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

(bifunctional enzyme/gene fusion/pET vectors/translation reinitiation site)

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ABSTRACT **The fructose-2,6-bisphosphatase domain of rat liver 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (EC 2.7.1.105/EC 3.1.3.46) was expressed in *Escherichia coli* by using an expression system based on bacteriophage T7 RNA polymerase. The protein was efficiently expressed (*i*) as a fusion protein that starts at the T7 major capsid protein initiation site in a pET expression vector and (*ii*) as a protein that starts within the bisphosphatase sequence by translation reinitiation. Both proteins have similar properties. The protein was purified to homogeneity by anion-exchange chromatography and gel filtration. The purified fructose-2,6-bisphosphatase domain was active and no 6-phosphofructo-2-kinase activity was found associated with it. In contrast to the dimeric bifunctional enzyme, the fructose-2,6-bisphosphatase domain behaved as a monomer of 30 kDa. The turnover number and kinetic properties of the separate bisphosphatase domain were similar to those of the bisphosphatase of the bifunctional enzyme, including the ability to form a phosphoenzyme intermediate. These results support the hypothesis that the rat liver enzyme consists of two independent domains and is a member of a class of enzymes formed by gene fusion.**

The 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) catalyzes the synthesis and degradation of fructose 2,6-bisphosphate (Fru-2,6-P_2), a key signal molecule whose steady-state concentration determines the rate and direction of carbon flux in the glycolytic/gluconeogenic pathway in mammalian liver (1–4). Both activities of the enzyme are under acute control by hormones that act by altering the levels of cAMP, such as glucagon, insulin, and β-adrenergic agonists (5–8). The enzyme, a dimer with a 55-kDa subunit, is phosphorylated by cAMP-dependent protein kinase on a single serine residue per subunit with a concomitant decrease in the kinase activity and increase in the bisphosphatase activity (9).

Recently this laboratory (Stony Brook) has sequenced the bifunctional enzyme and its cDNA (10, 11). The enzyme contains 470 amino acids and can be divided into two domains: an amino-terminal kinase domain (residues 1–249) and a carboxyl-terminal bisphosphatase domain (residues 250–470) that contains the active-site histidine (9, 11). The 6-phosphofructo-2-kinase domain has no clear amino acid sequence similarity with other mammalian kinases (12). However, the bisphosphatase domain was found to be functionally and structurally similar to yeast phosphoglycerate mutase and human erythrocyte 2,3-bisphosphoglycerate mutase (12, 13): all these enzymes catalyze their reactions by means of a phosphoenzyme intermediate involving a histidine residue (14–16), and the active-site sequences of the three enzymes are strikingly similar (12). It was postulated (13) that this structural and functional similarity supports the concept that the bifunctional enzyme was formed by a gene-fusion event. If this were the case, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase would be expected to consist of two independent domains, both of which would be active in a separate form. Since it has not been possible to isolate the two separate domains of the liver bifunctional enzyme in active forms after limited proteolysis, we expressed the fructose-2,6-bisphosphatase cDNA coding region in a bacterial expression system and compared its properties with those of the bisphosphatase of the bifunctional enzyme.

MATERIALS AND METHODS

Material. Restriction enzymes and bacteriophage T4 DNA ligase were purchased from New England Biolabs. DEAE-Sephadex A-50, Sephadex G-75, and Q Sepharose Fast Flow were from Pharmacia. Color-development reagent for immunoblotting was obtained from Bio-Rad. [2-^{32}P]Fru-2,6-P_2 and antiserum to the purified rat liver bifunctional enzyme were prepared as described (17).

Construction of Plasmids. Plasmids pBP1, -2, and -3, containing the bisphosphatase cDNA sequence controlled by bacteriophage T7 expression signals, were made and manipulated by use of standard techniques (18). The initial transformation host was *Escherichia coli* HMS174 (19). Recombinant plasmids were identified by restriction analysis.

Expression System. Expression of the bisphosphatase sequence in the plasmids was directed by T7 RNA polymerase using the T7 expression system described by Studier and Moffat (19). The plasmids were used to transform *E. coli* BL21(DE3), where the gene for T7 RNA polymerase is located in the chromosome under control of the inducible *lac*UV5 promoter. Cultures were grown at 37°C in M9 medium containing ampicillin (20 μg/ml). T7 RNA polymerase was induced by adding isopropyl β-D-thiogalactoside (0.4 mM) when the culture OD_{600} reached 0.4.

Analysis of Protein Synthesis. Cells (50 μl) were labeled for 3 min at 37°C with [^{35}S]methionine (20 μCi/ml; 1 μCi = 37 kBq), collected by centrifugation, suspended in sample buffer containing 1% $NaDodSO_4$, and heated for 3 min in a boiling water bath, and the lysates were subjected to electrophoresis in 10–20% polyacrylamide gradient gels in the presence of 0.1% $NaDodSO_4$ (20). Unlabeled cells (100 μl) were analyzed in the same way. Labeled proteins were visualized by autoradiography and total proteins by staining with Coomassie blue. Crude extracts of cells were separated into a soluble

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Abbreviations: Fru-2,6-P_2, fructose 2,6-bisphosphate; mu, milliunit(s).

and an insoluble fraction before analysis of proteins (21). Immunoblot analyses were performed essentially as described by Towbin *et al.* (22), using a 1:500 dilution of the liver enzyme antiserum (2 hr at 37°C) and a 1:4000 dilution of the second antibody [goat anti-rabbit immunoglobulin coupled to horseradish peroxidase (Boehringer Mannheim) 2 hr at 37°C].

Purification of the Bisphosphatase Domain. BL21(DE3) cells (2 liters) containing either pBP1 or pBP3 were grown in M9 medium containing ampicillin (20 μg/ml) to OD_{600} = 0.4, induced with isopropyl β-D-thiogalactoside (0.25 mM), and harvested by centrifugation after 2.5 hr of induction. Cell pellets were resuspended in 20 ml of buffer A [20 mM *N*-[tris(hydroxymethyl)methyl]aminoethanesulfonic acid (Tes)/KOH buffer (pH 7.5) containing 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, and 1 mg of lysozyme per ml]. The solution was kept on ice for 30 min and then subjected to freeze–thaw cycles three times in a dry ice/ethanol bath. $MgSO_4$ (10 mM final concentration) and 2 mg of DNase I were added to the broken cells and the mixture was incubated at 4°C for 1 hr. The solution was centrifuged twice and the supernatant was applied to a DEAE-Sephadex A-50 column. The column was washed with buffer B (buffer A without lysozyme) and the enzyme was eluted at 500 mM KCl in buffer B. The fractions containing the activity were pooled and precipitated at 70% saturation with $(NH_4)_2SO_4$. The precipitate was dissolved in a minimal volume of buffer B and applied to a Sephadex G-75 column equilibrated with buffer B. The fractions containing fructose-2,6-bisphosphatase activity were pooled and applied to a small Q Sepharose column equilibrated with buffer C [20 mM Tes/KOH buffer (pH 7.5) containing 50 mM KCl, 1 mM dithiothreitol, 1 mM EDTA, and 10% (vol/vol) glycerol] and the bisphosphatase was eluted at 300 mM KCl. The enzyme was precipitated at 70% saturation with $(NH_4)_2SO_4$ overnight and the pellet was dissolved in buffer B containing 20% (vol/vol) glycerol.

Assay of Enzyme Activities. 6-Phosphofructo-2-kinase activity was measured by the production of Fru-2,6-P_2 with the phosphofructo-1-kinase activation assay (2). Fructose-2,6-bisphosphatase activity was assayed by following the rate of production of $^{32}P_i$ from 10 μM [2-^{32}P]Fru-2,6-P_2 containing 5 mM P_i (23). Phosphoenzyme formation was assayed as acid-precipitable radioactivity on phosphocellulose paper (17).

Protein Sequencing. Amino-terminal sequence analysis of the expressed bisphosphatase domain was performed according to Hunkapillar and Hood (24) with an Applied Biosystems 470A protein sequencer. Purified protein (150 pmol) was dialyzed against 2 mM ammonium bicarbonate and then lyophilized to dryness and applied to the sequencer. Insoluble pellets from crude extracts of induced cells (2 hr), containing about 500 pmol of protein, were washed twice with H_2O, taken up in 70% formic acid, and applied directly to the sequencer.

Other Methods. The bifunctional enzyme was purified to homogeneity from rat liver as described (23). Protein was determined by the method of Lowry *et al.* (25).

RESULTS

Expression of the Fructose-2,6-bisphosphatase Domain in *E. coli*. The bisphosphatase domain of the rat liver bifunctional enzyme was expressed in *E. coli* using a T7 expression system (19, 26). The coding sequence for the carboxyl-terminal 252 amino acids of the enzyme was fused in each reading frame after the 11th codon of the T7 major capsid protein (gene *10*) in pET-2a, -2b, and -2c to make pBP1, -2, and -3, respectively (Fig. 1). The in-frame fusion (pBP3) codes for a protein of 264 amino acids; the out-of-frame fusions code for proteins of 22 (pBP1) or 53 (pBP2) amino acids (Fig. 2).

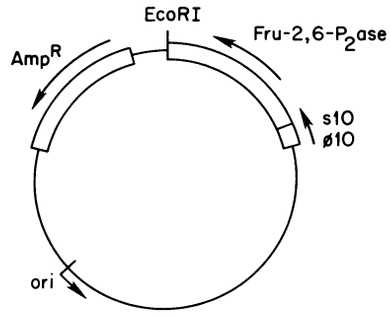

FIG. 1. Fructose-2,6-bisphosphatase expression plasmids. The 931-base-pair *Bgl* II–*Eco*RI fragment from p2K1100, containing nucleotides 704–1790 of the rat liver bifunctional enzyme cDNA (10), was inserted in the three reading frames of pET-2a, -2b, and -2c between the *Bam*HI and *Eco*RI sites (26). Expression of the bisphosphatase sequence is controlled by a promoter for T7 RNA polymerase (ϕ10) and the T7 gene *10* protein initiation site (s10), including the Shine–Dalgarno sequence and the first 11 codons of gene *10* followed by a *Bam*HI linker that determines the reading frame of the fusion. Positions (boxes) and orientations (arrows) are shown for the T7 signals, the bisphosphatase sequence, the β-lactamase gene conveying resistance to ampicillin (Amp^R), and the replication origin (ori) of the pBR322-derived plasmid.

Each plasmid was tested for the ability to express proteins when T7 RNA polymerase was provided in the host cell (Fig. 3). The in-frame fusion (pBP3, Fig. 3*C*) and, unexpectedly, one out-of-frame fusion (pBP1, Fig. 3*A*) directed the rapid synthesis of proteins in the 30-kDa range, about the size of the bisphosphatase domain. A small protein was induced from the other out-of-frame fusion (pBP2, Fig. 3*B*). The 30-kDa proteins accumulated to about half (pBP3) or somewhat less than half (pBP1) of total cell protein after 2 hr of induction (data not shown). Antiserum prepared against the rat liver enzyme reacted specifically with both 30-kDa proteins in immunoblot analysis of a gel similar to that shown in Fig. 3 (data not shown).

The proteins containing the bisphosphatase domain were further identified by amino-terminal sequence analysis. Both proteins were substantially insoluble in crude extracts of induced cells (data not shown). Cell pellets contained sufficient amounts of bisphosphatase polypeptide so that unambiguous sequences could be determined by using this material directly. The in-frame fusion protein (pBP3) was analyzed through 20 cycles of the sequencer and, as expected, starts with the 12 amino acid gene *10* leader sequence from the vector (26), Met-Ala-Ser-Met-Thr-Gly-Gly-Gln-Gln-Met-Gly-Arg-, followed by fructose-2,6-bisphosphatase sequence beginning with Ile-219 of the bifunctional enzyme (11),

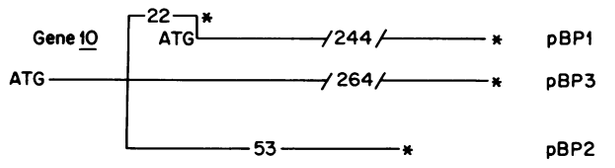

FIG. 2. Fusion proteins. Initiation (ATG) and termination (*) codons and the lengths in amino acids are shown for T7 gene *10*–fructose-2,6-bisphosphatase fusion proteins encoded by pBP1, -2, and -3 and for the protein induced from pBP1 that starts at Met-227. The termination codon of the 22 amino acid fusion protein encoded by pBP1 is 1 nucleotide downstream of the ATG codon for Met-227 (ATGGTAA). The fusions contain 11 gene *10* codons, followed by 1 codon from the linker (26) and then codons from the bisphosphatase cDNA, beginning with Ile-219 for the in-frame fusion (pBP3).

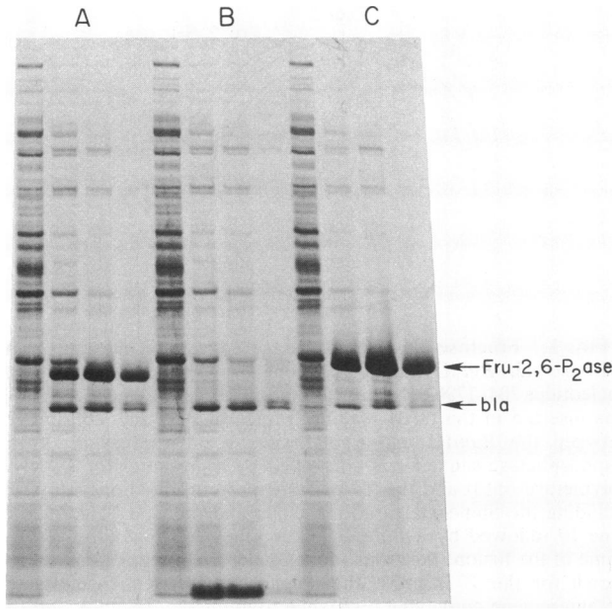

FIG. 3. Induction of fructose-2,6-bisphosphatase. Cultures of BL21(DE3) carrying pBP1 (*A*), pBP2 (*B*), or pBP3 (*C*) were grown, induced, labeled with [^{35}S]methionine, and processed for gel electrophoresis. For each culture, patterns are shown (lanes left to right) for proteins labeled immediately before and 30, 60, and 90 min after induction. The position of the fructose-2,6-bisphosphatase polypeptides (Fru-2,6-P_2ase) and β-lactamase (bla) are shown.

-Ile-Phe-Asp-Val-Gly-Thr-Arg-Tyr-. The protein produced from pBP1 was analyzed through 20 cycles and contained only bisphosphatase sequence, starting at Met-227 (11): Met-Val-Asn-Arg-Val-Gln-Asp-His-Val-Gln-Ser-Arg-Thr-Ala-Tyr-Tyr-Leu-Met-Asn-Ile-.

It seems likely that the bisphosphatase polypeptide that starts at Met-227 is made by translation reinitiation (27, 28). In pBP1, ribosomes that initiate at the T7 gene *10* ATG terminate at a TAA codon that starts 1 nucleotide downstream of the ATG codon for Met-227 (ATGGTAA) (Fig. 2). Further, this ATG is not used for initiation in the absence of the upstream gene *10* initiation site (data not shown) or in the other two reading frame fusions, where ribosomes translate through this ATG and terminate 98 (pBP2) or 732 (pBP3) nucleotides downstream (Fig. 3).

Although both proteins were largely insoluble, easily detectable levels of fructose-2,6-bisphosphatase activity and phosphoenzyme formation were found in the soluble fraction of extracts (Table 1). The bisphosphatase activity increased nearly 7-fold after 2 hr of induction in both the pBP1- and the pBP3-containing strains, and there was a parallel increase in formation of phosphoenzyme, an intermediate in the bisphosphatase reaction. The bisphosphatase activity measured in uninduced cells probably represents nonspecific phosphatase activity, since no phosphoenzyme formation was found in these cells.

Table 1. Induction of fructose-2,6-bisphosphatase activity and phosphoenzyme formation

Induction	Fructose-2,6-bisphosphatase, mu/ml	Phosphoenzyme formation, cpm
None	0.152	0
1 hr	0.595	427
2 hr	1.02	700

A culture of BL21(DE3) carrying pBP1 was grown in M9 medium to OD_{600} = 0.4 and induced with isopropyl β-D-thiogalactoside (0.4 mM). One milliliter of this culture was harvested by centrifugation and resuspended in 100 μl of buffer A. The cells were broken by the procedure described in *Materials and Methods* and enzyme activities were assayed in the soluble supernatant fraction. Cultures carrying pBP3 showed similar levels of induced activity. mu, Milliunit(s).

Purification of the Bisphosphatase Domain. Attempts to purify the separate bisphosphatase domain by the same procedures used for the wild-type bifunctional enzyme failed because the expressed protein could not be specifically eluted from phosphocellulose columns with substrate, a method that yields pure rat liver bifunctional enzyme (23). A simple and rapid method for purification of the bisphosphatase domain was developed that employed anion-exchange chromatography and gel filtration. The protein was purified 200-fold, and the yield was near 30%. Approximately 1 mg of purified protein was obtained from 2 liters of cells grown in minimal medium. The protein was more than 90% pure as judged by $NaDodSO_4$/polyacrylamide gel electrophoresis and had a subunit molecular mass of 30 kDa as compared to 55 kDa for the native bifunctional enzyme (Fig. 4, lanes 1 and 2). This procedure was used to purify soluble enzyme expressed by both pBP1 and pBP3. No 6-phosphofructo-2-kinase activity was found associated with the bisphosphatase domain in either case.

Properties of the Bisphosphatase Domain. The separately expressed domain was characterized by using the protein that starts at Met-227. Since only about half the native protein has been expressed in the *E. coli* system, it was of interest to determine whether the separate bisphosphatase domain would undergo dimerization. Fig. 5 shows a Sephadex G-75 gel-filtration profile after the first DEAE-Sephadex chromatography step of the purification scheme. Phosphoenzyme formation (Fig. 5*B*) and immunoreactivity (Fig. 5*C*) were coincident with a protein peak corresponding to a molecular size of 30 kDa. Thus, in contrast to the native enzyme (1), the bisphosphatase domain behaves as a monomer.

To determine whether the purified protein catalyzed the hydrolysis of Fru-2,6-P_2 via a phosphoenzyme intermediate, the bisphosphatase domain was incubated with [2-^{32}P]Fru-2,6-P_2 and subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis. The same was done with the native enzyme to provide a positive control. As in the case of the bifunctional native enzyme (17), the separate bisphosphatase domain was labeled upon incubation with [2-^{32}P]Fru-2,6-P_2 (Fig. 4). The

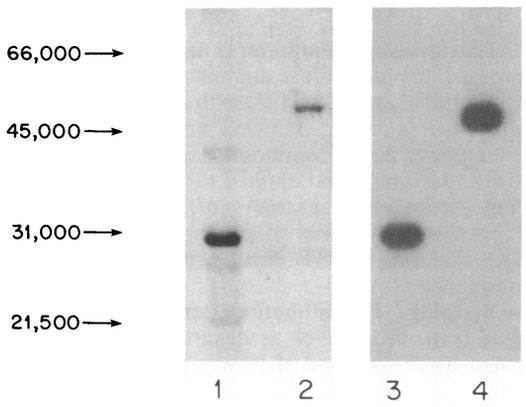

FIG. 4. $NaDodSO_4$/polyacrylamide gel electropherogram of ^{32}P-labeled bisphosphatase domain and bifunctional enzyme. ^{32}P-labeled bisphosphatase domain (8 μg) and ^{32}P-labeled kinase/bisphosphatase (1.5 μg) were prepared by incubation of the proteins with [2-^{32}P]Fru-2,6-P_2 according to published methods (17). The samples were subjected to electrophoresis in the presence of $NaDodSO_4$ and then either were stained with Coomassie brillant blue (lanes 1 and 2) or were autoradiographed (lanes 3 and 4). Lanes 1 and 3, bisphosphatase domain; lanes 2 and 4, bifunctional enzyme. Positions and sizes (Da) of standard proteins run in parallel are indicated at left.

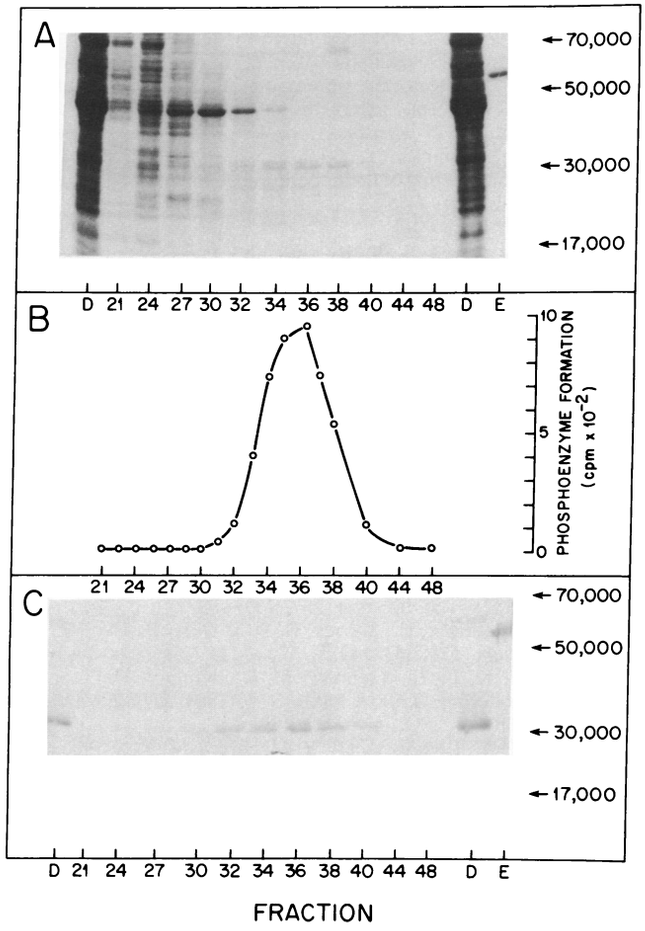

FIG. 5. Molecular size of the bisphosphatase. Pooled fractions from the DEAE-Sephadex step in the purification scheme were applied to a Sephadex G-75 column (1.5 × 90 cm) equilibrated in buffer B (see *Materials and Methods*). (*A*) Coomassie blue stain after $NaDodSO_4$/polyacrylamide gel electrophoresis of the fractions, aliquots of the applied sample (lanes D), and native bifunctional enzyme (lane E). (*B*) Bisphosphatase activity measured by phosphoenzyme formation. (*C*) Immunoblot of each fraction, probed with antiserum to the bifunctional enzyme. The column was calibrated with proteins of known molecular mass (aldolase was eluted at fraction 25, bovine serum albumin at 28, ovalbumin at 32, chymotrypsinogen A at 37, and ribonuclease A at 45).

autoradiogram shows a single band of radioactivity at 30 kDa in the case of the bisphosphatase domain (lane 3) and at 55 kDa in the case of the bifunctional enzyme (lane 4), both coincident with Coomassie blue-stained bands (lanes 1 and 2). The stoichiometry of ^{32}P labeling of the separately expressed bisphosphatase domain reached 0.09 mol of phosphate per mol of protein, half the value observed for the native enzyme (Table 2).

The bisphosphatase activity of the bifunctional enzyme is strongly inhibited by Fru-6-*P*. This inhibition can be eliminated either by adding a Fru-6-*P*-depleting system directly to the assay mixture or by the addition of P_i, which acts by decreasing Fru-6-*P* binding (29). The separate bisphosphatase domain behaved similarly: maximal velocity was obtained in the presence of either a Fru-6-*P*-depleting system or 10 mM P_i (Fig. 6).

In Table 2, the kinetic properties of the purified bisphosphatase domain are compared with the properties of the bifunctional enzyme from rat liver. The isolated bisphosphatase domain had a V_{max} of 220 mu/mg in the presence of a Fru-6-*P*-depleting system and 60 mu/mg in its absence. In the same experiment, the native enzyme had a V_{max} of 146

Table 2. Comparison of the activities of the native bifunctional enzyme and the separate bisphosphatase domain

Activity	Bifunctional enzyme	Bisphosphatase domain
6-Phosphofructo-2-kinase, mu/mg	50	0
Fructose-2,6- bisphosphatase, mu/mg		
Without depleting system	56	60
With depleting system	146	220
K_m for Fru-2,6-P_2, μM	0.1*	0.3
Phosphoenzyme formation, mol/mol of enzyme	0.20	0.09

The bisphosphatase domain expressed by pBP1 and the native enzyme were purified and their kinetic properties were determined. *Ref. 29.

mu/mg and 56 mu/mg in the presence or absence of a Fru-6-*P*-depleting system, respectively. The K_m of the bisphosphatase domain for Fru-2,6-P_2 was 0.3 μM, whereas that of the native enzyme was 0.1 μM (29). These results indicate that the kinetic properties of the separate bisphosphatase domain expressed in *E. coli* and the bisphosphatase activity of the bifunctional enzyme are similar.

DISCUSSION

This paper reports the expression in *E. coli*, purification, and characterization of the fructose-2,6-bisphosphatase domain of rat liver 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase. A T7 expression system was used to express the bisphosphatase domain at high levels, both as a 264 amino acid fusion protein (containing a 12 amino acid T7 gene *10* leader sequence) and as a 244 amino acid protein that starts at Met-227 of the bifunctional enzyme. Both proteins had similar properties. Although much of the bisphosphatase was insoluble in crude extracts, a significant fraction remained soluble and was found to have enzymatic activity. About 1 mg of soluble purified protein could be isolated from 2 liters of induced cells, a yield sufficient for structure–function studies.

The protein that starts at Met-227 appears to be made quite efficiently by translation reinitiation. The nucleotide sequence near the ATG codon for Met-227 is GTGGGCACACGCTACATGGTAA. Ribosomes that start at the upstream T7 gene *10* initiation site in pBP1 stop at the TAA

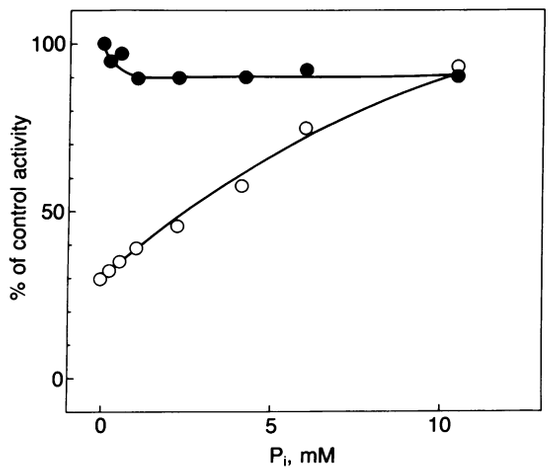

FIG. 6. Effect of P_i on the bisphosphatase domain. Fructose-2,6-bisphosphatase activity was measured in the absence (○) or presence (●) of a Fru-6-*P*-depleting system at the indicated concentrations of P_i.

termination codon close to the restart ATG, as occurs in other cases of efficient reinitiation (27, 28). In this case, reinitiation occurs at a site having only a minimal (GGG) Shine–Dalgarno (ribosome-binding) sequence, suggesting that reinitiation can take place efficiently without a good Shine–Dalgarno sequence. The efficient synthesis of the reinitiation protein may also be due to the high level of mRNA usually induced in the T7 expression system (19) and to the efficiency of primary initiation at the upstream T7 gene *10* site.

The functional properties of the bisphosphatase domain expressed in *E. coli* were similar to those of the bisphosphatase of the bifunctional enzyme. The separate bisphosphatase domain also exhibited phosphoenzyme formation, strongly suggesting that its reaction mechanism is identical to that of the bisphosphatase of the rat liver enzyme. Neither additional residues from the kinase domain and hinge region (31 in the case of the fusion protein, 23 in the reinitiation protein) nor the 12 T7 gene *10* amino acids on the amino-terminal end of the fusion protein appeared to affect the bisphosphatase activity. Since the fructose-2,6-bisphosphatase activity of the separate domain showed little or no difference in turnover number or substrate affinity compared to the bifunctional enzyme, it is unlikely that any region of the kinase domain is an important determinant for bisphosphatase activity.

A major difference between the liver bifunctional enzyme and the bisphosphatase domain expressed in *E. coli* is that the former enzyme is a dimer, whereas the latter is a monomer. This finding suggests either that the regions that interact to form dimers of the rat liver enzyme do not include the bisphosphatase domain or that interaction to form the dimer occurs between the bisphosphatase domain of one subunit and the kinase domain of the other. It should be possible to identify those regions of the subunit that are important for dimerization by extending the expressed protein in the amino-terminal direction.

On the basis of conservation of primary and secondary structural elements and similar kinetics, it was postulated that the bisphosphatase domains of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase, phosphoglycerate mutase, and 2,3-bisphosphoglycerate mutase are evolutionarily linked and that the bifunctional enzyme was formed by a gene-fusion event (13). In many cases of gene fusion, the single protein that is formed consists of two independent domains (30–32). Our demonstration that the fructose-2,6-bisphosphatase domain expressed in *E. coli* is active and has properties similar to those of the bisphosphatase of the bifunctional enzyme provides strong support for the gene-fusion hypothesis. However, the reciprocal effect of cAMP-dependent phosphorylation on kinase and bisphosphatase activities of the bifunctional enzyme suggests that the two domains can interact. To better understand the relation of the domains, it will be important to express the kinase domain separately.

The structures of fructose-2,6-bisphosphatase and phosphoglycerate mutase have been compared by molecular graphics (33). The structural similarity between these proteins, particularly at the active site, provides a strong rationale for studies involving site-directed mutagenesis. The studies reported in this paper establish the system for *in vitro* analysis of mutations of the bisphosphatase.

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