Canine Cardiac Myosin with Special Reference to Pressure Overload Cardiac Hypertrophy

I. SUBUNIT COMPOSITION*

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In studies of myosin from left and right ventricles of normal hearts and hypertrophic hearts at 5 weeks and 13 weeks after aortic banding, polyacrylamide gel electrophoresis shows intermediate molecular weight components which derive from heavy chains fragmented in the presence of dodecyl sulfate. The proportion of degraded heavy chains is greater in myosin from hypertrophic hearts than normal hearts, with comparable degradation in left and right ventricle myosin. The observed fragmentation of myosin results from proteolysis due to contaminant proteases or a thermally activated, heat-stable nonenzymatic process, or both. The susceptibility of heavy chains to crude myofibrillar proteases differs in normal and hypertrophic cardiac myosin; however, the kinetics of tryptic digestion are identical for both myosins.

With precautions to minimize proteolytic artifacts on dodecyl sulfate-polyacrylamide gel electrophoresis, preparations of myosin from left and right ventricles of normal and hypertrophic hearts exhibit comparable subunit composition, with approximately molar ratios of heavy chains, light chain L1, and light chain L2. Comparable stoichiometry for the light chain fraction is determined by high speed sedimentation equilibrium at pH 11 and direct fractionation of the different cardiac myosins. We do not confirm reports (e.g. Wikman-Coffelt, J., Fenner, C., Smith, A., and Mason, D. T. (1975) J. Biol. Chem. 250, 1257-1262) of different proportions of light chains in left and right ventricle myosin of normal and hypertrophic canine hearts. The light chains display microheterogeneity, with L1 generating two isoelectric variants and L2 generating two major and two minor variants, but identical mobilities and isoelectric values are obtained in the different myosin preparations.

The myosin molecule is composed of light and heavy polypeptide chains with isozymal variants in different muscles (1-6). In skeletal and cardiac muscle from a variety of sources, the physiological properties of intact muscle may be correlated with the specific activity of myosin ATPase1 (7, 8). In the case of cardiac myosin, there has been interest whether the subunit composition of myosin varies in response to physiological stress on the heart. In normal canine hearts, a relation between the intraventricular tension and the subunit composition of cardiac myosin was proposed from evidence that the proportion of light chains is greater and ATPase activity is less in right ventricle myosin than left ventricle myosin (9, 10).

There is also evidence that cardiac hypertrophy is accompanied by diminished contractility (11) and diminished myofibrillar ATPase (12, 13), raising the question whether myosin is altered or different myosin isoforms are produced in hypertrophic hearts. In studies involving left ventricular pressure overload, due to aortic banding, there are reports of altered ATPase activity (14-16) and an increased proportion of light chains in canine cardiac myosin (15), but no change in light chains of bovine cardiac myosin (17). In other studies involving right ventricle pressure overload in several animal models, there are conflicting reports of a diminished proportion of light chains (18), a new light chain (19), or no change in the light chains (20), as well as altered ATPase activity of myosin (20-22).

This paper describes the structural properties of myosin from left and right ventricles of normal canine hearts and hypertrophic canine hearts at 5 and 13 weeks after aortic banding. Canine cardiac myosin may exhibit extensive proteolysis on SDS-polyacrylamide gels, and we examine this effect in detail, and also the subunit composition of canine cardiac myosin in the absence of proteolytic artifacts.

EXPERIMENTAL PROCEDURES

Animals

Pressure overload cardiac hypertrophy was induced in healthy mongrel dogs (25 to 33 kg). Animals were anesthetized with sodium pentothal (12.5 mg/kg), intubated, and ventilated at 15 breaths/min, using an Engstrom respirator with 10% oxygen, 90% room air. Intra-ventricular and aortic pressures were recorded using a polyethylene catheter connected to a Stratham pressure transducer. Following right thoracotomy, a 1 cm cotton band was tied loosely around the aorta 1 cm distal to the aortic valve, and the band was constricted until left ventricular systolic pressure was increased by 50 mm Hg. Routine postoperative care was given, and animals were subsequently allowed to exercise ad libitum.

Studies were done at 5 to 7 and 12 to 14 weeks postoperatively and on control animals. At time of death, animals were weighed, anesthetized, and ventilated as above. Following right thoracotomy, intraventricular and aortic pressures were recorded, and the heart was extracardiac.

1 The abbreviations used are: ATPase, adenosine triphosphatase, (ATP phosphohydrolase EC 3.6.1.3); SDS, sodium dodecyl sulfate; EGTA, ethylene glycol bis(β-aminoethy ether)N,N'-tetraacetic acid; LV, left ventricle; RV, right ventricle.
The free walls of left ventricle and right ventricle were rapidly dissected and promptly immersed in distilled water at 4°C. All extraction procedures were initiated within 10 min after cardiectomy.

**Purification of Cardiac Myosin**

**Direct Extraction**—All procedures were carried out at 4°C. Excised tissue was added to 3 volumes of extraction solution (A), containing 0.3 M KCl, 0.1 M potassium phosphate, 8 mM Na$_3$SO$_4$, 0.2 µg/ml of pepstatin (generously provided by Dr. H. Umezawa), 1 mM dithiothreitol (Calbiochem), and 3.25 mM ATP (Sigma grade), pH 6.8. The mixture was homogenized by 5 to 10 pulses in a Waring Blender, until no large clumps of tissue could be observed; the homogenate was centrifuged at 4,000 x g for 5 min. The supernatant was diluted with 15 volumes of water, and a fluffly precipitate was allowed to settle over 30 min. The precipitate was centrifuged, re-disolved in an equal volume of 1.0 M KCl, 0.02 M potassium phosphate, pH 7.0, and dialyzed exhaustively over 36 h against Solution B (0.5 M KCl, 5 mM NaHCO$_3$, 3 mM NaN$_3$, 0.1 M EDTA, 0.1 M dithiothreitol, pH 7.6). The myosin solution was clarified by centrifugation at 45,000 rpm for 2 h.

**Extraction from Myofibrils**—The excised tissue was minced coarsely and added to 15 volumes of wash solution, containing 0.1 M KCl, 7.7 mM potassium phosphate, 8 mM Na$_3$SO$_4$, 7.5 mM EGTA, 1 mM dithiothreitol, 1 mM MgCl$_2$, 1% Triton X-100, pH 7.0. The suspension was homogenized in a Sorvall Omnimixer, and immediately centrifuged at 2,000 x g for 15 min. The supernatant volume was reduced to 1/30 of the homogenate volume by centrifugation at 45,000 rpm for 2 h. The precipitate was redissolved in 3 volumes of Extraction Solution A, and this material was treated identically as in the direct extraction procedure, except for use of only two precipitation cycles.

**Column Purification**—On occasion, cardiac myosin, as extracted directly from cardiac tissue or from Triton-washed myofibrils, was chromatographed on DEAE-Bio-Gel (Bio-Rad), essentially as described (23). Myosin was applied to columns equilibrated in 10 mM sodium pyrophosphate, 5 mM Tris, 3 mM NaN$_3$, pH 7.5. A linear salt gradient from 0.4 M KCl was used, and myosin eluted at KCl concentrations above 0.19 M.

**Anmionium Chloride/Ethanol Fractionation of Myosin**—Cardiac myosin (in Solution H) was mixed with 9 volumes of 1.2 M NH$_4$Cl, 20 mM Tris, 12 mM MgCl$_2$, 1 mM dithiothreitol, 50% ethanol, pH 7.4. After 15 min with constant stirring, 1 volume of ethanol was added, and the suspension was centrifuged at 40,000 x g for 15 min, yielding supernatant: (light chains) and precipitate: (heavy chains). The pellet was immediately redissolved in 10 volumes of 2 M KCl, 50 mM potassium phosphate, pH 7.5. Any appreciable delay in redissolving the packed pellet would result in irreversible denaturation of the heavy chains, with poor recovery of soluble protein. The light chains were concentrated from the supernatant fraction by adding an equal volume of 10% trichloroacetic acid and centrifuging at 40,000 x g for 15 min; the pellet was redissolved in a minimal volume of 1 M K$_2$HPO$_4$. The solutions of heavy chains and light chains were dialyzed against appropriate buffers for electrophoretic or stoichiometric analyses, or both.

**Analytical Methods**

**SDS-Gel Electrophoresis**—Electrophoresis on 5 or 10% polyacrylamide gels in the presence of dodecyl sulfate was performed essentially by the method of Shapiro et al. (24) and Weber and Osborn (25). Routinely, protein bands are be observed in dodecyl sulfate at pH 1.0, 0.5% mercaptoethanol, 0.5% sucrose, 3 mM Na$_3$NO$_3$, pH 7, for 18 h at 22°C. The samples were incubated with 5% glycerol and 0.02% bromphenol blue, and heated at 50°C for 45 min immediately prior to loading the gel. Gels were prefixed in 12.5% methanol, 7.5% acetic acid over 18 h. The gels were stained in 50% methanol, 7.5% acetic acid, 0.25% Coomassie brilliant blue R-250 for 1 h and destained in 12.5% methanol, 7.5% acetic acid, using the Hoefer diffusion developer. Each protein sample was analyzed at six different loads (2 to 70 µg). The gels were scanned using a Zeineth-Helena soft laser densitometer at 837 nm. Densitometric areas of each component were plotted against total protein load, and the limiting linear slopes were used to determine relative stoichiometry, assuming equal extinction for each component, as described (26).

**Electrophoresis in Tris/Glycine**—Protein samples were dialyzed over 18 h against 0.17 mM EDTA, 33 mM Tris, 0.228 mM glycine, 0.15% β-mercaptoethanol, 8 mM urea (Schwarz/Mann, ultrapure), pH 8.6, brought to 46% glycerol, 0.01% bromphenol blue; and subjected to electrophoresis on 10% polyacrylamide gels containing Tris/glycine/glycerol, according to the procedure of Porrie and Perry (27). An upper 5-mm layer of spacer gel (3.5% acrylamide) was used for analysis of whole myosin.

**Isoelectric Focusing**—Isoelectric focusing was performed using an LKB Multiphor, as described by Lansman (28). Samples of protein in 9 M urea (Schwarz/Mann, ultrapure), 1 mM dithiothreitol, 3 mM NaN$_3$, were adsorbed onto felt strips and placed near the cathode terminal of slab gels. Isoelectric values were estimated by concurrent electrophoresis of reference proteins. Two-dimensional electrophoresis was performed essentially by the method of Lansman (29), in which polyacrylamide disc electrophoresis in the presence of SDS (or Tris/glycine) was followed by isoelectric focusing on slab gels in a perpendicular dimension.

**Sedimentation Equilibrium**—High speed sedimentation equilibrium experiments, by the method of Yphantis (29), were done at 4°C using a Beckman model E analytical ultracentrifuge. An externally loaded, six-channel Rexolite centerpiece (29, 30) was used, with 0.11 ml of solution (or dialysate) layered over 0.01 ml of FC43 (Beckman), yielding a mean transaortic systolic pressure gradient of 57 mm Hg, with left ventricular systolic pressure of 155 mm Hg. Any appreciable delay in redissolving the precipitate was immediately upon attainment of rotor speed. Synthetic boundary experiments were done with twin capillary centerpieces, at protein concentrations of 2 to 4 mg/ml. Multicomponent analysis of myosin at pH 11 is based on interference data obtained at successive equilibria at rotor speeds of 12,000 rpm and 36,000 to 44,000 rpm, as described previously (1, 2, 31).

**Kinetics of Tryptic Digestion**—Four-milliliter samples of cardiac myosin in 0.5 M KCl, 2 mM NaHCO$_3$, at 10 mg/ml were incubated with 200/1 (w/w) trypsin (190 units/mg). Worthings at 22°C using the Radiometer pH-stat apparatus. The pH was maintained at pH 9.0, using 6 mM NaOH as titrant. Rates of protein production were calculated by numerical differentiation, using a least squares moving point method, from the pH-stat record of alkali uptake with respect to reaction time. The kinetic parameters for fast-reacting and slow-reacting classes of hydrolyzed bonds were calculated by the method of Leonis (32), assuming a pH of 7.85 for the evolved amino group (33).

**Protein Concentration**—Concentrations of cardiac myosin were determined by absorption at 280 nm, with correction for Rayleigh scattering, assuming $E_{1%}$ for 5.43. Concentrations of other protein samples were determined by the biuret reaction (34), calibrated against cardiac myosin.

**RESULTS**

**Effects of Aortic Banding**—In animals examined 5 and 13 weeks postbanding, hemodynamic measurements at the time of death indicate a mean transaortic systolic pressure gradient of 57 ± 6 mm Hg, with left ventricular systolic pressure of 155 ± 15 mm Hg. Left ventricular end diastolic pressures are within the normal range for control animals and experimental animals. In view of physiological differences inherent to hemodynamic measurements on anesthetized animals during thoracotomy, no attempt was made to measure pressure measurements to estimate the extent of cardiac hypertrophy among individual animals. In the experimental animals, there was no evidence of heart failure subsequent to aortic banding. At time of death, there was approximately a 75% decrease in cross-sectional area of the aortic lumen.

At 5 weeks postbanding (Table I), there is an increase of 

Portions of this paper (including Figs. 1, 3 to 5, 7 to 9, and 14 and Tables I to VI) are presented in manuscript at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass.
hearts, an increase of heart/body weight ratio from 7.0 to 8.9, and a concomitant increase in the ratio of left ventricle free wall/body weight, whereas the ratio of right ventricle free wall/body weight remains constant. At 13 weeks postbanding, there is partial return of left ventricle weight towards normal, whereas right ventricle weight is 20% greater than in the control group.

The heart/body weight ratio is known to vary with body weight (35), and this effect might complicate any analysis based on mean values. On plotting heart weight against body weight for the individual animals (Fig. 1), the control group shows a linear relationship of heart weight to body weight, with a slope of 3.79 g/kg and a zero intercept, $H_0$, of 80.5 g. At 5 weeks after banding, heart weights are about 1 S.D. above the control regression line for four animals and appreciably above the control regression line for the other nine animals. At 13 weeks after banding, heart weights are 1 S.D., or slightly more, above the control regression line in all four animals.

In order to normalize heart/body weight ratio for the dependence on body weight, the linear regression analysis for the control group would indicate that the ratio $(H - H_0)/B$ is constant, having a value of 3.79 g/kg, where $H$ is heart weight and $B$ is body weight. Any increase of this ratio would thus be a measure of cardiac hypertrophy in a particular animal. A normalized heart/body weight index, $r_n$, may be defined as the ratio of $(H - H_0)/B$ for a particular animal to $(H - H_0)/B$ from the regression analysis for the control group, assuming constant $H_0$ for canine hearts. That is,

$$r_n = \frac{H - H_0}{3.79 B}$$

Values of $r_n$ vary from 0.7 to 1.2 for control hearts, and from 1.2 to 2.0 for the banded hearts. Thus, despite the variation of heart/body weight ratio among individual animals, the control and experimental groups are clearly demarcated with respect to the normalized heart/body weight index.

**Purity and Recovery of Cardiac Myosin**—When myosin is isolated from canine cardiac tissue in the absence of protease inhibitors, there is extensive protein degradation, as shown on SDS-polyacrylamide gels. However, when extraction is done in the presence of 8 mM Na$_2$S0$_3$, 0.2 μg/ml of pepstatin, normal cardiac tissue yields preparations of myosin with minimal degradation (see below). These findings indicate the presence of contaminant proteases in cardiac myosin extracts, and to minimize their effects, all studies here reported were done including Na$_2$S0$_3$/pepstatin in the extraction solution.

For both control and experimental groups, preparations of myosin isolated by direct extraction from cardiac tissue display negligible contamination by actin, tropomyosin, and troponin, as shown on SDS-polyacrylamide gels. The direct extraction procedure yields 1.5 mg of myosin/g of cardiac tissue, whereas extraction from Triton-washed myofibrils yields about 6 mg of myosin/g of cardiac tissue, with comparable purity in both preparations.

When freshly prepared samples of myosin are chromatographed on DEAE-Bio-Gel, approximately 70% is recovered in the myosin peak. Unchromatographed myosin and chromatographed myosin display the same purity and subunit composition upon SDS-polyacrylamide electrophoresis. The $A_{280}/A_{260}$ absorbance ratio increases from 1.6 in unchromatographed myosin, to 2.0, or greater, in column-purified myosin, consistent with removal of contaminant nucleoproteins or nucleic acids during chromatography, or both.

**SDS-Polyacrylamide Gel Electrophoresis of Cardiac Myosin**—On SDS-5% polyacrylamide gels (Fig. 2A), normal cardiac myosin exhibits a characteristic pattern of heavy chains (apparent $M_r = 200,000$), light chains L1 and L2 (apparent $M_r = 27,000$ and 18,000, respectively), and intermediate molecular weight components ($M_r = 70,000$ to 180,000). As shown in Table II, myosin from normal left ventricle contains 72.8% heavy chains, 9.9% L1, and 6.7% L2. Taking molecular weight values as above, these polypeptides are present in approximately molar ratio. The intermediate molecular weight component comprises about 10.6% by weight, and presumably represents minor contaminants like C-protein, as found in

![Fig. 2. SDS-polyacrylamide gel electrophoresis of myosin from left ventricle (LV) and right ventricle (RV) of normal animals (N) and at 5 weeks postbanding (B). Samples were dialyzed for 18 h against SDS buffer at 22°C, and heated at 55°C for 45 min immediately prior to electrophoresis on 5% acrylamide gels at loads of 10 to 20 μg (Panel A), or 10% acrylamide at loads of 50 to 80 μg (Panel B). Gels were not run concurrently. Labeled are heavy chains (HC), intermediate molecular weight component (IMWC), and light chains L1 and L2.

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skeletal muscle myosin (36) or degraded heavy chains, or both. Comparable stoichiometric proportions are obtained for myosin from normal right ventricle, and, in general, paired preparations of myosin from left ventricle and right ventricle of the same heart exhibit similar proportions of heavy chains and light chains.

At 5 weeks postbanding, left ventricle myosin contains heavy and light chains of the same apparent molecular weights, as in normal cardiac myosin. Stoichiometric analysis reveals 60.7% heavy chains, 8.5% L1, and 6.9% L2, indicating apparent molar ratio of 1:1.03:1.27, respectively. There is considerable intermediate molecular weight material (Fig. 2A), and, in some preparations, traces of other low molecular weight components \( (M_r = 12,000 \text{ to } 30,000) \), which are more clearly demarcated on overloaded SDS-10% polyacrylamide gels (Fig. 2B). The intermediate molecular weight material varies among individual preparations, with respect to the distribution of distinct bands (Fig. 2) and to stoichiometric proportion, which ranges from 6.9 to 33.1% of total protein (Table II).

Essentially the same features are obtained in preparations of right ventricle myosin at 5 weeks after aortic banding (Fig. 2). The proportion of intact heavy chains is only 51% of the total protein, while the proportion of light chains remains about the same as in normal cardiac myosin, leading to an apparent increase in the molar ratio of light to heavy chains. Preparations of myosin from left and right ventricles of the same heart tend to show comparable proportions of intermediate molecular weight material, although the proportions vary among preparations from different hearts (Table II).

At 13 weeks after aortic banding, myosin from left and right ventricles shows similar stoichiometric proportion as in normal cardiac myosin in two cases; and in one case, myosin from left and right ventricles shows considerable intermediate molecular weight material, with a reciprocal decrease of intact heavy chains (Table II).

There is some evidence that the occurrence of intermediate molecular weight component is related to the extent of cardiac hypertrophy (Fig. 3). In the banded groups, most myosin preparations from hearts with \( r_t \) values above 1.3 show appreciable intermediate molecular weight component; however, most myosin preparations with \( r_t \) values of 1.2 to 1.3 show the same proportion of intermediate molecular weight component as in the control group.

The occurrence of intermediate molecular weight material is unrelated to the method of purification. Provided protease inhibitors are present during extraction, comparable intermediate molecular weight material is obtained in myosin preparations isolated from the same tissue by direct extraction, from Triton-washed myofibrils, or those subjected to further chromatographic purification on DEAE-Bio-Gel.

Dodecyl Sulfate-dependent Proteolysis of Heavy Chains—The evidence for intermediate molecular weight component in myosin preparations from hypertrophic hearts and, to a lesser extent, normal hearts derives from electrophoretic experiments in which myosin was exposed to dodecyl sulfate. However, it is uncertain whether the intermediate molecular weight component represents contaminant proteins or degraded heavy chains, and, if the latter, whether degradation occurs during exposure of myosin to dodecyl sulfate, or previously. The question was explored by experiments in which solutions of normal cardiac myosin with dodecyl sulfate were heated at 55°C for 45 min, and then incubated at 22°C until electrophoresis on SDS-polyacrylamide gels. In one such experiment (Fig. 4A), immediately after heating, there was 78% heavy chains, 2% intermediate molecular weight component, 11% L1, and 9% L2. During incubation of myosin in SDS buffer at 22°C, there is progressive accumulation of intermediate molecular weight component, a reciprocal decrease in the proportion of intact heavy chains, and no significant change in the proportions of light chains.

The findings from this and similar experiments demonstrate that incubation of normal cardiac myosin in SDS buffer at 22°C is accompanied by progressive fragmentation of heavy chains to components of \( M_r = 70,000 \text{ to } 180,000 \). Although it is difficult to be precise in comparing absorbance values from gel series done at different times, the total densitometric mass of protein components does not change significantly (±10%) during exposure to dodecyl sulfate at 22°C, suggesting that fragmentation involves hydrolysis of a small number of peptide bonds along the heavy chains.

The observed fragmentation of heavy chains might be due to contaminant proteases which are activated by dodecyl sulfate or nonenzymatic hydrolysis of cardiac myosin in the presence of dodecyl sulfate, or both. In general, proteases are denatured by heat. However, heat treatment does not prevent fragmentation of myosin in the presence of dodecyl sulfate, as shown in Fig. 4B. The same sample of myosin, as in Fig. 4A, was diluted in SDS buffer, heated at 96°C for 1 h, and then incubated for 22°C until electrophoresis, as above. Immediately after heat treatment at 96°C, the myosin sample contains 52% intact heavy chains, 27% intermediate molecular weight component, and 20% light chains. During subsequent incubation at 22°C, there is progressive increase in intermediate molecular weight material, a reciprocal decrease of intact heavy chains, and no significant change in the light chain fraction. The total mass of protein again does not change significantly during exposure to SDS buffer. It would thus appear that heat treatment of normal cardiac myosin in the presence of dodecyl sulfate results in augmented fragmentation while at 96°C, and does not abolish subsequent degradation at 22°C.

The kinetics of heavy chain degradation in the presence of dodecyl sulfate are shown in Fig. 5. On incubation of myosin at 96°C, heavy chains undergo fragmentation according to first order kinetics, with the same linear slope (solid line), irrespective of the duration of subsequent incubation at 22°C. Similarly, irrespective of the extent of prior heat treatment at 96°C, the slower rates of heavy chain fragmentation which are obtained at 22°C exhibit the same slope (dashed line), although the slope is now nonlinear. Comparable data were obtained in a number of experiments where normal cardiac myosin was incubated in SDS buffer at different temperatures. Fragmentation of heavy chains in the presence of dodecyl sulfate would appear to be a stochastic process, having initial first order rate constants of \( 1.6 \times 10^{-4} \text{ s}^{-1} \) at 96°C, \( 3.2 \times 10^{-4} \text{ s}^{-1} \) at 22°C, and \( 7 \times 10^{-6} \text{ s}^{-1} \) at 55°C.

The greatly augmented rate of fragmentation of heavy chains at 96°C, as well as the maintenance of this augmented rate as long as 5 h, would be unusual features for a reaction catalyzed by a mammalian protease, and, heavy chain fragmentation in SDS buffer at 96°C would seem to be nonenzymatic in origin. However, it is uncertain whether the slower fragmentation of heavy chains which occurs in SDS buffer at 22°C is nonenzymatic in origin, or in part due to action of contaminant proteases, although any such proteases would be unusual in retaining activity at 22°C, following prolonged exposure at 96°C (Fig. 5).

Similar findings are obtained in experiments involving myosin from 5-week banded hearts, and in several experiments involving paired preparations of myosin from normal hearts and 5-week banded hearts, rates of degradation and the distribution of degraded products appear comparable, although it should be noted that in these experiments, the banded...
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hearts are among those with \( r_2 \) values close to 1.2 (Fig. 3).

One next questions whether preparations from normal and banded hearts differ with respect to heavy chain degradation following minimal exposure of myosin to dodecyl sulfate. In several instances, samples of myosin were isolated simultaneously from left and right ventricles of control and 5-week banded hearts, and all analyses were done concurrently, using the same buffer solutions throughout. Samples of cardiac myosin were mixed with SDS buffer, heated at 55°C for 45 min, and promptly subjected to electrophoresis on SDS-polyacrylamide gels. Some prior exposure to dodecyl sulfate is required to ensure full solubilization of myosin, and 45-min exposure at 55°C was found consistently to provide complete dissociation of myosin polypeptide chains on subsequent electrophoresis. Identical patterns are obtained for myosin from left and right ventricles of control and 5-week banded hearts, with approximately 82.5% heavy chains (\( M_r = 200,000 \)), 9.0% L1 (apparent \( M_r = 27,000 \)), and 6.5% L2 (apparent \( M_r = 18,000 \)), along with 2% intermediate molecular weight component and traces of myosin dimers and low-\( n \)-mers. The small amount of intermediate molecular weight material which is consistently obtained, is presumably due to minor contaminants or fragmentation of heavy chains, or both. In this regard, preparations of rabbit skeletal myosin contain about 5% of C-protein, which can be removed by fractionation on DEAE-Sephadex (36). The appreciably smaller amount of intermediate molecular weight component in cardiac myosin is not removed by chromatography on DEAE-Bio-Gel.

Evidence for Heavy Chain Protease—It would thus appear that fragmentation of myosin in the presence of dodecyl sulfate largely accounts for the heavy chain degradation which is found on routine SDS-polyacrylamide gel electrophoresis of cardiac myosin (Table II). However, the evidence for substantially more heavy chain degradation in some of the preparations from hypertrophic hearts would suggest that those particular preparations may contain enhanced protease activity or heavy chains (or both) which are more susceptible to proteolysis in the presence of dodecyl sulfate.

In exploring these alternatives, experiments were performed in which paired preparations of purified myosin, isolated from Triton-washed myofibrils of normal and 5-week banded hearts, were seeded identically with small amounts of crude myofibrils, isolated in the absence of Triton from 5-week banded left ventricle. Upon incubation of unseeded myosin in SDS buffer at 22°C, the proportion of intermediate molecular weight component increases from 2% initially to 20 to 25% after 30 h, with identical findings for myosin from normal and banded hearts. The same results are obtained when the unseeded samples of myosin are heated in SDS buffer at 96°C for 15 min and then incubated at 22°C. However, when samples of myosin from normal or banded hearts are seeded with crude myofibrils, heated in SDS buffer at 96°C for 15 min, and then incubated at 22°C, there is an augmented rate of heavy chain degradation. Thus, it would appear that crude myofibrils from banded hearts contain a protease(s) catalyzing the hydrolysis of heavy chains in the presence of dodecyl sulfate. If so, the protease is heat-stable, since brief prior treatment at 96°C does not abolish proteolytic activity at 22°C, and also shows affinity for heavy chains and not light chains, at least initially.

When normal cardiac myosin is seeded with crude myofibrils and then incubated in SDS buffer at 22°C, without intervening heat treatment, the same rate of degradation is obtained as in the absence of seeding. When hypertrophic cardiac myosin is similarly treated, the rate of heavy chain degradation is greater than in the absence of seeding, although less than in the seeded and heated sample. Again, there is no evidence of light chain fragmentation during 70 h of incubation in the presence of dodecyl sulfate.

Thus, on seeding normal cardiac myosin with crude myofibrils from 5-week banded left ventricle, a short period of prior heat treatment at 96°C appears necessary for subsequent proteolysis in the presence of dodecyl sulfate.
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protease activity at 22°C; however, on seeding hypertrophic cardiac myosin, protease activity occurs at 22°C without prior heat treatment. That normal and hypertrophic cardiac myosin exhibit different susceptibility to initial fragmentation by the same protease would suggest some kind of difference in their constituent heavy chains.

The question remains whether cardiac myosin may undergo proteolysis in the absence of dodecyl sulfate. Samples of myosin obtained by direct extraction were stored under different solvent conditions at 22°C, and then subjected to SDS-polyacrylamide gel electrophoresis. In one such experiment (Fig. 7A), there is a marked pH effect on the extent of heavy chain degradation, with minimal degradation at pH 6. Under both acid and alkaline conditions, fragmentation of heavy chains is not accompanied by significant change in the light chain fraction or the total protein mass. The extent of proteolysis is not significantly affected by ionic strength, at least over the range from 50 mM to 0.5 M KCl at neutral pH (Fig. 7B). Identical results are obtained in the presence or absence of 10 mM Ca²⁺, indicating that proteolysis during storage of cardiac myosin is not Ca²⁺-sensitive.

These results would indicate that myosin preparations from hypertrophic hearts contain heavy chain protease(s) which may co-purify with myosin during direct extraction. Proteolysis occurs under mildly alkaline conditions, essentially those employed for purification of myofibrillar proteins. However, rates of heavy chain fragmentation are considerably less than obtained during incubation of myosin in the presence of dodecyl sulfate (Fig. 4A).

Sedimentation Equilibrium of Cardiac Myosin at pH 11—In order to determine subunit composition by some method which does not expose cardiac myosin to dodecyl sulfate, myosin from control hearts and 5-week postbanded hearts was analyzed by sedimentation equilibrium at pH 11, under conditions where the myosin molecule is known to dissociate into light chains and heavy chain core (1, 31). When sedimentation equilibrium is reached at rotor speeds of 36,000 and 44,000 rpm, plots of log (concentration) against r²/2 are linear throughout the concentration distribution (Fig. 8), and values of weight average molecular weight of the light component are the same or slightly greater at the lower rotor speed (Table III). In addition, the stoichiometric proportion of light component is nearly identical at the two rotor speeds. The minor heterogeneity between data at the two rotor speeds and at different loading concentrations of protein would appear to reflect the observed differences in molecular weight of L1 and L2, rather than contamination by intermediate molecular weight material. The molecular weight of heavy component is determined from analysis of equilibrium data at 12,000 rpm, after correction for the presence of light component using the high speed data (1, 31). These plots also appear relatively linear at fringe displacements above 0.4 (Fig. 8), consistent with the absence of substantial amounts of intermediate molecular weight material; however, the data from the difference curve are not sufficiently precise to warrant evaluation of heavy component heterogeneity.

Table III summarizes sedimentation equilibrium data at pH 11 for myosin from left and right ventricles of normal and 5-week postbanded hearts. For normal left ventricle myosin, the heavy component has a weight average molecular weight of 390,000, and the light chains have a weight average molecular weight of 20,800, comprising about 14.8% of the total protein. Comparable data are obtained on sedimentation equilibrium of myosin preparations from right ventricles of normal hearts and from left and right ventricles of 5-week banded hearts (Table III). In particular, there is no significant difference in the proportion or weight average molecular weight of light component in myosin from the different cardiac sources.

Several preparations of hypertrophic cardiac myosin (He, Hh) are among those which on SDS-gel electrophoresis exhibit extensive degradation of heavy chains and increased low molecular weight component other than light chains (Table II). However, on sedimentation equilibrium at pH 11, these preparations display the same molecular weights and stoichiometric proportion of light chains and heavy chain core, as in preparations without extensive degradation on SDS-polyacrylamide gels (Nd, Nh). These results are consistent with the evidence that the extensive heavy chain degradation which may be observed upon SDS-polyacrylamide gel electrophoresis of hypertrophic cardiac myosin occurs during exposure to dodecyl sulfate, and not during purification and storage of cardiac myosin.

Quantitative Separation of Light and Heavy Chains—Salting-out of myosin with ethanol in the presence of 1.2 M NH₄Cl provides a simple way to fractionate light and heavy chains, as shown in Fig. 9, where the precipitate fraction (b) contains heavy chains and heavy chain fragments, and the

Fig. 10. Electrophoresis on Tris/glycine/10% acrylamide gels of light chains obtained by ammonium chloride/ethanol fractionation of myosin from: a, normal left ventricle; b, left ventricle 5 weeks postbanding; c, mixture of a and b; d, left ventricle 13 weeks postbanding; e, mixture of a and d; f, normal left ventricle; g, normal right ventricle; h, left ventricle 5 weeks postbanding; i, corresponding right ventricle; j, left ventricle 13 weeks postbanding; and k, corresponding right ventricle. Loading levels, approximately 22 µg. Samples f to k run concurrently.
supernatant fraction (c) contains all of the light chains and traces of other low molecular weight material, including tropomyosin and troponin as trace contaminants. Preparations of normal cardiac myosin yield 15.0% (±1.3 S.D., n = 5) light chains, without significant difference between left and right ventricles. Myosin isolated from 5-week postbanded hearts yields 14.0% (±0.6 S.D., n = 6) light chains, also with similar proportions in left and right ventricles. That the procedure yields quantitative recovery of light and heavy chains is supported by the finding that the same procedure yields a value of 15.3% for the proportion of light chains in rabbit skeletal myosin.

Charge Electrophoresis of Cardiac Myosin Light Chains—Frearson and Perry (37) have shown that on polyacrylamide gel electrophoresis in Tris/glycine, cardiac myosins from a variety of mammalian sources yield a single band for L1 and a complex pattern for L2, including L2 proper, L2 satellite (or L2'), L2p, and L2p', listed in order of increasing mobility. The same pattern is here obtained for the light chains of normal cardiac myosin (Fig. 10). Trace contaminants on SDS-polyacrylamide gels (Fig. 9c) appear on Tris/glycine gels as minor bands of greater or lesser mobility than the major bands for L1 and L2. Identical patterns are obtained on Tris/glycine gels for the entire myosin sample and for purified light chains following fractionation by alkali, urea, or ammonium chloride/ethanol. Bands of identical mobility are obtained for light chains from left and right ventricles of normal, 5-week postbanded, and 13-week postbanded hearts. The total light chains include, on the average, approximately 52% L1, 27% L2, 18% L2', and 3% L2p,p' (Table IV), although L2p,p' is found in variable amount or not at all in different myosin preparations. In 13-week banded hearts, three of the six preparations display appreciable increase in the proportion of L2p,p' and a reciprocal decrease in the proportions of L2 and L2'. There is no correlation between the proportion of L2p,p' and the extent of heavy chain degradation in the same preparations of myosin. An increased proportion of L2p,p' may occur in myosin from left or right ventricle and does not seem to be correlated with the extent of cardiac hypertrophy as indicated by the r index.

Two-dimensional Polyacrylamide Electrophoresis of Myosin Light Chains—Isoelectric focusing might be expected to provide a more precise way to characterize the charge variants of myosin light chains. In order to demonstrate which light chain generates which isoelectric band(s), myosin was first subjected to disc gel electrophoresis in the presence of SDS, and the unstained gel containing fractionated polypeptide chains was subjected to isoelectric focusing in a perpendicular dimension. In a representative experiment on purified light chains (Fig. 11b) and the entire myosin sample (Fig. 11c), from normal left ventricle, the L1 component on SDS-gel generates two major isoelectric bands, having p1 5.7 and p1 5.6, designated L1 and L1', respectively. The L2 component generates a complex pattern of isoelectric bands, all of p1 less than 5.6. Isoelectric focusing achieves complete removal of light chains from the disc gel, whereas the heavy chains are not removed from the disc gel except for a small amount which is poorly focused.

In another experiment, light chains were subjected to polyacrylamide disc electrophoresis in Tris/glycine, followed by isoelectric focusing in a perpendicular dimension (Fig. 12). Again, the L1 band on disc gel generates two distinct isoelectric components, L1 and L1'. Although L1' does appear to have slightly greater mobility than L1, this difference is not sufficient to resolve the two components on Tris/glycine gels. The L2,L2' duplex, which is fractionated by charge electrophoresis, is shown to generate distinct isoelectric components at p1 5.35 and p1 5.20, respectively. Sample c contains a small amount of L2p,p' duplex on disc gel, and these components are shown to generate isoelectric components at p1 5.15 and p1 5.05, respectively. Comparable isoelectric values are obtained when samples of light chains, in 9 M urea, are applied directly to the slab gel, without prior disc gel electrophoresis (Fig. 12, a and d). Furthermore, equivalent p1 values for the light chain variants are obtained on direct isoelectric focusing and on two-dimensional isoelectric focusing following disc gel electrophoresis in the presence of SDS or Tris/glycine.

Isoelectric Focusing of Myosin Light Chains from Normal and Hypertrophic Hearts—Light chain preparations without appreciable L2p,p' variants were subjected to direct isoelectric focusing (Fig. 13). Identical isoelectric values are obtained for light chains from normal hearts, 5-week postbanded hearts, and 13-week postbanded hearts. The proportions of each light chain variant are comparable in left and right ventricles of normal and banded hearts (Table V). Artifactual bands may form upon storage of light chains in urea (27); however, the proportions of light chain variants here observed are the same after 18 h of dialysis in the presence or absence of urea.
normal and hypertrophic cardiac myosin: subunit composition

Kinetics of Tryptic Digestion of Cardiac Myosin—Canine cardiac myosin and rabbit skeletal myosin appear to differ in susceptibility to tryptic digestion (38), although pH-stat kinetics have been analyzed in detail only for rabbit skeletal myosin (33, 39). Tryptic digestion of myosin from left and right ventricles of a normal heart (a and b), left and right ventricles of another normal heart (c and d), left and right ventricles at 5 weeks postbanding (e and f), left and right ventricles of another heart at 5 weeks postbanding (g and h), left and right ventricles at 13 weeks postbanding (i and j), and left ventricle of another heart at 13 weeks postbanding (k). Loading level, 7.5 µg.

FIG. 13. Isoelectric focusing of light chains, as obtained by ammonium chloride/ethanol fractionation of myosin from normal left ventricle (a and b), left and right ventricles at 5 weeks postbanding (c and d), left and right ventricles of another normal heart (e and f), left and right ventricles of another heart at 5 weeks postbanding (g and h), left and right ventricles at 13 weeks postbanding (i and j), and left ventricle of another heart at 13 weeks postbanding (k). Loading level, 7.5 µg.

There is also evidence for protease-dependent proteolysis of myosin, and a slow-reacting class with about 45 more bonds, which are split at the same rate (k₁) as in rabbit skeletal myosin (Table VI). The number of bonds and rate constants for the fast-reacting class and the slow-reacting class are comparable in myosin preparations from left and right ventricles of control and 5-week postbanded hearts.

DISCUSSION

Exposure of cardiac myosin to dodecyl sulfate is accompanied by degradation of heavy chains to fragments of Mᵦ = 70,000 to 180,000, without significant change in the light chain fraction. Although this effect appears not to have been recognized in previous work on cardiac myosin, a number of published patterns do show the presence of intermediate molecular weight components (17, 40–42). The appearance of a small number of discrete intermediate fragments on SDS-polyacrylamide gels, as well as the absence of significant proteolysis of light chains, would suggest that fragmentation of myosin in the presence of dodecyl sulfate represents a selective process involving only a few peptide bonds. The intermediate fragments appear different from the meromyosins, which yield smaller fragments (Mᵦ = 30,000 to 115,000) on SDS-polyacrylamide gels (43, 44).

It appears well documented (45) that polypeptide chains may be fragmented during incubation with dodecyl sulfate, and the elimination of this artifact by heat treatment has been taken to imply the presence of contaminant proteases in diverse systems including yeast hexokinase (45), and Mercuraria paramyosin (46). Proteases, like chymotrypsin (47) and proteinase K (48), are known to exhibit enhanced activity in the presence of dodecyl sulfate at room temperature. There is also evidence that proteins may undergo nonenzymatic degradation in the presence of dodecyl sulfate. Steinhardt and Fugitt (49) early reported that in a variety of proteins, rates of hydrolysis by dilute acids are greatly augmented in the presence of dodecyl sulfate or other sulfate half-esters, and a mechanism involving acid hydrolysis of peptide bonds was proposed (49). The same phenomenon might possibly occur at pH close to neutral, albeit at a considerably slower rate.

Fragmentation of canine cardiac myosin in the presence of dodecyl sulfate is augmented at 96°C, and prolonged exposure at 96°C does not abolish proteolytic activity during subsequent incubation at 22°C. In contrast, mammalian proteases are generally rendered inactive upon incubation at high temperature, and prolonged heat treatment results in their irreversible denaturation. Thus, at least some fragmentation of heavy chains in the presence of dodecyl sulfate appears to be nonenzymatic in origin, presumably related to SDS-catalyzed acid hydrolysis (49). The phenomenon is not due to thermally dependent hydrolysis of dodecyl sulfate (49), since pH values remain constant throughout.

The rate of heavy chain degradation at 96°C is greater than expected from simple linear extrapolation of Arrhenius behavior in the 22–55°C region, suggesting a transition near 55°C, above which myosin exhibits enhanced sensitivity to fragmentation in the presence of dodecyl sulfate. In this respect, considerable α helical structure persists in dodecyl sulfate denatured proteins (50), including rabbit skeletal myosin (51), and Tanford (52) has concluded that dodecyl sulfate denaturation of proteins involves partial unfolding with ordered regions which interact tightly with a relatively few detergent molecules. Presumably, some higher order structure which exists in the presence of dodecyl sulfate at room temperature might be denatured at temperatures above 55°C, accounting for the enhanced fragmentation of SDS-treated heavy chains at higher temperatures.
of myosin in the presence of dodecyl sulfate. In experiments where cardiac myosin was seeded with crude myofibrils and briefly heated with dodecyl sulfate at 86°C, rates of heavy chain degradation on subsequent incubation at 22°C were augmented in seeded samples, as compared with unseeded samples. This would indicate the occurrence of contaminant proteases which are active in the presence of dodecyl sulfate and, significantly, survive brief heat treatment, unlike those contaminant proteases which are irreversibly denatured by brief heat treatment in the presence of SDS (43, 45). The evidence that such protease activity requires brief heat treatment of myosin with dodecyl sulfate for normal cardiac myosin, but not hypertrophic cardiac myosin, would indicate that the different myosin molecules display different susceptibility to the same myofibrillar proteases. Thus, the extensive degradation which occurs in hypertrophic cardiac myosin might conceivably arise from augmented activity by contaminant proteases, presumably released from lysosomes (53), or enhanced susceptibility of hypertrophic cardiac myosin to fragmentation in SDS buffer, or both.

With precautions to minimize artifacts related to proteolytic degradation, preparations of myosin isolated from left and right ventricles of normal and hypertrophic canine hearts exhibit comparable patterns on SDS-polyacrylamide gel electrophoresis, with close to molar ratios of heavy chains ($M_h = 200,000$), light chain L1 ($M_l = 27,000$), and light chain L2 ($M_l = 18,000$). According to the gel data, the light chains comprise approximately 15% by weight of canine cardiac myosin, and this stoichiometry is confirmed by sedimentation equilibrium of myosin at pH 11 (Table III) and direct fractionation of light and heavy chains. In all cases, myosin preparations from left and right ventricles of normal and hypertrophic hearts yield equivalent proportions of light chains. Our results disagree with prior reports that in normal canine hearts, the proportion of light chains is 18% in right ventricle myosin and 10% in left ventricle myosin (9), and that pressure overload hypertrophy, as induced by aortic banding, causes significant increase in the proportion of light chains in left ventricle myosin (15). These findings (9, 15) derive from analyses of eluted dye from single SDS-gels after destaining by transverse electrophoresis. In our hands, transverse electrophoresis does not provide a uniform field along the entire gel, and slight variations in the duration of destaining may lead to major artifacts. If the period of destaining is too short, incomplete elution of dye may cause an inhomogeneous background, whereas excessive destaining may remove dye from some of the protein bands. These difficulties were here avoided, as destaining was accomplished by diffusion, and a series of gels at multiple protein loads was directly scanned densitometrically for each stoichiometric estimate.

The ultracentrifugal studies indicate a molecular weight about 393,000 for the heavy chain core, and a weight average molecular weight of 21,400 for the light chains of canine cardiac myosin. Assuming molar ratios as above, canine cardiac myosin would have a molecular weight of approximately 480,000. Comparable data are obtained for myosin from left and right ventricles of normal and hypertrophic hearts, and the overall results are in accord with well established data on the subunit composition of rabbit skeletal myosin (1-3, 5, 6) and rabbit cardiac myosin (54, 55). The present results disagree with other data purporting to show molecular weights of 574,000 for right ventricle myosin, and 524,000 for left ventricle myosin of normal canine hearts (10). In that study (10), the limiting slope of log (concentration) against $r^2$ increases as loading concentration is diminished; and this finding was attributed to nonideality, yielding a relatively high value for the molecular weight of cardiac myosin. However, such non-linearity under meniscus depletion conditions actually implies high molecular weight heterogeneity, as previously shown in rabbit skeletal myosin (2, 6). Thus, the presence of dimers and other low $n$-mers presumably accounts for the high molecular weights reported by these workers (10).

The present studies indicate heterogeneity among each weight class of light chains. Although light chain L1 yields only one band on charge electrophoresis, isoelectric focusing shows two components, having $p_I$ values of pH 5.6 and 5.7, with relative proportion essentially the same in myosin from left and right ventricles of normal hearts and handed hearts. The two L1 forms would appear to represent a minor structural variation, without gross change in apparent molecular weight. Perhaps coincidentally, the two isoelectric forms of L1 are found in a 2:1 ratio, analogous to the observed ratio of L1 to L3 in myosin from rabbit fast muscle (3).

Frearson and Perry (37) have shown that phosphorylation of L2 proper and L2' leads to the occurrence of four bands on charge electrophoresis. The same phenomenon may account for the similar band heterogeneity here observed for L2; however, we did not conduct systematic experiments using ingested $^{32}$P in order to label the presumptive phosphorylated variants.

It was early reported (38) that canine cardiac myosin is more resistant to tryptic digestion than is skeletal cardiac myosin. According to the present evidence, cardiac myosin, like rabbit skeletal myosin (33, 39), exhibits a fast-reacting class and a slow-reacting class of susceptible peptide bonds on tryptic digestion. The fast-reacting class includes about 20 fewer bonds and the slow-reacting class includes about 45 more bonds, with both rate constants slightly greater than in rabbit myosin. These kinetic differences indicate some structural difference between the heavy chain core of canine cardiac myosin and rabbit skeletal myosin, but the present data do not warrant more explicit interpretation. While pH-stat kinetics of tryptic digestion are the same for myosin from left and right ventricles of normal hearts and hypertrophic hearts, hypertrophic cardiac myosin is more susceptible than normal cardiac myosin to fragmentation by a crude myofibrillar protease in the presence of dodecyl sulfate. The difference involves only a few large fragments, at least initially, and it would thus appear that several highly susceptible peptide bonds may vary in some way in normal and hypertrophic cardiac myosin.

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