Characterization of the separate kinase domain of chicken liver 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase*

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Abstract The bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase consists of two distinct domains which catalyze the synthesis and hydrolysis of fructose-2, 6-bisphosphate, respectively. In this work the properties of the separate 6-phosphofructo-2-kinase domain were investigated. Purification of the expressed separate domain or isolation of this domain from purified glutathione S-transferase (GST) fusion protein with thrombin cleavage led to the loss of its kinase activity. Thus the domain in the GST-tagged form was characterized. The two forms of the domain with different lengths (amino acids 1 ~ 249 and 1 ~ 286) were very similar in kinetic property and could catalyze the formation of fructose-2,6-bisphosphate with a k_{cat} 4-fold lower than that of the full-length enzyme. In addition, the domain was much more sensitive to guanidine inactivation and heat denaturation, and less stable at pH values below 7 than the full-length enzyme. The results suggest that the separate kinase domain of the bifunctional enzyme is far less perfect in structure in the absence of the bisphosphatase domain, though it still possesses the 6-phosphofructo-2-kinase activity.

Keywords: 6-phosphofructo-2-kinase, fructose-2,6-bisphosphatase, glutathione S-transferase fusion protein, kinetic property.

The synthesis and hydrolysis of fructose-2,6-bisphosphate (Fru-2,6-P_2), an important regulatory metabolite whose concentration determines glycolytic/gluconeogenic flux in the hepatocyte, is catalyzed by 6-phosphofructo-2-kinase (6PF-2K) and fructose-2,6-bisphosphatase (Fru-2,6-P_2ase), respectively[1]. The 6PF-2K and Fru-2,6-P_2ase activities coexist in one polypeptide which is called a bifunctional enzyme 6PF-2K/Fru-2,6-P_2ase. The respective kinase and bisphosphatase activities reside in two distinct domains: the N-terminal 6PF-2K domain and C-terminal Fru-2,6-P_2ase domain[1,2]. It has been hypothesized that this bifunctional enzyme is the result of gene fusion event of distinct primordial enzymes[3]. The Fru-2,6-P_2ase domain is homologous to mutase and acid phosphatase families[3~5]. Crystal structure analysis[5] and modelling[6] have revealed that 6PF-2K domain is related to mononucleotide binding proteins, such as adenylate kinase and the catalytic core of G proteins.

Previous works have shown that the two domains of the bifunctional enzyme exert some influences on each other. As manifested in rat liver bifunctional enzyme, deleting the last 30 residues from the

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C-terminus not only increased the bisphosphatase V_{max} by 9-fold, but also reduced the kinase V_{max} by 50%[7]. The C-terminal deletion mutations of bovine heart bifunctional enzyme, however, increased the V_{max} of kinase[8]. Removal of the initial 22 residues from rat liver isoform increased the V_{max} of bisphosphatase by 4-fold[9], and deletion of the N-terminal 24 or 30 amino acids from rat testis enzyme resulted in a 2-fold increase in the bisphosphatase activity[10]. The separate rat liver[11~13] and chicken liver[14] Fru-2, 6-P_2ase domains exhibited activities greater than that of the respective wild type enzymes, which suggested that in the intact enzyme the kinase domain plays an inhibitory role on the bisphosphatase domain, and that the Fru-2, 6-P_2ase activity of the bifunctional enzyme is independent of the kinase domain. However, little has been known of whether the 6PF-2K activity of the bifunctional enzyme is dependent on the Fru-2, 6-P_2ase domain or not. In this work the chicken liver 6PF-2K domain was expressed and characterized, and the properties of this domain were described.

1 Materials and methods

1.1 Materials

Restriction endonucleases, T7 DNA polymerase, Klenow fragment of DNA polymerase and other DNA modifying enzymes were obtained from Life Technologies, Inc. and New England Biolabs Inc. pGEX vector and related materials were the product of Amersham Pharmacia. ATP, Fru-6-P, Isopropyl-1-thio-β-D-galactopyranoside, glutathione (GSH) and guanidine hydrochloride (GdnHCl) were purchased from Sigma.

1.2 Construction of the expression plasmids

The expression plasmid for chicken liver 6PF-2K/Fru-2, 6-P_2ase (CKB) was previously reported[15]. Termination codon TGA was introduced into the expected sites of cDNA for CKB via PCR techniques to produce the separate 6PF-2K domain of CKB (CKD) of various lengths. GST-fusion expression plasmids were constructed by in-frame insertion of the cDNA for CKD into pGEX-4T-1, which encodes a thrombin recognition site between GST and target protein.

1.3 Enzyme expression and purification

GST-CKD fusion proteins were expressed *in Escherichia coli* DH5α in tryptone-phosphate medium[16]. The concentration of isopropyl-1-thio-β-D-galactopyranoside used for induction was either 20 μmol/L for GST-CKD$^{1-249}$ or 150 μmol/L for GST-CKD$^{1-286}$ and GST-CKD$^{1-221}$. The cells were harvested after induction for 22 h at 22℃ and lysed by freezing and thawing. Proteins were extracted with 40 mmol/L Tris-HCl, pH 8.0, 50 mmol/L KCl, 0.5 mmol/L EDTA and 2 mmol/L 2-mercaptoethanol. To the high-speed supernatant glycerol was added (20%). This crude sample was loaded directly onto a GSH-Sepharose affinity column which was equilibrated with buffer A (20 mmol/L Tris-HCl, pH 8.0, 50 mmol/L KCl, 0.5 mmol/L EDTA, 2 mmol/L 2-mercaptoethanol and 20% glycerol). After extensive washing with buffer A containing 150 mmol/L KCl, GST-CKD fusion proteins were eluted from the column with 5 mmol/L GSH in buffer A. The purified fusion proteins were dialyzed against buffer A to remove GSH and stored at −30℃. All the above purification procedure was performed at 4℃. Recombinant CKB was expressed and purified as described[15,17].

1.4 Separation of CKD domain from GST

CKD was separated from the GST tag with thrombin cleavage. The usual thrombin/substrate ratio was 1/500 (w/w). The fusion proteins to be cleaved were either in purified form or bound to GSH-Sepharose beads. In the former case, the cleaving was performed in the cleavage buffer containing 50 mmol/L Tris-HCl, pH 8.0, 150 mmol/L KCl, 2.5 mmol/L $CaCl_2$ and 20% glycerol for about 5 h at 15℃. The GST tag and uncut fusion proteins were then removed by GSH-Sepharose absorption. In the latter case, after extensive washing as described above, the affinity column was equilibrated with the cleavage buffer and the thrombin was added to the column as described[18]. The released CKD was washed out of the column and collected. The thrombin was then removed by heparin-Sepharose absorption.

1.5 6PF-2K activity determination

6PF-2K activity was assayed by the formation of Fru-2,6-P_2, which was quantified by the stimulation of potato tuber pyrophosphate: fructose-6-phosphate-1-phosphotransferase[19]. Unless otherwise stated, the reaction mixture contained 100 mmol/L Tris-HCl, pH 7.4, 5 mmol/L inorganic phosphate (P_i) 1 mmol/L DTT, 10 mmol/L ATP, 1 mmol/L $MgCl_2$ and 2 mmol/L fructose-6-phosphate (Fru-6-P) for CKB, or contained 100 mmol/L Tris-HCl, pH 7.4, 5 mmol/L P_i, 1 mmol/L DTT, 20 mmol/L ATP, 4 mmol/L $MgCl_2$ and 10 mmol/L Fru-6-P for GST-CKD, in a total volume of 50 μL. The reaction was initiated by addition of the enzyme. The mixture was incubated for 10 min at 30℃ and terminated with addition of 10 μL of 1.5 mol/L NaOH. The solution was heated for 1 h at 80℃ and diluted to 1 mL with water. Suitable aliquots of the diluted solution were then assayed for Fru-2, 6-P_2.

1.6 Inactivation by GdnHCl

The enzyme preparations (10 μg each) were incubated with various concentrations of GdnHCl in 20 μL of buffer containing 50 mmol/L Tris-HCl, pH 7.4, 5 mmol/L P_i, 1 mmol/L DTT and 20% glycerol for 10 min at 25℃. Aliquots were then taken and 6PF-2K activity was immediately assayed at 30℃.

1.7 Thermal inactivation

The stability of GST-CKD and CKB was determined by incubating enzymes (10 μg each) in 20 μL of buffer containing 50 mmol/L Tris-HCl, pH 7.4, 50 mmol/L KCl, 0.5 mmol/L EDTA, 1 mmol/L DTT and 20% glycerol for 10 min at the indicated temperatures. Aliquots were taken and residue 6PF-2K activity was immediately assayed for 5 min at 30℃.

1.8 pH dependency

The stability of different enzyme forms at various pH values was determined by diluting the enzyme (10 μg each) in a total volume of 20 μL of buffer containing 40 mmol/L MES, 40 mmol/L Bis Tris propane, 40 mmol/L HEPES, 40 mmol/L Tris and 1 mmol/L DTT, which were adjusted to the indicated pH. After incubating for 10 min at 25℃, aliquots were taken and 6PF-2K activities were measured at pH 7.4 as described above. The pH profile of the 6PF-2K activity was determined by

measuring the production of Fru-2,6-P_2 in 50 μL of buffer at the indicated pH. The reactions were initiated with addition of enzyme and performed for 10 min at 30℃.

2 Results

2.1 Expression and purification

Both CKD^{1-286} and CKD^{1-249} were expressed in active form in *Escherichia coli*, but their activities were lost after purification (data not shown). However, the 6PF-2K activity in purified GST-CKD^{1-286} and GST-CKD^{1-249} was well maintained. The three forms of GST fusion protein of the kinase domain with various lengths were purified to homogeneity by affinity chromatography as judged by SDS-PAGE (Fig. 1). Among the three fusion proteins, GST-CKD^{1-221} displayed only trace 6PF-2K activity, implying that its tertiary structure or catalytic core might be damaged owing to hefty truncation.

The kinase domain was effectively separated from the GST tag with thrombin cleavage. However, little 6PF-2K activity could be detected in the recovered CKD^{1-286} and CKD^{1-249}, though their GST-tagged counterparts were apparently active. Fig. 2 clearly shows that along with the separation from the GST tag was the quick loss of the 6PF-2K activity in CKD^{1-249}. Similar result was also obtained for CKD^{1-286} (data not shown). Thus the GST fusion protein was used to study the properties of the separate kinase domain.

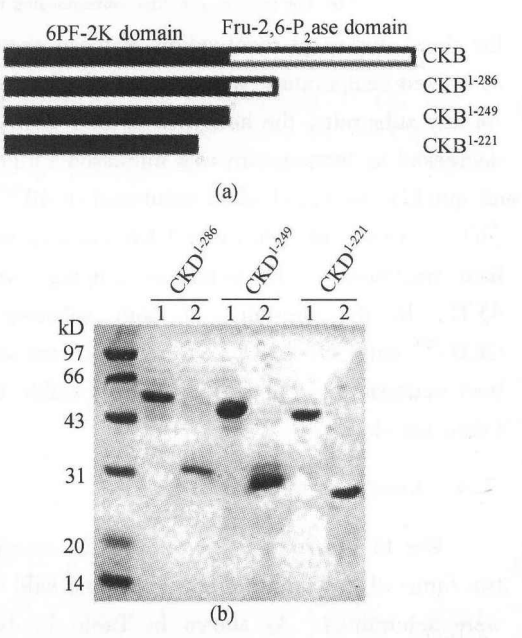

Fig. 1 SDS-PAGE of purified GST-CKD fusion proteins and isolated CKD domains. (a) Schematic representation of the wild type and various CKD forms of CKB. (b) The purified GST-CKD (lane 1) and the isolated CKD after thrombin cleavage (lane 2).

2.2 GdnHCl inactivation

The loss of 6PF-2K activity in CKD during purification or during isolation from GST tag suggested that the separate kinase domain was very unstable. Guanidine inactivation of the kinase domain, in comparison with that of the wild type bifunctional enzyme, was investigated. As shown in Fig. 3, the 6PF-2K activity in GST-CKD^{1-286} and GST-CKD^{1-249} decreased rapidly with increasing concentration of GdnHCl (0 ~ 0.6 mol/L). However, 6PF-2K of CKB was activated by GdnHCl in the same concentration range. When the concentration of GdnHCl exceeded 0.8 mol/L, the 6PF-2K activity in all enzyme forms was lost completely. GST-CKD^{1-249} was a little more liable to GdnHCl inactivation than GST-CKD^{1-286}, (Figure 3).

2.3 Heat denaturation

The 6PF-2K activities in CKB, GST-CKD^{1-286} and GST-CKD^{1-249} after incubating for 10 min at varying temperatures are shown in fig. 4. In order to compare their sensitivity to heat denaturation,

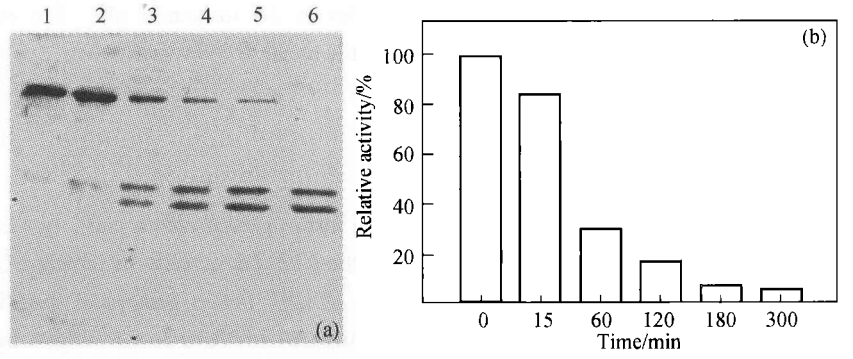

Fig. 2 Thrombin cleavage of GST-CKD$^{1\text{-}249}$. (a) Lane 1, control without thrombin cleavage; lanes 2 ~ 6, samples incubated with thrombin for 15, 60, 120, 240 and 300 min, respectively. (b) The relative activities corresponding to lanes 1 ~ 6 in (a).

the time courses of inactivation of these enzyme forms at a fixed temperature were measured. In the absence of any substrate, the activities of GST-CKD obviously decreased as temperature was increased (Fig. 4(a)), and quickly decreased when incubated at 40℃ (Fig. 4 (b)). In contrast, wild type CKB was less sensitive to heat treatment at temperatures ranging from 25 to 45℃. In the presence of both substrates, GST-CKD$^{1\text{-}286}$ and GST-CKD$^{1\text{-}249}$ were much less sensitive to heat inactivation, but still much less stable than CKB (data not shown).

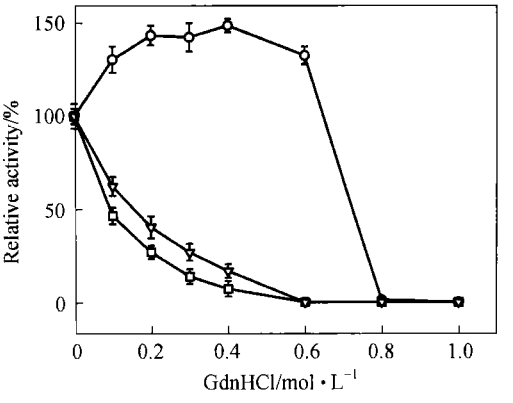

Fig. 3 Effect of GdnHCl on the 6PF-2K activities in CKB, GST-CKD$^{1\text{-}286}$ and GST-CKD$^{1\text{-}249}$. CKB (○), GST-CKD$^{1\text{-}286}$(▽) and GST-CKD$^{1\text{-}249}$(□). Data points are the means ± range from two separate experiments done in duplicate.

2.4 Kinetic properties

The kinetic properties of 6PF-2K activity of the two forms of the kinase domain and the wild type CKB were determined. As shown in Table 1, both GST-CKD$^{1\text{-}286}$ and GST-CKD$^{1\text{-}249}$ have a k_{cat} of 3.1 min^{-1}, which is about 1/4 of that of the wild-type CKB in the non-activated state. Compared with CKB, GST-CKD$^{1\text{-}286}$ and GST-CKD$^{1\text{-}249}$ have K_m values 20- and 17-fold higher for Fru-6-P in the absence of Pi, or 35 and 29-fold higher in the presence of 5 mmol/L Pi, respectively, and K_m values for ATP are 4 ~ 6-fold of that of the wild type enzyme.

Previously it has been reported that the 6PF-2K of either native or recombinant CKB exhibited an inhibition by magnesium ion at high concentration and the optimal concentration of $MgCl_2$ was in the range of 1 ~ 2 mmol/L[15, 20], which made it distinct from other documented 6PF-2K/Fru-2,6-P_2ase. As shown in Fig. 5, higher concentration of $MgCl_2$ significantly inhibited the 6PF-2K activity of CKB. Similar effects of Mg^{2+} were also observed on GST-CKD$^{1\text{-}286}$ and GST-CKD$^{1\text{-}249}$, but the optimal concentration of $MgCl_2$ was around 5 mmol/L and the inhibition was less significant than that on CKB (Figure 5).

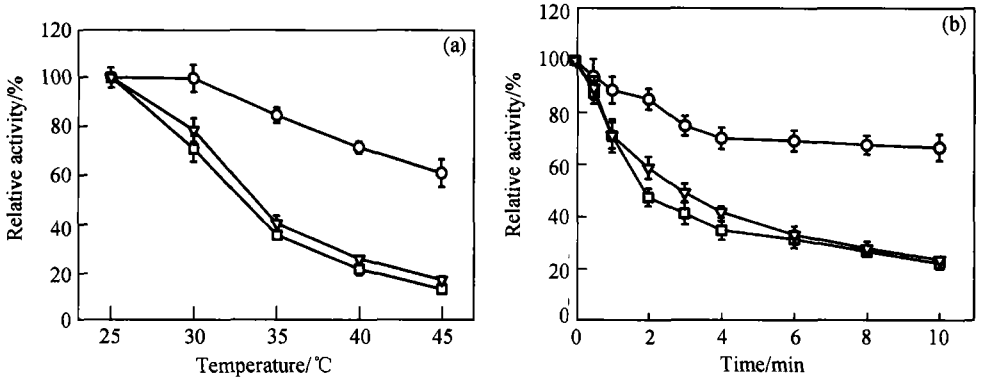

Fig. 4 Thermal inactivation of CKB, GST-CKD$^{1-286}$ and GST-CKD$^{1-249}$. CKB (○), GST-CKD$^{1-286}$ (▽) and GST-CKD$^{1-249}$ (□) were incubated for 10 min at varying temperatures as indicated (a) or incubated for the indicated time at 40℃ (b).

Previous work also revealed that the chicken liver 6PF-2K exhibited ATP substrate activation[15, 21]. From the ATP curve of the enzyme, two k_{cat} values could be obtained which represent the non-activated activity and the activated activity of the enzyme respectively (Table 1). In contrast to CKB, the two GST-CKD fusion proteins did not exhibit ATP activation.

Table 1 Kinetic properties of 6PF-2K in CKB and GST-CKD

Kinetic properties	Enzyme forms		
	CKB	GST-CKD$^{1-286}$	GST-CKD$^{1-249}$
k_{cat}/min^{-1} b)	12 ± 2	3.1 ± 0.2	3.1 ± 0.3
	37 ± 5		
$K_m^{Fru-6-P}$/mmol/L			
− Pi	0.86 ± 0.07	17 ± 2	15 ± 2
+ 5 mmol/L P_i	0.015 ± 0.002	0.52 ± 0.04	0.43 ± 0.05
K_m^{ATP}/ mmol/L b)	0.23 ± 0.02	0.97 ± 0.10	1.4 ± 0.2
	0.64 ± 0.06		
K_a^{Pi}/μmol/L a)	80 ± 7	38 ± 4	25 ± 3

a) K_a value for P_i was defined as the concentration of P_i at which K_m for Fru-6-P was reduced to half in the absence of P_i. V_{max} and K_m values were obtained using a "Hyperbolic regression analysis" program (http://www.liv.ac.uk/~jse/software.html). The values represent average ± ranges for three determinations. b) Double reciprocal plots of 6PF-2K activity of CKB versus ATP concentration revealed two slopes (Fig. 6). Fitting the data corresponding to the two ATP ranges (0.05 ~ 0.4 mmol/L and 0.4 ~ 4 mmol/L) separately into the above-mentioned software yielded two k_{cat}, and correspondingly two K_m values.

2.5 pH dependency

The stability and 6PF-2K activity of GST-CKD and CKB at various pH values were also compared. Fig. 6 shows that in the low pH range (pH 6 ~ 7), the two GST-CKD forms are less stable than CKB; and in the range of pH 7 ~ 9, both CKD and CKB are stable under the indicated condition (Fig. 6(a)). There is no significant difference in pH dependency of the 6PF-2K activity of the CKD and CKB in the range of pH 6 ~ 9 (Fig. 6(b)).

3 Discussion

Expression of the separate 6PF-2K domain has been performed previously on the rat liver en-

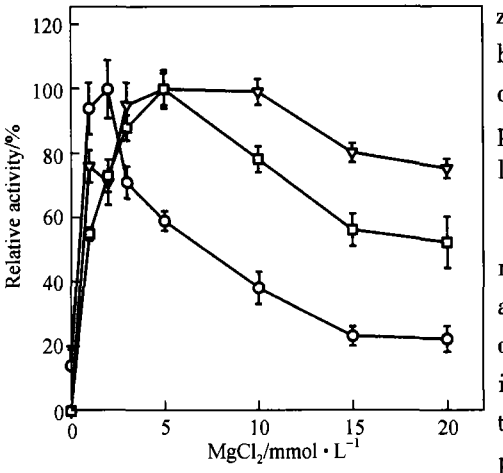

Fig. 5 Effect of Mg^{2+} on the 6PF-2K activities of CKB, GST-CKD$^{1-286}$ and GST-CKD$^{1-249}$. The results represent the mean ± ranges of 4 determinations. CKB (○), GST-CKD$^{1-286}$(▽), GST-CKD$^{1-249}$(□).

zyme[14]. Although the rat liver kinase domain has been shown to be an active form, the properties of this domain remain unknown. In this work we report the properties of the separate kinase domain from chicken liver bifunctional enzyme.

The separate kinase domain of CKB, including residues either 1 ~ 286 or 1 ~ 249, was expressed in active form in *Escherichia coli*. But purification of this domain resulted in nearly total loss of its activity though it was in homogeneous form (Figs. 1 and 2). In contrast, the GST fusion protein of the kinase domain was purified as an active enzyme. CKD fusion proteins were more sensitive towards GdnHCl inactivation, more liable to heat treatment, and slightly less stable in acidic pH range in the absence of any substrate than the intact CKB. In general, the separate kinase domain was not as stable as the kinase domain in the wild type enzyme. This suggests that the structure of the separate kinase domain might not be as perfect as that in CKB. The presence of the bisphosphatase domain seems necessary for the stability of the kinase domain. So far no separate 6PF-2K domain or Fru-2,6-P_2ase domain has been found in nature, implying their mutual dependence for stability, especially the dependence of the kinase domain on the bisphosphatase domain. Although the yeast 6PF-2K and Fru-2,6-P_2ase activities are possessed separately by two enzymes PFK26 and FBP26[22,23], both yeasts PFK26 and FBP26 contain kinase and bisphosphatase domains, with natural Fru-2,6-P_2ase-dead mutation and 6PF-2K-dead mutation, respectively.

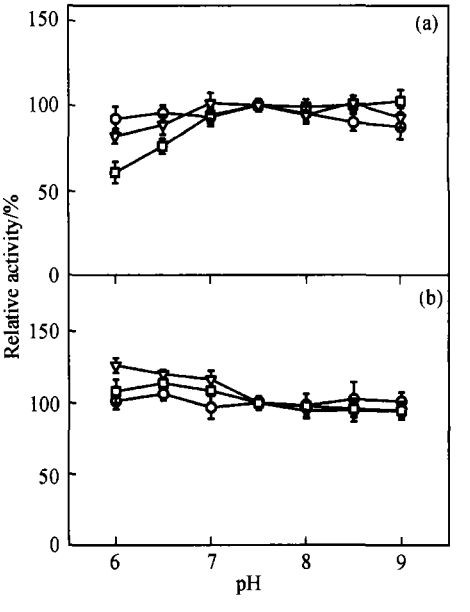

Fig. 6 pH dependency of 6PF-2K activity of CKB, GST-CKD$^{1-286}$ and GST-CKD$^{1-249}$. (a) The stability of different enzyme forms at various pH values. (b) The relative 6PF-2K activities assayed at various pH. Data points are the mean ± ranges from two separate experiments done in duplicate. CKB (○), GST-CKD$^{1-286}$(▽) and GST-CKD$^{1-249}$(□).

Both GST-CKD$^{1-286}$ and GST-CKD$^{1-249}$ specifically catalyzed the synthesis of Fru-2,6-P_2 with a k_{cat} of 3.1 min^{-1}, which was comparable to the k_{cat} of rat liver 6PF-2K/Fru-2, 6-P_2ase, 3.0 ~ 5.4 min^{-1} (calculated from the reported V_{max} values[24,25]). Although the K_m for Fru-6-P of the CKD form was much higher than that of CKB, the affinity for Fru-6-P of the CKD form could be increased by the physiological concentration of Pi with similar magnitude to that of CKB. The results suggest that the kinase domain might be functionally independent of the bisphosphatase domain. In addition, gel filtration determination revealed that both separate CKD domain and GST-CKD fu-

sion protein were oligomeric with molecular weight greater than 800 kD (data not shown). This was inconsistent with the report on rat liver kinase domain[14]. The low activity observed in the kinase fusion protein might be caused, at least partially, by the aggregation of the enzyme.

It was previously reported that CKB could be activated by substrate ATP, and the binding of ATP to CKB exhibited negative cooperativity[15,20,21]. This phenomenon could be caused by either the interaction between two kinase domains of the homodimers or the interaction between the kinase domain and the bisphosphatase domain, since a nucleotide binding motif and a site have been identified in the bisphosphatase domain[26,27]. Recently, we have revealed that the ATP activation of CKB was caused by ATP binding to the bisphosphatase domain, using site-directed mutagenesis (unpublished data). This was confirmed by the observation in this work that the separate kinase domain did not exhibit ATP activation. The characterization of the separate kinase domain not only supports the postulation that the bifunctional enzyme is formed as a result of gene fusion event, but also reflects the mutual structural and functional dependence between the kinase domain and bisphosphatase domain.

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