Involvement of the chicken liver 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase sequence His^{444}-Arg-Glu-Arg in modulation of the bisphosphatase activity by its kinase domain

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The bisphosphatase activity of the hepatic bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase is repressed by its kinase domain, and regulated by cAMP-dependent protein kinase (PKA)-catalysed phosphorylation. In the present study, the mechanism by which the bisphosphatase activity is repressed by the kinase domain and regulated by phosphorylation was investigated. We found that truncation of the C-terminus of the enzyme by 25, but not 20, amino acids dramatically enhanced the catalytic rate of the bisphosphatase, abrogated the inhibition by the kinase domain, and eliminated the effect of PKA-mediated phosphorylation on activity. In addition, mutation of His^{444}-Arg-Glu-Arg to Ala-Ala-Glu-Ala had similar effects as the deletion. Moreover, the mutations also significantly affected the phosphorylation-mediated regulation of the kinase activity of the enzyme. Furthermore, the mutations altered the pH-dependence of the bisphosphatase, and the mutant bisphosphatases were more sensitive to modification by diethyl pyrocarbonate and guanidine-induced inactivation than the wild-type enzyme. Taken together, these results demonstrate that the sequence His^{444}-Arg-Glu-Arg plays a critical role in repression of the bisphosphatase activity by both the N-terminal kinase domain and the C-terminal tail itself. These results also explain the activation of the bisphosphatase activity by PKA-catalysed phosphorylation, by suggesting that phosphorylation may relieve the inhibitory effect of the kinase domain that is mediated by the three basic residues in this sequence.

Key words: bifunctional enzyme, mutagenesis, phosphorylation, terminal region.

INTRODUCTION

The bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (6PF-2K/Fru-2,6-P_2ase) catalyses both the synthesis and the hydrolysis of the important regulatory metabolite fructose-2,6-bisphosphate (Fru-2,6-P_2). The enzyme consists of two independent catalytic domains, the N-terminal kinase domain and the C-terminal bisphosphatase domain, which catalyse the two opposite reactions [1,2]. Several tissue-specific isoforms of the enzyme, including those from the liver, heart, muscle, brain and testis, have been characterized [3–8]. There is a high degree of conservation in the core structures of both the kinase and bisphosphatase domains of all of these isoenzymes, but the various isoenzymes vary significantly in their lengths and amino acid sequences at their N- and C-terminal regions, which serve to adapt the kinetic properties of the catalytic cores to the metabolic exigencies of a particular tissue [1,2]. Among these isoenzymes, the liver, heart and brain isoforms were found to have protein kinase phosphorylation sites in their N-termini and/or C-termini, and phosphorylation regulates their kinase and/or bisphosphatase activities [9].

The hepatic bifunctional enzyme is the only isoform whose kinase and bisphosphatase activities are regulated reciprocally by cAMP-dependent protein kinase (PKA)-mediated phosphorylation at a single serine residue in the N-terminal tail [9]. Phosphorylation of rat liver 6PF-2K/Fru-2,6-P_2ase (RKB) at Ser^{32} at physiological pH increases the Fru-2,6-P_2ase activity by 2–3-fold and the K_m of 6PF-2K for fructose 6-phosphate (Fru-6-*P*) by 10–15-fold [10]. Previous studies have indicated that the regulation of the kinase and bisphosphatase activities by PKA-catalysed phosphorylation involves both the N-terminus and the C-terminus of this enzyme. For example, a 4-fold increase in the k_{cat} of Fru-2,6-P_2ase and a 20-fold decrease in the affinity of 6PF-2K for Fru-6-*P* compared with wild-type RKB were observed when the N-terminal 22 residues were deleted from RKB. The deletion also diminished the effect of PKA-mediated phosphorylation on the activation of Fru-2,6-P_2ase and the inhibition of 6PF-2K [10]. Furthermore, deletion of the C-terminal 30 amino acids of RKB enhanced the Fru-2,6-P_2ase k_{cat} by 9-fold and the K_m for Fru-6-*P* by approx. 2-fold, and completely abolished the effect of PKA-mediated phosphorylation on Fru-2,6-P_2ase activity [11]. These data suggest that the two opposite activities of the hepatic isoenzyme are modulated by both the N- and C-terminal sequences of the enzyme, and that PKA-dependent regulation of this enzyme may be mediated by these autoregulatory sequences.

The mutual regulatory nature of the two enzymic domains raises two interesting questions: how do these two domains inter-

Abbreviations used: 6PF-2K, 6-phosphofructo-2-kinase; Fru-2,6-P_2ase, fructose-2,6-bisphosphatase; CKB, chicken liver 6PF-2K/Fru-2,6-P_2ase; RKB, rat liver 6PF-2K/Fru-2,6-P_2ase; CBD, separate chicken liver Fru-2,6-P_2ase domain; CKB^{Cxdel}, C-terminal *x*-residue deletion mutant of CKB; Fru-2,6-P_2, fructose 2,6-bisphosphate; Fru-6-*P*, fructose 6-phosphate; PKA, cAMP-dependent protein kinase; DEP, diethyl pyrocarbonate; GdnHCl, guanidine hydrochloride.

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act with each other, and what is the role of the N- and C-termini of this enzyme in the interaction between the enzymic domains? Although the crystal structure of a mutant rat testis 6PF-2K/Fru-2,6-P_2ase has been solved [12], the structure of the N-terminal 36 amino acids of the enzyme was not visible, providing no answers to these questions. Recently, Kurland et al. [13] identified the region Gly^5-Glu-Leu as the locus of the interaction of the N-terminal tail with the kinase and bisphosphatase domains of RKB. However, how the C-terminal region interacts with the kinase domain and the bisphosphatase catalytic core still remains unclear. In the present study, we gained further insight into the structural basis for the mutual regulation of the two catalytic domains by characterizing kinetically various mutants with mutations in the C-terminus of chicken liver 6PF-2K/Fru-2,6-P_2ase (CKB). We found that the sequence His^{444}-Arg-Glu-Arg (specifically the three basic residues in this sequence) is responsible for the repression of the Fru-2,6-P_2ase activity exerted by the kinase domain.

EXPERIMENTAL

Oligonucleotide-directed mutagenesis

The deletion mutants of CKB were engineered by replacing the *Eco*RI/*Bam*HI fragment of wild-type CKB cDNA with that of corresponding deletion mutant forms of the separate bisphosphatase domain of CKB (CBD) [14,15]. All the site-directed mutations of CKB were generated by PCR using the Expand™ High Fidelity PCR System (Roche Molecular Biochemicals, Mannheim, Germany). The mutants CKB^{AEA} (in which Arg^{445} and Arg^{447} of CKB were mutated to Ala), CKB^{AAEA} (in which His^{444}, Arg^{445} and Arg^{447} were mutated to Ala), CKB^{444K} and CKB^{444D} (in which His^{444} was replaced by Lys and Asp respectively), and CKB^{445K} and CKB^{445D} (in which Arg^{445} was replaced by Lys and Asp respectively) were constructed by replacing the 760 bp *Kpn*I/*Bam*HI fragment of CKB with that digested from the PCR product. For construction of the CKB^{444A} and CKB^{446A} mutants (His^{444} and Glu^{446} respectively mutated to Ala), a pair of primers was used to generate the mutation and to introduce a restriction site with a silent mutation. The *Eco*RI/*Bam*HI fragment of wild-type CBD was substituted with that of the site-directed mutant forms of CKB to construct CBD^{444A}, CBD^{AEA} and CBD^{AAEA}. The nucleotide sequences of the mutant plasmids were determined to ensure that no other point mutations were introduced.

Expression and purification of the enzyme

All mutant forms of CKB and CBD were expressed in *Escherichia coli* BL21 (DE3) using the phage T7 RNA polymerase-based system, as described previously for wild-type CKB and CBD [14–16]. After induction with isopropyl β-D-thiogalactoside (Sigma; 0.01 μM for CBD and 100 μM for CKB) at 22 °C for 24 h [17], the protein was extracted and purified using the procedure for CKB [14] or CBD [16] described previously. All enzymes were purified to homogeneity, as judged by SDS/PAGE (results not shown).

Assay of 6PF-2K and Fru-2,6-P_2ase activities

6PF-2K activity was measured by monitoring the production of Fru-2,6-P_2 [18]. Fru-2,6-P_2ase activity was assayed by following the rate of release of [^{32}P]P_i from [2-^{32}P]Fru-2,6-P_2, as described previously [19].

Phosphorylation of CKB by PKA

The wild-type and mutant forms of CKB (100 μg each) were incubated in a final volume of 0.1 ml containing 50 mM Tris/HCl, pH 7.5, 5 mM P_i, 5 mM $MgCl_2$, 2 mM dithiothreitol and 1 mM [γ-^{32}P]ATP (200 c.p.m./pmol). The reaction was started by the addition of 2 μg of the catalytic subunit of PKA. After incubation at 30 °C for 20 min, the stoichiometry of the incorporation of ^{32}P into the enzyme was determined according to the method of Roskoski [20]. The incorportation of ^{32}P for all forms of CKB was greater than 0.9 mol/mol of subunit.

Chemical modification

The protein (10 μg) was incubated with 1 mM diethyl pyrocarbonate (DEP) (Sigma) at 30 °C in a total volume of 100 μl. The reaction was stopped by the addition of dithiothreitol to a final concentration of 10 mM, and the residual Fru-2,6-P_2ase activity was assayed as described above.

Effect of pH on Fru-2,6-P_2ase activity

A mixture of 100 mM Mes, 100 mM Bis-Tris propane, 100 mM Hepes and 100 mM Tris, titrated to the desired pH with HCl or NaOH, was employed as buffer for the pH range 5.0–10.0. Fru-2,6-P_2ase activity was measured at 30 °C in the presence of 5 mM P_i, 10 μM Fru-2,6-P_2 and 40 mM of the buffer mixture.

Fluorescence measurements and denaturation with guanidine hydrochloride (GdnHCl)

The proteins (30 μg/ml) were incubated with GdnHCl (Sigma) at 25 °C for 20 min in buffer containing 20 mM Tris/HCl, pH 8.0, 50 mM KCl and 1 mM dithiothreitol. Aliquots were then removed for fluorescence measurement and assay of Fru-2,6-P_2ase activity. Upon excitation at 295 nm, the intrinsic fluorescence of the samples was scanned from 300 to 380 nm with an Hitachi F4010 fluorescence spectrophotometer. The bandwidth for both excitation and emission was 5 nm.

RESULTS

Effects of C-terminal deletion on the Fru-2,6-P_2ase activity of the dephospho- and phospho-forms of CKB

Our previous study on the separate bisphosphatase domain CBD showed that successive deletion of the C-terminal region of CBD gradually increased the Fru-2,6-P_2ase activity [15]. Because PKA-catalysed phosphorylation of the intact hepatic bifunctional enzyme also activates the Fru-2,6-P_2ase, it is interesting to determine the role of the C-terminal tail in the phosphorylation-mediated activation of Fru-2,6-P_2ase. We generated a series of C-terminal deletion mutants of CKB (CKB^{C15del}, CKB^{C20del}, CKB^{C25del} and CKB^{C30del}, where the numbers indicate the numbers of residues deleted) by substituting the bisphosphatase domain of CKB with the corresponding deletion mutants of CBD. All of these mutants were expressed in *E. coli* BL21 (DE3) and purified to homogeneity (results not shown). In addition, the phosphorylated forms of these enzymes were prepared.

As shown in Table 1, deletion of the C-terminal 25 or 30 residues of CKB enhanced the V_{max} of Fru-2,6-P_2ase by approx. 20-fold, and increased the K_m for Fru-2,6-P_2 by approx. 2–3-fold. In addition, the Fru-2,6-P_2ase activity of CKB^{C25del} and CKB^{C30del} did not respond to PKA-mediated phosphorylation, because neither the V_{max} of Fru-2,6-P_2ase nor the K_m for Fru-2,6-P_2 of

Table 1 Kinetic properties of the dephospho- and phospho-forms of the truncation mutants of CKB

V_{max} and K_m values were calculated using a Hyperbolic Regression Analysis Program (http://www.liv.ac.uk/~jse/software.html). The V_{max} for 6PF-2K and the K_m value for Fru-6-*P* were determined in the presence of 2 mM Mg^{2+} and 10 mM ATP. The K_m for ATP was determined in the presence of 2 mM Fru-6-*P* and 10 mM Mg^{2+}. Double-reciprocal plots of 6PF-2K activity for CKB and CKB^{C15del} against ATP concentration revealed two slopes. Fitting the data corresponding to the two ATP ranges (0.05–0.4 mM and 0.4–4 mM) separately into the above-mentioned software yielded two K_m values. Fru-2,6-P_2ase activity was determined in the presence of 5 mM P_i. The values represent means ± ranges for at least three measurements. Statistically significant differences: $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ for mutants compared with wild-type CKB; †$P < 0.05$, ††$P < 0.01$, †††$P < 0.001$ for the phospho (P-) form compared with the corresponding dephospho-form.

	6PF-2K			Fru-2,6-P_2ase		
		K_m (mM)				
Enzyme form	V_{max} (m-units/mg)	Fru-6-*P*	ATP	V_{max} (m-units/mg)	K_m Fru-2,6-P_2 (μM)	K_i Fru-6-*P* (μM)
CKB	337 ± 38	0.022 ± 0.003	0.11 ± 0.04, 0.91 ± 0.10	14 ± 2	0.11 ± 0.02	10.3 ± 1.4
P-CKB	182 ± 17††	0.033 ± 0.008	0.17 ± 0.03, 1.57 ± 0.14	36 ± 5†††	0.12 ± 0.02	11.0 ± 1.5
CKB^{C15del}	282 ± 22	0.022 ± 0.004	0.13 ± 0.02, 0.54 ± 0.07	17 ± 3	0.12 ± 0.02	11.4 ± 1.0
P-CKB^{C15del}	153 ± 21††	0.031 ± 0.012	0.21 ± 0.03, 0.92 ± 0.13	40 ± 5†††	0.11 ± 0.03	11.8 ± 0.6
CKB^{C20del}	312 ± 20	0.026 ± 0.002	0.40 ± 0.08	31 ± 3**	0.11 ± 0.02	8.3 ± 0.9
P-CKB^{C20del}	188 ± 25††	0.041 ± 0.010	0.59 ± 0.05†	70 ± 9†††	0.15 ± 0.05	8.5 ± 0.8
CKB^{C25del}	536 ± 46**	0.040 ± 0.012	0.34 ± 0.05	282 ± 29***	0.25 ± 0.05*	6.4 ± 0.7*
P-CKB^{C25del}	225 ± 31†††	0.42 ± 0.14††	0.48 ± 0.04†	275 ± 22	0.28 ± 0.06	6.3 ± 0.4
CKB^{C30del}	514 ± 42**	0.045 ± 0.010*	0.41 ± 0.04***	271 ± 26***	0.30 ± 0.05**	5.8 ± 0.3**
P-CKB^{C30del}	232 ± 28†††	0.48 ± 0.15††	0.62 ± 0.07†	272 ± 19	0.29 ± 0.07	6.4 ± 0.6

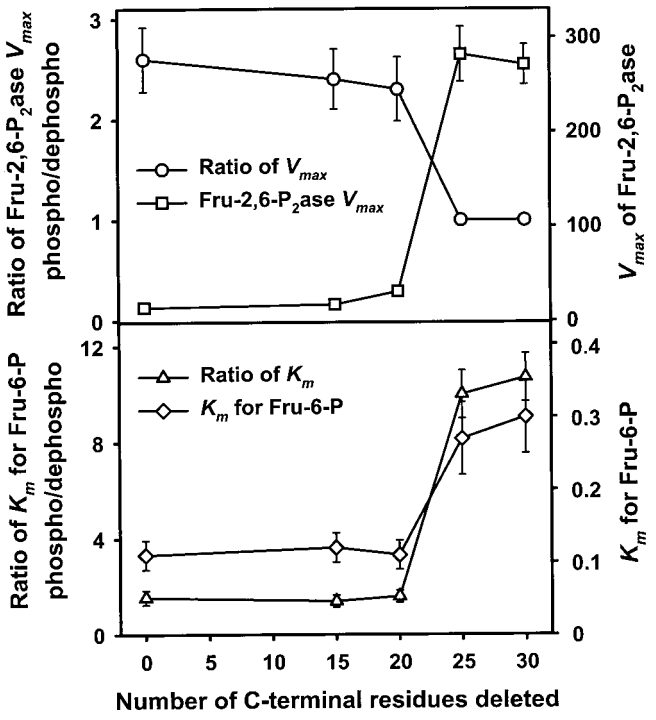

Figure 1 Effects of C-terminal deletions on the 6PF-2K and Fru-2,6-P_2ase activities of the phospho- and dephospho-forms of CKB

6PF-2K and Fru-2,6-P_2ase activities were measured at pH 7.4 in the presence of 5 mM potassium phosphate. Upper panel, phospho/dephospho V_{max} ratio (○) and absolute V_{max} (□) of the Fru-2,6-P_2ase activity of full-length CKB and its deletion mutants. Lower panel, phospho/dephospho K_m ratio (△) and absolute K_m for Fru-6-*P* (◇) of the 6PF-2K activity of the enzymes. The data represent means ± range of at least three determinations.

CKB^{C25del} or CKB^{C30del} was changed upon phosphorylation (Table 1, Figure 1). However, deletion of the C-terminal 15 or 20 amino acids of CKB had no significant effect on Fru-2,6-P_2ase activity, as the V_{max} for Fru-2,6-P_2ase was increased by even less than 2.5-fold (Table 1). The effect of PKA-catalysed phosphorylation on the activities of CKB^{C15del} and CKB^{C20del} was similar to that on the activity of wild-type CKB, i.e. an enhancement of the V_{max} of Fru-2,6-P_2ase by 2–3 fold (Figure 1), with no change in the K_m for Fru-2,6-P_2 (Table 1). These results suggest that C-terminal residues 21–25 play an important role in repression of the Fru-2,6-P_2ase activity of CKB, and that these residues are also involved in the regulation by PKA-catalysed phosphorylation.

Effects of point mutations in the region His^{444}-Arg-Glu-Arg on the Fru-2,6-P_2ase activity of the dephospho- and phospho-forms of CKB

C-terminal residues 25–21 are Thr^{443}-His-Arg-Glu-Arg, containing three basic residues and one acidic residue. To determine whether the acidic or basic residues are critical for the regulation of Fru-2,6-P_2ase activity, we constructed two site-directed mutants: CKB^{446A}, in which the acidic residue Glu^{446} was mutated to Ala, and CKB^{AAEA}, in which the three basic residues His^{444}, Arg^{445} and Arg^{447} were mutated to Ala. As shown in Table 2, CKB^{446A} showed no differences from wild-type CKB in any kinetic properties, whereas the V_{max} for Fru-2,6-P_2ase was approx. 11-fold greater and the K_m of Fru-2,6-P_2 approx. 3-fold greater for CKB^{AAEA} than for wild-type CKB. In addition, PKA-mediated phosphorylation had no effect on the Fru-2,6-P_2ase activity of CKB^{AAEA} (Table 2). The results demonstrate the critical importance of the three basic residues in the repression of Fru-2,6-P_2ase activity, and also indicate that these residues are involved in the effect of PKA-catalysed phosphorylation.

To ascertain further the role of specific basic residues in the repression of Fru-2,6-P_2ase activity and in the effect of phosphorylation, we generated another two mutant enzymes, CKB^{444A} ($His^{444} \rightarrow$ Ala) and CKB^{AEA} (Arg^{445}-Glu-Arg → Ala-Glu-Ala). CKB^{444A} and CKB^{AEA} show V_{max} values for Fru-2,6-P_2ase approx. 6–7-fold greater and K_m values for Fru-2,6-P_2 approx. 2–3-fold greater than those of wild-type CKB (Table 2). Similarly, the mutations $His^{444} \rightarrow$ Ala and Arg^{445}-Glu-Arg →

Table 2 Kinetic properties of the dephospho- and phospho-forms of the point mutants of CKB

See the legend to Table 1 for details. The values represent means ± range for at least three determinations. Statistically significant differences: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ for mutants compared with wild-type CKB; †$P < 0.05$, ††$P < 0.01$, †††$P < 0.001$ for the phospho (P-) form compared with the corresponding dephospho-form.

	6PF-2K			Fru-2,6-P_2ase		
		K_m (mM)				
Enzyme form	V_{max} (m-units/mg)	Fru-6-*P*	ATP	V_{max} (m-units/mg)	K_m Fru-2,6-P_2 (μM)	K_i Fru-6-*P* (μM)
CKB	337 ± 38	0.022 ± 0.003	0.11 ± 0.04, 0.91 ± 0.10	14 ± 2	0.11 ± 0.02	10.3 ± 1.4
P-CKB	182 ± 17††	0.033 ± 0.008	0.17 ± 0.03, 1.57 ± 0.14	36 ± 5††	0.12 ± 0.02	11.0 ± 1.5
CKB^{AAEA}	495 ± 42**	0.042 ± 0.008*	0.20 ± 0.05, 0.52 ± 0.10	152 ± 16***	0.35 ± 0.08**	7.6 ± 0.4*
P-CKB^{AAEA}	292 ± 33††	0.50 ± 0.12††	0.18 ± 0.04, 0.79 ± 0.09	150 ± 14	0.42 ± 0.11	7.4 ± 0.2
CKB^{AEA}	418 ± 30*	0.041 ± 0.012*	0.13 ± 0.03, 0.68 ± 0.07	88 ± 6***	0.30 ± 0.05**	7.8 ± 0.3*
P-CKB^{AEA}	271 ± 26††	0.45 ± 0.13††	0.19 ± 0.04, 1.02 ± 012	84 ± 6	0.40 ± 0.12	7.7 ± 0.5
CKB^{444A}	406 ± 35	0.040 ± 0.005**	0.22 ± 0.05, 0.61 ± 0.05	102 ± 5***	0.25 ± 0.04**	8.5 ± 0.4
P-CKB^{444A}	255 ± 28††	0.35 ± 0.15†	0.24 ± 0.04, 0.89 ± 0.09	100 ± 6	0.30 ± 0.04	8.2 ± 0.6
CKB^{444K}	388 ± 24	0.031 ± 0.004*	0.14 ± 0.02, 0.78 ± 0.08	37 ± 5**	0.18 ± 0.04	6.5 ± 0.5*
P-CKB^{444K}	214 ± 21†††	0.18 ± 0.05††	0.21 ± 0.02, 1.30 ± 0.10	43 ± 6	0.16 ± 0.03	7.2 ± 0.4
CKB^{444D}	412 ± 18*	0.039 ± 0.006*	0.23 ± 0.05, 0.58 ± 0.06	76 ± 9***	0.34 ± 0.06**	7.1 ± 0.5*
P-CKB^{444D}	236 ± 12†††	0.55 ± 0.09†††	0.29 ± 0.05, 0.76 ± 0.08	79 ± 11	0.33 ± 0.05	6.8 ± 0.6
CKB^{445K}	342 ± 22	0.027 ± 0.006	0.11 ± 0.02, 0.92 ± 0.09	22 ± 4*	0.12 ± 0.03	10.5 ± 0.9
P-CKB^{445K}	202 ± 17†††	0.082 ± 0.010††	0.26 ± 0.04, 1.63 ± 0.20	31 ± 5	0.12 ± 0.04	9.9 ± 0.8
CKB^{445D}	398 ± 20	0.045 ± 0.008**	0.16 ± 0.04, 0.83 ± 0.10	96 ± 8***	0.46 ± 0.12**	8.1 ± 0.5
P-CKB^{445D}	205 ± 15†††	0.61 ± 0.10†††	0.29 ± 0.03, 1.20 ± 0.10	90 ± 8	0.35 ± 0.10	7.6 ± 0.3
CKB^{446A}	328 ± 32	0.021 ± 0.002	0.14 ± 0.02, 0.82 ± 0.09	14 ± 2	0.12 ± 0.03	9.8 ± 1.6
P-CKB^{446A}	193 ± 23††	0.034 ± 0.005†	0.19 ± 0.04, 1.42 ± 0.13	34 ± 3†††	0.11 ± 0.03	10.2 ± 1.4

Ala-Glu-Ala eliminated the effect of PKA-mediated phosphorylation on the kinetic properties of Fru-2,6-P_2ase (Table 2). The results indicate that both His^{444} and the two Arg residues (or one of the two Arg residues) are important in the regulation of the Fru-2,6-P_2ase activity of CKB.

Sequence comparison reveals that His^{444} and Arg^{445} are highly conserved in all isoenzymes of the 6PF-2K/Fru-2,6-P_2ase family. Thus we further mutated these residues to Lys or Glu. The kinetic parameters of CKB^{444K}, CKB^{444D}, CKB^{445K} and CKB^{445D} are shown in Table 2. Mutation of His^{444} or Arg^{445} to Lys increased the V_{max} of Fru-2,6-P_2ase by 3-fold and 50% respectively, and mutation of either residue to Glu enhanced the Fru-2,6-P_2ase activity by 5–7-fold. In addition, mutation of His^{444} or Arg^{445} to Glu completely eliminated the activation of Fru-2,6-P_2ase by PKA-catalysed phosphorylation; however, phosphorylation of CKB^{444K} or CKB^{445K} could activate the Fru-2,6-P_2ase by 20–50% (Table 2). These results indicate that the electrostatic properties of the side chains of these two highly conserved residues play important roles in the regulation of the Fru-2,6-P_2ase activity, as well as in the effect of phosphorylation on the Fru-2,6-P_2ase.

Comparison of the k_{cat} values for Fru-2,6-P_2ase of wild-type CKB and CBD and their mutants

The Fru-2,6-P_2ase activity of CKB is repressed by both the N-terminal kinase domain and the C-terminal sequence [15,16]. It was proposed previously that PKA-catalysed phosphorylation of the hepatic bifunctional enzyme activates Fru-2,6-P_2ase in part through relieving the repression of Fru-2,6-P_2ase activity by the N-terminal kinase domain [9]. The findings that the mutations in CKB^{444A}, CKB^{AEA} and CKB^{AAEA} stimulated Fru-2,6-P_2ase activity and eliminated the phosphorylation-mediated effects prompted us to determine whether the N-terminal kinase domain still has repressive effects on the Fru-2,6-P_2ase activity of these mutant enzymes. In order to address this question, we introduced the same point mutations into the separate bisphosphatase domain of the enzyme. All the mutant forms of CBD were expressed and purified as described previously [15,16]. The k_{cat} values for Fru-2,6-P_2ase of these three mutants (CBD^{444A}, CBD^{AEA} and CBD^{AAEA}) were 30–40% higher than those of the corresponding full-length bifunctional enzymes (Figure 2). These results suggest that, in the full-length bifunctional enzyme with these mutations, repression of the bisphosphatase activity by the

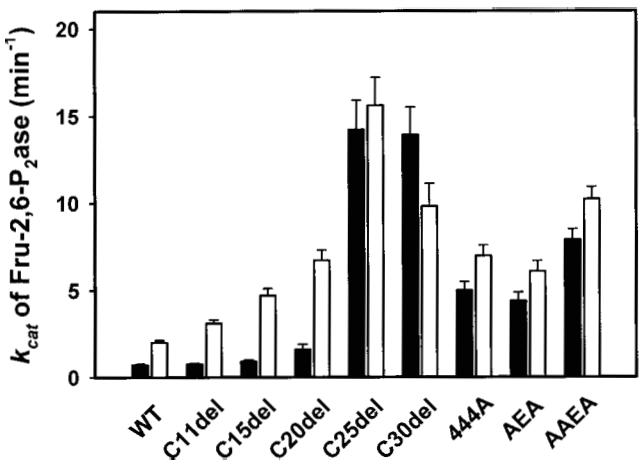

Figure 2 Comparison of the Fru-2,6-P_2ase activities of wild-type CKB and its mutants with the activities of the corresponding bisphosphatase domains

The activity of Fru-2,6-P_2ase is expressed in term of k_{cat} (min^{-1}). Solid bars represent the Fru-2,6-P_2ase activity of the bifunctional enzyme (CKB), and empty bars represent the Fru-2,6-P_2ase activity of the recombinant separate bisphosphatase domain (CBD). The values represent means ± range for at least three determinations.

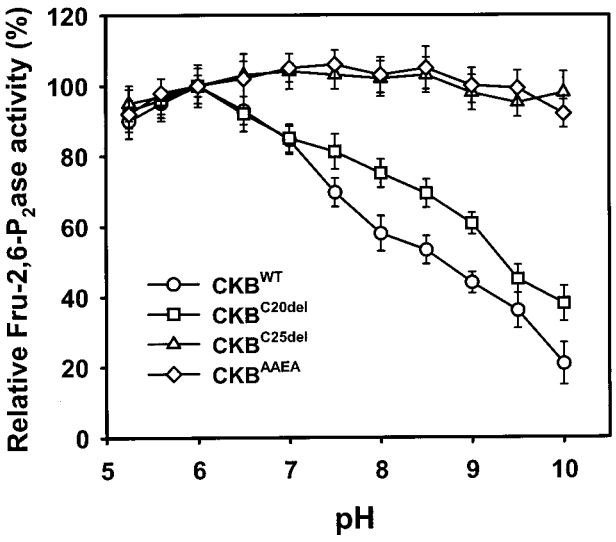

Figure 3 Effects of C-terminal deletions and point mutations on the pH sensitivity of Fru-2,6-P_2ase activity

Fru-2,6-P_2ase activity was measured in 10 μM Fru-2,6-P_2 at the pH values indicated. For each enzyme, the activity is expressed as the ratio of V_{max} at indicated pH to that at pH 6.0. WT, wild type. Each data point was calculated based on the average of at least three independent measurements; error bars indicate range.

N-terminal kinase domain still remains to some extent, but is much weaker than in wild-type CKB, as the k_{cat} of Fru-2,6-P_2ase of CBD was 2–3-fold higher than that of CKB. Thus we conclude that the three basic residues mediate the repressive effect of the N-terminal kinase domain on the Fru-2,6-P_2ase catalytic core. Furthermore, the Fru-2,6-P_2ase k_{cat} values of CBD^{444A} and CBD^{AEA} were approx. 3–4-fold greater, and that of CBD^{AAEA} was approx. 6-fold greater, than that of the original CBD. This indicates that, in addition to their role in mediating the repressive effect of the kinase domain on the Fru-2,6-P_2ase, the three basic residues are also responsible for the repression of Fru-2,6-P_2ase activity by the C-terminal tail itself.

Interestingly, although mutation of all three basic residues to Ala resulted in activation of Fru-2,6-P_2ase that was significantly greater than with the two individual mutations (His^{444} → Ala and Arg^{445}-Glu-Arg → Ala-Glu-Ala), simultaneous mutation of the three basic residues did not result in greater relief of repression of Fru-2,6-P_2ase activity by the N-terminal kinase domain: the Fru-2,6-P_2ase k_{cat} values of CKB^{AAEA}, CKB^{444A} and CKB^{AEA} were all approx. 30% lower than that of their corresponding separate bisphosphatase domains (Figure 2).

A comparison of the k_{cat} values for Fru-2,6-P_2ase between CKBs and CBDs with various C-terminal deletion mutations is also shown in Figure 2. The Fru-2,6-P_2ase k_{cat} value of CKB^{C25del} was almost identical with that of CBD^{C25del}, suggesting that truncation of the C-terminal 25 residues of CKB caused elimination of the influence of the kinase domain on Fru-2,6-P_2ase activity. However, for wild-type CKB, CKB^{C11del}, CKB^{C15del} and CKB^{C20del}, removal of the kinase domain enhanced Fru-2,6-P_2ase activity by 3–5-fold, since CBD, CBD^{C11del}, CBD^{C15del} and CBD^{C20del} had Fru-2,6-P_2ase k_{cat} values 3–5-fold higher than those of the corresponding CKBs. These results suggest that the C-terminal 20 amino acids of CKB do not contribute to the repression of Fru-2,6-P_2ase activity by the kinase domain at all, and that the sequence encompassing residues 21–25 is the only part of C-terminal sequence that is responsible for the repressive effect of the N-terminal kinase domain.

pH-dependence of the Fru-2,6-P_2ase activity of wild-type CKB and various mutants

The Fru-2,6-P_2ase activities of wild-type CKB and various mutants were determined at different pH values from 5.0 to 10.0. The stability of all the proteins at each pH was examined by incubating the enzyme at that pH for 30 min and then measuring the Fru-2,6-P_2ase activity at pH 7.4. There was no loss of enzyme activity after incubation at pH values between 5.0 and 10.0 (results not shown). As shown in Figure 3, the Fru-2,6-P_2ase activities of CKB^{C20del} and wild-type CKB were optimal at pH 6.0, and decreased to approx. 20–40% of the optimal activity at pH 10.0. However, pH sensitivity was not observed for CKB^{C25del}. Interestingly, the Fru-2,6-P_2ase activities of CKB^{444A}, CKB^{AEA} (results not shown) and CKB^{AAEA} (Figure 3) were also not affected by changes in pH. The pH profile of CKB^{446A} was similar to that of wild-type CKB (results not shown). Thus it is reasonable to conclude that the three residues His^{444}, Arg^{445} and Arg^{447} in the C-terminus play a role in the pH-dependent regulation of the Fru-2,6-P_2ase activity of CKB.

Effects of deletion and point mutations in the C-terminus of CKB on the 6PF-2K activity of the dephospho- and phospho-forms

The 6PF-2K activities of all CKB mutants were determined. As shown in Table 1, deletion of the final 20 residues of CKB had little effect on kinase activity. However, further truncation of an additional five amino acids induced approx. 2-fold increases in both the V_{max} and K_m for Fru-6-*P* of 6PF-2K. Phosphorylation of wild-type CKB only slightly modulated kinase activity, decreasing the V_{max} by approx. 40% and increasing the K_m for Fru-6-*P* by less than 2-fold. Phosphorylation of CKB^{C20del} had an effect on kinase activity similar to that for wild-type CKB (Table 1, Figure 1). However, for CKB^{C25del} and CKB^{C30del}, phosphorylation decreased the V_{max} of 6PF-2K by approx. 60% and reduced the affinity of the 6PF-2K for Fru-6-*P* by approx. 10-fold (Table 1, Figure 1).

The effects of point mutations of basic residues to Ala or Glu mimicked those of C-terminal 25- or 30-residue truncations: CKB^{444A}, CKB^{444D}, CKB^{445D}, CKB^{AEA} and CKB^{AAEA} showed slightly higher 6PF-2K V_{max} values and 2-fold lower affinities for Fru-6-*P* than wild-type CKB, and phosphorylation of these mutants increased the K_m for Fru-6-*P* by approx. 10-fold (Table 2). For the mutants CKB^{444K} and CKB^{445K}, the 6PF-2K activities did not differ obviously from that of wild-type CKB. However, phosphorylation of these two mutants decreased the affinity of 6PF-2K for Fru-6-*P* by 3–6-fold. The results suggest that these three basic residues in the C-terminal region are involved in the modulation of 6PF-2K activity by phosphorylation, and again indicate the important roles of specific properties of side chains of His^{444} and Arg^{445}.

Earlier work demonstrated that chicken liver 6PF-2K is activated by ATP, which reflects a negative co-operativity for binding of ATP to the enzyme [14,21]. Double-reciprocal plots of the 6PF-2K activity of CKB against ATP concentration clearly showed two slopes (results not shown), which yielded two K_m values for ATP (Table 1). Here we found that the C-terminal 30-, 25- or 20-residue deletions eliminated the activation by ATP of 6PF-2K. CKB^{C30del}, CKB^{C25del} and CKB^{C20del} exhibited typical Michaelis–Menten kinetics with respect to ATP. The data suggest that the C-terminal region of CKB is somehow involved in the activation of 6PF-2K activity by ATP. However, the region His^{444}-Arg-Glu-Arg is not involved in this activation, since all

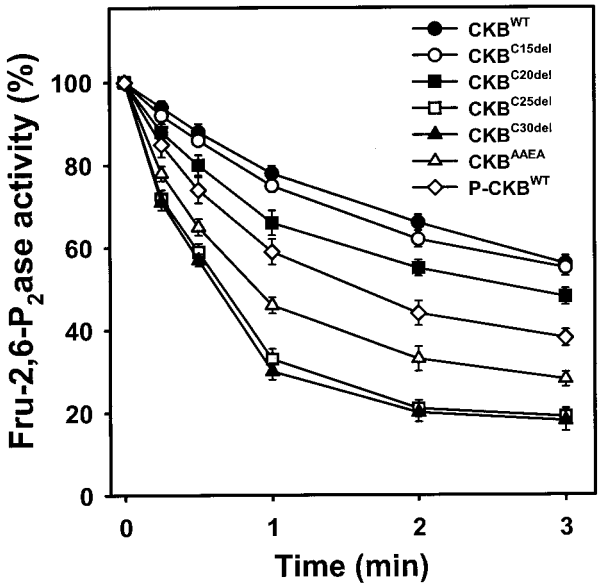

Figure 4 Time course of inactivation by DEP modification of the Fru-2,6-P_2ase activity of the dephospho- and phospho-forms of CKB and its mutants

A sample of 10 μg of each protein was incubated with 1 mM DEP at 30 °C, and the reaction was stopped at the indicated time by adding dithiothreitol to a final concentration of 10 mM. Fru-2,6-P_2ase activity was then assayed. WT, wild type; P-CKB, phospho-form of CKB. Data shown are means ± range from two separate experiments done in duplicate.

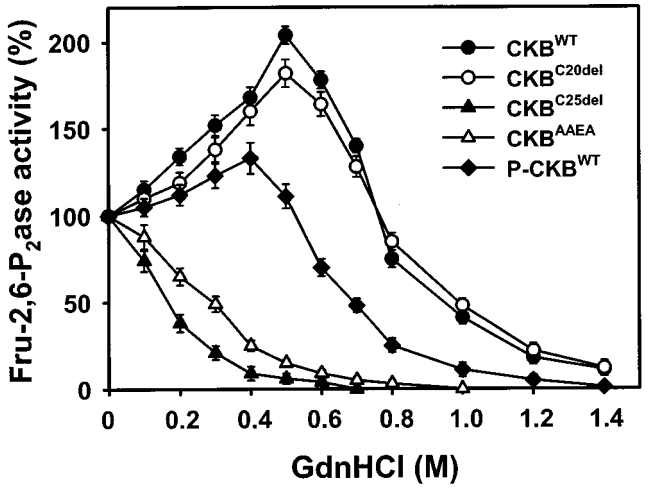

Figure 5 Effects of GdnHCl on the Fru-2,6-P_2ase activities of the dephospho- and phospho-forms of CKB and its mutants

The enzymes (90 μg/ml) were incubated at 25 °C for 20 min with the indicated concentrations of GdnHCl, and aliquots were then removed for the Fru-2,6-P_2ase activity assay. WT, wild type; P-CKB, phospho-form of CKB. The data shown are the averages of two separate experiments done in duplicate; error bars indicate range.

the point mutants still showed activation by ATP of the 6PF-2K activity (Table 2).

Effects of the mutations on inactivation of the enzyme by DEP modification

Previous work had suggested that the modification of His residues at the active site of Fru-2,6-P_2ase by DEP could result in inactivation [22,23]. The accessibility of the catalytic core to DEP may indicate the extent of opening of the catalytic site. We determined the time course of inactivation by DEP modification of wild-type CKB and its various mutants, as well as phosphorylated CKB. As shown in Figure 4, inactivation of CKB^{C25del} and CKB^{C30del} by DEP modification was much faster than that of wild-type CKB, CKB^{C15del} and CKB^{C20del}; the rates of inactivation of CKB^{AAEA} and phosphorylated CKB were intermediate. After being incubated in 1 mM DEP for 2 min, the residual activities of wild-type CKB, CKB^{C15del}, CKB^{C20del}, CKB^{C25del}, CKB^{C30del}, CKB^{AAEA} and phosphorylated CKB were approx. 66%, 62%, 55%, 21%, 20%, 35% and 44% respectively of their original activity. The higher accessibility of the active site of the Fru-2,6-P_2ase of CKB^{C25del} and CKB^{C30del} to DEP suggests a much more open conformation of the active sites in these two deletion mutants. The obvious difference in inactivation rates between CKB^{AAEA} and wild-type CKB suggests that there may be a conformational change in the Fru-2,6-P_2ase active site that is caused by mutation of the three basic residues to Ala. Moreover, the phospho-form of CKB was clearly more accessible to DEP than the dephospho-form, indicating that PKA-catalysed phosphorylation induces a more open conformation of the Fru-2,6-P_2ase catalytic core, which might account for the higher Fru-2,6-P_2ase activity of phosphorylated CKB.

Fluorescence spectra and GdnHCl-induced inactivation of wild-type CKB, CKB^{C20del}, CKB^{C25del} and CKB^{AAEA}

In order to detect whether the various mutations alter the global structure of the enzyme, we compared the intrinsic fluorescence spectra of the CKB^{C25del} and CKB^{AAEA} mutants with that of the wild-type enzyme in the presence of different concentrations of GdnHCl. The fluorescence spectra for these enzymes were very similar. There were no significant differences in the red shift of the emission maximum or in the change in emission intensity at 335 nm in the presence of various concentrations of GdnHCl among these enzyme preparations (results not shown). Therefore there are no significant changes in the global structures of these two mutant enzymes compared with the wild-type enzyme. However, the GdnHCl-induced inactivation of CKB^{C25del} and CKB^{AAEA} was dramatically different from that of wild-type CKB and CKB^{C20del}. As shown in Figure 5, after incubation in low concentrations of GdnHCl (0–0.6 M) at 25 °C for 20 min, the Fru-2,6-P_2ase of wild-type CKB and CKB^{C20del} was activated. The concentration of GdnHCl causing maximal activation was 0.5 M. At concentrations of GdnHCl exceeding 0.6 M, the Fru-2,6-P_2ase was gradually inactivated. In contrast, the Fru-2,6-P_2ase activity of CKB^{C25del} and CKB^{AAEA} decreased rapidly, and was completely lost at 0.6 and 1.0 M GdnHCl respectively. Inactivation of the phospho-form of CKB is considerably different from that of the dephospho-form. These observations, consistent with the DEP modification results, demonstrate that a C-terminal 25-residue deletion, mutation of His^{444}-Arg-Glu-Arg to Ala-Ala-Glu-Ala, or phosphorylation of the enzyme may result in a much more open conformation of the Fru-2,6-P_2ase catalytic core, although there are no significant changes to the global structure of the enzyme.

DISCUSSION

It is well known that, for the hepatic 6PF-2K/Fru-2,6-P_2ase, the N-terminal kinase domain has a repressive effect on the C-terminal bisphosphatase, and that PKA-catalysed phosphoryl-

ation at an N-terminal Ser residue activates the Fru-2,6-P_2ase activity. Deletion of either the N- or the C-terminus affects both the 6PF-2K and Fru-2,6-P_2ase activities. How the two catalytic domains interact with each other is of great interest. In the present study, we reveal that the sequence His^{444}-Arg-Glu-Arg within the C-terminus of CKB is the key structural element in the bisphosphatase domain that mediates the repressive effect of the N-terminal kinase domain on the bisphosphatase catalytic core. In addition, our data show that the region is also responsible for the inhibition of Fru-2,6-P_2ase activity by the C-terminal tail itself, and is involved in regulation of this activity by PKA-catalysed phosphorylation.

Mutation of the three basic residues within the sequence His^{444}-Arg-Glu-Arg almost eliminated the repression of Fru-2,6-P_2ase activity by the kinase domain, suggesting that the inhibitory effect of the N-terminal kinase domain is probably mediated by electrostatic interactions. Importantly, the Fru-2,6-P_2ase activity of the mutants CKB^{444A} (or CKB^{444D}), CKB^{445D}, CKB^{AEA} and CKB^{AAEA} was no longer regulated by PKA-catalysed phosphorylation (Table 2), although the repression of Fru-2,6-P_2ase activity by the N-terminal kinase domain still remained to some degree (Figure 2). Thus it is reasonable to believe that PKA-catalysed phosphorylation probably activates the Fru-2,6-P_2ase by relieving the repression by the kinase domain that is mediated by this sequence, or, more exactly, by the two basic residues His^{444} and Arg^{445}.

Interestingly, we found that mutation of the three basic residues to Ala or Glu also influenced the effect of PKA-mediated phosphorylation on the 6PF-2K activity of CKB. The PKA-catalysed phosphorylation of CKB^{444A} (or CKB^{444D}), CKB^{445D}, CKB^{AEA} and CKB^{AAEA} increased the K_m of 6PF-2K for Fru-6-*P* by approx. 10-fold, while increasing that of the wild-type enzyme by only approx. 2-fold (Figure 1, Table 2). These results suggest that there may exist some direct or long-range interaction between the sequence His^{444}-Arg-Glu-Arg and the kinase domain.

Our results show that the C-terminal 25 amino acids of CKB are responsible for the pH sensitivity of its Fru-2,6-P_2ase activity (Figure 3). This is consistent with a previous report by Lin et al. [11] that the pH sensitivity of the Fru-2,6-P_2ase activity of wild-type RKB was not observed with RKB^{C30del}. Interestingly, the muscle isoenzyme, which differs from the hepatic isoenzyme only in its N-terminal sequence, also exhibits pH-insensitive Fru-2,6-P_2ase activity, suggesting a possible interaction between the N- and C-terminal regions [11]. The finding that mutations in the region His^{444}-Arg-Glu-Arg of CKB resulted in a loss of sensitivity of Fru-2,6-P_2ase to changes in pH suggests that an interaction occurs between this region and the kinase domain.

All of our data demonstrate that the sequence His^{444}-Arg-Glu-Arg of CKB is a key element that links the two catalytic domains of the enzyme. The fact that mutation of His^{444}-Arg-Glu-Arg to Ala-Ala-Glu-Ala renders the enzyme much more susceptible to DEP treatment (Figure 4) and to GdnHCl-induced inactivation (Figure 5) demonstrates that the three basic residues in this region are very important in maintaining the Fru-2,6-P_2ase catalytic core in a tight conformation. This tetrapeptide sequence probably interacts directly with residues in the catalytic core of Fru-2,6-P_2ase and reduces the rate of breakdown of the phosphoenzyme intermediate or the rate of product release. Based on the crystal structures of the rat testis bifunctional enzyme and the rat liver bisphosphatase domain [12,24], we postulated previously that the spatial proximity of the C-terminal tail and loop 2 of the Fru-2,6-P_2ase catalytic core, which contains 11 acidic amino acids, might underlie the inhibitory effect of the C-terminal tail on the Fru-2,6-P_2ase activity [15]. The results of the present work further support our idea, by suggesting that electrostatic interactions between the three basic C-terminal residues (His^{444}, Arg^{445} and Arg^{445}) and the acidic residues within the loop 2 underlie the repression of Fru-2,6-P_2ase activity by the C-terminal tail.

We do not know how the sequence His^{444}-Arg-Glu-Arg interacts with the N-terminal kinase domain. The crystal structure of the rat testes isoenzyme showed no direct interaction between the C-terminal tail and the kinase catalytic core [12]. Because the location of the N-terminal 36 residues was not visible in the crystal structure [12], the possibility cannot be ruled out that an interaction might exist between the N- and C-terminal regions. However, it is also possible that the kinase and bisphosphatase domains affect each other mutually only through a long-range effect, and that the sequence His^{444}-Arg-Glu-Arg plays a role in such a long-range interaction. We may not know this for sure until the complete structure of the enzyme is available.

As stated above, we propose that phosphorylation activates the Fru-2,6-P_2ase by relieving the repression by the N-terminal kinase domain, which is mediated by the tetrapeptide sequence. An interesting question is whether the tetrapeptide sequence containing three positively charged residues could be the phosphate recognition site. However, according to the crystal structure referred to above, the distance between the first located residue (residue 37) and the tetrapeptide sequence is approx. 42 Å. For hepatic isoenzymes, the phosphorylation site is at N-terminal residue 30 (for CKB) or 32 (for RKB). If we assume that the crystal structure of the testis isoenzyme basically reflects that of the hepatic isoenzyme, there seems no possibility that the phosphate group could interact directly with the tetrapeptide sequence. In our opinion, the mechanism of regulation of the hepatic Fru-2,6-P_2ase by phosphorylation might be somewhat similar to the case of glycogen phosphorylase, in which the phosphorylation of Ser^{14} promotes a long-range conformational change by introducing new charge-to-charge interactions, and alters the catalytic site to a catalytically competent one [25,26]. For the hepatic bifunctional enzyme, phosphorylation of the N-terminal serine residue probably activates the Fru-2,6-P_2ase by inducing an overall change in the tertiary structure of the enzyme and by partly disrupting the interaction of the two domains.

Comparison of the kinetic properties of CKB^{444K} and CKB^{444D}, or CKB^{445K} and CKB^{445D}, revealed that the electrostatic properties of His^{444} and Arg^{445} are responsible in large part for the role of these two residues. However, since mutation of the two residues to lysine also had some significant effects on the enzyme's properties, especially with regard to the effect of phosphorylation on the two activities of the enzyme, the specific properties of the side chains of the two residues must also be important. It is noteworthy that the tetrapeptide sequence His^{444}-Arg-Glu-Arg is well conserved in all isoforms of 6PF-2K/Fru-2,6-P_2ase. The first two basic residues (His^{444} and Arg^{445} of CKB) are completely conserved, whereas Glu^{446} and Arg^{447} are replaced in some isoforms by asparagine and lysine respectively. Thus it is reasonable to postulate that this sequence might play similar roles in the modulation of Fru-2,6-P_2ase activity in various members of the bifunctional enzyme family.

Compared with the rat liver bifunctional enzyme, the chicken liver enzyme behaves much more like a kinase. The V_{max} ratio of 6PF-2K to Fru-2,6-P_2ase of CKB is approx. 18-fold greater than that of RKB [14]. The phosphorylation of RKB greatly reduces the affinity of the 6PF-2K for Fru-6-*P* by 10–15-fold [10]. However, phosphorylation of CKB did not significantly change the K_m for Fru-6-*P* (Table 1). For CKB the ratio of the k_{cat}/K_m value of 6PF-2K to that of Fru-2,6-P_2ase decreased 8–12-fold upon phosphorylation, while that for RKB decreased 60–70-fold [10], indicating that the phosphorylation of RKB switches

the enzyme from a kinase to a bisphosphatase [9], whereas phosphorylated CKB still retains significant kinase activity. These facts are consistent with the particular metabolic requirements of the two species: in chicken and other domestic birds, the liver has a higher capacity for glucose utilization than rat liver, and at least 90 % of body fat synthesis in birds occurs in the liver [27,28]. The C-terminal regions of the two species-specific isoenzymes seem to contribute critically to the distinct effects of phosphorylation on the affinity of 6PF-2K for Fru-6-*P*. For RKB^{C30del}, the K_m for Fru-6-*P* of the kinase only increases 3-fold upon phosphorylation [11], whereas for wild-type RKB it increases 15-fold [10]. However, the K_m for Fru-6-*P* of CKB^{C30del} was elevated by 10-fold upon phosphorylation, while that of wild-type CKB was increased by no more than 2-fold (Figure 1). These observations indicate that the C-terminus of RKB intensifies the repressive effect of phosphorylation on the kinase activity, while that of CKB greatly weakens the inhibitory effect of phosphorylation on the kinase activity. This supports the hypothesis that the terminal regions of the various isoforms of the bifunctional enzyme regulate the kinase and bisphosphatase activities to suit their respective physiological needs.

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