Light Chain 2 Profile and Activity of Human Ventricular Myosin During Dilated Cardiomyopathy
Identification of a Causal Agent for Impaired Myocardial Function

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Background. A number of parameters reflecting the effects of idiopathic dilated cardiomyopathy (IDC) on the structure and function of myosin from the human myocardium were analyzed.

Methods and Results. The content of the regulatory light chain, LC2, was reduced in myopathic heart myosin in contrast to the controls in which it was present in stoichiometric amounts relative to the essential light chain, LC1. In IDC hearts, the absence or significant reduction in amount of LC2 was related to the presence of an active protease, which was isolated and purified about 130-fold. The protease exhibited a significant degree of specificity: It cleaved LC2 almost totally (but not the heavy chains) in human control heart myosin but only partially cleaved LC2 in canine heart or in rabbit skeletal muscle myosin. The protease was present at a very low level or was inactive in control heart tissue. When the LC1/LC2 molar ratio was calculated, it was found to be 1:1.0 in control heart myosin and remained constant in various samples analyzed, whereas in myopathic myosin from different individuals, this ratio varied from 1:0.1 to 1:0.69. The rates of ATP binding to control and myopathic myosins were similar, whereas the $V_{max}$ of actin-activated ATPase of myopathic myosin was about 25% less than that of the control. However, ATP binding and its hydrolysis by control S1, i.e., the myosin head, were faster by a factor of 2 than that of the myopathic S1. In addition, control myosin synthetic thick filament length as well as turbidity in solution, measured by light scattering, were twice as large as those of the myopathic heart myosin. These effects induced by myopathy in both filament assembly and turbidity were reversed upon reassociation of IDC myosin with LC2.

Conclusions. The changes in myosin structure and function were linked to a protease-mediated cleavage of LC2 in myosin; a possible role for the protease in the degenerative effects of idiopathic dilated cardiomyopathy is thus defined. (Circulation 1992;85:1720–1733)

Key Words • light chains • cardiomyopathy • myosin

Cardiac myosin isoforms1,2 are under a variety of adaptive and developmental control systems.3–6 The expression of individual phenotypes is regulated by the functional demands imposed on the specific tissue in which they are found.

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Cardiac myosin contains one mole each of essential light chain (LC1) and regulatory light chain (LC2) on each of the two myosin heads.7,8 These light chains are also subject to transitions from one isotype to another by external factors.9,10 One of the light chains, the phosphorylatable LC2, appears to be sensitive to the presence of proteolytic enzymes under certain conditions. For example, slow degradation of skeletal muscle myosin LC2 occurred over a period of storage.11 More significantly, LC2 is greatly reduced or absent in freshly prepared cardiac myosin from myopathic hamsters.12

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Frequent attempts have been made to evaluate myosin from cardiac tissue in humans suffering from a variety of heart diseases to comprehend the molecular basis of the pathophysiology of this disorder. The findings have been inconsistent. A possible explanation for conflicting reports based on human heart experiments may be the diversity or complexity of the cardiomyopathy being investigated. Idiopathic dilated (or congestive) cardio-
myopathy (IDC) is the result of a variety of insults and is by definition a disease of unknown etiology as opposed to hypertrophic cardiomyopathy, which may in some cases be associated with a genetic defect in myosin heavy chain. Regardless of the inciting cause, the natural history of the disease is not well-defined because the presenting symptoms are those of heart failure, basically the end stage of the condition. Histologically, the picture reveals diffuse fibrosis and in some cases, small focal scars. The progression of the disease from onset of symptoms to terminal heart failure is quite variable, and in some cases occurs within months and in others takes years, with the same final appearance of the heart. As a result, patients with IDC usually are diagnosed when symptoms of heart failure occur and other causes of heart failure are eliminated; thus, the disease tends to appear well past the initial insult. Furthermore, recent reports have shown a decrease in the myofibrillar content of myocytes from patients with IDC.

On the basis of the above considerations, we undertook an examination of the contractile proteins in hearts excised at transplant time, which provided fresh tissue for analysis of the contractile apparatus. In this communication, we report that the LC1-to-LC2 ratio, which is normally 1 in cardiac myosin, is reduced to 1/0.1 to 1/0.69 in patients with IDC. Furthermore, we have identified a neutral protease present at a threefold to fivefold higher activity in IDC samples over control hearts. This particular neutral protease had a high degree of substrate specificity for human heart myosin LC2, unlike that found in the hamster cardiomyopathy model, which cleaved LC2 equally extensively in rabbit skeletal or cardiac myosin. The modified, LC2-deficient myosin has been shown to possess a lower V_m for the actin-activated MgATPase, and synthetic thick filaments prepared from it tended to be shorter by 50% or more when compared with control myosin filaments.

**Methods**

**Description of Cardiomyopathy and Treatment of Human Cardiac Tissue**

In all, 17 hearts were obtained at the time of transplant (see Table 1). All patients except No. 29 were in New York Heart Association class III or IV heart failure and receiving appropriate medications. Case 29 had intractable ventricular tachycardia, and case K had Eisenmenger's complex with severe pulmonary hypertension for which a heart-lung transplant had been performed. Upon excision, the hearts were placed in Krebs-Henseleit solution buffered with 10 mM HEPES to pH 7.4 at 4–5°C. The epicardial arteries, veins, and when present, bypass grafts were removed for other studies. The remainder of the left and right ventricles were diced into 1–1.5-cm² sections, the epicardial fat was dissected away, and the cubes were placed in a buffer consisting of 50% glycerol, 0.03 M KCl, 0.01 M K$_2$HPO$_4$, 5 mM MgCl$_2$, and 1 mM dithiothreitol (DTT) (pH 6.5). At this time, tissue for scanning and transmission electron microscopy and light microscopy was prepared for diagnosis.

The samples were stored at 4°C for 24 hours and were then placed in fresh 50% glycerol buffer, quick-frozen in liquid nitrogen, and stored at −70°C until shipment on dry ice. At the time of dissection, obvious scars and areas of grossly visible diffuse fibrosis were avoided. All of the hearts available were hypertrophied to varying degrees. The hearts diagnosed as IDC were the experimental group, and all others were considered controls. In some cases, a single heart did not supply enough tissue and had to be combined with others. This is indicated in Table 1. Twenty to 40 arterial rings were obtained from each heart and subjected to a variety of functional tests. All rings were embedded in paraffin, and the amounts of intimal thickening, atherosclerosis, and medial thickness were evaluated using a Bioquant system. Regions of atherosclerosis when present were noted. The IDC patients had either no or minimal atherosclerosis, i.e., no plaque region involving more than 20% of the lumen. Of the atherosclerotic patients, all had at least 90–100% occlusive old plaques corresponding to the region or regions of infarction.

**Preparation of Human Cardiac Myofibrils and Myosin**

Human cardiac tissue was washed several times with 0.03 M KCl, 0.01 M potassium phosphate (pH 7.0), 1 mM DTT, 1 mM EDTA, and was then ground. Myofibrils were prepared from ground tissue according to the method of Solaro et al., and myosin was extracted by the procedure described before. Phenylmethylsulfonyl fluoride (PMSF, 0.1 mM) and N-p-tosyl-lysine chloromethyl ketone (TLCK, 0.015 mM) were included in buffers used for myofibrillar protein preparations from control tissues. Myosin was further purified by chromatography on a DE52 column equilibrated in 0.02 M sodium pyrophosphate to remove actin and tropomyosin. Myosin subfragment 1 (S1) was prepared by α-chymotryptic digestion, and rabbit skeletal muscle actin was prepared from acetone-dried muscle residue by the method of Spudich and Watt.

**SDS-Polyacrylamide and Agarose Gel Electrophoresis and Western Blotting**

Sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described by Laemmli, using a 4% stacking gel. Samples were treated with Laemmli's sample buffer containing 0.062 M Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 8 M urea, 0.001% bromphenol blue, with or without 5% DTT (reduced), or 10 M iodoacetamide (nonreduced). Samples were incubated for 10 minutes at 96°C. Either 7% and 10% or 4–18% acrylamide gradient slab gels were used. To calculate the relative molar ratios of LC1 to LC2, the gels were scanned by a two-dimensional scanning densitometer with a laser light source (Biomed Instruments, Fullerton, Calif.). The nitrocellulose (Schleicher and Schuell, Keene, N.H.) used for electroblocting was incubated and autoradiographed as described below. Molecular weights were calibrated by using prestained molecular weight standards (BRL, Bethesda, Md.) and included fibrinogen, thyroglobulin, and ferritin.

Control and myopathic myosin S1s at 2 mg/ml were subjected to limited tryptic digestion as described. Aliquots were removed at regular intervals, digestion was stopped with soybean trypsin inhibitor (twice the amount of trypsin by weight), and samples were treated.
for SDS-PAGE. Equivalent amounts (micrograms) of samples were run on 12% slab gels and the amount of S1 heavy chain remaining was determined by scanning the gels with a computerized, soft laser scanning, two-dimensional densitometer as described above. Gels were traced for quantitative analysis by computerized true integration of the area times density of each band. The calculated area densities were then plotted as log% of control against time (minutes) and the kinetic rate constants of heavy chain hydrolysis were determined from the slopes.

Blocked electroblots were treated with specific 125I-labeled IgG (0.5–1.0×10⁶ cpm/ml in Blotto solution),32 washed with blot buffer (Tris-buffered saline with 0.1% Triton X-100) until background count was achieved, and were then dried and autoradiographed at −80°C. In some instances, antigen recognition was achieved using a double detection technique: The blots were incubated with specific primary rabbit antibody, and recognition was achieved with a second 125I-labeled antibody33 conjugate of goat anti-rabbit antibody. Rabbit polyclonal anti-LC2 antibody was not affinity purified because of the unavailability of pure antigen. Instead, it was adsorbed by adding pure LC1 for use in blotting experiments only. In some cases, the autoradiogram was superimposed on the blot, and the required regions were thus specifically excised, counted, and compared. Polyclonal rabbit IgG directed against LC1 or LC2 was prepared according to a modified procedure.34

Preparation of Synthetic Thick Filaments and Rod Paracrystals

Synthetic thick filaments were prepared by stepwise dilution of normal and myopathic heart myosins at 2 mg/ml with 10 mM imidazole (pH 6.9) and 1 mM DTT.

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**Table 1. Diagnoses and Analyses of the Available Hearts**

<table>
<thead>
<tr>
<th>Case</th>
<th>Sex</th>
<th>Age (yr)</th>
<th>Heart weight</th>
<th>Reason for transplant</th>
<th>Pathological findings</th>
<th>Use</th>
<th>Protease activity</th>
<th>LC1:LC2 ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>M</td>
<td>59</td>
<td>405</td>
<td>HF</td>
<td>AS with HI</td>
<td>Control for ATPase and filament assembly; LC1:LC2 ratio assay</td>
<td>ND</td>
<td>1:1.0</td>
</tr>
<tr>
<td>10</td>
<td>F</td>
<td>25</td>
<td>250</td>
<td>HF</td>
<td>AS with H ant I</td>
<td>Control for ATPase and filament assembly; LC1:LC2 ratio assay</td>
<td>ND</td>
<td>1:1.0</td>
</tr>
<tr>
<td>12</td>
<td>M</td>
<td>58</td>
<td>527</td>
<td>HF</td>
<td>AS with H ant I</td>
<td>Control for ATPase and filament assembly; LC1:LC2 ratio assay</td>
<td>ND</td>
<td>1:1.0</td>
</tr>
<tr>
<td>11</td>
<td>M</td>
<td>54</td>
<td>443</td>
<td>HF</td>
<td>AS with H ant I</td>
<td>Control for ATPase and filament assembly; LC1:LC2 ratio assay</td>
<td>ND</td>
<td>1:1.0</td>
</tr>
<tr>
<td>29</td>
<td>M</td>
<td>50</td>
<td>480</td>
<td>VT</td>
<td>AS, CABG</td>
<td>29 and 30 combined for protease assay</td>
<td>Control level*</td>
<td>ND</td>
</tr>
<tr>
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<td>56</td>
<td>472</td>
<td>HF</td>
<td>AS, CABG</td>
<td>29 and 30 combined for protease assay</td>
<td>Control level*</td>
<td>ND</td>
</tr>
<tr>
<td>44</td>
<td>M</td>
<td>52</td>
<td>506</td>
<td>HF</td>
<td>AS, CABG</td>
<td>Protease assay</td>
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</tr>
<tr>
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<td>42</td>
<td>425</td>
<td>HF</td>
<td>PMVP</td>
<td>Control for ATPase and filament assembly</td>
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<td>1:1.2 Myofibrils</td>
</tr>
<tr>
<td>K</td>
<td>M</td>
<td>...</td>
<td>...</td>
<td>EC</td>
<td>PH</td>
<td>Protease assay</td>
<td>Control level</td>
<td>ND</td>
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<td>77</td>
<td>F</td>
<td>10</td>
<td>385</td>
<td>HF</td>
<td>CHL</td>
<td>LC1:LC2 ratio</td>
<td>ND</td>
<td>1:0.9</td>
</tr>
<tr>
<td>IDC</td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>21</td>
<td>F</td>
<td>20</td>
<td>440</td>
<td>HF</td>
<td>IDC</td>
<td>Protease assay</td>
<td>3.6×Control</td>
<td>ND</td>
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<tr>
<td>22</td>
<td>M</td>
<td>39</td>
<td>...</td>
<td>HF</td>
<td>IDC</td>
<td>ATPase assay and filament assembly</td>
<td>ND</td>
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</tr>
<tr>
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<td>34</td>
<td>405</td>
<td>HF</td>
<td>IDC</td>
<td>Protease assay</td>
<td>3.2×Control</td>
<td>ND</td>
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<tr>
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<td>F</td>
<td>52</td>
<td>408</td>
<td>HF</td>
<td>IDC</td>
<td>Protease assay</td>
<td>4.5×Control</td>
<td>ND</td>
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<tr>
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<td>M</td>
<td>61</td>
<td>499</td>
<td>HF</td>
<td>IDC</td>
<td>LC1:LC2 ratio</td>
<td>ND</td>
<td>1:0.69</td>
</tr>
<tr>
<td>48</td>
<td>M</td>
<td>24</td>
<td>896</td>
<td>HF</td>
<td>IDC</td>
<td>LC1:LC2 ratio</td>
<td>ND</td>
<td>1:0.6</td>
</tr>
</tbody>
</table>

LC: light chain; M, male; F, female; HF, heart failure; VT, ventricular tachycardia; EC, Eisenmenger's complex; AS, severe atherosclerosis; H, healed and I, infarct; ant, anterior; CABG, coronary artery bypass graft; PMVP, prior mitral valve prostheses; PH, pulmonary hypertension; CHL, congenital heart lesion; IDC, idiopathic dilated cardiomyopathy; ND, not determined, preparation used for another purpose. All cases had one or more 90–100% old occlusive plaques. None of these cases had plaques of 20% occlusion or greater.

*Activity calculated as cpm/OD₂₅₀, the values for cases 29 and 30 combined, 44, and K were 1.8, 1.6, and 2.0×10⁶ averaged to 1.8×10⁶ cpm/OD₂₅₀ with which IDC samples were compared.
to a final concentration of 0.5 mg/ml.\textsuperscript{35} Conditions for the preparation of rod paracrystals are similar to those used to generate three-dimensional quasicrystalline aggregates of gizzard myosin,\textsuperscript{36} i.e., precipitating the rod preparations (at 1–2 mg/ml) by dialysis against 0.2 M KCl, 2 mM Tris-HCl, and 1 mM DTT (pH 8.0).

\textbf{ATPase Assays}

Steady-state rates of ATP hydrolysis were measured by the colorimetric determination of phosphate\textsuperscript{37} at the indicated actin concentrations using the method of initial rates. The dependence of rate on actin concentration was fitted using a computer program that uses a nonlinear least-squares simplex method.

\textbf{Kinetics of ATP Binding}

The kinetics of ATP binding to myosin and S1 were measured in 0.6 M NaCl, 20 mM imidazole (pH 7.0), and 2 mM MgCl\textsubscript{2} at 15°C from the increase in tryptophan fluorescence observed upon mixing protein with ATP in a stopped-flow fluorometer.\textsuperscript{37} The excitation wavelength was 295 nm, and emission wavelength was 320–380 nm. Data were collected with a Nicolet III oscilloscope and then transferred to a Zenith 148 personal computer for analysis and long-term storage. Observed rate constants, $k_{obs}$, were obtained by fitting the equation $I(t) = I_0 e^{-k_{obs} t} + C$ by the method of moments, where $I$ is the observed fluorescence intensity, and $I_0$ is the amplitude of fluorescence change.\textsuperscript{38}

\textbf{Light-Scattering Measurements}

The rate and extent of myosin aggregation caused by self-association into filamentous polymers was measured by injecting 0.2 ml of 2.0-mg/ml stock myosin in 0.6 M KCl, 20 mM imidazole (pH 7.0), 0.5 mM DTT, and 5 mM MgCl\textsubscript{2} with a spring-loaded Hamilton syringe into a rapidly stirring solution of 1.6 ml of 0.1 M KCl in the same buffer. Aggregation was quantified with a Spex fluorolog spectrofluorometer using the amount of 340-nm light scattered at 90°.

\textbf{Isolation of a Neutral Protease From Myopathic Human Hearts}

The procedure used to isolate the crude protease from myopathic heart tissue was similar to that used to isolate it from hamster hearts.\textsuperscript{12,39} After eluting the protein from a Sephacryl S-200 column equilibrated in 0.4 M KCl, 0.05 M potassium phosphate (pH 7.0), 2 mM EDTA, and 1 mM DTT, the fractions containing proteolytic activity were pooled and sometimes used as such to digest human and canine cardiac myosin. The crude preparation was further purified using the following steps: It was first dialyzed against 0.05 M sodium acetate, pH 5.5, 1 mM DTT, and 1 mM EDTA, at which point a noticeable amount of protein precipitated. After spinning, both the supernatant and precipitate were assayed for activity. All the activity remained in the supernatant, which was applied to a CM52 column in the sodium acetate/EDTA buffer. After washing with the starting buffer, the retarded proteins were eluted with a linear NaCl gradient in the same buffer. The eluted active fractions were pooled and further purified on a heparin–Sepharose-Cl affinity column (bed volume, 3.5 ml) equilibrated in 0.05 M potassium phosphate (pH 7.0), 2 mM EDTA, and 1 mM DTT. Heparin–Sepharose-Cl has been shown to be useful in the purification of a large number of proteins and in particular enzymes either by binding impurities or by binding the specific protein desired, releasing individual proteins at characteristic ionic strength.\textsuperscript{40,41} The column was washed with this buffer, and the retarded proteins were eluted with stepwise application of 0.1, 0.3, 0.6, 1.0, and 2.0 M NaCl in starting buffer. The active fractions were not retained by the column. Although several contaminating proteins were separated, the final preparation was still not pure, and a number of protein bands were still visible on SDS gels stained with silver. However, the purity of the protease at this stage was sufficient to demonstrate its presence in the myopathic heart and its specificity for LC2 in human cardiac myosin.

\textbf{Protease Assays}

Fractions from the chromatographic columns during purification were monitored for protease activity using \textsuperscript{3H}-casein as substrate. Relative specific activities were expressed as cpm/OD\textsubscript{280}. Assays were done at 40°C, and liberated \textsuperscript{3H} was counted after stopping the reaction with TCA.\textsuperscript{12} To test the activity of the protease on LC2, it was incubated overnight with canine and control human heart and rabbit skeletal myosins in 0.45 M KCl, 0.01 M imidazole (pH 7.0), 2 mM EDTA, and 1 mM DTT as described before.\textsuperscript{26,42} The following day, the mixture was dialyzed against a low-ionic strength buffer containing PMSF to precipitate the digested myosin. Both the supernatant and the precipitate were run on SDS-PAGE to monitor the extent of digestion.

\textbf{Results}

\textbf{Light Chain Profile of Normal and Myopathic Human Cardiac Myosin}

Table 1 summarizes the characteristics of the control and IDC cases used in this study. The major difference between the structures of myosins isolated from control and myopathic heart tissue was in the content of LC2 present as revealed by quantitative SDS-PAGE (Figure 1). Because of the limited amount of material available, cardiac tissue from cases 9, 10, and 11 (see Table 1) were combined for the preparation of control myosin. The relative molar ratio of LC1 to LC2 in this preparation was 1:1.0 (Figure 1A and Table 1), i.e., a full complement of both light chains was present in myosin. The content of LC2 in myopathic myosin, on the other hand, was reduced appreciably (Figure 1B). The relative molar ratio of LC1 to LC2 in the myosin preparation of case 22 was 1:0.2, i.e., LC2 was reduced to 20% of LC1. In almost all of the control myosin preparations from cases 11, 24, and 77, the LC1/LC2 ratio remained nearly constant (1:1.0, 1:1.2, 1:0.9, and 1:0.9, respectively; Table 1). In case 24, the ratio was 1:1.2 in myofibrils and remained essentially the same at 1:0.9 when myosin was purified from the same sample. However, the LC1/LC2 ratio in all of the IDC cases not only varied but was always less than 1. In case 48, the ratio was less than 1:0.1 (Table 1), i.e., the myosin contained less than 10% of the normal LC2 complement. In cases 49 and 36, the ratio was 1:0.6 and 1:0.69, respectively. In one IDC sample (case 31), the tissue was used to
obtain the protease, but when the myofibrils from this particular tissue were analyzed for LC1/LC2 ratio, it was calculated to be 1:0.3 (Table 1). The reduced level of LC2 in myopathic myosin was further confirmed by Western blots. Autoradiograms of normal and myopathic heart myosins blotted with anti-LC2 antibodies paralleled the results obtained by SDS-PAGE. The intensity of the LC2 band revealed by the autoradiogram was barely detectable in myopathic myosin (Figure 1D) when compared with control myosin (Figure 1C). When Western blots were semiquantified in one case (case 22), it was found that the LC2 level in myopathic myosin was reduced to background level, whereas control levels of LC2 remained positive and comparable to LC1 levels.

**Kinetics of ATP Binding to Normal and Myopathic Human Heart Myosins**

The fluorescence increase observed upon mixing human cardiac myosin with ATP in a stopped-flow fluorometer were fitted to a single rate constant, \( k_{\text{obs}} \). The dependence of \( k_{\text{obs}} \) on the rate of ATP binding to normal and myopathic human cardiac myosin on ATP concentration is shown in Figure 2A. There is no significant difference in the rate of binding between the data observed for the normal and myopathic myosins. The combined sets of data were fitted by the values \( k_{\text{max}} = 58 \pm 8 \text{ sec}^{-1} \) and \( K_{\text{app}} = 415 \pm 130 \mu M \). The maximum rate was similar to the value measured by Smith and Cusanovich\(^{43} \) for ATP binding to bovine cardiac S1 under similar conditions.

These same parameters were calculated using S1 from both myosins (Figure 2B). The rate of binding (\( k_{\text{max}} \)) of ATP to normal S1 was 43.2 sec\(^{-1} \) with a \( K_{\text{app}} \) of...
7.5 μM, and for myopathic S1, the rate was calculated to be 18.8 sec⁻¹, and the $K_{\text{app}}$ was 3.7 μM.

Steady-State ATP Hydrolysis by Normal and Myopathic Myosins

The dependence of the steady-state rate of ATP hydrolysis on [actin] is shown in Figure 3. The data are fitted reasonably well by a hyperbolic equation. The $K_{\text{act}}$ was 7.5 μM for normal and 6.3 μM for myopathic human cardiac S1. However, the maximum rate was observed to be more than twice as large, 0.69 sec⁻¹ for normal S1, as for myopathic S1, which was 0.29 sec⁻¹ (Figure 3B). The value obtained for control human heart S1 was approximately one half the value measured under the same conditions for bovine and porcine cardiac S1: 1.0–1.5 sec⁻¹. The difference in activity could be attributed to either an intrinsic difference between the normal and myopathic S1 or partial activity of the human cardiac preparations. Difference in actin-activated rates of ATP hydrolysis by control (0.08 sec⁻¹) and myopathic (0.06 sec⁻¹) myosins were, however, not significant within experimental error (Figure 3A), which indicates that the observed differences may be due to a difference in stability of the proteins to proteolysis.

Assembly and Aggregation of Human Heart Myosin

Because LC2 was shown to have an effect on determining the length of thick filaments, myosin from myopathic heart offered an ideal system in which to determine whether this was the case with myopathic myosin from which LC2 was absent by the time it was purified in contrast to the LC2-deficient myosin used in

Figure 4. Graphs show light-scattering measurements of filament formation by normal (panel A), myopathic (panel B), and light chain 2 (LC2)-recombined (panel C) human cardiac myosin. A 0.2-ml solution of 2.5 mg/ml cardiac myosin in 0.6 M KCl, 20 mM imidazole (pH 7.0), 5 mM MgCl₂, and 0.5 mM DTT was injected into a 3-ml fluorescence cuvette containing 1.8 ml of a rapidly stirred solution containing the same buffer except that the KCl concentration was 0.14 M. Formation of myosin filaments was measured from the increase in 340-nm light scattered at 90°.
earlier studies in which LC2 was removed by chemical or proteolytic treatment. Two different approaches were used: one was to follow the increase in light-scattering at 340 nm and the other was to prepare and analyze the morphology of synthetic thick filaments from both myosins.

The results of light-scattering measurements are shown in Figure 4. The initial rate of increase in turbidity was very fast and apparently similar in all of the myosins tested. However, the extent of turbidity (maximum absorbance at 340 nm, 1.0 OD unit) in normal (Figure 4A) or myopathic myosin recombined with LC2 (Figure 4C) was about double that of the myopathic myosin (maximum absorbance at 340 nm, 0.6 OD unit, Figure 4B). Because intensity of turbidity is directly related to size of the scattering particles (i.e., the bigger the particle, the larger the turbidity), similar results should be obtained by actually observing the filaments formed. This was confirmed by electron microscopic analysis of synthetic thick filaments formed by each type of myosin (Figure 5). Comparison of various filaments stained with uranyl acetate indicates that synthetic thick filaments from normal (Figure 5A) human cardiac myosin were 0.7–2.0 μm in length, with a clear bare zone and crossbridges on either side of the central bare zone. The positively contrasted control filaments (Figure 5B) tend to appear relatively thinner, but again, the crossbridges seem to project almost at right angles to the filament shaft. Myopathic filaments, on the other hand, were generally shorter, about 0.35–0.5 μm long (Figure 5C), with a relatively stubby appearance caused by the clustering of crossbridges at opposite sides of the bare zone instead of the more tapered tips seen in normal myosin filaments. Myopathic filaments after LC2 reassociation were essentially 0.5–1.8 μm long (Figure 5D), i.e., as long as the controls, and also normal in shape with pointed tips rather than the more knobby, blunt tips seen in myopathic filaments.

The changes in filament size appeared to be regulated by the composition of the myosin head; thus, to determine whether the tail of myosin had any influence on assembly, the ability of myosin rod to form ordered aggregates was monitored using a comparative approach involving rod preparations from both control and myopathic heart myosin. For this, we chose conditions favoring generation of three-dimensional quasicrystalline assemblies found in gizzard myosin, which produced highly ordered aggregates. Figure 6 shows the results of electron microscopy of epoxy-embedded paracrystals formed from rod preparations of both control and myopathic myosin sectioned in various planes. When these new paracrystalline forms were sectioned through the planes indicated in Figure 6B, neither the control (Figures 6A and 6C) nor the myopathic (Figures 6D and 6E) rod paracrystals revealed any difference in structure or in their packing order. In Figure 6C, the section through plane A-A reveals an axial repeat of about 14 nm. The same periodicity is found in sections through the B-B plane of the myopathic rod paracrystal shown in Figures 6D and 6E. In Figure 6D, small arrows indicate the micropart of 14 nm, and the large arrow indicates the wider spacing at 21–24 nm, the main structural feature of the paracrystals that appears to be a fiber formed of many rod elements.

**Susceptibility of Myosin Heavy Chain to Trypsin**

The kinetic results of ATP binding and hydrolysis and the differences among the calculated values of V₀ and Kᵦ suggested the existence of subtle differences in the structure of myosin heavy chain other than the absence of LC2 alone in myopathic myosin. One way to monitor subtle differences was to use limited tryptic digestion and measure the kinetic rates of proteolysis of S1 heavy chain from control and myopathic myosin. The end result of limited tryptic digestion is the generation of three structural domains with Ms of 25–50-20 kDa, as indicated in the gel pictures of trypsin-cleaved S1 from normal (Figure 7A) and myopathic (Figure 7B) myosins. Although the gel pictures do not reveal a striking difference in the susceptibilities of normal and myopathic S1 to trypsin, when they were traced, the myopathic S1 heavy chain revealed somewhat greater resistance to trypsin cleavage. The rate of disappearance of S1 heavy chain obtained from densitometer traces of gels seen in Figure 7 was calculated from graphs of the areas of S1 heavy chain remaining at each time interval (Figure 8). The rate constants of heavy chain hydrolysis computed from the slopes of the plots were 0.10 min⁻¹ and 0.08 min⁻¹ for control and myopathic S1, respectively, suggesting some variability either within the structures or the conformation of S1 heavy chains.

**Isolation of a Neutral Protease From Myopathic Human Hearts**

It seemed very likely that the lack of LC2 in purified myopathic myosin could be a result of endogenous proteolysis. Consequently, an attempt was made to isolate and purify the responsible protease. The crude protease was obtained after chromatographing the muscle extract (from case 21, for example: Table 1) on a Sephacryl S-200 column. The elution profile is shown in Figure 9A. The eluted fractions across the protein peak were assayed for activity, most of which was associated with fractions 29–31. These fractions were pooled, concentrated, clarified by centrifugation, and dialyzed against 0.45 M KCl, 0.01 M imidazole (pH 7.0), 2 mM EDTA, and 1 mM DTT. To determine whether such a protease was present in control heart tissues, the extract from control heart tissues (from cases 29 and 30, Table 1) was subjected to a similar purification step. Figure 9B shows the elution profile. There was some proteolytic
activity in fractions 30 and 31; however, the activity associated with these fractions was not only one fourth that shown in Figure 9A (from the IDC case), but it also did not digest LC2 in control myosin. The protease was also isolated from two other samples, cases 28 and 31, which had threefold and fivefold higher activity than that present in control tissues from case 44, case K, and the combined cases 29 and 30 (Table 1).

The crude protease from myopathic heart was then used to establish its specificity for LC2 by incubating it with control human and canine heart myosin as well as rabbit skeletal muscle myosin (at a protease-to-myosin weight ratio of 1 to 30) in 0.45 M KCl, 0.01 M imidazole (pH 7.0), 2 mM EDTA, and 1 mM DTT. The digested myosins were monitored by SDS-PAGE (Figure 10). The striking result was that the protease was more
specific for human heart myosin than with the others: LC2 visible in control heart myosin (Figure 10A) was almost totally digested (Figure 10B). In the case of canine heart myosin (Figure 10I), there was a substantial amount of LC2 present after digestion (Figure 10J).

The same was true for LC2 or the DTNB light chain (Figure 10C) in rabbit skeletal muscle myosin: Its level appeared to decrease (Figure 10D) but did not totally disappear. Under similar conditions, the protease from hamster hearts or skeletal muscle totally digested LC2 from both canine heart and rabbit skeletal muscle myosin. In none of the samples shown in Figure 10 was there any evidence of an appreciable degree of heavy chain cleavage, as was the case with the hamster protease.

To evaluate further the possibility of the protease presence in controls, tissues from two separate non-IDC cases (44 and K) were used for protease preparation. Fractions with proteolytic activity were incubated with human heart myosin to determine whether LC2 would be cleaved. The extent of LC2 cleavage was monitored by blotting the gels onto nitrocellulose paper and revealing LC2 transfer by anti-LC2 antibody. LC2 was prominently revealed in control myosin (Figure 10E); however, when myosin was incubated with protease fractions eluted early from the column from the extract of case 44, there was very little digestion of LC2 (Figure 10F). The extent of digestion increased with a later fraction from the same preparation (Figure 10G). The degree of degradation of LC2 was somewhat less when using fractions with protease activity from case K (Figure 10H).

Further Purification of Protease

The steps required to partially purify the protease included the following steps: the crude preparation (from case 28) was dialyzed against 0.05 M NaOAc (pH 5.5), 2 mM EDTA, and 1 mM DTT, which resulted in the precipitation of some of the proteins present. Both the supernatant and the precipitated material were assayed for activity. The activity remained in the supernatant, which was applied to a MonoS (HR 10/10) FPLC column equilibrated in the same buffer. The bound proteins were eluted with a linear NaCl gradient (0–0.5 M in the starting buffer), and fractions were monitored at 235 nm to optimize detection. Proteolytic activity was confined to fractions 32–35 eluted between 0.2 and 0.29 M NaCl. These fractions were pooled, dialyzed against 0.05 M potassium phosphate (pH 7.0), 2 mM EDTA, and 1 mM DTT, and applied to a heparin-Sepharose-Cl affinity column; the protein was eluted with stepwise NaCl gradient, and active protease fractions were eluted unretracted. At this point, there was about 130-fold purification with respect to the crude preparation. Fractions with protease activity were pooled and dialedized against 0.02 M Tris-HCl (pH 8.0), 2 mM EDTA, and 1 mM DTT and chromatographed on a MonoQ (HR 5/5) FPLC column. Two major protein peaks were eluted, but neither demonstrated proteolytic activity, suggesting that the protease was either denatured or extremely diluted such that no activity could be measured.

Discussion

The question of linking a number of pathological states to an altered function or dysfunction of the actin myosin system remains unresolved.
myocardial contractile apparatus has been the basis of numerous studies\textsuperscript{19,20} that have reported a significant reduction in myofibrillar composition. Such a reduction in myofilament content would impair cardiac performance and could well represent one component of IDC. In some disorders, it has been specifically shown that the synthesis of myosin heavy chain at the transcriptional level is affected, because transitions were demonstrated from \(\alpha\alpha\) to \(\beta\beta\) myosin heavy chains.\textsuperscript{3,6} On the other hand, in individuals with tetralogy of Fallot, a defect in the temporal sequence of the light chains was observed without change in the heavy chains.\textsuperscript{47} A major drawback in these studies is the wide diversity and multiplicity of factors that result in a given pathological state, and the pathology of idiopathic cardiomyopathy is no exception.

In IDC, the myofilaments have been shown to disintegrate progressively.\textsuperscript{19,20} This is in contrast to hypertrophic cardiomyopathy, in which no striking changes in the structure and activity of contractile proteins were observed.\textsuperscript{48}

All of the cases of IDC studied here exhibited cardiac hypertrophy, and all were in severe heart failure (Table 1). The only human control tissue available in a state permitting valid examination of myosin were hearts excised at transplant and thus, all abnormal. However, these are considered useful controls because all were clearly hypertrophied and all were in severe heart failure. All patients were receiving a variety of medications for heart failure, although the usual regimen for the atherosclerotic patients did vary from those with

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure8.png}
\caption{Plot shows rate of disappearance of S1 heavy chain during limited tryptic digestion of normal (open circles) and myopathic (open squares) heart myosin S1. The amounts of heavy chain left at each time interval are plotted as log\% of the zero time point against time (minutes). The rates of heavy chain hydrolysis calculated from the slopes of the plots were 0.1 min\(^{-1}\) and 0.08 min\(^{-1}\) for normal and myopathic S1, respectively.}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure9.png}
\caption{Graphs show chromatographic profile of the neutral protease from myopathic (panel A) and normal (panel B) heart tissue on a Sephacryl S-200 column (2.5 x 110 cm) equilibrated in 0.4 M KCl, 0.05 M potassium phosphate (pH 6.9), 1 mM DTT, and 2 mM EDTA. Solid lines, optical densities at 280 nm; dashed lines, radioactivity counts corresponding to proteolytic activity. Note high activity in fractions from myopathic hearts in comparison with normal hearts in which radioactivity of fractions was slightly above background.}
\end{figure}
FIGURE 10. Coomassie-stained SDS-PAGE and autoradiograms of Western blots of a number of myosins before and after treatment with human myopathic heart protease and an extract from normal heart tissue having some proteolytic activity. Lanes A–D and I–J, Coomassie-stained SDS-PAGE: lane A (25 μg): control human heart myosin; lane B (20 μg): control human heart myosin after treatment with protease (isolated from idiopathic dilated cardiomyopathy [IDC] case 31) showing removal of light chain 2 (LC2); lane C (30 μg): rabbit skeletal muscle myosin, control; lane D (20 μg): same as lane C, after incubation with the same protease; note in lane D that there was either partial or no cleavage of LC2; lane I (30 μg): canine cardiac myosin, control; lane J (30 μg): canine cardiac myosin after incubation with protease showing partial cleavage of LC2. Lanes E–H, Western blots revealed with anti-LC2 and using protein loads of equivalent weight (40 μg in each case): lane E, control human heart myosin revealing LC2 prominently; lanes F and H, normal heart myosin after incubation with fractions from normal heart tissue (case 44) eluted early from the column; lane G, human heart myosin after incubation with fractions eluted later from the column (same case as in lane F); lane H, same as in lane G, incubated with protease from a different normal heart preparation (case K). Neither of the preparations shown in lanes G and H totally hydrolyzed LC2, whereas protease from the IDC heart tissue shown in lane B did.

IDC, as one would expect. Cases 28 and 31 both had left ventricular assist devices. None of the patients with atherosclerotic heart disease had received a left ventricular assist device. Though no strictly normal hearts were available, our findings are clearly not induced by either hypertrophy or heart failure.

The major structural change immediately noticeable in IDC tissues analyzed (cases 22, 31, 36, 48, and 49; Table 1) was the absence or appreciable reduction of LC2 content in myosin purified from these tissues (see Figures 1B and 1D). In one instance (case 31), the LC1/LC2 ratio was determined from the traces of myofibrils run on SDS gels, i.e., at the earliest stage of preparation; this same material was subsequently used for protease preparation. The reduced level of LC2 found in this IDC case confirms the conclusion that the reduction in LC2 content correlates with the substantial presence of active protease. Also, this result argues against proteolysis of LC2 occurring during later stages of the protein preparation during which proteolysis might conceivably be facilitated. Thus, the modification in the structure of myopathic myosin provided impetus to analyze its functional properties, namely, its ability to bind and hydrolyze ATP, its affinity for actin, and its self-assembly into ordered aggregates or thick filaments.

Several of these properties were influenced by cardiomyopathy: The rate of ATP binding (Figure 2), steady-state ATP hydrolysis (Figure 3), and affinity for actin were all diminished.

Some assembly properties were also destabilized: There was no difference between the ability of control and myopathic myosin rod to form the paracrystalline structure shown in Figure 6. Synthetic thick filaments from myopathic myosin, on the other hand, despite having the same outward morphology (i.e., bipolar structures with a bare zone, myosin heads projecting outward from the filament surface on either side of the bare zone), were consistently and significantly shorter than those from control myosin, terminating in a knobby structural feature. The absence of LC2 in myopathic myosin thus appeared to lead to the formation of shorter, stunted filaments, because when myopathic myosin was recombined with LC2, the filament lengths were comparable to those of normal filaments (Figure 5), as reported earlier and subsequently confirmed. These changes were confirmed by light-scattering measurements in which turbidity is a direct function of particle size. Thus, two independent methods generated fundamentally identical results. Myopathic myosin is thus judged to be defective in the globular S1 head,
suggesting either that the presence of LC2 causes myofilaments to form normally, resulting from a specific interaction involving LC2 with other LC2s, or else that LC2 has a stabilizing effect on the globular head of myosin, which is in turn able to stabilize filament structure. That there was some change within myosin S1 was further supported by the observation of a 20% reduction in the rate of heavy chain cleavage of cardiomyopathic S1 during limited trypsin digestion (Figure 8). Given the relatively small difference in the cleavage rates, this may reflect a conformational change rather than an amino acid substitution within the primary structure at the 25–70 kd or the 50–20 kd linker region where trypsic attack occurs. A crucial point is that the decrease in turbidity (Figure 4) and in filament length and the reduction in the steady-state rate of ATP hydrolysis were reversible upon recombination with LC2, as reported before.

There have been a number of studies of the genetic composition of patients with IDC implicating the immune system. There would appear to be an excess of specific human leukocyte antigen molecules in the population with the disease, although there is not complete agreement as to which specific haplotypes are involved. The presence of specific haplotypes occurs in one third to one half of the cases and in a small percentage of control subjects. Besides involvement of the cellular component of the immune system, humoral antibodies to a variety of myocyte intracellular antigens are demonstrable in patients with IDC. Studies involving the immune system are generally retrospective, and the incidence of a prior insult, especially viral myocarditis, is unknown but assumed to be high. However, the immune system is thought to be involved in one third to one half of the cases of IDC. If it is accepted that a specific insult occurring in a properly prepared host (i.e., the correct haplotype) results in a higher probability of IDC than in an individual with a dissimilar HLA makeup, it then follows that there could be other underlying host conditions predisposing development of IDC after an insult. The underlying condition may involve the cardiac myocyte and be sufficiently benign so as to be unnoticeable until an initiating cause occurs, as with the HLA subtypes prone to develop IDC.

In the cases described here, the absence of LC2 was due to the presence of a protease that was either synthesized de novo during cardiomyopathy or present in reduced amount or in an inactive state until it was activated. The latter possibility seems to be more likely in humans, because its presence was detectable at low levels in the preparation shown in Figure 9B from the combined tissues of control cases 29 and 30 and from control cases 44 and K (Table 1). There are other proteases present in the heart or skeletal muscle tissues, but some of these lack a clear specificity and/or are involved in regulating normal protein turnover in these tissues. The protease described here is unique in that it is even more specific for human LC2 than that isolated from hamsters. It does not attack the myosin heavy chain at all, as shown in Figure 1B, or else heavy-chain cleavage is negligible. Furthermore, it did not hydrolyze LC2 as readily in canine heart or rabbit skeletal myosin (Figure 10).

What emerges, therefore, is a comprehensive picture of a possible molecular mechanism of interdependent changes (as indicated by the parameters evaluated in this study and summarized in a simplified manner in Table 2) upon onset of cardiomyopathy: Probably the activation of the protease or its de novo synthesis is the critical step. Regardless of the mechanism of activation and/or synthesis of the protease, once active, it hydrolyzes LC2. Eventually, the rate of LC2 breakdown surpasses that of its synthesis, resulting in myosin with substoichiometric amounts of LC2 that subsequently form shorter filaments in in vitro preparations and have altered ATPase activity. If a similar reduction in size were to occur in the sarcomere, it would most likely lead to a generalized destabilization within the lattice geometry of the sarcomere, eventually leading to the collapse of contractile function and distinct morphological changes as reported.

The results discussed here would allow the inference of such a sequence of changes to explain the molecular basis of cardiomyopathy at the level of the major contractile protein, myosin, identifying it directly as being the critical target upon which the degenerative effects of the protease are impacted.

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