Assembly of Avian Skeletal Muscle Myosins: Evidence that Homodimers of the Heavy Chain Subunit Are the Thermodynamically Stable Form

Bruce Kerwin and Everett Bandman

Department of Food Science and Technology, University of California, Davis, California 95616

Abstract. Using a double antibody sandwich ELISA we examined the heavy chain isoform composition of myosin molecules isolated from chicken pectoralis major muscle during different stages of development. At 2- and 40-d posthatch, when multiple myosin heavy chain isoforms are being synthesized, we detected no heterodimeric myosins, suggesting that myosins are homodimers of the heavy chain subunit. Chymotryptic rod fragments of embryonic, neonatal, and adult myosins were prepared and equimolar mixtures of embryonic and neonatal rods and neonatal and adult rods were denatured in 8 M guanidine. The guanidine denatured myosin heavy chain fragments were either dialyzed or diluted into renaturation buffer and reformed dimers which were electrophoretically indistinguishable from native rods. Analysis of these renatured rods using double antibody sandwich ELISA showed them to be predominantly homodimers of each of the isoforms. Although hybrids between the different heavy chain fragments were not detected, exchange was possible under these conditions since mixture of biotinylated neonatal rods and fluoresceinated neonatal rods formed a heterodimeric biotinylated-fluoresceinated species upon renaturation. Therefore, we propose that homodimers are the thermodynamically stable form of skeletal muscle myosin isoforms and that there is no need to invoke compartmentalization or other cellular regulatory processes to explain the lack of heavy chain heterodimers in vivo.

MYOSIN is the major contractile protein of vertebrate skeletal muscle. It is a hexameric protein composed of two heavy chains and four light chains. The COOH-terminal domains of two MHCs fold into an α-helical coiled coil known as the rod that can be purified after proteolytic cleavage of whole myosin. The rod is involved in myosin thick filament formation and may also play some role in contraction. The NH_2-terminal domain of each MHC is folded into a globular structure that along with the myosin light chains form the myosin head containing the ATPase and actin binding sites intrinsic to myosin (for review see Cooke, 1986; Harrington and Rogers, 1984; McLachlan, 1984).

In the chicken, as in other vertebrates, MHCs are encoded by a multigene family (Nguyen et al., 1982; Buckingham, 1985; Robbins et al., 1986). As a result of differential gene expression during development distinct MHC isoforms are expressed (Robbins et al., 1986; Moriarity et al., 1987). The MHCs expressed in the pectoralis major (PM)[1] have been characterized by peptide mapping and mAb analysis and termed embryonic, neonatal, and adult isoforms (Bader et al., 1982; Bandman et al., 1982; Winkelmann et al., 1983; Cerny and Bandman, 1987; Bandman and Bennett, 1988; Van Horn and Crow, 1989). However, recent studies have shown that embryonic and neonatal MHCs are expressed in other adult muscles of the chicken (Crow and Stockdale, 1986; Bandman and Bennett, 1988). It is likely that the extent of MHC protein diversity has not been fully characterized since genomic studies of the chicken have found >20 myosin-like genes (Robbins et al., 1986; Moriarity et al., 1987).

During certain periods of muscle maturation, multiple MHC isoforms are simultaneously expressed in the developing PM. This occurs just before hatching when both embryonic and neonatal MHC isoforms are expressed and again from days 20 to 80 after hatching when both the neonatal and adult MHC isoforms are expressed. Recent evidence indicates that when multiple MHC isoforms are present within the PM, coexpression of multiple isoforms occurs within the same muscle cell (Gauthier, 1990; Cerny and Bandman, 1987). Experiments in our lab using differentially labeled mAbs to either the embryonic and neonatal or neonatal and adult MHCs have also been used to demonstrate the colocalization of multiple MHC isoforms within the same myofibril during periods of coexpression (unpublished observations). Furthermore, it has also been demonstrated that the majority of native thick filaments isolated from PM myofibrils of 19-d embryonic and from 44-d chickens contain multiple MHCs (Taylor and Bandman, 1989). Similar results have been found in *Caenorhabditis elegans* where two different myosin isoenzymes have been shown to be differentially distributed

1. *Abbreviations used in this paper*: DASE, double antibody sandwich ELISA; PM, pectoralis major.

within the same thick filament (Miller et al., 1983). Together, these studies suggest that different MHC isoforms can reside within the same thick filament and myofibrils of a single cell.

While colocalization of different PM MHC isoforms to the same thick filament and myofibril has been observed, it is unclear whether these different MHC isoforms can reside within the same myosin molecule. Since myosin molecules are dimers of the heavy chain, it is possible for myosins in PM cells expressing multiple MHCs to be heterodimers of the heavy chain subunit as has been shown for rat cardiac myosin (Hoh et al., 1979; Dechesne et al., 1987). However in studies of myosin isoforms from the body wall of *C. elegans*, two different myosin heavy chain subunits expressed in the same cell were found only as homodimers (Schachat et al., 1977, 1978). In an accompanying paper (Lowey et al., 1991), immunoaffinity chromatography and immunoelectron microscopy were used to demonstrate that chicken PM myosins were also predominantly homodimers. In this report we have used MHC isoform-specific mAbs to develop a double antibody sandwich ELISA (DASE) that enabled us to study the heavy chain subunit composition of myosin molecules and myosin rods. We also found that at 2-d posthatch when embryonic and neonatal MHC isoforms were coexpressed and at 40-d posthatch when neonatal and adult MHC isoforms were present essentially no heavy chains heterodimers were detected. We investigated the molecular basis for this observation by preparing chymotryptic rod fragments of embryonic, neonatal, and adult myosins. We found that equimolar mixtures of embryonic and neonatal rods and neonatal and adult rods denatured in guanidine failed to form heterodimeric rods upon renaturation by either dialysis or quick dilution. That subunit exchange could occur under these conditions was shown by denaturing a mixture of biotinylated neonatal rods and fluoresceinated neonatal rods and producing heterodimeric biotinylated-fluoresceinated neonatal rods upon renaturation. Thus, based on these results we propose that the MHC homodimer is the thermodynamically stable form of avian skeletal myosin and that there is no need to invoke compartmentalization or other cellular regulatory processes to explain the lack of heavy chains heterodimers in vivo.

Materials and Methods

DASE

Monoclonal antibodies 2E9, AB8, EB165, and B103 were purified with either a protein G column (Genex Corp., Gaithersburg, MD) or a protein A column (Pierce Chemical Co., Rockford, IL) according to the manufacturers instructions. Purified monoclonal antibodies were biotinylated with sulfosuccinimidyl 6-(biotinamido) hexanoate (Pierce Chemical Co.) according to the method of Gretch et al. (1987) and stored at 1 mg/ml in PBS.

DASE was performed according to the following method. Purified antibodies were diluted to 10 μg/ml in panacoat which was diluted 1:49 with water according to the manufacturers instructions (Panbaxy Laboratories, McLean, VA). 50 μl of the diluted antibody solution was placed into a well of a polystyrene microtiter plate and incubated overnight at room temperature in a humidified chamber. The next day each well was washed three times with 100 μl per well of PBS/Tween (PBS, 0.05% [vol/vol]Tween-20). Whole myosin, or myosin rod, was diluted to a concentration of 10 μg/ml in HSB/Tween (40 mM $Na_4P_2O_7$, 1 mM $MgCl_2$, 1 mM EGTA, 0.05% [vol/vol]Tween-20, pH 7.5 at 4°C). 50 μl of the diluted myosin was added to the wells of the microtiter plate containing the bound antibody and incubated 1–2 d at 4°C in a humidified chamber. Subsequently, the wells were washed three times with PBS/Tween as before and 50 μl of biotinylated mAb, diluted in PBS/Tween, was added. The microtiter plate was then incubated for 30 min at 37°C. After the incubation the wells were washed three times with PBS/Tween and 50 μl of HRP-streptavidin (Pierce Chemical Co.) (1 μg/ml in PBS/Tween) was added to each well and the plate was incubated for 15 min at 37°C. The plate was then washed twice with PBS/Tween for 30 s each and once for 5 min. After the washes 100 μl per well of ABTS peroxidase substrate (Kirkegard & Perry Laboratories, Gaithersburg, MD) was added to each well and incubated in the dark. The absorbance was read at 405 nm at 5-min intervals with a Titertek Multiscan MC ELISA plate reader (Flow Laboratories, McLean, VA). While the rate of color development differed for each of the biotinylated mAbs, the data shown is from a time when all data points were within the linear range of the assay. All data points are the average of triplicate determinations and error bars are given as 95% confidence levels.

In the sandwich ELISA for detection of biotinylated-fluoresceinated heterodimeric rod, affinity-purified polyclonal anti-fluorescein antibody (Biodesign Int., Kennebunkport, ME) was diluted to 20 μg/ml in 1× panacoat and adsorbed to the microtiter plate as described above for mAbs. Modified rod mixtures were diluted to 20 μg/ml in PBS/Tween and incubated in the wells for 30 min at 37°C. After washing the wells three times in PBS/Tween, bound biotinylated subunits were detected with streptavidin horseradish peroxidase (4 μg/ml) as described above.

Purification of Myosin

Myosin was purified from the pectoralis major muscle of White Leghorn chickens according to the method of Bandman et al. (1982) and further purified on a diethylaminoethyl-cellulose column as described by Margossian and Lowey (1982). Myosins were prepared from the PM of 15-d embryos (predominantly embryonic myosin), from the PM of 2-d chickens (a mixture of embryonic and neonatal myosin) from the PM of 8- and 12-d chickens (predominantly neonatal myosin), from the PM of 40-d-old chickens (a mixture of neonatal and adult myosin), and from the PM of 1- and 2-yr-old chickens (adult myosin).

Preparation and Purification of Myosin Rods

Embryonic, neonatal, and adult myosins were used to prepare myosin rod fragments. Digestion of the myosin was performed with α-chymotrypsin as described by Margossian and Lowey (1982). After digestion the myosin was either purified according to the method of Margossian and Lowey (1982) or the method of Bertazzon and Tsong (1989). When the method of Margossian and Lowey (1982) was used solution A was replaced by HSB (40 mM $Na_4P_2O_7$, 1 mM $MgCl_2$, 1 mM EGTA, pH 8.0 at 4°C). When the method of Bertazzon and Tsong (1989) was used the rods were resuspended in HSB. All myosin rod fragment preparations used were stored at 4°C in HSB.

Biotinylation of Myosin Rods

Myosin rods were biotinylated according to the method of Gretch et al. (1987) using sulfosuccinimidyl 6-(biotinamido)hexanoate (Pierce Chemical Co.) at a rod to sulfosuccinimidyl 6-(biotinamide)hexanoate molar ratio of 1:1 in HSB, pH 8.0.

Fluoresceination of Myosin Rods

Myosin rods were fluoresceinated with FITC isomer I. FITC was dissolved in acetone at a molar concentration of 50–100 times that of the rod solution. FITC was then added to the myosin rods to yield a final FITC-myosin rod molar ratio of ~2:1. The solution was allowed to incubate overnight at 4°C in the dark followed by extensive dialysis for 3–4 d against many changes of HSB, pH 8.0.

Denaturation and Renaturation Procedures

Equal quantities of either embryonic and neonatal or neonatal and adult myosin rod fragments were mixed and dialyzed against a solution of 8 M guanidine hydrochloride, 10 mM Tris, 10 mM DTT, pH 8.0 at room temperature in a microdialyzer (Pierce Chemical Co.). The final concentration of total myosin rods after dialysis was adjusted to 0.4 mg/ml for the dialysis experiments and 1 mg/ml for the dilution experiments assuming 100% recovery of protein from the microdialyzer. The myosin rods, in 8 M guanidine buffer, were incubated overnight at room temperature then either dialyzed or diluted into HSB containing 10 mM DTT, pH 8.0 at 4°C. Dialysis

of the myosin rod fragments was carried out for 3–4 h at 4°C using the microdialyzer. The final protein concentration after dialysis was adjusted to 0.1–0.2 mg/ml. In the dilution renaturation experiments myosin rod fragments in 8 M guanidine buffer were diluted 20-fold into HSB buffer containing 10 mM DTT, pH 8.0 at 4°C while gently vortexing. After either dialysis or dilution of the guanidine denatured myosin rods, the solutions were stored at 4°C until they were analyzed.

Polyacrylamide Gel Electrophoresis

SDS-PAGE was performed according to the method of Laemmli (1970). Native gel electrophoresis was performed on 1.5-mm slab gels run for 16–24 h at 33 mA according to the method of Waller and Lowey (1985). SDS-PAGE gels were stained overnight in Coomassie blue, destained in 10% acetic acid, 40% methanol until a major portion of the background disappeared, and then soaked in water until scanned with a laser densitometer or dried. Native gels were stained for 2–3 h in Coomassie blue, destained for 10 min in 10% acetic acid, 40% methanol, then further destained in 7% acetic acid until the background disappeared.

Results

Specificity of the Double Antibody Sandwich ELISA

To determine the MHC isoform composition of a myosin molecule we developed a double antibody sandwich ELISA (DASE) using mAbs to embryonic, neonatal, and adult MHC isoforms (Bandman, 1985; Cerny and Bandman, 1987; Bandman and Bennett, 1988). In this assay, a mAb was adsorbed onto the wells of a microtiter plate and myosin was retained only if it contained the epitope recognized by the mAb. Subsequently, a biotinylated mAb that recognized a different epitope on the same MHC was added and detected with streptavidin peroxidase. The specificity of this assay.is illustrated in Fig. 1. When an adult specific mAb was used to coat the plate, myosin isolated from the adult PM was bound, while myosin isolated from the PM of 15-d embryos was not and myosin isolated from PM of neonatal chickens gave a weak signal (Fig. 1 *C*). Similarly, when a neonatal specific mAb was used, myosin from the PM of neonatal chickens was bound while myosin from the PM of adult chickens was not and myosin from PM of 15-d embryos was weakly positive (Fig. 1 *B*). When an antibody to embryonic MHC was coated on the plate, myosin from PM of 15-d embryos was bound, to a lesser extent myosin from the PM of 12-d chickens was bound, and myosin from the PM of adult chickens gave a weak signal only at very high secondary antibody concentrations (Fig. 1 *A*). These results are essentially identical to standard ELISA studies that have shown the predominance of embryonic, neonatal, and adult myosins at similar stages of development (Bader et al., 1982; Winkelmann et al., 1983; Cerny and Bandman, 1987). Our results also confirm previous observations that some embryonic myosin persists in the PM of neonatal chickens (Cerny and Bandman, 1987) and suggest that low levels of neonatal myosin may be present in the PM of 15-d embryos and also persist in the PM of the adult chicken.

Heavy Chain Composition of Myosins from the Developing PM

The heavy chain composition of myosins from the developing PM was analyzed using DASE. When either the anti-embryonic mAb or anti-neonatal mAb was used to coat the plate, myosin isolated from the PM of 2-d chickens was bound (Fig. 2 *A*) indicating that both the embryonic and neo-

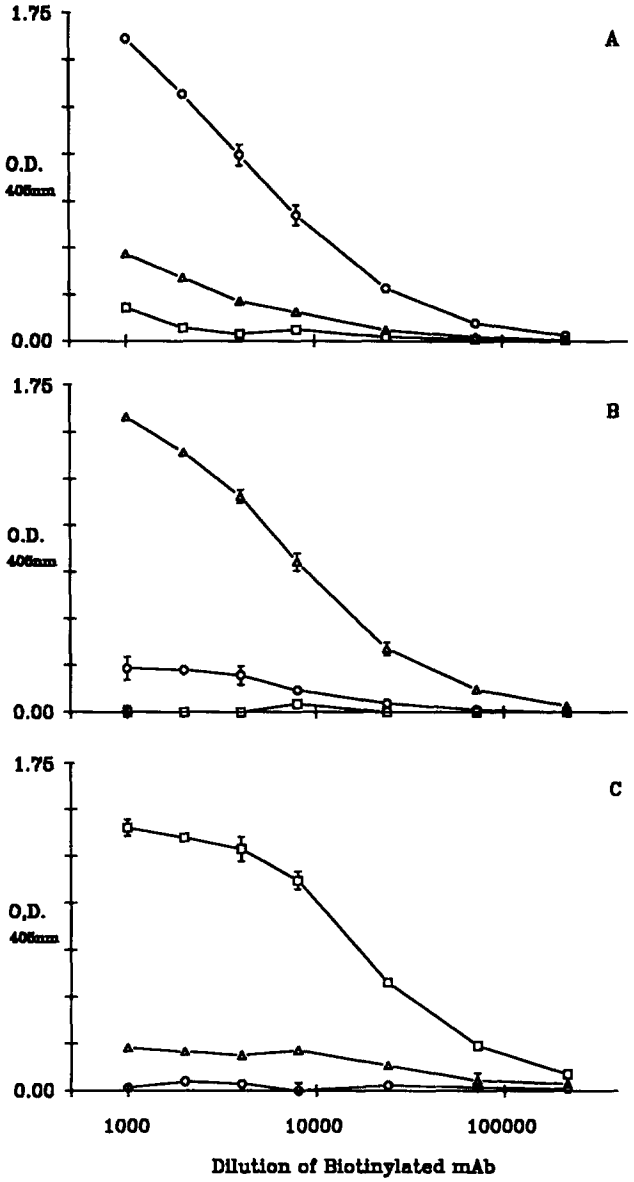

Figure 1. Specificity of double antibody sandwich ELISA. Polystyrene ELISA plates were coated with (*A*) mAb EB165, (*B*) mAb 2E9, and (*C*) mAb AB8 as described in Materials and Methods. Myosin from the PM of 15-d embryo (○), from the PM of 8- (in *C*) or 12-d-old (in *A* and *B*) chicken (△), or from the PM of adult chicken (□), was incubated in the wells overnight at 4°C. Biotinylated B103 mAb was used to detect bound myosin in *A* and *B*, and biotinylated EB165 mAb was used to detect bound myosin in *C*. The ELISA was developed with HRP-streptavidin and ABTS and read in a microtiter plate reader as described in Materials and Methods. To determine nonspecific binding of myosin, chromatographically purified mouse IgG was used to coat the wells in place of the anti-MHC mAbs. Values obtained from these controls were subtracted from those obtained with the mAbs. All data points are the average of triplicate determinations and error bars are given as 95% confidence intervals. The results demonstrate the specificity of the DASE employing EB165 and biotinylated B103 for embryonic myosin, 2E9 and biotinylated B103 for neonatal myosin, as well as AB8 and biotinylated EB165 for adult myosin.

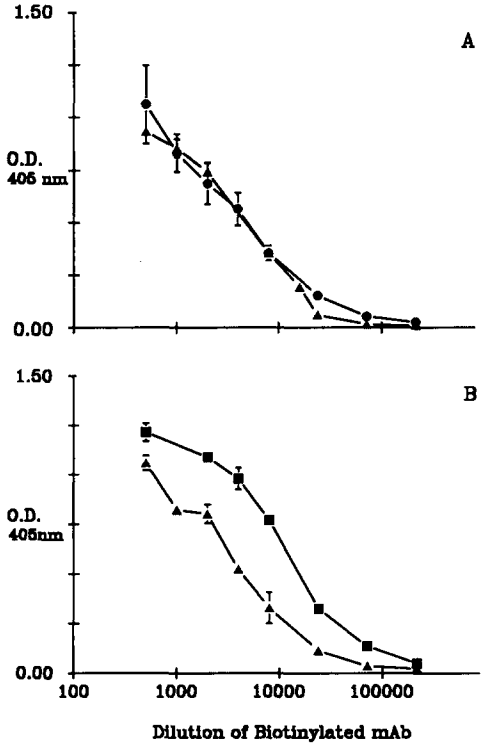

Figure 2. DASE of myosin from the 2- and 40-d PM. (*A*) Myosin from the PM of 2-d-old chicken was incubated in wells coated with either EB165 mAb (●), or 2E9 mAb (▲), and detected with biotinylated B103 mAb. (*B*) Myosin from the PM of 40-d old chicken was incubated in wells coated with either 2E9 mAb and detected with biotinylated B103 mAb (▲), or in wells coated with AB8 mAb and detected with biotinylated EB165 mAb (■). Non-specific binding was determined with nonimmune mouse IgG as described in the legend to Fig. 1 and all data points are the average of triplicate determinations. The results demonstrate that both embryonic and neonatal MHC epitopes are present in myosin from the PM of 2-d chicken and both neonatal and adult MHC epitopes are present in myosin from the PM of 40-d chicken.

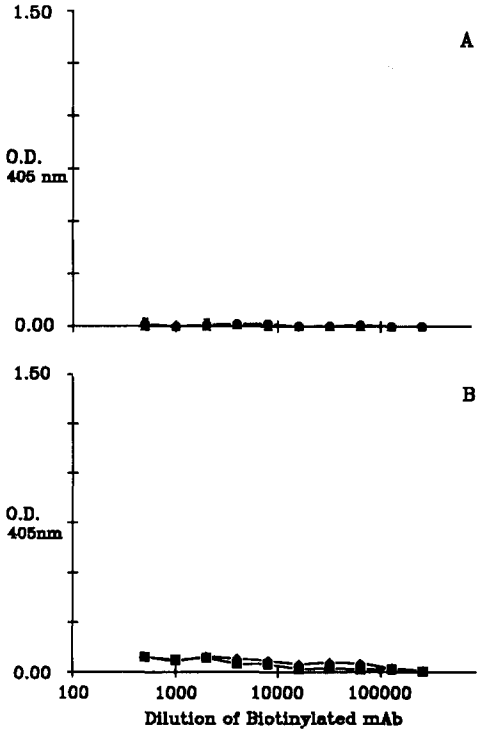

Figure 3. Myosins containing two different MHC isoforms are not present in the 2- and 40-d PM. DASE was used to determine whether myosin from the 2- and 40-d PM was composed of two different MHC isoforms. (*A*) EB165 mAb (●) or 2E9 mAb (▲) was adsorbed to the wells of a microtiter plate. After incubation with myosin from the 2-d PM, the presence of heterodimeric myosins was determined by incubating biotinylated 2E9 mAb in wells coated with EB165 mAb or biotinylated EB165 mAb in wells coated with 2E9 mAb. Nonspecific binding of biotinylated 2E9 mAb to embryonic MHC was determined by incubating myosin from the 15-d embryonic PM in wells coated with EB165 mAb and subtracting the values from those obtained using myosin from the PM of 2-d chicken. Nonspecific binding of biotinylated EB165 mAb to neonatal MHC was determined by incubating myosin from the 12-d chicken PM in wells coated with 2E9 mAb and subtracting the values from those obtained using myosin from the PM of 2-d chicken. All data points are the average of triplicate determinations and error bars are given as 95% confidence intervals. The results demonstrate that myosins bound by EB165 mAb do not react with 2E9 mAb and that myosins bound by 2E9 mAb do not react with EB165 mAb. (*B*) 2E9 mAb (♦) or AB8 mAb (■) was adsorbed to the wells of a microtiter plate. After incubation with myosin from the PM of 40-d chickens biotinylated AB8 mAb was used to detect myosin in wells coated with 2E9 mAb and biotinylated 2E9 mAb was used to detect myosin in wells coated with AB8 mAb. Nonspecific binding of biotinylated 2E9 mAb to adult MHC was determined by incubating myosin from the 2-yr adult chicken PM in wells coated with AB8 mAb and subtracting the values from those obtained using myosin from the PM of 40-d chicken. Nonspecific binding of biotinylated AB8 mAb to neonatal MHC was determined by incubating myosin from the 8-d chicken PM in wells coated with 2E9 mAb and subtracting the values from those obtained using myosin from the PM of 40-d chicken. All data points are the average of triplicate determinations and error bars are given as 95% confidence intervals. The results demonstrate that myosins bound by 2E9 mAb do not react with AB8 mAb and that myosins bound by AB8 mAb do not react with 2E9 mAb.

natal MHC isoforms were present. Similar qualitative results were obtained when myosin isolated from the PM of 0-, 1-, and 4-d chickens was used, although with increasing age less myosin was bound in wells coated with the anti-embryonic antibody and more myosin was bound in wells coated with the anti-neonatal antibody (data not shown). The heavy chain composition of myosin from PM of 40-d chickens was examined with DASE using the anti-neonatal and anti-adult mAbs. As shown in Figure 2B myosin was bound when either mAb was used to coat the microtiter plate, indicating that both the neonatal and adult MHC isoforms were present at this stage of PM development. When myosin samples from PM of 24-, 29-, 34-, 50-, and 60-d chickens were assayed, both mAbs bound myosins. With increasing age the anti-neonatal mAb bound less myosin while the anti-adult mAb bound more (data not shown). These results are in agreement with previous studies using standard and competition ELISAs to determine the MHC isoform content of chicken PM at different stages of development (Winkelmann et al., 1983; Cerny and Bandman, 1987).

The concentrations of the different MHC isoforms present in the 2- and 40-d myosin samples were estimated using DASE. Quantitation was performed by comparing results

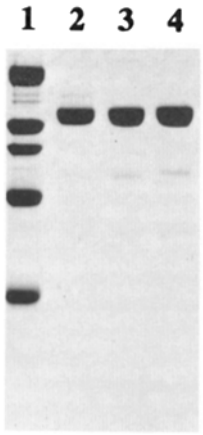

Figure 4. SDS-PAGE of purified myosin rods from embryonic, neonatal, and adult PM. Myosin rods were isolated as described in Materials and Methods and subjected to SDS-PAGE on 7.5% polyacrylamide gels. Lane *1*, molecular mass standards 200 kD (myosin heavy chain), 116.25 kD (β-galactosidase), 97.4 kD (phosphorylase B), 66.2 kD (bovine serum albumin), 45.0 kD (ovalbumin). Lane *2*, myosin rod prepared from myosin isolated from 15-d embryonic PM. Lane *3*, myosin rod prepared from the myosin isolated from PM of 12-d chicken. Lane *4*, myosin rod prepared from myosin isolated from PM of 1-yr adult chicken. Calculated molecular masses are 128.8 kD for the embryonic myosin rod, 127.6 kD for the neonatal myosin rod, and 127.2 kD for the adult myosin rod. The results demonstrate that myosin rods prepared from different MHC isoforms are of similar molecular mass and are >80% pure.

obtained from the 2- or 40-d myosin samples against standard curves of either the embryonic, neonatal, or adult myosins. In the myosin sample from the PM of 2-d chickens, embryonic MHC represented ~70% and the neonatal isoform ~30% of the total MHC present, while in the myosin sample from the PM of 40-d chickens, approximately equal quantities of neonatal and adult isoforms were present (data not shown). If MHC dimers were formed by random association of the different MHC isoforms present, then the PM of 40-d chickens would contain ~25% neonatal MHC dimers, approximately 25% adult MHC dimers, and ~50% neonatal-adult heterodimers. Based on the 70:30 ratio of embryonic and neonatal MHCs in the myosin sample from PM of 2-d chickens, ~50% would be dimers of the embryonic heavy chain, ~10% dimers of the neonatal heavy chain, and ~40% would be embryonic-neonatal heterodimers.

We determined whether heavy chain heterodimers were present in myosin samples from the PM of 2- and 40-d chickens using DASE. As before a mAb to either embryonic, neonatal, or adult MHC was used to coat the wells of a microtiter plate. After addition of the myosin sample a second biotinylated mAb was added that recognized a different MHC isoform than the one recognized by the mAb bound to the plate. For example, with the 2-d sample an anti-embryonic mAb was bound to the plate, and the biotinylated secondary mAb was anti-neonatal. Alternatively, the anti-neonatal mAb was bound to the plate and the biotinylated secondary mAb was anti-embryonic. Similarly, with the 40-d sample anti-neonatal mAb was coated in the well and biotinylated anti-adult mAb was the detecting antibody. Additionally, the anti-adult mAb was coated in the well and the biotinylated anti-neonatal mAb was the secondary antibody. As demonstrated in Fig. 3 *A* no myosins were detected in the sample from the PM of 2-d chickens that reacted with both the anti-embryonic and the anti-neonatal mAbs. This was true if the anti-embryonic mAb was used to coat the plate and biotinylated anti-neonatal mAb was used for detection or if the anti-neonatal mAb was used to coat the plate and biotinylated anti-embryonic mAb was used for detection. Similarly, very little myosin from the 40-d sample was detected that reacted with both anti-neonatal and anti-adult mAbs irrespective of which antibody was used to coat the plate (Fig. 3 *B*). Quantitative estimates of the amount of myosin that reacted with both anti-neonatal and anti-adult mAbs was <3% of the total myosin present (data not shown). Taken together with the results shown in Figs. 1 and 2, these observations indicate that essentially all myosins are composed of identical heavy chain subunits. Furthermore, the lack of heterodimeric myosins suggests that the MHC composition of myosin molecules is not the result of random aggregation of the two heavy chain subunits.

Mixtures of Myosin Rods Composed of Different MHC Isoforms Do Not Exchange

The lack of heterodimeric myosins in vivo could be the result of a cell-mediated process or the inability of different MHC isoforms to form stable dimers. To investigate the latter possibility we isolated and purified chymotryptic rod fragments of myosin from the PM of 15-d embryos, 12-d chickens, and adult chickens. The rod is composed of two subunits derived from MHC, each with an approximate size of 130-kD (Margossian and Lowey, 1982). As shown in Fig. 4, the molecular masses determined by SDS-PAGE for each isoform fragment are similar and each was >80% pure as determined by laser densitometry. The mobility of each of the native rods was also identical under native conditions (Fig. 5). To determine whether the subunits of the myosin

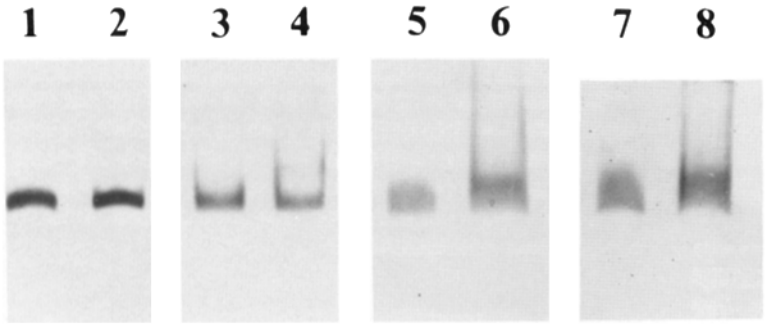

Figure 5. Nondenaturing PAGE of native and renatured myosin rods. Nondenaturing 4% polyacrylamide gels were run as described in Materials and Methods. Lane *1*, an equimolar mixture of embryonic and neonatal myosin rods. Lane *2*, an equimolar mixture of embryonic and neonatal myosin rods which were denatured in 8 M guanidine and subsequently renatured by dialysis. Lane *3*, an equimolar mixture of neonatal and adult myosin rods. Lane *4*, an equimolar mixture of neonatal and adult myosin rods which had been denatured in 8 M guanidine and renatured by dialysis. Lane *5*, an equimolar mixture of embryonic and neonatal myosin rods. Lane *6*, an equimolar mixture of embryonic and neonatal myosin rods that were denatured in 8 M guanidine and renatured by quick dilution. Lane *7*, an equimolar mixture of neonatal and adult myosin rods. Lane *8*, an equimolar mixture of neonatal and adult myosin rods which were denatured in 8 M guanidine and renatured by quick dilution. The results demonstrate that all myosin rods have similar electrophoretic mobilities on nondenaturing gels. In addition, myosin rod samples denatured in 8 M guanidine and subsequently renatured either by dialysis or dilution are electrophoretically indistinguishable from native rods.

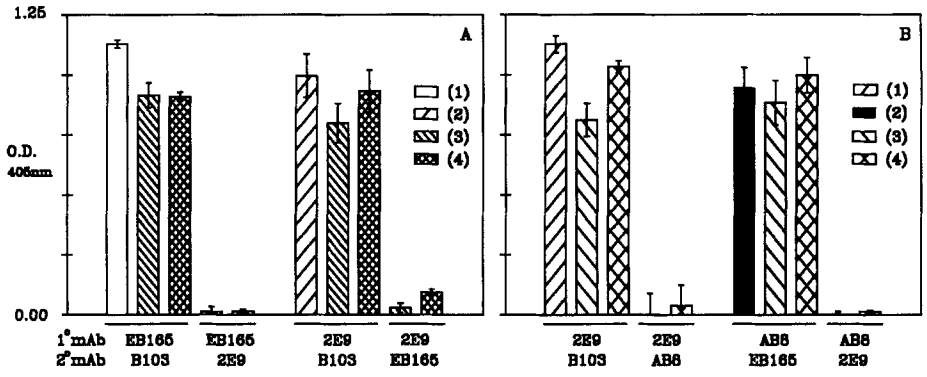

Figure 6. DASE of native and denatured myosin rod mixtures renatured by dialysis. DASE was used to analyze myosin rods, mixtures of myosin rods, and mixtures of myosin rods denatured in 8 M guanidine and renatured by dialysis as described in Materials and Methods. All data presented are the average of triplicate determinations. Error bars are given as 95% confidence intervals. 1° mAb indicates the antibody adsorbed to the microtiter plate and 2° mAb indicates the biotinylated mAb used to detect myosin bound. (*A*) Experiments with myosin rods from the embryonic and neonatal PM. (*1*) Rods prepared from myosin isolated from the PM of 15-d embryos; (*2*) rods prepared from myosin isolated from the PM of 12-d chicken, (*3*) an equimolar mixture of embryonic and neonatal myosin rods; (*4*) an equimolar mixture of embryonic and neonatal myosin rods denatured in 8 M guanidine and renatured by dialysis. (*B*) Experiments with myosin rods from the PM of 12-d chicken and 2-yr adult. (*1*) Rods prepared from myosin isolated from the PM of 12-d chicken; (*2*) rods prepared from myosin isolated from the PM of adult chicken; (*3*) an equimolar mixture of neonatal and adult myosin rods; (*4*) an equimolar mixture of neonatal and adult myosin rods denatured in 8 M guanidine and renatured by dialysis. Nonspecific binding was determined as described in the legend to Fig. 3 except myosin rods were used in place of whole myosin. The results demonstrate that the same antibodies used in DASE of whole myosin can be used to analyze the composition of myosin rods. Very little, if any, myosin rods which reacted with both EB165 and 2E9 in *A* and with 2E9 and AB8 in *B* were detected in the mixed rod sample and the denatured and renatured rod mixture. These results indicate that no subunit exchange has occurred under these conditions.

rods could exchange we mixed together embryonic and neonatal rods as well as neonatal and adult rods in equimolar quantities and dialyzed them into 8 M guanidine buffer containing 10 mM DTT. We kept these solutions at room temperature for at least 18 h to ensure the denaturation of the myosin rods and reduction of any disulfide bonds. The denatured myosin rod mixtures were then either dialyzed or rapidly diluted 20-fold into HSB, 10 mM DTT at 4°C. After this, the concentration of recovered rods was determined by either SDS-PAGE analysis or DASE. Recovery was routinely in excess of 90% for the dialyzed rods and from 50 to 90% for the diluted rods (data not shown).

Nondenaturing polyacrylamide gel electrophoresis was used to determine if the rods refolded into structures with the same electrophoretic mobility as native rods. As shown by nondenaturing gel electrophoresis in Fig. 5, the rods that had been denatured and renatured migrated identically to native rods not exposed to guanidine. This was true for both the embryonic and neonatal and the neonatal and adult myosin rod mixtures. Furthermore, we saw no additional protein that was unable to enter the gel in the renatured myosin rod samples as compared to the native rod samples. This suggested that no large aggregates were formed upon renaturation by dialysis or dilution of the denatured myosin rods. On occasion a slower migrating band was observed in some electropherograms (see Fig. 5, lane *4*). While we cannot account for the appearance of this band, its presence was not reproducible, and was also found in native rod samples indicating it was not a result of the renaturation procedure.

Previous studies demonstrated that the mAbs used in this study reacted with epitopes within the myosin rod (Bandman et al., 1989). As shown in Fig. 6, the same antibodies used in DASE of whole myosins could also be used to detect myosin rods composed of embryonic, neonatal, and adult MHC fragments (samples 1 and 2 in Fig. 6, *A* and *B*). In a sample composed of an equimolar mixture of embryonic and neonatal rods, DASE could detect embryonic rods and neonatal rods, but did not detect rods which reacted with both anti-embryonic and anti-neonatal mAbs irrespective of which was the primary antibody coated in the well. Essentially identical results were obtained with an equimolar mixture of embryonic and neonatal rods that had been denatured and renatured by dialysis. When the anti-neonatal antibody was used to coat the well a small signal above background was observed when using the biotinylated anti-embryonic antibody. However, no significant signal above background was found when the anti-embryonic antibody was coated in the well and the biotinylated anti-neonatal antibody was the secondary detecting antibody. Thus while we do not rule out the possibility of the formation of some rods which react with both antibodies, the vast majority of renatured rods contain either embryonic epitopes or neonatal epitopes. In the sample composed of an equimolar mixture of neonatal and adult rods, DASE could detect the homodimeric neonatal and adult rods, but essentially no rods were detected which reacted with both the anti-neonatal and anti-adult mAbs. Again identical results were obtained with the equimolar mixture of neonatal and adult rods that had been denatured and renatured by dialysis. Equivalent results were also obtained when renaturation was carried out by quick dilution (data not shown). Assuming that in the presence of guanidine random exchange is possible, 50% of the renatured rods would have been heterodimeric when an equimolar mixture of two isoforms was denatured. Since we observed very little, if any, heterodimers, either subunit exchange was not possible under these conditions, or only homodimers could be formed upon renaturation.

Subunits of Myosin Rods of the Same MHC Isoform Can Exchange

To determine whether subunit exchange was possible in guanidine buffer, an aliquot of neonatal rods was biotinylated and a second aliquot of neonatal rods was fluoresceinated.

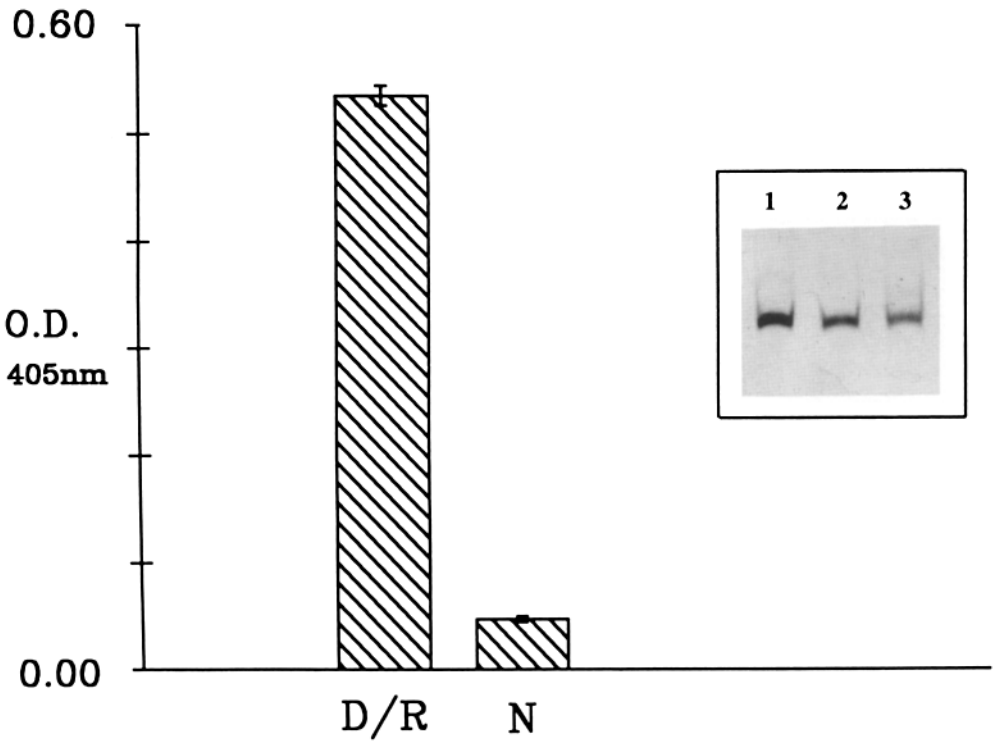

Figure 7. Subunits of biotinylated neonatal myosin rods and subunits of fluoresceinated neonatal myosin rods can exchange in 8 M guanidine. Neonatal rods were either biotinylated or fluoresceinated and mixtures were analyzed by DASE as described in methods. (*N*) An equimolar mixture of biotinylated rods and fluoresceinated rods mixed immediately before the assay. (*D/R*) An equimolar mixture of biotinylated rods and fluoresceinated rods was denatured in 8 M guanidine and renatured by dialysis. Nonspecific binding of HRP-streptavidin to fluoresceinated rods was subtracted from all values. All data presented are the average of triplicate determinations and error bars are given as 95% confidence intervals. (*Inset*) Nondenaturing PAGE of modified neonatal myosin rods. Lane *1*, unmodified neonatal myosin rods; lane *2*, a mixture of biotinylated neonatal rods and fluoresceinated neonatal rods; lane *3*, a mixture of biotinylated neonatal rods and fluoresceinated neonatal rods denatured in 8 M guanidine and renatured by dialysis. Neither modification significantly affected the electrophoretic mobility of the rod. The bar graph demonstrates that subunit exchange occurred in the mixed sample denatured in 8 M guanidine and renatured by dialysis, while little exchange occurred without denaturation.

We then mixed equal quantities of the biotinylated and fluoresceinated rods and subjected them to the denaturation and dialysis procedure described above. After renaturation the samples were subjected to nondenaturing polyacrylamide gels electrophoresis. As shown in the inset of Fig. 7, the modified rods were electrophoretically similar to the native neonatal rods and the modified rods subjected to denaturation and renaturation were indistinguishable from either the native or modified rods.

If exchange of a myosin rod subunit could occur in guanidine, a mixture of biotinylated and fluoresceinated neonatal rods would result in the formation of a population of myosin rods composed of one biotinylated subunit and one fluoresceinated subunit. We assayed for the formation of heterodimeric biotinylated and fluoresceinated rods using DASE. Fluoresceinated rods were captured by coating the well with an anti-fluorescein polyclonal antibody and the biotinylated rods detected using HRP-streptavidin. Thus this assay would not detect dimers of biotinylated subunits since they would not bind to the plate, nor would dimers of fluoresceinated subunits be detectable since they would not bind HRP-streptavidin. As shown in Fig. 7, the presence of biotinylated-fluoresceinated rods was only barely detectable when a mixture of biotinylated neonatal rods and fluoresceinated neonatal rods were mixed together. However, if the mixture of biotinylated and fluoresceinated neonatal rods were denatured in 8 M guanidine buffer and then allowed to renature by dialysis there was a large increase in the presence of biotinylated-fluoresceinated rods. The identical result was also obtained if adult rods were biotinylated and fluoresceinated and subjected to the same analysis, indicating that the ability to exchange was not a property specific to the neonatal isoform (data not shown). Some subunit exchange, albeit to a lesser degree, was also observed if biotinylated and fluoresceinated rods were incubated overnight at room temperature, but no exchange was observed if the modified rods were incubated overnight at 4°C (data not shown). These results demonstrate that myosin rod subunits are competent to exchange in guanidine and suggests that the inability to detect heterodimeric rods composed of different MHC isoforms in Figure 6 is due to the inability of dissimilar subunits to form myosin rods.

Discussion

Our conclusion that MHC isoforms coexpressed in the same fiber of chicken PM exist as homodimers is based on the DASE results presented in Figs. 1–3. In Fig. 1 and 2 we demonstrated that myosin molecules bound by a mAb adsorbed to a microtiter plate could react with a second mAb to a different epitope on the same MHC isoform. However, as shown in Fig. 3, when two mAbs which react with different MHC isoforms were used in DASE essentially no myosin was detected. Although we concluded that no heterodimeric myosins were present, an alternative explanation is that the proximity of the binding sites for the mAbs used in Fig. 3 interfered with the DASE assay. However, this explanation is unlikely because we found that the same mAb can be used for both capture and detection of myosin in a single DASE, albeit with reduced sensitivity (data not shown). In addition,

we are mapping the positions of the epitopes in MHCs and our studies indicate that the anti-neonatal and anti-adult mAb epitopes are separated by at least 600 amino acids (unpublished observations). Since the antibodies used to demonstrate the specificity of the DASE in Fig. 1 react with epitopes that are closer than 600 amino acids (unpublished observations), the lack of myosin molecules that react with anti-neonatal and anti-adult mAbs can not be explained by the proximity of the two epitopes. Furthermore, similar conclusions regarding the lack of neonatal and adult MHC heterodimers in developing PM have been reached using different techniques (Lowey et al., 1991). All of these observations support our conclusion that during periods of MHC isoform transition in avian skeletal muscle, myosins are homodimeric in their heavy chain subunit composition.

The lack of MHC heterodimer formation in vivo could have been the result of a cell-mediated process such as cotranslational assembly (Isaacs and Fulton, 1987) or mRNA compartmentalization (Lawrence and Singer, 1986; Hall and Ralston, 1989; Pavlath et al., 1989). However, based on our studies of the myosin rod there is no need to invoke cellular regulatory processes to explain the lack of MHC heterodimers in vivo. We have demonstrated that homodimers are the thermodynamically stable form of the myosin rod since no subunit exchange was detected when mixtures of denatured rods were renatured either by dialysis or dilution. Extending the properties of the myosin rods to the intact myosin molecule would explain the lack of heterodimeric myosins in vivo. Unfortunately, it is not possible to test this hypothesis since intact myosin molecules have not been successfully renatured (Bertazzon and Tsong, 1989; unpublished observations). Nevertheless, there is no evidence to implicate the myosin head in rod assembly. In addition to our results (Fig. 5), it has been shown that thermally denatured myosin rods could refold correctly (Bertazzon and Tsong, 1989). Furthermore, recent studies demonstrated that MHC tail fragments expressed in *Escherichia coli* formed structures with similar properties to native myosin filaments (De Lozanne et al., 1987) lending further support to the proposal that the structural information for correct assembly of the myosin dimer lies principally within the amino acid sequence of the rod.

Other α-helical coiled-coil proteins also contain subunits that exist as different isoforms. In the case of tropomyosin, one of the factors that regulates the composition of the dimer is the sequence similarity of the isoforms. The existence of $\alpha\beta$ tropomyosin heterodimers observed in vivo was attributed to the 87% sequence similarity of α and β isoforms (Mak et al., 1980). A similar process may also regulate the assembly of the α-helical coiled-coil of the myosin rod. The lack of myosin heterodimers composed of MHC A and MHC B in nematode muscle (Schachat et al., 1978) is likely related to relatively low sequence homology ($\sim$61%) of the isoforms (Dibb et al., 1989). Conversely, the 93% similarity of the rat α and β cardiac MHCs, can explain the existence of heterodimeric myosins in the rat heart (Hoh et al., 1979; Dechesne et al., 1987; McNally et al., 1989). However, factors other than sequence similarity must also play a role in dimer stability. The preponderance of the $\alpha\beta$ tropomyosin heterodimer in frog and rabbit skeletal muscle was explained by observations that the $\beta\beta$ homodimer was thermodynamically less stable than the $\alpha\beta$ heterodimer at physiological temperatures (Bronson and Schachat, 1982; Brown and Schachat, 1985; Lehrer et al., 1989). Thus sequence similarity is necessary but not sufficient to form stable dimers in vivo. Similar conclusions have also been reached concerning other α-helical coiled-coil proteins (O'Shea et al., 1989). Given the high homology of skeletal muscle myosin rods in vertebrates (Stedman et al., 1990) and our observations, factors other than sequence similarity must also play a role in myosin assembly.

Different enzymatic activities of myosin could provide an explanation for the diversity of MHC isoforms. It is clear that motility is proportional to myosin ATPase activity in fast and slow muscle in vivo (Barany, 1967) and in vitro (Sheetz and Spudich, 1983; Sheetz et al., 1984). However, the various myosins expressed in the chicken PM have been found to exhibit identical enzymatic activities (Lowey, 1986). If the unique amino acid composition in the rod of each MHC subunit regulates myosin assembly, then the sequence diversity that exists in the rod likely has functional ramifications in myosin filament assembly as well. Previous observations that certain myosin isoforms are localized in specific regions of the thick filament in nematodes (Epstein et al., 1986) and in developing chicken muscle (Taylor and Bandman, 1989) would support this hypothesis.

We propose that the inability of skeletal MHCs to form heterodimers provides a mechanism for compartmentalizing isoforms which may be important for fibrillogenesis in vivo. Studies on the assembly of *Acanthamoeba* myosin-II minifilaments demonstrated that protomeric bipolar filaments arose from successive dimerization of anti-parallel dimers (Sinard et al., 1989). Studies on synthetic filaments from skeletal muscle have shown that a parallel dimer was involved in the elongation phase of assembly (Davis et al., 1982). Assuming that an equilibrium exists between myosin monomers and the assembly intermediates, differences in the composition of the rod regions among the various isoforms could effect the equilibria and hence the proportion of the different forms. If this mechanism is correct for thick filament assembly in vivo, this could explain our previous observation of the non-random distribution of the neonatal MHC isoform in thick filament isolated from the developing PM (Taylor and Bandman, 1989). The capability to capture these intermediates (Davis et al., 1982) combined with our ability to analyze their MHC composition by DASE provides the basis for testing this hypothesis.

That myosins in the PM are restricted to being homodimers may also be necessary for their specific interaction with myosin binding proteins. Recent studies have shown that C protein is a member of the immunoglobulin superset of proteins (Einheber and Fischman, 1990) and has a precisely defined position along the thick filament in the myofibril in the PM (Bennett et al., 1986). C protein also exists as multiple isoforms and undergoes isoform transitions in the chicken PM similar to myosin (Takano-Ohmuro et al., 1989). If there are specific interactions between myosin binding proteins and different myosin isoforms, the lack of heterodimeric myosins and the coordinate switching among other contractile proteins (Masaki et al., 1982; Obinata, 1984) may facilitate transitions within the myofibril. Clearly, further studies are necessary in order to clarify the role of dif-

ferent MHC isoforms in fibrillogenesis and turnover before we may understand the advantage that the homodimeric structure of myosin provides in the PM.

The authors would like to thank Macdonald Wick for his assistance with development of the double antibody ELISA procedure, Dr. Susan Lowey for her assistance with the preparation of the chymotryptic rods and other helpful discussions, Dr. Laurie Quinn for her helpful suggestions, and Dr. Laurie Quinn, Lauri Herman, Maria Arrizubieta, and William Tidyman for supplying us with the locations of the mAb epitopes.

This research was supported by National Institutes of Health (NIH) grant AG08573 and a grant from the Muscular Dystrophy Association to E. Brandman. B. Kerwin was supported by predoctoral traineeship NIH grant GM07377.

Received for publication 10 December 1990 and in revised form 23 January 1991.

References

Bader, D., T. Masaki, and D. A. Fischman. 1982. Immunochemical analysis of myosin heavy chain during avian myogenesis in vivo and in vitro. *J. Cell Biol.* 95:763–770.

Bandman, E. 1985. Continued expression of neonatal myosin in adult dystrophic skeletal muscle. *Science (Wash. DC).* 227:780–782.

Bandman, E., and T. Bennett. 1988. Diversity of fast myosin heavy chain expression during development of gastrocnemius, bicep brachii, and posterior latissimus dorsi muscles in normal and dystrophic chickens. *Dev. Biol.* 130:220–231.

Bandman, E., R. Matsuda, and R. C. Strohman. 1982. Developmental appearance of myosin heavy and light chain isoforms *in vivo* and *in vitro* in chicken skeletal muscle. *Dev. Biol.* 93:508–518.

Barany, M. 1967. ATPase activity of myosin correlated with speed of muscle shortening. *J. Gen. Physiol.* 50:197–218.

Bennett, P., R. Craig, R. Starr and G. Offer. 1986. The ultrastructural location of C-protein, X-protein, and H-protein in rabbit muscle. *J. Muscle Res. Cell Motil.* 7:550.

Bertazzon, A., and T. Y. Tsong. 1989. High-resolution differential scanning calorimetric study of myosin, functional domains, and supramolecular structures. *Biochemistry.* 28:9784–9790.

Bronson, D. D., and F. H. Schachat. 1982. Heterogeneity of contractile proteins. Differences in tropomyosin in fast, mixed, and slow skeletal muscles of the rabbit. *J. Biol. Chem.* 257:3937–3944.

Brown, H. R., and F. H. Schachat. 1985. Renaturation of skeletal muscle tropomyosin: implications for in vivo assembly. *Proc. Natl. Acad. Sci. USA.* 82:2359–2363.

Buckingham, M. E. 1985. Actin and myosin multigene families: their expression during the formation of skeletal muscle. *Essays Biochem.* 20:77–109.

Cerny, L., and E. Bandman. 1987. Expression of myosin heavy chain isoforms in regenerating myotubes of innervated and denervated chicken pectoral muscle. *Dev. Biol.* 119:350–362.

Cooke. R. 1986. The mechanism of muscle contraction. *CRC Crit. Rev. Biochem.* 21:53–118.

Crow, M. T., and F. E. Stockdale. 1986. The developmental program of fast myosin heavy chain expression in avian skeletal muscle. *Dev. Biol.* 118:333–342.

Davis, J. S., J. Buck, and E. P. Greene. 1982. The myosin dimer: an intermediate in the self-assembly of the thick filament of vertebrate skeletal muscle. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 40:293–297.

Dechesne, C. A., P. Bouvagnet, D. Walzthony, and J. J. Leger. 1987. Visualization of cardiac ventricular myosin heavy chain homodimers and heterodimers by monoclonal antibody epitope mapping. *J. Cell Biol.* 105:3031–3037.

De Lozanne, A., C. H. Berlot, L. A. Leinward, and J. A. Spudich. 1987. Expression in *Escherichia coli* of a functional *Dictyostelium* myosin tail fragment. *J. Cell Biol.* 105:2999–3005.

Dibb, N. J., I. N. Maruyama, M. Krause, and J. Karn. 1989. Sequence analysis of the complete *Caenorhabditis elegans* myosin heavy chain gene family. *J. Mol. Biol.* 205:603–613.

Einheber, S., and D. A. Fischman. 1990. Isolation and characterization of a cDNA clone encoding avian skeletal muscle C-protein: an intracellular member of the immunoglobulin superfamily. *Proc. Natl. Acad. Sci. USA.* 87:2157–2161.

Epstein, H. F., I. Ortiz, and T. Mackinnon. 1986. The alteration of myosin isoform compartmentation in specific mutants of *Caenorhabditis elegans*. *J. Cell Biol.* 103:985–993.

Gauthier, G. 1990. Differential distribution of myosin isoforms among the myofibrils of individual developing muscle fibers. *J. Cell Biol.* 110:693–701.

Gretch, D. R., M. Suter, and M. F. Stinski. 1987. The use of biotinylated monoclonal antibodies and streptavidin affinity chromatography to isolate herpesvirus hydrophobic proteins or glycoproteins. *Anal. Biochem.* 163:270–277.

Hall, Z. W., and E. Ralston. 1989. Nuclear domains in muscle cells. *Cell.* 59:771–772.

Harrington, W. F., and M. E. Rodgers. 1984. Myosin. *Annu. Rev. Biochem.* 53:35–73.

Hoh, J. F. Y., G. P. S. Yeoh, M. A. W. Thomas, and L. Higginbottom. 1979. Structural differences in the heavy chains of rat ventricular myosin isoenzymes. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 97:330–334.

Isaacs, W. B., and A. B. Fulton. 1987. Cotranslational assembly of myosin heavy chain in developing cultured skeletal muscle. *Proc. Natl. Acad. Sci. USA.* 84:6174–6178.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.

Lawrence, J. B., and R. H. Singer. 1986. Intracellular localization of messenger RNAs for cytoskeletal proteins. *Cell.* 45:407–415.

Lehrer, S. S., Y. Qian, and S. Hvidt. 1989. Assembly of the native heterodimer of *Rana esculenta* tropomyosin by chain exchange. *Science (Wash. DC).* 246:926–928.

Lowey, S. 1986. Cardiac and skeletal muscle myosin polymorphism. *Med. Sci. Sports Exerc.* 18:284–291.

Lowey, S., G. S. Waller, and E. Bandman. 1991. Neonatal and adult myosin heavy chains form homodimers during avian skeletal muscle development. *J. Cell Biol.* 113:303–310.

Mak, A. S., L. B. Smillie, and G. R. Stewart. 1980. A comparison of the amino acid sequences of rabbit skeletal muscle α- and β-tropomyosins. *J. Biol. Chem.* 255:3647–3655.

Margossian, S. S., and S. Lowey. 1982. Preparation of myosin and its subfragments from rabbit skeletal muscle. *Methods Enzymol.* 85:55–71.

Masaki, T., D. Bader, F. C. Reinach, T. Shimizu, T. Obinata, S. A. Shafiq, and D. A. Fischman. 1982. Monoclonal antibody analysis of myosin heavy chain and C-protein isoforms during myogenesis. In Muscle Development: Molecular and Cellular Control. M. L. Pearson and H. F. Epstein, editors. Cold Spring Laboratory, Cold Spring Harbor, NY. 405–417.

McLachlan, A. D. 1984. Structural implications of the myosin amino acid sequence. *Annu. Rev. Biophys. Bioeng.* 13:167–189.

McNally, E. M., R. Kraft, M. Bravo-Zehnder, D. A. Taylor, and L. A. Leinward. 1989. Full-length rat alpha and beta cardiac myosin heavy chain sequences. Comparisons suggest of molecular basis for functional differences. *J. Mol. Biol.* 210:665–671.

Miller, D. M., III, I. Ortiz, G. C. Berliner, and H. F. Epstein. 1983. Differential localization of two myosins within nematode thick filaments. *Cell.* 34:477–490.

Moriarity, D. M., K. J. Barringer, J. B. Dodgson, H. E. Richter, and R. B. Young. 1987. Genomic clones encoding chicken myosin heavy chain genes. *DNA (NY).* 6:91–99.

Nguyen, H. T., R. M. Gubits, R. M. Wydro, and B. Nadal-Ginard. 1982. Sarcomeric myosin heavy chain is coded by a highly conserved multigene family. *Proc. Natl. Acad. Sci. USA.* 79:5230–5234.

Obinata, T., F. C. Reinach, D. M. Bader, T. Masaki, S. Kitani, and D. A. Fischman. 1984. Immunochemical analysis of C-protein isoform transitions during development of chicken skeletal muscle. *Dev. Biol.* 101:116–124.

O'Shea, E. K., R. Rutkowski, W. F. Stafford III, and P. S. Kim. 1989. Preferential heterodimer formation by isolated leucine zippers from fos and jun. *Science (Wash. DC).* 245:646–648.

Pavlath, G. K., K. Rich, S. G. Webster, and H. M. Blau. 1989. Localization of muscle gene products in nuclear domains. *Nature (Lond.).* 337:570–573.

Robbins, J., T. Horan, J. Gullick, and K. Kropp. 1986. The myosin heavy chain gene family. *J. Cell Biol.* 261:6606–6612.

Schachat, F., R. L. Garcea, and H. F. Epstein. 1978. Myosins exist as homodimers of heavy chains: demonstration with specific antibody purified by nematode mutant myosin affinity chromatography. *Cell.* 15:405–411.

Schachat, F. H., H. E. Harris, and H. F. Epstein. 1977. Two homogeneous myosins in body-wall muscle of *Caenorhabditis elegans*. *Cell.* 10:721–728.

Sheetz, M. P., and J. A. Spudich. 1983. Movement of myosin-coated fluorescent beads on actin cables *in vitro*. *Nature (Lond.).* 303:31–35.

Sheetz, M. P., R. Chasan, and J. A. Spudich. 1984. ATP-dependent movement of myosin in vitro: characterization of a quantitative assay. *J. Cell Biol.* 99:1867–1871.

Sinard, J. H., W. F. Stafford, and T. D. Pollard. 1989. The mechanism of assembly of *Acanthamoeba* myosin-II minifilaments: minifilaments assemble by three successive dimerization steps. *J. Cell Biol.* 109:1537–1547.

Stedman, H. H., M. Eller, E. H. Jullian, S. H. Fertels, S. Sarkar, J. E. Sylvester, A. M. Kelly, and N. A. Rubinstein. 1990. The human embryonic myosin heavy chain: complete primary structure reveals evolutionary relationships with other developmental isoforms. *J. Biol. Chem.* 265:3568–3576.

Takano-Ohmuro, H., S. M. Goldfine, T. Kojima, T. Obinata, and D. A. Fischman. 1989. Size and charge heterogeneity of C-protein isoforms in avian skeletal muscle. Expression of six different isoforms in chicken muscle. *J. Muscle Res. Cell Motil.* 10:369–378.

Taylor, L. D., and E. Bandman. 1989. Distribution of fast myosin heavy chain

isoforms in thick filaments of developing chicken pectoral muscles. *J. Cell Biol.* 108:533–542.

Van Horn, R., and M. T. Crow. 1989. Fast myosin heavy chain expression during the early and late embryonic stages of chicken skeletal muscle development. *Dev. Biol.* 134:279–288.

Waller, G. S., and S. Lowey. 1985. Myosin subunit interactions: localization of the alkali light chains. *J. Cell Biol.* 269:14368–14373.

Winkelmann, D. A., S. Lowey, and J. L. Press. 1983. Monoclonal antibodies localize changes on myosin heavy chain isozymes during avian myogenesis. *Cell.* 34:295–306.