Separate bisphosphatase domain of chicken liver 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase: the role of the C-terminal tail in modulating enzyme activity

Lin LI[1], Song LING, Chun-lai WU, Wei-zhe YAO and Gen-jun XU

Shanghai Institute of Biochemistry, Chinese Academy of Sciences, 320 Yue Yang Road, Shanghai 200031, China

The separate bisphosphatase domain (amino acid residues 243–468) of the chicken liver bifunctional enzyme 6-phosphofructo-2-kinase–fructose-2,6-bisphosphatase was expressed in *Escherichia coli* and purified to homogeneity. The fructose-2,6-bisphosphatase activity of the separate domain was 7-fold higher than that of the native bifunctional enzyme, and exhibited substrate inhibition characteristic of the native enzyme. The inhibition of the enzymes by fructose 2,6-bisphosphate could be overcome by P_i, glycerol 3-phosphate and GTP. Deletion of 30 amino acid residues from the C-terminus of the separate domain resulted in around a 5-fold increase in the V_{max} of the bisphosphatase. Also, the truncated form was more accessible to chemical modification by diethyl pyrocarbonate and *N*-ethylmaleimide, suggesting a more open structure than the wild-type form. In addition, the mutation of cysteine-389 to alanine increased bisphosphatase activity by 20% and the K_m value for fructose 2,6-bisphosphate by 3-fold, and both the point mutation at cysteine-389 and the deletional mutation led to the predominantly insoluble expression of the enzyme. The results indicated that the C-terminal tail plays a role in modulating the enzyme activity and suggested that the difference in the C-terminal tail sequence is responsible for the difference in activity of the chicken and rat liver fructose-2,6-bisphosphatases. It is postulated that an interaction between the C-terminal tail and the active site might be present.

INTRODUCTION

The bifunctional enzyme 6-phosphofructo-2-kinase(6PF-2K)–fructose-2,6-bisphosphatase (Fru-2,6-P_2ase) has a chimaeric structure consisting of an N-terminal kinase domain and a C-terminal bisphosphatase domain [1,2]. Three different forms of the separate Fru-2,6-P_2ase domain of the rat liver bifunctional enzyme (RBD), $RBD^{(227–470)}$, $RBD^{(235–470)}$ and $RBD^{(251–470)}$, have been expressed in *Escherichia coli* and characterized [3–5]. The cDNA of chicken liver bifunctional enzyme (CKB) was isolated and the enzyme was expressed in *E. coli* using the T7 RNA polymerase system [6,7]. Based on the deduced amino-acid sequence, the Fru-2,6-P_2ase domain of the chicken liver enzyme (CBD) has 95% similarity with that of RBD [6]. However, the activity of the chicken Fru-2,6-P_2ase was only one third that of the rat enzyme [7]. In order to determine if the difference in activity was due to difference in kinase/bisphosphatase interactions, CBD was expressed and its activity was compared with those of CKB and RBD. Moreover, the effect of a C-terminal 30-residue deletion from CBD (CBD^{C30del}) on the activity of Fru-2,6-P_2ase was also examined to ascertain if the difference in the activity of the chicken and rat enzymes was attributable to the difference in their C-terminal tail sequences.

MATERIALS AND METHODS

Materials

Restriction enzymes and DNase I were obtained from Boehringer-Mannheim (Mannheim, Germany). Q-Sepharose Fast Flow was from Pharmacia, Piscataway, NJ, U.S.A. Isopropyl β-D-thiogalactoside (IPTG), fructose 6-phosphate (Fru-6-P), fructose 2,6-bisphosphate (Fru-2,6-P_2), ATP, GTP, *N*-ethylmaleimide (NEM) and diethyl pyrocarbonate (DEP) were from Sigma, St. Louis, MO, U.S.A. [γ-^{32}P]ATP and [α-^{35}S]dATP were from Amersham (Little Chalfont, Bucks, U.K.).

Expression system

The plasmid pCBD for expression of the separate CBD was constructed as described previously [7]. pCBD was transformed into *E. coli* BL21(DE3) and proteins were induced by the addition of 0.4 mM IPTG at 22 °C for approx. 24 h. The same system was used for the expression of the mutant forms of CBD.

Oligonucleotide-directed mutagenesis

Site-directed mutation at cysteine-389 and the C-terminal 30-residue deletional mutation were engineered by the PCR method [8]. The $Cys^{389} \rightarrow Ala$ mutant was constructed by substituting the 360-base-pair *Eco*RI–*Sac*I fragment of CBD with that digested from the PCR product, and CBD^{C30del} was engineered by introducing a terminal codon (TGA) between valine-438 and glutamate-439. The double mutant $Cys^{389} \rightarrow Ala$ of CBD^{C30del} was directly constructed by substituting the 210-base-pair *Sac*I–*Bam*H I fragment of $Cys^{389} \rightarrow Ala$ with that of CBD^{C30del}. The nucleotide sequence of the mutant plasmid was determined

Abbreviations used: CBD, separate chicken liver fructose-2,6-bisphosphatase domain; CBD^{C30del}, C-terminal 30-residue deletional mutant of CBD; C389A/CBD, $Cys^{389} \rightarrow Ala$ mutant of CBD; CKB, chicken liver 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase; RBD, rat liver fructose-2,6-bisphosphatase domain; Fru-6-P, fructose 6-phosphate; Fru-2,6-P_2, fructose 2,6-bisphosphate; Fru-2,6-P_2ase, fructose-2,6-bisphosphatase; 6PF-2K, 6-phosphofructo-2-kinase; IPTG, isopropyl β-D-thiogalactoside; GdnHCl, guanidine hydrochloride; DEP, diethyl pyrocarbonate; DTT, dithiothreitol; NEM, *N*-ethylmaleimide.

[1] To whom correspondence should be addressed.

on the alkali-denatured double-stranded DNA to ensure that there were no other point mutations.

Enzyme purification

CBD purification

Proteins were extracted from the expressed cells as described by Lin et al. [9]. The buffer used for purification was 20 mM Tris/HCl (pH 8.0)/50 mM KCl/0.5 mM EDTA/1 mM dithiothreitol (DTT) or 2 mM 2-mercaptoethanol and 5% (v/v) glycerol. The protein extracted from 1 litre of culture medium was loaded directly on to a Q-Sepharose Fast Flow column (1.8 cm × 15 cm). The proteins were eluted by a 50–500 mM KCl gradient; CBD was eluted at about 280 mM KCl. The fractions containing CBD were pooled and the proteins were precipitated by the addition of $(NH_4)_2SO_4$ to 60% saturation. The precipitated proteins were dissolved in a small volume of buffer, the sample was loaded on to an Ultrogel ACA-44 column (2.0 cm × 120 cm) and eluted with the same buffer. CBD gave a peak at the elution volume of about 260 ml.

Mutant CBD purification

The mutant enzymes mainly existed in insoluble forms in the expressed cells (Figure 1). However, the soluble enzymes reached up to 15–20 mg/l of culture medium. All mutant forms of CBD were purified from the soluble extracts by the procedure described above for wild-type CBD.

CKB purification

Purification of CKB and the rat liver bifunctional enzyme 6PF-2K–Fru-2,6-P_2ases were purified to homogeneity as described previously [7].

Determination of CD spectra

All far UV spectra were collected on a JASCO 720 spectrophotometer in a 0.01-cm cell at 25 °C. The scan was collected at a speed of 50 nm/min with a response of 2 s and resolution of 0.2 nm. The band width was 1.0 nm and every datum was the average of 4 scans. Samples were dissolved in 20 mM Tris/HCl (pH 8.0)/0.5 mM EDTA/1 mM DTT/50 mM KCl and centrifuged at 25000 ***g*** for 10 min to remove any precipitated protein. The A_{280} was measured to scale the CD data to the same concentration.

Fluorescence measurements

Wild-type and mutant CBD (30 μg/ml) were incubated at 25 °C for 20 min with the indicated concentrations of guanidine hydrochloride (GdnHCl) (see Figure 3) in 50 mM Tris/HCl (pH 7.5)/0.5 mM DTT. Protein fluorescence was measured in a Hitachi fluorescence spectrophotometer (model F-4010) from 300 nm to 400 nm with a excitation wavelength of 295 nm, the excitation and the emission bandpass were each 5 nm. The emission fluorescence intensity at 335 nm and the maximum emission wavelength were recorded.

Assay of Fru-2,6-P_2ase activity

Fru-2,6-P_2ase activity was measured at pH 7.4 by following the rate of production of [^{32}P]P_i from [2-^{32}P]Fru-2,6-P_2 as described by El-Maghrabi et al. [10].

Chemical modification of CBD

CBD or CBD^{C30del} (10 μg) was incubated at 30 °C with various concentrations of DEP or NEM in a total volume of 100 μl. After 2 min, the reactions were stopped by addition of DTT to the incubation mixture to a final concentration of 10 mM DTT, and the enzyme activities were assayed as described above.

Preparation of [2-^{32}P]Fru-2,6-P_2

Radioactively-labelled Fru-2,6-P_2 was prepared by incubating 0.1 mM [γ-^{32}P]ATP (0.5 mCi) and 10 mM Fru-6-P in 20 mM Tris/HCl (pH 8.0)/100 mM KCl/1 mM $MgCl_2$/5 mM P_i/0.5 mM EDTA/1 mM DTT buffer containing 10 μg of purified CKB in a total volume of 100 μl for 1 h at 30 °C. The reaction was stopped by the addition of 5 μl of 5N NaOH. After treatment with charcoal, the sample was loaded on to a DEAE–Sephadex A-25 column (2 ml). The column was washed with approx. 20 ml of 0.2 M triethylamine/HCO_3, pH 8.2, and [2-^{32}P]Fru-2,6-P_2 was eluted from the column by 1 M KCl in 20 mM Tris/HCl, pH 8.0. Protein concentration was determined by the method of Lowry et al. [11].

RESULTS

Expression and purification of wild-type and mutant forms of CBD

Previously, an expression vector encoding residues 243–468 of CBD was constructed and it was reported that CKB was expressed in *E. coli* at a very low level, probably due to rapid degradation after induction by IPTG [7]. In contrast, CBD was expressed efficiently in *E. coli* to a level of approx. 100 mg/l, as determined by the phospho–enzyme intermediate assay [10] using CKB as a standard. The expression reached a maximum level at 20–24 h after induction by IPTG (results not shown). As shown in Figure 1, CBD was expressed as a major soluble protein.

The Cys^{389} → Ala mutant of CBD (C389A/CBD) and the truncated mutant, CBD^{C30del} were engineered by PCR as described in the Materials and methods section. An interesting finding was that, in contrast to wild-type CBD, both expressed C389A/CBD and CBD^{C30del} mainly existed in insoluble forms, although the expression levels were also high (Figure 1). Each of CBD, C389A/CBD, CBD^{C30del} and C389A/CBD^{C30del} was

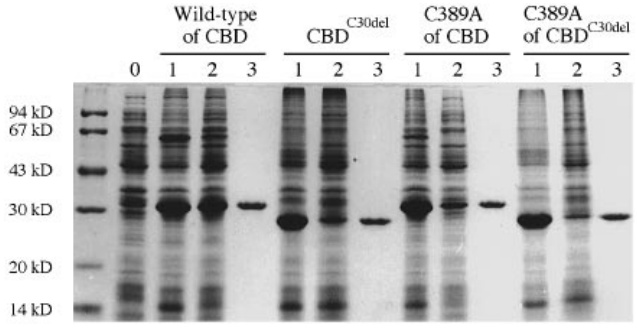

Figure 1 SDS/PAGE analysis of whole cell and soluble extracts of expression cultures and purified forms of wild-type and mutant CBD

The wild-type and mutant forms of CBD were expressed in BL21(DE3) by induction by IPTG for 24 h at 22 °C, and were extracted and purified to homogeneity as described in the Materials and methods section. The whole cell extract, soluble extract and the purified proteins were treated by boiling in sample buffer and then separated by SDS/PAGE (12% gel). Lane 0, control without induction by IPTG; and lanes 1, whole cells; lanes 2, soluble extract; lanes 3, purified enzyme, for each CBD form.

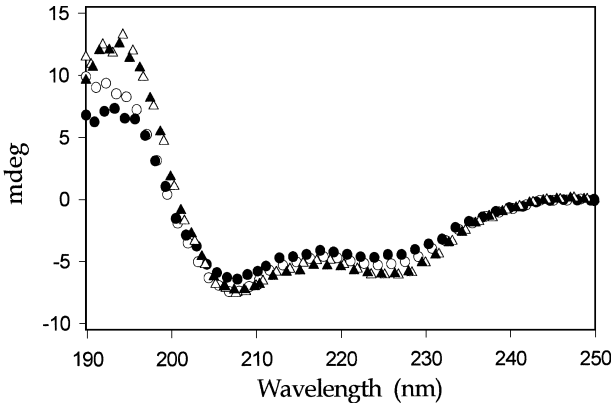

Figure 2 CD spectra of the wild-type and mutant forms of CBD

The CD data were corrected for the blank reading and scaled for concentration. The mol degree (mdeg) of signal corresponds to measurements conducted in a 0.01-cm cell with a protein solution equal to 0.8 A_{280} in a 1-cm light path. CBD (●), CBD^{C30del} (▲), C389A/CBD (○) and C389A/CBD^{C30del} (△). For clarity only selected data points are presented.

purified by anion-exchange (Q-Sepharose FF) and gel-filtration (Ultrogel ACA-44) chromatography from extracts of the expressed cells. The four purified enzymes were homogeneous, shown by SDS/PAGE (Figure 1). The apparent molecular mass of CBD was about 32 kDa, determined by gel filtration on Ultrogel ACA-44 (results not shown), and the elution volumes for C389A/CBD, CBD^{C30del} and C389A/CBD^{C30del} were similar to that of wild-type CBD. The result indicates that, in contrast to the full-length bifunctional enzyme which behaves as a homodimer, CBD is monomeric in solution.

CD spectra and change in fluorescence properties of wild-type and mutant forms of CBD

To compare the secondary structure of the C-terminal deletional mutant with full-length CBD, and to determine whether mutation of cysteine-389 to alanine changed the secondary structure of the enzyme, the CD spectra of the four forms of CBD were determined. As shown in Figure 2, the deletional mutation led to a slight change in the far UV CD spectra of CBD, and the results of CD experiments demonstrate that the point mutation at cysteine-389 has no appreciable effect on the secondary structure of CBD. Estimations of secondary structure for CBD are: $29.9\pm0.5\%$ α-helix, $29.3\pm0.7\%$ β-structure and $7.2\pm1.7\%$ turn; for C389A/CBD are: $27.7\pm0.5\%$ α-helix, $29.7\pm0.3\%$ β-structure and $10.0\pm0.6\%$ turn; and for CBD^{C30del} are: $44.8\pm0.7\%$ α-helix, $19.5\pm1.9\%$ β-structure and $8.0\pm0.8\%$ turn (means$\pm$S.D.). The results indicate that the mutation at cysteine-389 does not significantly change the secondary structure of the enzyme, and suggest that the CBD^{C30del} has a more orderly structure than CBD and/or the C-terminal tail of CBD exhibits a random structure.

The fluorescence of the four forms of CBD in various concentrations of GdnHCl was measured and, as shown in Figure 3 (upper panel), the red shifts of the emission maximum of the four forms are almost the same, indicating that their polarity change in the hydrophobic environment of tryptophan are similar. The results also suggest that there is no significant change in the structure of the three mutant CBD when compared with the wild-type. Both CBD and CBD^{C30del} showed an increase in fluorescence intensity to about 140% of the original value and reached a maximum at 1.5 M GdnHCl (Figure 3, lower panel).

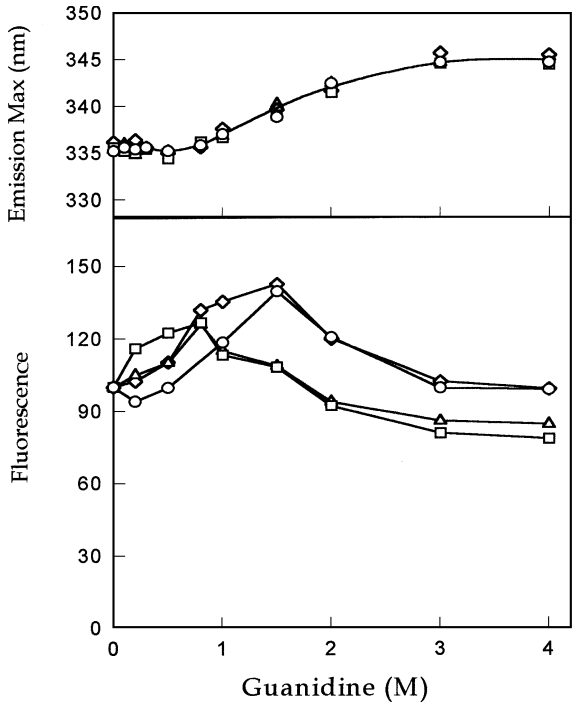

Figure 3 Change in fluorescence of the wild-type and mutant CBD in various concentrations of GdnHCl

The conditions used are described in the Materials and methods section. The emission maximum (upper panel) and fluorescence intensity at 335 nm (lower panel) are shown. CBD (○), CBD^{C30del} (□), C389A/CBD (△) and C389A/CBD^{C30del} (◇).

Table 1 Comparison of kinetic properties of Fru-2,6-P_2ase of CKB, wild-type and mutant forms of CBD

The Fru-2,6-P_2ase activity was measured in the presence of 5 mM P_i, as described in the Materials and methods section. The V_{max} and K_m values were obtained from Lineweaver–Burk plots and the K_i values ($1/v$ versus Fru-6-P concentrations) were obtained from Dixon plots. The values represent the average of at least three determinations.

	Enzyme forms				
Kinetic Parameters	CKB	CBD	CBD^{C30del}	C389A/CBD	C389A/CBD^{C30del}
V_{max} (m-units/mg of protein)	12	87	445	106	420
K_m, Fru-2,6-P_2 (μM)	0.21	0.23	0.32	0.71	0.32
K_i, Fru-6-P (μM)	10	16	6	20	8

Those for C389A/CBD and C389A/CBD^{C30del} increased to about 126% and reached a maximum at 0.8 M GdnHCl. Based on these results, we postulate that the increases in fluorescence are due to the departure of one or more quenching residue(s) in the hydrophobic environment of tryptophan during denaturation of the enzymes by GdnHCl, and that cysteine-389 might be a quenching residue, since cysteine residues have been reported to be efficient quenchers of tryptophan fluorescence [12].

Kinetic properties of wild-type and mutant forms of CBD

As shown in Table 1, the Fru-2,6-P_2ase activity of CBD was about 7-fold higher than that of the CKB, indicating that

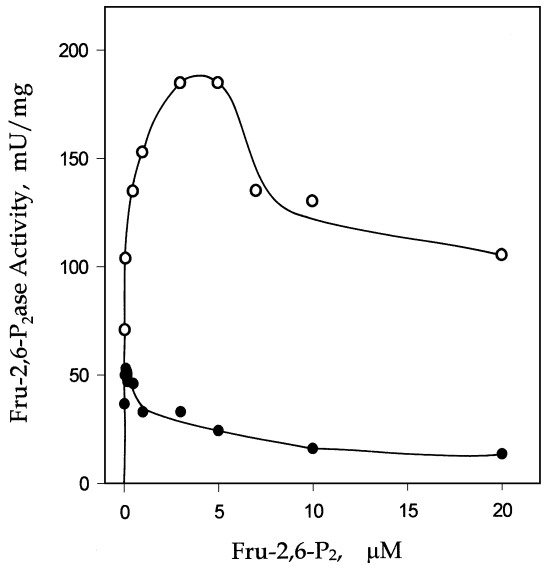

Figure 4 Fru-2,6-P_2ase activity of CBD and CBD^{C30del} with Fru-2,6-P_2 as substrate

The activities were measured, in the absence of P_i, as described in the Materials and methods section. CBD (●); CBD^{C30del} (○).

Table 2 Effect of P_i, glycerol 3-phosphate and GTP on the Fru-2,6-P_2ase activities of CKB, CBD and CBD^{C30del}

Fru-2,6-P_2ase activity was assayed in the presence of 5 mM P_i, 5 mM glycerol 3-phosphate and 1 mM GTP respectively. The enzymes were preincubated with the effectors and the reaction was started by the addition of Fru-2,6-P_2. The values are the means ± S. E. of three to four determinations.

	Relative Fru-2,6-P_2ase activity	
Enzyme forms	Fru-2,6-P_2 (0.05 μM)	Fru-2,6-P_2 (10 μM)
CKB		
control	1	1
+ P_i	0.50 ± 0.12	5.2 ± 0.4
+ glycerol 3-phosphate	0.88 ± 0.11	7.0 ± 0.7
+ GTP	1.0 ± 0.1	4.1 ± 1.5
CBD		
control	1	1
+ P_i	0.63 ± 0.04	3.9 ± 0.7
+ glycerol 3-phosphate	0.57 ± 0.09	5.3 ± 0.4
+ GTP	0.97 ± 0.07	3.1 ± 0.7
CBD^{C30del}		
control	1	1
+ P_i	0.66 ± 0.11	3.0 ± 0.4
+ glycerol 3-phosphate	0.85 ± 0.09	3.3 ± 0.4
+ GTP	0.65 ± 0.05	2.7 ± 0.1

interactions between the N-terminal 6PF-2K domain and the C-terminal Fru-2,6-P_2ase domain results in inhibition of Fru-2,6-P_2ase activity. The V_{max} of CBD^{C30del} was 445 m-units/mg of protein, which is about 5-fold higher than that of CBD and 37-fold that of CKB. The results demonstrate that, in addition to suppression by the N-terminal kinase domain in 6PF-2K–Fru-2,6-P_2ase, Fru-2,6-P_2ase activity is also modulated by the C-terminal tail in CBD.

Previously, it has been proposed that cysteine-256 and cysteine-391 of the rat liver 6PF-2K–Fru-2,6-P_2ase may form a disulphide bond [13]. The lack of effect when cysteine-256 is replaced by serine or alanine residues argues against this hypothesis [7]. Cysteine-391, which is adjacent to a catalytic-site residue histidine-392, is conserved in the chicken enzyme (cysteine-389) [6]. It was still interesting to identify the role this residue plays in the catalysis and/or substrate binding of CBD. As shown in Table 1, the substitution of cysteine-389 with alanine in the CBD resulted in 20% increase in V_{max} and an approx. 3-fold increase in K_m value for Fru-2,6-P_2. However, the cysteine-389 mutation of CBD^{C30del} did not significantly affect the kinetic properties of Fru-2,6-P_2ase. The results suggested that cysteine-389 contributed to the binding of Fru-2,6-P_2 in CBD to some extent, but the C-terminal 30-amino-acid deletion eliminated this role of the cysteine-389 residue.

Substrate inhibition of Fru-2,6-P_2ase of CBD and CBD^{C30del}

It has been reported that the bisphosphatase of the rat liver bifunctional enzyme exhibits substrate inhibition at saturated Fru-2,6-P_2 concentrations [13], and that the three basic residues, arginine-352, lysine-356 and arginine-360, are responsible for the inhibition of the rat liver enzyme [14,15]. Since these three residues are conserved in CBD (the corresponding residues are arginine-351, lysine-355 and arginine-359) [6], it is interesting to determine if the chicken enzyme is also inhibited by the substrate. The substrate saturation curves for CBD and CBD^{C30del} are shown in Figure 4. The curve for CKB was very similar to that for CBD (results not shown). The results clearly demonstrate that the Fru-2,6-P_2ases of both CKB and CBD exhibit substrate inhibition. However, unlike CBD or CKB, which were inhibited at Fru-2,6-P_2 concentrations greater than 0.1 μM, CBD^{C30del} was not inhibited by Fru-2,6-P_2 concentrations < 5 μM (Figure 4).

Glycerol 3-phosphate, P_i and GTP are known effectors of Fru-2,6-P_2ase [13,16–18]. These three effectors are not only competitive inhibitors with the substrate Fru-2,6-P_2, but also activators at saturated Fru-2,6-P_2 concentrations by relieving substrate inhibition. The effect of the three effectors on the chicken enzymes was investigated. As shown in Table 2, all three effectors activated the Fru-2,6-P_2ase activities of CKB, CBD and CBD^{C30del} at a Fru-2,6-P_2 concentration of 10 μM, indicating that P_i, glycerol 3-phosphate and GTP could overcome the inhibition of the enzymes by Fru-2,6-P_2. In addition, at low Fru-2,6-P_2 concentration (0.05 μM), both P_i and glycerol 3-phosphate inhibited the activity of CKB, CBD and CBD^{C30del}, and GTP at a concentration of 1 mM also inhibited the activity of CBD^{C30del} but not those of CKB and CBD. The results suggested that GTP is a poor effector with respect to inhibition of Fru-2,6-P_2ase of both wild-type CKB and CBD, and that CBD and CBD^{C30del} have distinct properties with respect to the effect of GTP.

Effect of chemical modification on the activities of CBD and CBD^{C30del}

In order to determine further if two different active-site conformations for CBD and CBD^{C30del} existed, two chemical modifiers DEP and NEM were used to explore these two forms of the enzyme. As shown in Figure 5, CBD^{C30del} was more accessible to DEP and NEM than the wild-type CBD. Under similar conditions, the loss of activity of CBD^{C30del} occurred more quickly than that of CBD as the concentration of DEP or NEM was increased. Two histidine residues (histidine-258, histidine-392) have been identified as catalytic residues of Fru-2,6-P_2ase of the rat liver 6PF-2K–Fru-2,6-P_2ase [19], and previous work suggested that the modification of histidine residues at the active

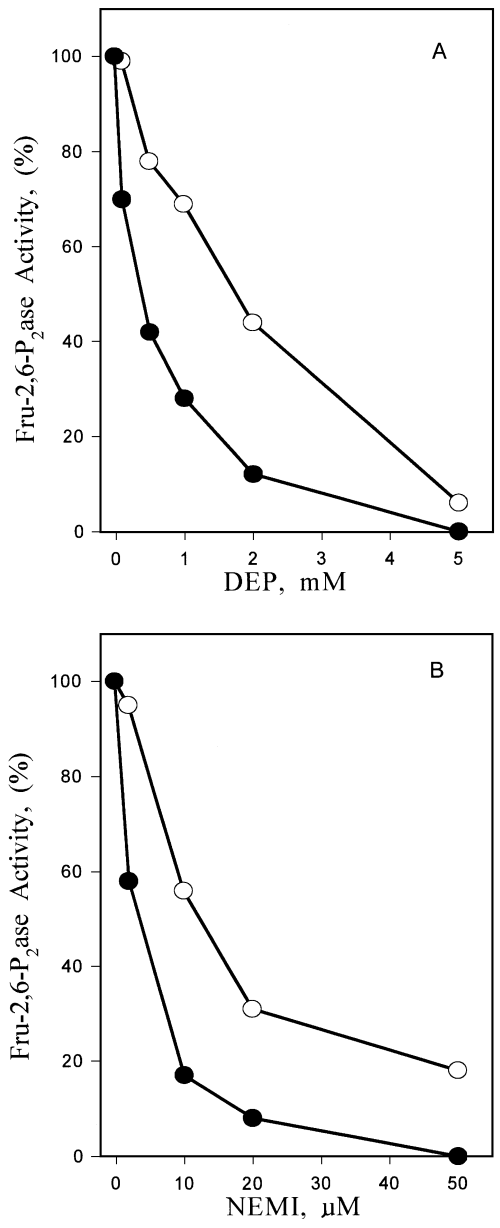

Figure 5 Fru-2,6-P_2ase activities of CBD and CBD^{C30del} as functions of DEP and NEM concentrations

CBD (○) or CBD^{C30del} (●) (10 μg) was incubated with various concentrations of (**A**) DEP or (**B**) NEM (NEMI) for 2 min and assayed for Fru-2,6-P_2ase activity as described in the Materials and methods section.

site by DEP inactivated Fru-2,6-P_2ase [20]. The two residues are conserved in the chicken enzyme (the corresponding residues, are histidine-257 and histidine-390) [6]. Deletion of the C-terminal tail of CBD led to an increase in accessibility of chemical reagents to the active site (Figure 5), which suggested that CBD^{C30del} has a more open structure than CBD.

It is worth noting that, although NEM inactivated Fru-2,6-P_2ase of CBD, it did not affect the rat liver 6PF-2K–Fru-2,6-P_2ase [21]. Since all cysteine residues of CBD are conserved in the rat liver enzyme [6], this raised the question whether the presence of the kinase domain in 6PF-2K–Fru-2,6-P_2ase prevented cysteine residue(s) located within the bisphosphatase domain from reacting with the reagent.

DISCUSSION

CBD was expressed efficiently in *E. coli* with a 7-fold increase in Fru-2,6-P_2ase activity when compared with CKB (Table 1). A similar phenomenon was observed in the rat liver enzyme [4,5]. The separate bisphosphatase domains of the rat liver enzymes, $RBD^{(235–470)}$ [4] and $RBD^{(251–470)}$ [5], also had higher activities than that of 6PF-2K–Fru-2,6-P_2ase. Recently, we found several monoclonal antibodies against CKB which activated Fru-2,6-P_2ase of CKB by 4-fold without affecting the activity of CBD [22]. The results suggested that the conformation/activity state of Fru-2,6-P_2ase in CKB and CBD are distinct. It seems that the effect of the monoclonal antibodies on CKB was to relieve the interaction between the two domains.

Comparison of the amino-acid sequences revealed that the Fru-2,6-P_2ase domains of CKB and the rat liver 6PF-2K–Fru-2,6-P_2ases are 95% similar [6]. However, the Fru-2,6-P_2ase activity of the rat bifunctional enzyme is 3-fold higher than that of CKB [7]. The V_{max} of CBD was 87 m-units/mg of protein (Table 1), which was only 25% of the V_{max} of $RBD^{(251–470)}$ (360 m-units/mg of protein) [5]. A number of residues, which previously have been shown to be critical in substrate binding and/or catalysis in the Fru-2,6-P_2ase of the rat liver 6PF-2K–Fru-2,6-P_2ase [1], are all conserved in the chicken liver enzyme [6]. Cysteine-256, which is adjacent to two critical residues arginine-257 and histidine-258, is not conserved in the chicken enzyme where the corresponding residue is serine-255 [6]. However, it has been shown by site-directed mutagenesis that the variation at this position is not responsible for the difference in activity of Fru-2,6-P_2ases [7].

It is obvious that the major difference in the primary sequences of the chicken and mammalian liver Fru-2,6-P_2ase domains is in the C-terminal tail [6]. Since the deletion of 30 amino acid residues from the C-terminus resulted in approx. 30% activation of Fru-2,6-P_2ase in RBD (V_{max} of $RBD^{(251–440)}$ was 430 m-units/mg of protein [5]), and the V_{max} of CBD^{C30del} was similar to that of $RBD^{(251–440)}$, it is reasonable to infer that the difference in activities of CBD and RBD is because of the differences in the C-terminal amino-acid sequence.

Cysteine-389 is adjacent to a catalytic-site residue, histidine-390. The results of mutagenesis experiments suggested that this residue may play a role in substrate binding as mutation at cysteine-389 resulted in a 3-fold increase in K_m when compared with the wild-type enzyme (Table 1). However, mutation by amino-acid deletion at the C-terminal eliminated the effect of mutation at cysteine-389, and mutation of cysteine-389 resulted in an obvious increase of aggregate expression of CBD, which was quite similar to that of the C-terminal deletional mutation. The results implied a relationship between the C-terminal tail and the cysteine-389 residue. It is possible that the C-terminus might overhang the active site of Fru-2,6-P_2ase and/or there exists an interaction between the C-terminal tail and the cysteine-389 residue. There is evidence that the conformational states of CBD^{C30del} and CBD might be quite different: the CD measurements suggest that CBD^{C30del} has a more orderly structure than CBD; CBD is much more sensitive to substrate inhibition than CBD^{C30del} (Figure 4); GTP is an efficient inhibitor of CBD but not of CBD^{C30del} (Table 2); and covalent modification experiments suggested that CBD^{C30del} has more open structure than CBD (Figure 5), which may be why the C-terminus can inhibit Fru-2,6-P_2ase activity. Crystal structures of C-terminal truncated RBD and a mutant form of the rat testis 6PF-2K–Fru-2,6-P_2ase

have been obtained [23,24] and it was noted that the absence of the C-terminus of RBD led to a much more open active site than was seen in the testis isoenzyme [24]. Since the C-terminus of the enzyme plays an important role in regulating the activity of Fru-2,6-P_2ase, it is important to obtain the structures of the full-length bisphosphatase domain and of the wild-type 6PF-2K–Fru-2,6-P_2ase in order to investigate how the C-terminal tail modulates Fru-2,6-P_2ase activity and whether there exists direct interaction between the C-terminal tail and the active site. This work is in progress.

Comparison of the amino-acid sequences of various 6PF-2K–Fru-2,6-P_2ase isoforms, such as the liver [25], skeletal muscle [26], heart [27,28] and testis [29] forms, reveals a highly conserved kinase and bisphosphatase domain core with isoform specific N- and C-terminal tails attached. The bovine heart form has a C-terminal tail 61 residues longer than that of the liver form [28], which might contribute to the much lower Fru-2,6-P_2ase activity in heart form. The deletion of 78 amino acid residues from the C-terminal of the heart 6PF-2K–Fru-2,6-P_2ase resulted in a 3-fold increase in Fru-2,6-P_2ase activity [30]. Our results indicate that deletion of 30 residues from the C-terminal inhibits Fru-2,6-P_2ase activity in CBD. It is believed that interactions between the N- and C-terminus determine the kinase/phosphatase ratio among various isoforms from different species.

We are grateful for the constructive criticism of Dr. Kai Lin (Department of Biochemistry, U.C.S.F., San Francisco, CA 94143, U.S.A.) during preparation of this manuscript. The project was supported by the National Natural Science Foundation of China and the Pandeng Project of the China Commission for Science and Technology.

REFERENCES

1 Pilkis, S. J., Claus, T. H., Kurland, I. J. and Lange, A. J. (1995) Annu. Rev. Biochem. **64**, 799–835
2 Rousseau, G. G. and Hue, L. (1993) Prog. Nucleic Acid Res. **45**, 99–127
3 Tauler, A., Rosenberg, A. H., Colosia, A., Studier, F. W. and Pilkis, S. J. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 6642–6646
4 Lin, K., Kurland, I. J., Li, L., Lee, Y. H., Okar, D., Marecek, J. F. and Pilkis, S. J. (1994) J. Biol. Chem. **269**, 16953–16960
5 Lee, Y. H., Lin, K., Okar, D., Alfano, N. L., Sarma, R., Pflugrath, J. W. and Pilkis, S. J. (1994) J. Mol. Biol. **235**, 1147–1151
6 Li, L., Lange, A. J. and Pilkis, S. J. (1993) Biochem. Biophys. Res. Commun. **190**, 397–405
7 Li, L., Yao, W.-Z., Lange, A. J., Pilkis, S. J., Dong, M.-Q., Yin, Y. and Xu, G.-J. (1995) Biochem. Biophys. Res. Commun. **209**, 883–893
8 Nelson, R. M. and Long, G. L. (1989) Anal. Biochem. **180**, 147–151
9 Lin, K., Kurland, I. J., Xu, L. Z., Lange, A. J., Pilkis, J., El-Maghrabi, M. R. and Pilkis, S. J. (1990) Protein Expression Purif. **1**, 169–176
10 El-Maghrabi, M. R., Correia, J. J., Heil, P. J., Pate, T. M., Cobb, C. E. and Pilkis, S. J. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 5005–5009
11 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. **193**, 265–275
12 Steiner, R. F. and Kirby, E. P. (1969) J. Phys. Chem. **73**, 4130–4135
13 Stewart, H. B., El-Maghrabi, M. R. and Pilkis, S. J. (1985) J. Biol. Chem. **260**, 12935–12941
14 Li, L., Lin , K., Correia, J. J. and Pilkis, S. J. (1992) J. Biol. Chem. **267**, 16669–16675
15 Li, L., Lin, K., Pilkis, J., Correia, J. J. and Pilkis, S. J. (1992) J. Biol. Chem. **267**, 21588–21594
16 Van Schaftingen, E., Davies, D. R. and Hers, H. G. (1982) Eur. J. Biochem. **124**, 143–149
17 El-Maghrabi, M. R., Claus, T. H., Pilkis, J., Fox, E. and Pilkis, S. J. (1982) J. Biol. Chem. **257**, 7603–7607
18 Lee, Y. H., Okar, D., Lin, K. and Pilkis, S. J. (1994) J. Biol. Chem. **269**, 11002–11010
19 Tauler, A., Lin, K. and Pilkis, S. J. (1990) J. Biol. Chem. **265**, 15617–15622
20 Pilkis, S. J., Walderhaug, M., Murray, K., Beth, A., Venkataramu, S. D., Pilkis, J. and El-Maghrabi, M. R. (1983) J. Biol. Chem. **258**, 6135–6141
21 El-Maghrabi, M. R., Pate, T. M., Pilkis, J. and Pilkis, S. J. (1984) J. Biol. Chem. **259**, 13104–13110
22 Li, T.-Y., Ding, J.-F., Li, L. and Xu, G.-J. (1996) Sci. China Ser. C, **39**, 342–349
23 Lee, Y. H., Ogata, C., Pflugrath, J. W., Levitt, D. G., Sarma, R., Banaszak, L. J. and Pilkis, S. J. (1996) Biochemistry **35**, 6010–6019
24 Hasemann, C. A., Istvan, E. S., Uyeda, K. and Deisenhofer, J. (1996) Structure **4**, 1017–1029
25 Lively, M. O., El-Maghrabi, M. R., Pilkis, J., D'Angelo, G., Colosia, A. D., Ciavola, J. A., Fraser, B. A. and Pilkis, S. J. (1988) J. Biol. Chem. **263**, 839–849
26 Darville, M. J., Crepin, K. M., Hue, L. and Rousseau, G. G. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 6543–6557
27 Kitamura, K. and Uyeda, K. (1987) J. Biol. Chem. **262**, 679–681
28 Tsuchya, Y. and Uyeda, K. (1994) Arch. Biochem. Biophys. **310**, 467–474
29 Sakata, J., Abe, Y. and Uyeda, K. (1991) J. Biol. Chem. **266**, 15764–15770
30 Abe, Y, Minami, Y., Li, Y., Nguyen, C. and Uyeda, K. (1995) Biochemistry **34**, 2553–2559

Received 27 May 1997/19 August 1997; accepted 3 September 1997