Phosphorylation of chicken brain-type creatine kinase affects a physiologically important kinetic parameter and gives rise to protein microheterogeneity in vivo

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Abstract

In addition to the two monomer subunits of chicken brain-type creatine kinase (B-CK, EC, 2.7.3.2), termed Bb (basic) and Ba (acidic), another subspecies called Bb* was identified by chromatofocussing in the presence of 8 M urea (Quest et al., ). The latter low abundance protein species, isolated from tissue extracts, comigrated on 2D-gels with three minor species (Bb1-3), initially identified in immunoprecipitated, [35S]methionine labeled in vitro translation products of cDNA coding for the basic monomer Bb. During in vitro translation experiments in the presence of [32P]-gamma-ATP, Bb1-3 were labeled while phosphatase treatment eliminated these minor species. It is concluded that Bb* is identical to Bb1-3 and represents phosphorylated derivatives of Bb. B-CK dimer populations from different tissues were separated by ion-exchange chromatography and the Km values of the resulting fractions were determined under phospho-creatine (CP)-limiting conditions. In fractions containing only Bb and Bb* two kinetically different enzyme species were detected (Km values for CP = 1.6 mM and 0.8 mM), while fractions containing B-CK [...]
Phosphorylation of chicken brain-type creatine kinase affects a physiologically important kinetic parameter and gives rise to protein microheterogeneity in vivo

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In addition to the two monomer subunits of chicken brain-type creatine kinase (B-CK, EC, 2.7.3.2), termed Bb (basic) and Ba (acidic), another subspecies called Bb* was identified by chromatofocussing in the presence of 8 M urea (Quest et al., [20]). The latter low abundance protein species, isolated from tissue extracts, comigrated on 2D-gels with three minor species (Bbl-3), initially identified in immunoprecipitated, p35S-methionine labeled in vitro translation products of cDNA coding for the basic monomer Bb. During in vitro translation experiments in the presence of [33P]-y-ATP, Bbl-3 were labeled while phosphatase treatment eliminated these minor species. It is concluded that Bb* is identical to Bbl-3 and represents phosphorylated derivatives of Bb. B-CK dimer populations from different tissues were separated by ion-exchange chromatography and the $K_a$ values of the resulting fractions were determined under phospho-creatine (CP)-limiting conditions. In fractions containing only Bb and Bb* two kinetically different enzyme species were detected ($K_a$ values for CP = 1.6 mM and 0.8 mM), while fractions containing B-CK dimers composed of the major Ba and Bb monomers, but no Bb*, were homogeneous in this respect ($K_a$ for CP = 1.6 mM). Phosphorylation of Bb to yield Bb* is concluded to reduce the $K_a$ of B-CK dimers for CP by about 50%. This $K_a$ shift is within the range of CP concentrations found in tissues expressing the B-CK isoenzyme and may therefore be of physiological relevance.

1. INTRODUCTION

Creatine kinase (CK) belongs to a class of enzymes called phosphagen kinases, with representatives in phyla throughout the animal kingdom, which catalyze the reversible transfer of high-energy phosphate groups from ATP to guanidino compounds, termed phosphagens, and vice versa [1]. The direction of the reaction favoured by CK depends on substrate concentrations, pH, on the isoforms involved and their subcellular location. Both, cytosolic brain-type B-CK and muscle-type M-CK, as well as the mitochondrial isoenzymes (Mi-CK) readily catalyze the reaction from CP to ATP (the so-called 'reverse reaction' [2]) under in vitro assay conditions at the appropriate pH and substrate concentrations resembling the cytosolic milieu [3,4]. However, studies of mitochondrial respiration in rat heart and sea urchin sperm provide strong evidence that energy channeling from the mitochondria into the cytoplasm depends upon the preferential catalysis of the forward reaction by Mi-CK from ATP to CP [5,6]. Enzymes catalyzing reversible reactions are generally not considered as targets for regulatory control [7]. Clearly, however, constraints imposed upon the mitochondrial isoenzyme of CK by its environment determine the direction of the phosphate-group-transfer reaction catalyzed [4-6]. This observation emphasizes that the performance of enzymes catalyzing reversible reactions may also be regulated. Subcellular compartmentation does not provide the only possibility for such a control. Recently, it has been demonstrated that enolase, an enzyme catalyzing a reversible step in the glycolytic pathway, is phosphorylated by protein kinase C (PKC) and that this posttranslational modification has inverse effects on both reaction directions [7]. Potentially, mechanisms controlling the performance of cytosolic CK isoenzymes together with mechanisms specifying their subcellular distribution could determine local intracellular ATP levels, for the latter nucleotide is not homogeneously distributed within cells, but rather exists in compartmentalized pools [8,9]. Microheterogeneity of ATP distribution throughout the cytosol, however, also can occur independent of compartmentation by membranes [10], possibly due to the

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non-uniform distribution of ATP-generating and ATP-consuming reactions [11]. On the other hand, ATP may fulfill multiple regulatory functions in a cell. For instance, it is an allosteric inhibitor of phosphofructokinase, a key enzyme in the glycolytic pathway [12]. Furthermore, it modulates potassium channel [13,14] as well as EGF receptor [15] activity. The latter examples are typically membrane-bound functions. For certain cell types, evidence exists that the ATP concentration in the immediate vicinity of the cell membrane may be lower than that in the bulk cytoplasm [11]. In this context B-CK, which has been found associated with synaptic vesicles [16,17] seemed of particular interest.

Chicken B-CK is particularly heterogeneous, as initially revealed by 2D-gel analysis [18]. The two major monomer species present, designated Ba- and Bb-CK [19], were shown to be distinct proteins [20] which are encoded by different mRNAs [21]. An additional species termed Bb* was also isolated, although its nature remained unresolved [20]. Some minor species identified in cDNA-derived in vitro translation products [22,23], termed Bb1, Bb2 and Bb3 [24], are shown here to be phosphoproteins, which comigrate with Bb*.

We thus addressed the question whether post translational modification of B-CK might influence the enzymes' kinetic properties under CP-limiting conditions, for limitation of CP seems to be an important parameter for the CK reaction and for brain energetics in general (see Discussion).

Evidence is provided that the presence of Bb*, which can be partially enriched by chromatofocusing [20], is responsible for the kinetic heterogeneity observed, with respect to the $K_m$ for CP, in those fractions of native, dimeric B-CK, containing Bb* monomers. It is concluded that chicken Bb-CK is phosphorylated and that this modification induces a 50% decrease in the $K_m$ value of the enzyme for CP.

2. MATERIALS AND METHODS

2.1. B-CK purification

Active B-CK dimers were purified as described [19]. The final separation of active dimer populations was achieved on an FPLC MonoQ anion exchange column (Pharmacia), while B-CK monomers were separated on an FPLC MonoP chromatofocusing column (Pharmacia) in the presence of 8 M urea [20].

2.2. 2D gel analysis

Two-dimensional gel analysis was carried out according to O'Farrell with minor modifications as described [24]. After electrophoresis proteins in the gels were stained with Serva Blue G or transferred onto nitrocellulose [25] and reversibly stained for protein with Ponceau Red S. In comigration experiments with $[^{35}S]$-labeled H4 Bb-CK cDNA-derived in vitro translation products [22,24], gels stained with Serva Blue G were subsequently prepared for fluorography using Amplify (Amersham). Protein positions were indicated on dry gels with minor modifications as described [24].

2.3. In vitro translation of B-CK mRNA

Aliquots of frozen mRNA derived from in vitro transcription (kindly performed by T. Wirz) from a chicken Bb-CK cDNA full length clone [22] called H4 were in vitro translated in reticulocyte lysate (Promega) containing [$^{35}$S]methionine (Amersham) as described by the manufacturer, except for the addition of 40 U of RNasin (Promega) per 50 µl assay. The translated radioactive B-CK was immunoprecipitated with a specific rabbit anti-chicken B-CK antibody [16], the immune complexes collected on fixed Staphylococcus aureus (lg-sorb) and the precipitated radioactive CKs were coelectrophoresed with biochemically purified protein on 2D gels. After developing the autoradiogram on X-ray film, the radioactively labeled B-CK was used as an external standard for the identification of B-CK subunit spots stained with Serva Blue G or by silver staining.

2.4. Cotranslational phosphorylation assays

Phosphorylation of B-CK was demonstrated by using a protein kinase activity residing in rabbit reticulocyte lysates (Soldati, unpublished). Synthetic transcripts of Bb-CK were translated as described above except for the absence of labeled [$^{35}$S]methionine, but including 100 µCi of [$^{32}$P]-γ-ATP. Unfortunately, it was not possible to use lysates depleted of ATP and CP by gel filtration through Sephadex G25 to achieve a higher specific radioactivity, for lysates treated in such a manner have been shown to be translation incompetent [26]. Therefore, untreated lysates had to be used for cotranslational phosphorylation and incorporation of $^{32}$P yielded only very faint signals due to the high endogenous phosphate pools. Incubation was followed by immunoprecipitation [23,27] and extensive washing before preparation of the samples for 2D gel analysis.

2.5. Treatment of purified B-CK fractions or in vitro translated Bb-CK with phosphatases

Aliquots of translation reactions or purified Bb-CK, enriched in Bb* from MonoQ columns, were immunoprecipitated as described above. Immune complexes were collected with Protein-A or Protein-G Sepharose CL-4B beads (Pharmacia). The beads were then suspended in CTAP buffer (50 mM Tris-HCl, pH 8.0, containing 0.1 mM EDTA) and incubated in the presence of 50-100 U of calf intestinal alkaline phosphatase (CIAP, Pharmacia) at 30°C for 2 h [28]. The beads were then washed twice and the samples prepared for 2D gel analysis.

2.6. Kinetic analysis of B-CK subspecies

Creatine kinase activity was measured by pH-stat as described [3] and was expressed in IU (one international unit corresponds to 1 µmol of CP converted per min at pH 7.0 and 25°C). Individual fractions of the two B-CK peaks (type-I and type-II) obtained on MonoQ columns were subjected to kinetic analysis under CP-limiting conditions, but in the presence of excess ADP and Mg$^{2+}$ (3 mM and 10 mM, respectively) (for details, see [3]). Aliquots of the same fractions were assayed for CK activity at different CP concentrations and the initial slopes ($V$) of the different curves recorded were compared to those obtained in the presence of excess (10 mM) CP ($V_{max}$). Results were linearized by plotting ($V/V_{max}$) versus ($V/V_{max} 	imes S$) (modified Eadie-Hofstee plots). The negative slopes of these curves are numerically corresponding to the $K_m$ values for CP. Kinetically different species present in a mixture can be resolved by these plots. The $K_m$ values were only considered statistically significant if at least four independent values of a plot could be used for regression analysis and if the correlation coefficient was 0.99 or higher. Statistical comparison of the regression coefficients ($K_m$) of single fractions was performed using the two-sided Student's $t$-test, the resulting error probabilities were indicated as $P$-values ($P$, see Table I).

3. RESULTS

3.1. Bb* is a phosphorylated form of Bb-CK

The basic main monomer (Bb), three minor species (Bb1, Bb2 and Bb3), the M12-Bb species, as well as a spot on the acidic side of M12-Bb (M12-Bb1) were
found as $^{35}$S-labeled in vitro translation products using mRNA derived from the full length B-CK cDNA clone H4 (Fig. 1a). The M12-Bb species was shown recently to arise by alternative ribosomal initiation of the Bb-CK mRNA at methionine 12 [24]. Two minor species (Bb2 and Bb3) were also directly visible by protein staining in Fig. 1c, when purified B-CK protein was resolved on 2D gels using a higher voltage and different buffers during isoelectric focussing than before [20] (compare Fig. 2). The in vitro translation products themselves are not visible by protein staining methods. Phosphatase treatment of these products eliminated Bb1, Bb2 and Bb3, and the spot on the acidic side of the M12-Bb species (Fig. 1b). Only Bb (large arrowhead) and M12 (small arrow) remain after such treatment (Fig. 1b), indicating that the minor species, Bb1-3 and the more acidic M12-Bb1 satellite specified above, were the phosphorylation products of Bb- and M12-Bb-CK, respectively. This was confirmed in a complementary experiment by in vitro translation in the presence of $^{32}$P-$\gamma$-ATP. Under these conditions phosphorylation occurred and only the above specified Bb1,2,3- and M12-Bb1-CK minor monomer species were radioactively labeled (Fig. 1d).

An additional minor component, termed Bb*, was found enriched in the intermediate peak fractions during separation of subunits on the MonoP column in the presence of urea [20]. This component migrated to a position proximal to Bb indicated by an asterisk (Fig. 2), coinciding with that of the three minor species Bb1, Bb2 and Bb3 (Fig. 1). Using the identical method of 2D gel electrophoresis [19] these three minor species were not individually resolved (Fig. 2). Recent improvements of the 2D gel system for the purified protein indicate that Bb* contains all of these three minor species observed in vitro (Fig. 1c).

![Fig. 1. Effect of phosphatase treatment on the migration behaviour of the Bb-CK protein subspecies and incorporation of radioactive phosphate into Bb-CK during in vitro translation. Immunoprecipitated in vitro translation products (reticulocyte lysate including $^{35}$S)methionine) from mRNA derived from synthetic H4 B-CK cDNA were resolved on 2D gels and then transferred onto nitrocellulose. (a, h) Autoradiograms of blotted proteins before (a), and after (b) treatment with CIAP. (c) A sample translated in vitro in the presence of cold amino acids, but with $^{32}$P-$\gamma$-ATP after co-electrophoresis with 2 pg of purified B-CK, transfer onto nitrocellulose and protein staining with Ponceau Red S. (d) An autoradiogram of the sample analyzed in panel (c), stained for protein, shows incorporation of radioactive phosphate label into Bb1-, Bb2- and Bb3- as well as M12-Bb1-CK. Long arrowheads point to the main B-CK monomer species, Bb and Ba, whereas the short arrows indicate the position of the M12-Bb in vitro translation product arising from alternative initiation of translation at the methionine residue 12 [24].]
Fig. 2. Comigration of MonoP fractions of B-CK with in vitro translation products from a full length cDNA clone coding for Bb-CK. Samples from different fractions of a MonoP chromatofocussing column containing either Bb; Bb and Bb*; or Ba monomers, are shown in panels (a), (d), and (g) respectively, representing the corresponding 2D gels after staining for proteins with Serva Blue G. The samples were comigrated with H4 B-CK cDNA-derived, radioactively labeled in vitro translation products. (b), (e) and (h) show the same gels after soaking with Amplify, drying and marking visible protein spot positions with arrows of radioactive ink. Large and small arrows indicate positions of the major and minor protein species respectively, which were identified within the various fractions. (c), (f) and (i) show the autoradiograms on X-ray film of corresponding gels. The M12-Bb spot results from internal initiation (see Fig. 1). The spots marked by stars are phosphatase-sensitive in vitro phosphorylation products of Bb and M12-Bb (Fig. 1). Clearly, the enriched Bb* monomers migrate at the same position as the phosphatase-sensitive synthetic Bb1-3 species (compare Fig. 1).

3.2. Phosphorylated Bb (Bb*) is responsible for kinetic diversity of B-CK dimer populations in different tissues

A MonoQ column profile of B-CK derived from chicken gizzard extracts is shown in Fig. 3, panel A. Similar profiles have been discussed previously for heart and brain derived B-CK [20]. Two major peaks of activity, defined as type-I and type-II B-CK, representing different B-CK dimer populations are typically resolved on this column [19]. As representative examples for type-I B-CK and initial fractions of type-II B-CK, results of the kinetic analysis with respect to the $K_m$ for CP of fractions 43 and 47, respectively, are shown in Fig. 3B. Clearly, the plots of type-I B-CK fractions were biphasic, while initial fractions of type-II B-CK were homogeneous in this respect. Since in these plots the negative slopes of the straight lines represent the $K_m$ values for CP of the respective species, it is obvious that two distinctly different kinetic species were present in type-I B-CK fractions (Fig. 3B). Analysis of the corresponding fractions on 2D gels (Fig. 3C) show that, while type-II B-CK fractions contain both Ba and Bb monomers, only Bb and Bb* monomers are present in type-I fractions. The correlation between the presence of Bb* monomer and the existence of two kinetically different B-CK species was shown with B-CK derived from chicken brain, gizzard and heart (Table I). Only type-I fractions contained two kinetic species with statistically different $K_m$ values for CP, whereas initial fractions of type-II peaks did not show this kinetic heterogeneity. The average $K_m$ values for CP of the two species in type-I B-CK fractions were 0.8 mM and 1.6 mM, while in initial type-II fractions only the higher $K_m$ value was found (Table I). These results show that phosphorylation process of B-CK, similar to those found by in vitro phosphorylation experiments (Bb1,2, and 3, see Fig. 1), are also found in vivo (Bb*, see Fig. 2) and that B-CK dimers containing phosphorylated Bb-CK monomers (Bb*, see fig. 3C) display a distinctly different $K_m$ for CP (see Fig. 3B). A low $K_m$ value (0.8 ± 0.2 mM CP) was only found in fractions containing Bb* whereas the high $K_m$ value (1.6 ± 0.3 mM CP) was found in all fractions (Table I).
4. DISCUSSION

In previous experiments designed to resolve the nature of the two predominant chicken B-CK monomer species, Ba and Bb, an additional component Bb* was identified [20]. Its pI shift of about 0.1 unit of 2D gels with respect to Bb supported the hypothesis that this species may result from phosphorylation of Bb. Posttranslational modification of brain-type CK was proposed to occur in rat brains [29], where this CK isoenzyme was shown to be phosphorylated by an unknown protein kinase present in crude brain microtubule preparations. However, the role of this modification, which to this point has not been studied in purified enzyme preparations, remained unclear.

In this paper, Bb* is shown to comigrate on 2D gels with those in vitro translated products of H4 mRNA, termed Bb1, Bb2 and Bb3, which are phosphatase-sensitive and can be labeled with [32P]-γ-ATP during in vitro translation experiments (for nomenclature of the different B-CK subspecies, see [24]). Therefore, Bb*, a B-CK component purified from tissue extracts, is iden-
Kinetische Analyse von Einzel-Mono Q Peak Fraktionen von B-CK aus Hühnerhirn, Gänsegabel- und Herzmuskel: Vergleich von $K_m$-Werten

<table>
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<th>Fraktion</th>
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<th>Herz B-CK</th>
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<td>43</td>
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<td>Anwesenheit von Bb*</td>
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<td>Mittel</td>
<td>höhere $K_m$-Werte (mM CP) $= 1.6 \pm 0.3$ $(n = 26)$</td>
<td>niedrigere $K_m$-Werte (mM CP) $= 0.8 \pm 0.2$ $(n = 23)$</td>
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Hieraus ergibt sich, dass es sich um eine Kinetikanalyse von Einzel-Mono Q Peak Fraktionen von B-CK aus Hühnerhirn, Gänsegabel- und Herzmuskel handelt, mit dem Ziel, den Vergleich von $K_m$-Werten zu untersuchen. Die Tabelle zeigt die Fraktionen 43 und 47 für Hirn B-CK, 43 und 47 für Gänsegabel B-CK und 22 und 25 für Herz B-CK. Die Anwesenheit von Bb* und die $K_m$-Werte für die hohen und niedrigen Fraktionen sind aufgeführt. Die statistischen Signifikanzen ($P <$) sind ebenfalls angegeben. Die mittleren $K_m$-Werte für höhere und niedrigere Fraktionen sind mit $1.6 \pm 0.3$ für hoch und $0.8 \pm 0.2$ für niedrig angegeben, wobei $n$ die Anzahl der Messungen bezeichnet.
isoenzymes [34] were found in brain by non-invasive $^{31}$P-NMR measurements and immunofluorescence staining, respectively. In the present work, the in vitro CK enzyme reaction was measured in the direction of ATP-synthesis, for in brain the physiologically relevant brain [32]. The importance of CP as a relevant energy flux from CP to ATP, as shown directly by the $^{31}$P-NMR methods in intact human brain [34]. The regulation of CK activity by phosphorylation together with factors affecting intracellular partitioning (e.g. the association of CK with membranes which might be influenced by phosphorylation itself) may determine ATP levels at, and in the vicinity of, these membranes [31] and thereby directly modulate ATP-sensitive, membrane-associated functions [14,15,31,37].

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