FEBS Letters 475 (2000) 22–26 FEBS 23741

Creatine kinase isoenzymes specificities: histidine 65 in human CK-BB, a role in protein stability, not in catalysis

Tamara Mourad-Terzian[a], Jean-Paul Steghens[a,*], Kyung-Lyum Min[b], Christian Collombel[a], Dominique Bozon[c]

[a] *Laboratoire de Biochimie C, Hôpital Edouard Herriot, 5 place d'Arsonval, 69437 Lyon Cedex 03, France*
[b] *Department of Microbiology, College of Natural Sciences and Research Center for Microbiology, Seoul National University, Seoul 151-742, South Korea*
[c] *Laboratoire de Biochimie pédiatrique, Hôpital Debrousse, 29 rue Sœur Bouvier, 69322 Lyon Cedex 05, France*

Received 24 March 2000; received in revised form 10 May 2000

Edited by Pierre Jolles

Abstract Creatine kinases (CK) play a prominent role in cell energy distribution through an energy shuttle between mitochondria and other organelles. Human brain CK was cloned and overexpressed in COS-7 cells. We then deleted His-65 and/or Pro-66 situated near the center of a flexible loop as shown by X-ray crystallography on mitochondrial and cytosolic CK. The ΔH65 mutant had nearly the same affinity for its substrates as wild isoenzyme, but its stability was very low. Unlike ΔH65, ΔH65P66 had a eightfold decreased affinity for creatine phosphate and was unable to dephosphorylate cyclocreatine phosphate. Our results demonstrate that, despite an overall similar shape of the proteins, this loop accounts for some subtle differences in isoenzyme functions.

Key words: Brain-type creatine kinase; Expression; Site-directed mutagenesis; Protein stability

1. Introduction

Creatine kinases (ATP:creatine phosphotransferase, EC 2.7.3.2; CK) are key enzymes in the regulation of cellular energy homeostasis. They are the two ends of a cell energy shuttle between mitochondria and cellular substructures. The mitochondrial isoform of CK, CK-Mi, which is bound to the outer surface of the inner mitochondrial membrane, phosphorylates creatine with ATP to export energy as creatine phosphate (CP) from the mitochondria. Then ADP is phosphorylated in the presence of CP by cytosolic CK isoenzymes which are associated with subcellular structures requiring ATP, e.g. microtubules, membrane structure of sarcoplasmic reticulum and myofibrils [1–5].

Two different genes encode two cytosolic CK subunits: B (brain) and M (muscle), with a molecular mass of ~41 kDa and a high amino acid sequence homology (78–80%) among species [6]. The subunits associate to form homodimeric (MM, BB) and heterodimeric (MB) isoenzymes. The MM and MB forms are characteristic of skeletal and heart muscle whereas the BB form is predominant in brain. There are also two mitochondrial forms: ubiquitous (CK-Mi_a) and sarcomeric (CK-Mi_b) mitochondrial CK [3,5,7], encoded by two different genes [8]. Each mitochondrial subunit has a molecular weight of approximately 42 kDa and the active forms are dimeric and octameric. Recently, chicken muscle CK-Mi_b was crystallized without its substrates and its X-ray structure analyzed [9]. More recently, the same group published the crystal structure at 1.41 Å resolution [10].

The function of some amino acid residues has been investigated by site-directed mutagenesis. Since it had been suggested that a histidine residue may act as a general acid–base catalyst [11,12], the Kenyon group tried to identify this residue by mutagenesis of conserved His-96, His-105, His-190, His-223 and His-295, in rabbit muscle CK. Neither of these amino acids was essential for catalysis [13].

In mitochondrial isoenzymes, a reactive cysteine, Cys-278, conserved among the guanidine kinases has been shown to play an important role in the synergism of substrate binding, but not to be essential for catalysis [14].

The mutation of six Trp residues of chicken sarcomeric mitochondrial CK by site-directed mutagenesis identified Trp-223 as an active site residue and Trp-206 as important for conformation and active site stabilization [15]. In a similar manner, Trp-210 was shown to be essential for stabilizing the dimeric active state of CK-MM [16]. According to the X-ray crystallographic structure, three of the five conserved histidines surrounding the active site (His-61, 92, and 186 of chicken CK-Mi_b) were mutated [17]. The first, corresponding to His-65 in CK-BB, seemed to be important for the catalytic reaction but did not serve as an acid–base catalyst in the transphosphorylation reaction. Forstner et al. [17] suggested the involvement of His-92 and His-186 in the binding of creatine and ATP in the active site. They also suggested the role of a flexible loop bearing this His-61, highly conserved through all of the CKs. This loop moves towards the guanidine substrate binding site and contributes to catalysis. In our laboratory, we worked on synthesizing selective and specific inhibitors of CK isoenzymes [18]. Further data indicated noticeable differences between inhibition of cytosolic isoenzymes and different catalytic mechanisms [19]. These results strongly suggested some differences in the creatine binding domain of the human CK-BB and CK-MM. These data emphasize that CK isoenzyme specificity relies more on the CP than on the nucleotide binding domain. In order to determine the CP bind-

*Corresponding author. Fax: (33)-4-72 11 06 75.
E-mail: jean-paul.steghens@chu-lyon.fr

Abbreviations: CK, creatine kinase; CK-BB, brain-type creatine kinase; CK-BB$(His)_6$, C-terminal 6 histidine-tagged CK-BB; BCK, gene encoding CK-BB; CP, creatine phosphate; CCr, cyclocreatine; CCrP, cyclocreatine phosphate; ArgK, arginine kinase

0014-5793/00/$20.00
PII: S0014-5793(00)01614-8

ing domain in CK-MM, we synthesized a photoactivatable bisubstrate analog, BzPG (*N*-dibenzylphospho-*N'*-(4-benzoyl)-benzylguanidine) [20]. After photoaffinity labeling and CNBr cleavage of rabbit CK-MM, this photoprobe allowed us to sequence a peptide Ala-30–Val-60 belonging to the CP binding site [20]. We therefore decided to use site-directed mutagenesis to focus on the potential role of His-65 in CK-BB in comparison to CK-Mi. We performed a deletion of His-65 and/or Pro-66 on a histidine-tagged human isoenzyme. As mutants were expressed in COS-7 cells containing their own CK-BB, the tag avoided any contamination by endogenous CK.

Despite the similar shape of these two isoenzymes revealed by crystallography, we show that the flexible loop plays a different role in CK-BB. Our results also suggest that nucleotidic and CP binding sites interact differently.

2. Materials and methods

2.1. Materials

The following items were obtained from the indicated sources: ADP, NAD, NADH, hexokinase (EC 2.7.1.1), glucose-6-phosphate dehydrogenase (EC 1.1.1.49), pBH vector (ref. 1814605) (Roche Molecular Biochemicals); cellular RNA was extracted with RNeasy Mini kit (Qiagen, ref. 74106), Qiagen Plasmid Maxi kit (Qiagen, refs. 74106 and 12263); human brain mRNA (Clontech, ref. 6543-1); *Escherichia coli* strain DH5α competent cells, Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, refs. 18265-017 and 32430-027 respectively), expression vector pcDNA3.1(+), pZeoSV40 (Invitrogen, refs. V790-20 and V850-01 respectively), CK isoenzyme electrophoresis kit (Beckman, ref. 655930), Enzyline CK standardized 10 (Biomerieux, ref. 63151).

2.2. BCK cDNA and construction of expression vectors

The single-stranded cDNA of the BCK gene was synthesized from human brain mRNA using the first-strand cDNA synthesis kit (Pharmacia, ref. 27-9261-01). The BCK cDNA was amplified by PCR using two primers containing a *Hin*dIII restriction site (forward: 5′-CGG CCC AAG CTT GCC ATG CCC TTC TCC AAC AG-3′ and reverse: 5′-GGG TGT AAG CTT GGC TTC ATT TCT GGG CAG GC-3′, accession: M16451, nt 21–40 and nt 1173–1153 respectively of the cDNA sequence) and *Pfu* polymerase (Stratagene, ref. 600153). These PCR products of 1153 bp were inserted into the *Hin*dIII site of a Bluescript II KS vector (Stratagene, ref. 212205). Clones were sequenced by the dideoxynucleotide technique using Thermo Sequenase (Amersham Pharmacia Biotech, ref. US7950). The correct BCK cDNA was subcloned into two eukaryotic expression vectors, pZeoSV40 and pcDNA3.1, to obtain pZe-BCK and pcD-BCK respectively.

2.3. Histidine tag addition to the 3′-terminus of the BCK cDNA

In order to construct a novel BCK including a six histidine tag sequence on the 3′-terminus, we performed a PCR by changing the reverse primer (5′-ACG AGA AGC TTT CAA TGA TGA TGA TGA TGA TGC TTA TCG TCG TCG TCT TTC TGG GCA GGC ATG AGG TCG TC-3′). The reverse primer contains the *Hin*dIII restriction site, an enterokinase cleavage site and a sequence encoding 6His. This newly synthesized B6H-CK cDNA of 1191 bp was inserted into the *Hin*dIII site of a pcDNA3.1 vector. The DNA sequence of PCR products was verified. The pcD-BCK6His clone was selected for expression study.

2.4. Site-directed mutagenesis of BCK

The deletion of His-65 and Pro-66 and the double deletion His-65–Pro-66 was performed by the megaprimer method [21] on the B6H-CK cDNA. We used a modification of the megaprimer technique proposed by Ekici et al. [22]. This in vitro mutagenesis is based on a one tube, two stage PCR reaction using a set of three primers and *Tfu* polymerase (Appligene, ref. 120241). For the first two single amino acid deletions, in the first step, the 3′ universal BGH reverse primer (5′-TAG AAG GCA CAG TCG AGG-3′) and the 5′ mutagenic primer (Table 1) generate and amplify a double-stranded, mutated fragment of 1140 bp for 16 cycles. Each cycle consisted of 45 s of denaturation at 95°C, 45 s at 60°C and 90 s at 72°C. This synthesized fragment carrying the mutation is used as a megaprimer together with the 5′ universal T7 primer in the second step PCR reaction, using the same cycling conditions to obtain the desired 1.2 kb mutated fragment.

For the double deletion His-65–Pro-66, the 3′ primer in the first step and the 5′ primer of the second step of the megaprimer reaction, we used the *Nhe*I-pcDNA primer (5′-AAGCTGGCTAGCGTTTAAACTTAA-3′, nt 889–912 of pcDNA3.1 vector) and the *Bam*HI-pcDNA primer (5′-AGTGGATCCGAGCTCGGTACC-3′, nt 916–937 of pcDNA3.1 vector) which generated a fragment of approximately 1.2 kb. The mutated fragment was then purified on agarose 0.8% and reintroduced into the pcDNA3.1 vector. The deletion was confirmed by sequencing with the USB Sequenase kit (Amersham Pharmacia Biotech, ref. US78500).

2.5. Cell culture and transfection

COS-7 cells were cultured in a DMEM–25 mM HEPES medium supplemented with 12% (v/v) fetal calf serum under 5% CO_2 at 37°C. Plasmid was introduced in COS cells with 0.3% (v/v) FuGENE-6 transfectant reagent (Roche Molecular Biochemicals, ref. 1814443) and expressed for 48 h. Each transfection experiment was repeated three times.

2.6. Extraction of the recombinant protein CK-BB

After 48 h, cells were harvested by trypsinization in TE and centrifugation at 1500 rpm at 4°C for 5 min. Cells were washed three times with phosphate-buffered saline and frozen at −80°C until assayed. The pellets were resuspended on ice in a lysis buffer (100 mM Tris–HCl, 200 mM NaCl, pH 7.0). Three cycles of freezing-thawing were carried out, and insoluble cell debris was removed by centrifugation at 12 000 rpm at 4°C for 6 min. The extracts were used for CK catalytic and gel analysis and for total protein measurement.

2.7. Purification of CK-BB6His

The His-tagged CK-BB protein (CK-BB$(His)_6$) expressed in COS was purified on a nickel affinity column (Ni-NTA superflow, Qiagen, ref. 30410) under native conditions. After washing with 100 mM imidazole, CK-BB$(His)_6$ was eluted with 250 mM imidazole, 1 M NaCl, 50 mM phosphate buffer (pH 8.0). The fractions containing CK activity were pooled and dialyzed against a buffer (100 mM Tris–HCl, 50 mM NaCl, 6 mM dithiothreitol (DTT), pH 8.0). The purified enzyme was stored at −20°C after the addition of 4 g/l bovine serum albumin and 50% glycerol.

2.8. Mi-CK cDNA and construction of expression vectors

Total RNA was prepared from MCF-7 cells. The single-stranded cDNA was synthesized with the Omniscript RT kit (Qiagen, ref. 205111) using oligo-dT as a primer. The Mi-CK cDNA was amplified for 20 cycles, each consisting of 30 s at 94°C for denaturation, 1 min at 45°C for annealing and 2 min at 72°C for primer extension by the polymerase. The primers used were CK-Mi for (5′-GTA CGA AAG CTT AGC TGC CAG TGA ACG ACG G-3′) and CK-Mi rev (5′-GGG GAG GAA TTC TTA ATG CTT GGT GTG GAT-3′). These primers contain the *Hin*dIII and *Eco*RI restriction sites and are positioned on nt 518–535 and nt 5582–5570, respectively, of the genomic sequence (accession number J04469). The 1.2 kb products were purified with the Qiaquick PCR purification kit, subcloned into pBluescript II KS vector, followed by transformation into *E. coli* DH5α competent cells. Clones obtained were sequenced (ESGS/Genopole,

Table 1
List of mutagenic primers for the site-directed mutagenesis

Amino acid deletion	Mutagenic primer (5′-3′)
His-65	GTG GAC AAC CCG GGC … CCG TAC ATC ATG ACC
His-65–Pro-66	GTG GAC AAC CCG GGC … TAC ATC ATG ACC GTG
CK-MiΔHis61	GTG GAC AAC CCT GGC … CCC TTC ATC AAG ACT

The deletion of amino acids is represented by three dots.

Evry, France). The correct Mi-CK cDNA was subcloned into the prokaryotic expression vector pBH, to obtain pBH-MiCK.

2.9. Deletion of His-61 by site-directed mutagenesis of human Mi-CK

The deletion was performed on Mi-CK cDNA as described previously by the megaprimer method. The three primers used were CK-Mi for, CK-Mi rev and the mutagenic oligonucleotide CK-MiΔHis61 (Table 1). The two PCR reactions were performed for 16 cycles each consisting of 45 s at 94°C, 45 s at 46°C for the first PCR reaction, 55°C for the second one and 1 min at 72°C for the extension. The resulting 1.2 kb DNA fragment was purified, cloned in pBH vector and sequenced as described for the wild-type Mi-CK cDNA.

2.10. CK-Mi protein purification

E. coli DH5α cells were transformed with plasmids encoding the mutant or the wild-type enzymes, and proteins were expressed in *E. coli* and purified. The expression of CK-Mi protein was induced by the addition of 1 mM IPTG. After 5 h induction, cells were pelleted (10 min, 5000 rpm, 4°C), washed, centrifuged a second time and resuspended in 10 ml of an ice-cold extraction buffer (Tris–HCl 50 mM, EDTA 10 mM, NaCl 100 mM, pH 8) supplemented with a protease inhibitor cocktail tablet (complete, Mini, EDTA, Roche Biochemicals, ref. 1836170) and 0.5 mg/ml lysozyme. Cells were incubated on ice for 15 min, lysed by sonication for 10 s and placed on ice for 2 min. This was repeated three times, followed by three freeze-thaw cycles and centrifugation for 20 min at 12 000 rpm at 4°C. The supernatant was then transferred to be dialyzed against a 25 mM sodium phosphate, 3 mM DTT, 0.2 mM EDTA, pH 7 buffer for 4 h through a 100 kDa membrane. Finally, we concentrated the protein in a Centriplus-100 concentrator (Amicon). The extracted protein was then used for CK analysis.

2.11. CK activity measurements

For the reverse reaction (ATP synthesis), CK catalytic activities were measured with the hexokinase–glucose-6-phosphate dehydrogenase coupled method (Enzyline CK standardized 10, Biomerieux). The reaction was started by adding CP (30 mM).

2.12. CK isoenzyme analysis by agarose gel electrophoresis

CK isoenzymes in the lysate were analyzed on commercial 1% agarose gels according to the manufacturer's instructions. The final quantification was done with an integrator under fluorescent light (Sebia Preference).

2.13. Protein analysis

Total proteins were measured with the BCA Protein Assay Reagent (Pierce, ref. 23225). The purified protein was analyzed by SDS–PAGE according to the method of Laemmli on a 12% acrylamide gels.

2.14. K_m determination

For the reverse reaction, plots were constructed from the activities measured with CP or cyclocreatine phosphate ranging from 1 to 10 mM, while the ADP concentration was held at 2 mM, and for ADP (0.1–0.5 mM) with a CP concentration at 30 mM. The K_m values were evaluated by a linear regression method, using a double reciprocal plot of velocity versus substrate concentration.

2.15. Synthesis of cyclocreatine phosphate

1-Carboxymethyl-2-iminoimidazolidine (cyclocreatine) was chemically phosphorylated in our laboratory according to the published procedure [23].

Table 2
Overexpression of recombinant CK-BB enzymes in COS-7 cells (mean ± S.D., $n = 3$)

Vector	CK activity (U/mg total protein)	Expression level
pZeOSV40	0.25 ± 0.01	–
pcDNA3.1	0.26 ± 0.02	–
pZe-BCK	2.15 ± 0.02	7.89 ± 0.01
pcD-BCK	50 ± 3.6	200 ± 30
pZe-BCK6His	1.18 ± 0.03	5 ± 1
pcD-BCK6His	44.33 ± 3.74	177 ± 25

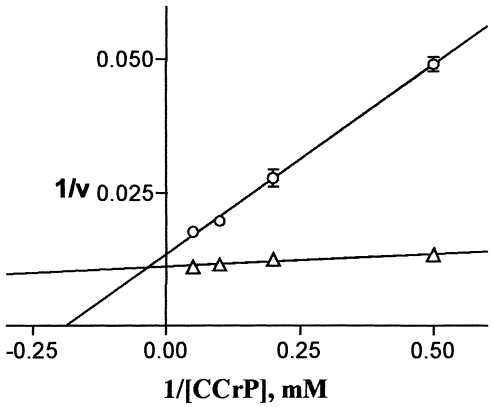

Fig. 1. Double reciprocal plots of velocity versus CCrP concentration for CK-BB(His)$_6$ (triangles) and ΔHis65 (circles) ($n = 3$ for each data point). The error bars are standard deviations, not shown when smaller than the symbol. CK activities were measured by a hexokinase–glucose-6-phosphate dehydrogenase coupled method in the direction of ATP formation. ADP concentration was held at 2 mM.

3. Results

3.1. cDNA cloning and overexpression of wild-type CK-BB

After sequencing 20 clones, we chose the clone CKB*h2* cDNA for expression study. To avoid the formation of inclusion bodies in *E. coli*, we decided to produce our protein in a mammalian cell line, COS-7, previously used by the Perryman group to express CK [24]. pZe-BCK with SV40 promoter expressed a low level of recombinant protein, approximately eightfold that of the endogenous CK-BB in COS-7 cells, while the pcD-BCK vector with the CMV promoter increased the expression level up to approximately 200-fold (Table 2). Electrophoretic analysis of native proteins on agarose gel showed a basal level of BB isoenzyme in non-transfected COS and a high level of overexpressed CK-BB in transfected cells.

3.2. His-tagged CK-BB

To allow rapid and efficient purification of the recombinant protein, we added six histidines and an enterokinase cleavage site at the C-terminus of CK-BB and obtained pZe-BCK6His and pcD-BCK6His vectors respectively. The overexpression level of histidine-tagged CK-BB in COS-7 cells was not affected by the tag and we chose to use pcD-BCK6His for further experiments (Table 2). The tagged protein was purified through a Ni-NTA superflow column and resulted in one homogeneous ∼42 kDa band on the SDS polyacrylamide gel. Thus the one step purified enzyme was directly used for kinetic studies without further purification.

K_m values for CP and ADP of CK-BB(His)$_6$ were 650 and 280 μM respectively and were comparable to the literature data for CK-BB (660 and 110 μM respectively).

3.3. Deletion of His-65 in human CK-BB6His

We deleted the nucleotides corresponding to His-65 in the pcD-BCK6His vector by the megaprimer method to obtain the ΔHis65 mutant. The mutant protein was expressed in COS-7 cells, purified, and characterized.

As shown in Table 3, the K_m values of ΔH65 mutant for CP and ADP are 710 and 200 μM respectively. The affinity for its

two natural substrates is identical to that of CK-BB(His)$_6$. Further kinetic studies were also performed for CCrP, a known analog of CP, which has a conformationally restricted and bulky cyclic structure. The affinity of the ΔH65 mutant for CCrP decreased approximately 12-fold in comparison to that of recombinant 6His (Table 3) or wild-type enzyme (750 μM, personal results) although the maximal velocity of ΔH65 for CCrP was not greatly changed (Fig. 1).

3.4. Stability of the ΔHis65 mutant

For stability studies, we checked the catalytic activities over 7 months. CK-BB(His)$_6$ showed no significant loss of activity for 5 months and retained a residual activity of approximately 70% after 210 days, whereas ΔHis65 activity decreased drastically to 10% of its initial activity in a short period, 70 days (Fig. 2).

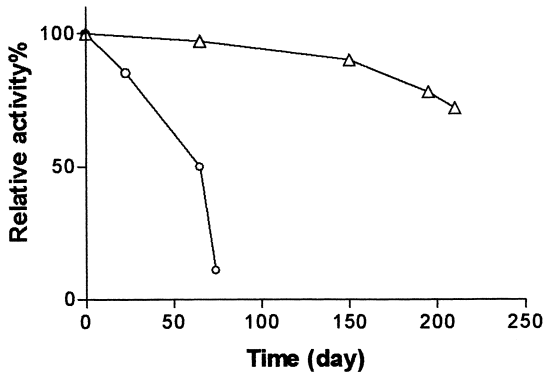

Fig. 2. Stability of CK-BB(His)$_6$ (triangles) and ΔH65 (circles). CK activities were measured by a hexokinase–glucose-6-phosphate dehydrogenase coupled method in the direction of ATP formation. ADP and CP concentrations were held at 2 and 30 mM respectively.

3.5. Deletion of Pro-66

Whereas the affinity of this mutant (ΔP66) for ADP was not significantly changed, the K_m value for CP was eightfold in comparison to CK-BB(His)$_6$. It was not possible to determine the K_m value for CCrP since no activity was detected in the presence of this substrate (Table 3).

3.6. Deletion of His-65–Pro-66

In the cell extract, the double mutant, ΔH65ΔP66, exhibited less than 3% (with CP) of the CK-BB(His)$_6$ activity control. It exhibited the same affinity for CP and ADP as ΔP66. The determination of the K_m value for CCrP also failed (Table 3).

3.7. cDNA cloning and expression of wild-type and mutant CK-Mi (MiΔH61)

In order to compare our results on His-65 in CK-BB to those on His-61 in CK-Mi described by Wallimann's group, we decided to clone the Mi-CK cDNA. The sequence obtained was 100% homologous to that found by Haas et al. [25]. After expression in *E. coli* and purification, the Michaelis–Menten constants for this wild-type enzyme, in the reverse reaction (K_m(CP) = 753 ± 25 μM, K_m(ADP) = 145 ± 6 μM), were in good agreement with those of Forstner et al. [17] (K_m(CP) = 1210 ± 110 μM, K_m(ADP) = 166 ± 37 μM). We then deleted His-61 of the Mi-CK by site-directed mutagenesis, and expressed the mutant protein in the same conditions as the wild-type. No activity could be detected with either of the natural substrates, CP or ADP (Table 3).

4. Discussion

Several histidine residues conserved throughout the phosphagen kinases including CK have been studied either by site-directed mutagenesis or by chemical modification. Recently, Forstner et al. [17] substituted three histidines in the sarcomeric mitochondrial isoenzyme, CK-Mi$_b$, of chicken muscle (His-61, His-92, and His-186) with Ala and Asp. The most prominent effects were obtained with the H61D mutant.

According to crystallographic studies, this His (corresponding to His-65 in the cytosolic isoenzymes) is in a flexible loop [9]. On the one hand, it has been suggested that this loop moves towards the active site in order to bring His-61 near the γ-phosphate of ATP. On the other hand, by photoaffinity labeling or computational sequence analysis, the loop was demonstrated to be at least a part of the CP binding site [20,26].

To investigate the probable role of this loop in CK-BB catalysis, we deleted His-65 and Pro-66 successively. The deletion of His-65 has no apparent effect on the kinetic parameters. This unexpected result prompted us to check our results. The wild-type CK-Mi enzyme we obtained showed similar characteristics to that prepared by the Wallimann group. Instead of mutating the CK-Mi His-61, we decided to delete it. The mutant MiΔH61 is nearly inactive and any trial to characterize this enzyme failed, a result which is in excellent agreement with that for H61D (Table 3). Our results most probably account for structural differences due to the isoenzyme type.

From the comparison of 18 amino acid sequences of different phosphagen kinases, Suzuki et al. [26,27] proposed the His-65–Ile-68 region as a possible candidate for the guanidine recognition site. They suggested an inverse relationship between the size of the loop and that of the guanidine substrate. The deletion of the two amino acids, His-65 and Pro-66, may alter the loop's shape and decrease the affinity for relatively larger substrates such as CCrP. In fact, the deletion of His-65 increased the K_m for CCrP about 12-fold without any significant effect on CP binding. Unlike His-65, ΔP66 showed a sevenfold decrease in CP affinity, and CCrP affinity was so low that the catalytic activity was undetectable. ΔH65ΔP66 showed the same characteristics as the ΔP66 mutant, confirming that the deletion of Pro disturbed the loop's shape, since a Pro residue often plays an important role in bending a structure. This explains the weak binding of CP. It seems that, in the double deletion, Pro-66 is accountable for the K_m(CP) increase. It is noteworthy that, while CP affinity was modified, K_m(ADP) decreased. This fact has to be related to the ordered

Table 3
Kinetic constants for wild-type and mutant CK-BB enzymes in the direction of ATP formation (mean ± S.D., $n = 3$)

	K_m (μM)		
	CP	CCrP	ADP
CK-Mi	753 ± 25	–	145 ± 6
CK-BB(His)$_6$	650 ± 185	432 ± 24	280 ± 39
ΔH65	710 ± 43	5340 ± 500	190 ± 12
ΔP66	4910 ± 118	n.d.	83 ± 6
ΔH65ΔP66	4910 ± 240	n.d.	100 ± 7
MiΔH61	n.d.	–	n.d.

n.d.: not detected.

catalytic mechanism we previously described at pH 6.6 for CK-BB [19]. Further experiments are necessary to understand better how the deletions modulate the interaction between the two substrate sites.

Although His-65 deletion did not affect in any way the natural substrates binding to CK-BB, apparently it did have an effect on the interaction with larger substrates such as CCrP.

However, an obvious effect of its deletion is a dramatic decrease in enzyme stability (Fig. 2). While His-65 is not crucial for catalysis in CK-BB, the flexible loop as a whole altered the binding of the guanidine substrate. In this case, the main role of His-65 may be to stabilize the general conformation of the protein.

So far, the resolved structures of some members of the guanidine kinase family have been based on molecular refinement by replacement using the coordinates of CK-Mi (3.0 Å resolution) [9]: horseshoe crab ArgK [28], rabbit CK-MM [29], chicken CK-BB [11]. Perhaps this refinement tends to increase the likeness of the shape of these guanidine kinases. Moreover, the crystals of CK-BB were obtained in the absence of any substrate or transition state analog and weak electron density was reported in the region of the flexible loop. This leads us to propose that, despite an overall similar shape, some subtle structural differences exist and contribute to isoenzyme specificities and functions yet to be clarified.

Acknowledgements: Financial support for this study was provided by Region Rhône-Alpes (Contract 99029224). We thank Sarah Somerville for carefully reading the manuscript and Prof. Christian Vial for helpful discussion.

References

[1] Bessman, S.P. and Carpenter, C.L. (1985) Annu. Rev. Biochem. 54, 831–862.
[2] Bessman, S.P. and Geiger, P.J. (1981) Science 211, 449–452.
[3] Wallimann, T., Wyss, M., Brdiczka, D., Nicolay, K. and Eppenberger, H.M. (1992) Biochem. J. 281, 21–40.
[4] Wallimann, T. (1994) Curr. Biol. 4, 42–46.
[5] Wyss, M., Smeitink, J., Wevers, R.A. and Wallimann, T. (1992) Biochim. Biophys. Acta 1102, 119–166.
[6] Mühlebach, S.M., Gross, M., Wirz, T., Wallimann, T., Perriard, J.-C. and Wyss, M. (1994) Mol. Cell. Biochem. 133/134, 245–262.
[7] Stachowiak, O., Schtlattner, U., Dolder, M. and Wallimann, T. (1998) Mol. Cell. Biochem. 184, 141–151.
[8] Haas, R.C. and Strauss, A.W. (1990) J. Biol. Chem. 265, 6921.
[9] Fritz-Wolf, K., Schnyder, T., Wallimann, T. and Kabsch, W. (1996) Nature 381, 341–345.
[10] Eder, M., Schlattner, U., Becker, A., Wallimann, T., Kabsch, W. and Fritz-Wolf, K. (1999) Protein Sci. 8, 2258–2269.
[11] Pradel, L.A. and Kassab, R. (1968) Biochim. Biophys. Acta 167, 317–325.
[12] Clarke, D.E. and Price, N.C. (1979) Biochem. J. 181, 467–475.
[13] Chen, L.H., Borders, C.L., Vasquez, J.R. and Kenyon, G. (1996) Biochemistry 35, 7895–7902.
[14] Furter, R. and Furter-Graves, E.M. (1993) Biochemistry 32, 7022–7029.
[15] Gross, M., Furter-Graves, E.M., Wallimann, T., Eppenberger, H.M. and Furter, R. (1994) Protein Sci. 3, 1058–1068.
[16] Perraut, C., Clottes, E., Leydier, C., Vial, C. and Marcillat, O. (1998) Proteins 32, 43–51.
[17] Forstner, M., Müller, A. and Stolz, M. (1997) Protein Sci. 6, 331–339.
[18] Min, K.L., Steghens, J.P., Henry, R., Doutheau, A. and Collombel, C. (1996) Eur. J. Biochem. 238, 446–452.
[19] Min, K.L., Steghens, J.P., Henry, R., Doutheau, A. and Collombel, C. (1997) Biochim. Biophys. Acta 1342, 83–89.
[20] Min, K.L., Steghens, J.P., Henry, R., Doutheau, A. and Collombel, C. (1998) Biochim. Biophys. Acta 1387, 80–88.
[21] Sommer, S.S. and Sarkar, G. (1990) BioTechniques 8, 404–407.
[22] Ekici, A.B., Park, O.S., Fuchs, C. and Rautenstrauss, B (1997) Elsevier Trends Journals Technical Tips On Line, T01122.
[23] Struve, G.E., Gazzola, C. and Kenyon, G.L. (1977) J. Org. Chem. 42, 4035–4040.
[24] Lin, L., Perryman, M.B., Friedman, D., Roberts, R. and Ma, T.S. (1994) Biochim. Biophys. Acta 1206, 97–104.
[25] Haas, R.C., Korenfeld, C., Zhang, Z., Perryman, B., Roman, D. and Strauss, A.W. (1989) J. Biol. Chem. 264, 2890–2897.
[26] Suzuki, T., Kawasaki, Y., Furukohri, T. and Ellington, W.R. (1997) Biochim. Biophys. Acta 1348, 152–159.
[27] Suzuki, T., Kamidochi, M., Inoue, N., Kawamichi, H., Yazawa, Y., Furukohri, T. and Ellington, W.R. (1999) Biochem. J. 340, 671–675.
[28] Zhou, G., Somasundaram, T., Blanc, E., Parthasarathy, G., Ellington, W.R. and Chapman, M.S. (1998) Prot. Natl. Acad. Sci. USA 95, 8449–8454.
[29] Rao, J.K., Bujacz, G. and Wlodawer, A. (1998) FEBS Lett. 439, 133–137.