Regulation of α-Helical Coiled-coil Dimerization in Chicken Skeletal Muscle Light Meromyosin*

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The dimerization specificity of the light meromyosin (LMM) domain of chicken neonatal and adult myosin isoforms was analyzed by metal chelation chromatography. Our results show that neonatal and adult LMMs associate preferentially, although not exclusively, as homodimeric coiled-coils. Using chimeric LMM constructs combining neonatal and adult sequences, we observed that a stretch of 183 amino acids of sequence identity at the N terminus of the LMM was sufficient to allow the adult LMM to dimerize in a non-selective manner. In contrast, sequence identity in the remaining C-terminal 465 amino acids had only a modest effect on the dimerization selectivity of the adult isoform. Sequence identity at the N terminus also promoted dimerization of the neonatal LMM to a greater degree than sequence identity at the C terminus. However, the N terminus had only a partial effect on the dimerization specificity of the neonatal sequence, and residues distributed throughout the LMM were capable of affecting dimerization selectivity of this isoform. These results indicated that dimerization preference of the neonatal and adult isoforms was affected to a different extent by sequence identity at a given region of the LMM.

The two heavy chains (MyHC) of a myosin molecule dimerize through their C-terminal 1098 amino acids, forming an α-helical coiled-coil structure known as myosin rod. In all vertebrates, sarcomeric MyHCs are represented by multiple isoforms that are differentially expressed at various developmental stages and in different muscle fiber types (1, 2). Frequently, several MyHC isoforms are expressed in a single muscle cell (3–5), giving rise to the possibility of these heavy chains associating as either homo- or heterodimeric myosin molecules.

At least five fast MyHC isoforms are alternatively expressed in developing chicken fast skeletal muscle (6–10), and it has been shown that at stages when either embryonic and neonatal isoforms or neonatal and adult isoforms are being simultaneously expressed in the same muscle fiber, chicken myosin molecules are found as dimers of identical MyHC isoforms (11). However, dimerization selectivity at the C terminus. However, the N terminus had only a partial effect on the dimerization specificity of the neonatal sequence, and residues distributed throughout the LMM were capable of affecting dimerization selectivity of this isoform. These results indicated that dimerization preference of the neonatal and adult isoforms was affected to a different extent by sequence identity at a given region of the LMM.

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The amino acid sequence of the rod follows the seven-amino acid repeat (abcdefg), characteristic of α-helical coiled-coil proteins, in which positions a and d are mostly occupied by hydrophobic residues and charged residues are often found at positions e and g (13, 14). Experiments with model peptides have shown that hydrophobicity and packing effects of residues at a and d positions affect the stability and oligomerization state of the coiled-coil (15–19), while electrostatic interactions appear to control dimerization specificity and chain orientation (parallel versus antiparallel) (20). A large body of experimental evidence implicates charged amino acids at g and e positions in the regulation of dimerization specificity of Fos and Jun and other leucine zipper proteins (21–28). Besides interhelical ionic interactions between g and e residues, general unfavorable electrostatic effects in the dimer interface have been suggested to modulate preferential heterodimerization of coiled-coil proteins (24, 29, 30). However, it is not clear if these interhelical electrostatic interactions control the association of peptide sequences that preferentially form homodimeric coiled-coils (31).

Despite the extensive work on dimerization specificity of leucine zippers and model coiled-coils, little is known about the molecular mechanism that controls myosin dimerization specificity. The ability of two isoforms to form heterodimeric myosin molecules has been correlated with sequence conservation; therefore, it has been suggested that sequence homology controls dimerization specificity (11). However, the amino acid sequences of chicken myosins are highly conserved (>95% identical) (32), and yet they do not form stable heterodimeric coiled-coils.

To investigate the sequence elements that determine the preference of chicken myosin isoforms to associate as homodimers, we have analyzed the dimerization of the chicken LMM domain, a rod fragment encompassing the C-terminal 650 amino acids of the MyHC. Recombinant LMM constructs were expressed with or without a polyhistidine tag at the N terminus, and their ability to dimerize was analyzed by metal chelation chromatography. Homo- and heterodimers between histidine-tagged and untagged LMM constructs could be fractionated based on the different affinity exhibited by LMM dimers containing zero, one or two histidine-tagged strands for binding to the Ni-NTA resin. Our results showed that denatured neonatal and adult LMMs could refold into heterodimeric coiled-coils although they preferentially associated as homodimeric molecules. The contribution of the amino acid differences present in the LMM region of these isoforms to dimerization selectivity was studied by construction of chimeric proteins combining neonatal and adult sequences. Our results indicated that, although sequence homology indeed stabilized the dimer, not all amino acid differences had an equivalent
effect on heterodimer stability. Sequence identity at the N terminus of the LMM had a greater effect on dimer stability than sequence identity at the C terminus. In addition, our experiments indicated that dimerization selectivity of the neonatal and adult isoforms was affected differently by sequence identity at a given region of the LMM.

**MATERIALS AND METHODS**

**Cloning of Recombinant LMMs**—Recombinant LMMs were expressed using the T7 RNA polymerase-based (33) ampicillin-resistant pET expression vectors (Novagen, Madison, WI).

**Neonatal and Adult LMM Clones**—cDNA clones encoding the rod sequences of chicken neonatal and adult MyHC isoforms have been previously characterized (9). The cDNA clone E1 (9) was digested with *SspI*, *DraI*, and *EcoRI* restriction enzymes, and the *SspI*-EcoRI 2-kilobase pair fragment coding for the neonatal LMM sequence subcloned into pBluescript vector (digested with *SmaI* and *EcoRI*) resulting in the LNbs clone. The 2-kilobase pair BamHI-EcoRI fragment of LNbs clone was ligated into the pET-5c expression vector (Novagen). The resulting clone, termed pET-5cNeoLMM, encoded the 648 C-terminal amino acids of the chicken neonatal LMM and at its N terminus contained 15 amino acids (MASMTGGNNMGRIPH) translated from vector sequences. An analogous strategy was used to subclone the adult LMM from clone AA-4 (9) resulting in the clone pET-5cAdLMM. These clones were used to express neonatal (Neo) and adult (Ad) LMM constructs.

**Histidine-tagged LMM Clones**—The 2-kilobase pair BamHI-EcoRI insert of either pET-5cNeoLMM or pET-5cAdultLMM, coding for the LMM sequence of the neonatal or adult MyHC isoforms, respectively, was subcloned into the pET-15b vector (Novagen). Polymerase chain reaction was used to delete one nucleotide at the 5' end of the insert in order to correct the reading frame of the insert. The sequence of the forward primer containing the deletion was 5'-GTCGAGAATCCCATCACGCGAAGC-3'. The reverse primer within the LMM sequence was 5'-AGGGCTCCATCCATCTGCTAGT-3'. The polymerase chain reaction-amplified region was subcloned as a BamHI-StuI fragment into the parental vector, replacing the corresponding wild-type BamHI-StuI fragment. The resulting clones were termed pET-15b-NeoLMM-StuI and pET-15b-AdultLMM-StuI, and were used to express histidine-tagged neonatal (HNeo) and histidine-tagged adult (HAD) LMMs, respectively. DNA sequencing confirmed the reading frame change and the absence of additional mutations. The N-terminal 25 amino acids of the fusion proteins (MGSSHHHHHHHHSSGLVPRGSHHGDP) were expressed by vector sequences and included a hexahistidine tag flanked by a thrombin proteolytic site.

**Chimeric LMM Clones**—Chimeric LMM proteins A386, A278, and A183 were constructed by substituting 386, 278, or 183 amino acids at the N terminus of the neonatal LMM with the corresponding adult LMM sequences. Plasmids pET5c-NeoLMM and pET5c-AdLMM were digested with BamHI and either Neol, StuI, or Stal restriction enzymes. BamHI-NeoI (1158 base pairs), BamHI-StuI (384 base pairs), and BamHI-StalI (549 base pairs) fragments of the adult LMM sequence were ligated to the double-digested pET5c-NeoLMM plasmid to substitute the corresponding neonatal BamHI-NeoI, BamHI-StalI, or BamHI-StuI fragments. An analogous strategy was used to produce construct N183. N386 was constructed by replacing the StalI-NeoI fragment at the 3' end of the neonatal sequence in pET5c-NeoLMM with the corresponding adult sequence. The correct clones were identified by their restriction digestion patterns.

**Expression and Purification of Recombinant LMMs**—BL21(DE3)pLysS E. coli cells (33) transformed with recombinant pET plasmids were cultured according to manufacturer's directions (Novagen). The bacterial cultures were harvested 3 hours after induction, centrifuged at 6000 × g for 10 minutes in a GSA rotor, and the pellet stored at −80 °C. The frozen bacterial pellets were thawed on ice, resuspended in low salt buffer (20 mM KCl, 2 mM KH₂PO₄, 1 mM EDTA, pH 6.8), 10 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride in 1/40 of the volume of the bacterial culture, and sonicated on ice to decrease viscosity. The LMM proteins were purified according to the method previously described to purify myosin from chicken muscle (34), and further purified by gel permeation chromatography under denaturing conditions.

**LMM Denaturation and Renaturation**—The LMM preparations were denatured by dialysis against 4 M GdmCl, 40 mM NaPP₃, pH 7.5, 10 mM DTT, centrifuged (14,000 × g, 15 minutes, 4 °C), and the supernatant loaded onto a Bio-Gel A-1.5m (Bio-Rad) column (20 × 50 cm) equilibrated with the same buffer. The column was eluted with the equilibration buffer at 10 ml/h at room temperature. The fractions with the highest degree of homogeneity were pooled, renatured, and stored at −20 °C in 80 mM NaPP₃, pH 7.5, 10 mM DTT, 50% glycerol.

LMM constructs containing a polyhistidine tag at the N terminus were purified by Ni-NTA chromatography under denaturing conditions. The procedure was similar to the one described below for Ni-NTA column except that 4 M GdmCl was added to all the buffers and that after washing with 10 mM imidazole buffer, the column was eluted with 100 mM imidazole. The eluted fractions were pooled, concentrated, and further purified by gel permeation chromatography under denaturing conditions.

**Determination of Protein Concentration**—Concentration of protein preparations was estimated by scanning densitometry (Pharmacia LKB, Bromma, Sweden) of Coomassie Blue-stained SDS-PAGE gels. Increasing amounts of the protein preparations were loaded, and the slope obtained for each protein preparation was compared with the slope of a standard LMM preparation of known concentration. The concentration of the standard had been estimated by lyophilization of the protein and resuspension to a known concentration.

**Circular Dichroism**—Circular dichroism measurements were carried out on a Jasco-700 spectropolarimeter (Jasco Inc., Easton, MD) at 25 °C under nitrogen flush, in a 0.1-mm cell, with protein concentration of 0.1–0.4 mg/ml. The instrument was calibrated with ammonium d(+)-10-camphor sulphonate at 290.5 nm. CD spectra were the average of five scans obtained by collecting data at 10 nm/min, with a time constant of 2 min. The mean residue molar ellipticity ([θ]°) was calculated as described by Zhou et al. (35). Denaturation curves of the recombinant LMMs were obtained at 25 °C by following the decrease in ellipticity at 222 nm in the presence of increasing concentrations of GdmCl, as described by Pace et al. (36). The molarity of the GdmCl solutions was determined by refractive index measurements as described by Nozaki (37). Parameters of the unfolding reaction were calculated as described by Pace et al. (36).

**Polyacrylamide Gel Electrophoresis**—Chicken dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (38). Native gel electrophoresis was carried out on 1.5-mm slab gels according to the method of Waller and Lowey (39).

**LMM Denaturation and Renaturation**—Equal amounts of two protein preparations (10 μM NaPP₃, buffer (1–1.5 mg of total protein), only one of which contained a polyhistidine tag, were mixed and denatured by addition of denaturant buffer (8 M GdmCl, 40 mM NaPP₃, pH 7.5, 10 mM DTT) to a final 5 M GdmCl concentration. Protein concentration was adjusted to 1 mg/ml and, after incubation at room temperature for 2 hours, the preparation was renatured by dialysis in 40 mM NaPP₃, pH 7.5, 10 mM DTT for 4 h at 4 °C. The renatured samples were dialyzed overnight against Ni-NTA loading buffer (10 mM imidazole, 0.5 M NaCl, 0.4 M GdmCl, pH 7.8, 5 mM β-mercaptoethanol) at 4 °C. After centrifugation (10,000 × g for 30 min at 4 °C), the sample was loaded onto a Ni-NTA column.

**Ni-NTA Column chromatography**—The renatured sample equilibrated in the Ni-NTA loading buffer was loaded onto 0.8 ml of packed Ni-NTA resin. All exchange experiments were carried out with Ni-NTA resin (Quigaen, lot nos. R9419 and RA97008). The column was run in a continuous flow mode at 10 ml/h. The loading buffer was 10 mM imidazole, 0.5 M NaCl, 0.4 M GdmCl, 5 mM β-mercaptoethanol, pH 7.8. The column was washed with 12 column volumes of loading buffer and eluted with 18 column volumes of 50 mM imidazole buffer (50 mM imidazole, 0.5 M NaCl, 0.4 M GdmCl, pH 7.8, 5 mM β-mercaptoethanol) at 4 °C. After centrifugation (10,000 × g for 30 min at 4 °C), the sample was loaded onto a Ni-NTA column.

**LMM Chromatography**—The renatured sample equilibrated in the Ni-NTA loading buffer was loaded onto 0.8 ml of packed Ni-NTA resin. All exchange experiments were carried out with Ni-NTA resin (Quigaen, lot nos. R9419 and RA97008). The column was run in a continuous flow mode at 10 ml/h. The loading buffer was 10 mM imidazole, 0.5 M NaCl, 0.4 M GdmCl, 5 mM β-mercaptoethanol, pH 7.8. The column was washed with 12 column volumes of loading buffer and eluted with 18 column volumes of 50 mM imidazole buffer (50 mM imidazole, 0.5 M NaCl, 0.4 M GdmCl, pH 7.8, 5 mM β-mercaptoethanol) at 4 °C. After centrifugation (10,000 × g for 30 min at 4 °C), the sample was loaded onto a Ni-NTA column.
RESULTS

Expression, Purification, and Structural Characterization of LMM Constructs—Recombinant LMM sequences encompassing the C-terminal 648 amino acids of the chicken fast neonatal and adult isoforms were expressed either with or without a hexahistidine tag at the N terminus (Fig. 1), and purified as described under "Materials and Methods." Fig. 2A shows samples of these preparations analyzed by SDS-PAGE. The proteins were estimated to be >95% homogeneous by scanning densitometry.

Non-denaturing electrophoresis and Circular Dichroism analysis of the recombinant LMM preparations were consistent with these constructs being stabilized in a dimeric coiled-coil conformation. The mobility of the recombinant LMMs on NDE gels was intermediate between that of the rod and the LMM fragments obtained by chymotryptic digestion of native myosin molecules (Fig. 2B). This was expected for dimers of these constructs since their $M_r$ was higher than that of LMM fragments obtained by proteolysis (Fig. 2A). The CD spectra of the recombinant LMM preparations presented a double minima at 222 and 208 nm as well as a maximum close to 190 nm. The high molar ellipticity value at 222 nm indicated that the protein was predominantly $\alpha$-helical (Fig. 3A). The molar ellipticity ratio was greater than 1 ($[\theta]_{222}/[\theta]_{208} = 1.044$), which has been described to be characteristic of $\alpha$-helical coiled-coils (18).

In the dimerization experiments described in this report, strand exchange between LMM constructs was promoted by denaturant-mediated unfolding of the coiled-coil followed by refolding upon removal of the denaturing agent. As shown in Fig. 3B, GdmCl concentrations below 1 M had no effect on the folding state of the LMM, whereas the unfolding was practically complete above 2 M GdmCl. The transition midpoint was at about 1.5 M GdmCl ($([\text{GdmCl}])_{c}$) (Table I). The conformational stability of the LMM constructs in the absence of denaturant (Table I) was estimated assuming a two-state transition, as described by Pace et al. (36). No significant differences were found in the denaturation of the Neo, Ad, and HNeo LMM constructs.

Analysis of Dimerization between Histidine-tagged and Untagged LMM Constructs by Ni-NTA Chromatography—The hexahistidine tag confers upon the LMM constructs the ability to selectively bind to the Ni-NTA resin. In the presence of 10 mM imidazole, untagged LMMs do not bind to the column and elute in the wash fraction. In contrast, histidine-tagged LMMs bind to the column at 10 mM imidazole and elute at 80–100 mM imidazole. This different affinity is shown in Fig. 4A, where a mixture of HNeo and Ad LMMs was fractionated by the metal chelation column into two protein components.

When the histidine-tagged and untagged constructs were denatured together and then renatured prior to loading, three protein peaks were recovered from the column, indicating the presence of a new protein species of intermediate affinity (Fig. 4B and C). The fact that this new species, eluting in the 50 mM imidazole fraction, was recovered only when strand exchange between tagged and untagged LMMs had been promoted by denaturing and refolding of the coiled-coil structure indicated that it probably corresponded to heterodimers between tagged and untagged constructs containing a single histidine tag per dimer. The Western blot and SDS-PAGE analysis of Ni-NTA
Since the free energy of unfolding, $\Delta G^\circ$, varies linearly with GdnCl concentration in the limited region where $\Delta G^\circ$ can be measured, the conformational stability of the protein in the absence of GdnC ($\Delta G^\circ_{H_2O}$) was estimated by extrapolation of the regression line defined by the $\Delta G^\circ$ measured at the various [GdnC]. $\Delta G^\circ_{H_2O}$, and the $m$ values represent the $y$ axis intercept and the slope of that regression line, respectively (36).

![Figure 3](image)

**Figure 3.** Characterization of recombinant LMMs. A, CD profile of Neo-LMM construct in 40 mM NaPi, buffer (pH 7.5) in the absence (thick line) or presence (thin lines) of increasing guanidinium chloride concentration. B, denaturation curve of recombinant neonatal LMM.

### Table I

Summary of denaturation experiments of recombinant LMMs

| LMM construct | $[\text{GdmHCl}]_{1/2}$ | $\Delta G^\circ_{H_2O}$ | $m$  |
|---------------|----------------|----------------|-----|
| NeoLMM        | 1.44           | -4.02E+3       |     |
| HisNeoLMM     | 1.50 ± 0.02    | -3.97E+3 ± 0.148E+3 |     |
| AdultLMM      | 1.50 ± 0.03    | -3.87E+3 ± 0.178E+3 |     |

Therefore, the heterodimers formed during exchange experiments between different isoforms was about half that of the 50 mM imidazole fraction in exchange experiments between identical LMM sequences.

**Exchange Experiments between Chimeric LMMs and Either Neonatal or Adult His-tagged LMMs**—The LMM sequences of the neonatal and adult isoforms differ in 35 amino acids, only 18 of which are at heptad positions a, d, g, and e, which participate in interhelical contacts and thus can have the largest contribution to dimer stability and specificity (41, 42). In order to investigate which of these amino acid differences were relevant to the preferential formation of homodimeric LMMs, three chimeric LMM proteins were constructed that combined neonatal and adult sequences (Fig. 1). The N-terminal 183, 278, and 386 amino acids of the neonatal LMM were substituted with the corresponding adult sequences, resulting in the chimeric LMMs termed A183, A278, and A386, respectively. The three chimeric LMMs were virtually identical to the neonatal and adult recombinant LMMs, as judged by NDE mobility (Fig. 2B). Their reactivity with isoform-specific mAb reflected that their sequence contained both neonatal and adult epitopes (9).

In exchange experiments between HNeo and the chimeric LMMs, the proportion of eluted protein present in the 50 mM imidazole fraction was 28.9 (± 2.7), 37.1 (± 2.8), 38.4 (± 2.3%), for chimeric LMMs A386, A278, and A183, respectively (Fig. 6). The amino acid sequences of these three chimeric LMMs contained 22, 15, and 11 differences (10, 6, and 4 at a, d, g, and e positions) with respect to the neonatal sequence.

In exchange experiments with HAd LMM, the proportion of heterodimers formed was 47.3 (± 2.6), 49.0 (± 3.2), and 49.6 (± 4.1%), for chimeric constructs A183, A278, and A386, respectively. The amino acid sequences of these three chimeric LMMs contained 24, 20, and 13 differences (12, 6, and 4 at a, d, g, and e positions) with respect to the adult sequence. The differences in amount of heterodimers formed in exchange experiments between HAd and either one of these chimeric LMMs or the Ad LMM were not statistically significant at the 5% level.

The fact that replacement of the 183 N-terminal amino acids of the neonatal LMM with the corresponding adult sequence was sufficient to obtain virtually random exchange between chimera A183 and HAd LMM suggested a predominant role of these residues in the determination of the dimerization selec-
tivity. To test if absence of substitutions in the N-terminal 183 amino acids was sufficient to stabilize LMM dimers, we built construct N183 (N-terminal 183 residues corresponded to the neonatal sequence and the remaining 465 C-terminal amino acids to the adult sequence) and tested its ability to form dimers with the HNeo and HAd LMMs.

Chimera N183 formed 36.1% heterodimers with HNeo LMM. Although this amount was considerably higher than that obtained in exchange experiments between Ad and HNeo LMMs, it did not represent random dimerization. In exchange experiments with HAd LMM, N183 formed about 28.0% heterodimers, virtually the same values obtained in exchange experiments between HAd and Neo LMMs. Further incrementing the extent of neonatal sequence at the N terminus (chimera N386) resulted in an additional increase in the amount of heterodimers formed between the chimera and HNeo (41.5% heterodimers), without reaching values expected for non-selective dimerization.

**DISCUSSION**

The present work addressed the problem of which sequence elements control dimerization specificity in the coiled-coil of chicken skeletal muscle Light Meromyosin. Neoblast LMM fragments do form heterodimers. Pairwise exchange experiments between neonatal or adult LMMs with either HisNeo or HisAdult LMMs were carried out as described under “Materials and Methods.” The three protein fractions recovered from the Ni-NTA column at 10 mM, 50 mM, and 1 M imidazole were precipitated and quantitated. The percentage of recovered protein present in the 50 mM imidazole heterodimer fraction is presented for each pair. The bars represent the average values of at least three determinations, and the error bars indicate standard deviation.
the chicken myosin rod. We have developed a method using Ni-NTA chromatography that is capable of fractionating coiled-coil dimers according to the number of histidine-tagged strands they contain. Subsequently, we have used this method to study dimerization specificity of chicken neonatal and adult myosin LMM fragments by monitoring dimerization between histidine-tagged and untagged LMM constructs.

We showed that, during in vitro refolding of denatured neonatal and adult LMMs, dimerization between sequences of identical isoforms was favored. The 35 amino acid differences between the neonatal and adult LMMs reduced by 50% the extent of strand exchange between histidine-tagged and untagged LMM constructs. It is not likely that the preferential homodimer formation between neonatal and adult LMMs is caused by trapping of folding intermediates at low temperature, as described for α and β tropomyosin isoforms (43), since increasing the temperature of refolding did not favor heterodimer formation (data not shown). In addition, in the case of α and β tropomyosin the relative instability of the ββ homodimers favored the formation of the αβ heterodimer (44). In our case, no differences in the free energy of dissociation of neonatal and adult LMMs were observed (Table I).

In studies on the dimerization specificity of full-length chicken neonatal and adult myosin rods, heterodimers represented less than 5% of the refolded dimers (12). Our observation that neonatal and adult myosin LMMs could form up to 28% heterodimers could be explained if amino acid differences outside the LMM region contribute to further destabilization of the heterodimeric myosin rod. However, we cannot rule out the possibility that the sensitivity of the two methods used to quantify heterodimers is different. Nevertheless, the neonatal and adult LMMs still exhibit preferential dimerization with the homologous sequence.

Exchange experiments conducted between HNeo and chimeric LMMs A386, A278, or A183 indicated that the lower the number of amino acid differences between the two polypeptides, the higher the degree of heterodimerization. These results were consistent with the proposal that sequence homology plays a key role in myosin dimerization specificity (11). However, any of these chimeric LMMs was able to dimerize with HAd to virtually the same extent as the adult LMM (Ad), despite all the amino acid differences present. The extent of dimerization between A183 and HAd was especially remarkable, since the sequences of the two constructs differed in 24 positions out of the 35 amino acid differences present between neonatal and adult LMMs. The observation that removing 11 amino acid differences in the N terminus of the molecule remarkably increased the amount of heterodimers formed (compare HAd × A183 versus HAd × Neo), whereas removing 13 amino acid differences from the C terminus had little effect on dimerization (compare HNeo × A386 versus HNeo × Ad), indicated that not all substitutions had the same contribution to destabilization of heterodimers and suggested that the N terminus of the LMM played a crucial role in dimerization selectivity. However, chimeric construct N183, where the N-terminal 183 amino acids corresponded to the neonatal sequence, did not dimerize randomly with HNeo. HNeo formed about the same amount of heterodimers with N183 as with A278. Thus, a stretch of 183 amino acids of identity at the N terminus of the molecule stabilized the heterodimer to the same extent as a stretch of 372 amino acids of identity at the C terminus. These results were also consistent with the idea of sequence identity at the N terminus of the LMM being more relevant to dimerization than sequence identity at the C terminus.

The amount of heterodimers formed for each pair of proteins is a function of the difference between the free energy of dissociation of the heterodimer and the half sum of the free energy of the homologous sequences, as described for α and β tropomyosin isoforms (43), since increasing the temperature of refolding did not favor heterodimer formation (data not shown). In addition, in the case of α and β tropomyosin the relative instability of the ββ homodimers favored the formation of the αβ heterodimer (44). In our case, no differences in the free energy of dissociation of neonatal and adult LMMs were observed (Table I).

In order to gain further insight into LMM dimerization specificity, we converted neonatal and adult LMM constructs to histidine-tagged forms and let them refold at different temperatures. The results obtained (data not shown) indicated that removing 11 amino acid differences in the N terminus of the molecule remarkably increased the amount of heterodimers formed (compare HAd × A183 versus HAd × Neo), whereas removing 13 amino acid differences from the C terminus had little effect on dimerization (compare HNeo × A386 versus HNeo × Ad), indicating that not all substitutions had the same contribution to destabilization of heterodimers and suggested that the N terminus of the LMM played a crucial role in dimerization selectivity. However, chimeric construct N183, where the N-terminal 183 amino acids corresponded to the neonatal sequence, did not dimerize randomly with HNeo. HNeo formed about the same amount of heterodimers with N183 as with A278. Thus, a stretch of 183 amino acids of identity at the N terminus of the molecule stabilized the heterodimer to the same extent as a stretch of 372 amino acids of identity at the C terminus. These results were also consistent with the idea of sequence identity at the N terminus of the LMM being more relevant to dimerization than sequence identity at the C terminus.
of dissociation of the two homodimeric species. Since the untagged homodimer species in exchange experiments (HAd × A183) and (HNeo × A183) is the same (Fig. 7), if we assume similar free energy of dissociation for HNeo and HAd dimers (as would be expected based on the data in Table 1), then the higher amount of heterodimers formed between HAd and A183 indicated that the HAd-A183 heterodimer is more stable than HNeo-A183 heterodimer, despite the fact that the former pair of constructs differ at 24 positions and the latter pair of constructs only at 11 positions. Similarly, the higher amount of heterodimers obtained in exchange experiments (HNeo × N183) versus (HAd × N183) indicated that the HNeo-N183 dimer is more stable than HAd-N183 dimer, despite the higher number of amino acid differences. Thus, in either case heterodimers between pairs of constructs with no amino acid differences at the N-terminal 183 residues are more stable than heterodimers between constructs with no amino acid differences at the C-terminal 465 residues.

The fact that amino acid substitutions in the N- and C-terminal affect dimerization selectivity differently indicate that the residues in these regions contribute in a non-uniform manner to dimer stability. Positional effects of amino acid mutations have been noticed both in globular and in α-helical coiled-coil proteins (15, 18), and in general it is accepted that mutations are less detrimental to protein stability when they occur in regions that are not closely packed within the interior of the native molecule. Similarly, it is possible that the LMM coiled-coil is more flexible at its C terminus with a less closely packed dimer interface than at its N terminus. Examination of the sequence of the N-terminal 183 amino acids and C-terminal 264-amino acid stretch reveals regional differences in the amino acid composition. The proportion of alanine at a and d positions in the C-terminal-264 region (22.2%) is double of that of the whole myosin rod (12.15%, (13)), and triple of that of the N-terminal-183 region (7.6%). In addition, the density of potential interhelical salt-bridges (45) in the C-terminal-264 region is only half of the density in the N-terminal-183 region. These features could result in a less stable interface at the C-terminal region and underlie the low effect that amino acid differences in this region exert on dimerization specificity.

The different effect of the N versus C terminus of the molecule in dimerization selectivity was more marked in exchange experiments with HAd than in experiments with HNeo. The dimerization selectivity of HAd was determined almost exclusively by the N-terminal 183 residues of the LMM. On one hand, HAd dimerized virtually in a non-selective manner with A183, and on the other hand exchange experiments between HAd and construct N183 resulted in as much inhibition of heterodimerization as exchange experiments between HAd and Neo LMM. In contrast, sequence identity at the N terminus had only a partial effect on dimerization selectivity of HNeo LMM. The amount of heterodimers formed between HNeo and N183 was significantly higher than the values obtained in exchange experiments with the heterologous isoform (HNeo × Ad), but did not reach the values obtained in exchange experiments with the homologous isoform (HNeo × Neo). Even 386 amino acids of sequence identity at the N terminus was not able to produce random exchange between HNeo and construct N386, indicating that amino acid differences at the C terminus conferred some destabilization to the HNeo-N386 heterodimer. The different effect of sequence identity at the N terminus on the dimerization selectivity of adult and neonatal sequences might indicate that a different contribution of this region to stabilization of the adult and neonatal structures either during folding or in the native state. This difference could underlie the observed preference of these isoforms to associate as homodimeric coiled-coils.

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