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Autophosphorylation of creatine kinase: characterization and identification of a specifically phosphorylated peptide

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Abstract

We report that several different chicken and rabbit creatine kinase (CK) [1] isoenzymes showed an incorporation of ^{32}P when incubated with [γ-^{32}P]ATP in an autophosphorylation assay. This modification was shown to be of covalent nature and resulted from an intramolecular phosphorylation reaction that was not dependent on the CK enzymatic activity. By limited proteolysis and sequence analysis of the resulting peptides, the autophosphorylation sites of chicken brain-type CK could be localized within the primary sequence of the enzyme to a 4.5 kDa peptide, spanning a region that is very likely an essential part of the active site of creatine kinase. Homologous peptides were found to be autophosphorylated in chicken muscle-type CK and a mitochondrial CK isoform. Phosphopeptide as well as mutant enzyme analysis provided evidence that threonine-282 [2], threonine-289 and serine-285 are involved in the autophosphorylation of CK. Thr-282 and Ser-285 are located close to the reactive cysteine-283. Thr-289 is located within a conserved glycine-rich region highly homologous to the glycine-rich loop of protein kinases, which is known to be important for ATP binding. Thus, it seems likely that the described region constitutes an essential part of the active site of CK.

Keywords: ATP-binding site; Peptide analysis; Glycine-rich loop; Recombinant expression; Creatine kinase; Autophosphorylation

1. Introduction

Creatine kinase (CK, EC 2.7.3.2) is a key enzyme of eukaryotic energy metabolism, catalyzing the reversible phosphoryltransfer from phosphocreatine to ADP to generate ATP and creatine. So far, two mitochondrial (Mi_a- and Mi_b-CK) as well as two cytosolic subunit isoforms, the muscle-type CK (M-CK) and brain-type CK (B-CK) have been described (for review see Ref. [2]. In chicken and other vertebrate species, the dimeric B- and M-CK isoenzymes, when analyzed by 2D-gel analysis, each give rise to a number of subforms with different isoelectric points but almost identical apparent molecular weights [3,4]. The two main subunit forms of chicken B-CK arise from alternative splicing of a common RNA transcript [5], whereas additional microheterogeneity of B-CK was partly attributed to internal ribosomal initiation [6]. Other subforms seem to arise from covalent modifications of the enzyme. For example, there are subforms of chicken B-CK [7], [4] and rat B-CK [8] that show slightly shifted isoelectric points and seem to arise from phosphorylation. Autophosphorylation of rabbit M-CK is likely to be responsible for some of the M-CK heterogeneity [9].

Autophosphorylation has been shown to have important consequences for protein kinase active-site architecture

Abbreviations: CK, creatine kinase; DNFB, dinitrofluorobenzene; Mi-CK, mitochondrial creatine kinase; M-CK, muscle-type creatine kinase; B-CK, brain-type creatine kinase; EDTA, ethylene diamine tetraacetic acid; PMSF, phenylmethanesulfonylfluoride; PVDF, polyvinylidene difluoride; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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[1] CK, creatine kinase. Mutants with amino acid substitutions are denoted in the standard one-letter code by the wild-type residue and numbered position within the sequence, followed by the amino acid substitution.

[2] Residue numbers are given with respect to the cDNA-derived sequence of chicken B-CK [1], thus assigning the first number to the initiating methionine, which is cleaved off in the native as well as in the recombinantly expressed enzyme.

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were incubated with [γ-^{32}P]ATP in an autophosphorylation assay Section 2, separated by SDS-PAGE, blotted onto PVDF membranes, stained (Fig. 1a) and subsequently autoradiographed (Fig. 1a*). All CK isoenzymes were radioactively labeled whereas none of the control proteins, including ATP binding proteins such as adenylate kinase and hexokinase, showed any detectable incorporation of radioactivity. The incorporation of radioactivity was temperature-dependent. Incubation at temperatures higher or lower than 37°C led to increased or decreased incorporation, respectively. At temperatures above 50°C, labeling decreased sharply, indicating that the native enzyme conformation was a prerequisite for incorporation of radioactivity (not shown). The incorporated radioactivity remained stably bound to CK during SDS-PAGE and withstood treatment with 8 M urea, 4 M guanidine hydrochloride or trichloroacetic acid (not shown). This strongly suggests that the labeling is of covalent nature. The radioactivity incorporated into chicken M-CK (Fig. 1b,1b*) and B-CK [29] could be removed by alkaline phosphatase treatment in a dose-dependent manner, indicating that the observed modification is a phosphorylation of CK.

Preliminary information about the nature of the amino acids phosphorylated in B-CK was obtained by determining the acid and base stability of the phosphoryl groups. Treatment of autophosphorylated, immobilized chicken B-CK with 1.0 M KOH reduced the amount of label to approximately 10% of the value obtained under neutral conditions (control). In contrast, the incorporated radioactivity was completely resistant against $HClO_4$ treatment. Similar results were obtained with chicken M-CK and chicken Mi_b-CK. Alkaline phosphatases can dephosphorylate phosphoserine and phosphothreonine as substrates and were also reported to possess protein tyrosine phosphatase activity [30]. However, since phosphoserine and phosphothreonine are the only known phosphoamino acids which are acid-stable but alkaline-labile, this strongly suggests that at least 90% of the labeling is due to phosphorylated serine and/or threonine residues. Furthermore, phosphoamino-acid analysis [21] indicated the presence of both phosphoserine and phosphothreonine (but not phosphotyrosine) in hydrolysates of autophosphorylated chicken B-CK (not shown).

Several lines of evidence indicate that the observed phosphorylation is a result of an autophosphorylation reaction rather than a consequence of a contaminating protein kinase activity in the various CK preparations: (1) All three chicken isoenzymes, Mi_b-, M- and B-CK, have been purified to apparent homogeneity (Fig. 1a, lanes 1, 2 and 3), using different purification schemes. Purification of Mi_b-CK utilized FPLC Mono S cation exchange chromatography [14], while FPLC Mono Q anion exchange chromatography was used in the purification of M-CK and B-CK [16], [15]. Mono Q-purified B_b-CK fractions, further purified by chromatofocussing on a FPLC Mono P column, still exhibited the same autophosphorylation activity (not shown). (2) B_b-CK and Mi_b-CK, expressed in *Escherichia coli* and purified to apparent homogeneity, showed the same incorporation of radioactivity in the autophosphorylation assay as did the enzymes purified from tissue (see below). (3) All commercially available bovine and rabbit CK isoforms tested were also modified in autophosphorylation assays, as shown for rabbit M- and rabbit B-CK (Fig. 1a, lanes 4 and 5). (4) No heterologous phosphorylation could be observed when autophosphorylation-competent M-CK was co-incubated in an autophosphorylation assay with B-CK that was made autophosphorylation-incompetent by heat inactivation or by 8 M urea treatment (not shown). Furthermore, an intramolecular reaction mechanism is strongly suggested by the linear dependency of the autophosphorylation rate of CK on either ATP or CK concentration [9,29].

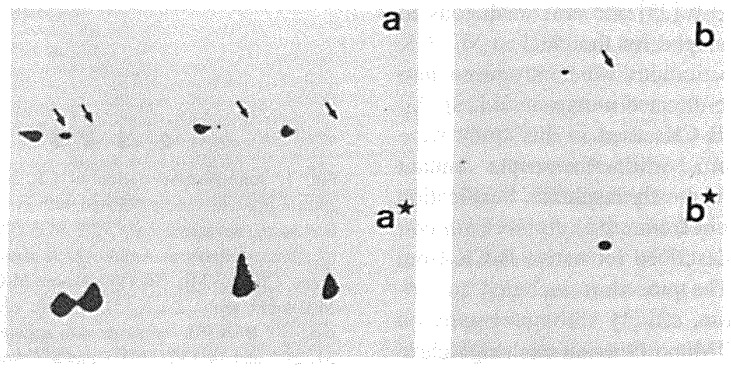

Fig. 2. Two-dimensional gel analysis of autophosphorylated chicken B- and M-CK. Panel a: Amido black staining. Chicken B- and M-CK were autophosphorylated and separated on a 2D-gel. M-CK migrated in the basic (left) part of the gel, B-CK in the acidic (right) part of the gel. Arrows in panel a indicate positions where the labeled protein species are seen in the corresponding autoradiogram, depicted in panel a*. Note that for all CK isoforms the number of the autophosphorylated species corresponds to the number of major species. Panel b: Amido black staining. Homodimeric B_b-CK was autophosphorylated and separated on a 2D-gel. The arrow indicates the position where the unique labeled protein was seen in the autoradiogram as depicted in panel b*). In panel b, a carbamylated marker protein (see weak, evenly spaced spots at bottom of b) was co-migrated to unambiguously identify the position of a single protein spot in relation to the other gel.

3.2. *Identification of a small peptide which is labeled upon autophosphorylation*

On two-dimensional gels, B- and M-CK isolated from chicken tissue both display two main subforms which have different isoelectric points but very similar molecular weights [3,4]. The two major B-CK isoforms, B_a- and B_b-CK, arise by differential RNA splicing in the *N*-terminal region, resulting in different *N*-terminal protein sequences [5]. The molecular basis of the two M-CK subforms is unknown. The *N*-terminal protein sequence of the two M-CK subforms was determined by directly sequencing the two major protein species from the blotted 2D-gels. Since the two M-CK subforms have an identical protein sequence (PFSSTHNKHKLKFS), we could directly demonstrate that the M-CK heterogeneity does not arise by differential splicing at the *N*-terminus.

When autophosphorylated chicken B- and M-CK are subjected to 2D gel electrophoresis, the two main subforms are easily visible by protein staining (Fig. 2a). On autoradiograms, however, the two main subforms are not labeled but instead two new subforms are seen for each isozyme, all showing identical p*I* shifts towards the acidic side as compared to the unphosphorylated forms (Fig. 2a★). The assumption that each autophosphorylated subspecies corresponds to one major, nonphosphorylated subspecies could be directly tested for the chicken B-CK. From a heterogenous mixture of dimeric chicken B-CK, consisting of B_a- and B_b-CK subunits, a homodimeric population of B_bB_b-CK can be separated by Mono Q anion exchange chromatography [7,15]. Autophosphorylation of the homogenous B_bB_b-CK fraction (Fig. 2b) resulted in a single radioactive species (Fig. 2b★), demonstrating a one-to-one relation between the major subforms and the autophosphorylated species of B-CK; it is likely that the same relation holds for M-CK. The presence of a single radioactive species for each B-CK subform indicates that every B-CK molecule is phosphorylated at the same number of sites; the shift in p*I* is consistent with phosphorylation at a single site. It should be noted that the autophosphorylation labeling was substoichiometric, even in the presence of a 100-fold excess of ATP. Less than 10% of the protein displayed a shifted p*I* (below the detection limit of the protein staining method used in Fig. 2). Attempts to separate the phospho forms from unphosphorylated enzyme were impeded by the substoichiometric labeling, in combination with the fact that CK isoforms are multimeric enzymes [2].

In order to locate the phosphorylated amino acids within the primary sequence of CK, proteolytically generated peptides of autophosphorylated B-CK were separated on tris-tricine gels, blotted onto PVDF membranes and visualized by staining and subsequent autoradiography. Particularly informative peptides (peptide set A) were obtained with endoproteinase Lys-C under native conditions (Fig. 3, lanes 4 and 5). In this case, cleavage of phosphorylated B-CK for 6 h resulted in peptides of sizes 15, 13 and 7 kDa, of which only the 13 kDa peptide (indicated by an arrowhead) was labeled in the autoradiogram (Fig. 3, lane 4). Upon longer proteolysis (Fig. 3, lane 5), additional peptides of sizes 11.5, 8 and 4.5 kDa were generated; the 4.5 kDa peptide (marked by an arrowhead) was derived from the 13 kDa peptide (not shown) and was also a phosphopeptide (Fig. 3a★, lane 5). Additional peptides

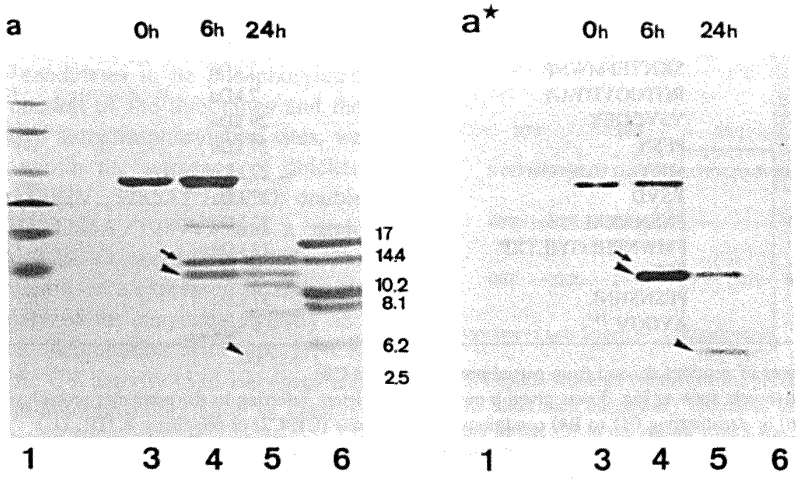

Fig. 3. Identification of a specifically labeled peptide by limited proteolysis. Autophosphorylated chicken B-CK was partially proteolyzed with endoproteinase Lys-C under native conditions for the indicated time periods. Proteinase digests were separated on a tris-tricine gel, blotted onto a PVDF membrane and autoradiographed. Panel a represents the protein-stained blot and panel a★ the corresponding autoradiogram. Molecular weight standards as in Fig. 1 (1); proteolyzed B-CK (3–5); peptide marker from myoglobin (6) for which the apparent molecular masses are indicated. Arrowheads mark radioactively labeled peptides, arrows mark an unlabeled peptide. Individual peptide bands were sequenced directly from the membrane by Edman degradation. The 15, 13, 11.5, 8, 7 and 4.5 kDa peptides, seen in lane 5 from top to bottom are referred to as bands A3, A1, A4, A6, A5 and A2, respectively. The amino acid sequences of the corresponding peptides in lane 4 were identical to those in lane 5.

were generated with Lys-C under denaturing conditions (peptide set B), with V8 proteinase (set C), and with proteinase K (set D) (not shown). To determine the identity of the different peptides, both the unlabeled and labeled proteolytic fragments were directly sequenced. The amino-acid sequence and location of the peptides are shown in detail in Table 1 and in Fig. 4. The sequence analysis revealed that all of the labeled peptides corresponded to an overlapping region starting with the Asn-268 of the B_b-CK sequence (fragments A1, A2, B1). Proteolytic cleavage with V8 proteinase revealed unique labeling of a peptide (C1) starting with amino-acid residue Phe-271. Since the proteinase K fragment D2 was not radioactively labeled and this cleavage site has been mapped between Ala-328 and Ala-329 [31], the *C*-terminal boundary of the phosphopeptide cannot reach beyond residue Ala-328. Thus, the region which is specifically labeled by autophosphorylation could be narrowed with certainty to the amino-acid stretch between Phe-271 and Ala-328. Furthermore, considering the estimated length of the labeled peptide A2 (Table 1) of 4.5 kDa (Fig. 3, lane 5, arrowhead), it is very likely that the *C*-terminal boundary of this labeled Lys-C peptide is either at amino-acid residue Lys-298 or Lys-313, both representing a possible Lys-C cleavage site (Fig. 5). Densitrometric analysis of autoradiograms and peptide stainings with amido black revealed that at least 90% of the radioactivity which incorporated into B-CK during autophosphorylation is located within the peptides that overlap this specific region. Proteolysis of chicken M- and Mi_b-CK revealed peptide and autophosphorylation patterns similar to those seen with B-CK, and sequence analysis of these peptides strongly suggested that the same specific region in these sequences was labeled (not shown). The sequence of this region of B-CK, and the corresponding sequences of Mi_b-CK and M-CK are shown in Fig. 5.

3.3. Mutant analysis

Within the fragment Phe-271 to Lys-313, four residues were found that could serve as putative autophosphorylation sites, namely Tyr-279, Thr-282, Ser-285 and Thr-289. These four residues are all conserved among B-, M- and Mi_b-CK (Fig. 5; see Ref. [32]). Based on the acid and base stability and the phosphoamino-acid analysis by thin-layer electrophoresis described above, only the serine and threo-

Table 1
Summary of sequence analysis of peptides, derived from enzymatic proteolysis of autophosphorylated B-CK

No. [a]	Labeling [b]	Peptide sequences [c]	Molecular mass [d]	Amino acid No. [e]
A1	+	NYEFMWNP	13 kDa	(268)
A2	+	NYEFMWNPHLGYILTXPSNLGTG	4.5 kDa	(268)
	(+)	PVSPLLLASGMARDWPDARG	4.5 kDa	(197)
A3	–	KLXDRQTSSG	15 kDa	(41)
	–	LRDRQTSSGF	15 kDa	(42)
A4	–	ELFDPVI	11.5 kDa	(87)
A5	–	YYAL	7 kDa	(173)
A6	–	LRDRQTSSGFT	8 kDa	(42)
	–	TDLNADNLQGGDDLD	8 kDa	(108)
B1	+	NYEFMWNPHL	7 kDa	(268)
	+	SKNYEFMWNP	7 kDa	(266)
	(+)	RGTGGVDTAA	7 kDa	(320)
B2	–	YSVDDEY	29 kDa	(14)
B3	–	PFSN	27 kDa	(2)
	–	MKY	27 kDa	(12)
	–	YSVD	27 kDa	(14)
B4	–	PXSNXXXL	25 kDa	(2)
CL	+	FMWNPHLGYILTXP	12 kDa	(271)
C2	–	PFS	8 kDa	(2)
D1	+	PFSNSHNL	37 kDa	(2)
D2	–	AVGGV [1)]	6 kDa	(329)

N-terminal amino acid sequences of peptides derived from partial proteolysis of B-CK.

[a] Each band seen on preparative gels such as Fig. 3 was given a number and a letter, referring to the particular proteolysis experiment: endoproteinase Lys-C under native (A1 to A6) or denaturating (B1 to B4) conditions, V8 proteinase (C1, C2) or proteinase K (D1, D2).

[b] Radioactive labeling, indicated as + or –, was determined by autoradiography. For A2 and B1 the sequences of two and three peptides, respectively, migrating at very similar molecular weights, were determined from one excised radioactively labeled 'band'. '(+)' indicates peptides which by themselves were not labeled, but co-migrated with labeled fragments.

[c] Amino acid sequences determined by micro-sequencing are specified by the single letter code (X stands for unresolved), and numbering (e) indicates the amino acid position within intact B-CK corresponding to the first amino acid of the individual sequenced peptides.

[d] The sizes of the respective peptides were judged from their apparent mobilities in tris-tricine gels. The molecular mass of the proteinase K fragments of B-CK (D1;D2) was calculated from the single specific cleavage site of proteinase K within the primary sequence of creatine kinase, located between ala-328 and ala-329 in chicken B-CK.

[1] Sequence taken from Ref. [31].

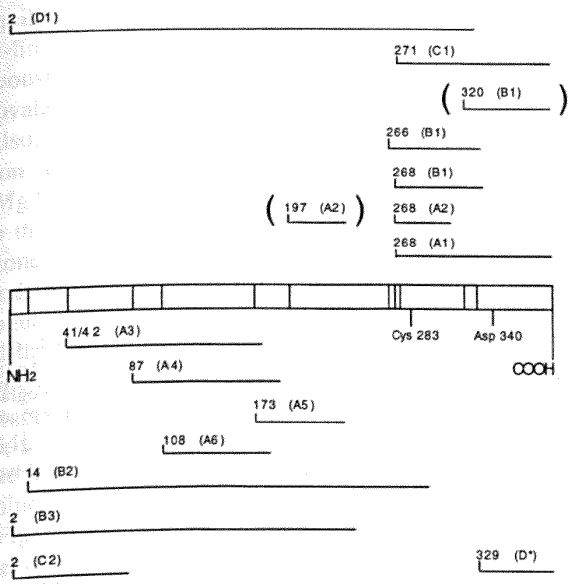

Fig. 4. Summary of the peptide sequence analyses of labeled and unlabeled B-CK peptides. The B-CK fragments listed in Table 1 are illustrated with respect to their location within the primary sequence of B_b-CK [1]. All the peptides which were radioactively labeled are positioned above the B_b-CK sequence (open bar in the middle), whereas unlabeled B-CK fragments are illustrated below this bar. Peptides derived from limited proteolysis with endoproteinase Lys-C under native conditions are depicted by lines indicated with A1 to A6, whereas Lys-C fragments resulted under denaturing conditions are marked by B1 to B3, V8 fragments by C1 and C2, and proteinase K fragments by D1 and D2. The reactive cysteine residue of CK and the affinity labeled Asp-340 (see text), both likely to be close to the active site of CK, are indicated below the B-CK sequence. The *N*-terminal ends of the peptides were determined by direct sequencing, and the *C*-terminal boundary was estimated based on their electrophoretic mobility within tris-tricine gels (approximate error of 10–20%). Fragments shown in parentheses co-migrated with a labeled fragment of the same size but were themselves unlabeled.

nine residues are likely candidates to be phosphorylated for CK. To probe the function of the threonines and the serine identified as putative autophosphorylation sites, we mutated these residues within the sequence of chicken B_b-CK. Single mutants (T282V; S285A; T289V), double mutants (T282V/T289V; S285A/T289V) and a triple mutant (T282V/S285A/T289V) were generated. The wild-type and mutant proteins were expressed in and purified from *E. coli* and analyzed for enzymatic activity and autophosphorylation. The recombinant wild-type B_b-CK exhibited the same specific CK activity (334 EU/mg) and the same characteristics in autophosphorylation as genuine B-CK, purified from chicken tissue. In all mutants except those where Ser-285 was changed, the amino-acid substitutions had no apparent effect on the enzymatic activity. The specific activities for T282V, T289V and T282V/T289V were determined to be 331, 366, and 348 EU/mg, respectively. S285A and S285A/T289V both had specific activities of only 7 EU/mg protein. All single point mutations exhibited autophosphorylation rates comparable to wild-type, although the mutant protein T282V showed a slightly reduced incorporation of ^{32}P in several experiments and exhibited a somewhat increased electrophoretic mobility in SDS-gels. Interestingly, S285A, which had a 98% reduced specific CK activity, still exhibited the same extent of autophosphorylation as wild-type B-CK (Fig. 6a,6a*). Both double mutants, however, showed substantially decreased autophosphorylation (Fig. 6b, 6b*); T282V/T289V showed more than 90% reduction in autophosphorylation while S285A/T289V showed about 65% reduction (as determined by liquid scintillation counting). It is important to note that T282V/T289V had a specific CK activity similar to the wild-type B-CK, indicating that these mutations did not cause gross conformational changes in the protein. Co-incubation of wild-type B-CK with the T282V/T289V mutant protein (which had slightly increased mobility in SDS-PAGE, like T282V) clearly showed that the reduced autophosphorylation was an inherent feature of this mutant enzyme and not the result of some inhibitory activity within the preparation of T282V/T289V (Fig. 6c, 6c*). The fact that both double mutants showed drastically reduced autophosphorylation, while the single mutants did not, argues that more than one amino acid is involved in autophosphorylation. Since it is possible that both threonine 282 and 289 as well as serine 285 are involved in this process, a triple mutant T282V/S285A/T289V was analyzed. This T282V/S285A/T289V protein was barely soluble and could not be purified to a satisfactory level. A partially purified preparation of the triple mutant did not show any autophosphorylation; however, the absence of ^{32}P incorporation could be partly due to competition for the [γ-^{32}P]ATP by other ATP-binding proteins present in the preparation. Taken together, the characterization of au-

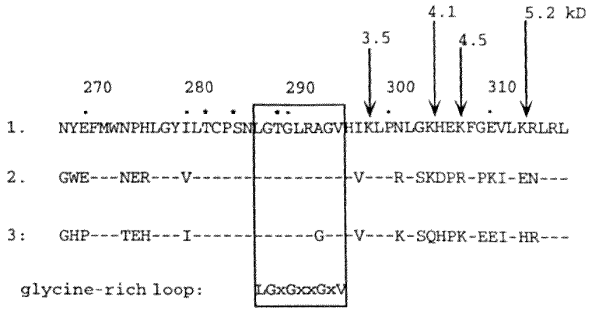

Fig. 5. Peptide sequence containing the putative autophosphorylation sites of CK. Shown are the primary sequences of chicken B-CK (1), Mi_b-CK (2) and M-CK (3) in the region which was labeled by autophosphorylation. The numbering is according to the B_b-CK sequence [1]. Residues identical in all CK isoforms are indicated by a horizontal dash (-). Possible Lys-C cleavage sites are indicated by vertical arrows and the corresponding calculated molecular masses of these putative peptides (starting at asn-268) are given in kDa. Note that this region contains the reactive cysteine residue (cys-283) and a sequence motif that is highly homologous to the glycine-rich loop of protein kinases (see text). Putative autophosphorylation sites are thr-282, ser-285 and thr-289, all indicated by asterisks.

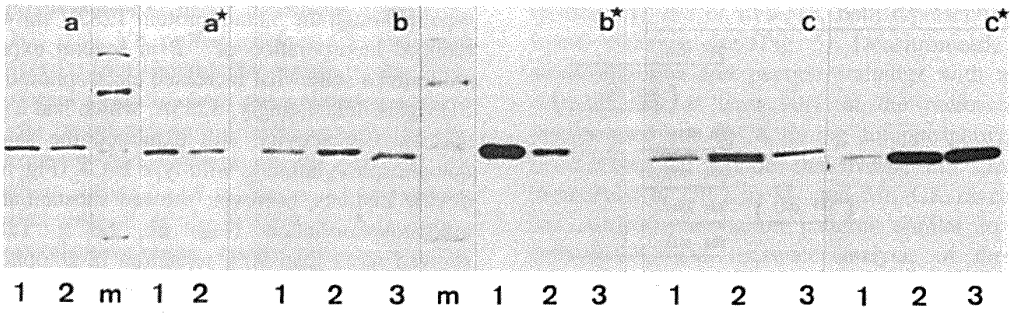

Fig. 6. Mutant analysis of autophosphorylation sites in recombinant B-CK. Wild-type and mutant chicken B-CKs, expressed in and purified from *E. coli*, were autophosphorylated as described in Section 2 and Fig. 1. Standards with apparent molecular mass of 97, 66, 43 and 31 kDa (m). Panel a: Amido black staining of proteins on PVDF membrane. 2 μg recombinant wild-type B-CK (1); mutant enzyme S285A (2). Panel a*: Corresponding autoradiogram of PVDF membrane shown in panel a). Panel b: 2 μg of mutant protein S285A (1); mutant enzyme S285A/T289V (2); mutant enzyme T282V/T289V (3). Panel b*: Corresponding autoradiogram of PVDF membrane shown in panel b. Panel c: Amido black staining of proteins on PVDF membrane. 2 μg of mutant protein T282V/T289V (1); co-incubation of 2 μg wild-type B-CK together with 2 μg mutant protein T282/T289V (2); 2 μg of wild-type B-CK (3). Panel c*: Corresponding autoradiogram of PVDF membrane shown in panel c.

tophosphorylated CK isoenzymes and the mutant analysis of putative phosphorylation sites suggest that autophosphorylation of CK involves several amino acids located within a small region of the *C*-terminal moiety of CK. Strikingly, this region contains a motif that is highly homologous to the so-called glycine-rich loop, an ATP binding motif in protein kinases [33]. The reactive cysteine 283 is also included within this region.

3.4. CK autophosphorylation is independent of transphosphorylation

Since the region involved in autophosphorylation overlaps the putative active site of CK, it was of interest whether CK transphosphorylation activity, in which a phosphoryl group is reversibly transferred from ATP to the substrate creatine, is a prerequisite for autophosphorylation. It was previously reported that the incorporation of ^{32}P into CK isoenzymes, like the transphosphorylation reaction of CK [34], is dependent on divalent metal ions like magnesium, manganese or calcium and could be reversibly inhibited by addition of EDTA [9]. However, several earlier observations suggested that CK autophosphorylation might be independent of the transphosphorylation reaction. The radioactive label could not be released from autophosphorylated CK by addition of the other CK substrates [9]. This is in agreement with the reaction mechanism of CK which has been classified as rapid

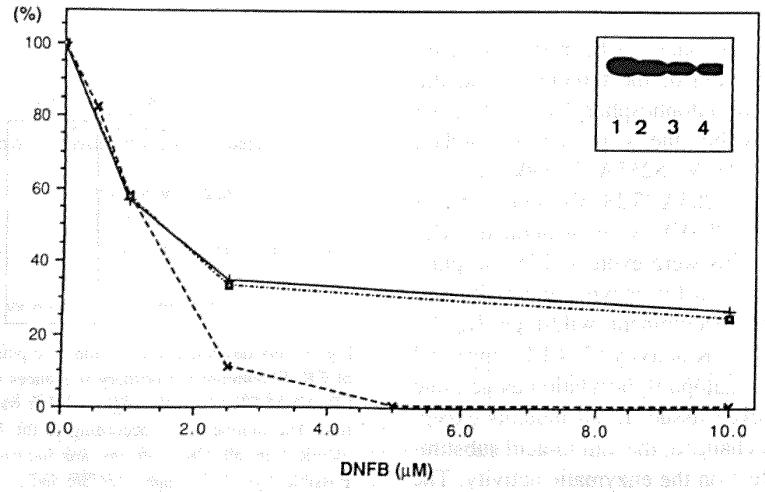

Fig. 7. Inhibition of CK transphosphorylation but not autophosphorylation activity by alkylation. Rabbit M-CK (0.2 mg/ml, corresponding to 5 μM of monomeric CK) was inactivated with increasing concentrations of DNFB. CK transphosphorylation activity after alkylation was determined by the pH-stat method (dashed line). Autophosphorylation activity was determined by incubating the alkylated M-CK in the autophosphorylation assay, followed by SDS-PAGE and blotting onto PVDF membranes. To determine incorporation of ^{32}P, autoradiograms were measured densitometrically (solid line) and excised bands were quantified by liquid scintillation counting (dotted line). Activities are expressed as a percentage of the activity of unalkylated enzyme. Inset: Autoradiogram of autophosphorylated CK, treated with 0 μM (1), 1.0 μM (2), 2.5 μM (3) and 10.0 μM (4) DNFB.

equilibrium random with all evidence pointing to a direct, in-line transfer of the phosphoryl group between simultaneously bound substrates [34]. So far, no evidence for a covalent enzyme-phosphate intermediate was reported. Also, co-incubation of [γ-^{32}P]ATP in the autophosphorylation assay with the transition state analogue substrates (MgADP, KNO_3, creatine) inhibited autophosphorylation to the same limited extent as addition of ADP or creatine alone. In contrast, transphosphorylation activity was inhibited to a much greater extent by the transition state analogue substrates than by ADP or creatine alone [35]. To further investigate this question, rabbit M-CK was incubated with dinitrofluorobenzene (DNFB), an agent known to react specifically with the 'reactive cysteine' of CK [34], and transphosphorylation activity was determined by the pH-stat assay while autophosphorylation was determined by autoradiography and liquid scintillation counting (Fig. 7). Incubation of rabbit M-CK with 5 μM of DNFB decreased the transphosphorylation activity by more than 99.5% of the original activity. However, autophosphorylation activity persisted at about 30% of the original value even at higher concentrations of DNFB, demonstrating that autophosphorylation of CK is considerably less sensitive to alkylation than is CK transphosphorylation activity. The fact that alkylation of Cys-283 reduced autophosphorylation to some extent indicates that autophosphorylation may take place in the vicinity of this residue.

Analysis of Mi-CK and B-CK containing certain point mutations provided further support that the transphosphorylation activity is not necessary for autophosphorylation of CK. Recombinant mutant Mi_b-CK in which the reactive cysteine was substituted by an alanine showed less than 0.3% of the wild-type transphosphorylation activity at pH 7.0 [27] but still showed about 25% autophosphorylation (not shown). A similar observation was made with the S285A B-CK mutant enzyme, which exhibited less than 2% CK transphosphorylation activity as compared to the wild-type enzyme but autophosphorylated to the same extent as the wild-type enzyme (Fig. 6a, a*).

4. Discussion

In this work we have shown that B-CK, M-CK and Mi_b-CK can be autophosphorylated. Our data suggest that the autophosphorylation of CK is not dependent on substrate turnover and is not the result of the transphosphorylation reaction. We have used peptide analysis and mutagenesis to locate the phosphoamino acids in B-CK. The phosphopeptide analysis showed that the autophosphorylation sites are all located within a small region of the protein that maximally spans from amino acids 271 to 313 and contains the putative autophosphorylated residues Thr-282, Ser-285 and Thr-289. Thr-282 and Ser-285 are located close to the reactive cysteine (cys-283). Although this cysteine can be affinity labeled with the ATP analogue [^{14}C]ATP-γ-*p*-azidoanilide [36] and with the creatine analogue [^{14}C]epoxycreatine [37], modification of the reactive cysteine with *S*-methyl methanethiosulfonate has indicated that the reactive cysteine might not be directly involved in ATP or creatine binding but might rather serve as a flexible hinge between the two substrate binding sites [38]. The hinge hypothesis, while not universally accepted [39], is strongly supported by an extensive mutational study which showed that substitution of the reactive cysteine in chicken Mi_b-CK by glycine or serine led to the complete loss of synergism in substrate binding while not dramatically affecting substrate binding and catalysis [27].

The other putative autophosphorylated residue, Thr-289, is located within a glycine-rich region (LGTGLRAGV). Many nucleotide binding proteins contain glycine-rich regions that are associated with nucleotide binding (for review see Ref. [40]). The sequence found in CK is homologous to the type of glycine-rich loop (LGxGxxGxV) that is conserved within the entire family of protein kinases [33]. Sequence comparison of CK isoenzymes shows that this motif (^{287}*LGTGLRGGV*) is perfectly conserved in all known M-CK isoenzymes and that all known B- and Mi-CK isoforms [32] contain a very similar motif, with a single conservative amino-acid exchange (LGTGLR*A*GV). A similar replacement of glycine by alanine is also seen in some members of the protein kinase family such as PKCζ and PHO85 [33]. The glycine-rich loop has been shown to be an essential part of the ATP binding pocket in the crystal structure of the cAMP-dependent protein kinase and other recently solved crystal structures of protein kinases (for review see Ref. [12]). Thus, it is tempting to speculate that this sequence motif is also involved in ATP binding for CK. In the protein kinase family, residues located within the glycine-rich loop of the cdc2 protein kinase can be phosphorylated, and such phosphorylation is an additional means to negatively regulate this kinase (for references see Refs. [12,41]).

In the protein kinase family, the glycine-rich loop is located within the very *N*-terminal region of the catalytic core of these enzymes [12,33]. In contrast, the analogous glycine-rich motif of CK is located in the *C*-terminal part of its sequence. This suggests that the ATP binding site is located in the *C*-terminal moiety of CK and implies that the same motif is used for ATP binding in CK isoenzymes and protein kinases, but in a different structural context. Another indication that the ATP binding site of CK is located in the *C*-terminal moiety of CK is that the conserved residue Asp-335 of chicken Mi_a-CK (homologous to Asp-340 in B-CK) could be labeled with the ATP analogue CIRATP [42]. A high resolution crystal structure of CK will be necessary to definitively locate the ATP binding site and to see where the putative autophosphorylation sites are located with respect to the nucleotide binding site and with respect to each other. A crystal structure is also needed to show that the glycine-rich motif in CK constitutes an essential part of the active site that

wraps around the nucleotide as the homologous loop does in the catalytic core of protein kinases [12,40].

Phosphorylation and autophosphorylation have been shown to play important roles in protein kinases. Phosphorylation of Thr-197 of the cAMP-dependent protein kinase, which clearly occurs as an autocatalytic process in the recombinantly expressed enzyme [10], seems to play an essential structural role for the formation of the catalysis-competent conformation of the enzyme [12]. Many other members of the protein kinase family are activated either by autophosphorylation [11] or through phosphorylation at the homologous sites by heterologous kinases (for review see Ref. [41]). In vitro phosphorylation by protein kinase C has been shown to decrease the K_m value for phosphocreatine in B-CK by a factor of two [43]. While the substoichiometric labeling of CK, in combination with the enzyme's multimeric nature, impeded purification and kinetic analysis of the phosphorylated species, the overlap of the putative active site with the putative autophosphorylation sites makes it attractive to speculate that phosphorylation, and in particular autophosphorylation could play an important structural or even regulatory role within the active site of CK.

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