 Binding of ATP to the Fructose-2,6-bisphosphatase Domain of Chicken Liver 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase Leads to Activation of Its 6-Phosphofructo-2-kinase

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To understand the mechanism by which the activity of the 6-phosphofructo-2-kinase (6PF-2K) of chicken liver 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase is stimulated by its substrate ATP, we studied two mutants of the enzyme. Mutation of either Arg-279, the penultimate basic residue within the Walker A nucleotide-binding fold in the bisphosphatase domain, or Arg-359 to Ala eliminated the activation of the chicken 6PF-2K by ATP. Binding analysis by fluorescence spectroscopy using 2’(3’)-O-(N-methylanthraniloyl)-ATP revealed that the kinase domains of these two mutants, unlike that of the wild type enzyme, showed no cooperativity in ATP binding and that the mutant enzymes possess only the high affinity ATP binding site, suggesting that the ATP binding site on the bisphosphatase domain represents the low affinity site. This conclusion was supported by the result that the affinity of ATP for the isolated bisphosphatase domain is similar to that for the low affinity site in the wild type enzyme. In addition, we found that the 6PF-2K of a chimeric enzyme, in which the last 25 residues of chicken enzyme were replaced with those of the rat enzyme, could not be activated by ATP, despite the fact that the ATP-binding properties of this chimeric enzyme were not different from those of the wild type chicken enzyme. These results demonstrate that activation of the chicken 6PF-2K by ATP may result from allosteric binding of ATP to the bisphosphatase domain where residues Arg-279 and Arg-359 are critically involved and require specific C-terminal sequences.

The bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (6PF-2K/Fru-2,6-P₂ase)† is a homodimer, and each subunit contains an N-terminal kinase domain and a C-terminal bisphosphatase domain (1, 2). The C-terminal Fru-2,6-P₂ase domain is homologous to the glycerate mutase and acid phosphatase families (3–5). Fru-2,6-P₂ase catalyzes the hydrolysis of Fru-2,6-P₂ by the formation of a phosphoenzyme intermediate through phosphorylated His-258. Residue His-258 along with Glu-327 and His-392 comprise the catalytic triad in rat liver Fru-2,6-P₂ase (reviewed in Ref 2). Recent work using x-ray crystallography and NMR supports the importance of these residues and provides further insights into the reaction mechanism (4, 6, 7). In addition, the structural study by Hasemann et al. (5) and the modeling work by Bertrand et al. (8) have demonstrated independently that the N-terminal 6PF-2K domain is structurally related to mononucleotide-binding proteins, such as adenylate kinase and the catalytic cores of G proteins. Based on the structural comparison, Hasemann et al. (5) further proposed that the 6PF-2K domain, like G proteins, operates via a transition state stabilization mechanism and predicted additional residues important for substrate binding or catalysis, which were subsequently confirmed by mutation (9, 10).

It has long been recognized that both the N and C termini of the enzyme play important roles in the regulation of the 6PF-2K and the Fru-2,6-P₂ase activities (11). Recently, Wu et al. (12) found that the charged residues as well as Ser²⁰–Ser²⁴ in the N-terminal tail might be involved in interactions with the catalytic domains. The interaction may lead to increased 6PF-2K and reduced Fru-2,6-P₂ase activities, as disruption of this N-terminal interaction resulted in the reduction in both kinase and phosphatase activities. The work of Kurland et al. (13) revealed that residues Gly²⁰–Glu³⁰–Leu³¹ of the liver isoform are responsible for the increase in the affinity of 6PF-2K for Fru-6-P, the inhibition of Fru-2,6-P₂ase activity, and the effects of cAMP-dependent protein kinase phosphorylation on the two activities. The C terminus of the bifunctional enzyme has a regulatory function on both the kinase and bisphosphatase activities, and it is generally regarded as having an inhibitory effect on the bisphosphatase activity (14–17).

The kinase domain of the bifunctional enzyme has a Walker A motif (Gly-X-X-X-Gly-Lys-Thr) and B motif (Z-Z-Z-Z-Asp), which are typical of nucleotide-binding proteins (3, 18, 19). Mutation of the first Gly of the A motif abolished the kinase activity of the rat liver bifunctional enzyme (20). The Lys residue in the A motif is critical for ATP binding, and the Thr residue is necessary for catalysis (21, 10). The Asp residue in the B motif is also important for the binding of ATP, as it coordinates the ATP-bound Mg²⁺ (18). Interestingly, a sequence characteristic of the Walker A motif was also identified in the Fru-2,6-P₂ase domain (22). Early work on rat hepatic enzyme revealed that the isolated Fru-2,6-P₂ase domain can be regulated by GTP, ATP, and other nucleotide triphosphates, and Arg-360 was important for the regulation of Fru-2,6-P₂ase activity by nucleotide triphosphates (22). These results suggest...
that the nucleotide triphosphates might regulate the activity of Fru-2,6-P₉ase by binding specifically to this domain. In addition, it has been found that 6PF-2K of chicken hepatic enzyme was activated by substrate ATP. The Hill plot of the kinase activity against the concentrations of ATP yielded a coefficient of 0.56 (23). Such a phenomenon might be either the result of negative cooperativity between the two identical ATP binding sites in the kinase domains of the homodimeric enzyme or the allosteric binding of ATP to the Fru-2,6-P₉ase domain. In this work, we provide evidence to support the latter possibility by using site-directed mutagenesis and biochemical approaches.

**EXPERIMENTAL PROCEDURES**

**Materials**—Restriction endonucleases and other DNA modifying enzymes were obtained from Life Technologies, Inc. or New England Biolabs. ATP, Fru-6-P, and Fru-2,6-P₂ were purchased from Sigma. N-methylisatoic anhydride was purchased from Aldrich.

**Construction of Expression Plasmids and Protein Preparation**—Point mutations were made using standard polymerase chain reaction techniques and checked by double strand DNA sequencing. The construct for chimeric enzyme, where the last 25 residues from the C terminus of CKB was replaced with that of RKB (CKB-RCT) was generated as reported elsewhere (17). All forms of CKBR were expressed in Escherichia coli BL21(DE3) using pET3a plasmid and purified to homogeneity by the same procedures for the preparation of the wild type CKB (24, 25).

**Preparation of Mat-ATP**—The N-methan-threonyl derivative of ATP was synthesized according to Hiratsuma (26). After reaction, the product was purified according to Woodward et al. (27), except that a DEAE-Sephadex A-25 column was used instead of a DEAE-cellulose column. The purified Mant-ATP was verified by absorption spectra, fluorescence spectra, and thin-layer chromatography (data not shown).

**Determinations of Circular Dichroism Spectra**—All far UV spectra were collected on a Jasco 720 dichrograph in a 0.01-cm cell at 25 °C. Scans were collected at 50 nm/min between 190 and 250 nm with a response of 2 s and resolution of 0.2 nm. The bandwidth was 1 nm. All data were the average of four blank corrected samples. The proteins were diluted with buffer containing 0.1 M Tris-HCl, pH 7.5, and 1 mM EDTA, 50 mM KCl, and 1 mM DTT by mini-gel filtration. Samples were centrifuged for 10 min at 15,000 × g to remove any precipitates, and the A₂₈₀ was measured to scale the CD data to the same concentration.

**Fluorescence Measurements**—Different forms of CKB (final concentration, 20 μg/ml) were incubated for 20 min at 25 °C with various concentrations of GdnHCl in 50 mM Tris-HCl, pH 7.5, and 1 mM DTT. Protein fluorescence was measured with an Hitachi F-4010 fluorescence spectrophotometer from 300 to 400 nm with an excitation wavelength of 295 nm. Protein fluorescence intensity at 355 nm and the maximum emission wavelength were recorded.

**Fluorescence Determination of Ligand Binding**—The increase in the intrinsic fluorescence was employed to measure the binding of Mant-ATP to different enzyme forms at 20 °C with an Hitachi F-4010 fluorescence spectrophotometer. The spectral bandwidths were 3 and 5 nm, respectively, for excitation and emission. Mant-ATP (50 μM-5 mm) was added dropwise (1–2 μl) to 90–100 μg of enzyme diluted in 1 ml of buffer containing 100 mM Tris-HCl, pH 7.4, 0.5 mM EDTA, 5 mM MgCl₂, and 5 mM MgCl₂. The excitation wavelength was 350 nm, and the fluorescence was recorded from 400 to 500 nm. The fluorescence of free Mant-ATP in the absence of enzyme was also recorded under the same conditions. The differential spectrum resulted from subtracting the fluorescence of Mant-ATP from that in the presence of enzyme. All measurements were corrected for dilution (less than 3%) and inner filter effects.

**6PF-2K Activity Determination**—6PF-2K activity was assayed by the formation of Fru-2,6-P₉, which was quantified by the stimulation of potato tuber pyrophosphatase (fructose-6-phosphate phosphotransferase) (28). Unless stated otherwise, the reaction mixture contained 100 μg Tris-HCl, pH 7.4, 5 mM P₄, 1 mM EDTA, 10 mM ATP, 10 mM MgCl₂, and 2 mM MgCl₂. The final volume of 50 μl of reaction was increased 2-fold by the addition of enzyme. The mixture was incubated for 10 min at 30 °C and terminated with the addition of 1.5 μl NaOH. The solution was heated for 1 h at 80 °C and diluted to 1 ml with water. Suitable aliquots of the diluted solution were then assayed for Fru-2,6-P₉.

**Fru-2,6-P₉ase Activity Determination**—The activity of Fru-2,6-P₉ase was assayed at pH 7.4 by following the rate of production of [13C]Fru from [2-32P]Fru in 280 was measured to scale the CD data to the same concentration.

**RESULTS**

**Effects of Mutation of Arg-279 and Arg-359 of CKB on the Properties of Fru-2,6-P₉ase**—Our early work showed that the 6PF-2K activity of the chicken liver bifunctional enzyme was regulated by its substrate ATP, indicating an apparent negative cooperativity for the enzyme in binding of ATP (23, 24). Because 6PF-2K/Fru-2,6-P₉ase is a homodimer, such cooperativity might result from the interaction between the two ATP binding sites of the kinase domains of the CKB homodimer (23, 24). Alternatively, the cooperativity may result from the interaction between the ATP binding site of the kinase domain and that of the bisphosphatase domain. To determine which mechanism underlies the ATP activation of chicken 6PF-2K, we examined whether elimination of the binding of ATP to the bisphosphatase domain affected the activation of chicken 6PF-2K.

Previously, it was reported that the Fru-2,6-P₉ase activities of the hepatic bifunctional enzyme was regulated by triphosphate nucleotides such as GTP or ATP (22, 28). Lee et al. (22) reported that the activation of the Fru-2,6-P₉ase by GTP or ATP involved a direct interaction of the triphosphate nucleotides with the active site of the bisphosphatase domain, and the activation is caused by the phosphate moieties of the triphosphate nucleotides competing with the 2-phosphogroup of Fru-2,6-P₂ for the phosphoenzyme interaction, thus relieving substrate inhibition. Arg-360 of rat liver enzyme was demonstrated as a critical residue responsible for the substrate inhibition (30) and is important for the binding of GTP to the bisphosphatase domain (22). As shown in Table I, this residue is highly conserved among various isoforms of the bifunctional enzymes; the corresponding residue of the chicken liver enzyme is Arg-359. In addition, the nucleotide-binding motif (Gly-274-Leu-Ser-Ala-Gly-Lys-Gln) found in the bisphosphatase domain of RKB (22) is also conserved in CKB (Table I). The penultimate basic residue was shown previously to be critical for nucleotide binding (31). The corresponding residue in the chicken liver enzyme is Arg-359 (Table I). To investigate whether Arg-279 and/or Arg-359 are involved in the binding of ATP to the bisphosphatase domain of CKB, two mutants, CKBR279A and CKBR359A, were produced and analyzed.

| Enzyme species | Sequence alignment |
|----------------|-------------------|
| Chicken liver | GLSVEKGYA...RDQKRYYRPK |
| Rat liver     | GLSVEKGYA...RDQKRYYRPK |
| Rat testis    | GLSVEKGDFA...RDQKLYRYPK |
| Rat skeletal muscle | GLSVEKGYA...RDQKRYYRPK |
| Rat brain     | GLSVEKGYA...RDQKLYRYPK |
| Human liver   | GLSVEKGYA...RDQKRYYRPK |
| Bovine heart  | GLSVEKGYA...RDQKLYRYPK |

The Walker A motif in the bisphosphatase domain of CKB with the corresponding sequences in other isoforms of the bifunctional enzyme

The Walker A motif in the bisphosphatase domain of CKB is underlined, and the two basic amino acid residues, Arg-279 and Arg-359, are in bold face type. The corresponding residues in RKB are Lys-280 and Arg-360, respectively.

**Table I**

**Comparison of the Walker A motif and the sequence around Arg-359 in the bisphosphatase domain of CKB with the corresponding sequences in other isoforms of the bifunctional enzyme**

The Walker A motif in the bisphosphatase domain of CKB was replaced with that of RKB (CKB-RCT), was generated as described previously by El-Maghrabi et al. (24). Alternatively, the cooperativity may result from the interaction between the ATP binding site of the kinase domain and that of the bisphosphatase domain. To determine which mechanism underlies the ATP activation of chicken 6PF-2K, we examined whether elimination of the binding of ATP to the bisphosphatase domain affected the activation of chicken 6PF-2K.

**Table II**

**Properties of Fru-2,6-P₂ase**

Our early work showed that the 6PF-2K activity of the chicken liver bifunctional enzyme was stimulated by its substrate ATP, indicating an apparent negative cooperativity for the enzyme in binding of ATP (23, 24). Because 6PF-2K/Fru-2,6-P₉ase is a homodimer, such cooperativity might result from the interaction between the two ATP binding sites of the kinase domains of the CKB homodimer (23, 24). Alternatively, the cooperativity may result from the interaction between the ATP binding site of the kinase domain and that of the bisphosphatase domain. To determine which mechanism underlies the ATP activation of chicken 6PF-2K, we examined whether elimination of the binding of ATP to the bisphosphatase domain affected the activation of chicken 6PF-2K.
ATP Activation of Chicken Liver 6-Phosphofructo-2-kinase

The 6PF-2K activity was measured in buffer containing 100 mM Tris-HCl, pH 7.4, 5 mM P<sub>i</sub>, 1 mM DTT, and 10 mM MgCl<sub>2</sub>. The double regression analysis program. For the wild type CKB, fitting the data corresponding to the two ATP ranges (0.05–0.4 and 0.4–4 mM) separately into the above mentioned software yielded two V<sub>max</sub> and, correspondingly, two K<sub>m</sub> values. The values represent the mean ± S.D. for three determinations.

| Kinetic properties | Enzyme forms |
|--------------------|--------------|
|                    | CKB         | CKB<sup>R279A</sup> | CKB<sup>R359A</sup> | CKB-RCT | RKB |
| 6PF-2K V<sub>max</sub> (milliunits/mg) | 97 ± 16 | 197 ± 20 | 74 ± 8 | 130 ± 11 | 120 ± 15 |
| K<sub>ATP</sub> (mM) | 0.11 ± 0.03 | 0.19 ± 0.05 | 0.10 ± 0.03 | 0.17 ± 0.06 | 0.077 ± 0.013 |
| K<sub>Fru-6-P</sub> (mM) | 0.86 ± 0.07 | 0.63 ± 0.05 | 0.72 ± 0.07 | 0.97 ± 0.11 | 0.91 ± 0.10 |
| Fru-2,6-P<sub>2</sub>ase V<sub>max</sub> (milliunits/mg) | 16 ± 3 | 15 ± 3 | 18 ± 5 | 39 ± 4 | 37 ± 4 |
| K<sub>m</sub> Fru-2,6-P<sub>2</sub> (mM) | 0.13 ± 0.04 | 0.11 ± 0.02 | 0.85 ± 0.11 | 0.17 ± 0.08 | 0.17 ± 0.03 |

CKB<sup>R279A</sup> showed V<sub>max</sub> and K<sub>m</sub> values for Fru-2,6-P<sub>2</sub> similar to those of the wild type CKB and acted similarly to the wild type enzyme with regard to substrate inhibition (Fig. 1). However, the Fru-2,6-P<sub>2</sub>ase activity of CKB<sup>R279A</sup>, unlike the wild type enzyme, could not be activated by ATP (Fig. 2), suggesting that Arg-279 of CKB is required for ATP binding to the Fru-2,6-P<sub>2</sub>ase domain.

Effects of Mutation of Arg-279 and Arg-359 on ATP Activation of Chicken Liver 6PF-2K—Knowing that the two Arg residues are important for the Fru-2,6-P<sub>2</sub>ase domain to bind ATP, an important question is whether the two mutations affect the activation of the 6PF-2K of CKB by ATP. The 6PF-2K of the wild type CKB exhibited substrate activation by ATP, as reported earlier when assayed in the presence of 1-2 mM of MgCl<sub>2</sub> (23, 24). Although MgCl<sub>2</sub> at concentrations over 2 mM inhibits the chicken liver 6PF-2K, it does not affect the substrate activation of 6PF-2K of wild type CKB by ATP, as the ATP activation was also observed in the presence of 10 mM MgCl<sub>2</sub> (Fig. 3). For convenience, all of the following kinetic analyses were carried out in buffers containing 10 mM MgCl<sub>2</sub>. The double reciprocal plot of 6PF-2K activity of CKB versus the ATP concentrations revealed two slopes (Fig. 3), which yielded two pairs of K<sub>m</sub> and V<sub>max</sub> values corresponding to the nonactivated and the ATP-activated activities of the 6PF-2K of CKB, respectively (Table II). In contrast, neither CKB<sup>R279A</sup> nor CKB<sup>R359A</sup> exhibited ATP activation (Fig. 3) Because these two mutations did not affect the K<sub>m</sub> of the 6PF-2K for Fru-6-P (Table II), it is likely that the ATP activation of the chicken liver 6PF-2K is caused by allosteric binding of ATP to the Fru-2,6-P<sub>2</sub>ase domain.

CD Spectra and Fluorescence Spectra of CKB<sup>R279A</sup> and CKB<sup>R359A</sup>—To determine whether any of these two mutations grossly alters the secondary structure of the enzyme, the circular dichroic spectra of the mutant and wild type forms of CKB were examined. As shown in Fig. 4, the circular dichroic spectra of various forms of CKB were very similar: The wild type CKB showed 29.1 % a-helix, 26.9 % 310-helix, and 11.3 % 10.2 % 3.1-helix. These data indicated that the mutations are unlikely to cause significant changes in the secondary structure of the enzyme. This conclusion was further confirmed by the fluorescence spectroscopic analysis. Fig. 5 shows that there were no significant differences between either of the mutant forms and the wild type CKB in fluorescence quenching or red shift of the emission maximum caused by Dn-HCl.

Binding Analysis Employing Mant-ATP—Although the analysis of the kinetic parameters of the two mutants suggested that the binding of ATP to the Fru-2,6-P<sub>2</sub>ase domain regulates the 6PF-2K activity allosterically, direct measurement of ATP binding of the enzyme should provide more straightforward evidence. Thus the ATP binding properties of these different forms of CKB were investigated by fluorescence spectroscopy, using Mant-ATP as ligand.
Mant-ATP had been previously used to study the ATP binding properties of RKB and the rat testis enzyme (21, 32). CKB could utilize Mant-ATP as phosphate donor with a $K_m$ value of 14 $\mu$M and $V_{max}$ of 75 milliunits/mg. In addition, the 6PF-2K activities were inhibited by Mant-ATP at higher concentrations (>0.1 mM) (data not shown), consistent with the report on the rat testis enzyme (32).

The addition of CKB resulted in an increase of the fluorescence of the Mant-ATP under the conditions described under "Experimental Procedures." Such an increase caused by enzyme was used to analyze the binding of enzymes to Mant-ATP. A Scatchard plot of $\Delta F$ (the fluorescence change) versus $\Delta F/\text{Mant-ATP}$ (the fluorescence change over the concentration of Mant-ATP) showed that the wild type CKB exhibits an apparent negative cooperativity for binding Mant-ATP. As shown in Fig. 6, it seems that the wild type enzyme has two kinds of sites.
with different affinities for binding of ATP, as there were the two distinct slopes for ATP binding showing two different dissociation constants \(K_d\) (Table III). However, CKB\(^{R279A}\) and CKB\(^{R359A}\) did not show cooperativity in binding Mant-ATP (Fig. 6), and there is only one \(K_d\) value of CKB\(^{R279A}\) or CKB\(^{R359A}\), which is similar to the high affinity value of the wild type CKB (Table III). We also tested the binding of the isolated bisphosphatase domain of CKB (CBD) with Mant-ATP. As expected, CBD bound Mant-ATP with one \(K_d\) value equivalent to the low affinity value of CKB (Fig. 6, Table III). These data indicate that the mutation of Arg-359 or Arg-279 to Ala eliminates both the binding of ATP to the bisphosphatase domain of the bifunctional enzyme and the activation of 6PF-2K by ATP in CKB and that the Walker A motif in the bisphosphatase domain is critical for the binding of ATP. Considering these together with the kinetic data, it is reasonable to conclude that the ATP activation of the chicken liver 6PF-2K is caused by the allosteric binding of ATP to the Fru-2,6-P\(_2\)ase domain of the enzyme.

**Effect of the Replacement of C-terminal Tail of CKB with That of RKB in the 6PF-2K Properties of the Enzyme**—Despite the high homology between RKB and CKB, ATP activation of 6PF-2K was not observed in RKB. It raised the question of whether this difference is due to the divergence in their amino acid sequences at the C and N termini, in which the majority of amino acid sequence differences between the two enzymes are found. Recently, we found that a chimeric enzyme, CKB-RCT, in which the C-terminal sequence of CKB was replaced with that of RKB, mimicked RKB in all of the kinetic properties of the Fru-2,6-P\(_2\)ase activity (17). It was interesting to see whether the replacement of the C-terminal tail also affects the kinetic behavior of the N-terminal 6PF-2K. In comparison with the wild type CKB and RKB, CKB-RCT was more like RKB than CKB in the 6PF-2K properties; particularly, the 6PF-2K of CKB-RCT could not be activated by substrate ATP (Table II and Fig. 7A).

Regardless of their different kinetic properties in the 6PF-2K activity, CKB, RKB, and CKB-RCT had similar properties in binding to Mant-ATP, as shown in Fig. 7B and Table III. Thus, the difference in the C-terminal tails between these two hepatic enzymes may be accounted for by the differences in the kinetic properties of the 6PF-2K in respect to ATP. The two \(K_d\) values of RKB for Mant-ATP were in the same ranges as those of CKB (Table III), and the \(K_d\) value for the high affinity binding was also similar to that of the rat testis enzyme (32). However, these values were different from those of RKB as reported by Vertommen et al. (21), probably because of different assay conditions.

**DISCUSSION**

Two models, the pre-existent asymmetric model and the ligand-induced sequential model, have been proposed to explain the apparent negative cooperativity (33). The present study supports the pre-existent asymmetric model for the binding of ATP to the bifunctional enzymes. It is the allosteric binding of ATP to the bisphosphatase domain that causes the activation of 6PF-2K in CKB. The binding of the bisphosphatase domain to ATP is specific because it has a Walker A motif, which is typical of nucleotide-binding. In addition, Arg-279, a basic residue within this motif, has here been proved vital for binding of ATP. Another basic residue, Arg-359, is equally important for the binding of ATP to the bisphosphatase domain; this was suggested initially by Lee et al. (22) and confirmed by our present work. Although the Fru-2,6-P\(_2\)ase domain shares homology with proteins of the acid phosphatases and phosphoglycerate mutase families, neither acid phosphatases and phosphoglycerate mutases possess the nucleotide-binding motif (3). The appearance of the nucleotide-binding sequence \(^{273}\)Gly-Leu-Ser-Thr-Arg-Gly-Agl\(^{280}\) in CKB in the bisphosphatase domain of the bifunctional enzyme might be an event that occurs later in evolution to meet the demand of the multiple and subtle regulations of the critical bifunctional enzyme.

High concentrations of ATP favor the binding of ATP to the allosteric binding site in the bisphosphatase domain, which might induce a global structural change of that domain, and this in turn affects the catalytic core of the kinase domain, resulting in the activation of 6PF-2K. CKB is highly homologous to all documented mammalian 6PF-2K/Fru-2,6-P\(_2\)ases.
For example, CKB and RKB share about 88% amino acid sequence identity (34). Although RKB also exhibited negative cooperativity in ATP binding as revealed by a Mant-ATP binding analysis (Fig. 7B), this isoform did not show ATP activation of 6PF-2K (Fig. 7A). Interestingly, ATP activation of 6PF-2K was not observed on the chimeric enzyme CKB-RCT, where the C-terminal tail of CKB was replaced with that of RKB (Fig. 7). Therefore, it is reasonable to believe that the C-terminal region of CKB is involved in the activation of the 6PF-2K by the binding of ATP to the bisphosphatase domain and that the differences in the C-terminal amino acid sequences between the chicken and rat enzyme are responsible for the differences in their kinetic properties in respect to ATP. In addition, our recent work had revealed a role of the C-terminal tail of CKB in the mediation of the repressive effect of the kinase domain on the Fru-2,6-P2ase activity of the enzyme.2 Taken together, these data demonstrate the importance of the C-terminal tail in the modulation of the 6PF-2K activity by the bisphosphatase domain, and vice versa. Although the N-terminal tail of bifunctional enzyme is also critically involved in the regulation of enzyme activities, its role in the ATP-activation of chicken liver 6PF-2K remains to be determined.

The \( V_{\text{max}} \) ratio of 6PF-2K to Fru-2,6-P2ase was 6–13 for CKB, which was 2–4-fold higher than that for RKB (Table II), indicating that CKB behaves more like a 6PF-2K comparing with RKB. In addition, the 6PF-2K of CKB is activated by high concentrations of ATP whereas RKB cannot, and the inhibition of 6PF-2K activity of CKB by cAMP-dependent protein kinase is also critically involved in the regulation of the C-terminal tail of bifunctional enzyme is also critically involved in the regulation of enzyme activities, its role in the ATP-activation of chicken liver 6PF-2K remains to be determined.

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