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Expression of rat liver 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase and its kinase domain in *Escherichia coli*

(bifunctional enzyme/gene fusion/pET vectors)

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ABSTRACT **The rat liver bifunctional enzyme, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (ATP:D-fructose-6-phosphate 2-phosphotransferase/D-fructose-2,6-bisphosphate 2-phosphohydrolase, EC 2.7.1.105/EC 3.1.3.46) and its separate kinase domain were expressed in *Escherichia coli* by using an expression system based on bacteriophage T7 RNA polymerase. The bifunctional enzyme (470 residues per subunit) was efficiently expressed as a protein that starts with the initiator methionine residue and ends at the carboxyl-terminal tyrosine residue. The expressed protein was purified to homogeneity by anion exchange and Blue Sepharose chromatography and had kinetic and physical properties similar to the purified rat liver enzyme, including its behavior as a dimer during gel filtration, activation of the kinase by phosphate and inhibition by α-glycerol phosphate, and mediation of the bisphosphatase reaction by a phosphoenzyme intermediate. The expressed 6-phosphofructo-2-kinase also started with the initiator methionine but ended at residue 257. The partially purified kinase domain was catalytically active, had reduced affinities for ATP and fructose 6-phosphate compared with the kinase of the bifunctional enzyme, and had no fructose-2,6-bisphosphatase activity. The kinase domain also behaved as an oligomeric protein during gel filtration. The expression of an active kinase domain and the previous demonstration of an actively expressed bisphosphatase domain provide strong support for the hypothesis that the hepatic enzyme consists of two independent catalytic domains encoded by a fused gene.**

The synthesis and degradation of fructose 2,6-bisphosphate (Fru-2,6-P_2) in liver are catalyzed by the bifunctional enzyme, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (6-PF-2-K/Fru-2,6-P_2ase; ATP:D-fructose-6-phosphate 2-phosphotransferase/D-fructose-2,6-bisphosphate 2-phosphohydrolase, EC 2.7.1.105/EC 3.1.3.46) (1–3). The enzyme is a dimer of identical subunits, each composed of 470 amino acids and containing three discrete domains: an amino-terminal regulatory domain (residues 1–36), a kinase domain (residues 37–249), and a C-terminal bisphosphatase domain (residues 250–470) (4, 31). The regulatory region contains a cAMP-dependent protein kinase phosphorylation site (Ser-32) (4, 5). Phosphorylation at this site inhibits the kinase and activates the bisphosphatase, which suggests that the regulatory domain must somehow interact with the other two domains. The Fru-2,6-P_2ase reaction proceeds via a phosphoenzyme intermediate (6, 7), whereas 6-PF-2-K catalyzes transfer of the γ-phosphate of ATP to fructose 6-phosphate (Fru-6-*P*) by a direct in-line transfer not involving a covalent intermediate (8). On the basis of these results and various protein modification experiments that demonstrated differential effects on the two reactions (9–11), it has been suggested that the bifunctional enzyme was formed as a result of a gene fusion event (3, 12, 13, 31). Consistent with this hypothesis, an active separate bisphosphatase domain has recently been expressed in *Escherichia coli* and shown to have properties similar to the bisphosphatase domain in the bifunctional structure (14). We report here the expression in *E. coli* and characterization of an enzymatically active 6-PF-2-K domain and of an active 6-PF-2-K/Fru-2,6-P_2ase.

MATERIALS AND METHODS

Materials. Restriction enzymes and bacteriophage T4 DNA ligase were obtained from New England Biolabs. Sephadex G-75 and G-100 superfine and Q-Sepharose Fast Flow were from Pharmacia. [^{35}S]Methionine was from DuPont. Color-development reagent for immunoblotting was obtained from Bio-Rad.

DNA Manipulation. Standard DNA manipulations were carried out as described (15). Oligodeoxynucleotides were synthesized by phosphoramidite chemistry on an Applied Biosystems model 380A synthesizer and gel-purified as described (13). Plasmids were propagated in *E. coli* strain HB 101 following standard transformation procedures.

Expression System. The proteins were expressed by using the phage T7 expression system described by Studier and Moffat (16).

Purification of the Expressed 6-PF-2-K/Fru-2,6-P_2ase and Fru-6-*P*-2-K. The 6-PF-2-K/Fru-2,6-P_2ase was expressed as described for the bisphosphatase domain (14) and purified by a modification of the method used to purify the rat liver enzyme‡ (17). The kinase domain was extracted as described above. The extract was applied to a Blue-Sepharose column equilibrated in buffer A (20 mM 2-{[tris(hydroxymethyl)-methyl]amino}ethane sulfonic acid, pH 7.5/100 mM KCl/1 mM dithiothreitol/2 mM EDTA/5 mM potassium phosphate/0.5 mM phenylmethanesulfonyl fluoride/2.5 μg of leupeptin per ml). The enzyme was recovered in the flow-through volume, precipitated with $(NH_4)_2SO_4$ (0.47 g/ml), dissolved in buffer A, and applied to a Sephadex G-100 column equilibrated with buffer A. The fractions containing activity were pooled and concentrated by ultrafiltration. The kinase domain was purified 15-fold by this procedure.

Assay of Enzyme Activities. Fru-2,6-P_2 levels, 6-PF-2-K, and Fru-2,6-P_2ase activities were assayed as described (17–

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Abbreviations: Fru-6-*P*, fructose 6-phosphate; Fru-2,6-P_2, fructose 2,6-bisphosphate; Fru-2,6-P_2ase, fructose-2,6-bisphosphatase; 6-PF-2-K, 6-phosphofructo-2-kinase; IPTG, isopropyl thiogalactoside.

‡During the Q-Sepharose step, bisphosphatase activity but no kinase activity was detected in the void volume. The active material was eluted from a Sephadex G-75 column as a 30-kDa protein and had the same mobility on $NaDodSO_4$/polyacrylamide gels as the expressed bisphosphatase domain (14).

19). Phosphoenzyme formation was assayed as acid-precipitable radioactivity on phosphocellulose paper after incubation on ice with [2-^{32}P]Fru-2,6-P_2 (20).

Analysis of Protein Synthesis. The rate of induced protein synthesis was measured by pulse-labeling with [^{35}S]-methionine essentially as described (14, 16). Immunoblot analyses were performed essentially as described by Towbin *et al.* (21) with a 1:500 dilution of the liver enzyme antiserum (2 hr at 37°C) (20) and a 1:4000 dilution of the second antibody (goat anti-rabbit immunoglobulin coupled to horseradish peroxidase; 2 hr at 37°C).

Protein Sequencing. The amino-terminal sequences of the expressed proteins were determined with an Applied Biosystems 470A protein sequencer by the method of Hunkapillar *et al.* (22) as described previously (14).

Other Methods. [2-^{32}P]Fru-2,6-P_2 was prepared enzymatically from [γ-^{32}P]ATP and Fru-6-P as described (19). The separate bisphosphatase domain was expressed in *E. coli* and purified as described (14). Protein was determined by the method of Lowry *et al.* (23).

RESULTS

Construction of Plasmids. The strategy used to construct a vector for expression of rat liver 6-PF-2-K/Fru-2,6-P_2ase in *E. coli* is summarized in Fig. 1. A fragment of the coding-length cDNA of the bifunctional enzyme isolated by Colosia *et al.* (24), cDNA^{2K500}, contains 205 base pairs (bp) of 5′ untranslated region and codes for the first 90 amino acids of the protein. In order to express only the coding sequence and not a fusion protein, the 5′ untranslated region was deleted by digestion of cDNA^{2K500} with *Bgl* II and *Eco*RI. The *Eco*RI restriction site corresponds to the internal site of the coding-length cDNA (24). These operations removed the start codon and the sequence encoding the first 13-amino acid residues of the protein. To reintroduce the start codon and the coding sequence for the missing amino acids, a linker of two complementary oligonucleotides was ligated to the 5′ end of the *Bgl* II–*Eco*RI fragment (Fig. 1). Ligation of this fragment into the *Nde* I/*Eco*RI-cut pET-3a vector yielded a construct, pPKBa, that contained a 6-PF-2-K/Fru-2,6-P_2ase cDNA attached directly to the translation initiation codon of pET-3a. The remainder of the coding region (including the stop codon) was added by introducing into the *Eco*RI site of pPKBa the 1422-bp *Eco*RI–*Eco*RI clone fragment (cDNA^{2K1400}, that codes for amino acids 91–470, the stop codon, and 280 bp of 3′ untranslated region (24). The resulting expression plasmid, designated pPKB, contained the entire 6-PF-2-K/Fru-2,6-P_2ase coding sequence under the control of the ϕ10 bacteriophage T7 promoter.

The expression vector for the kinase domain was achieved by digesting pPKB with *Nco* I/*Hin*dIII, thereby removing the bisphosphatase-encoding region and the 3′ untranslated region, and then adding a linker at the 3′ end to reintroduce a stop codon (Fig. 2). The resulting expression plasmid was designated pPKa.

To determine the role of the regulatory domain on the enzymes' two activities, we constructed an expression vector for the bifunctional enzyme without the coding region for the phosphorylation domain. pPKB was digested with *Xho* I/*Nde* I, which deleted the sequence coding for the phosphorylation domain and part of the kinase (residues 1–52), and the deleted sequence was replaced by ligating a linker that codes for amino acids 37–52 to the digested plasmid (Fig. 2). The resulting expression plasmid was designated pKBa. Fig. 2 *Lower* schematically summarizes the pertinent restriction sites of cDNA^{2K500} and cDNA^{2K1400} and the respective domain structures of 6-PF-2-K/Fru-2,6-P_2ase.

Expression of the Recombinant Proteins. Plasmids pPKB, pPKa, and pKBa were used to transform BL21(DE3) cells.

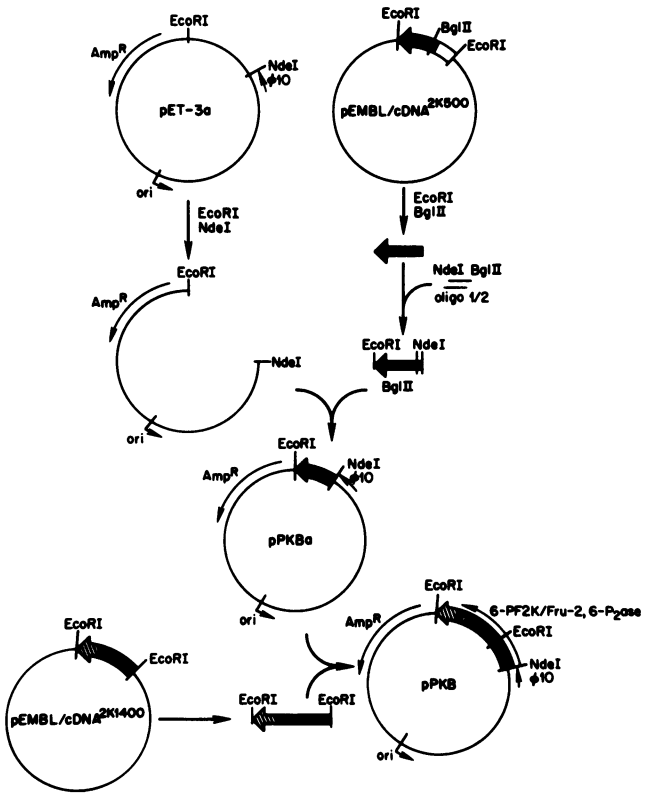

FIG. 1. Construction of rat liver 6-PF-2-K/Fru-2,6-P_2ase expression plasmid pPKB. pPKB, containing the full coding region of rat liver 6-PF-2-K/Fru-2,6-P_2ase controlled by bacteriophage T7 expression signals, was engineered by using the pET-3a expression vector and segments of cDNA^{2K500} and cDNA^{2K1400} (24). A *Bgl* II–*Eco*RI fragment from cDNA^{2K500} was isolated, and its 5′ *Bgl* II site ligated with a synthetic fragment formed by the annealing of two complementary oligonucleotides designed to give *Nde* I and *Bgl* II cohesive ends: 5′-TATGTCTCGAGAGATGGGAGAACTCACTCAAAC-CAGGTTACAGAA-3′, and 5′-GATCTTCTGTAACCTGGTT-TGAGTGAGTTCTCCCATCTCTCGAGACA-3′. This ligated fragment was introduced into pET-3a expression vector previously digested with *Nde* I and *Eco*RI. The new plasmid, pPKBa, was amplified, and an *Eco*RI–*Eco*RI fragment from cDNA^{2K1400} was introduced into its *Eco*RI restriction site. The solid regions indicate coding regions of 6-PF-2-K/Fru-2,6-P_2ase; the open regions indicate the 5′ untranslated flanking sequence, and the hatched regions indicate the 3′ untranslated sequence. Positions and orientations (arrows) are shown for the T7 RNA polymerase promoter (ϕ10) and T7 gene *10* translation initiation signals, the β-lactamase gene conveying resistance to ampicillin (Amp^R), the 6-PF-2-K/Fru-2,6-P_2ase cDNA, and the replication origin (ori) of the pBR322-derived plasmid. All the recombinant plasmids were identified by restriction analysis.

Each plasmid was tested for its ability to express the appropriate proteins when T7 RNA polymerase was induced. Fig. 3 shows the isopropyl thiogalactoside (IPTG)-induction time courses of [^{35}S]methionine pulse-labeled cultures containing pPKB and pPKa. Upon induction, cells that contained pPKB produced increased amounts of a protein that migrated with the same mass (55 kDa) as the bifunctional enzyme (Fig. 3, lanes 1–4). In cells that contained pPKa, a 27-kDa protein was induced that corresponds to the size of the 6-PF-2-K domain (Fig. 3, lanes 5–8). In cells containing the pKBa plasmid, a protein slightly smaller than the bifunctional enzyme was induced (not shown). Analysis of total protein by Coomassie blue staining of $NaDodSO_4$/polyacrylamide gels showed that the induced protein accounted for nearly 60% of total synthesized protein in each case.

Although a large fraction of the expressed bifunctional enzyme and the kinase domain were insoluble, both proteins could be detected in the soluble fraction of extracts by

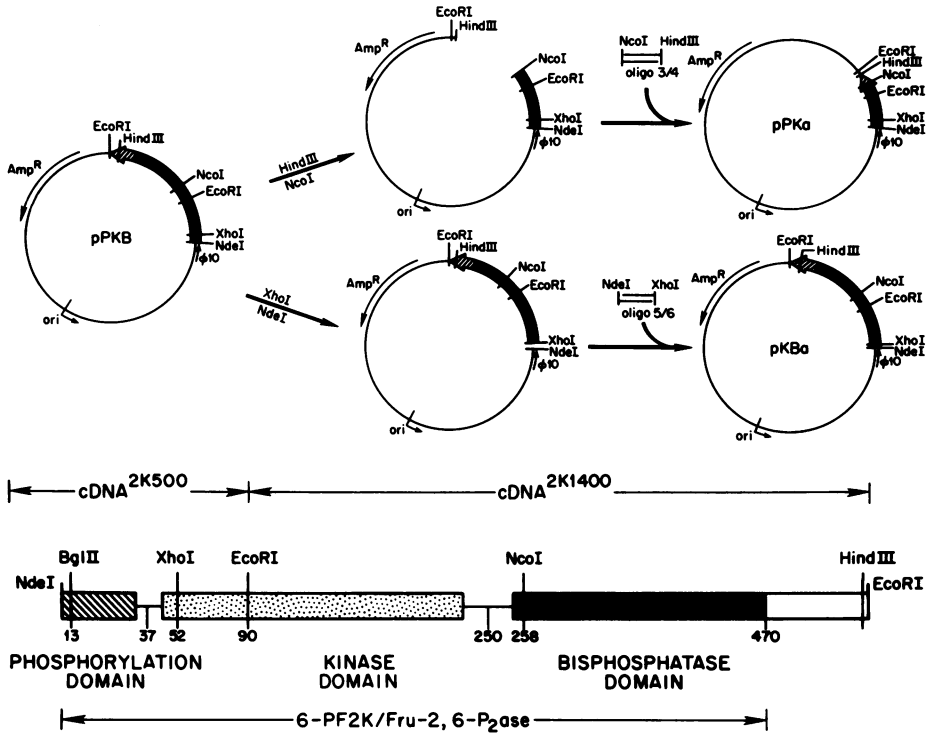

FIG. 2. Construction of expression vectors for the separate 6-PF-2-K domain and for 6-PF-2-K/Fru-2,6-P_2ase without the phosphorylation domain. (*Upper*) pPKa plasmid, coding for the kinase domain (residues 1–257), was constructed by digestion of the pPKB vector with *Nco* I and *Hin*dIII. A synthetic *Nco* I–*Hin*dIII linker formed by annealing of two complementary oligonucleotides, 5′-CATGGTTGA-3′ and 5′-AGCTTCAAC-3′, containing a stop codon, was ligated into the isolated *Nco* I/*Hin*dIII-digested vector. pKBa plasmid, coding for the full protein without the phosphorylation domain (deletion at amino acid residue 37), was constructed by digestion of the pPKB vector with *Nde* I and *Xho* I. A synthetic linker formed by hybridizing two complementary oligonucleotides designed to give *Nde* I and *Xho* I cohesive ends 5′-TATGTTCACTAATTCTCCCACGATGGTGATCATGGTGGGTTTACCAGC-3′ and 5′-TCGAGCTGGTAAACCCACCATGATCAC-CATCGTGGGAGAATTAGTGAACA-3′, which code for amino acid residues 37–52, was ligated to the isolated *Nde* I/*Xho* I-digested vector. The solid regions indicate coding regions of 6-PF-2-K/Fru-2,6-P_2ase and the striped regions indicate the 3′ untranslated flanking region. Positions and orientations are shown as described in Fig. 1. (*Lower*) Boxes delineating the location of the phosphorylation domain (hatched), the 6-PF-2-K domain (stippled), the Fru-2,6-P_2ase domain (solid), and the 3′ untranslated sequence (open) are shown. Numbers show positions of the pertinent restriction sites, and arrows define the lengths of the 6-PF-2-K/Fru-2,6-P_2ase cDNA plasmids used to engineer the expression vectors. The coding region of 6-PF-2-K/Fru-2,6-P_2ase is also indicated with an arrow.

immunoblot analysis with an antibody to the purified rat liver enzyme (Fig. 4). The less intense immunoreaction of the kinase domain may reflect the decreased immunoreactivity of the kinase region relative to the bifunctional enzyme with the polyvalent antibody (S.J.P., unpublished results) and/or less efficient expression. The lower band (30 kDa) in lanes 3–5 corresponds to the bisphosphatase domain of the bifunctional enzyme, which was produced during the induction of bifunctional enzyme expression, probably as a result of translation reinitiation at a ribosome-binding site preceding the bisphosphatase-coding sequence (14). The correct processing of the expressed proteins was confirmed by amino-terminal sequence analysis though 20 cycles on a gas-phase sequencer.

6-PF-2-K activity and phosphoenzyme formation (an intermediate in the bisphosphatase reaction) were assayed in the soluble fraction of these cells before and after IPTG induction. Negligible kinase activity and phosphoenzyme formation were detected before induction, but parallel increases in 2-kinase activity and phosphoenzyme formation were found after induction in cells that contained the pPKB plasmid (Table 1). In contrast, kinase activity but no phosphoenzyme formation was detected when the kinase domain was expressed. The product formed in the 6-PF-2-K reactions for both proteins was acid labile and was eluted from an anion-exchange column in the same position as Fru-2,6-P_2 (data not shown). The bifunctional enzyme expressed without its phosphorylation domain (residues 37–470) was completely insoluble (data not shown).

Characterization of the Bifunctional Enzyme and Its Kinase Domain Expressed in *E. coli*. The expressed bifunctional enzyme was greater than 90% pure after chromatography on the Blue Sepharose column. The protein was purified 150-fold, and the yield was 30%. About 1 mg of purified protein was obtained from 2 liters of culture cells. The enzyme migrated on a $NaDodSO_4$/polyacrylamide gel with the same subunit molecular weight as the native enzyme (Fig. 5). Some properties of this enzyme and of the partially purified kinase domain were compared with the properties of the bifunctional enzyme purified from rat liver (Table 2). 6-PF-2-K activity of the expressed bifunctional enzyme had a V_{max} of 20 milliunits/mg compared with 31 milliunits/mg for the rat liver enzyme. Inorganic phosphate had no effect on the V_{max} of the 6-PF-2-K of either bifunctional enzyme but greatly decreased the K_m for Fru-6-*P* for both enzymes. The K_m values for Fru-6-*P* and ATP for 6-PF-2-K of the expressed protein and for the rat liver enzyme were similar. The expressed protein was also inhibited by α-glycerol phosphate in a competitive manner with regard to Fru-6-*P* (data not shown). The bisphosphatase activity of the expressed bifunctional enzyme had a slightly higher V_{max} than did the bisphosphatase of the rat liver enzyme in either the absence or presence of P_i or a Fru-6-*P*-depleting system. The K_m for Fru-2,6-P_2 was the same for both proteins. Phosphoenzyme formation was also readily detected upon incubation of either protein with [2-^{32}P]Fru-2,6-P_2. The expressed bifunctional enzyme was eluted from a Sephadex G-100 column (in buffer A) with an

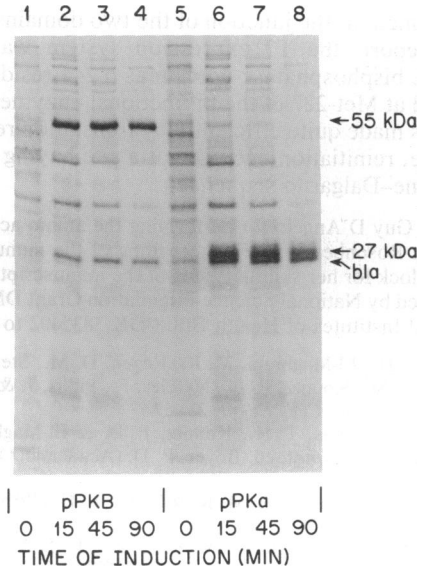

FIG. 3. Induction of the 6-PF-2-K/Fru-2,6-P_2ase and the separate kinase domain. Polyacrylamide gel autoradiograph of cultures of BL21(DE3) carrying pPKB and pKBa plasmids. The cells were grown, induced, labeled with [^{35}S]methionine, and processed for gel electrophoresis as described. Lanes: 1–4, cells carrying pPKB plasmid before and 15, 45, and 90 min after induction; 5–8, the same time course of induction but with cells carrying the pKBa plasmid. The position of 6-PF-2-K/Fru-2,6-P_2ase (55 kDa) and 6-PF-2-K (27 kDa) polypeptides and β-lactamase (bla) are shown.

apparent molecular mass of 110 kDa, indicating that under these conditions it behaves as a dimer. The native liver enzyme behaves in an identical manner (2, 3, 11).

The K_m for Fru-6-*P*, in the absence or presence of P_i, of the partially purified kinase domain expressed in *E. coli* was higher than that of the kinase of the bifunctional enzyme. In the presence of inorganic phosphate, the K_m for Fru-6-*P* of both kinases was 1/50th that in the absence of P_i. Similarily, the affinity for ATP was greatly reduced in the case of the expressed kinase domain. When the partially purified kinase domain was applied to a Sephadex G-100 column, 6-PF-2-K activity and immunoreactivity were coincident with a protein peak that was eluted at 120 kDa (data not shown). Thus, in contrast to the bisphosphatase domain expressed in *E. coli*, which is active as a monomer (14), the kinase domain is oligomeric, since its subunit is 27 kDa (Fig. 4).

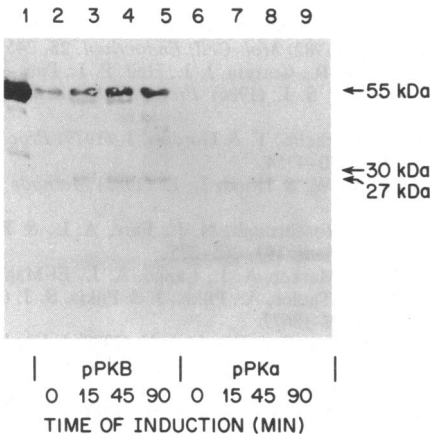

FIG. 4. Immunoblot of soluble expressed 6-PF-2-K/Fru-2,6-P_2ase and 6-PF-2-K domain. Lanes: 1, rat liver 6-PF-2-K/Fru-2,6-P_2ase; 2–5, cells carrying pPKB plasmid before and after 15, 45, and 90 min of induction; 6–9, cells carrying pPKa plasmid at the same times of induction. After transfer to nitrocellulose, the blot was probed with antiserum to the purified rat liver bifunctional enzyme.

Table 1. Induction of 6-PF-2-K activity and phosphoenzyme formation by IPTG in cells containing pPKB or pPKa

Enzyme	6-PF-2-K activity, milliunits/ml	Phosphoenzyme formation, cpm
6-PF-2-K/Fru-2,6-P_2ase		
Uninduced	0	100
Induced		
30 min	0.011	3,650
1 hr	0.023	5,960
2 hr	0.060	17,700
6-PF-2-K domain		
Uninduced	0	0
Induced		
30 min	0	0
1 hr	0.0010	0
2 hr	0.0025	0

Cultures of BL21 (DE3) carrying pPKB or pPKa were grown in M9 medium to A_{600} = 0.4 and were induced with 0.25 mM IPTG. At the indicated times, 10-ml aliquots of the cultures were centrifuged, and the cells were resuspended in 200 μl of buffer A. The cells were lysed and the samples were assayed for enzyme activities as described.

DISCUSSION

This paper reports the expression of rat liver 6-PF-2-K/Fru-2,6-P_2ase and its kinase domain in *E. coli* by using the phage T7 expression system described by Studier and Moffat (16). In this system, the expression of the protein is controlled by the ϕ10 promoter of T7 RNA polymerase and the strong translation initiation signals for the major capsid protein of bacteriophage T7. The T7 expression system has been used to express a growing number of eukaryotic proteins (25–28). Despite limited solubility, the high efficiency of this expression system makes it possible to obtain milligram quantities of structurally and functionally intact proteins. For example, expression of the bifunctional enzyme, its kinase domain, or its bisphosphatase domain (14) in *E. coli* represented >60% of the total amount of protein synthesized.

Although the enzyme lacking the phosphorylation site domain (residues 1–37) was efficiently expressed, this protein was not soluble, suggesting that some of the structural elements essential for maintaining the bifunctional enzyme

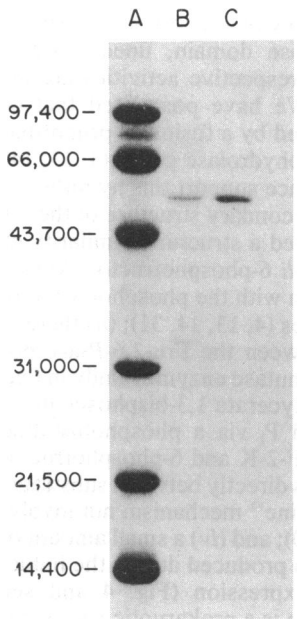

FIG. 5. NaDodSO$_4$/polyacrylamide gel of purified expressed 6-PF-2-K/Fru-2,6-P_2ase and rat liver bifunctional enzyme. Expressed 6-PF-2-K/Fru-2,6-P_2ase (0.5 μg) and rat liver enzyme (3 μg) were subjected to polyacrylamide electrophoresis in the presence of NaDodSO$_4$ and visualized by Coomassie blue staining. Lanes: A, molecular weight markers; B, expressed 6-PF-2-K/Fru-2,6-P_2ase; C, rat liver enzyme.

Table 2. Comparison of kinetic properties of the native rat liver bifunctional enzyme with those of the bifunctional enzyme and its kinase domain expressed in *E. coli*

Activities	6-PF-2-K/Fru-2,6-P_2ase		Kinase domain expressed
	Expressed	Rat liver	
6-PF-2-K			
V_{max} (mU/mg)			
− P_i	20.0	31.0	—
+ 5 mM P_i	20.0	31.0	—
K_m ATP (mM)*	0.15	0.10	10
K_m Fru-6-*P* (mM)			
− P_i	0.7	0.40	>20
+ 5 mM P_i	0.02	0.015	0.4
Fru-2,6-P_2ase			
V_{max} (mU/mg)			
− DS	15.0	8.0	ND
+ DS	45.0	22.0	ND
+ 5 mM P_i	50.0	40.0	ND
K_m Fru-2,6-P_2 (nM)	10.0	15.0	—
Phosphoenzyme formation (mol/mol)	0.2	0.15	ND

ND, not detectable; DS, Fru-6-*P*-depleting system; mU, milliunits.
*The K_m for ATP was unaffected by the presence of P_i.

and its kinase domain in a soluble form are localized in the first 37 amino acid residues. Consistent with this notion, limited proteolysis removed an N-terminal peptide containing the cAMP-dependent phosphorylation site, resulting in loss of kinase activity (9, 10, 29), and the cleaved protein was insoluble (9).

The expressed kinase domain had different kinetic properties than the kinase of the bifunctional enzyme and, in contrast to the dimeric nature of the latter, the kinase behaved in solution as an oligomeric protein. These results suggest that, although the kinase domain possesses the catalytic elements necessary for activity, association with the C-terminal bisphosphatase in the bifunctional structure affects its kinetic and structural properties. The results are also consistent with the hypothesis, put forth by structural analysis of the kinase and bisphosphatase domains (31) and by the monomeric behavior of the expressed bisphosphatase (14), that kinase–kinase interactions are responsible for the dimerization of the bifunctional enzyme.

The finding that the separate kinase domain was enzymatically active, together with the previous report of the expression of an active bisphosphatase domain, unequivocally proves the hypothesis that the respective activities are localized in different domains. We have postulated that 6-PF-2-K/Fru-2,6-P_2ase was formed by a fusion of primordial phosphotransferase and phosphohydrolase genes (3, 13, 14, 31). Several other lines of evidence support this hypothesis: (*i*) analysis of the primary and secondary structure of the rat liver bifunctional enzyme revealed a structural similarity of its 6-PF-2-K domain with *E. coli* 6-phosphofructo-1-kinase and of its bisphosphatase domain with the phosphoglycerate mutase/acid phosphatase families (4, 13, 14, 31); (*ii*) there is also a functional homology between the Fru-2,6-P_2ase domain and the phosphoglycerate mutase enzyme family in that they all catalyze hydrolysis of glycerate 1,3-bisphosphate to glycerate 3-monophosphate and P_i via a phosphohistidine intermediate (13); (*iii*) both 6-PF-2-K and 6-phosphofructo-1-kinase transfer phospho groups directly between substrates in a ternary complex by an "in-line" mechanism not involving a covalent intermediate (8, 30); and (*iv*) a small amount of bisphosphatase domain was also produced during the induction of bifunctional enzyme expression (Fig. 4 and see footnote ‡), suggesting that there is a prokaryotic ribosome-binding element at the junction of the two domains (14). In a previous report, the T7 expression system was used to express the bisphosphatase domain as a 244-residue protein that started at Met-227 of the bifunctional enzyme (14). This protein was made quite efficiently by translation reinitiation. In this case, reinitiation occurred at a site having a minimal (GGG) Shine–Dalgarno sequence.

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