Isoenzyme-specific Interaction of Muscle-type Creatine Kinase with the Sarcomeric M-Line Is Mediated by NH$_2$-terminal Lysine Charge-Clamps

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Abstract. Creatine kinase (CK) is located in an isoenzyme-specific manner at subcellular sites of energy production and consumption. In muscle cells, the muscle-type CK isoform (MM-CK) specifically interacts with the sarcomeric M-line, while the highly homologous brain-type CK isoform (BB-CK) does not share this property. Sequence comparison revealed two pairs of lysine residues that are highly conserved in M-CK but are not present in B-CK. The role of these lysines in mediating M-line interaction was tested with a set of M-CK and B-CK point mutants and chimeras. We found that all four lysine residues are involved in the isoenzyme-specific M-line interaction, acting pair-wise as strong (K104/K115) and weak interaction sites (K8/K24). An exchange of these lysines in MM-CK led to a loss of M-line binding, whereas the introduction of the very same lysines into BB-CK led to a gain of function by transforming BB-CK into a fully competent M-line–binding protein. The role of the four lysines in MM-CK is discussed within the context of the recently solved x-ray structures of MM-CK and BB-CK.

Key words: creatine kinase • isoenzyme-specific association • sarcomeric M-line • electrophoretic mobility shift • muscle energetics

Introduction

Cells and tissues with intermittently high and fluctuating energy requirements, such as skeletal and cardiac muscle, brain, retina, and spermatozoa, depend on the immediate availability of large amounts of energy. In these cells, the enzyme creatine kinase (CK[1]; ATP, creatine *N*-phosphoryl transferase, EC 2.7.3.2) plays a key role in cellular energy metabolism (Wallimann et al., 1992) by replenishing ATP through the reversible transfer of the *N*-phosphoryl group from phosphocreatine (PCr) to ADP (Kenyon and Reed, 1983). Creatine kinases constitute a family of different oligomeric isoforms with tissue-specific expression and isoenzyme-specific subcellular localization. Three cytosolic isoforms, ubiquitous brain-type BB-CK, sarcomeric muscle-type MM-CK, and the MB-CK heterodimer (Eppenberger et al., 1967), as well as two mitochondrial isoforms, ubiquitous Mi$_a$-CK and sarcomeric Mi$_b$-CK, are synthesized in a tissue-specific manner (Schlegel et al., 1988,

1990). The cytosolic M-CK and B-CK subunits ($\sim$43 kD) combine to enzymatically active homo- and heterodimers, MM-CK, MB-CK, and BB-CK, whereas the mitochondrial protomer (Mi-CK) combines preferentially to homooctamers (Schlegel et al., 1988). In fully differentiated skeletal muscle, MM-CK is the predominant isoform, occurring in appreciable amounts together with mitochondrial Mi$_b$-CK. BB-CK is the more widely distributed ubiquitous isoform present in brain, smooth muscle, heart and a variety of other tissues (Eppenberger et al., 1967; Trask and Billadello, 1990). All three cytosolic isoforms coexist together only during myogenesis (Caravatti et al., 1979) and to some extent also in mammalian heart, while MB-CK and BB-CK are undetectable in mature skeletal muscle (Turner et al., 1973; Wallimann et al., 1977, 1983b). Biochemical fractionation (Wallimann et al., 1977, 1978) and in situ immunolocalization techniques (Wegmann et al., 1992) on skeletal and cardiac muscle have shown that cytosolic MM-CK is not evenly distributed within muscle cells. A small but significant amount of cytosolic MM-CK (5–10%, depending on the muscle fiber type and preparation) is specifically bound to the myofibrillar M-line, whereas the soluble main fraction can be extracted by buffers of physiological ionic strength (Turner et al., 1973). This property is unique to MM-CK and is not shared by BB-CK or the heterodimeric MB-CK (Wallimann et al.,

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1983b; Schäfer and Perriard, 1988; Stolz and Wallimann, 1998). The M-line–bound fraction of MM-CK is functionally coupled to the myofibrillar actin-activated Mg^{2+}-ATPase as an efficient intramyofibrillar ATP regenerator (Wallimann et al., 1984; Ventura-Clapier et al., 1994).

Beside its enzymatic function, immunoelectron microscopic data also suggest a structural role for the M-line–bound MM-CK, by the formation or association with the interlinking M4 (M4′) m-bridges (Strehler et al., 1983; Wallimann et al., 1983a), which are important for the structural assembly and stability of the myosin filaments within the myofibrillar filament lattice (Luther and Squire, 1978; Luther et al., 1981; Thornell et al., 1987). The fact that M-line–bound MM-CK can be extracted by an excess of specific high-affinity monovalent anti–MM-CK Fab fragments (Wallimann et al., 1978) and that rebinding of MM-CK needs intact thick filaments (Bähler et al., 1985) indicates that the interaction of MM-CK within the M-line, is of dynamic nature with moderate strength and requires a native supramolecular structure. Reconstitution of M-line–bound MM-CK by incubation of chemically skinned muscle fibers (Kraft et al., 1995) or extracted myofibrils with externally added MM-CK has been used before to localize MM-CK and to study its function in the M-line (Wallimann and Eppenberger, 1985). Recently, it was shown that the responsible region is located somewhere within the NH_2-terminal half of the MM-CK isoforms (Stolz and Wallimann, 1998). In the work presented here, we succeeded in characterizing the molecular interaction sites in more detail and identifying the key amino acid residues on the dimeric MM-CK molecule that are responsible for the interaction with the myofibrillar M-line structure. To study the M-line interaction, we used an in situ biochemical approach with single muscle fibers from which the sarcolemma membrane had been removed by detergent treatment (Stolz and Wallimann, 1998). In contrast to isolated myofibrils, these fibers are structurally and functionally intact. They show most of the sarcomeric superstructure and normal contraction and calcium regulation properties (Kraft et al., 1995). The incapacity of BB-CK to bind into the M-line, observed when added as extrinsic protein into skeletal muscle or when present during myogenesis (Wallimann et al., 1977, 1983b; Stolz et al., 1998), provided us with a suitable reference protein to evaluate the functional role of different CK domains and putative key residues for the isoenzyme-specific binding to sarcomeric subregions.

In this work we show that four highly conserved lysine residues, that is, two lysine pairs each are responsible and entirely sufficient for the isoenzyme-specific binding of the MM-CK dimer to the sarcomeric M-line.

Materials and Methods

Escherichia coli Strains, Plasmids, and DNA Manipulation

E. coli strain BL21 (DE3) pLysS and expression vector pET3b (Studier et al., 1990) were used as described earlier for mitochondrial CK (Furter et al., 1992) or M-and B-CK (Stolz and Wallimann, 1998). pRF5 is identical to pET3b except a deleted EcoRV-EcoRI fragment. *E. coli* XL1 blue (Bullock et al., 1987), media, and standard DNA manipulations were used as already described (Ausubel et al., 1994). The construction of plasmid pT17 containing the chicken M-CK cDNA (Kwiatkowski et al., 1984; Or-

dahl et al., 1984) and pT23 containing the chicken B_b-CK cDNA (Hossle et al., 1986) has also been described earlier (Stolz et al., 1998).

Site-directed Mutagenesis and Construction of CK Point Mutations

Polymerase chain reaction was used for site-directed mutagenesis (Kadowaki et al., 1989) of chicken M-CK and chicken B-CK. The site-specific mutations were introduced by using the inverse PCR method (Jones and Howard, 1990) and appropriate oligonucleotides that were 5′ phosphorylated. As template for the M-CK and B-CK mutants, plasmid pT17 and plasmid pT23, respectively, were used (Stolz and Wallimann, 1998). Site-directed mutagenesis was performed using Pfu DNA Polymerase (Stratagene) containing a 3′→5′ proof-reading ability to prevent further mutations during the PCR amplification. The DMSO concentration was varied between 0 and 10% to increase yield and specificity of the PCR reaction. The resulting PCR products were extracted by phenol/chloroform (Ausubel et al., 1994) and subsequently purified by agarose gels using the GENECLEAN KIT (Bio101 Inc.). The purified PCR products were self-ligated using T4 DNA ligase (FPLCpure; Amersham Pharmacia Biotech) and transformed into *E. coli* XL1 blue. All CK mutants were finally checked by DNA sequencing. A summary of the CK mutant constructs is given in Fig. 2.

Protein Sources and Fluorescently Labeled Antibodies

A mouse monoclonal antibody against the 185-kD M-line protein, myomesin, from chicken (Grove et al., 1984) was generated by standard protocols at the Institute of Cell Biology (ETH Zürich, Switzerland) and kindly provided by Drs. H.M. Eppenberger and J.C. Perriard. It was used at a 1:100 dilution for M-line reference labeling. Cyanin-5–conjugated goat anti–mouse IgG (The Jackson Laboratory; Dianova) was used as secondary antibody.

Chicken MM-CK, chicken BB-CK, and the mutant derivatives thereof were expressed in *E. coli* similar as described elsewhere (Furter et al., 1992). Active BB-CK and the B-CK–derived mutants could be purified out of the soluble fraction of the cell lysates in a two-step procedure similar to an earlier published protocol (Quest et al., 1989). In the first step, the crude cell extract was absorbed to Blue Sepharose CL6-B (Amersham Pharmacia Biotech) and equilibrated in loading buffer (50 mM sodium phosphate pH 5.8, 1 mM $MgCl_2$, 0.2 mM EGTA, and 1 mM β-mercaptoethanol) after having adjusted the pH to 5.8 with 1 M $NaH_2PO_4 \times H_2O$. After extensive washing with loading buffer the protein was eluted specifically from the affinity matrix with elution buffer (50 mM sodium phosphate, pH 8.5, 1 mM $MgCl_2$, 0.2 mM EGTA, 1 mM β-mercaptoethanol, and 10 mM ADP). The pooled peak fractions were concentrated by ultrafiltration and dialyzed against anion buffer (10 mM Tris-HCl, 10 mM bis-Tris-propane, and 0.5 mM β-mercaptoethanol, pH 8.5). The second purification step consisted of an anion-exchange chromatography on a Poros 20 HQ column (PerSeptive Biosystems Inc.) equilibrated with anion buffer, pH 8.5. Proteins were eluted with a linear NaCl gradient from 15 to 500 mM. The peak of wild-type BB-CK elutes at ∼150 mM NaCl. Peak fractions, containing CK activity, were pooled, concentrated by ultrafiltration, and stored in storage buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.2 mM NaCl, 0.2 mM EDTA, 2 mM β-mercaptoethanol, and 0.02% NaN_3) at 4°C.

In contrast to BB-CK and variants thereof, MM-CK and the M-CK–derived mutants were obtained by solubilization of the insoluble cell fractions in urea and subsequent refolding. In brief, the inclusion body containing pellet was washed three times or more with ice-cold lysis buffer (50 mM Tris-HCl pH 8.0, 10 mM EDTA, 50 mM NaCl, 15% [wt/vol] sucrose, and 1% Triton X-100) supplemented with 25 units Benzonase (Merck) by homogenizing the paste-like pellet with a glass rod. After sedimentation by centrifugation (25,000 *g*, 10 min, 4°C), the supernatant containing mainly contaminating proteins was removed and discarded. The washed pellet was then redissolved in ∼10 ml solubilization buffer (10 mM Tris-HCl, pH 8.0, 7 M urea, 5 mM EDTA, and 10 mM β-mercaptoethanol) by incubation overnight with slow stirring at 22°C. The solubilized inclusion bodies were then renatured by slow dilution into a 50-fold excess of renaturation buffer (50 mM Tris-HCl, pH 9.5, 150 mM NaCl, 10 mM EDTA, and 1 mM PMSF) at 22°C and subsequent incubation at 4°C overnight for further refolding of the protein. The renatured protein was then upconcentrated by a fractionated ammonium sulfate precipitation at 40%, pH 7.5, and 75%, pH 8.4, saturation, respectively. The 75% ammonium sulfate pellet containing the bulk CK amount was then dissolved in a small

amount of anion buffer, pH 9.5, and extensively dialyzed against the same buffer. The dialyzed protein was further purified by anion exchange chromatography on a Poros 20 HQ column (PerSeptive Biosystems Inc.) equilibrated with anion buffer, pH 9.5. Proteins were eluted with a linear NaCl gradient from 0 to 250 mM. The peak of wild-type MM-CK elutes at ~40 mM NaCl. Peak fractions containing CK activity were pooled, upconcentrated by ultrafiltration, and stored in storage buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.2 mM EDTA, 2 mM β-mercaptoethanol, and 0.02% NaN_3) at 4°C.

The specific CK activities of wild-type and mutant enzymes were assayed photometrically in the reverse reaction, using the Glu6P-DH/Hexokinase–coupled enzyme assay (Eisenthal and Danson, 1995) at room temperature (22°C). Protein concentrations were determined according to Bradford (Bradford, 1976) using BSA as standard.

Fluorescent Labeling of MM-CK, BB-CK, and the CK Mutants thereof

Specific labeling of CK wild-type and mutant proteins at their accessible sulfhydryl groups via cysteine residues was done with iodoacetamide-based fluorescent dyes, rhodamine iodoacetamide (Rhod-IA; Molecular Probes Inc.), or 5′ iodoactamide fluorescein (5′ IAF; Molecular Probes). In brief, 2–4 mg protein in CK storage buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.2 mM EDTA, and 2 mM β-mercaptoethanol) was incubated in the presence of 10 mM DTT for 1 h at 37°C to activate the cysteine sulfhydryl groups. Subsequently, DTT was removed and the buffer exchanged against 100 mM H_3BO_3, pH 8.5, 0.5 mM EDTA by gel filtration on a Sephadex G25 column. The protein was then concentrated using a CENTRICON 30 ultrafiltration unit (Amicon Corp.) to a final volume of 0.5 ml and incubated with a 2.2-fold molar excess of dye over the total content of cysteine residues for 1 h in the dark under gentle agitation. The labeling reaction was quenched by adding 1 mM DTT. Unconjugated fluorescent dye was removed and the buffer exchanged against CK storage buffer, using a second gel filtration step on a Sephadex G-25 column. The dye/protein ratio was evaluated spectrophotometrically at pH 7.9 (Simon and Taylor, 1986), using a molar extinction coefficient of 20,000 $M^{-1}cm^{-1}$ at 575 nm for Rhod-IA and 72,200 $M^{-1}cm^{-1}$ at 490 nm for 5′ IAF. E_{280nm} at 1 mg/ml was 0.88 and 0.83 for MM-CK and BB-CK, respectively.

Functional In Situ Binding Assay

All binding experiments were performed on chemically skinned single-muscle fibers of rabbit *psoas major* prepared according to Yu and Brenner (Yu and Brenner, 1989) and stored in chilled skinning solution (5 mM KH_2PO_4, 3 mM Mg acetate, 5 mM EGTA, 3 mM Na_2ATP, 50 mM CrP, 2 mM DTT, and 0.02% azide, pH 6.8, at 4°C) supplemented with a protease inhibitor cocktail (10 μM leupeptin, 10 μM pepstatin, 10 μM E64, 10 μM antipain, 1 μg/ml aprotinin, and 1 mM PMSF, all from Sigma-Aldrich). The fibers were mounted in a flat, self-made, flow-through chamber on a microscopic slide as described in detail earlier (Stolz et al., 1998). All subsequent washing steps, incubations and dilutions were done with LSR-buffer (10 mM imidazole, 2 mM $MgCl_2$, 3 mM EGTA, 2 mM DTT, 2 mM MgATPγS [Calbiochem], 0.25 mM p1,p5-di[adenosine.5′] pentaphosphate [adenylate kinase inhibitor; Boehringer Mannheim, Germany], 100 mM glucose, and 0.1 units hexokinase [Boehringer; Kraft et al., 1995]). The ionic strength was adjusted to 120 mM with potassium propionate and the pH stabilized to 6.8 at 25°C. Fluorescently labeled proteins were used in a concentration of 50–100 μg/ml in relaxing solution. To qualitatively compare the binding strength of the different CK constructs, we used a standardized binding protocol consisting of an initial binding phase of 5 min followed by three washing steps with 100 μl LSR-Buffer and 2-min pause between each step. The laser confocal system consisted of a Leica DM RBE fluorescence microscope and a Leica TCS NT confocal scanner unit.

Results

M-CK and B-CK Differ by Four Highly Conserved Lysines in the NH₂-terminal Region Responsible for the M-Line Binding

In a systematic quest to identify those epitopes of chicken MM-CK that are responsible for its specific M-line binding, genetically engineered M/B-CK chimeric constructs had been used previously. This approach led to the unambiguous identification of the M-line–binding epitope within a NH₂-terminal stretch from amino acids 1–234 (Stolz and Wallimann, 1998). The identified region was subsequently further characterized by the exchange of different NH₂-terminal regions between M-CK and B-CK. Two endogenous restriction sites, MscI at amino acid position 32 and BsaAI at amino acid position 134, present in both cytosolic CK isoforms, were used to subdivide the identified region. Analyzing the ability of the generated chimeras to interact with the M-line showed a strong binding of the construct containing the amino acid region 32–134 of M-CK (chi10, Figs. 2 and 4 B). Also a weak interaction could be observed with the chimera, containing the amino acids 1–32 of M-CK, whereas no binding was seen with the construct containing the M-CK stretch from amino acid 134–234 (data not shown; Stolz, 1997). The amino acid sequence comparison between M-CK and B-CK (Fig. 1) over a range of six animal species showed within the so identified region from amino acid 1–134, only a few highly conserved amino acid conversions between the M-type and the B-type. In three positions (8, 24, and 104) uncharged amino acids in B-CK are replaced by basic lysine residues in M-CK, whereas in one case an acidic glutamate (Q115) in B-CK is replaced also by a lysine (K115) in M-CK. Further conserved amino acid conversions can be found at position 45, where an acidic glutamate residue in M-CK is exchanged by uncharged residues in B-CK, as well as in position 93, where an uncharged polar residue in M-CK is replaced by an acidic glutamate in B-CK (Fig. 1). All other identified amino acid exchanges within this region are either conservative replacements or not well conserved across different species. The four highly conserved charged lysine residues, present in M-CK but not in B-CK, are therefore likely candidates for being directly involved in the specific M-line binding of the MM-CK isoenzyme.

K104 and K115 Are Responsible for the Isoenzyme-specific Mobility in SDS-PAGE

To test the role of the identified lysines in mediating the M-line binding of MM-CK, we created a set of CK point mutants (Fig. 2), in which the lysines of M-CK were replaced by the homologous B-CK amino acid residues and, vice versa, another set in which the M-CK–specific lysines were introduced into B-CK. Furthermore, we reconverted the chimeric M/B-CK construct (Fig. 2, chi10), which showed a strong M-line interaction by remutating the residues K104 and K115 to the homologous B-CK residues E104 and Q115 (Fig. 2, chi11). The different CK variants were heterologously expressed in *E. coli* purified to homogeneity (Fig. 3), as outlined in Materials and Methods.

The calculated molecular mass (M_r) and specific activities of the generated mutants are indicated in Fig. 2 and a SDS-PAGE of the purified mutants is seen in Fig. 3. It is a known phenomenon that the apparent molecular mass of the brain isoform (M_r 42.745 Da) on SDS-PAGE appears to be ~2 kD bigger than the calculated molecular mass, whereas the muscle isoform (M_r 43.202 Da) runs according to its calculated molecular mass (Caravatti et al., 1979). The analysis of the SDS-PAGE migration pattern of the

Figure 1

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                  8              24                45
MCk (dog)      1 PFGNTHNKFK LNYKPEEEYP DLTKHNNHMA KALTPEIYKK LRDKETPSGF TLDDVIQTGV  60
MCk (human)    1 PFGNTHNKFK LNYKPEEEYP DLSKHNNHMA KVLTLELYKK LRDKETPSGF TVDDVIQTGV  60
MCk (mouse)    1 PFGNTHNKFK LNYKPQEEYP DLSKHNNHMA KVLTPDLYNK LRDKETPSGF TLDDVIQTGV  60
MCk (rabbit)   1 PFGNTHNKYK LNYKSEEEYP DLSKHNNHMA KVLTPDLYKK LRDKETPSGF TLDDVIQTGV  60
MCk (rat)      1 PFGNTHNKFK LNYKSQEEYP DLSKHNNHMA KVLTPDLYNK LRDKETPSGF TLDDVIQTGV  60
MCk (chicken)  1 PFSSTHNKHK LKFSAEEEFP DLSKHNNHMA KVLTPELYKR LRDKETPSGF TLDDVIQTGV  60
BCk (chicken)  1 PFSNSHNLLK MKYSVDDEYP DLSVHNNHMA KVLTLDLYKK LRDRQTSSGF TLDDVIQTGV  60
BCk (rat)      1 PFSNSHNTQK LRFPAEDEFP DLSSHNNHMA KVLTPELYAE LRAKCTPSGF TLDDAIQTGV  60
BCk (rabbit)   1 PFSNTHNTLK LRFPAEDEFP DLSAHNNHMA KVLTPEMDAE LRAKSTPSGF TLDDVIQTGV  60
BCk (mouse)    1 PFSNSHNTQK LRFPAEDEFP DLSSHNNHMA KVLTPELYAE LRAKCTPSGF TLDDVIQTGV  60
BCk (human)    1 PFSNSHNALK LRFPAEDEFP DLSAHNNHMA KVLTPELYAE LRAKSTPSGF TLDDVIQTGV  60
BCk (dog)      1 PFSNSHNTLK LRFPAEDEFP DLSAHNNHMA KVLTPELYAE LRAKSTPSGF TLDDVIQTGV  60

                                    93        104        115
MCk (dog)     61 DNPGHPFIMT VGCVAGDEES YQVFKDLFDP IIQDRHGGYK PTDKHKTDLN HENLKGGDDL  120
MCk (human)   61 DNPGHPFIMT VGCVAGDEES YEVFKELFDP IISDRHGGYK PTDKHKTDLN HENLKGGDDL  120
MCk (mouse)   61 DNPGHPFIMT VGCVAGDEES YTVFKDLFDP IIQDRHGGYK PTDKHKTDLN HENLKGGDDL  120
MCk (rabbit)  61 DNPGHPFIMT VGCVAGDEES YTVFKDLFDP IIQDRHGGFK PTDKHKTDLN HENLKGGDDL  120
MCk (rat)     61 DNPGHPFIMT VGCVAGDEES YTVFKDLFDP IIQDRHGGFK PTDKHKTDLN HENLKGGDDL  120
MCk (chicken) 61 DNPGHPFIMT VGCVAGDEES YEVFKELFDP VIQDRHGGYK PTDKHKRTDLN HENLKGGDDL  120
BCk (chicken) 61 DNPGHPFIMT VGCVAGDEES YEVFKELFDP VIEDRHGGYK PTDEHKTDLN ADNLQGGDDL  120
BCk (rat)     61 DNPGHPYIMT VGAVAGDEES YDVFKDLFDP IIEDRHGGYQ PSDEHKTDLN PDNLQGGDDL  120
BCk (rabbit)  61 DNPGHPFIMT VGCVAGDEES YEAFKELFDP IIEDRHGGYK PSDEHKTDLN PDNLQGGDDL  120
BCk (mouse)   61 DNPGHPYIMT VGAVAGDEES YDVFKDLFDP IIEERHGGYQ PSDEHKTDLN PDNLQGGDDL  120
BCk (human)   61 DNPGHPYIMT VGCVAGDEES YEVFKDLFDP IIEDRHGGYK PSDEHKTDLN PDNLQGGDDL  120
BCk (dog)     61 DNPGHPYIMT VGCVAGDEES YDVFKELFDP IIEDRHGGYK PSDEHKTDLN PDNLQGGDDL  120
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Figure 1. Amino acid sequence comparison between the muscle isoform (M-CK) and the brain isoform (B-CK) over a range of the first 120 residues. This NH$_2$-terminal stretch that was identified to be responsible for the M-line binding of MM-CK. The CK sequences of six different animal species are compared. The highly conserved lysine residues in M-CK (K8/K24/K104 and K115) and the homologous less conserved amino acid residues at the corresponding positions of B-CK are outlined in gray, as well as residues E45 and Q93 which are also highly conserved in M-CK but differ in B-CK.

different mutant isoforms (Fig. 3) showed that this electrophoretic behavior is obviously dependent on the amino acid residues present in position 104 and 115. All mutant and chimeric constructs containing the M-CK lysines K104 and K115 showed an electrophoretic mobility similar to the M-CK wild-type, whereas the presence of the B-CK residues E104 and Q115 shifted the electrophoretic mobility to that of the B-CK isoform. The introduction of the lysine pair K8/K24 had no influence on the electrophoretic behavior.

The specific activities of the mutant CK constructs (Fig. 2) were comparable to the wild-type protein except for the M-CK mutant, mck3. This mutant protein showed a reduced specific activity of ~20% of the wild-type activity. Since this mutant, like all the other M-CK based mutants, was renatured from solubilized inclusion bodies, it can not

Figure 2

CK construct	calc.molec. weight	spec. activity (IU/mg)	M-line binding
M-CKwt	43,202 kDa	105	+++
B-CKwt	42,745 kDa	130	-
chi10	42,833 kDa	110	+++
chi11 (K104E K115Q)	42,861 kDa	91	+/-
mck1 (K8L K24V)	43,158 kDa	97	++
mck2 (K104E K115Q)	43,203 kDa	89	+
mck3 (K8L K24V K104E K115Q)	43,159 kDa	21	-
bck1 (L8K V24K)	42,773 kDa	107	-
bck2 (E104K Q115K)	42,744 kDa	120	++
bck3 (L8K V24K E104K Q115K)	42,772 kDa	94	+++

Figure 2. Summary of chimeric constructs and point mutations of MM-CK and BB-CK. The M-CK and B-CK derived parts are represented as hatched and open white bars, respectively. The isoenzyme-specific diagnostic box region (amino acids 258–270) is marked by a dark rectangle: M-260, highly conserved motive in M-CK; B-260, corresponding region in B-CK. Wild-type M-CK, mck1, mck2, and mck3 were purified from inclusion bodies. Wild-type B-CK, bck1, bck2, bck3, chi10, and chi11 could be purified from the cell lysate. The molecular mass was calculated according to the amino acid sequences. The intensity of the M-line fluorescence obtained with the different constructs after three washing steps is indicated: +++, very strong; ++, strong; +, weak; +/−, traces; −, none.
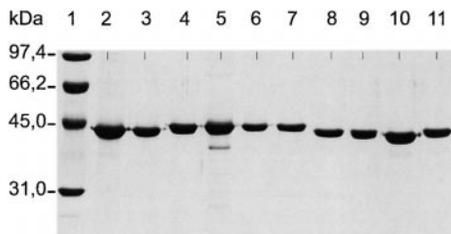
Figure 3. SDS-PAGE of heterologously expressed CK wild-type isoproteins, chimeras and point mutants. Individual fractions, each 2 μg of purified recombinant protein (lines 2–12) were separated on a 12% SDS–polyacrylamide gel and stained by Coomassie blue. The proteins were expressed in an inducible bacterial system, followed by purification either from inclusion bodies (lines 2 to 5) or as soluble protein from cell lysate (lines 6 to 11). Lane 1, molecular size markers; lane 2, wild-type chicken M-CK; lane 3, mck1; lane 4, mck2; lane 5, mck3; lane 6, wild-type chicken B-CK; lane 7, bck1; lane 8, bck2; lane 9, bck3; lane 10, chimera10; lane 11, chimera11.

be excluded that under the chosen conditions, the refolding of this particular mutant resulted in partly misfolded protein. However, the refolded mutant behaved similar to the active M-CK–based constructs on the subsequent purification steps.

In MM-CK, All Four Lysines Differing between M-CK and B-CK Are Responsible for Mediating the Full, Isoenzyme-specific Interaction with the M-Line

The purified chicken proteins were fluorescently labeled using a cysteine-specific fluorescent dye. The average labeling ratio of dye to the dimeric protein was 1.5–3.3 for the M-CK variants (8 cysteines) and 1.8–4.1 for the B-CK variants (10 cysteines). The capacity of the mutant proteins to bind to the sarcomeric M-line structure was tested with chemically skinned single muscle fibers using the established in situ binding assay (Kraft et al., 1995; Stolz et al., 1998). As a M-line marker, we used a monoclonal antibody against myomesin (Grove et al., 1984), a protein known to be exclusively present in the sarcomeric M-line. To qualitatively quantify the affinities of the different CK constructs we subjected each mutant to a standardized binding protocol, consisting of an initial binding phase, followed by three subsequent washing steps. The stainings that were obtained after this procedure with the different CK mutants are shown in Fig. 4.

The wild-type MM-CK protein displayed a strong M-line labeling, as it was expected and had also been shown earlier (Kraft et al., 1995; Stolz et al., 1998). Earlier work showed that this staining could be reverted by a following incubation with a large excess of unlabeled enzyme (Stolz and Wallimann, 1998) and demonstrated the absence of a specific M-line labeling with fluorescently labeled BB-CK. In contrast to this, the chimeric B-CK construct that contains the M-CK region from amino acids 32–124 (Fig. 2, chi10) showed a clean M-line–labeling pattern (Fig. 4 B), similar to that of wild-type MM-CK (Fig. 4 A). Both interactions were stable against further subsequent washing steps. This remarkable gain of function with a predominantly B-CK–like construct could be reverted by

exchanging the two lysines K104 and K115 within the M-CK–specific stretch to the original B-CK–specific residues E104 and Q115 (Fig. 2, chi11). This mutant protein did lose its M-line interaction capability almost completely (Fig. 4 C). However, a faint staining was still visible after three washing steps, suggesting a further M-line interaction site which is still present in this mutant. However, the interaction seems to be weak, since the remaining staining could be completely removed by a few further washing steps, whereas this was not the case with the wild-type protein or the chi10 construct. Both chimeric proteins showed an enzymatic activity similar to the wild-type protein (Fig. 2), so that major structural changes of the protein structure can be excluded.

To examine the role of the different conserved lysines in mediating the M-line interaction, the set of point mutants was tested (Fig. 4, D–I). The M-CK mutant in which the lysine pair (K8/K24) was converted to the corresponding B-CK residues (mck1) was still able to bind into the M-line (Fig. 4 D). However, the binding of this mutant was less intense compared with the wild-type protein and the pattern somewhat less sharp. Introducing this lysine pair (K8/K24) into B-CK led to a mutant which showed a faint M-line staining during the binding period, but was completely removed by the subsequent three washing steps (Fig. 4 G). The exchange of the second lysine pair (K104/K115) in M-CK, led to a mutant with a reduced but specific M-line binding (Fig. 4 E). This staining could also be washed out by further washing steps. The interaction seemed to be, however, stronger than that of the bck1 mutant (Fig. 4 G), which shares the same pattern of NH_2-terminal lysines (K8/K24). On the other hand, led the introduction of the very same lysine pair (K105/K115) into B-CK to a mutant with a strong capability to bind into the M-line (Fig. 4 H). This confirms the outstanding role of K105 and K115 in mediating the major part of the observed binding, a finding fully consistent with the binding of the chimeric M/B-CK constructs chi10 and chi11 (Fig. 4, B and C). In spite of the strong interaction, the staining pattern of bck2 appeared to be somewhat more diffuse. The whole picture was accomplished by introducing all four lysines examined here into B-CK (bck3). The observed M-line interaction was fully comparable to that of M-CK wild-type (Fig. 4 I) and also stable against further washing steps. Therefore, we conclude that all four lysines are necessary to convey the full MM-CK–like binding ability to the BB-CK protein. This was corroborated by the M-CK mutant (mck3) in which all four lysines were converted to the corresponding B-CK residues. No M-line binding could be observed with this mutant anymore (Fig. 4 F). Since this construct also shows a reduced specific activity, an alteration in the secondary structure cannot be excluded. However, the results obtained with this particular mutant, although fully supporting the general picture, are not crucial for the interpretation of our data. The much more convincing result is the gain of M-line binding capacity by upmutating the BB-CK isoform.

All Four Lysines Are Located on the Surface of the Protein and Are Exposed in a Prominent Manner

The recently solved three-dimensional x-ray structures of
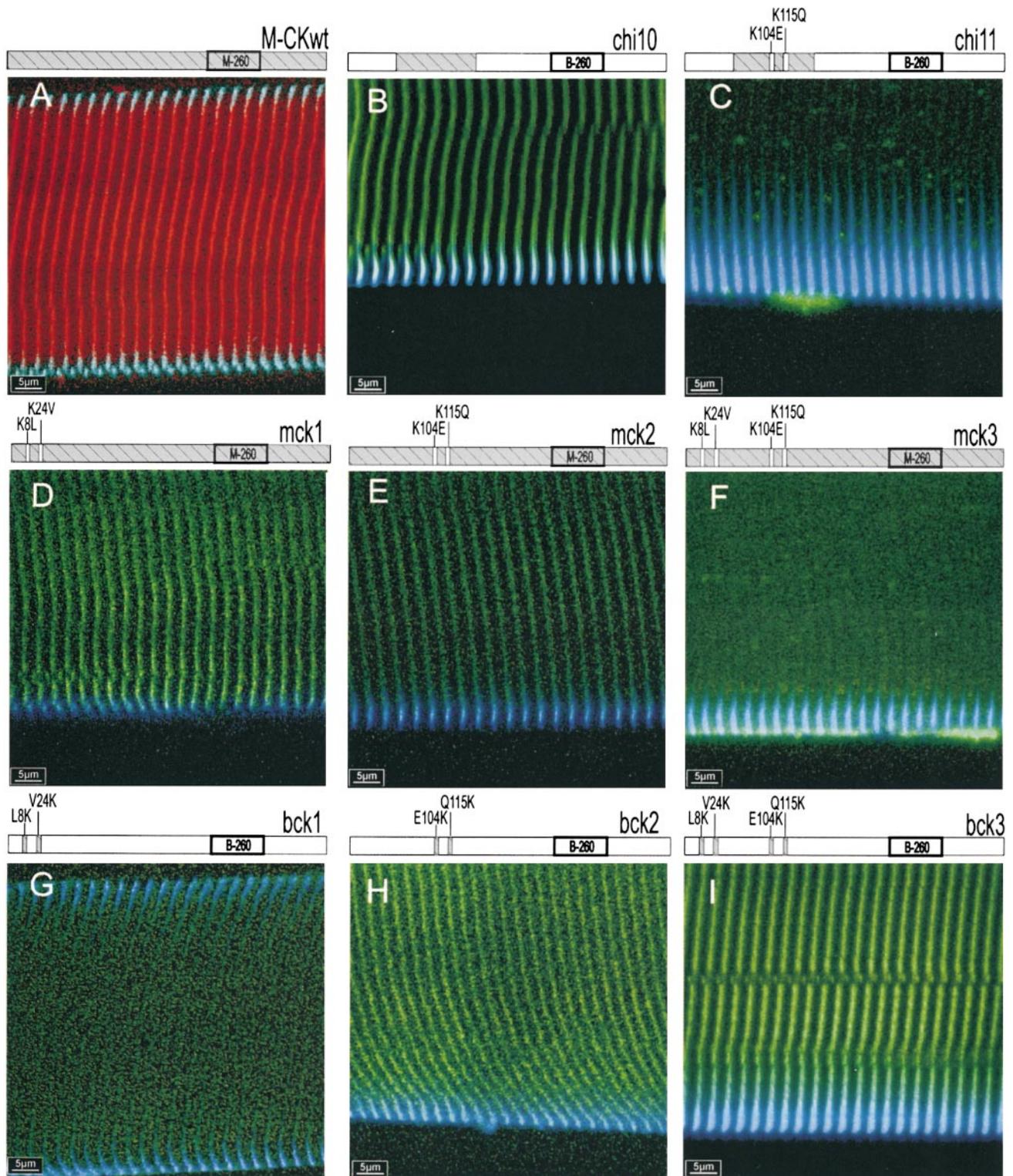
Figure 4. In situ labeling of the M-line by wild-type MM-CK and mutant constructs (Fig. 2) in unfixed, chemically skinned muscle fibers. Confocal images of longitudinal optical sections of muscle fibers after equilibration with fluorescently labeled protein (50 μg/ml) in LSR-buffer followed by three subsequent washing steps with LSR-buffer. For reference the staining pattern of a M-line–specific anti-myomesin antibody, visualized with a secondary cyanin-5–labeled antibody is overlaid. The schematic drawings on top of each panel depicts the corresponding construct: gray tinted areas, parts derived from M-CK; white areas, parts derived from B-CK; M-260, highly conserved motive in M-CK; B-260, corresponding region in B-CK. Fluorescence channels: green, 5′IAF-labeled CK variants; red, rhodamine-labeled wild-type MM-CK; blue, anti-myomesin antibody. The pictures show the binding pattern of (A) rhod-IA–labeled wild-type MM-CK; (B) chi10, a 5′IAF-labeled B-CK construct containing the high-affinity M-line binding region (amino acids 32–124) of M-CK; (C) chi11, a 5′IAF-labeled K104E/K115Q point mutant of chi10; (D) mck1, a 5′IAF-labeled K8L/K24V M-CK point mutant; (E) mck2, a 5′IAF-labeled K104E/K115Q M-CK point mutant; (F) mck3, a 5′IAF-labeled K8L/K24V/K104E/K115Q M-CK point mu-

rabbit MM-CK (Rao et al., 1998) and chicken BB-CK (Eder et al., 1999) are shown in Fig. 5. The structures (Fig. 5, A and B) show an extremely high similarity between the two isoenzymes. The topological position of the identified residues K8, K24, K105, and K115 in MM-CK (Fig. 5 A) are almost identical to the position of the corresponding residues L8, V24, E104, and Q115 in BB-CK (Fig. 5 B). In a spacefill representation of the structures (not shown) it is clearly visible that all identified residues are positioned on the surface of the protein. The lysines K104/K115 are pairwise symmetrically located in a surface-exposed loop at the two very ends of the dimeric CK structure (Fig. 5 A). The arrangement of the two lysine pairs at the opposing ends of the dimeric enzyme forms a charge-clamp–like conformation. Interestingly, the temperature factors determined for this region indicate a much higher flexibility in MM-CK compared with that of BB-CK.

The other lysine pair (K8/K24) is located closer to the monomer–monomer interface. K8 is exposed at the back of the protein and is also easily accessible for protein interactions, whereas K24 is located more in the bend region of the protein but should also be accessible for larger molecules from the side. Due to the dimeric structure of MM-CK, the two lysines K8 of each monomer are located close to each other and form a positively charged patch at the exposed back of the dimer, which seems also suitable for being involved in mediating protein–protein interactions.

Discussion

MM-CK and BB-CK isoenzymes have similar biochemical properties and enzyme kinetics (Stolz and Wallimann, 1998). Within the same isoform group of MM-CK and BB-CK, amino acid sequence homology is as high as 98 and 95%, respectively, across vertebrate species. Between MM-and BB-CK, the homology is ~80%, which is still very high (Mühlebach et al., 1994). However, both isoenzymes are expressed in a tissue-specific manner and show a very distinct intracellular targeting to specific sites within subcellular structures (Wallimann et al., 1983b). MM-CK is specifically bound to the myofibrillar M-line structure, whereas neither BB-CK nor the heterodimeric MB-CK binds into this sarcomeric region (Wallimann et al., 1983b; Stolz and Wallimann, 1998). Sequence comparison between M-CK and B-CK reveals only a few residues that are conserved among vertebrates and differ between the two cytosolic isoforms. The COOH-terminal half of M-CK containing a prominent and highly conserved distinct region between residues 235–285, the so-called isoenzyme-specific diagnostic box, was reported earlier to be responsible for M-line binding (Schäfer and Perriard, 1988). Later work, however, allocated the relevant myofibrillar-binding epitope of MM-CK unambiguously within the NH₂-terminal part (amino acids 1–234; Stolz and Wallimann, 1998). By further subdividing this region we could

identify a stretch from amino acid 1–134, containing two regions that seemed to be responsible for the M-line interaction. The first region is located in a stretch from amino acids 1–32, mediating a rather weak interaction with the M-line since the bound protein could be washed out easily by further washing steps. The second region is located in the stretch from amino acids 33–134 and mediates a strong interaction with the M-line (Fig. 4 B) which was resistant against subsequent washing steps. Comparing the amino acid sequence of M-CK and B-CK within this region over many different species (Fig. 1), we identified two pairs of highly conserved lysine residues in M-CK, not present in B-CK. These lysines turned out to be necessary and completely sufficient to mediate the isoenzyme-specific binding of CK into the M-line structure. The mutation of these lysine residues in M-CK led to a loss of myofibrillar binding function. Conversely, a gain of specific function by introducing these very four M-CK–specific lysines into the BB-CK isoenzyme could be demonstrated. This latter BB-CK mutant (bck3) displayed the full characteristics of a genuine M-line–binding protein. Due to their differential resistance of the mediated interaction against further washing steps, the regions containing the K104/K115 and the K8/K24 lysine pairs can be attributed to a strong and a weak M-line interaction domain of MM-CK, respectively.

The x-ray structure of MM-CK (Fig. 5 A) shows that the sites K104 and K115 are located in an exposed loop at the two ends of the dimeric enzyme forming two symmetrical, opposite, positively charged anchorage sites. The presence of two symmetrical, charge-clamp–like interaction sites seems to be crucial for a proper M-line interaction since the MB-CK heterodimer, exposing only one interaction site per dimer, is not able to bind into the M-line (Wallimann et al., 1983b). The obvious necessity of two binding sites exposed at both ends of the protein fits well into the proposed model of MM-CK, to form a protein interlinking crossbridge within the M-line of the sarcomeric muscle (Wallimann and Eppenberger, 1985). It was observed that K104 and K115 are also responsible for the isoenzyme-specific mobility on the SDS-PAGE. This might be explained by a changed hydrodynamic diameter of the unfolded polypeptide, influencing the separation due to molecular sieving effects in the polyacrylamide gel matrix (Nielsen and Reynolds, 1978). In this context it is interesting that the temperature factors in the x-ray structure of MM-CK indicate a significantly enhanced flexibility of the exposed loop containing the lysines (K105/K115) compared with the same region in BB-CK. Although the flexibility of a peptide chain in the crystal packing does not necessarily reflect the situation in solution, it is possible that these loops show also an enhanced flexibility under soluble conditions. The latter could lead to the observed lower apparent M_r in SDS-PAGE since a flexible peptide chain would migrate more easily through the gel matrix than a more rigid one. The enhanced flexibility could con-

tant; (G) bck1, a 5′IAF-labeled L8K/V24K B-CK point mutant; (H) bck2, a 5′IAF-labeled E104K/Q115K B-CK point mutant; (I) bck3, 5′IAF-labeled L8K/V24K/E104K/Q115K B-CK point mutant. Note that introducing the two high-affinity M-line interaction mediator lysines K104 and K115 into wild-type BB-CK renders this BB-CK mutant to bind specifically to the sarcomeric M-line in a gain of function mode (H). The same holds true for the four-lysine BB-CK mutant (I) turning this variant into an even more potent M-line binding protein.
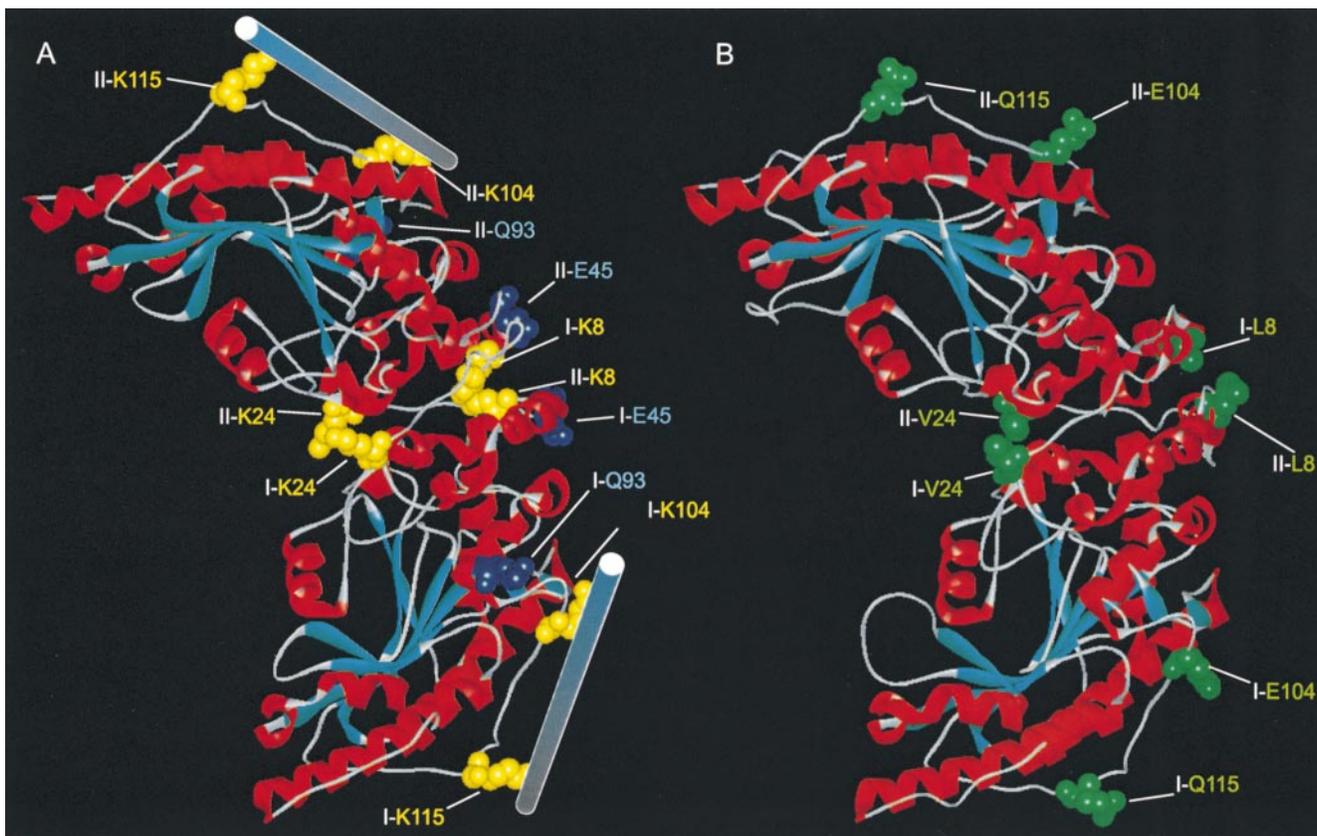
Figure 5. A side view onto the recently solved x-ray structure of the (A) rabbit MM-CK isoenzyme dimer (Rao et al., 1998) and the (B) chicken BB-CK isoenzyme dimer (Eder et al., 1999). The MM-CK dimer was generated from the monomeric data set by using the diagonal two-fold axis in the (001) plane according to (Rao et al., 1998). Since residues 1–7 could not be solved in the MM-CK structure we decided to omit the start methionine and to renumber the residues according to the BB-CK nomenclature for better comparability. The secondary structure is shown in a cartoon representation (α-helix structures are shown in red; β-sheet structures are shown in blue). The two monomeric chains of a dimer are marked by I and II. The lysine residues K8, K24, K104, K115, which have been identified to be responsible for the isoenzyme-specific interaction of MM-CK with the M-line (see Results) are shown in yellow (spacefill mode) in the MM-CK structure (A) and the homologous residues in the BB-CK isoform L8, V24, E104, and Q115 are depicted in green (spacefill mode) in the BB-CK structure (B). The residues E45 and Q93, which are also highly conserved and might contribute to the binding in a minor way are shown in blue. MM-CK and BB-CK isoenzymes display an extremely high overall structural similarity. The identified residues show the same topology in both isoforms and differ only in the type of amino acid. Clearly visible are the high-affinity binding residues K104, K115 of MM-CK (A) corresponding to E104, Q115 in B-CK (B). They are located in a flexible loop, symmetrically arranged at both ends of the dimeric enzyme consisting of monomers I and II. The topology of this distal positive lysine "charge-clamp" in MM-CK seems to be well suited to form two opposing interaction sites with two identical or differing partner molecules, which might be sandwiched together via one MM-CK dimer. The proposed structural role of the MM-CK dimers as linker molecules is demonstrated schematically by connecting the two rods (A). Also visible is the close, neighboring position of the two K8 residues in the dimeric MM-CK (I-K8, II-K8). These form a well accessible, charged patch which could also serve as an additional anchor site and thus is likely to be involved in forming the low-affinity site identified in this work. The two residues I-K24 and II-K24 are located more in the bend region and protrude out sideward on each side of the dimeric enzyme. The images were created with the program WebLab Viewer V3.0 (Molecular Simulations, Inc.).

tribute, besides the introduced charges, additionally to the interaction of MM-CK with the M-line, either by an improved accessibility of the loop region or by enabling a specific structural conformation upon binding to the M-line.

Whereas the outstanding role of the lysines K104/K115 in mediating the M-line interaction could be clearly demonstrated, the exact role of the lysines K8/K24 remains less clear. The introduction of this pair into B-CK led to a mutant that interacted only weakly with the M-line and could be washed out easily by the following washing steps (Fig. 4 G). On the other hand, the mutant mck2, which lacks the lysines K104/K115 but contains K8/K24, showed a faint but specific staining of the M-line. This observation suggests that besides the identified lysine residues some additional residues might contribute to the binding in a minor way. This was also supported by the mutant chi11, which lacks the four lysines but still shows a very weak interaction. Further candidates for being possibly involved in the binding are the additionally conserved amino acids E45 and Q93 (Fig. 5). It is also possible that slight structural differences between BB-CK and MM-CK, which could not fully be mimicked by the introduction of the lysines, contribute to the binding.

The M-line region is a complex structure that transverses the center of the A-band in cross-striated muscle (Luther and Squire, 1978). It appears to be the only myofibrillar structure that connects thick filaments directly to each other, providing physical stability between thick filaments during contraction. In electron micrographs of striated muscle, the M-line appears to be made up of several transverse elements connecting the thick filaments through the bare zone region and gives rise to the typical hexagonal thick filament lattice (Franzini-Armstrong and Porter, 1964). Ultrathin frozen sections of muscle show up to nine symmetrically arranged transverse elements (M1-M9 and M1′-M9′) in each half of the M-line structure (Thornell and Sjöström, 1975; Sjöström and Squire, 1977; Carlsson et al., 1990). Immunoelectron microscopic data suggest that MM-CK is part or associated with the prominent M4 and M4′ cross-bridges, also visible by conventional electron microscopy (Wallimann et al., 1983a; Wallimann and Eppenberger, 1985). This is consistent with the observation of a loss of these bands after low salt treatment or the use of high-affinity anti-M-CK Fab, which parallels the concomitant extraction of the M-line bound MM-CK (Turner et al., 1973; Wallimann et al., 1977). Electron micrographs of transverse sections of the M-line zone show an alternating arrangement of thick filaments and m-filaments with a distance of 100–160 nm depending on the sarcomere length (Luther et al., 1981). The dimeric MM-CK molecule (92×42 nm) is just the size to form half of a M4 or M4′ m-bridge structure, interlinking a thick filament with an m-filament. However, this size would not be sufficient in a contracted sarcomere. Thus, a dynamic rearrangement of m-bridges, depending on the contraction state, is likely to take place in working muscle. Up till now, five components of the sarcomeric M-line structure have been identified unambiguously so far. These are, besides the bare zone of the myosin rods, the 185-kD protein myomesin (Grove et al., 1984), the 250-kD COOH-terminal region of titin (Obermann et al., 1996), and the 165-kD M-protein (Trinick and Lowey, 1977), as well as MM-CK (Turner et al., 1973). Furthermore, recent findings indicate that the glycolytic enzyme β-enolase is also bound to the M-line structure and could be chemically cross-linked to MM-CK (Foucault et al., 1999). Additional M-line components might still be unidentified. The creation of specific gain and loss of M-line–binding mutants of CK isoenzymes should provide us with an optimal tool to identify the specific interaction partner(s) and structural position of MM-CK within the complex M-line structure.

Although the most consistent feature in all the different main fiber types of vertebrates is the marked density at the M4 and M4′ cross striations, the exact structural role of MM-CK within the M-line remains to be determined. On the level of conventional electron microscopy, M-CK knockout mice did not show an obviously altered sarcomeric structure on the ultrastructural level, but showed a physiological relevant phenotype (van Deursen et al., 1993). However, the high conservation of the lysine residues identified in M-CK, in contrast to B-CK indicates an evolutionary advantage of conveying to MM-CK, but not BB-CK, the ability to specifically bind into the M-line structure. It was demonstrated earlier that a proper isoenzyme-specific intracellular localization is crucial for maintaining the full cellular functionality (Wojtas et al., 1997). Besides a possible structural role, the enzymatic role of MM-CK to regenerate ATP at sites of high energy consumption and thus providing nearby located myosin ATPases with sufficient ATP to work even under strenuous conditions (Wallimann and Eppenberger, 1985; Wallimann et al., 1992; Ventura-Clapier et al., 1994; Veksler et al., 1997), might be the critical physiological advantage for muscle function, based on the isoenzyme-specific interaction of MM-CK with the sarcomeric M-line.

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