate phosphotransferase

Abbreviations used: 6PF2K, 6-phosphofructo-2-kinase; Fru-2,6-P₂ase, fructose-2,6-bisphosphatase; PK-A, cAMP-dependent protein kinase; Fru-6-P, fructose 6-phosphate; Fru-2,6-P₂, fructose 2,6-bisphosphate; ND, N-terminal deletion; CD, C-terminal deletion; SMCD, skeletal muscle isoform CD; PP_i-PF1K, pyrophosphate:fructose-6-phosphate phosphotransferase; DTT, dithiothreitol.

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WILD TYPE LIVER:

- ATG TCT CGA GAG ATG GGA GAA CTC ACT CAA ACC AGG TTA CAG AAG ATC TGG ATT CCA CAC AGC AGC AGT AGC AGC GTG CTG CAA CGG CGA AGG GGC TCC TCC ATA CCA
 M S₁ R E M G E L T Q T₁₀ R L Q K I W I P H S₂₀ S S S S V L Q R R R₃₀ G S S₃₃ I P

MUTATION PRIMERS:**ND4:**

5' ATG TCT CGA CAT ATG GGA GAA CTC ACT CAA 3'
 M G E L T Q

ND7:

5' TCT CGA GAG ATG GGA CAT ATG ACT CAA ACC AGG TTA C 3'
 M T Q T R L

ND12:

5' TC ACT CAA ACC CAT ATG CAG AAG ATC TGA ATT CC 3'
 M Q K I W I P

ND23:

5' T ATG TCC GTG CTG CAA CGG CGC CGC GGC TCC TCC ATA CCA CAG

TTC ACT AAT TCT CCC ACG ATG GTG ATC ATG GTG GGT TTA CCA GC 3'

M S V L Q R R R₃₀ G S S₃₃ I P Q

F T N S P T M V I M V G L P A

WILD TYPE SKELETAL MUSCLE:

- ATG GAA GAA AAA GCC TCT AAG AGA ACA GCC TCC ATA CCA
 M E₁ E K A S K R T A S₁₀ I P

Figure 1 Partial nucleotide and encoded amino acid sequences of wild-type liver and skeletal muscle isoforms and site-specific mutation primers for their N-terminal truncation mutants

Mutated nucleotide bases are underlined.

(PP_i-PF1K) were from Sigma. Homogeneous PK-A catalytic subunit (specific activity 15 μ mol/min per mg) from bovine heart was from Promega. Protein/SDS molecular-mass standards were obtained from Bio-Rad. [γ -³²P]ATP was from ICN.

DNA manipulations

Standard DNA manipulations were carried out as described previously [14]. Oligonucleotides were synthesized by phosphoramidite chemistry on an Applied BioSystems (Foster City, CA, U.S.A.) model 381A synthesizer, and purified on oligonucleotide purification cartridges (OPC[®]), according to the Applied BioSystems manual.

Engineering of expression plasmids for the various truncated forms of the bifunctional enzyme

Figure 1 shows liver- and muscle-specific sequences of the N-terminus of hepatic 6PF2K/Fru-2,6-P₂ase, the regions that were deleted to form the N-terminal truncation mutants, as well as the primers used in the oligonucleotide-directed PCR mutagenesis. The amino acid regions deleted were chosen based on their content of acidic and basic residues, since these charged residues may modulate substrate binding in the kinase region, or affect N-terminal regulation of the bifunctional enzyme's activities by phosphorylation at Ser³². The truncation mutant in which four N-terminal residues were deleted (ND4) was initiated at the naturally occurring Met⁴. The remaining deletion mutants were generated by mutations of the codons for Leu⁷ and Leu¹² into codons for methionine, to give ND7 and ND12. These mutants

were made using PCR oligonucleotide-directed mutagenesis of the wild-type liver isoform and 5'- primers that introduce initiation codons for methionine as part of *Nde*I restriction sites at the positions mentioned above (Figure 1). The opposing 3'-primer flanked the codon for residue 300, past an *Nco*I restriction site. Expression plasmids for the liver isoform N-terminal truncation mutants were constructed by replacing the coding sequence of the kinase domain, between the *Nde*I and *Nco*I sites, with those of the truncated forms. The ND23 liver mutant was constructed by cassette mutagenesis [15], using the sense strand of the cassette shown in Figure 1, and a complementary strand (not shown). The annealed double-stranded DNA was digested with *Nde*I and *Xho*I restriction enzymes, and the fragment generated was used to replace wild-type sequences in the expression plasmid. A 30 residue C-terminal truncation mutant (CD30) was made by mutating the codon for residue 441 into a stop codon [16]. Expression plasmids for the liver (residues 23–440) and skeletal muscle (residues 1–418) catalytic cores were engineered by ligating the coding sequence of the bisphosphatase domain of the CD30 form (*Nco*I to *Hind*III region) to the kinase domain of the ND23 or the skeletal muscle wild-type.

Purification and expression of wild-type and mutant bifunctional enzymes in *Escherichia coli*

All constructs were expressed using the T7 RNA polymerase-based pET-3a expression system of Studier et al. [17], in the *Escherichia coli* BL21(DE3)-pLysS strain. The enzymes were induced by isopropyl β -D-thiogalactoside addition (0.2 mM) after

growth in Luria–Bertani ('LB') media to mid-exponential phase [attenuance (D) at 600 nm = 0.6–0.8], and the cells were harvested after 20 h of induction at 22 °C. The cells were lysed, and the enzymes were extracted as described previously [18]. The extract was applied to a Blue-Sepharose column (2.5 cm × 15 cm), equilibrated with 20 mM Tes/50 mM KCl/1 mM dithiothreitol (DTT)/0.1 mM EDTA/0.5 mM PMSF/2.5 mg/l leupeptin, pH 7.5, then washed with 7 column vol. of the same buffer containing 200 mM KCl. The enzyme was eluted with 5 mM ATP/10 mM MgCl₂/5 mM KH₂PO₄/K₂HPO₄, pH 7.4. The enzymes were then precipitated with 20% (w/v) poly(ethylene glycol) 8000, then dissolved in phosphocellulose loading buffer (20 mM Tes/10 mM KCl/1 mM DTT/0.1 mM EDTA/0.5 mM PMSF/2.5 mg/l leupeptin, pH 7.0) and applied to the phosphocellulose column (2.5 cm × 10 cm) equilibrated with the same buffer. The column was washed with 25 column vol. of buffer, the pH of the buffer was raised to 8.0 and the column was then washed with five additional column vol. The wild-type liver isoform was eluted with 2 mM Fru-6-P. All other forms were eluted with linear KCl gradients between 10 mM and 300 mM. The yields of the wild-type liver and skeletal muscle isoforms were 12 mg and 27 mg/l of culture, respectively. The yields of purified ND4, ND7, ND12, ND23, CD30, ND23CD30, and SMCD30 (C-terminal deletion from the skeletal muscle isoform) truncated forms were between 2 and 11.0 mg/l of culture.

Measurement of 6PF2K and Fru-2,6-P₂ase activities

Since all bifunctional enzyme forms contain an associated bisphosphatase activity, 6PF2K activity measurements are invariably underestimated, particularly at lower Fru-6-P concentrations, where the bisphosphatase is more active. Therefore we routinely assayed 6PF2K activity both as net activity, in which only accumulated Fru-2,6-P₂ was monitored by the activation of potato PP_i-PF1K [19], or as total activity, by measuring the sum of [2-³²P]Fru-2,6-P₂ and [³²P]P_i produced from [γ -³²P]ATP. In the former assay, the enzyme (1 μ g) was incubated with Fru-6-P at concentrations from 1.5 μ M to 20 mM, and 5 mM ATP, 10 mM MgCl₂, 5 mM KH₂PO₄/K₂HPO₄, in buffer [100 mM Tes (pH 7.0)/100 mM KCl/1 mM DTT/1 mg/ml BSA/0.1 mM EDTA] at 30 °C for 10 and 20 min. The reactions were terminated by the addition of NaOH to a final concentration of 0.1 M and heating at 90 °C for 10 min, before the determination of the Fru-2,6-P₂ produced [19]. In the radioactive assay, the enzyme (1 μ g) was incubated with 1.5 μ M to 20 mM Fru-6-P, 0.4 mM [γ -³²P]ATP (75 c.p.m./pmol), 1 mM MgCl₂ and 5 mM KH₂PO₄/K₂HPO₄ in 100 μ l of buffer. After 7 and 14 min, 40 μ l aliquots were transferred to Eppendorf tubes containing 160 μ l of 0.15 M NaOH to stop the reaction. Remaining [γ -³²P]ATP was removed by the addition of 200 μ l of a suspension of alkaline charcoal (200 mg/ml) in 1 mM K₂HPO₄, 50 μ M Fru-2,6-P₂, and 20 mM ammonium bicarbonate, pH 8.2, and subsequent centrifugation. An aliquot (180 μ l) of each supernatant was diluted to 5 ml with 20 mM ammonium bicarbonate, pH 8.2, and applied to a 1 ml column of DEAE-Sephadex A-25, equilibrated with the same buffer [20]. The [³²P]P_i was eluted with 7 ml of 150 mM ammonium bicarbonate, and the [2-³²P]Fru-2,6-P₂ was eluted with 5 ml of 1 M ammonium bicarbonate, and the radioactivity was counted as Cerenkov radiation.

Bisphosphatase $V_{\max}$ was measured by following the rate of production of [³²P]P_i from 10 μ M [2-³²P]Fru-2,6-P₂ with 5 mM KH₂PO₄/K₂HPO₄ [21], in buffer [100 mM Tes (pH 7.0), 100 mM KCl, 1 mM DTT, 1 mg/ml BSA and 0.1 mM EDTA] at 30 °C with aliquots taken at 4.8 and 12 min and processed as described above.

For measurements of 6PF2K $V_{\max}$ activities at various pH values (6.0–10.0), using the potato PP_i-PF1K activation assay, controls assays to determine the recovery of Fru-2,6-P₂ at the different pH values showed greater than 95% recovery of Fru-2,6-P₂ at all pH values tested. In addition, since BSA was found to have no effect on the kinetics of the enzyme, but appeared to confer a protective effect against extreme pH values, it was included in all assay mixtures.

Kinetic analysis

Fru-2,6-P₂ data obtained by the potato PP_i-PF1K activation assay, and as [2-³²P]Fru-2,6-P₂, were defined as net 6PF2K activity, while the combined [2-³²P]Fru-2,6-P₂ + [³²P]P_i data were defined as 'total' 6PF2K. Calculations of $V_{\max}$ and $[S]_{0.5}$ for 6PF2K and Fru-2,6-P₂ase were made using the linear and non-linear regression subroutines of the GraphPAD InPlot or Prism program (Graph PAD Software, San Diego, CA, U.S.A.). Each kinetic parameter was the result of at least three separate measurements.

PK-A-catalysed phosphorylation of the bifunctional enzyme

Wild-type and mutant enzyme forms (40 μ g) were incubated in a final volume of 100 μ l that contained 20 mM Tes, pH 7.0, 35 mM KH₂PO₄/K₂HPO₄, 4 mM MgCl₂, 0.8 mM ATP, 0.3 mM DTT. The reaction was started by the addition of 10 m-units (0.66 μ g) of the catalytic subunit of PK-A. Incorporation of ³²P into the enzyme was measured by substituting 0.2 mM [γ -³²P]ATP (2000 c.p.m./pmol) for the non-radioactive ATP, as described previously [15].

Determination of circular dichroism spectra of the native and truncated bifunctional enzyme forms

All spectra were collected on a Jasco (Tokyo, Japan) 500A spectropolarimeter as described previously [21,22]. Secondary structures were estimated by the method outlined in the Jasco manual, using seven reference spectra. Only data between 196 nm and 250 nm were used in these fits [22,23].

Other methods

DNA sequence analysis was performed with an ABI Model 373 Automated DNA Sequencer. [2-³²P]Fru-2,6-P₂ was prepared enzymically as described previously [4]. Protein was determined by the bicinchoninic acid ('BCA') assay (Pierce Chemical Co., Rockford, IL, U.S.A.).

RESULTS

Expression, purification and physical properties of the bifunctional enzyme's catalytic cores

To determine how the N- and C-termini of 6PF2K/Fru-2,6-P₂ase regulate its activities, it was important to determine the limits of the catalytic core, i.e. how much of each terminus could be deleted without loss of kinase or bisphosphatase activity. Liver and skeletal muscle 6PF2K/Fru-2,6-P₂ase differ only in the amino acid composition encoded by the first exon [7]. The skeletal muscle-specific exon-1 encodes a unique nine amino acid domain, while the liver-specific exon-1 encodes a 32 amino acid domain that ends with a PK-A phosphorylation site. The expressed recombinant liver 6PF2K/Fru-2,6-P₂ase isoform, lacking the first 32, or the last 40, amino acid residues, does not appear to be soluble or catalytically active (results not shown). A

Figure 2 SDS/PAGE of purified wild-type liver enzyme, its ND4, ND7, ND12, ND23, CD30 and ND23CD30 deleted forms, wild-type skeletal muscle enzyme, and its CD30-deleted form

Approx. 1.5–3 μ g of each purified protein were separated by electrophoresis on a 10% (w/v) polyacrylamide gel in the presence of SDS. Lane 1 of gel (A), and lanes 1 and 8 of gel (B) contain molecular mass markers (kDa). Lanes 5 and 8 of gel (A), and 2 of gel (B) contain wild-type (WT) liver 6PF2K/Fru-2,6-P₂ase, and lane 2 of gel (A) contains wild-type skeletal muscle enzyme. The subunit molecular masses of the wild-type liver, ND4, ND7, ND12, ND23, CD30 and ND23CD30 polypeptides are 55, 54.5, 54, 53.5, 52.5, 51 and 49.2 kDa, respectively. The skeletal muscle (M.)-WT isoform and skeletal M.-CD30 subunits migrated as 52.5 and 49.2 kDa polypeptides, respectively.

longer N-terminal construct, ND23, with a 30 amino acid C-terminal deletion (CD30), was soluble and retained both kinase and bisphosphatase activities, but, interestingly, had a diminished

kinase/bisphosphatase activity ratio (see Table 1). Therefore it was reasoned that the double truncation mutant, ND23CD30, might constitute the catalytic core of the liver bifunctional enzyme. Furthermore, the postulated role of N-terminal interactions in mediating the effects of phosphorylation could still be tested, since this form retained the PK-A phosphorylation motif and site (Arg²⁸-Arg-Arg-Gly-Ser³²). Similarly, deletion of the 30 C-terminal amino acid residues from the skeletal muscle enzyme should generate a catalytic core for that enzyme, which would differ from the hepatic catalytic core by the composition of the nine N-terminal amino acid residues, lacking a phosphorylation motif. If these truncated forms (418 residues each) represent true catalytic cores, their kinetic properties should be similar, but divergent from their wild-type precursors.

Figure 1 contains the sequences of the liver and muscle-specific domains, and the various truncated forms generated in this study. The enzymes were purified from *E. coli* extracts as described in the Experimental section. Figure 2 shows that the purified native liver enzyme, its truncation mutants ND4, ND7, ND12, ND23, CD30, ND23CD30 and the skeletal muscle isoform and its SMCD30 truncated form were homogeneous, as judged by SDS/PAGE, migrating as 55, 54.5, 54, 53.5, 52.5, 51, 49.2, 52.5 and 49.2 kDa subunits, respectively. The bisphosphatase reaction of 6PF2K/Fru-2,6-P₂ase proceeds via a covalent intermediate [1–3], and all of the enzymes listed above formed a phosphoenzyme intermediate when incubated with [2-³²P]Fru-2,6-P₂ (results not shown).

No significant differences in circular dichroism spectra were observed between any of the truncated and their respective wild-type enzymes (results not shown), which suggests, first, that the core secondary structure was not affected by the N- and C-terminal truncations; and secondly, that these domains do not appear to contribute any significant helical or coil structures to the overall conformation.

Effect of N- and C-terminal deletions on the kinetic properties of the bifunctional enzyme

Maximal 6PF2K and Fru-2,6-P₂ase activities as a function of the number of N-terminal residues deleted are shown in Figure 3(A), and the kinase $[S]_{0.5}$ for Fru-6-P for the same mutants is shown in Figure 3(B). Deletion of the first four amino acid residues from the N-terminus (ND4) did not affect maximal kinase or bisphosphatase activity. However, deletion of the first 7 amino acids (ND7) decreased the kinase V_{max} by 75%, caused a 10-fold increase in the $[S]_{0.5}$ for Fru-6-P, a 2-fold increase in the bisphosphatase V_{max} , and a 9-fold increase in the bisphosphatase/kinase V_{max} ratio in comparison with the wild-type liver isoform. The deletion of 12 (ND12) or 23 (ND23) amino acid residues caused 7- and 12-fold increases in the $[S]_{0.5}$ for Fru-6-P, respectively, over wild-type increases in the $[S]_{0.5}$ for Fru-6-P, without very much further effect on either of the V_{max} values. The skeletal muscle isoform, assayed at pH 7.0 (which should more closely approximate the muscle cell pH [24]), exhibited a 40-fold higher $[S]_{0.5}$ for Fru-6-P, a 93% lower kinase V_{max} and a 2-fold higher bisphosphatase V_{max} , in comparison with the wild-type liver isoform (Table 1). The liver ND23 and CD30 truncated mutants exhibited enhanced bisphosphatase and decreased kinase activity (Table 1), and the removal of the C-terminal 30 residues (CD30) from ND23 resulted in an additional 2-fold increase in the kinase $[S]_{0.5}$ for Fru-6-P, and a 50% increase in the V_{max} of the bisphosphatase. The 6PF2K V_{max} of both ND23CD30 and ND23 were decreased to 20% that of the native isoform, whereas the same C-terminal deletion from the muscle form (SMCD30) had a 2.5-fold higher $[S]_{0.5}$ for Fru-6-P and a similar kinase V_{max}

Figure 3 Effect of N-terminal deletions on 6PF2K and Fru-2,6-P₂ase maximal velocities and affinity of 6PF2K for Fru-6-P

(A) Enzyme $V_{\max}$ values are in m-units (mU; nmol/min) per mg of enzyme protein. Both 6PF2K (●) and Fru-2,6-P₂ase (■) $V_{\max}$ activities were assayed in the presence of 5 mM potassium phosphate, and the bisphosphatase/kinase ratio is represented by (x---x). (B) The $[S]_{0.5}$ for Fru-6-P (●) and catalytic rates in (A) were determined by the potato PP₁-PF1K activation assay as described in the Experimental section.

(Table 1). The observations that the ratio of bisphosphatase/kinase activity was similar for both the liver and skeletal muscle catalytic cores, and that neither form could be truncated further without loss of activity (ND33, CD40), is consistent with the hypothesis that these forms represent the minimal liver/muscle 6PF2K/Fru-2,6-P₂ase catalytic core.

The results also suggest that the amino acids residues following Met⁴ in the liver isoform, which are not essential for catalysis of the 6PF2K reaction, are responsible for maintaining the repression of the bisphosphatase. The C-terminal 30 residues, on the other hand, have little effect on 6PF2K catalysis, but appear to be necessary for this repression (see below).

The 6PF2K measurements described in Table 1 reflect the net Fru-2,6-P₂ remaining at the end of the incubation, as determined by the extent of activation of PP₁-PF1K [19], with no consideration for the amount hydrolysed by the associated Fru-2,6-P₂ase. Wild-type liver Fru-2,6-P₂ase is inhibited by micromolar concentrations of Fru-6-P [20], consequently measurements of

Table 1 Comparison of the kinetic properties of the wild-type liver and skeletal muscle isoforms and their various truncated mutant forms

Steady-state kinetic analyses were performed at pH 7.0 at 30 °C. Enzyme velocity units are in m-units (= nmol/min) per mg of enzyme protein. 6PF2K and Fru-2,6-P₂ase activities were assayed in the presence of 5 mM potassium phosphate. The bisphosphatase/kinase ratio represents the ratio of Fru-2,6-P₂ase to 6PF2K activities measured under $V_{\max}$ conditions [15,22]. Unless otherwise stated, the properties of the dephosphorylated forms were measured. Abbreviations: WT, wild-type; P, phosphorylated form.

Enzyme form	6PF2K		Fru-2,6-P ₂ ase	
	$V_{\max}$ (m-units/mg)	$[S]_{0.5}$ Fru-6-P (μM)	$V_{\max}$ (m-units/mg)	Bisphosphatase/kinase
WT-liver	129.1 ± 6.9	32 ± 4.9	85.6 ± 25	0.67
P-WT-liver	44.3 ± 2.2	492 ± 92	177 ± 34	4.0
ND4	120 ± 7	48 ± 7.4	76.3 ± 17	0.64
ND7	31.2 ± 1.2	355 ± 58	183.1 ± 10.7	5.9
ND12	26.4 ± 8.2	673 ± 64	158.3 ± 8	6.0
ND23	28.5 ± 4.1	735 ± 90	222.1 ± 11.5	7.8
CD30	83.5 ± 2.1	55 ± 4.8	344.5 ± 76.8	4.1
P-CD30	31.5 ± 0.5	530 ± 26	226.8 ± 18.1	7.2
ND23CD30	26.1 ± 1.1	1205 ± 163	386.7 ± 39.2	14.8
P-ND23CD30	17.7 ± 1.8	1565 ± 278	353.9 ± 87	20
WT-skeletal muscle	8.9 ± 0.23	1285 ± 80	219.3 ± 25.2	24.6
SMCD30	10.6 ± 1.1	3227 ± 907	294.4 ± 12.2	27.8

6PF2K activity at these concentrations are consistently underestimated, because of the concomitant hydrolysis of the product. Furthermore, in almost all cases, N-terminal deletions result in substantial changes in Fru-2,6-P₂ase activity (Figure 3 and Table 1). In order to determine the total 6PF2K activity, and at the same time the contribution of the bisphosphatase reaction in each of the deletion mutants, [γ -³²P]ATP was substituted for the ATP in the reaction mixture [20]. The sum of the [³²P]Fru-2,6-P₂ and [³²P]P_i produced from its hydrolysis, or the [³²P]P_i produced alone, was taken to represent the total 6PF2K activity and the Fru-2,6-P₂ase activity, respectively. The results in Figure 4(A) are consistent with these observations; a 2-fold higher apparent $[S]_{0.5}$ for Fru-6-P for total versus net WT-liver 6PF2K activity at pH 7. This shift can be accounted for by a progressive decrease in Fru-2,6-P₂ase activity from its maximum of 25 % of the total 6PF2K $V_{\max}$ at 3 μM Fru-6-P, to full bisphosphatase inhibition at ≈ 50 μM (Figure 4A). In contrast, the bisphosphatase activity of the skeletal muscle isoform is 50 % of the total 6PF2K $V_{\max}$, and unlike its liver counterpart, is not significantly inhibited by Fru-6-P (Figure 4B). Unlike the wild-type liver isoform, the skeletal muscle isoform has a relatively constant Fru-2,6-P₂ase activity that results in the same apparent $[S]_{0.5}$ for Fru-6-P for the net or total 6PF2K activity (Figure 4B). Similarly, the ND23CD30 catalytic core also shows a total lack of Fru-6-P inhibition of its bisphosphatase activity, resulting in a Fru-2,6-P₂ase activity that is 25 % of the total 6PF2K $V_{\max}$ that is sustained throughout the Fru-6-P concentration-dependence curve at pH 7 (Figure 4C). Again, in contrast with the wild-type liver isoform, ND23CD30 has a relatively constant Fru-2,6-P₂ase activity that results in the same apparent $[S]_{0.5}$ for Fru-6-P for the net or total 6PF2K activity (Figure 4C).

The short length (nine residues) of the N-termini of both forms, wild-type skeletal muscle and liver ND23 (or ND23CD30), appears to be responsible for the loss of Fru-6-P inhibition of their respective bisphosphatase activities. However, the unique composition of the nine amino acid N-terminal head of the skeletal muscle enzyme, also appears to be responsible for

Figure 4 Comparison of net and total 6PF2K activity of wild-type and ND23CD30 forms of hepatic 6PF2K/Fru-2,6-P₂ase

Each 6PF2K/Fru-2,6-P₂ase form (1.0 μ g) was incubated at 30 °C with concentrations of Fru-6-P between 1.56 μ M and 2 mM [wild-type liver and skeletal muscle isoforms (A) and (B) respectively] or between 1.56 μ M and 20 mM (ND23CD30, panel C). The incubations also contained 0.4 mM [γ -³²P]ATP (75 c.p.m./pmol), 1 mM MgCl₂, 5 mM KH₂PO₄/K₂HPO₄, 100 mM Tes (pH 7.0), 100 mM KCl, 1 mM DTT, 1 mg/ml BSA and 0.1 mM EDTA in a total vol. of 100 μ l. Aliquots of the reaction mixture were removed at 10 and 20 min, and [2-³²P]Fru-2,6-P₂ and [³²P]P_i were measured as described in the Experimental section. Total 6PF2K velocity (●) was calculated from the sum of the [2-³²P]Fru-2,6-P₂ and [³²P]P_i, and net 6PF2K velocity (○) was calculated from the [2-³²P]Fru-2,6-P₂ data alone. The bisphosphatase (BP) activity (□) was calculated from the [³²P]P_i data. The enzyme activities (Vel.) for total and net kinase and bisphosphatase are expressed as fractions of the total 6PF2K V_{max}, which was set at unity. Values represent means $\pm$ S.E.M. for 3–5 measurements. (D) Bisphosphatase/total 6PF2K activity ratio as a function of Fru-6-P concentration. Values for wild-type liver (wt-liver, ▼), ND23CD30 (□), and wild-type skeletal muscle (wt-skel. musc., ■) were derived from the total kinase and bisphosphatase values shown in (A)–(C).

the reducing its net kinase activity by 70 %, from the liver ND23 (or ND23CD30) V_{max}, despite their similar bisphosphatase activities (Table 1). This is underscored further by comparing the bisphosphatase to kinase ratios of the wild-type liver, skeletal muscle and ND23CD30 forms as a function of Fru-6-P dependence (Figure 4D). Whereas the bisphosphatase/kinase ratio of the wild-type liver isoform decreased to a negligible level above 25 μ M Fru-6-P, the ND23CD30 bisphosphatase/kinase ratio was only reduced by 50 % at 500 μ M Fru-6-P and that of the skeletal muscle isoform remained at almost unity at 1 mM Fru-6-P (Figure 4D).

Role of N- and C-terminal residues in the regulation of 6PF2K/Fru-2,6-P₂ase activities by PK-A-catalysed phosphorylation

The effects of cAMP-dependent phosphorylation on the activities of 6PF2K/Fru-2,6-P₂ase as a function of N-terminal deletions are shown in Figure 5. There was a progressive loss of

phosphorylation-dependent reduction of 6PF2K [S]_{0.5} for Fru-6-P, that started with the deletion of the N-terminal four residues (ND4) (18 %), decreased significantly (75 %) in ND7, and maximally (85 %) in ND12 and ND23 (Figure 5A). In contrast, deletion of the first four residues had no effect on the ratio of phospho- to dephospho-6PF2K V_{max} ($\approx$ 0.3), which approached unity in the ND7 to ND23 mutants (Figure 5B). Fru-2,6-P₂ase V_{max} activity of ND4, on the other hand, showed a 20 % reduction in its phosphorylation-induced activation, which was reduced only slightly more by further N-terminal deletions (Figure 5B). The results confirm the significance of the first seven amino acid residues in the binding of Fru-6-P to the kinase domain, and of their role in mediating the effects of PK-A phosphorylation on both bifunctional enzyme domains. Interestingly, all of the kinetic properties of the ND7 mutant are indistinguishable from those of phosphorylated wild-type enzyme (Table 1 and Figure 5). This suggests that the introduction of a phosphate group at Ser³² induces a conformational change in the enzyme that could be mimicked by deleting the first seven amino acid residues.

Figure 5 Effect of N-terminal deletions on the 6PF2K and Fru-2,6-P₂ase activities of dephosphorylated and PK-A-phosphorylated enzymes

One μ g of dephosphorylated enzyme or enzyme that had been phosphorylated to the extent of 1 mol of 32 P/mol of enzyme subunit (see the Experimental section) was incubated for 10 min in 100 μ l of 100 mM Tris, at pH 7.0, containing 1 mg/ml BSA, 100 mM KCl, various Fru-6-P concentrations, 5 mM ATP, 10 mM MgCl₂ and 5 mM KH₂PO₄/K₂HPO₄. (A) The 6PF2K $S_{0.5}$ for Fru-6-P of the phosphorylated relative to that of the dephosphorylated enzyme (●) was measured by the potato PP₁-PF1K activation assay, as was the 6PF2K V_{max} (B). (B) The Fru-2,6-P₂ase V_{max} was measured as the rate of [32 P]P_i production from 10 μ M [2- 32 P]Fru-2,6-P₂ in the presence of 5 mM KH₂PO₄/K₂HPO₄, as described in the Experimental section. The ratio of phosphorylated/dephosphorylated 6PF2K V_{max} (●) and Fru-2,6-P₂ase V_{max} (■) are plotted versus the number of N-terminal residues deleted. The values are the means $\pm$ S.E.M. of three to five measurements.

The 6PF2K kinetic parameters of the liver CD30 truncation mutant were similar to those of the native liver isoform ($\approx 70\%$ the V_{max} of the dephospho native enzyme), but in contrast with any of the N-terminal deletions, it retained sensitivity to phosphorylation. The C-terminal truncation did, however, relieve the inhibition of the bisphosphatase activity (V_{max} was 4-fold higher) (Table 1). Phosphorylation of CD30 resulted in a 65% decrease in 6PF2K V_{max} , and a 10-fold increase in the $S_{0.5}$ for Fru-6-P. Interestingly, phosphorylation had no further effect on the bisphosphatase activity of CD30, suggesting that the deletion had mimicked its effects.

Although the deletion of the C-terminal 30 residues from ND23 (ND23CD30) increased the 6PF2K $S_{0.5}$ for Fru-6-P by approx. 60%, and the bisphosphatase by 50%, without changing the 6PF2K V_{max} , there was no further effect of PK-A catalysed

Figure 6 Effect of N-terminal deletions on the pH profile of 6PF2K V_{max}

One μ g of the wild-type (WT) liver isoform (■), ND4 (□), ND7 (◆), and ND12 (▼) mutants (panel A), or the ND23 mutant (○), and the wild-type skeletal muscle isoform (▲) (panel B), were incubated for 5 min in 100 μ l of 100 mM Bis-Tris propane buffer, at the indicated pH, and containing 1 mg/ml BSA, 100 mM KCl, 10 mM Fru-6-P, 5 mM ATP, 10 mM MgCl₂ and 5 mM KH₂PO₄/K₂HPO₄. 6PF2K activity was measured by the potato PP₁-PF1K activation assay, as described in the Experimental section. Values represent the means $\pm$ S.E.M. of three to five determinations.

phosphorylation, at Ser³², on any of these kinetic parameters (Table 1). This lack of responsiveness of the ND23CD30 form to phosphorylation supports the hypothesis that this form represents a liver bifunctional enzyme catalytic core.

pH-dependence of 6PF2K activity of the N- and C-terminal truncated mutants

The affinity of hepatic 6PF2K for Fru-6-P decreases as the pH decreases [22]. Since the ND7, ND12 and ND23 already had significantly reduced affinities for substrate (Table 1), it was of interest to investigate whether these truncations would affect the kinase pH-dependence (Figure 6). The wild-type enzyme exhibited a biphasic pH profile with two optima at pH 6.8 and 10.0, and a minimum at pH 8.5 (Figure 6). The ND4 mutant form exhibited a similar biphasic pH profile (Figure 6A), whereas the ND7, ND12 (Figure 6A) and ND23 (Figure 6B) forms exhibited a single pH optimum at pH 10. The pH-dependence of the 6PF2K activity of the skeletal muscle isoform was nearly identical with that of the ND23 mutant of the liver enzyme (Figure 6B).

The kinetic properties of the various bifunctional enzyme forms were evaluated at the pH 6.8 optimum and the pH 8.2

Table 2 Comparison of 6PF2K kinetic properties of the wild-type liver and skeletal muscle isoforms, their catalytic cores (ND23CD30 and SMCD30) and the ND23 liver mutant, at pH 6.8 and pH 8.2

Steady-state kinetic analyses were performed in 100 mM Tes, at pH 6.8 or pH 8.2, at 30 °C. Enzyme activities (m-units per mg of enzyme protein) were determined in the presence of 5 mM potassium phosphate. WT, wild-type.

Enzyme form	6PF2K, pH 6.8		6PF2K, pH 8.2	
	$V_{\max}$ (m-units/mg)	$[S]_{0.5}$ Fru-6-P (μ M)	$V_{\max}$ (m-units/mg)	$[S]_{0.5}$ Fru-6-P (μ M)
WT-liver	210.0 $\pm$ 7.2	87 $\pm$ 9.7	97.6 $\pm$ 8.1	22.5 $\pm$ 4.8
ND23	22.2 $\pm$ 1.3	1130 $\pm$ 194	90.7 $\pm$ 3.7	40.2 $\pm$ 5.6
ND23CD30	21.7 $\pm$ 1.6	3114 $\pm$ 552	80.7 $\pm$ 5.6	107.6 $\pm$ 26
WT-skeletal muscle	10.4 $\pm$ 0.6	1578 $\pm$ 284	55.8 $\pm$ 3	107.4 $\pm$ 18
SMCD30	17.6 $\pm$ 0.9	2322 $\pm$ 315	43.7 $\pm$ 1.7	171.0 $\pm$ 27

minimum (Table 2). As expected, the 6PF2K $V_{\max}$ of the wild-type liver form was less than half as large at pH 8.2 than at pH 6.8, and the $[S]_{0.5}$ for Fru-6-P was decreased 4-fold at pH 8.2 in comparison with pH 6.8. In contrast, all of the N-terminal truncated forms had 3- to 5-fold higher 6PF2K $V_{\max}$ values at the higher pH value, as well as significantly lower (20- to 30-fold) $[S]_{0.5}$ values for Fru-6-P (Table 2). This includes the wild-type skeletal muscle isoform, and its CD30 deletion mutant (SMCD30), as well (Table 2). The changes in affinity for substrate at pH 6.8 represent the greatest divergence between the various truncated forms and the wild-type. This divergence is reduced dramatically with an upward shift of $\approx$ 1 pH unit. For example, ND23 has a 12-fold higher $[S]_{0.5}$ for Fru-6-P than the wild-type at pH 6.8, but only a 2-fold higher value at pH 8.2.

The results indicate that a synergistic interaction occurs for the effects of N-terminal truncation and decreased pH to increase the net kinase Fru-6-P $[S]_{0.5}$. This synergism may imply a direct effect of the protonation state on the affinity for Fru-6-P of all the 6PF2K forms examined, independent of the elevated bisphosphatase activity that may be present secondary to N- and/or C-terminal truncations.

DISCUSSION

The mammalian 6PF2K/Fru-2,6-P₂ase enzyme family is encoded by at least four distinct genes and consists of at least five different tissue specific isoforms, which have a highly conserved two-domain chimeric structure. However, they differ dramatically in their kinase/bisphosphatase activity ratios and in their regulation by protein kinase-catalysed phosphorylation [4–12]. The results of the present study indicate functional roles for distinct portions of the N- and C-termini. The proximal portion of the liver enzyme N-terminus (amino acids 1–23) has five functions: (1) it represses the bisphosphatase activity; (2) it increases the affinity of the kinase for Fru-6-P; (3) it enhances the kinase catalytic rate; (4) it mediates the effect of PK-A phosphorylation on the two activities; and (5) it is responsible for Fru-6-P inhibition of the bisphosphatase. Different regions of this segment are responsible for modulating different kinetic parameters. For example, changes in kinase or bisphosphatase catalytic rates are regulated by the first seven residues, whereas the high affinity binding of Fru-6-P to the kinase requires the first 12 residues. The initial portion of the N-terminus also plays a key role in the pH-dependence of the kinase activity. The wild-type hepatic isoform, as well as the ND4 mutant, have a second pH maximum at 6.8, in addition to that at pH 10, that is abolished after the

removal of seven or more residues (ND7, ND12 and ND23), suggesting a clear dependence on the residues Gly⁵-Glu⁶-Leu⁷-. This region may interact directly with the kinase active site and/or alter the pK_a of important catalytic residues to promote a higher kinase catalytic rate and Fru-6-P affinity at physiological pH (Figure 6). It could be argued that the enhanced bisphosphatase activities of the ND7, 12 and 23 truncation mutants at values of pH < 8 are responsible for the apparently low kinase activities. However, comparison of the kinase activity of the wild-type skeletal muscle isoform with its His²³⁶ → Ala mutant, which has an inactive bisphosphatase, showed only a 70% increase in the kinase $V_{\max}$, and a 2-fold increase in the affinity for Fru-6-P (results not shown). This is more consistent with the hypothesis that the amino acid composition encoded by the first exon of the liver/skeletal muscle isoform determines the 6PF2K $V_{\max}$ and $[S]_{0.5}$ for Fru-6-P, independent of its function to regulate the bisphosphatase $V_{\max}$. N-terminal regulation of the kinase and bisphosphatase activities is important for different physiological situations. The $[S]_{0.5}$ for Fru-6-P of liver 6PF2K/Fru-2,6-P₂ase in the dephospho- and phospho-states, mirrors the Fru-6-P concentration in the fed and starved liver, respectively. Effectively, only a minimal amount of hepatic Fru-2,6-P₂ can be produced in the starved state, because the available Fru-6-P is far below the $[S]_{0.5}$ for Fru-6-P. The converse situation exists in the fed state, in which the mean Fru-6-P concentration not only exceeds the $[S]_{0.5}$ of the 6PF2K, but also almost totally inhibits the bisphosphatase activity. Skeletal muscle 6PF2K/Fru-2,6-P₂ase, on the other hand, does not have a PK-A phosphorylation site, its bisphosphatase is not inhibited by Fru-6-P, even in the millimolar range, and its catalysis of Fru-2,6-P₂ production appears to be controlled solely by substrate (Fru-6-P) availability. The mean Fru-6-P concentration in rat muscle at rest is between approx. 0.23–0.39 μ mol/g of tissue [25], which is equivalent to $\approx$ 0.5 mM. The pH of skeletal muscle varies between 7.0 at rest, to 6.2 with exhaustive exercise [24]. Since, as we have shown, that the $[S]_{0.5}$ for Fru-6-P increases as the pH decreases (Table 2), in the pH range that exists in skeletal muscle, 6PF2K activity is most likely submaximal, supporting the hypothesis of substrate control of Fru-2,6-P₂ production. The virtual elimination of Fru-6-P inhibition of skeletal muscle Fru-2,6-P₂ase, as well as the lower 6PF2K $V_{\max}$ (Figure 4) suggests that Fru-2,6-P₂ accumulation in muscle cells is kept to a minimum until Fru-6-P exceeds 0.5 mM, thereby preventing excessive lactic acid production even in 'fight or flight' situations.

The distal portion of the N-terminal first exon (amino acids 24–32) appears to contribute significantly to the enhancement of the kinase catalytic rate. This is manifested by a 3-fold higher $V_{\max}$ for the ND23 mutant than the skeletal muscle isoform, which differ from each other only in the sequence of their initial nine amino acid residues (Table 1). The distal portion of the liver N-terminus also contains the PK-A phosphorylation site at Ser³², which reciprocally regulates the two activities of the bifunctional enzyme. While PK-A phosphorylation of the liver isoform allows an 'on-off switch' for Fru-2,6-P₂ production, N- and C-terminal interactions appear to be only partially uncoupled by phosphorylation. This conclusion was based on the observation that the bisphosphatase activity is greater with deletion of either the 23 N-terminal or the 30 C-terminal residues, than with phosphorylation of the native enzyme (Table 1). It should be noted that the C-terminal 30 amino acids have only a minimal effect on the kinase catalytic rate, or its affinity for Fru-6-P.

The importance of the N- and C-terminal interactions in determining the balance of kinase and bisphosphatase activities for a given tissue is more apparent with the characterization of

the bifunctional enzyme ND23CD30 catalytic core. This core, defined as a 418 residue protein, comprised a kinase domain of 228 residues and a 190 residue bisphosphatase domain, has a much higher bisphosphatase/kinase activity ratio (≈ 15) than the 0.6 ratio of the native liver isoform. Virtual elimination of the Fru-6-P inhibition of the bisphosphatase is seen for this ND23CD30 core, as opposed to the very sensitive response of the bisphosphatase activity to Fru-6-P inhibition seen for the native liver isoform. The inherent nature of the catalytic core as a bisphosphatase unregulated by Fru-6-P inhibition underscores the importance of how evolution determined the amino acid composition of the N- and C-termini for this bifunctional enzyme.

The inhibition of the bisphosphatase in the dephospho-liver isoform may be due to a steric and conformational blocking mechanism, possibly by a mechanism involving the anchoring of the C-terminal tail in the bisphosphatase active site by the N-terminal. This is consistent with the observation that the bisphosphatase of the separately expressed domain (residues 251–470) and its CD30 deletion (residues 251–440) have the same $V_{\max}$ [26]. However, the finding that kinase activity of the CD30 mutant, but not the ND23CD30 mutant, is still responsive to phosphorylation, suggests that the interaction of the N-terminal region with the kinase active site is not dependent on the presence of the C-terminal region, and that an electrostatic effect of the negatively charged phosphoserine also plays a role.

The site of interaction of the N-terminus with the remainder of the molecule is unclear. The recent crystal structure of the testes 6PF2K/Fru-2,6-P₂ase shows that the first 36 residues (the equivalent of the first 39 residues of the liver isoform's N-terminal regulatory domain) were disordered and could not be visualized [13]. However, the presence of a loop between the fifth and sixth β -strand of the bisphosphatase domain, which comes close to the ATP-binding site of the 6PF2K domain and is adjacent to the initial unassigned portion of the N-terminus, suggests that the first seven amino acid residues may be interacting with this region. Surprisingly, mutation of Glu⁶ to Ala did not significantly affect the Fru-6-P affinity or $V_{\max}$ of the kinase (results not shown), indicating that the initial seven N-terminal amino acid residues may affect the overall conformational state of the bifunctional enzyme.

The end of the C-terminus is predicted to occlude the wide base of the funnel shaped active site of the liver CD30 bisphosphatase domain [27]. If these predictions are correct, phosphorylation of Ser³² might be expected to cause a restructuring of the interaction between the N- and C-termini that would result in an opening of the entrance to the bisphosphatase active site and a decrease in the kinase domain's affinity for Fru-6-P. It is not as yet clear whether this is due to a purely electrostatic interaction, or if hydrophobic residues in the N-terminal head may have a role.

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