

Visualization of Cardiac Ventricular Myosin Heavy Chain Homodimers and Heterodimers by Monoclonal Antibody Epitope Mapping

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Abstract. Two mAbs, one specific for cardiac α -myosin heavy chains (MHC) and the other specific for cardiac β -MHC, were used to investigate the heavy-chain dimeric organization of rat cardiac ventricular myosin. Epitopes of the two mAbs were mapped on the myosin molecule by electron microscopy of rotary shadowed mAb-myosin complexes. mAbs were clearly identifiable by the different locations of their binding sites on the myosin rod. Thus, myosin molecules could be directly discriminated according to their α - or β -MHC content. $\alpha\alpha$ -MHC and $\beta\beta$ -MHC homodimers were visualized in complexes consisting of two molecules of the same mAb bound to one myosin molecule. By simultaneously using the α -MHC-specific

mAb and the β -MHC-specific mAb, $\alpha\beta$ -MHC heterodimers were visualized in complexes formed by one molecule of each of the two mAbs bound to one myosin molecule. Proportions of $\alpha\alpha$ - and $\beta\beta$ -MHC homodimers and $\alpha\beta$ -MHC heterodimers were estimated from quantifications of mAb-myosin complexes and compared with the proportions given by electrophoreses under nondenaturing conditions. This visualization of cardiac myosin molecules clearly demonstrates the arrangement of α - and β -MHC in $\alpha\alpha$ -MHC homodimers, $\beta\beta$ -MHC homodimers, and $\alpha\beta$ -MHC heterodimers, as initially proposed by Hoh, J. F. Y., G. P. S. Yeoh, M. A. W. Thomas, and L. Higginbottom (1979).

THE myosin molecule is a hexamer, consisting of two heavy chains and four light chains, and is highly polymorphic. The evidences of the multiple forms of myosin in the heart were revealed in the rabbit by peptide mapping (Flink and Morkin, 1977), and in the rat by immunological techniques (Schwartz et al., 1978) and nondenaturing electrophoresis (Hoh et al., 1978). The polymorphism of cardiac myosin arises from the occurrence of two heavy-chain isoforms, called α and β , which were first demonstrated in the rat (Hoh et al., 1979). It has been found later that α - and β -myosin heavy chains (MHCs)¹ are encoded by two different genes in the rat (Mahdavi et al., 1982) and rabbit (Sinha et al., 1982). No complete amino acid sequence of cardiac MHC has yet been published, but partial α - and β -MHC primary sequences have either been deduced from nucleotide sequences or determined from peptide fragments in various species (Mahdavi et al., 1982; Sinha et al., 1982; Flink and Morkin, 1984; Kavinsky et al., 1984; Mahdavi et al., 1984; Sinha et al., 1984; Lichter et al., 1986; Liew et al., 1986). These sequences indicate a great homology between α - and β -MHC. Functionally speaking, myosin with α -MHC has a Ca²⁺ ATPase activity that is three times higher than that of

myosin with β -MHC (Pope et al., 1980). The physiological importance of the two MHC isoforms, which differ in their primary structures and enzymatic activities, is suggested by the fact that their expression varies according to animal species, development, hormonal state, hemodynamic characteristics, and intramyocardial localization (for a review see Swynghedauw, 1986).

A series of reports using immunolabeling in different species indicate that α - and β -MHC can be expressed not only in separate fibers but also in the same cardiac fiber (Samuel et al., 1983; Bouvagnet et al., 1984; Gorza et al., 1984; Tsuchimochi et al., 1984; Yazaki et al., 1984; Bouvagnet et al., 1985; Dechesne et al., 1985b; Everett, 1986; Kuro-o et al., 1986; Dechesne et al., 1987). The coexpression of α - and β -MHC in the same fiber raises the question of the relative arrangement of these MHC in such fibers. On the basis of electrophoretic separation of rat ventricular myosin under nondenaturing conditions into three bands, V₁, V₂, and V₃, Hoh et al. (1979) have proposed that α - and β -MHC can be arranged in three forms: $\alpha\alpha$ -MHC homodimers, $\alpha\beta$ -MHC heterodimers, and $\beta\beta$ -MHC homodimers, respectively. Thus, according to these results, when α - and β -MHC are expressed in separate fibers, they are necessarily expressed in homodimeric forms; but when α - and β -MHC are coexpressed in the same fiber, it is not known whether myosin occurs in the form of a mixture of $\alpha\alpha$ - and $\beta\beta$ -MHC homodimers, or only $\alpha\beta$ -MHC heterodimers, or both. In addition, the existence of $\alpha\beta$ -MHC heterodimers raises the problem

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1. Abbreviation used in this paper: MHC, myosin heavy chain.

of the functioning and role of such myosin molecules within thick filaments. Therefore the homodimeric and/or heterodimeric organization of α - and β -MHC is a very important aspect of the understanding of the functional role of cardiac myosin isoforms at the molecular level.

mAbs raised against cardiac MHC isoforms were used in electron microscopy after rotary shadowing (Slyter and Lowey, 1967; Walzthöny et al., 1983) to investigate the homodimeric and heterodimeric MHC organization of rat cardiac ventricular myosin. By simultaneously labeling myosin molecules with one α -MHC-specific mAb (designated here as anti- α) and one β -MHC-specific mAb (designated here as anti- β), we were able to visualize both $\alpha\alpha$ - and $\beta\beta$ -MHC homodimers and $\alpha\beta$ -MHC heterodimers. The proportions of $\alpha\alpha$ -, $\beta\beta$ -, and $\alpha\beta$ -MHC dimers were determined in three myosins exhibiting either one V_1 band, one V_3 band, or three $V_1V_2V_3$ bands on electrophoresis under nondenaturing conditions. This work constitutes a visual demonstration of the existence of the three possible molecular arrangements of α - and β -MHC proposed by Hoh et al. (1979).

Materials and Methods

Protein Preparation

Cardiac ventricles from Wistar male rats were frozen in liquid nitrogen and stored at -80°C until use. Ventricular myosin was prepared according to Kielly and Bradley (1956) from three kinds of hearts: hearts from 20- to 25-d-old rats, hearts from 6-mo-old rats, and hearts from rats thyroidectomized at 3 mo and killed at 7 mo of age. The three corresponding myosin preparations produced one V_1 band, three $V_1V_2V_3$ bands, and one V_3 band, respectively, on nondenaturing electrophoretic gels (data not shown), in agreement with the original results of Hoh et al. (1978). These myosins will here be referred to as V_1 , $V_1V_2V_3$, and V_3 myosins, respectively. Densitometric scanning of the $V_1V_2V_3$ myosin showed 51, 29, and 20% of the proteins in the V_1 , V_2 , and V_3 bands, respectively.

Production and characterization of mAb have been described previously (Bouvagnet et al., 1984; Dechesne et al., 1985a). The two mAbs used here belong to mAb populations whose α -MHC or β -MHC specificities have been demonstrated by various independent methods: Western blot, competitive radioimmunoassay, immunofluorescence, and immunoaffinity chromatography (Bouvagnet et al., 1985; Dechesne et al., 1985a; Dechesne et al., 1985b; Léger et al., 1985; Dechesne et al., 1987).

Immunoelectron Microscopy and Statistical Analysis

Preparation of myosin and mAb samples, rotary shadowing of mAb-myosin complexes, and measurements of myosin molecules have been described previously (Claviez et al., 1982; Dechesne et al., 1985a). mAb concentrations (200–500 $\mu\text{g}/\text{ml}$) always exceeded the myosin concentrations (100–200 $\mu\text{g}/\text{ml}$). Epitope location is designated by the distance between the head-tail junction of the myosin molecule and the mAb binding site, expressed as the mean \pm SD (nm). To eliminate the nonspecific mAb binding background, mAb binding sites were included in statistical calculations only if they were found at least three times at the same distance from the head-tail junction.

The theoretical randomly distributed proportions of the different possible types of mAb-myosin complexes were calculated under the assumption that the binding of two mAbs to the same myosin molecule constituted two independent events. When the anti- α and anti- β were incubated together with the $V_1V_2V_3$ myosin, the proportions were determined using the following probability equations:

$$\begin{aligned} q_{00} &= Q_{\alpha\alpha} (1 - p_\alpha)^2 = Q_{\beta\beta} (1 - p_\beta)^2 + (1 - Q_{\alpha\alpha} - Q_{\beta\beta}) (1 - p_\alpha)(1 - p_\beta); \\ q_{10} &= 2 Q_{\alpha\alpha} p_\alpha (1 - p_\alpha) + (1 - Q_{\alpha\alpha} - Q_{\beta\beta}) p_\alpha (1 - p_\beta); \\ q_{20} &= Q_{\alpha\alpha} p_\alpha^2; \\ q_{01} &= 2 Q_{\beta\beta} p_\beta (1 - p_\beta) + (1 - Q_{\alpha\alpha} - Q_{\beta\beta}) p_\beta (1 - p_\alpha); \\ q_{02} &= Q_{\beta\beta} p_\beta^2; \\ q_{11} &= (1 - Q_{\alpha\alpha} - Q_{\beta\beta}) p_\alpha p_\beta, \end{aligned}$$

where q_{ij} is the proportion of myosin molecules labeled by i anti- α molecules and j anti- β molecules per myosin molecule (i and $j = 0-2$), $Q_{\alpha\alpha}$ is

the proportion of $\alpha\alpha$ -MHC, $Q_{\beta\beta}$ is the proportion of $\beta\beta$ -MHC, p_α is the probability of anti- α binding to its epitope, and p_β is the probability of anti- β binding to its epitope. The probabilities p_α and p_β were obtained by labeling V_1 or V_3 myosin with only anti- α or anti- β , respectively (see Results). The proportions $Q_{\alpha\alpha}$ and $Q_{\beta\beta}$ were determined by densitometric scanning of the V_1 and V_3 bands of myosin separated on denaturing electrophoretic gels (see above), because these bands are considered to consist of $\alpha\alpha$ - and $\beta\beta$ -MHC homodimers, respectively (Hoh et al., 1979). Thus, for $V_1V_2V_3$ myosin, $Q_{\alpha\alpha} = 0.51$ and $Q_{\beta\beta} = 0.20$.

When only anti- α was incubated with V_1 myosin, the same calculations were done with $Q_{\alpha\alpha} = 1$ and $Q_{\beta\beta} = 0$. When only anti- β was incubated with V_3 myosin, the same calculations were done with $Q_{\alpha\alpha} = 0$ and $Q_{\beta\beta} = 1$.

The theoretical and observed proportions were compared by a chi-square test.

Results

Binding Characteristics of mAbs to Myosin Molecules

Two mAbs raised against cardiac MHC isoforms were selected on the basis of their MHC specificities and epitope locations. One mAb is specific for α -MHC, the other is specific for β -MHC (see Materials and Methods). They are designated here as anti- α and anti- β , respectively. To locate their epitopes, myosin molecules from rat cardiac ventricles were examined by electron microscopy after rotatory shadowing of mAb-myosin complexes. Fig. 1 shows electron micrographs of single-myosin molecules, complexed with either anti- α or anti- β . The binding characteristics of anti- α were determined on V_1 myosin and the binding characteristics of anti- β were determined on V_3 myosin. As expected, one or two mAb molecules were able to bind to one myosin molecule, because of the heavy-chain dimeric structure of myosin. The locations of the mAb binding sites on myosin molecules shown in the histograms in Fig. 1 were statistically analyzed. The epitope of anti- α was located at 62.7 ± 5.3 nm ($n = 54$) from the head-tail junction and the epitope of anti- β was located at 36.3 ± 4.5 nm ($n = 45$) from the same origin. Thus, anti- α and anti- β were easily distinguished because of the distance between their epitopes on the myosin molecules. For each mAb the same epitope location was found with one or two mAb molecules bound to a myosin molecule.

The distribution of myosin molecules observed with zero, one, or two epitopes occupied by either mAb is shown in Table I. These data indicate a lower affinity of anti- α for V_1 myosin than of anti- β for V_3 myosin, under these conditions. The probability of a mAb occupying its epitope is given by the ratio $(2n_2 + n_1)/2n$, where n_2 and n_1 indicate the number of myosin molecules with two or one epitopes occupied, respectively, and n is the total number of myosin molecules observed. The probabilities for anti- α and anti- β were $p_\alpha = 0.51$ and $p_\beta = 0.93$, respectively. Previous results have evidenced a slight contamination of rat V_1 myosin by $8 \pm 5\%$ of myosin with β -heavy chains (Dechesne et al., 1987). This observation was confirmed here. Consequently, when calculating the probability of anti- α binding to an α -MHC, n was decreased by 8% to account for the small amount of β -MHC. Using the probability equations described in Materials and Methods, we calculated the randomly distributed proportions of uncomplexed myosin molecules and mAb-myosin complexes with one and with two mAbs per myosin molecule. There is no significant difference ($P = 0.05$) between the theoretical values and the ob-

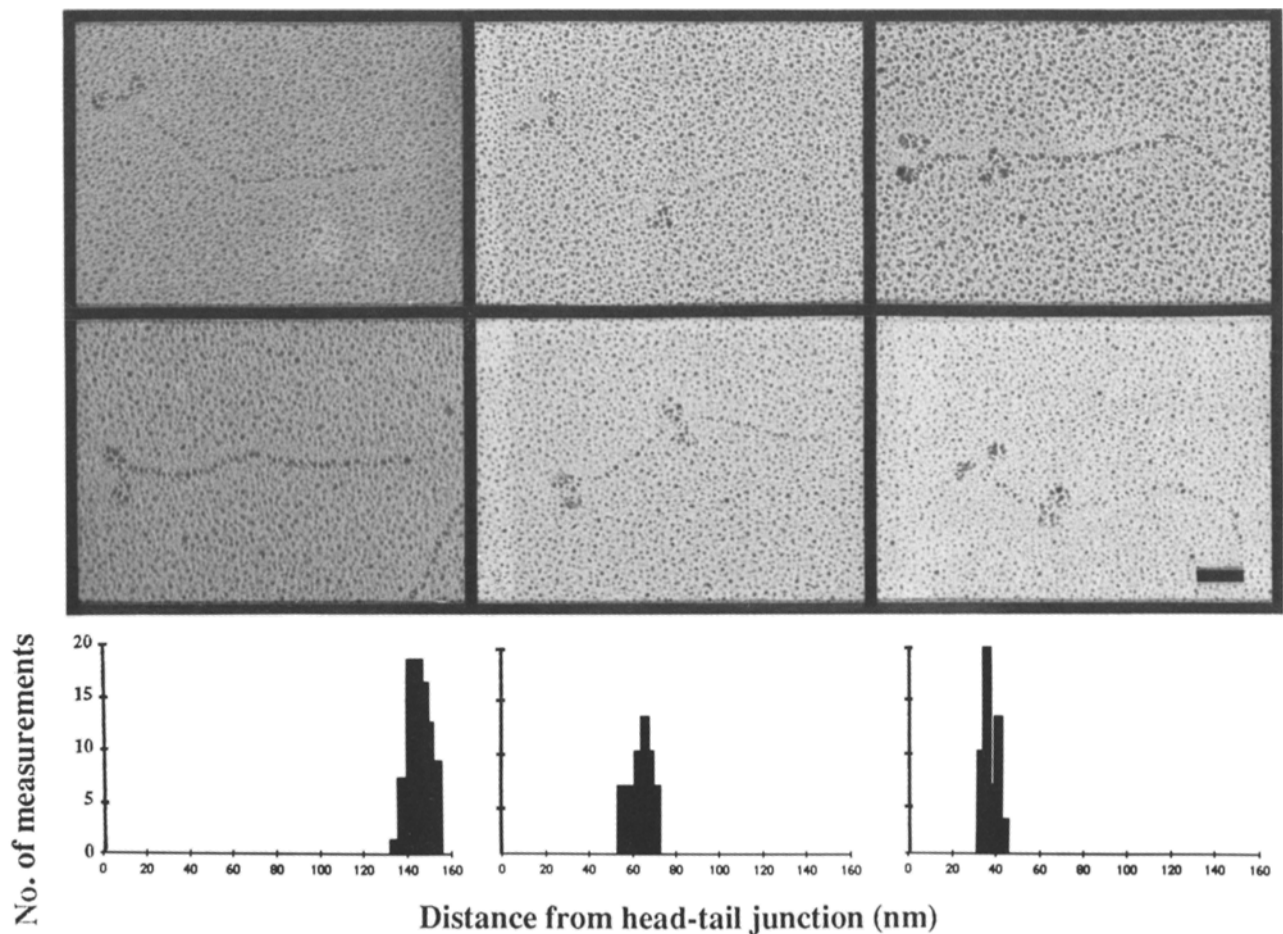


Figure 1. Electron micrographs of rotary-shadowed replicas of ventricular myosin molecules. On the left two myosin molecules are shown with no mAb labeling, in the middle they are labeled by anti- α , and on the right they are labeled by anti- β . One (*top*) or two (*bottom*) mAb molecules can bind to myosin molecules. Histograms represent, from left to right, the distribution of measurements of the rod lengths (146.5 ± 2.8 nm), the distances from the head-tail junction to the anti- α binding site (62.7 ± 5.3 nm), and anti- β binding site (36.3 ± 4.5 nm). Bar, 25 nm.

served values, either for anti- α or anti- β (see Table I). This supports the conclusion that anti- α or anti- β binding is independent of previous binding to myosin by another molecule of the same mAb.

Visualization of Myosin Heavy Chain Heterodimers

To obtain a double immunolabeling of myosin, anti- α and

Table I. Binding Characteristics of mAbs to Myosin Molecules

Number of mAbs per myosin molecule	0	1	2
Anti- α /V1 myosin			
Observed	30	45	25
(Theoretical)	(24)	(50)	(26)
Anti- β /V3 myosin			
Observed	2	9	89
(Theoretical)	(1)	(13)	(86)

The table indicates the observed and theoretical distributions of percentages of rotary-shadowed myosin molecules bound by zero, one, or two mAbs. The binding of anti- α and anti- β was analyzed in V₁ ($n = 157$) and V₃ ($n = 125$) myosins, respectively. The theoretical randomly distributed proportions are in parentheses.

anti- β were incubated together with V₁V₂V₃ myosin. Only myosin molecules bound with two mAbs can provide information about the homodimeric or heterodimeric heavy-chain structure of myosin. Three types of rotary shadowed mAb-myosin complexes with two mAbs per myosin molecule were detected by electron microscopy. The first and second types were myosin molecules bound with two anti- α or two anti- β , respectively, as shown above (Fig. 1). The two anti- α mAb molecules on the same site were identified by the location of their binding sites at 63.5 ± 5.1 nm ($n = 38$), and the two anti- β mAb molecules on the same site by the location of their binding sites at 37.4 ± 3.3 nm ($n = 46$). Myosin molecules labeled with two anti- α were consequently $\alpha\alpha$ -MHC homodimers and myosin molecules labeled with two anti- β were $\beta\beta$ -MHC homodimers. The third type corresponded to myosin molecules bound with one anti- α and one anti- β . The latter type of mAb-myosin complex is illustrated in the gallery and chart of Fig. 2. The anti- α mAb and anti- β mAb molecules bound together to the same myosin were identified by the location of their binding sites at 61.3 ± 5.7 nm ($n = 37$) and 34.3 ± 4.4 nm ($n = 37$), respectively. Labeling of myosin molecules with one anti- α and one anti- β indicated that these myosin molecules were $\alpha\beta$ -MHC heterodimers.

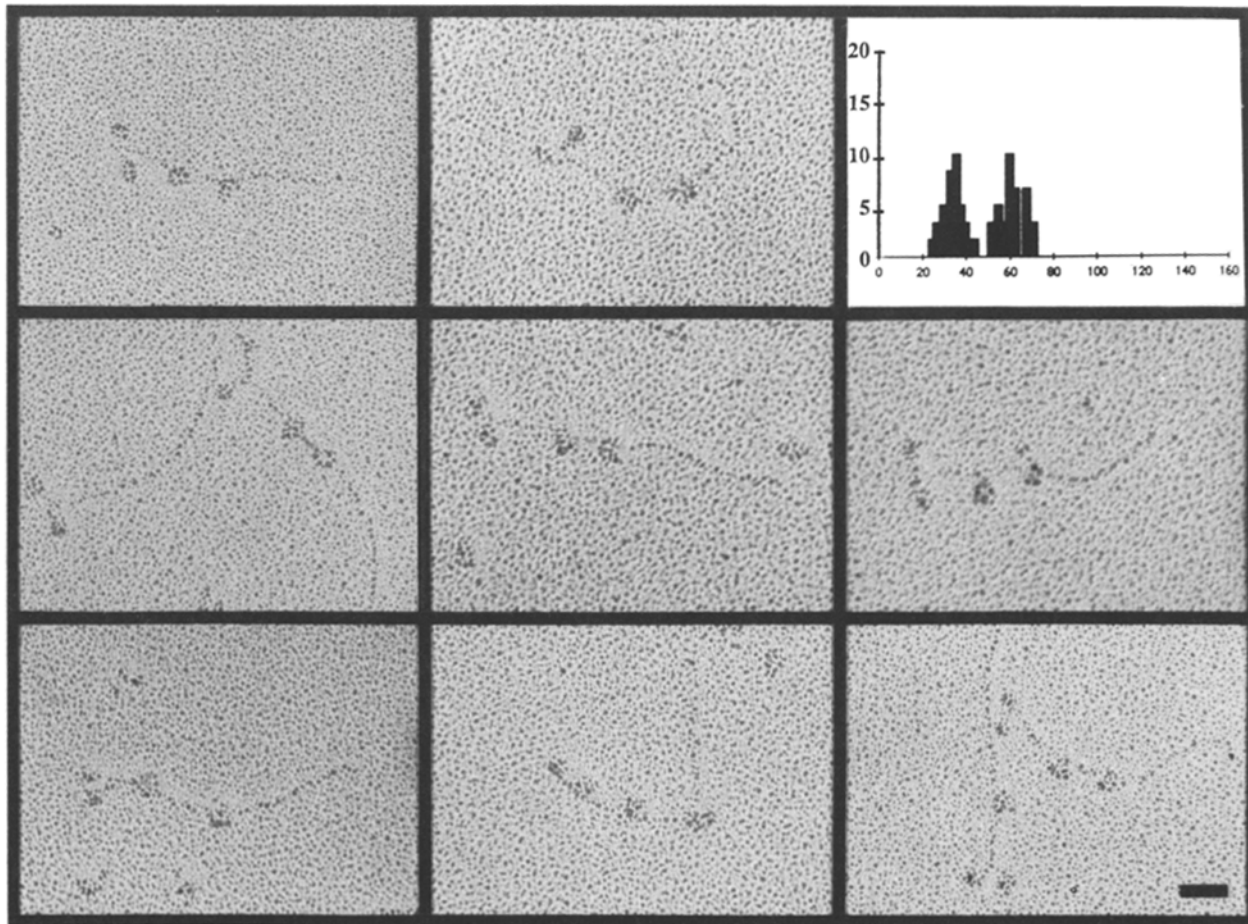


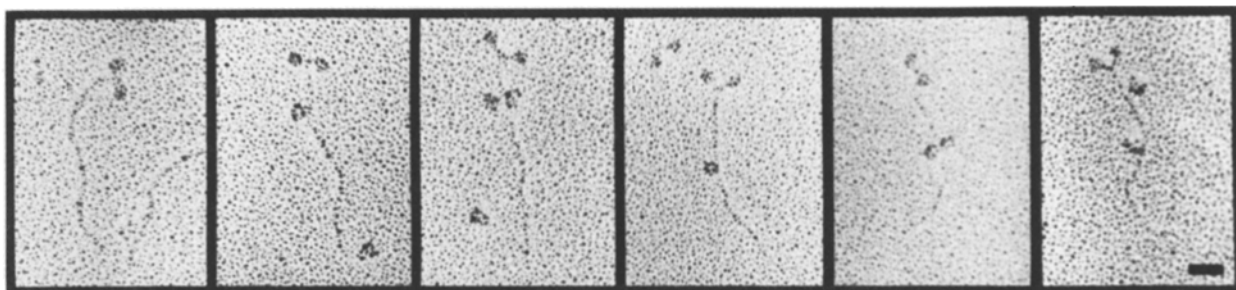
Figure 2. Gallery of rotary-shadowed $\alpha\beta$ MHC heterodimers, labeled by both anti- α and anti- β . The chart indicates, as in Fig. 1, distribution of measurements of the distances from the head-tail junction to the two mAb binding sites: 61.3 ± 5.7 nm in the case of anti- α , and 34.3 ± 4.4 nm in the case of anti- β . Bar, 25 nm.

There were no significant differences in the binding sites of either anti- α or anti- β in this experiment compared with the experiments above, in which the mAbs were used separately.

Quantitative Analysis of V_1 , $V_1V_2V_3$, and V_3 Myosins

The proportions of MHC homodimers and heterodimers in

V_1 , $V_1V_2V_3$, and V_3 myosins were investigated by simultaneous anti- α and anti- β labeling of myosin molecules. Six types of rotary-shadowed mAb-myosin complexes were found based on the numbers of each mAb bound per myosin molecule (Fig. 3): complexes with no mAb, a single anti- β , two anti- β , a single anti- α , two anti- α , and an anti- α plus an



Anti- β	0	1	2	0	0	1
Anti- α	0	0	0	1	2	1

Figure 3. The six types of rotary-shadowed mAb-myosin complexes after simultaneous labeling by anti- α and anti- β . Number of each mAb bound per myosin molecule is indicated under the type of complex. Bar, 25 nm.

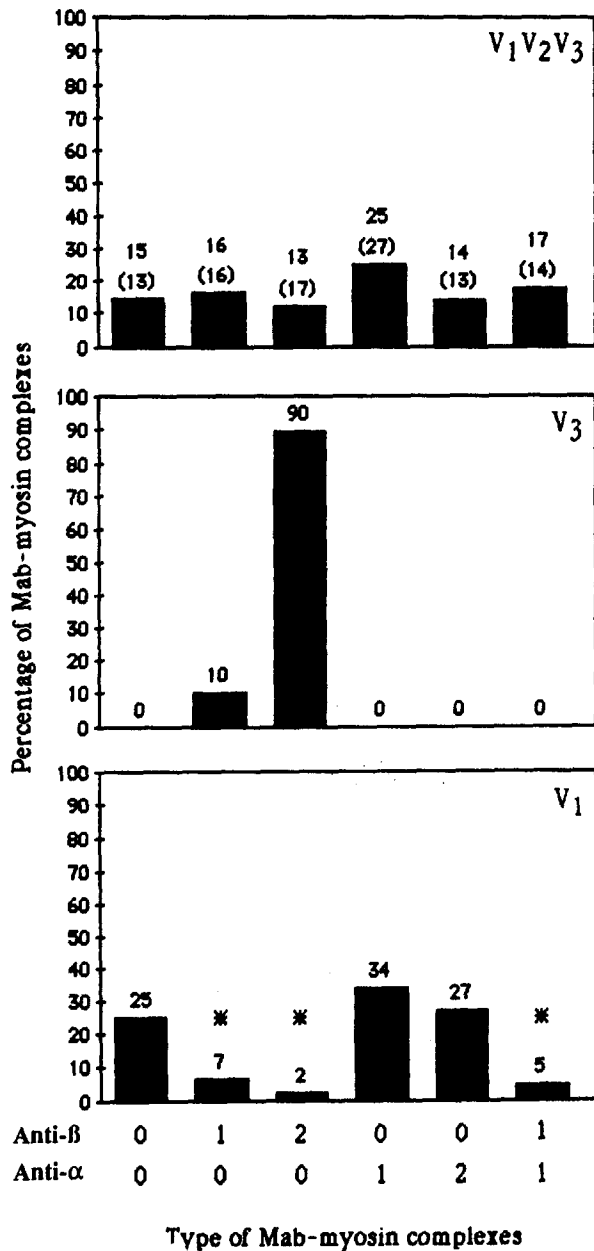


Figure 4. Distribution of percentages of the six types of rotary-shadowed mAb-myosin complexes observed after simultaneous labeling by anti- α and anti- β . Each type is designated below its bar by the number of anti- α and anti- β molecules bound per myosin molecule, as in Fig. 3. The three histograms represent the results from V₁V₂V₃ ($n = 130$), V₃ ($n = 38$), and V₁ ($n = 44$) myosins, respectively. For V₁V₂V₃ myosin, the numbers in parentheses indicate the theoretical randomly distributed proportions of mAb-myosin complexes. For V₁ myosin, the asterisks (*) indicate the three types of anti- β -myosin complexes observed in small amounts.

anti- β . These six types were quantitated on randomly taken micrographs. The results are illustrated histographically in Fig. 4. All six mAb-myosin complex types were present in V₁V₂V₃ myosin, and, in different proportions, in V₁ myosin. However, only complexes with one or two anti- β were detected in V₃ myosin. There were no significant differences in any of the mAb binding locations compared with the previous experiments using single mAbs.

In the case of V₁V₂V₃ myosin, the theoretical randomly distributed proportions of the six types of mAb-myosin complex were calculated according to the binding characteristics of anti- α and anti- β just determined, and on the basis of the amounts of $\alpha\alpha$ -, $\alpha\beta$ -, and $\beta\beta$ -MHC determined by densitometric scanning (see Materials and Methods). The theoretical proportions were not significantly different ($P = 0.05$) from the observed values (see Fig. 4). They were thus in very good agreement with the proportions of MHC homodimers and heterodimers shown by electrophoresis of myosin under nondenaturing conditions. Moreover, this suggests that the binding of anti- α and anti- β to the same myosin molecule represents two independent events.

Rotary-shadowed molecules of V₃ myosin were only labeled with one or two anti- β . Taking into account the binding characteristics of anti- β , both this finding and the proportions of the two types of mAb-myosin complex observed are also in very good agreement with the pure $\beta\beta$ -MHC homodimer content indicated by electrophoresis under nondenaturing conditions.

By contrast, the values observed with V₁ myosin do not correspond to a pure $\alpha\alpha$ -MHC homodimer content, as indicated by electrophoresis under nondenaturing conditions, since small amounts of anti- β -myosin complexes were observed. An analysis of the distribution of the six mAb-V₁-myosin complex types using a least squares linear regression curve, taking into account the binding characteristics of anti- α and anti- β previously determined, gives the following proportions: 87% $\alpha\alpha$ -MHC homodimers, 9% $\alpha\beta$ -MHC heterodimers, and 4% $\beta\beta$ -MHC homodimers. Thus, there was a small amount of β -MHC in V₁ myosin, and it was present in both homodimeric and heterodimeric forms.

Discussion

In this electronmicroscopic study we directly visualized cardiac $\alpha\alpha$ -MHC homodimers, $\beta\beta$ -MHC homodimers, and $\alpha\beta$ -MHC heterodimers, using one anti- α MHC mAb and one anti- β MHC mAb directed against different antigenic sites on α - and β -MHC. This visualization demonstrates the existence of homodimeric and heterodimeric arrangements of α - and β -MHC in cardiac myosin molecules. The proportions of $\alpha\alpha$ -MHC homodimers, $\alpha\beta$ -MHC heterodimers, and $\beta\beta$ -MHC homodimers found in V₁V₂V₃ myosin by our analysis of mAb-myosin complexes are in agreement with the quantification of the corresponding bands on nondenaturing electrophoretic gels, which completely supports the hypothesis of Hoh et al. (1979): V₁ and V₃ bands correspond to $\alpha\alpha$ - and $\beta\beta$ -MHC homodimers, respectively, and V₂ band corresponds to $\alpha\beta$ -MHC heterodimers.

Thus, the occurrence of a third type of ventricular rat MHC producing the V₂ band is highly unlikely. However, the possibility cannot be totally excluded. A few works do in fact suggest the presence of a third cardiac MHC type. In man, a very small amount of ventricular fibers with a special MHC content, named β' , has been evidenced by immunofluorescence (Bouvagnet et al., 1984). In bovine heart heterogeneities found in the primary sequence of β -MHC also suggest the existence of a β' -MHC (Flink and Morkin, 1984). Lastly in the rat, Watras (1981) has suggested a heterogeneity of V₃ myosin based on an analysis of myosin ATPase activity during postnatal development. Although the heterogene-

ity observed in these three papers only concerns β -MHC it remains to be demonstrated that the same phenomenon is responsible for the three results. In any case, our present results do not support the hypothesis of a third MHC type in rat ventricular myosin.

With the high sensitivity of electron microscopy, a small amount of β -MHC, not detected by electrophoresis under nondenaturing conditions, was found in V_1 myosin, in the form of homodimeric and heterodimeric MHC. In contrast, V_3 myosin was found to contain only β -MHC. These data are in agreement with our previous results on rat ventricles (Dechesne et al., 1987): (a) a persistence of β -MHC in 3-wk-old rat ventricles containing V_1 myosin, and (b) only β -MHC in fibers of ventricles containing V_3 myosin. In the former ventricles, myosin with at least one β -heavy chain per molecule was found essentially in the conductive cells. It was estimated to amount to $8 \pm 5\%$ of total myosin, using immunoaffinity chromatography, which is in good agreement with the 9% $\alpha\beta$ -MHC heterodimers and 4% $\beta\beta$ MHC homodimers detected here. Thus, there is always β -MHC expression in the rat ventricle during development, in contrast with what has been indicated by electrophoreses under nondenaturing conditions.

The existence of $\alpha\beta$ -MHC heterodimers raises the puzzling questions of their role and their molecular assembly, considering the functional and structural differences between cardiac α - and β -MHC. Moreover, there are the questions of how a molecule with two different MHCs and a thick filament containing a mixture of MHC homodimers and heterodimers could function. It is very likely that MHC heterodimers have a particular function because they can represent as much as 30% of total rat ventricular myosin. A partial solution may be provided by a determination of the spatial distribution of MHC heterodimers at the fiber and myocardial levels. However, the ultrastructural organization of α - and β -MHC coexpressed in the same cardiac fiber is not yet known, and immunological approaches used to evidence regional distribution of α - and β -MHC cannot discriminate homodimeric and heterodimeric MHC molecules (Gorza et al., 1981; Sartore et al., 1981; Bouvagnet et al., 1984; Yazaki et al., 1984; Dechesne et al., 1985b; Eisenberg et al., 1985; Kuro-o et al., 1986; Bouvagnet et al., 1987). Further elucidation of the function of MHC heterodimers could be provided by determining the mechanisms by which α - and β -MHC are arranged into homodimers or heterodimers. No such factors have yet been found. By contrast, two factors regulating levels of α - and β -MHC expression in a given cardiac fiber have been evidenced; i.e., the endogenous thyroid hormone level (Chizzonite and Zak, 1984) and the wall stress gradient within the myocardium (Gorza et al., 1981; Bouvagnet et al., 1985; Eisenberg et al., 1985; Kuro-o et al., 1986; Dechesne et al., in press). This suggests that physiological regulation determines the relative proportions of α - and β -MHC in each fiber, and that these MHCs are then arranged in the three possible dimeric structures, essentially according to the overall affinity of a given α - or β -MHC for another α - or β -MHC. To test this hypothesis we are at present searching $\alpha\beta$ -MHC heterodimers in another cardiac model: the human cardiac atrium. Like rat ventricle, this muscle contains a mixture of α - and β -MHC even within the same fibers (Dechesne et al., 1985a). If no atrial $\alpha\beta$ -MHC heterodimers are found, although α - and β -MHC are coex-

pressed in the same fiber, this would indicate that a precise mechanism induces the heterodimer arrangement of α - and β -MHC in the rat ventricle or represses it in the human atrium.

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