Dimerization Specificity of Adult and Neonatal Chicken Skeletal Muscle Myosin Heavy Chain Rods†

Sheetal Singh and Everett Bandman*

Department of Food Science and Technology, University of California, Davis, One Shields Way, Davis, California 95616

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ABSTRACT: The dimerization specificity of the recombinantly expressed and purified rod domain of adult and neonatal chicken myosin heavy chain was analyzed using metal chelation chromatography. Our results indicate that full-length adult and neonatal rods preferentially formed homodimers when renatured from an equimolar mixture of the two isoforms denatured in guanidine hydrochloride. The contribution made toward the dimerization specificity by subdomains of the rod has been addressed by making a chimeric protein consisting of the subfragment 2 (S2) region of the adult isoform and the light meromyosin region of the neonatal isoform. The proportion of heterodimers formed in exchange experiments between the chimera and the neonatal and adult rods rose with increase in the sequence homology between the two exchanging proteins. This suggests that multiple regions of the rod domain of chicken MyHC including S2 can contribute toward dimerization specificity.

Myosin is a major contractile protein found in muscle cells. It is a large (∼500 kDa) hexameric protein consisting of two heavy chains, two regulatory light chains, and two alkali light chains. The N-terminal 840 amino acids of the myosin heavy chain (MyHC)[1] are folded to form a globular domain, and the remaining 1098 amino acids dimerize with the corresponding region of another MyHC to form an α-helical coiled-coil referred to as the rod. The rod domain of the MyHC participates in the assembly of myosin into functional thick filaments. The globular domain along with the light chains forms the myosin head that contains the enzymatically active site and the actin binding domain inherent to myosin (*1*, *2*).

A multigene family encodes MyHC in chickens, as in other vertebrates (*3*, *4*). The amino acid sequence and the organization of seven different isoforms of chicken fast skeletal myosin heavy chains at a single gene locus have been identified (*5*). The different isoforms have been categorized as embryonic, neonatal, and adult on the basis of the age at which they appear in the pectoral muscle of the chicken. It has been shown that multiple isoforms of MyHC are expressed in a single muscle cell (*6*−*8*), leading to the possibility that dimerization of rod domains can result in both homodimers of identical isoforms or heterodimers of two different isoforms.

The question whether neonatal and adult isoforms of chicken skeletal myosin associate to form a heterodimer or homodimer has been previously addressed using immunological and electron microscopic techniques based on stage-specific monoclonal antibodies (*6*). During that study it was shown that MyHC preferentially existed as homodimers in vivo, as less than 10% of the isolated MyHC molecules were found to be heterodimers. These results were further supported by an in vitro study in which the chymotryptic rod fragments of neonatal and adult isoforms of myosin rods were denatured together in guanidine hydrochloride, and removal of denaturing agent resulted in the re-formation of homodimeric coiled-coils (*9*). This result leads to two inferences. First, the homodimers of MyHC are thermodynamically preferred, and no cellular regulatory processes or mRNA compartmentalization is required to explain the lack of chicken MyHC heterodimers in vivo. Second, amino acid differences in the rod domain of the MyHC isoforms are responsible for the dimerization selectivity of the molecule.

The amino acid sequences of the rod portion of rat cardiac α and β myosin heavy chains are >93% identical (*10*). This high degree of homology has been proposed to be responsible for an approximately equal number of homodimers and heterodimers of rat cardiac myosin found in vivo (*11*). However, the rod domains of MyHC A and B of *Caenorhabditis elegans* that are 61% similar predominantly form homodimers, likely because heterodimers are not stable (*12*, *13*). These studies suggest that sequence homology between the MyHC isoforms may permit the formation of heterodimers, but this has not been observed with chicken smooth or skeletal muscle myosin heavy chains. The two smooth muscle isoforms of chicken, SM1 and SM2, have identical amino acid sequences throughout the myosin rod region but have different nonhelical tailpieces. These isoforms preferentially form homodimers in vivo (*14*). The amino acid sequence of the rod region of chicken adult and

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* Corresponding author. Tel: (530) 752-2490. Fax: (530) 752-4759. E-mail: edbandman@ucdavis.edu.

[1] Abbreviations: S2, subfragment 2; MyHC, myosin heavy chain; LMM, light meromyosin; HNR, His-tagged neonatal rod; HAR, His-tagged adult rod; HFNR, His-FLAG-tagged neonatal rod; HFAR, His-FLAG-tagged adult rod; HANR, His-tagged chimera rod; FNR, FLAG-tagged neonatal rod; FAR, FLAG-tagged adult rod; NR, untagged neonatal rod; AR, untagged adult rod; ANR, untagged chimera rod; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; DTT, dithiothreitol; GuHCl, guanidine hydrochloride; Ni-NTA, nickel nitrilotriacetate; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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neonatal isoforms of skeletal MyHC is >95% homologous, with a total of 53 differences in the 1098 amino acid long rod domain (*15, 16*). Despite the high homology between the adult and neonatal isoforms of chicken MyHC, these isoforms have been shown to predominantly form homodimers (*6, 9*). Similar observations have been made for another α-helical coiled-coil protein, tropomyosin. The nonmuscle tropomyosin isoforms TM-2 and TM-3 have >94% homology (*17*). Investigation of the dimerization preference of these isoforms in rat fibroblasts showed that these isoforms exist as homodimers (*17, 18*). All of these studies suggest that the preference to form homodimers as opposed to heterodimers is not dictated purely by overall differences between the amino acid composition of the protein but by the particular residues responsible for the stability of the α-helical coiled-coil protein that drive dimerization specificity.

The amino acid sequence of the rod follows the seven amino acid repeat $(abcdefg)_n$, which is characteristic of α-helical coiled-coil proteins (*19*). The a and d positions are generally occupied by hydrophobic residues, and charged residues are often present at positions e and g (*19, 20*). The stability of the α-helical coiled-coil proteins is mainly provided by hydrophobic interactions at the interface of the two helices between the side chains of the residues present in the a and d positions (*21*). The secondary structure is probably further stabilized by the interhelical salt bridges of the charged residues present at the e and g positions (*22*). Work done with Fos and Jun leucine zippers and other synthetic peptides has indicated that these electrostatic interactions are important for dimerization specificity (*23*−*25*). But, with respect to chicken skeletal myosin, electrostatic interactions are not thought to be important for dimerization specificity although their role in myosin filament morphology has been proposed (*26*). Previous work done in our laboratory has indicated that dimerization specificity of chicken neonatal light meromyosin (LMM) could be changed by mutations of noncharged residues in the hydrophobic core of the molecule (*27*). In another study, the energetic contribution of different amino acids at a position of the leucine zippers toward dimerization specificity showed that homotypic a−a′ interactions were preferred (*28*). Thus it is possible that maintenance of stronger hydrophobic interaction in the core α-helical coiled-coil rod domain of chicken MyHC drives the dimerization process.

An analytic approach was taken for studying the dimerization specificity of LMM, comprising 650 C-terminal amino acids of MyHC. In those experiments equimolar amounts of neonatal and adult LMM were mixed, denatured, and renatured. Subsequently, the amount of homodimers and heterodimers was determined. The results indicated that neonatal and adult LMMs preferentially formed homodimers but ∼25% of heterodimers were also detected (*29*). However, in another study using chymotryptic full-length rods the amount of heterodimers formed was significantly less (*9*). This suggests that the sequences responsible for the dimerization specificity are not restricted to just the LMM region of the MyHC but amino acid residues in the S2 region, consisting of the N-terminal 448 residues of the MyHC, play a role in the process as well. In the present study the dimerization specificity of a recombinantly expressed and purified full-length myosin rod produced in the same manner as LMM in our previous study (*29*) has been investigated. The contribution made toward the dimerization specificity by the S2 region of the MyHC has been addressed by making a chimeric protein in which the S2 region of the adult isoform was combined with the LMM region of the neonatal isoform. Our results indicate that full-length chicken MyHC rods preferentially formed homodimers. The exchange experiments between the chimera and the neonatal and adult rods indicated that the proportion of heterodimers formed was related to the sequence homology between the two exchanging proteins.

MATERIALS AND METHODS

Histidine-Tagged Rod Clones. cDNA clones encoding neonatal and adult chicken MyHC have been characterized as GenBank accession numbers ab021180 and u87231, respectively. The full-length myosin rod of both isoforms along with 18 bp from the upstream globular domain of MyHC was cloned into expression vector pET-15b (Novagen) within the restriction sites *Bam*HI and *Eco*RI. During translation an additional 25 residues (MGSSHHHHHHSSGLVPRGSHMLEDP) were added at the N-terminus of the myosin rod from the vector sequences. These vector sequences encoded the hexahistidine tag followed by a thrombin cleavage site. The neonatal rod expression vector was designated pET-15b-HNR, and the adult rod expression vector was designated pET-15b-HAR. These two constructs were used to express His-tagged neonatal (HNR) and His-tagged adult (HAR) rods.

Histidine-FLAG-Tagged Rod Clones. The DNA sequence encoding the FLAG tag, an octapeptide (DYKDDDDK), was engineered into the myosin rod expression vectors pET-15b-HNR and pET-15b-HAR. Polymerase chain reaction was used to add the octapeptide upstream of the rod sequence but downstream of the His tag and thrombin cleavage site in the expression vectors. The forward primer used for mutagenesis was 5′-CGGCTTGTCATCGTCGTCCTTGTAGTCATCCTCGAGCATATGGCTGCC-3′, and the reverse primer used was 5′-GATGACTACAAGGACGACGATGACAAGCCGCTGTTCTTTAAGATCAAG-3′. The expression vectors containing the FLAG tag in addition to His tag were designated pET-15b-HFNR and pET-15b-HFAR for neonatal and adult rod proteins, respectively. The FLAG tag was inserted downstream of the His tag so that the same purification protocol using Ni-NTA chromatography could be used for His-tagged and His-FLAG-tagged rod proteins.

Chimera Rod Clone. The chimera rod protein expression clone, pET-15b-HANR, was constructed by combining the N-terminal 1347 bp of the adult rod sequence encoding the 449 residues of the S2 region with the C-terminal 1950 bp of the neonatal rod sequence encoding the 650 residues of the LMM region. The clones pET-15b-HNR and pET-15b-HAR were digested with the restriction enzyme *Ssp*I. The nucleotide sequences encoding the C-terminal 650 amino acids of the neonatal rod were transferred to pET-15b-HAR, replacing the corresponding adult sequences. The clones with the correct orientation were identified by restriction enzyme pattern and then further confirmed by sequencing. The His-tagged chimera rod protein generated with this newly constructed clone was designated HANR.

Expression and Purification of Recombinant Histidine-Tagged or Histidine-FLAG-Tagged Rod Proteins. BL21-(DE3) pLysS *Escherichia coli* cells were transformed with various pET expression vectors. The cultures were grown according to the instructions of the manufacturer (Novagen). The bacterial cultures were harvested after 4 h of induction with isopropyl l-thio-β-D-galactopyranoside added to a final concentration of 1 mM and centrifuged at 5000*g* using a GSA rotor (Sorvall Instruments) for 10 min, and the pellets were stored at −80 °C. The pellets obtained from 1 L culture were thawed on ice, resuspended in 8 mL of low salt buffer (LSB: 20 mM KCl, 2 mM KH_2PO_4, 1 mM EGTA, 10 mM DTT, 0.1 mM phenylmethanesulfonyl fluoride, pH 6.8), 400 μL of protease inhibitor cocktail III (Calbiochem), 200 μL of RNase (0.5 μg/μL), and 50 μL of DNase I (1 μg/μL), and then sonicated on ice in order to reduce the viscosity. The LSB soluble proteins were removed by centrifugation at 14000*g* for 20 min at 4 °C. The pellets were washed with just LSB four more times. Then the pellets were suspended in binding buffer (100 mM $NaH_2PO_4 \cdot H_2O$, 10 mM Tris base, 5 mM β-mercaptoethanol, 10 mM imidazole, 4 M GuHCl, pH 7.8). The proteins HNR and HAR were purified by an additional step of dialysis against LSB as described for purification of myosin from chicken muscle before being suspended in binding buffer (*30*). Proteins soluble in binding buffer were separated by centrifugation as described above and subjected to Ni-NTA chromatography (Qiagen) under denaturing conditions. The constitution of the buffer used for elution of purified protein was the same as that of the binding buffer; however, the concentration of imidazole was increased to 500 mM. The elution fractions were pooled, concentrated, and loaded onto a 50−150 μm 4% cross-linked agarose bead (Agarose Bead Technologies) column (2.5 × 50 cm) for further purification by gel permeation chromatography under denaturing conditions. The gel permeation column was equilibrated with 40 mM $Na_4P_2O_7 \cdot 10H_2O$, 10 mM DTT, 4 M GuHCl, pH 7.5, buffer, and the same buffer was used for the elution of pure protein at a flow rate of 300 μL/min. The fractions containing pure protein were pooled and renatured by dialysis against high salt buffer (HSB: 40 mM $Na_4P_2O_7 \cdot 10H_2O$, 10 mM DTT, pH 7.5) when they were to be used for the exchange experiments, and the proteins were renatured against thrombin buffer (1 M NaCl, 20 mM imidazole, 10 mM β-mercaptoethanol, pH 7.8) when the His tag was going to be removed with thrombin.

Untagged Rod Protein. The untagged neonatal rod (NR), adult rod (AR), and chimera rod (ANR) were generated by cleaving off the His tag with biotinylated thrombin (Novagen). Pure His-rod protein in the thrombin buffer was diluted at the time of thrombin reaction so that the concentration of thrombin buffer ingredients would be reduced to half. The thrombin reaction was set up according to the protocol provided by Novagen with some modifications. The amount of biotinylated thrombin used was increased to 1 unit for cleaving off His tag from 300 μg of protein. The reaction was then incubated overnight at 10 °C. After the removal of the His tag, the rod protein was treated with streptavidin resin (100 μL of the 50% slurry) to remove the biotinylated thrombin in the presence of 1% Tween-20 from the pure protein. The protein was then subjected to a second round of Ni-NTA chromatography under native conditions in order to remove uncleaved His-tagged rod from the untagged rod protein. Buffers used were the same as those used for the Ni-NTA column chromatography described above except GuHCl was not added in these buffers. Most of the untagged rod protein was found in the flow-through and the first three column washes with binding buffer. The untagged rod protein was then dialyzed against HSB and kept at 4 °C. The removal of His-tagged protein and the biotinylated thrombin from the protein preparation was confirmed by a western blot. The non-His-tagged proteins NR, AR, and ANR had eight (GSHMLEDP) residues added at the N-terminus of the rod protein by the sequences of the expression vector.

FLAG-Tagged Rod Protein. FLAG-tagged neonatal rod (FNR) and FLAG-tagged adult rod (FAR) were generated by expressing the protein from the expression vectors pET-15b-HFNR and pET-15b-HFAR and then cleaving off the His tag with biotinylated thrombin and removing the uncleaved His-FLAG-tagged rod by Ni-NTA chromatography as described above. The FLAG-tagged proteins have 16 (GSHMLEDDYKDDDDKP) residues added at the N-terminus of the rod protein by the expression vector sequences.

Determination of Protein Concentration. The concentration of various rod protein preparations was estimated by scanning densitometry (Pharmacia LKB, Bromo, Sweden) of SDS−PAGE gels. SDS−PAGE was performed as described by Laemmli (*31*). Pure rod protein preparations were run in triplicate on the SDS gel along with increasing known amounts of standard rod protein preparations. The concentration of the standard was estimated by measuring the absorbance at 280 nm and using an extinction coefficient of 0.22 mL mg^{-1} cm^{-1} (*32*). The concentration of the protein preparations was calculated on the basis of standard curves obtained from a known amount of rod protein samples.

Rod Denaturation and Renaturation. Five hundred micrograms each of two proteins in HSB, only one of them containing the polyhistidine tag, was mixed. The protein concentration of the rod protein mixture was adjusted to 2 μg/μL, and then it was denatured by addition of denaturation buffer (8 M GuHCl, 40 mM $Na_4P_2O_7 \cdot 10H_2O$, 10 mM DTT, pH 7.5) to a final concentration of 5 M GuHCl. After incubation at room temperature for 2 h, the preparation was renatured by dialysis against HSB for 4 h at 4 °C. Renatured samples were dialyzed against loading buffer (500 mM NaCl, 20 mM imidazole, 0.4 M GuHCl, 5 mM β-mercaptoethanol, pH 7.8) overnight at 4 °C. After centrifugation at 14000*g* for 20 min at 4 °C the supernatant was loaded onto a Ni-NTA column.

Ni-NTA Column Chromatography. The use of Ni-NTA column chromatography to separate homodimers and heterodimers on the basis of the different affinity of zero, one, or two histidine-tagged LMM molecules for the Ni-NTA resin has been established before (*29*). A similar approach was taken here to study the amount of heterodimers or homodimers formed during the exchange experiments with His-tagged and FLAG-tagged or untagged MyHC rod. The renatured samples in the loading buffer were loaded onto a 2.5 mL packed Ni-NTA resin column equilibrated with the same buffer and running at a flow rate of 10 mL/h. The column was eluted with 5 column volumes of loading buffer (500 mM NaCl, 20 mM imidazole, 0.4 M GuHCl, 5 mM β-mercaptoethanol, pH 7.8), then with 10 column volumes of 80 mM imidazole buffer (500 mM NaCl, 80 mM imidazole, 0.4 M GuHCl, 5 mM β-mercaptoethanol, pH 7.8),

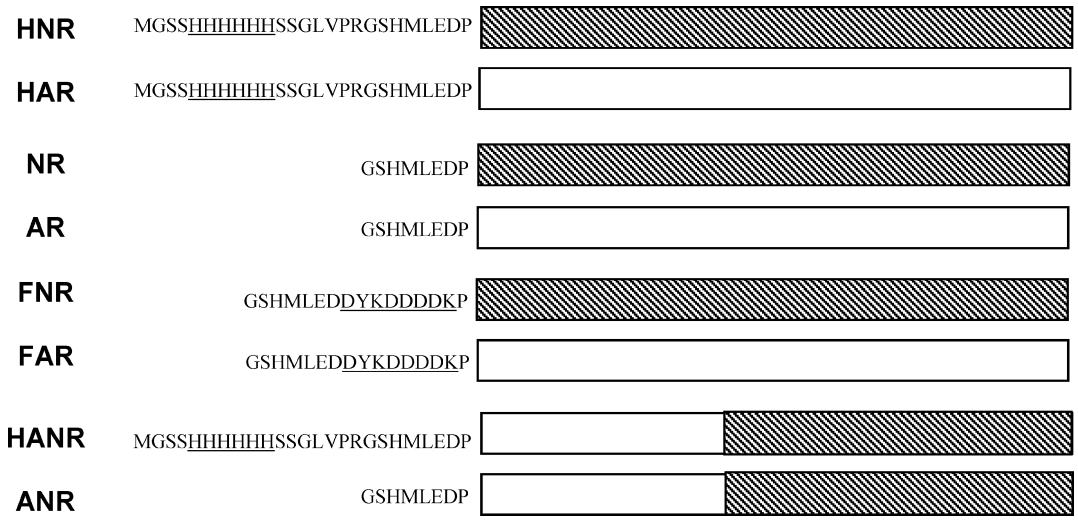

FIGURE 1: Illustration of various rod constructs. The rod domain is formed of the C-terminal 1098 amino acids of MyHC. The rod proteins used in the study had an additional six amino acids encoded from the sequences N-terminal of the rod region. The amino acids at the N-terminus of the rods that were derived from the vector sequences are shown. The His tag and the FLAG tag are underlined to indicate their location. The chimera, HANR, is comprised of the N-terminal 449 residues of the adult isoform and C-terminal 650 residues of the neonatal isoform.

and finally with 5 column volumes of 1 M imidazole buffer (500 mM NaCl, 1 M imidazole, 0.4 M GuHCl, 5 mM β-mercaptoethanol, pH 7.8). The amount of protein eluted in each peak was estimated as described earlier (*29*) with the difference that 5 μL of bovine serum albumin (0.5 μg/μL in sterile H_2O) was used as the internal standard for trichloroacetic acid precipitation efficiency. The statistical significance between different sets of exchange experiments was analyzed with ANOVA (single factor).

RESULTS

Expression and Purification of Rod Protein Constructs. The recombinant expression of the full-length rod region of chicken MyHC in *E. coli* with His tag or His-FLAG tag was done as described in the previous section. The additional amino acids added to the amino-terminal end of each rod construct arising from vector sequences are shown in Figure 1. We observed that the expression of full-length His-FLAG-tag adult or His-FLAG-tag neonatal rod was 2−3 fold less than the corresponding His-tag rods without the FLAG-tag sequence. This could have been due to increase in proteolysis or initiation of internal translation from the constructs used to express the double-tagged molecules. It has been shown previously that the protease recognition site inherent to the FLAG-tag sequence can be vulnerable during expression in bacteria and lead to cleavage of the FLAG tag from the expressed fusion protein (*33*). Also, the presence of sequences that can act as prokaryotic translation initiation sites in various contractile proteins including myosin has been defined (*34*, *35*). These internal translation initiation sites can lead to production of truncated rod protein that is missing the N-terminus. We found that it was necessary to process twice as much bacterial culture in order to produce equivalent amounts of rod protein at the end of the purification process for His-FLAG-tagged constructs compared to His-tagged constructs.

The untagged and the FLAG-tagged rod proteins were generated by removing the His tag with biotinylated thrombin. Using biotinylated thrombin facilitated the subsequent removal of thrombin after the reaction. The addition of Tween-20 at the time of removal of biotinylated thrombin decreased the loss of rod protein due to nonspecific binding to the streptavidin agarose resin.

The various pure proteins were analyzed by SDS−PAGE. The proteins were estimated to be >95% homogeneous by scanning densitometry (Figure 2). The dimerization specificity of rod proteins and chimera was studied by conducting "exchange experiments". In these experiments random strand exchange between rod constructs was promoted by unfolding the coiled coil with addition of a denaturant followed by refolding upon gradual removal of the denaturant. The different protein species generated during the denaturation−renaturation process were analyzed using Ni-NTA chromatography.

Separation of Homodimers and Heterodimers Using Ni-NTA Chromatography. Due to the presence of the His tag, chicken myosin rod protein can selectively bind to the Ni-NTA resin. We have previously demonstrated that when two LMM proteins with only one of them containing the His tag are mixed, denatured, and renatured, three protein peaks are obtained from the Ni-NTA column containing zero, one, or two histidine tags, respectively (*29*). Similarly, in the case of rod protein, when equal amounts of untagged and His-tagged rods were denatured and then renatured and then subjected to Ni-NTA chromatography, three peaks were observed when using an imidazole gradient to elute the protein. The intermediate fraction began eluting at 62.5 mM imidazole with a peak at 80 mM imidazole. A peak eluting at 80 mM imidazole was not observed when a His-tagged rod and untagged rod were mixed and applied to the column without denaturation and renaturation (data not shown), similar to the results in the LMM experiments (*29*). This observation indicated that a new class of rod dimers, containing only a single His-tagged myosin rod, was formed during the process of denaturation and renaturation. To clearly define the conditions for the exchange experiments, FLAG-tagged rod protein was introduced in the study. The binding properties of FLAG-tagged rods with the Ni-NTA

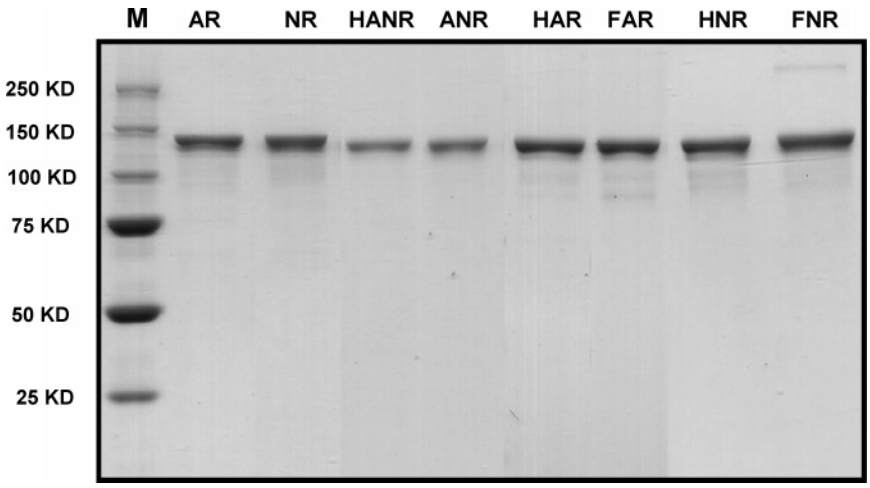

FIGURE 2: SDS−PAGE of recombinant rods. All of the proteins used in the study were estimated to be >95% homogeneous with scanning densitometry. M = protein molecular mass standards.

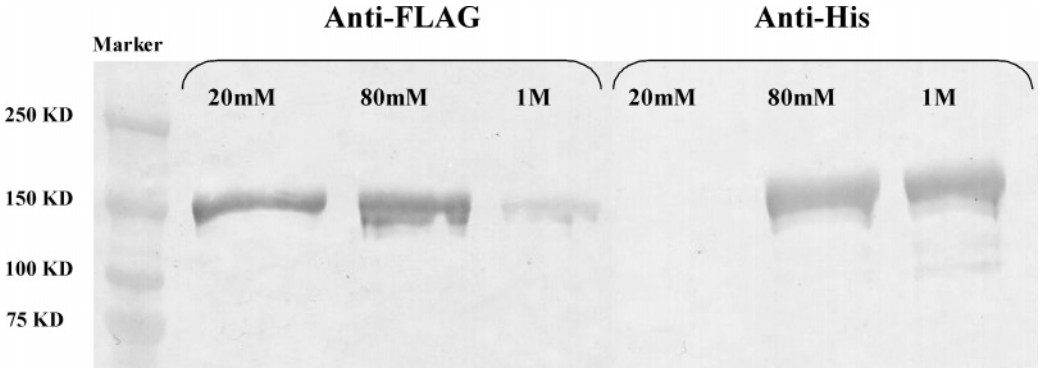

FIGURE 3: Western blot analysis of three protein peaks following Ni-NTA chromatography to separate heterodimers and homodimers. Equal amounts of His-tagged and FLAG-tagged neonatal rod proteins were mixed, denatured, and renatured. The proteins formed were separated with Ni-NTA column chromatography. The three peaks obtained were subjected to western blot analysis using anti-FLAG and anti-His antibodies.

resin were similar to those of the untagged rods. The FLAG-tagged rods did not bind nonspecifically to the Ni-NTA column, and its recovery was the same as that of untagged rod protein with 20 mM imidazole buffer (data not shown). Conducting exchange experiments with FLAG-tagged instead of untagged rod protein facilitated visualization and characterization of the three elution peaks obtained following an exchange experiment done with equivalent amounts of His-tagged and FLAG-tagged rods of the same isoform. Figure 3 shows the western blot done with anti-FLAG (anti-FLAG M2 monoclonal antibody; Sigma) and anti-His (penta-His antibody; Qiagen) antibodies for the three peaks obtained with 20 mM, 80 mM, and 1 M imidazole buffers for the exchange experiment of His-tagged and FLAG-tagged neonatal rods. From the western blot it is clear that the FLAG-tagged homodimers eluted in the 20 mM fraction, FLAG-tagged/His-tagged heterodimers eluted mainly in 80 mM fractions, and His-tagged homodimers eluted in 1 M fractions.

After defining the conditions for exchange experiments, control experiments for both of the isoforms of MyHC rod were set up by denaturation and renaturation of an equal amount of His-tagged and FLAG-tagged rods of each isoform separately. The results of the various control experiments show that close to a random (~50%) number of heterodimers are formed between FLAG-tagged and His-tagged rod proteins when they contain the same amino acid sequence; i.e., they are the same rod isoform. All of the exchange experiments were done in triplicate. The heterodimers for the His-tagged and FLAG-tagged neonatal rod exchange experiments were 45.31 ± 2.8% (n = 3), and for similar exchange experiments of adult rods the value was 41.59 ± 1.3% (n = 3). The conditions established for the control experiments were used for His-tagged and untagged chimera exchange experiments; these experiments yielded a similar percentage of heterodimers, 45 ± 0.1% (n = 3). This result indicated that the chimera, HANR, had denaturation and exchange properties similar to those of the neonatal or adult rods (Figure 4).

Neonatal and Adult Rods of Chicken MyHC Preferentially Form Homodimers. With the aim of studying the dimerization specificity of neonatal and adult rod proteins of chicken MyHC, equal amounts of neonatal and adult rods were denatured by GuHCl and renatured by dialysis. The renatured mixture was loaded onto an equilibrated Ni-NTA column, and the amount of protein eluting in the 80 mM fraction, which corresponds to the heterodimer fraction, was quantified.

In contrast to the values seen for control experiments (His-tagged and FLAG-tagged neonatal rod or His-tagged and FLAG-tagged adult rod), the percentage of the heterodimers was found to be significantly less (p < 0.05) when two different isoforms were mixed, denatured, and renatured. The percentage of heterodimers formed by the exchange experi-

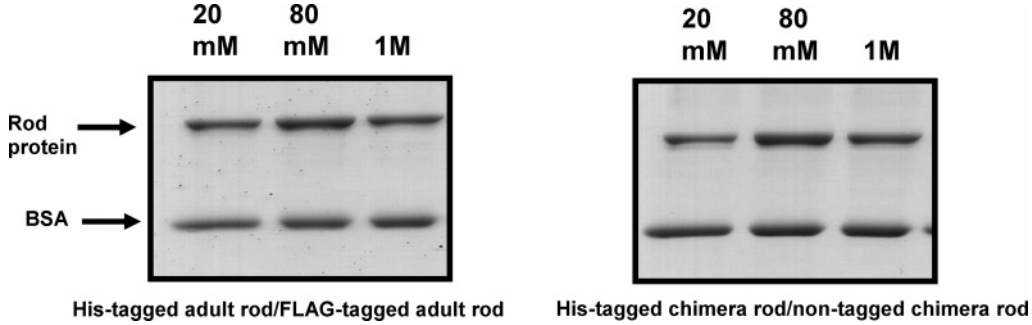

FIGURE 4: Comparison of control exchange experiments of adult and chimera rod proteins. SDS−PAGE of the three fractions obtained from the exchange experiments of His-tagged adult and FLAG-tagged adult along with His-tagged chimera and untagged chimera rod proteins are shown. From this figure it is evident that the chimera rod, HANR, forms similar levels of heterodimers during a control exchange experiment as the adult rod, suggesting that the denaturation and exchanging properties of both of the proteins are similar.

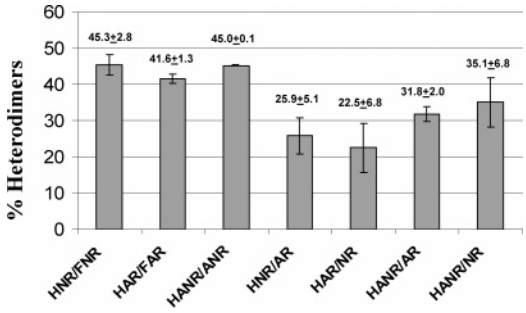

FIGURE 5: Percentage of heterodimers formed in different exchange experiments. The amount of protein present in the three fractions (20 mM, 80 mM, and 1 M) obtained from the Ni-NTA column following an exchange experiment was estimated by laser densitometry. The percentage of protein obtained in the 80 mM heterodimer fraction has been presented for each set of exchange experiments. The bars represent the average of three sets of experiments done for each pair of proteins, and the error bars indicate the standard deviation. The values above the bars represent the average ± standard deviation for each. Abbreviations: HNR, His-tagged neonatal rod; FNR, FLAG-tagged neonatal rod; HAR, His-tagged adult rod; FAR, FLAG-tagged adult rod; NR, untagged neonatal rod; AR, untagged adult rod; HANR, His-tagged chimera rod; ANR, untagged chimera rod.

ment of His-tagged adult and untagged neonatal rods was 22.47 ± 6.8% (n = 3), and for His-tagged neonatal and untagged adult rods the value was 25.86 ± 5.1% (n = 3). These experiments were also done in triplicate, and statistical analysis established that the percentage of heterodimers formed in these two sets of experiments was not significantly different ($p > 0.05$) from each other but was approximately half of what was observed for the exchange experiments between identical rod molecules (Figure 5). This observation indicated that neonatal and adult rod preferentially refolded to form homodimers following the denaturation−renaturation protocol.

The Chimeric Protein Forms Heterodimers with both Adult and Neonatal Rods. The percentage of heterodimers formed in the study done with recombinant LMM proteins was significantly greater than that observed previously for chymotryptic rod proteins (*9*, *29*). These results suggested that the S2 region of MyHC might be involved in dictating the dimerization specificity of the chicken MyHC. To address this question, a chimeric protein, HANR, was recombinantly expressed and purified. In this chimera the S2 region was that of the adult isoform, and the LMM region was that of the neonatal isoform (Figure 1). There are 53 amino acid differences between the neonatal and adult rod sequences, of which 18 are present in the S2 region. Of these 18 differences, 6 are present at the a and d positions and 3 at the e and g positions of the α-helical heptad repeat.

To address the question of whether differences in the S2 region of neonatal and adult rod can affect dimerization specificity, exchange experiments were conducted between His-tagged chimera and untagged adult rod proteins. The percentage of heterodimers formed for these experiments was 31.78 ± 2.0% (n = 3) (Figure 5) in comparison to that observed for exchange experiments of His-tagged adult and untagged neonatal rods (22.47 ± 6.8%) or His-tagged neonatal and untagged adult rods (25.86 ± 5.1%). In the exchange experiments with the chimera and the untagged adult rod, the number of amino acid differences between the two exchanging rods was 35. The percentage of heterodimers was 35.11 ± 6.8% (n = 3) for the exchange experiments between His-tagged chimera and untagged neonatal rod proteins. In this set of experiments the difference in the amino acid residues of the exchanging rod proteins was reduced to only 18 residues. The percentage of heterodimers formed in exchange experiments of the His-tagged chimera rod with either untagged adult or untagged neonatal rod was not significantly different ($p > 0.05$) than the exchange experiment between two different isoforms of the MyHC rods. Comparing the percentage of heterodimers formed for the exchange experiments of the chimera protein with the control experiments (His-tagged and FLAG-tagged neonatal rod or His-tagged and FLAG-tagged adult rod), it was observed that the heterodimers formed for the His-tagged chimera and untagged adult experiment were significantly less ($p < 0.05$) than those for the control experiments, but for His-tagged chimera and untagged neonatal exchange experiments the values were not significantly different ($p > 0.05$).

DISCUSSION

The objective of the present work was to study the dimerization specificity of the rod domain of chicken MyHC. Multiple isoforms of MyHC are frequently expressed in a single muscle cell (*6*−*8*), leading to the possibility that MyHC protein may be either heterodimers or homodimers. Both in vivo and in vitro studies have shown that chicken MyHC prefers to form homodimers (*6*, *9*). An analytical approach was followed to study the dimerization specificity of the LMM domain of MyHC, and it was established that although the LMMs preferentially formed homodimers, a small but significant number of heterodimers also were formed (*29*). The percentage of heterodimers formed in the

LMM study was significantly more than that previously observed in the dimerization study of chicken MyHC rods prepared by chymotryptic digestion (*9*). The present study using recombinant MyHC rod was undertaken to understand the role of the S2 and LMM domains in dimerization specificity.

Our results indicated that when the rods of neonatal and adult isoforms of chicken MyHC are denatured and renatured, the rods preferentially dimerized as homodimers. However, significant percentages of heterodimers also were formed in these experiments. The percentage of heterodimers observed in these experiments was ~24%, about half of that observed in control experiments done with equal amounts of His-tagged and FLAG-tagged rods of the same isoform. These results highlight the preference of rods to form homodimers; however, the percentage of heterodimers formed in these experiments is more than the ~5% heterodimers observed in another study where chymotryptic rods were used instead of recombinant rods (*9*). This disparity between the results of these two studies could be due to at least two reasons. First, the experimental design used to determine the percentage of heterodimers in the studies was different, as the percentage of heterodimers formed in the study using the chymotryptic rod was determined by the double antibody sandwich ELISA technique, whereas in the present study of recombinant rods Ni-NTA chromatography was used to segregate homodimers from heterodimers. There is a difference in the manner in which the amount of heterodimers is determined in two techniques. In the ELISA technique the percentage of heterodimers formed is determined from an amplified signal whereas with Ni-NTA chromatography the percentage of heterodimers formed is measured directly. Second, there was a difference in the method of rod protein preparation in the two studies. It has been shown that during chymotryptic preparation of rod protein the C-terminal 16 residues (nonhelical tail) are removed due to proteolysis (*36*, *37*). The removal of the tail has been shown to affect the solubility and assembly properties of both LMM and rod domains (*14*, *38*, *39*); this may alter the dimerization properties of these proteins and account for the differences in the percentage of heterodimers observed in the two rod dimerization studies. In a study of recombinant LMMs it was shown that neonatal and adult LMMs could form up to 28% heterodimers as opposed to less than 5% observed with chymotryptic rods. To determine if the sequence differences outside the LMM region contributed to the dimerization specificity of the MyHC, the present study was conducted. We found that the percentage of heterodimers formed when neonatal and adult rods were denatured and renatured together was similar to that observed with the corresponding LMMs. This could be due to the similar experimental design used in these two studies which was different from the ELISA-based technique used for the work done with chymotryptic rods. To determine if the residues outside the LMM region of the MyHC rod contributed to the dimerization specificity, exchange experiments with a chimeric protein were also carried out.

The importance of the S2 region in dimerization specificity was addressed by conducting experiments with a chimera, HANR, which had the S2 region of the adult isoform and the LMM region of neonatal isoform. The exchange experiment of His-tagged chimera and untagged adult rod generated additional heterodimers compared to the exchange experiments between different isoforms. This variation could be due to reduction in the differences between the amino acid sequences of His-tagged chimera and the untagged adult rod pair to 35 compared to 53 between the different isoform rods. This result also indicated that the S2 region contributed to the dimerization specificity of the chicken MyHC. The percentage of heterodimers increased further in exchange experiments of His-tagged chimera and untagged neonatal rod compared to the exchange experiments between different isoforms ,suggesting that residues in the LMM domain also affect the dimerization specificity. In this set of experiments the difference in the amino acid residues of the exchanging rod proteins was reduced from 53 to only 18. These results suggest that reducing the differences between the exchanging rod proteins promoted the formation of heterodimers.

The role of different regions of the MyHC rod toward dimerization specificity was further analyzed by comparing the results obtained with His-tagged chimera experiments and the control experiments (His-tagged and FLAG-tagged neonatal rod or His-tagged and FLAG-tagged adult rod). The percentage of heterodimers formed in His-tagged chimera and untagged adult exchange experiments was significantly less than that seen for the control exchange experiments. However, in exchange experiments of His-tagged chimera and untagged neonatal rod the percentage of heterodimers formed was not significantly different from those found in the control exchange experiments. This could be attributed to the fact that the number of amino acid differences in the His-tagged chimera/untagged neonatal sequences is only 18 compared to 35 in the His-tagged chimera/untagged adult pair. These results indicate that although the S2 region contributes to the dimerization specificity of the rod domain of chicken MyHC, the other regions of the protein are also involved in the process. Thus dimerization specificity is not restricted to one particular region of the rod protein.

There are 53 differences between the amino acid sequence of adult and neonatal chicken myosin rods with 27 found at the a, d, e, and g positions, which have been shown to be important for the stability of coiled-coil proteins (*21*, *22*). The analysis of 16 different sarcomeric myosins has shown that the sites of interhelical ion pairing of the residues present at the e and g positions were almost identical in all myosin rods, irrespective of whether they formed heterodimers or homodimers (*26*). This suggests that the dimerization specificity of chicken skeletal MyHCs is probably not dictated by the amino acids present in positions e and g and that the amino acids present in the a and d positions might have a greater effect on the stability of these coiled-coil proteins. The difference in the residues present in the a and d positions is spread throughout the entire sequence of the neonatal and adult rods except for one group that is present in heptads 16 and 17 that represent part of the hinge region of the MyHC rod. Similarly, the differences in the amino acid sequence of tropomyosin isoforms TM-2 and TM-3 are grouped in a 25 amino acid stretch as these are derived from the same gene by alternative RNA processing (*17*, *40*). These isoforms of tropomyosin were found to form homodimers in vivo (*18*). A similar observation was made for another set of isoforms of tropomyosin, TM-5a and TM-5b (*40*). It is possible that the differences in the amino acid sequence of neonatal and adult MyHC grouped in heptads 16 and 17 by a mechanism

similar to that observed for tropomyosin are responsible for the dimerization properties of chicken MyHC. This question can be addressed by generating a chimeric protein in which heptads 16 and 17 of the neonatal rod are replaced by corresponding adult sequences and then studying its ability to form heterodimers with both neonatal and adult rods.

Dimerization specificity has been shown to be altered by a single mutation. Previous work done in our laboratory had indicated that dimerization specificity of chicken neonatal LMM could be changed by mutations of residues in the hydrophobic core of the molecule. In that study substitution of amino acids at the a or d positions of neonatal LMM with corresponding adult LMM amino acids had changed the dimerization specificity of the neonatal LMM to that of adult LMM (*27*). In another study, the energetic contribution of different amino acids at a position of the leucine zippers toward dimerization specificity showed that aliphatic amino acids Leu, Ile, and Val preferred homotypic a−a′ interactions (*28*). Single mutations could be responsible for disruption of the coiled-coil structure and eventually myosin filament assembly as shown by a finding that a missense mutation, Ser1776Gly, at a position in the LMM region of human cardiac β-MyHC was responsible for familial hypertrophic cardiomyopathy (*41*). Similar selectivity of the hydrophobic interactions of the core residues might be responsible for the homodimer formation of chicken MyHC. Recently Kwok et al. have defined stabilizing clusters, which consist of three or more consecutive core residues of stabilizing amino acids, in tropomyosin and the rod domain of MyHC (*42*). Some of these stabilizing clusters might get disrupted during the formation of the heterodimer, making dimerizing as homodimers a more energetically efficient option. These various studies indicate that maintenance of stronger hydrophobic interactions in the core of the α-helical coiled-coil rod domain of chicken MyHC might be the key driving force behind dimerization and that this process can be disrupted by even single mutations. To understand the contribution of various residues present in the core of the rod domain of the chicken MyHC, point mutations in the rod domain of one of the isoforms should be generated and its effect on dimerization specificity should be studied. Even though the present study has indicated that the S2 region of the MyHC rod contributes to dimerization specificity, these extended experiments will help in understanding whether the preference to form homodimers or heterodimers is a function of the whole chicken MyHC rod or of a particular region.

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