The isoenzyme-diagnostic regions of muscle-type creatine kinase, the M-260 and M-300 box, are not responsible for its binding to the myofibrillar M-band

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Creatine kinase – protein targeting – skeletal muscle association – confocal microscopy – site-directed mutagenesis

Muscle-type creatine kinase is known for its unique interaction with the myofibrillar M-band, but the molecular origin for this structural relationship is not well understood. A systematic sequence comparison between the highly homologous cytosolic isoforms, muscle-type and brain-type creatine kinase, yielded two isoenzyme-specific regions in the muscle-type creatine kinases, the M-260 box (residues 258-270) and the M-300 box (residues 300–315). These particular regions were conspicuous for the specific interaction of this CK isoenzyme, but not of brain-type creatine kinase, with the sarcomeric M-band. In situ diffusion assays with fluorescently labeled native, as well as mutated muscle-type creatine kinase variants, were used to study by laser confocal microscopy their association with the M-band of chemically skinned muscle fibers. Neither a set of charge mutants of the M-260 box and/or the M-300 box nor a hybrid construct of both isoforms with the entire C-terminal region derived from the brain-type isoform showed any significant alteration in the in situ M-band-binding properties when compared to the wild-type form of muscle-type creatine kinase. This indicates that in the intact protein of muscle type creatine kinase, these C-terminal isoenzyme-specific regions are not important for M-band interaction and that the actual M-band interaction domain(s) lay mostly within the N-terminal half of the molecule. The highly conserved motives (M-260 box and M-300 box) may serve an isoenzyme-specific purpose yet to be identified.

Introduction

Creatine kinase (CK; ATP: creatine N-phosphoryl transferase, EC 2.7.3.2) plays a crucial role in the energy metabolism of cells with high and fluctuating energy requirements such as skeletal and cardiac muscle, brain, photoreceptor cells, electrocytes, and spermatozoa (for review see [30, 31]). The enzyme is found in two cytosolic isoforms, "ubiquitous" BB-CK and "sarcomeric" MM-CK [4], and two mitochondrial isoforms, "ubiquitous" Mi_a-CK, and "sarcomeric" Mi_b-CK (for review see [9, 11, 35]) which are all expressed in a tissue-specific manner. The subunits (≈ 43 kDa) of the cytosolic isoforms associate to form homo- and heterodimers, whereas the mitochondrial subunits associate to form predominantly homooctamers which are interconvertible into homodimers. This enzyme family catalyzes the reversible exchange of high-energy phosphates in the reaction: Phosphorylcreatine (PCr^{2-}) + $MgADP^- + H^+ \rightleftarrows$ Creatine (Cr) + $MgATP^{2-}$ (for review see [13, 33]). The physiological role of this enzyme family has been extensively studied in skeletal muscle (for review see [31]), as well as in a variety of non-muscle cells [32]. In differentiated sarcomeric muscle, MM-CK is the specific cytosolic isoform expressed [4], whereas BB-CK is the predominant isoform in brain and in a variety of other tissues. It is a particular feature of MM-CK that a small but significant amount of this otherwise soluble isoenzyme is specifically associated with the myofibrillar M-band (5–10% of the total CK present, depending on the fiber type [24, 26], where it is functionally coupled

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to the myofibrillar actin-activated Mg^{2+}-ATPase as an efficient intramyofibrillar ATP regenerator [25, 28]. An additional association of MM-CK with the sarcoplasmic reticulum, where it is functionally coupled with the ATP-dependent Ca^{2+}-pump, and with the sarcoplasma membrane, where it is functionally coupled to the ATP-dependent Na^{+}/K^{+}-pump, have also been described (for review see [31]). The M-band-bound fraction has been proposed to play a major structural role in this sarcomeric structure, located in the center of each sarcomer, that keeps the thick filaments in register [22, 27, 29]. Remarkably, this interaction with the M-band is isoenzyme-specific, that is, only muscle-type MM-CK, but neither brain-type BB-CK nor the heterodimer MB-CK, which occurs naturally to some extent during myogenesis [17] and in the mammalian heart, binds to the M-band [17, 18, 21, 24, 26, 27]. In an earlier study, it was suggested that molecular binding sites in the C-terminal half of the MM-CK isoenzyme might be responsible for this M-band-binding property [21]. Apart from this single study, there was no further progress in elucidating the structural differences that cause this isoform-specific interaction of MM-CK with the M-band.

In here, we have looked for prominent sequence differences particularly in the C-terminal half of the cytosolic isoforms, as this region was proposed to be responsible for the binding property of MM-CK. Two interesting regions, a promising isoform-characteristic sequence motive (the 260-box, residues 258–270) of 13 amino acids length, and another region with a number of conspicuously conserved sequence differences (the 300-box, residues 300-313), were investigated in more detail. We compared the results of specific MM-CK mutants of both regions with those of wild-type MM-CK. Conclusive evidence is given that none of the highly conserved motives (M-260 box or M-300 box), studied in the C-terminal half of MM-CK, nor the entire C-terminal half of the molecule are responsible for the isoenzyme-specific M-band interaction of MM-CK and that the domain(s) for association of MM-CK with the M-band are situated within the N-terminal half of the protein molecule.

Materials and methods

Bacterial strains, plasmids and DNA manipulation

E. coli strain BL21(DE3)pLysS and expression vector pET-3b have been described elsewhere [23]. Media and standard DNA manipulations were as described in [1]. The construction of plasmid pT17 and pT23, containing the full-length cDNA for chicken muscle-type CK and chicken brain-type CK, both a gift from Dr. J.-C. Perriard (Institute of Cell Biology, ETH Zürich, Switzerland), will be described elsewhere.

Protein sources and used antibodies

A monoclonal mouse antibody against the 185 kDa M-band protein myomesin from chicken [8] was generated by standard protocols at the Institute of Cell Biology (ETH Zürich, Switzerland) and kindly provided by Dr. J.-C. Perriard. A polyclonal cyanin-5-conjugated goat anti-mouse IgG (Jackson, Immuno Research, Dianova, Hamburg, Germany) was used as secondary antibody. Chicken MM-CK and specific variants thereof were overexpressed in *E. coli* and purified to homogeneity, as will be described in a separate work. Recombinant chicken MM-CK or commercially available cytosolic MM-CK from rabbit muscle (Boehringer, Mannheim, Germany) was used for chase experiments; the latter without further purification.

Fluorescent labeling of MM-CK and derivates thereof

Specific labeling of proteins at accessible sulfhydryl groups of their cysteine residues was carried out with iodoacetamide-based fluorescent dyes. Four mg protein was incubated in the presence of 10 mM DTT at 37°C for 1 h to activate the cysteine sulfhydryl groups. Subsequently, DTT was removed and buffer exchanged against 100 mM H_3BO_3, pH 8.5, by size exclusion chromatography on a fast desalting FPLC column (Pharmacia, Uppsala, Sweden). The protein was then concentrated using a Centricon-30 ultrafiltration unit (Amicon Corp., Lexington, MA, USA) to a final volume of 0.5 ml and incubated with a 2.2-fold molar excess of rhodamine × iodoacetamide (XRIA) or 5-iodoacetamidofluorescein (5-IAF; both Molecular Probes Inc., Eugene, OR, USA) at room temperature for 2 h in the dark under gentle agitation. The labeling reaction was then quenched by adding 1 mM DTT to the reaction. Unconjugated dye was removed and the buffer exchanged for 50 mM NH_4HCO_3, pH 7.9, a volatile buffer system, by size exclusion chromatography. Small aliquots were then lyophilized and stored at –20°C, protected from light.

Site-directed mutagenesis

Polymerase chain reaction (PCR) was used for site-directed mutagenesis of pT17, containing the full-length cDNA for chicken muscle-type CK under control of a T7 promotor. Synthetic oligonucleotides were designed to change the codon of particular charged amino acids within a conserved sequence motive (amino acid 258–270, the 260-box) into that of uncharged counterparts of similar structure (Lys to Met, Glu to Gln). Other oligonucleotides were designed to convert the codon of muscle-type CK specific amino acids in another region (amino acid 300–313, the 300-box) into that of the homologous brain-type CK residues (Pro 306 to Glu, Glu 309 to Gly, His 313 to Lys). A subsequent 260-box chimera and a M/B-CK hybrid were generated by exchanging corresponding fragments between pT17 and pT23, containing the full-length cDNA of chicken brain-type CK, using suitable commonly conserved restriction sites: Mlu I and Sfu I, corresponding to a cut after amino acid 234 and 285, respectively, in the translated sequence (see Fig. 2). The obtained variants (see Tab. I) were verified by sequencing and subsequently expressed in an inducible system in *E. coli* BL21(DE3)pLysS, as described elsewhere [6].
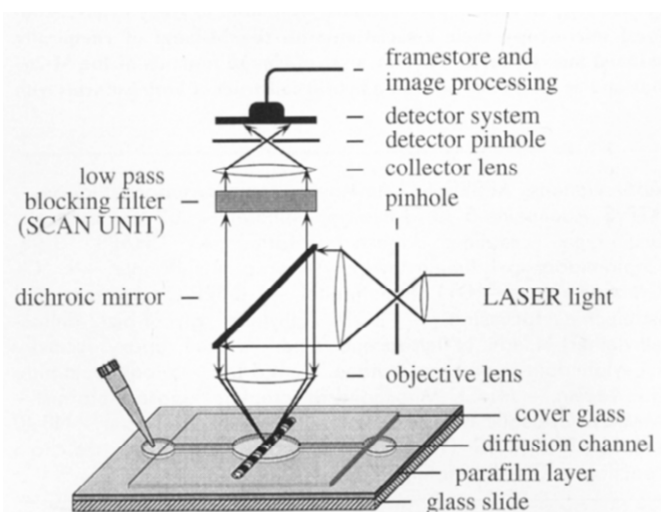
Fig. 1. Skinned muscle fiber diffusion assay set-up. This arrangement was used to study the diffusion and interaction of fluorescently labeled proteins with the sarcomeric structures of the skinned muscle fiber. In this assay a single muscle fiber is put down in a flow-through chamber such that it crosses over a central diffusion channel. This channel serves to exchange the media surrounding the fiber. The optical part of the confocal microscope, used for visualization, is depicted in the upper part.

One-dimensional isoelectric focussing (1-D IEF)

The 1-D IEF gel (7.4% acrylamide/bisacrylamide (49.5:3), 9M urea (Schwarzmann, ultra pure), 2% carrier ampholytes, pH 5–8 (Pharmacia, Uppsala, Sweden), 3% detergents (CHAPS/NP-40)) was overlayed with a slot gel (7.4% acrylamide/bisacrylamide (49.5:3) in the corresponding upper buffer) such that the comb was in contact with the IEF gel surface. Samples were prepared as followed: 25 mg purified protein in a total volume of 15 ml, 3.5 ml 200 mM DTT, 7 ml detergent mix (30% CHAPS, 15% NP-40), were incubated at 37°C for 15 min, followed by addition of 29 mg urea and 2.5 ml ampholyte pH 3–10 (Iso-DALT, Serva, Heidelberg, Germany). The incubation was continued at 37°C until urea was dissolved and the samples were then loaded into the gel slots. The separation by isoelectric focussing was performed in a Mini-PROTEAN II cell (Bio-Rad, Glattbrugg, Switzerland) in 20 mM cathode buffer (5 mM lysine, 5 mM arginine, 10 mM ethylenediamine) and 20 mM anode buffer (10 mM glutamic acid, 10 mM aspartic acid) at 4°C for 3700 V·h. Protein bands were visualized by silver staining [10].

Functional in situ binding assay

All binding experiments were performed on single, chemically skinned muscle fibers of rat musculus psoas major, prepared according to [2, 36]. Fibers were stored in chilled skinning solution (5 mM KH$_2$PO$_4$, 3 mM Mg-acetate, 5 mM EGTA, 3 mM Na$_2$ATP, 50 mM PCr, 2 mM DTT, 8 mM NaN$_3$, pH 7.0 at 4°C) supplemented with protease inhibitors (10 μM leupeptin, 10 μM pepstatin A, 10 μM E-64, 10 μM antipain, 1 μg/ml aprotinin, all purchased from Sigma, St. Louis, MO, USA; 1 mM AEBSF, Calbiochem-Novabiochem, La Jolla, CA, USA) at the day of the experiment [14]. Single fibers were prepared from a 15 mm muscle bundle, pinned down in a tray inlayed with the siliconelastomere SYLGARD® (Dow Corning, Bruxelles, Belgium) and covered with chilled skinning solution, by gently pulling them out of the muscle unit with the help of a pair of jeweller's forceps. These fibers, in average 100 μm in diameter, were put down in the tray by sticking their ends into the SYLGARD® matrix for intermittent storage. With aid of a fiber carrier single fibers were mounted on a flat, homemade, flow-through chamber on a microscope slide (Fig. 1). The chamber, about 200 μm deep and 6 mm in diameter (5.7 mm^3), was closed with a cover-slip so that the fiber, corresponding to less then 1% of the chamber volume, was completely surrounded by solution, which was then exchanged for relaxation solution (10 mM imidazole, 2 mM MgCl$_2$, 3 mM EGTA, 2 mM DTT, 2 mM MgATPγS (Calbiochem-Novabiochem), 0.25 mM p$_1$,p$_5$-di(adenosine-5')-pentaphosphate (Boehringer, Mannheim, Germany), 100 mM glucose, 0.1 units/ml hexokinase (Boehringer), pH 6.8, and ionic strength adjusted to 120 mM with potassium-propionate) [14] by sucking it through the diffusion channel, about 2 mm wide, with a piece of 3MM paper (Whatman, Maidstone, Great Britain) or a Kleenex® tissue. All subsequent incubation and washing steps of the mounted fibers were performed with relaxation solution. For the binding assay, fluorescently labeled proteins were diluted to 25–50 μg/ml in relaxation solution, i.e. 27–58 μM, and introduced in similar manner into the fiber, containing in vivo at least 100 μM MM-CK, while the fiber remained in place on the microscope. We used a Bio-Rad MRC-600 laser confocal system (Bio-Rad, Glattbrugg, Switzerland), equipped with a Zeiss AXIOPHOT fluorescence microscope (Carl Zeiss, Oberkochen, Germany) to follow in real-time the equilibration and interaction of fluorescently labeled molecules with the myofibrillar structure in longitudinal optical sections through the core region of a muscle fiber. Unfixed skinned fibers, as used in the experiments of the present study, were still able to contract, but did not tolerate the use of reagents to reduce photobleaching. Therefore, the observation time was kept as short as possible and the excitation light intensity was also kept as low as possible. If possible, following images were taken at neighbouring spots within the same region of the muscle fiber to avoid photobleaching artefacts.

Results

### Sequence comparison of cytosolic CK isoforms

The sequence compilation used (Fig. 2) led to the identification of a highly conserved isoenzyme-characteristic amino acid sequence motive (residue 258–270), the 260-box, in the C-terminal half of the enzyme, which represents the most striking difference between the two cytosolic isoforms, MM-CK and BB-CK. The amino acid homology in this stretch of 13 amino acids is only 30.8% among the chicken cytosolic isoforms, whereas the homology is over 80% in adjacent parts (83.3% in N-terminal and 80.2% in more C-terminal parts). As reference, the overall amino acid homology between chicken MM- and BB-CK is 80.6% and 77–82% among all known cytosolic CK isoforms. Between members of the same isoform the 260-box remains highly conserved. Thus this region had to be a putative candidate for the M-band-binding domain in MM-CK. A second conspicuous isoform-characteristic sequence motive (amino acid 300–313), the 300-box in CK, consists of an amino acid stretch which displays only 50% homology between chicken MM- and BB-CK and 46.2% among all cytosolic CK isoforms known so far. For chicken MM-CK, the conserved differences to BB-CK within this region, resulting in a change of the conveyed charge property, were Pro 306, Glu 309 and His 313. The latter is substituted by Thr 313 in the muscle-specific CK isoform of most other species (see Fig. 2). Other minor sequence differences could be found next to the 260-box and next to the C-terminal end of the enzyme.

Model of the "diagnostic-box" structure

Since conserved primary structures often act as a pointer for functional domains in related proteins, we looked more closely at the peculiar 260-box region. Popular secondary structure prediction algorithms [3, 7], both based on statistical methods, suggest an amphoteric α-helix for the 260-box in chicken MM-CK showing a prominent cluster of 6 charged amino acids (respectively 5 in other species) on one side and a cluster of hydrophobic residues on the other side of the α-helix (Fig. 3A). In contrast, a similar model for the corresponding BB-CK region did not show such an accumulation and arrangement of charged residues (Fig. 3B). The structural predictions for the 260-box in MM-CK were supported by the recent X-ray structure of intact chicken mitochondrial Mi$_b$-CK crystals [5]. The structural similarity (66–67% overall amino acid homology) has already allowed others to solve by molecular replacement the structure of rabbit MM-CK [12] and makes therefore this structure a suitable model to describe the approximate structure of other CK isoforms. The homologous 260-box region of the mitochondrial isoenzyme (30.8% identity with BB-CK and MM-CK) forms a similar α-helix at the surface of the molecule which is well exposed to the surrounding environment (Fig. 4A). Hence, the three-dimensional structure of Mi$_b$-CK was applied to create a putative model of the 260-box in chicken MM-CK (Fig. 4B) by molecular replacement, as the overall structure of the different CK isoforms is expectedly evolutionary well conserved. This procedure resulted in a similar arrangement of the residues as predicted from the primary amino acid sequence. According to this model, residues Pro 306, Glu 309 and His 313 were also located at the molecule surface (Fig. 4A). They are arranged close together in a parallel line relative to a long C-terminal α-helix (Glu 346 to Glu 368) and relative to the basis of a flexible
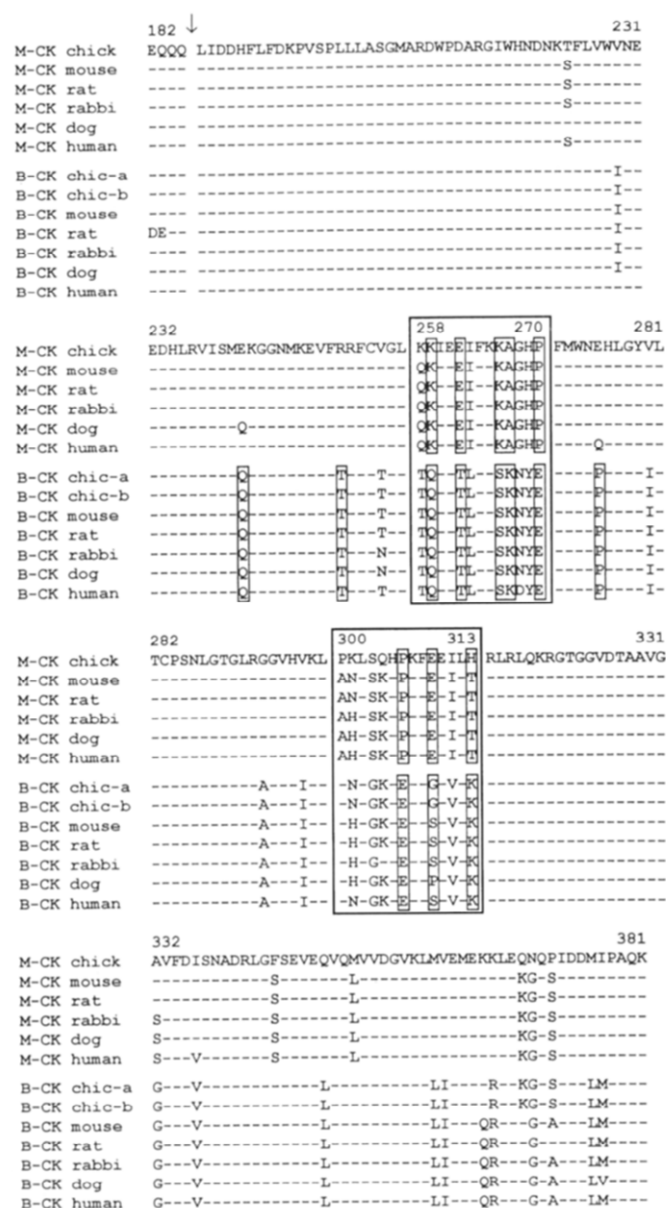
Fig. 2. Amino acid sequence alignment of known cytosolic CK isoforms. Only the C-terminal halves of the CK sequences are shown and compared with the chicken muscle-type CK sequence. The *vertical arrow* indicates the begin of the enzyme part supposedly responsible for the M-band binding [21]. The *boxed regions* are: the isoform-characteristic 260-box (amino acid 258–270) and the isoform-characteristic 300-box (amino acid 300-313). Sequence differences within both regions are printed. Conspicuous differences affecting the charge property of conserved residues are highlighted by *separate boxes*. The amino acid sequences are given in standard single letter code. Consensus residues are indicated by a *dash*. Residues outside the boxed regions which do not match with the chicken muscle-type CK sequence are printed. M-CK, indicates the muscle-type subunit isoforms; B-CK, brain-type subunit isoforms. Database access numbers: M-CK chicken, P00565, A00675; M-CK mouse, A23590; M-CK rat, P00564, A00674; M-CK rabbit, P00563, A00673; M-CK dog, P05123, A24686; M-CK human, P06732, A35238; B-CK chicken form-a, A37059, A24793; B-CK chicken form-b, B37059; B-CK mouse, Q04447, A42078; B-CK rat, P07335, A35682; B-CK rabbit, P00567, A00678; B-CK dog, P05124, B24686; B-CK human, P12277, S15935.
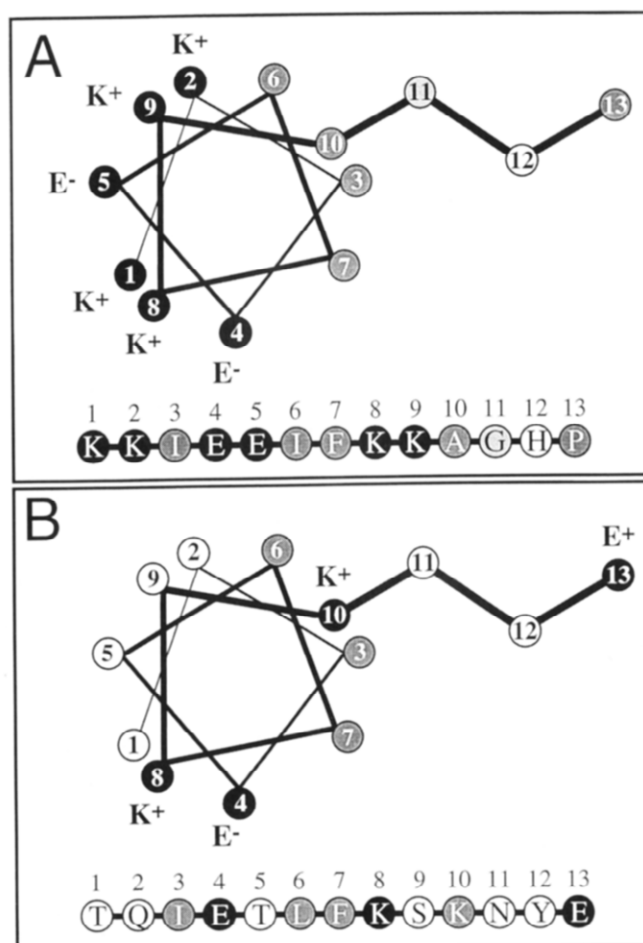

Fig. 3. Predicted secondary structure of the 260-box region. Common secondary structure predicting algorithms proposed consistently an amphoteric α-helix for the isoform-characteristic 260-box sequence motive in muscle-type CK (residues 258–270). A model for that region in both cytosolic isoforms, (**A**) chicken MM-CK region (M-260), and (**B**) chicken BB-CK region (B-260), is depicted according to the predicted structure for the muscle-type CK motive. The respective primary amino acid sequence is indicated below, numbered in a consecutive manner. Note the different distribution of charged and hydrophobic amino acids in both, the M-260 and B-260 model. *Black spheres*, charged residues; *dark grey*, hydrophobic residues; *white*, polar residues; *light grey*, glycine residue.

loop region (residue 321–331), which forms a lid structure that is thought to close upon substrate binding in order to exclude excess water from the active site during catalysis.

Site-specific variants of chicken MM-CK

The fact that the charged residues in the M-260 box are entirely exposed on the MM-CK molecule surface made this cluster a favourable candidate for being a molecular recognition site that interacts with other sarcomeric components. Site-directed mutagenesis was used to generate a set of mutant proteins in which the charged amino acids of this region were exchanged by uncharged counterparts of similar structure (Tab. I). In this approach, muscle-type CK-specific charges were removed in a successive manner, such that in its extreme the generated MM-CK variant had an uncharged M-260 box, only with those charges retained that are conserved between
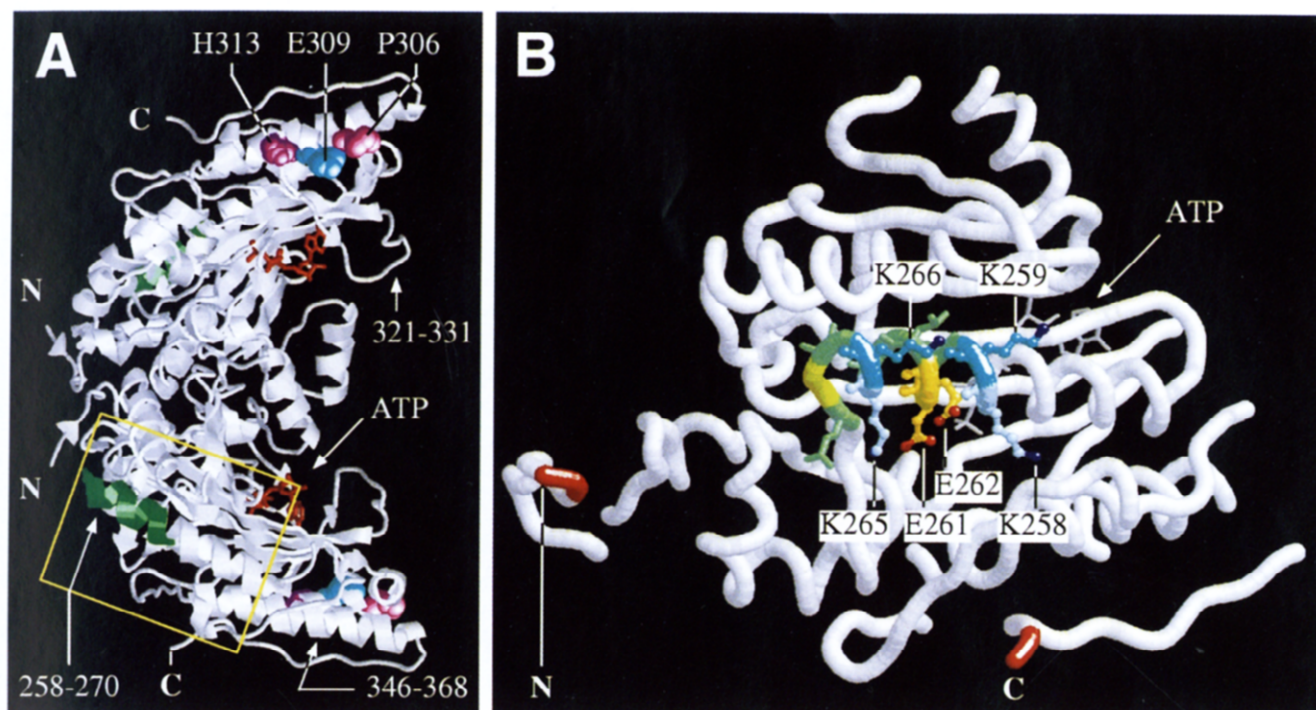
Fig. 4. Topology of the isoform-characteristic regions in the putative MM-CK structure. (**A**) Dimer structure of chicken MM-CK, derived by analogy to the solved X-ray structure of chicken Mi_b-CK [5], visualizing the corresponding location of the isoform-characteristic regions in chicken MM-CK. The course of the polypeptide chain is shown in the cartoon representation, provided by the rendering program RASMOL [20]. The molecule is shown in a sideview. For better orientation, bound ATP is shown in *red,* identifying the catalytic center in each monomer. The region of the 260-box is shown in *green.* The conserved residues in the second isoform-characteristic region (Pro 306, Glu 309,

His 313), the 300-box, are depicted in the spacefill presentation in *magenta, cyan* and *violet,* respectively. (**B**) A more detailed view of the putative structure in the 260-box region of chicken MM-CK. The amino acids of the charge cluster are shown in the ball-and-stick presentation in different colors. Positive charge, *dark blue;* negative charge, *red.* Side chains of hydrophobic residues are shown in *dark green* in the stick representation. N- and C-termini are shown in *red* and bound ATP is depicted in the background in *dark grey.* The numbering is according to the amino acid sequence of chicken muscle-type CK.

MM-CK and BB-CK isoforms (Glu 261 and Lys 265). Site-directed mutagenesis was also used to generate MM-CK variants in which the residues of the second isoform-characteristic region (Pro 306, Glu 309 and His 313), the 300-box, were transformed into those of the homologous BB-CK isoform, which lacks the M-band-binding property [18, 21, 27]. To evaluate the combined effect of both regions together, a subsequent chimeric MM-CK variant was generated in which additionally the entire M-260 box (residue 235–285) was exchanged for the homologous brain-type CK region and a complementary M/B-CK hybrid with a brain-type C-terminal region (Tab. I).

Characterization of generated MM-CK variants

All MM-CK variants were heterologously expressed in *E. coli* and purified from inclusion bodies, with exception of the M/B-CK hybrid which was purified from the cell lysate. They all showed a single protein band at about 43 kDa, which appeared nearly homogeneous by Coomassie blue staining (Fig. 5). One-dimensional IEF confirmed the concomitant overall charge shift in the different variant proteins, calculated from their amino acid sequence (Fig. 6). They all shared the special feature of wild-type MM-CK, to be focussed by IEF in multiple isoelectric isoforms [19], resulting in a typical pattern of 5 or more main bands as seen with the wild-type protein. The nature of this multibanded IEF pattern will be subject of
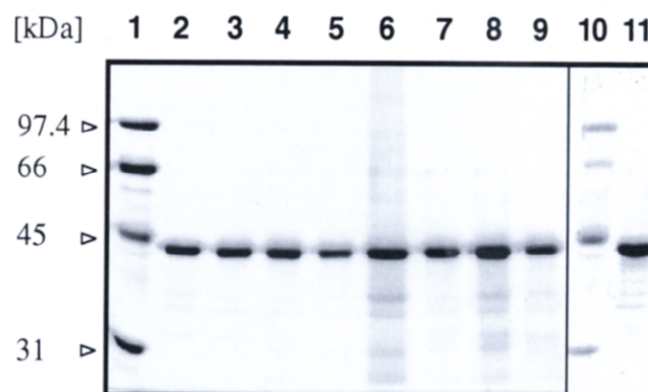
Fig. 5. SDS-PAGE of the site-specific chicken MM-CK variants. Individual fractions, each 2.5 μg of purified mutant protein, were separated on a 11 % SDS-polyacrylamide gel and stained with Coomassie Brilliant Blue R-250. All recombinant mutant proteins were isolated from inclusion bodies formed in *E. coli* strain BL21(DE3)pLysS. As reference, the separation of the wild-type protein is shown in *lane 2.* *Lane 1,* positions of molecular size markers (Bio-Rad, low M_r standards); *lane 2,* chicken MM-CK; *lane 3,* K259M-K266M; *lane 4,* K259M-E262Q-K266M; *lane 5,* K258,259M-E262Q-K266M; *lane 6,* K258,259M-K265,266M; *lane 7,* E309G-H313K; *lane 8,* P306E-E309G-H313K; *lane 9,* 260-box chimera, *lane 10,* positions of molecular size markers (Bio-Rad, low M_r standards); *lane 11,* M/B-CK hybrid.

Tab. I. Summary of the generated chicken MM-CK variants.

M-260 box variants:

chicken MM-CK(wild-type)

K259M-K266M

K258,259M-K265,266M

K259M-E262Q-K266M

K258,259M-E262Q-K266M
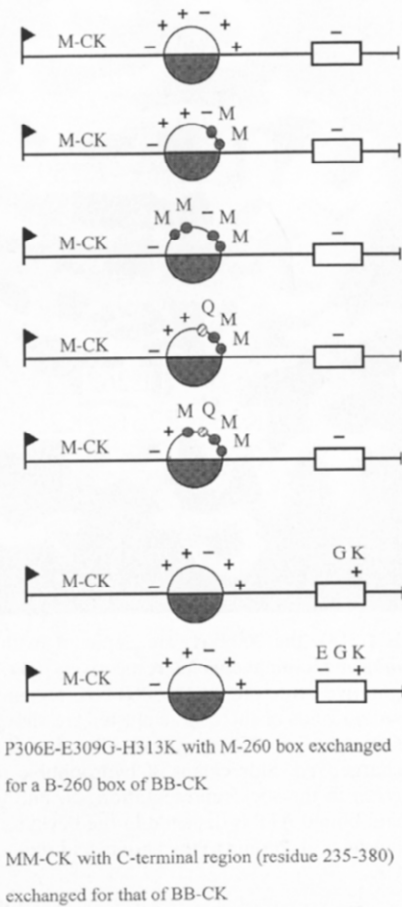
M-300 box variants:

E309G-H313K

P306E-E309G-H313K

260-box chimera   P306E-E309G-H313K with M-260 box exchanged for a B-260 box of BB-CK

C-terminal variant:

M/B-CK hybrid   MM-CK with C-terminal region (residue 235-380) exchanged for that of BB-CK

C-terminal variant:

M/B-CK hybrid   MM-CK with C-terminal region (residue 235-380) exchanged for that of BB-CK

Fig. 6. One-dimensional IEF gel of site-specific chicken MM-CK variants. Individual fractions, each 25 µg of purified mutant protein, were electrofocussed on a 1-D IEF, pH 8 to 6 gradient gel (*lanes 2–8*). The expected relative charge shift of each sample is noted in the box on top of the gel. The focussing pattern of wild-type chicken MM-CK (*lane 1*) is used as internal reference for the relative charge shift of the mutant proteins towards the acidic or basic end of the gel. The site-specific mutations resulted in a shift of the whole pattern in discrete steps according to the overall charge change. The IEF pattern of the proteins were visualized by silver staining. *Lane 1*, wild-type chicken MM-CK; *lane 2*, K259M-K266M; *lane 3*, K259M-E262Q-K266M; *lane 4*, K258,259M-E262Q-K266M; *lane 5*, K258,259M-K265,266M; *lane 6*, E309G-H313K; *lane 7*, P306E-E309G-H313K; *lane 8*, 260-box chimera.

The abbreviations used for the mutant proteins are a descriptive terminology, composed of the mutated amino acid, its location and its replacing amino acid. This is accompanied by a schematic drawing of the mutant construct, where possible, for visual assistance. The white and dark grey *hemicircle* in the center of the drawing corresponds to the charged and hydrophobic face of the M-260 box α-helix, respectively (see also Fig. 3). Changed charges are indicated by the replacing amino acid and a small sphere at its place to notify the conveyed character by the replacing amino acid. *Dark grey sphere*, hydrophobic amino acid; *hatched sphere*, polar amino acid. The *box* towards the end of the drawing corresponds to the M-300 box region. Changes of amino acids and concomitant charge changes are indicated. The N-terminus of the protein is symbolised by a small *flag*. Standard single letter code was used for the labeling of amino acids.

Tab. II. Summary of specific enzyme activities.

MM-CK variant	specific activity reverse reaction (units/mg)	specific activity forward reaction (units/mg)
M-260 box variants:		
chicken MM-CK(wild-type)	200	26.9
K259M-K266M	139	19.4
K258,259M-K265,266M	87.4	12.7
K259M-E262Q-K266M	165	19.2
K258,259M-E262Q-K266M	90.7	14.1
M-300 box variants:		
E309G-H313K	120	17.0
P306E-E309G-H313K	96.0	15.6
260-box chimera	75.7	13.0
C-terminal variant:		
M/B-CK hybrid	282	48

The table shows representative specific enzyme activities of purified chicken MM-CK variants, expressed in *E. coli*. The protein concentration was determined by Bio-Rad protein assay, using BSA as standard. The enzyme activities were determined with the pH-STAT assay [16, 28] in the reverse (ATP production) and the forward (PCr production) enzyme direction. One unit of enzyme activity is equal to 1 mmol of phosphorylcreatine transphosphorylated per minute at pH 7.0 and 25°C, defined for the reverse direction.

a separate study and will be presented elsewhere (in preparation). The whole pattern of the charge mutant proteins was shifted in discrete steps either more towards the acidic or basic end of the gel, relative to the wild-type protein (Fig. 6, lane 1), indicative for the charge change generated by site-directed mutagenesis. The specific activities of the recombinant mutant proteins, are shown in Tab. II. All mutant proteins were enzymatically active in both catalyzed enzyme directions, although most were somewhat less active then the wild-type protein.

Myofibrillar binding of site-specific MM-CK variants

In this approach, we used fluorescently labeled MM-CK variants at substoichiometric concentrations, with respect to the total MM-CK content in vivo, in a functional in situ binding assay with skinned muscle fibers to evaluate the putative functional role of both identified peculiar sequence motives for the isoform-characteristic targeting of MM-CK to the myofibrillar M-band. Skinned fibers served as a model system, since contractility and calcium regulation properties [15], as well as sarcomeric superstructure remain intact but become permeable to molecules [14]. With the help of confocal light microscopy, it was possible to follow the diffusion of fluorescently labeled proteins into the myfibrillar compartment and to observe directly their interaction with the subsarcomeric structure with a high spatial and temporal resolution. The resulting pattern was directly compared with that obtained with wild-type, rhodamine-labeled MM-CK. As internal reference, we used an antibody labeling directed against the M-band-specific protein myomesin [8], in order to unambiguously identify the corresponding sarcomer structure. In this in situ binding assay, a whole set of MM-CK variants was used to validate the functional role of the conspicuously charged residues in the M-260 box for the M-band-binding property of MM-CK. Surprisingly, all the different charge variants of the M-260 box region showed exactly the same labeling pattern as wild-type chicken

Tab. III. Summary of the binding characteristics of defined MM-CK variants in chemical skinned muscle fibers.

heterologously expressed proteins	M-band binding after diffusion	M-band binding after wash	M-band binding after reference labeling with XRIA MM-CK
wild-type CK proteins:			
chicken MM-CK	+++	++	++
chicken BB-CK*	–	–	–
M-260 box variants:			
K259M-K266M	+++	++	++
K258,259M-K265,266M	+++	++	++
K259M-E262Q-K266M	+++	++	++
K258,259M-E262Q-K266M	+++	++	++
M-300 box variants:			
E309G-H313K	+++	++	++
P306E-E309G-H313K	+++	++	++
260 box chimera	+++	++	++
C-terminal variant:			
M/B-CK hybrid	+++	++	++

The resulting labeling pattern was stable after the fiber was equilibrated with the fluorescently labeled protein, without any obvious differences between wild-type MM-CK and mutant protein. Note, that the 260-box chimera with both regions, the 300-box and the 260-box changed simultaneously, as well as the C-terminal variant still bind to the M-band, thus making these regions unlikely to play a role in the isoenzyme-specific association of MM-CK to the sarcomeric M-band. Intensity of fluorescence: +++, very strong; ++, strong; +, weak; ±, trace; –, none.
* Throughout the experiment with BB-CK, serving as a control, a more or less weak but specific interaction of BB-CK with the I-band, but never with the M-band, was observed (see Fig. 7 E).

MM-CK, even when all isoform-specific charges had been removed (see Tab. III). The additional MM-CK variants of the second isoform-characteristic region (residues 300–313), the 300-box, were also used to investigate a putative involvement
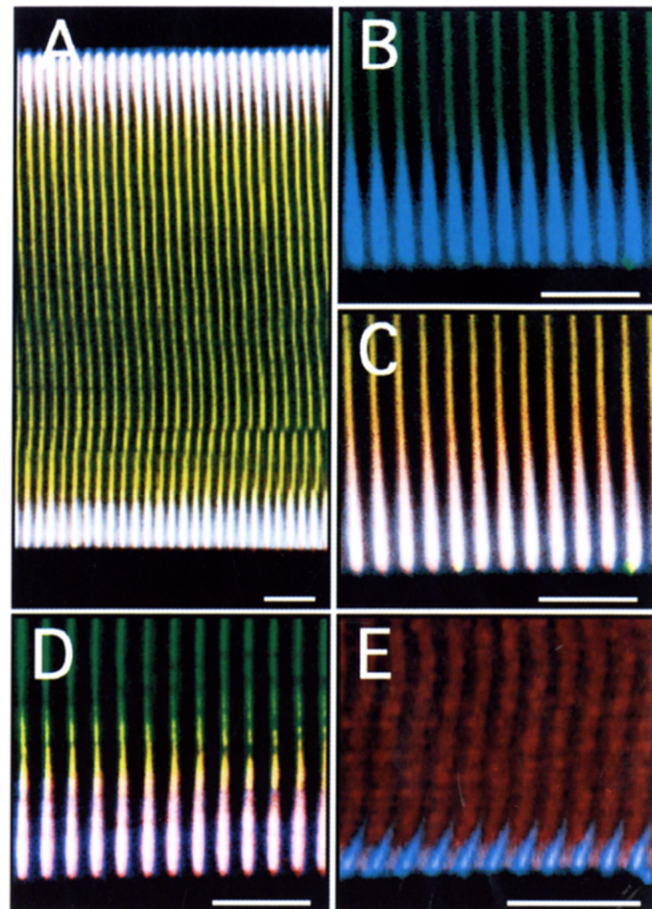
Fig. 7. Representative in situ labeling pattern of chicken MM-CK variants in a skinned muscle fiber. Confocal images of longitudinal optical sections through the core region of an unfixed chemically skinned muscle fiber after equilibration with fluorescently labeled protein of the charge mutant K258,259M-E262Q-K266M (**A–C**) and of the M/B-CK hybrid (**D**). Images were taken after washing and reference labeling with rhodamine-labeled chicken MM-CK. The fiber was preincubated with an antibody against the M-band-specific protein myomesin for at least 2h, which was subsequently visualized with a second, cyanin-5 labeled antibody. (**A**) Triple fluorescence pattern of 5-IAF K258,259M-E262Q-K266M, wild-type XRIA MM-CK and cyanin-5 anti-myomesin; (**B**) double fluorescence pattern of the same fiber with 5-IAF K258,259M-E262Q-K266M and cyanin-5 anti-myomesin at higher magnification; (**C**) corresponding triple-fluorescence pattern at higher magnification; (**D**) triple fluorescence pattern of 5-IAF M/B-CK hybrid, wild-type XRIA MM-CK and cyanin-5 anti-myomesin; (**E**) double fluorescence pattern of XRIA BB-CK and cyanin-5 anti-myomesin. The 260-box mutant protein, as well as the M/B-CK hybrid, still show the same localization as wild-type chicken MM-CK in the M-band, whereas wild-type BB-CK is confined to the I-band. This indicates that neither the 260-box region nor the C-terminal region, as a whole, are important for the isoenzyme-specific binding of MM-CK to the M-band. Similar results were also obtained with the MM-CK variants of the 300-box, as well as with a chimeric construct in which both regions had been altered simultaneously (not shown; see Tab. III).*Green fluorescence:* 5-IAF-labeled charge mutant; *red fluorescence:* rhodamine-labeled wild-type MM-CK or BB-CK; *blue fluorescence:* anti-myomesin antibody. Bars 10μm.

or contribution of that region. The same result as before was also found for the MM-CK variants of the M-300 box region (Tab. III). Even in a combination with both regions mutated, the 260-box chimera, as well as in the M/B-CK hybrid where the entire C-terminal region of MM-CK was exchanged against that of BB-CK, there was no alteration in the observed binding pattern. All these MM-CK variants showed the same strong binding to the M-band, as wild-type chicken MM-CK, with no obvious change in the binding strength. This was corroborated by examplary competition experiments with excess unlabeled native MM-CK (5 mg/ml), wherein the specificity of the bound fluorescent MM-CK variants remained unchanged, only the fluorescence intensity decreased signifcantly within 10–30 min (data not shown). Similarly, a presaturation of the fiber with excess unlabeled native MM-CK (5 mg/ml) did not affect the observed M-band specificity of the variants, although the equilibration process was slowed down, as reported before [14]. A representative result of this functional in situ assay, valid for all the MM-CK charge variants, is shown in Fig. 7A–C and for the complementary M/B-CK hybrid in Fig. 7D. In additional experiments, BB-CK showing a more or less prominent interaction with the sarcomeric I-band, but never with the M-band, was used as an internal control (Fig. 7E). This isoenzyme-specific interaction pattern of BB-CK is in full agreement with earlier histochemical results [14].

Discussion

Interactions of MM-CK variants with subsarcomeric structures

Confocal microscopy and skinned muscle fiber technology [14] provided a tool to study interesting regions in MM-CK which were thought to be of importance for the unique binding property of this isoform to the myofibrillar M-band. Perhaps, the most interesting region, in this respect, was the M-260 box (amino acid 258–270). All, its favourable position on the molecule surface, its location in the C-terminal half of the enzyme, which was in agreement with an earlier study [21], and its peculiar cluster of charged amino acids as part of on an amphoteric α-helix, made it a promising candidate for being a molecular M-band-binding site.

To study the functional role of this 260-box in the intact protein, we have altered those charged amino acid residues in the corresponding region of MM-CK, which were anticipated to represent functional elements therein. A common concern in protein engineering are global structural changes that could alter particular protein functions. We have therefore used only structurally related amino acids to replace the specific charged residues. In addition, the charges had been removed step-by-step, to balance for possible concomitant, unavoidable structural changes. The results of a detailed biochemical characterization ascertained us that the structural and functional integrity of the different mutant proteins remained intact, i.e. the enzymatic activity was largely retained and the behaviour of mutant proteins during purification did not change. With these charge variants of MM-CK we could show, in the functional in situ binding assay, that the charges of the 260-box region are not involved in the isoenzyme-specific M-band binding of MM-CK. Although we had expected a gradual effect from the alterations in the peculiar charged amino acid cluster, the M-band-binding ability of all MM-CK mutants was not affected

even if all isoform-specific charges had been removed. The same was also true for the MM-CK variants of the M-300 box, where we had converted conserved muscle-type CK-specific amino acids into those of the brain-type. Even when both regions were altered simultaneously in the 260-box chimera, the M-band-binding ability remained unchanged. We therefore conclude from these observations that neither the M-260 box nor the M-300 box is important for the isoenzyme-specific interaction of MM-CK with the M-band. This was finally confirmed by the result with the M/B-CK hybrid, which implies furthermore that none of the C-terminal sequence differences determine the M-band interaction. Wild-type BB-CK showed clearly no intrinsic affinity towards the M-band in the same assay, indicating that the binding properties of the MM-CK variants are not artefactual [14]. Furthermore, the binding properties of the wild-type isoforms have been shown before by other means [26, 27, 29, 34], as well as by immunodetection with isoenzyme-specific anti-CK antibodies against unlabeled protein in preincubated skinned fibers (data not shown), ruling out an artefact of the fluorescent tag.

Amino acid residues conserved in an isoprotein-specific fashion

It was to be expected, that the isoform-specific binding of MM-CK to the myofibrillar M-band [24, 26, 27] will find its reflection in distinct differences in the primary amino acid sequence. We have therefore used amino acid sequence comparison between the known cytosolic CK isoforms, MM-CK and BB-CK, to identify amino acid stretches which are highly conserved in an isoprotein-specific fashion, especially in the C-terminal half of the enzymes where the M-band interaction site of MM-CK had been proposed to reside in an earlier study [21]. Apart from both isoform-characteristic regions (M-260 box and M-300 box) studied, only a small number of less inter-related individual residues next to the 260-box region could be found as similarly isoenzyme-specifically conserved alterations. However, here, we unambiguously demonstrated that neither of both regions, the M-260 box nor the M-300 box, are involved in the M-band binding phenomenon. Besides that, as demonstrated with the M/B-CK hybrid, the remaining differences in the vicinity of these regions and other differences, especially near to the C-terminal end of the CK isoenzymes, are neither responsible for this property. Thus, the results presented make it rather unlikely that sequences in the C-terminal part of the enzyme are involved in the M-band binding. Current work in progress is therefore focused to allocate the responsible molecular interaction site(s) in the N-terminal half of the MM-CK molecule.

The work presented is a first part of a systematic study trying to identify the molecular basis of the isoenzyme-specific targeting of MM-CK to the myofibrillar M-band. Sequence comparison and subsequent analysis of the identified regions strongly suggest a more complex structure of the M-band binding site of MM-CK and a direct involvement of regions others than those in the C-terminal half of MM-CK. Additional studies, using the in situ binding assay presented here to identify such regions, are currently underway. However, considering the conspicuous features of the M-260 box region, a functional role of this domain in the targeting of MM-CK to other subcellular sites, as stated in the introduction, is likely.

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