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Myonase is Localized in Skeletal Muscle Myofibrils

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A novel chymotrypsin-like proteinase termed myonase was previously purified from MDX-mouse skeletal muscle [Hori *et al.* (1998) *J. Biochem.* 123, 650–658]. Western blots and immunohistochemical analyses showed that myonase was present within myocytes of both MDX-mouse and control mouse, and subcellular fractionation showed that it was associated with myofibrils. No significant difference was observed on Western blots between the amounts of myonase in myofibrils of MDX-mouse and control mouse, but the amount of myonase recoverable as a pure protein was 5–10-fold more when MDX-mouse was the source of the skeletal muscle. Myofibrils also possessed an endogenous inhibitor of myonase, whose inhibitory activity at physiological pH (pH 7.4) depended on salt concentration, stronger inhibition being observed at a low salt concentration. Inhibition at alkaline pH (pH 9) was weak and independent of salt concentration. Myonase in myofibrils was partially released at neutral pH by a high salt concentration (>0.6 M NaCl). However, even at 4 M NaCl, more than 80% of myonase remained within the myofibrils. Under alkaline conditions, release of myonase from myofibril was more extensive. At pH 12, myonase was almost completely present in the soluble fraction. Release of myonase under these conditions coincided with the solubilization of other myofibrillar proteins.

Key words: muscle, myofibril, myonase, serine proteinase.

Molecular events during progressive muscular dystrophy include myofibrillar degeneration followed by progressive muscle atrophy and weakness (*1, 2*). The cascade of phenomena involved in this myofibrillar degeneration should include the activation of intracellular and extracellular proteinases for degrading myofibrillar proteins (see Refs. *3* and *4*). Possible pathways of proteolysis related to degradation of myofibrillar proteins include Ca^{2+}-dependent cysteine proteinases (calpains), lysosomal cysteine proteinases (cathepsins), ATP/ubiquitin-dependent proteinases (proteasomes), and serine proteinases (chymases) (*3–6*).

It has been suggested that calpains may have an important role in the pathogenesis of muscular wasting diseases including muscular dystrophy (*7–9*). However, with the exception of a study of degradation of myofibrillar proteins during molt-induced atrophy in crustacean skeletal muscle (*10*), no data have been obtained *in vivo* that permit assessment of the importance of proteolysis by calpains in the muscle-wasting process (*7*). Calpains preferentially cleave substrates at restricted locations rather than causing wholesale muscle proteolysis (*7, 11*). Furthermore, the fact that loss of p94-calpain causes limb girdle muscular dystrophy in humans suggests that the enzyme has a regulatory role in muscle function and/or development (*10*), possibly related to early events in muscular dystrophy pathogenesis.

Lysosomal proteinases degrade many membrane-bound proteins such as cell surface receptors in lysosomes after endocytosis, or soluble intracellular proteins and some organellar proteins also in lysosomes by an analogous nonselective process, macroautophagy, whereby cellular contents are engulfed and sequestered within membrane-bound structures that ultimately fuse with lysosomes (*6*). An ATP/ubiquitin-dependent proteasome pathway also has an important role in myofibrillar degeneration (*3, 5*). The ATP/ubiquitin-dependent proteolysis is activated during muscle atrophy (*6, 10, 12*), but the dissociation of free myofibrillar proteins from the contractile filaments is primarily required for this pathway (*13*), and soluble myofibrillar proteins can be readily ubiquitinated and degradated by proteasomes to small fragments or amino acids (*14, 15*). For these reasons, myofibrillar degeneration would require other factors to dissociate myofibrillar proteins from the structure of contractile apparatus prior to the action of three intracellular proteolytic pathways: Ca^{2+}-dependent proteolysis, lysosomal proteolysis and the ATP/ubiquitin-dependent proteasome pathway. The dissociation of myofibrillar proteins may be induced *in vitro* by increasing ionic strength (*16, 17*) and pyrophosphate (*16, 18*). Alkaline

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Abbreviations: HRP, horseradish peroxidase; MDX-mouse, X-chromosome linked muscular dystrophic mouse (C57BL/10ScSn-mdx); IDC, idiopathic dilated cardiomyopathy.

Enzymes: Horseradish peroxidase [EC 1.11.1.7]; cathepsin B [EC 3.4.22.1]; cathepsin L [EC 3.4.22.15]; chymase [EC 3.4.21.39]; calpains [EC 3.4.22.17].

pH also stimulates the dissociation of myofibrillar proteins (*16*). It is unclear, however, how dissociation by ionic strength, pyrophosphate and pH can relate to muscle structures *in vivo*.

Chymotrypsin-like serine proteinase, chymase, has been proposed as another proteinase that may be related to myofibrillar degeneration in muscular dystrophy (*4*). In dystrophic muscle, it was reported to localize mainly in invading cells such as macrophages and mast cells (*4*). This implies that it may not be related to the initial event in degradation of myofibrillar proteins. However, there are two hypotheses concerning its origin in muscle. One proposes that it derives from invading mast cells (*19–21*), from which it is released and attaches to myofibrils (*20*). Mast cells appear in the skeletal muscle of genetic muscular dystrophy in human, dog and mouse (*21*), and this observation supports the first hypothesis. The second hypothesis is that the proteinase is an endogenous intrinsic proteinase in myocytes (*22, 23*). If the proteinase is directly involved in the initial event of the dissociation of myofibrillar proteins, this hypothesis might be reasonable. During muscle atrophy by disuse/denervation, myofibrillar proteins may be degraded at different rates in slow and fast muscle-fibers (*24, 25*). Even under steady-state conditions, there is rapid turnover of the contractile apparatus. Half-lives of 5.4 days for cardiac actomyosin (*26*) and 10 days (*27*) for skeletal actomyosin have been reported. Under these conditions, it seems unlikely that the proteinase is released from the mast cells which localize around myocytes and penetrates into myocytes to dissociate myofibrillar proteins from the structure of the contractile apparatus.

Recently, alkaline serine proteinases have been purified from mouse skeletal muscle [myonase (*28*)] and hamster skeletal muscle [mekratin (*29*)]. These proteinases have chymotrypsin-like activities and a similar primary structure to mast cell proteinase (*28, 29*). In the present study, we report that myonase is localized in myofibrils as an inactive form in both normal and MDX-mouse, and that mRNA of myonase is present in the myocyte itself. We also propose a physiological role for myonase in skeletal muscle.

MATERIALS AND METHODS

Materials—Lima bean trypsin inhibitor–conjugated agarose (LBTI-agarose) was purchased from Sigma-Aldrich. Pharmalyte was obtained from Amersham-Pharmacia Biotech. Diphenyl (1-((((6-(5-fluoresceinyl (thiocarbamoyl) amino) caproyl) phenylalanyl) leucyl) amino)-2-phenylethyl) phosphate (FITC-aca-phe-leu-pheP(OPh)$_2$) was a generous gift from Dr. J.C. Powers. Polyclonal anti–myonase IgG from rabbit and monoclonal anti–myonase specific peptide IgG were produced by Iatron Lab., Tokyo. All other reagents were of analytical grade or higher.

Purification of Myonase—Myonase was extracted and purified as previously described (*28*). Briefly, skeletal muscle was homogenized in 0.6 M NaCl/10 mM Tris-HCl, pH 7.8. The precipitate formed by centrifugation at 10,000 ×*g* for 20 min was washed by repeating two cycles of homogenization and centrifugation. The final suspension of the precipitate was passed through a nylon cloth to remove fibrous materials before centrifugation. The resulting precipitate was suspended in 2 M NaCl/10 mM Tris and the pH was adjusted to pH 10.3. This suspension was then centrifuged at 356,000 ×*g* for 20 min. The extraction process was then repeated once. The pooled supernatant containing the solubilized myonase was passed through a pulp-sheet using a Buchner funnel under gentle suction to eliminate fatty materials. The filtrate was then subjected to affinity column-chromatography on two LBTI-agarose columns (each 16 mm i.d. × 4 mm) connected in series that had been pre-equilibrated with 2 M NaCl/10 mM Tris-HCl, pH 9. After application of the crude myonase sample and washing with the equilibration buffer, bound myonase was eluted with 2 M NaCl/10 mM HCl solution. The eluate was collected manually in a tube containing 1 M sodium acetate, pH 4.3 (final concentration 0.1 M) and bovine serum albumin (BSA; final concentration 0.1 mg/ml). The proteinase activity of each fraction was then assayed. The active peaks were pooled and dialyzed overnight against 1 mM sodium acetate, pH 4.3. All procedures were performed at 4°C. The dialyzate was stored at –80°C until use.

Assay of Myonase Activity—Enzyme activity was measured by hydrolysis of the synthetic peptide suc-leu-leu-val-tyr-AMC. The reaction mixture containing proteinase, 50 mM Tris/50 mM glycine, pH 9.0, 2 M NaCl and 30 μM suc-leu-leu-val-tyr-AMC in a final volume of 100 μl was incubated at 30°C for 20 min. The proteinase reaction was stopped by adding 1.4 ml of 2 M NaCl/0.1 M sodium acetate, pH 4.3. A fluorescence spectrophotometer model F-2000 (Hitachi Inc.) was used to monitor the production of free AMC ($\lambda_{excitation}$ 380 nm, $\lambda_{emission}$ 460 nm). One unit of proteinase activity was defined as the quantity of proteinase that would produce one nmole of AMC per min.

Isolation of Myofibrils from Skeletal Muscle—Myofibrils were prepared as previously described (*30*). Briefly, normal-mouse skeletal muscle (8.5 g wet weight tissue) stored at –80°C was homogenized in 20 volumes of buffer I [39 mM sodium borate buffer, pH 7.1/25 mM KCl/5 mM EGTA/1 mM dithiothreitol (DTT)/protease inhibitors] using a Cartridge Mill (Ikeda Scientific) for 15 s, twice. The cocktail of protease inhibitors in the above homogenization step comprised 1 mM benzamidine, 1 mM iodoacetamide, 1 μg/ml trypsin inhibitor (ovoinhibitor), 2 μg/ml leupeptin, 1 μg/ml pepstatin A, and 0.2 mM phenylmethanesulfonylfluoride. The resulting homogenate (177.5 ml) was centrifuged at 1,500 ×*g* for 12 min. The pellet was re-extracted once with buffer I by repeating a cycle of homogenization and centrifugation. The pellet was resuspended in 20 volumes of buffer II (39 mM sodium borate buffer, pH 7.1/25 mM KCl/5 mM EGTA/1 mM DTT). The suspension was then centrifuged at 1,500 ×*g* for 12 min. The resulting pellet was washed once with buffer II by repeating a cycle of homogenization and centrifugation. The pellet was next extracted for 30min with Triton X-100 buffer (39 mM sodium borate, pH 7.1/25 mM KCl/1 mM DTT/1% Triton X-100). This suspension was then filtered through a nylon cloth before centrifugation at 1,500 ×*g* for 12 min. The pellet was re-extracted once with Triton X-100 buffer by repeating a cycle of homogenization and centrifugation. The pellet was then washed with suspension buffer (10 mM Tris-HCl, pH 7.1/100 mM KCl/1 mM DTT) by carrying out two cycles of homogenization and centrifugation. Finally, the pellet containing myofibrils was resuspended in low-salt buffer (10 mM Tris-HCl, pH 7.1/100 mM KCl/1 mM DTT/20% glycerol) at a final protein concentration of 9.3 mg/ml.

Preparation of Endogenous Inhibitor—The myofibril

preparation was treated with 0.1 M HCl, and the endogenous inhibitor was recovered in the supernatant by centrifigation (1,500 ×*g* for 12 min). The supernatant was dialyzed against 5 mM HCl. The dialyzed supernatant did not contain myonase activity, but had an inhibitory activity of myonase.

Protein Content Determination—Protein concentration was determined by the Bradford method (*31*) using pre-prepared reagents (Bio Rad Lab.). The BioRad DC protein assay kit (Bio Rad Lab.) was also used to assay protein concentration following solubilization with detergent or NaOH. BSA was used for reference.

SDS/PAGE Analysis and Western Blotting—Protein was dissolved in SDS-treatment mixture (2% SDS/5% β-mercaptoethanol/62.5 mM Tris-HCl, pH 6.8/10% glycerol/0.002% bromophenol blue). Subsequent separation was carried out on discontinuous SDS/PAGE mini-slab gels [separation gel; 7 (W) × 8 (H) cm] according to the method of Laemmli (*32*). The separation gels contained either 12% acrylamide or a gradient of 5–12% acrylamide in 0.375 M Tris-HCl, pH 8.8/0.882% SDS. The 5–12% acrylamide gradient gels were stained by the picrate-Coomassie Brilliant Blue R250 method (*33*). Samples on the 12% acrylamide gels were electroblotted onto a nitrocellulose membrane (Nitro PlusTM 2000, Micron Separation) in transfer solution (48 mM Tris/39 mM glycine/1.3 mM SDS/20% methanol). The blotted protein was treated with polyclonal anti-myonase IgG from rabbit or monoclonal anti-myonase specific peptide IgG and detected using goat anti-(rabbit IgG + IgL) or goat anti-mouse IgG conjugated with HRP by the method of Towbin *et al.* (*34*).

Two-dimensional Gel Electrophoresis (TDGE)—The improved isoelectric focusing technique designed to isolate quantitatively muscle proteins with MMs in the 10–2,000 kDa range has been described elsewhere (*35*). Briefly, the sample was first dialyzed against a urea solution (5 M urea/1 M thiourea/0.17% β-mercaptoethanol/10 mM sodium pyrophosphate). A 1% agarose gel containing Pharmalyte cocktail in 10% sorbitol/5 M urea/1 M thiourea/1.6% NP-40 was prepared using a glass tube of dimensions 2.5 mm × 260 mm (*36*). An aliquot of dialyzate was applied to this gel and subjected to isoelectric focusing. After electrophoresis for 25 h at 500 V, the gel was fixed with a 10% TCA/5% sulfosalicylic acid solution. Electrophoresis in the second dimension was by SDS-PAGE. The fixed gel was placed along the top of a 5–12% acrylamide gradient slab gel [separation gel; 21 (W) × 11 (H) cm]. After electrophoresis, the gel was stained with Coomassie Brilliant Blue R250 as described above.

Conjugation of Monoclonal Anti-Myonase Specific Peptide IgG with HRP—Monoclonal anti-myonase specific peptide IgG was conjugated to HRP using the EZ-linkTM maleimide activated HRP kit (Pierce). Briefly, 1 ml of IgG solution [1 mg/ml in PBS (0.01 M sodium phosphate, pH 7.2/0.85% NaCl)] was mixed with 100 μl of maleimide conjugation buffer containing 6 μl of β-mercaptoethanol. This solution was then incubated for 90 min at 37°C to reduce all -SH groups within the IgG. The sample (1.1 ml) was then applied to a pre-equilibrated desalting column (polyacrylamide; 10 ml bed volume) and eluted with the same maleimide conjugation buffer. EZ-linkTM maleimide activated HRP (4 mg) was added to the reduced IgG fractions (1.5 ml, 0.5 mg protein/ml) and these were left to incubate overnight at room temperature. The resulting monoclonal anti-myonase specific peptide IgG conjugated with HRP (1.2 ml) was dialyzed against PBS. Finally, after addition of 2.4 ml of the super freezeTM peroxidase conjugate stabilizer (Pierce), anti-myonase specific peptide IgG conjugated with HRP was stored at –20°C.

Immunohistochemical Procedure—A frozen tissue section (10 μm thick) of mouse femoral muscle on a silane-coated slide was fixed with chilled acetone and pre-treated with 0.3% H_2O_2 in PBS to remove the endogenous peroxidase. The tissue section was blocked with BSA (1% in PBS) and then washed three times with PBS (each time for 3 min). Rabbit anti-myonase IgG (final concentration 0.3 mg/ml ; taking OD_{280nm} of 10 mg/ml IgG as 13.8) was then applied to the tissue section. To another tissue section was added a diluted solution of control rabbit IgG which had been prepared from serum prior to immunizing a rabbit with myonase. Both slides were placed in a moist chamber overnight, then removed, washed three times with PBS (each time for 3 min) to remove the excess IgG, and treated for 30

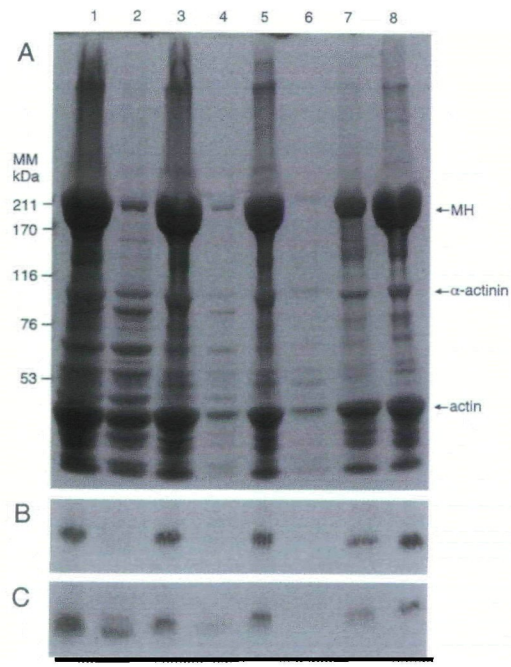

Fig. 1. **Presence of myonase in myofibrils.** A: Coomassie blue staining of SDS/PAGE gel loaded with muscle fractions obtained during the isolation of myofibrils. SDS/PAGE separation of myofibril proteins was performed using a separating gel with a 5–12% acrylamide gradient. The volume of each fraction was adjusted to produce the same final volume. By this method, each lane was loaded with an equal volume corresponding to 1 mg wet weight tissue. Lane 1, homogenate (H); 2, supernatant (S1) of the homogenate (buffer I wash); 3, pellet (P1) of the homogenate; 4, supernatant (S2) of the buffer II wash; 5, pellet (P2) of the buffer II wash; 6, supernatant (S3) of the Triton X-100 buffer wash; 7, pellet (P3) of Triton X-100 buffer wash; 8, the isolated myofibrils (MF) washed with the suspension buffer. Molecular mass standards used were myosin (MH) (211 kDa), α_2-macroglobulin (170 kDa), ß-galactosidase (116 kDa), transferrin (76 kDa), and glutamic dehydrogenase (53 kDa). The mobility of myosin, α-actinin and actin is indicated. B and C: Immunoblots obtained following electrotransfer onto nitrocellulose of myofibril proteins separated by SDS/PAGE on a 12% acrylamide gel. Panel B, rabbit anti-myonase IgG (0.3 mg protein/ml); Panel C, monoclonal anti-myonase specific peptide IgG (0.3 mg protein/ml).

min with a 1:100 dilution of goat anti-rabbit IgG conjugated with HRP. Finally, the slides were washed three times with PBS (each time for 3 min). In a similar immunohistochemical study, a 1:100 dilution of monoclonal anti-myonase specific peptide IgG conjugated with HRP was applied to tissue sections. As a control, monoclonal anti-myonase specific peptide IgG conjugated with HRP was absorbed with myonase specific peptide (final concentration 3.8 mM) prior to treatment (1:100 dilution) of another tissue section. After incubation overnight in a moist chamber, the slides were washed as previously described. All tissue sections were developed by incubation for several minutes in a solution of 50 mM Tris-HCl, pH 7.86/1.3 mM 3,3'-diaminobenzidine tetra-hydrochloride dehydrate (DAB)/3 mM H_2O_2. The slides were then washed with PBS. The sections on a glass slide were protected with a glass cover slip that was mounted with Entellan neu (Merck). Finally, the slides were observed under a microscope (Zeiss, Axioskop 50).

Histochemical Analysis with FITC-Aca-Phe-Leu-PheP-(OPh)$_2$—A frozen section (10 μm thick) of mouse femoral muscle on a silane-coated slide was fixed with chilled acetone as previously described. The slides were then blocked with BSA (1% in PBS). FITC-aca-phe-leu-pheP(OPh)$_2$ in PBS/1% BSA was applied to the tissue sections at a final concentration of 1 μM. As a control, FITC in PBS/1% BSA

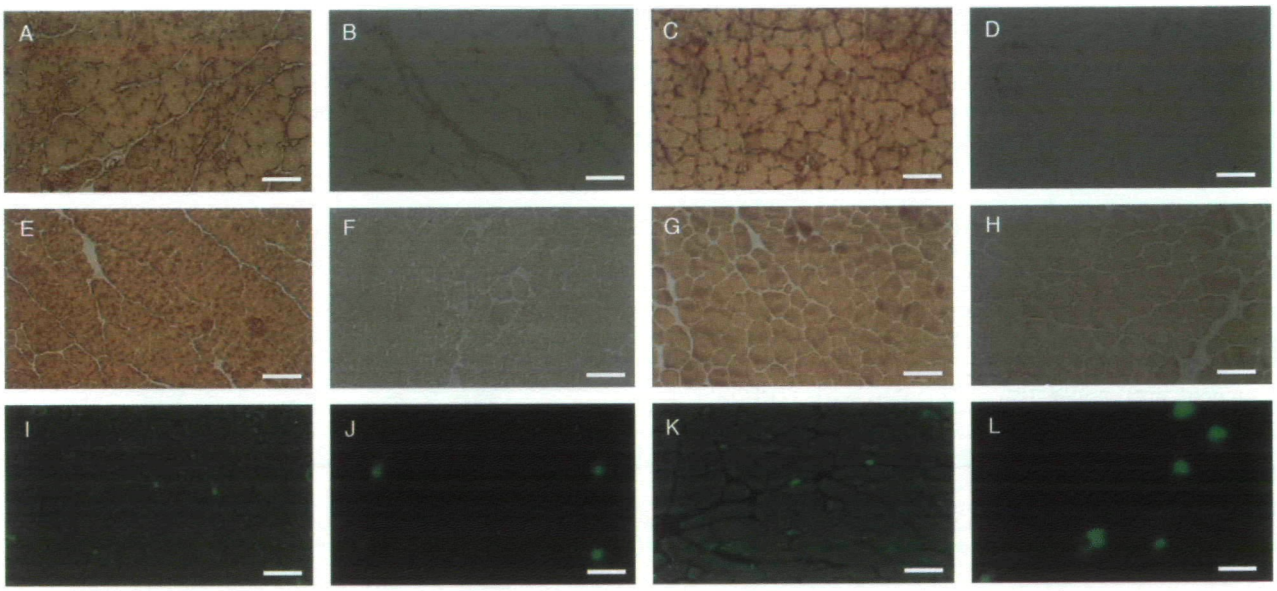

Fig. 2. **Immunohistochemical and histochemical localization of myonase in muscle.** A, B, E, F, I, J: femoral rectal muscle from MDX-mouse; C, D, G, H, K, L: femoral rectal muscle from control mouse. A, C: immunostaining with rabbit anti-myonase IgG; B, D: staining with control rabbit IgG; E, G: immunostaining with monoclonal anti-myonase specific peptide IgG conjugated with HRP; F, H: immunostaining with monoclonal anti-myonase specific peptide IgG conjugated with HRP, which was absorbed with myonase-specific peptide; I, K: staining with FITC-aca-phe-leu-phe $_P(OPh)_2$; J, L: staining with FITC. Original magnifications ×200. bar = 50 μm.

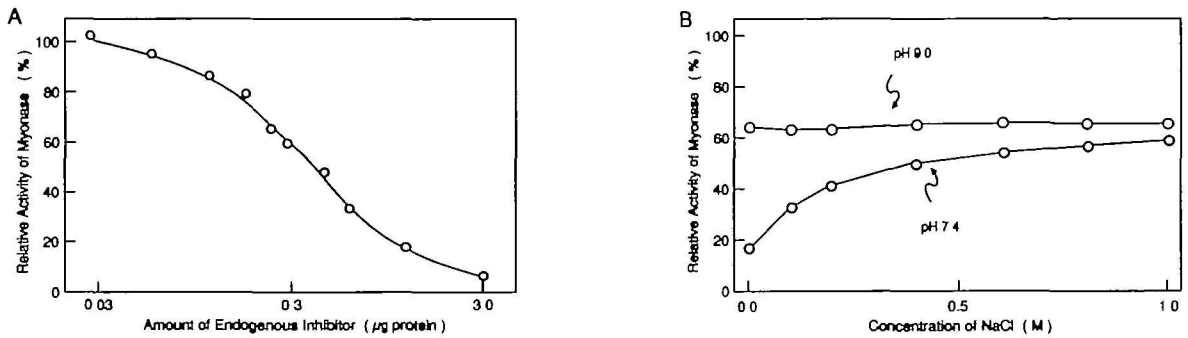

Fig. 3. **Inhibitory activity of the endogenous inhibitor.** The endogenous inhibitor was solubilized from myofibrils with 0.1 M HCl, then dialyzed against 5 mM HCl. A: Inhibition of myonase activity by the endogenous inhibitor. The activity of myonase was assayed in the presence of various amounts of the endogenous inhibitor (0–3 μg protein/100 μl) at pH 7.4 in the absence of NaCl. The activity is represented relative to the activity of myonase in the absence of the endogenous inhibitor. B: Effects of pH and salt concentration on inhibition of the endogenous inhibitor. The activity of myonase was assayed in the presence of the endogenous inhibitor (1.2 μg protein) at various concentrations of salt (0–1 M NaCl) and at pH 7.4 and 9.0. The activity is represented relative to the activity of myonase in the absence of the endogenous inhibitor under the same conditions. In the experiments performed at pH 7.4, 50 mM sodium phosphate was substituted for 50 mM Tris/50 mM glycine. Otherwise, reaction conditions were as described in "MATERIALS AND METHODS."

was also applied to a tissue section at a final concentration of 1 μM. The slides were placed in a moist chamber for 60 min, then washed three times with PBS/1% BSA (each time for 3 min). The tissue sections on a glass slide were protected with a glass cover slip that was mounted with Mounting Medium for fluorescent microscopy (Kirkegaard & Perry Lab.) and viewed immediately under a fluorescence microscope (Zeiss, Axioskop 50).

Cell Culture of Myoblast—Mouse myoblasts (cell line C_2C_{12}; ATCC) were cultured in 10% fetal calf serum/DMEM according to standard protocol. Differentiation to myotube was carried out by culturing the cells in 2% horse serum/DMEM.

mRNA Isolation—Extraction of total RNAs from cultured myoblasts and poly $(A)^+$ RNA selection were performed using the mRNA isolation kit (Stratagene).

Rapid Amplification of cDNA Ends (RACE) and Sequencing—3′RACE and 5′RACE of poly$(A)^+$ RNA extracted from mouse myoblasts were carried out using either the 3′RACE system or 5′RACE system kit (Life Technologies). The resulting first-strand cDNA was amplified by PCR between the gene-specific primer and the abridged universal amplification primer (AUAP). The gene-specific primer for 3′RACE (5′-ATT ATT GGT GGT GTT GAG TCT AGA-3′) was deduced from the N-terminal amino-acid sequence of myonase. The gene-specific primer for 5′RACE (5′-GGC TGA GGA CAG GCC GGG GG-3′) was in turn deduced from the sequence determined by 3′RACE. The products of PCR were separated by electrophoresis. The band comprising a fragment of the expected size was excised and purified using the Prep-A-Gene purification kit (Bio Rad Lab.). The nucleotide sequence was determined with an Applied Biosystem model 373 A DNA Sequencer using the ABI PRISM dye terminator cycle sequencing core kit.

RESULTS

Subcellular Fractionation Reveals that Myonase is Associated with the Myofibrillar Fraction—The subcellular fractions of mouse skeletal muscle were isolated as described in "MATERIALS AND METHODS." SDS-PAGE analysis of protein composition in each fraction is shown in Fig. 1. The muscle homogenate (H, lane 1) was centrifuged to obtain a supernatant fraction (S1, lane 2) and a pellet (P1, lane 3). The

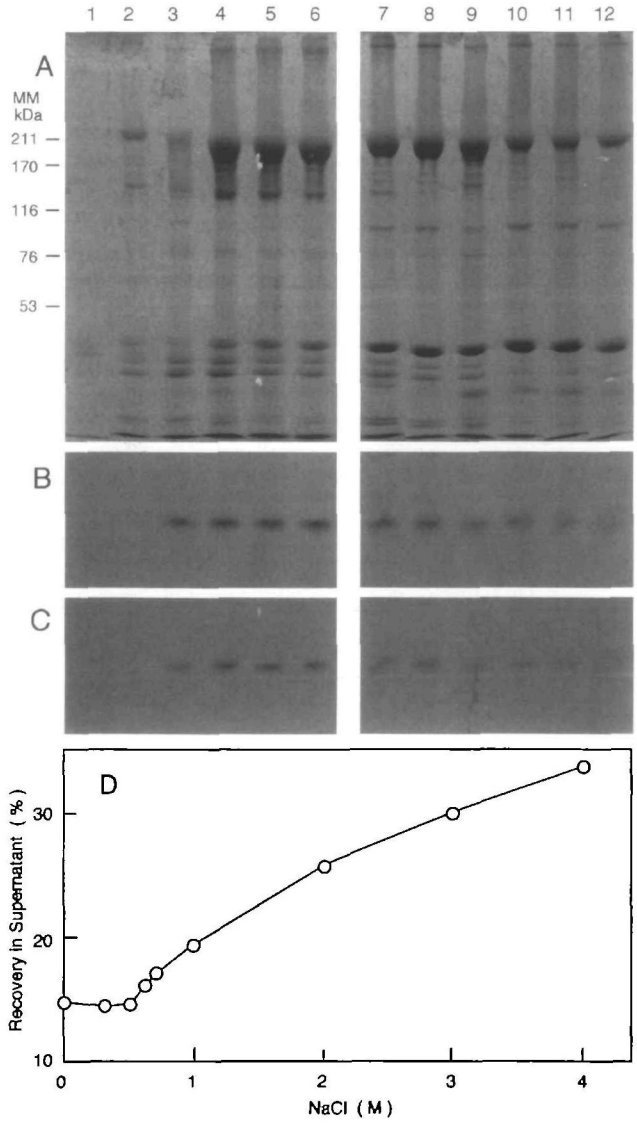

Fig. 4. **Effect of NaCl on solubility of myonase in myofibrils.** (1) SDS/PAGE and Western blotting: Myofibrils (1.2 mg protein) were incubated in buffer (50 mM sodium phosphate buffer, pH 7.4) containing various concentrations of NaCl (0, 0.5, 1, 2, 3, and 4 M). Following incubation, the volume of each sample was adjusted to produce a final volume of 500 μl. The samples were then centrifuged at 250,000 ×*g* for 25 min. After decanting, 100 μl of 7 M guanidine HCl was added to both the precipitate and the supernatant. The precipitate and supernatant fractions were then dialyzed against urea solution (5 M urea/1M thiourea/0.17% β-mercaptoethanol/10 mM sodium pyrophosphate) in the first instance and then against SDS-treatment mixture. Finally, after adjusting the volume of each fraction to 560 μl with SDS-treatment mixture, a 10-μl aliquot was subjected to SDS/PAGE for analysis. A: Coomassie blue staining of myofibril proteins separated by SDS/PAGE on a 5–12% acrylamide gradient gel. B and C, Immunoblots obtained following electrotransfer onto nitrocellulose of myofibril proteins separated by SDS/PAGE on a 12% acrylamide gel. Panel B, rabbit anti-myonase IgG (0.3 mg protein/ml); Panel C, monoclonal anti-myonase specific peptide IgG (0.3 mg protein/ml). Lanes 1, 2, 3, 4, 5, and 6 represent the supernatant fractions at 0, 0.5, 1, 2, 3, and 4 M NaCl, respectively, while lanes 7, 8, 9, 10, 11, and 12 represent the corresponding precipitate fractions. (2) Solubilization of FITC-aca-phe-leu-phe $_P(OPh)_2$ labeled myonase. D: Myofibril (3.7 mg protein) was suspended in reaction buffer (10 mM Tris-HCl, pH 7.1/1 mM DTT) containing 1 μM FITC-aca-phe-leu-phe $_P(OPh)_2$ in a final volume of 2 ml. The suspension was then centrifuged at 1,500 ×*g* for 12 min. After two cycles of washing by suspension in reaction buffer (2 ml) and centrifugation, the precipitate was suspended in 2 ml of reaction buffer. A 50-μl aliquot of the suspension (0.093 mg protein) was then incubated in 50 mM sodium phosphate buffer, pH 7.4 containing various concentrations of NaCl (0, 0.5, 1, 2, 3, and 4 M). The volume of each suspension was adjusted to produce a final volume of 500 μl. A 200-μl aliquot of this suspension was centrifuged at 250,000 ×*g* for 25 min, then 50 μl of 1 M NaOH was added to both precipitate and supernatant fractions. For comparison, 50 μl of 1 M NaOH was also added to another 200-μl aliquot of suspension. The volumes of suspension, supernatant and precipitate were then adjusted to 1.5 ml with 2 M NaCl/100 mM Na_2CO_3-$NaHCO_3$, pH 10.5. The fluorescence intensities of the suspension, supernatant and precipitate fractions were estimated ($\lambda_{excitation}$ 495 nm and $\lambda_{emission}$ 520 nm). In each experiment, 86.2–104% of the suspension fluorescence was recovered in the supernatant and precipitate fractions.

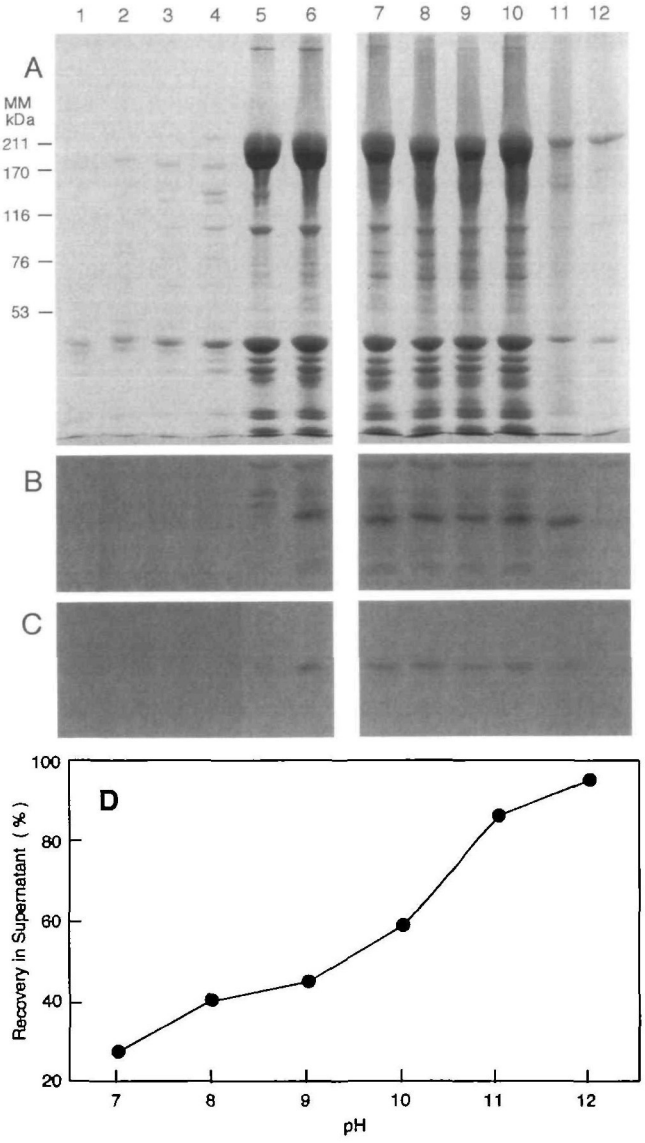

Fig. 5. **Effect of pH on solubility of myonase in myofibrils.** (1) SDS/PAGE and Western blotting. Myofibrils (4.8 mg protein) were incubated in a solution containing 10 mM sodium carbonate or 10 mM sodium phosphate at various pHs (7, 8, 9, 10, 11, and 12). The solution pH was adjusted with 1 M NaOH or 1 M HCl, then the volume was adjusted to produce a final volume of 2,000 μl. After centrifugation at 250,000 ×*g* for 25 min, 400 μl of 7 M guanidine HCl was added to the precipitate and supernatant fractions. The fractions were each dialyzed against urea solution, then against SDS-treatment mixture, and their volumes were adjusted with SDS-treatment mixture to produce a final volume of 2,240 μl. A 10-μl aliquot of each fraction was then subjected to SDS/PAGE for analysis. A: Coomassie blue staining of myofibril proteins separated by SDS/PAGE on a 5–12% acrylamide gradient gel. B and C: Immunoblots obtained following electrotransfer onto nitrocellulose of myofibril proteins separated by SDS/PAGE on a 12% acrylamide gel. Panel B, rabbit anti-myonase IgG (0.3 mg protein/ml); Panel C, monoclonal anti-myonase specific peptide IgG (0.3 mg protein/ml). Lanes 1, 2, 3, 4, 5, and 6 represent the supernatant fractions at pH 7, 8, 9, 10, 11 and 12, respectively, while lanes 7, 8, 9, 10, 11, and 12 represent the corresponding precipitate fractions. (2) Solubilization of FITC-aca-phe-leu-phe $_p(OPh)_2$ labeled myonase. D: All reaction conditions for the labeling of myonase were as described in the legend to Fig. 4. A 200-μl aliquot of the suspension (0.368 mg protein) containing labeled myonase was incubated in buffer at various pHs (7, 8, 9, 10, 11, and 12) in a final volume of 2 ml (as described above). The conditions used for the isolation of supernatant and precipitate fractions were also as described in the legend to Fig. 4. In each experiment, 94.1–119.3% of suspension fluorescence was recovered in the supernatant and precipitate fractions.

pellet (P1) was washed extensively with buffer II to obtain a second supernatant (S2, lane 4) and a second pellet (P2, lane 5). This pellet (P2) was further extracted with Triton X-100 to obtain a third supernatant (S3, lane 6) and a third pellet (P3, lane 7). The final product represented isolated myofibrils (MF, lane 8). The MF fraction predominantly consisted of myosin (MH) (211 kDa) and actin (42 kDa). Other cytoskeletal proteins, including α-actinin (105 kDa) and desmin (55 kDa), were also present in this fraction. The myonase content in each fraction was examined by immunoblotting using antibodies directed against myonase. Two gels loaded with samples identical to those shown in Fig. 1A were electroblotted onto nitrocellulose and immunostained with rabbit anti-myonase IgG (Fig. 1B) or monoclonal anti–myonase specific peptide IgG (Fig. 1C). The pellets of each suspension and the isolated myofibrils stained strongly for myonase (26 kDa) [Fig. 1, B and C, lanes 3 (P1), 5 (P2), 7 (P3), and 8 (MF)]. In contrast, the supernatant fractions stained only faintly for myonase [Fig. 1, B and C, lanes 2 (S1), 4 (S2), and 6 (S3)]. Furthermore, the nonionic detergent Triton X-100 failed to extract a significant amount of myonase from the pellet fraction [Fig. 1, B and C, lane 6 (S3)]. Hence, the isolated myofibrils contained a significant amount of myonase [Fig. 1, B and C, lane 8 (MF)]. The same immunostaining pattern for myonase was observed in three different myofibril preparations irrespective of whether the source was MDX-mouse or control mouse. The results indicate that myonase is strongly bound to the myofibril.

Immunohistochemical and Histochemical Localization of Myonase—Muscle fibres of MDX-mouse and control mouse were both stained by rabbit anti-myonase IgG (Fig. 2, A–D). In addition, a number of small cells scattered around the muscle fibre were also stained by rabbit anti-myonase IgG. The muscle fibres of MDX-mouse and control mouse were both also recognized by monoclonal anti–myonase specific peptide IgG conjugating with HRP (Fig. 2, E–H). However, this monoclonal antibody did not recognize the small cells scattered around the muscle fibres that were stained by the rabbit polyclonal antibody. When monoclonal anti-myonase specific peptide IgG conjugated with HRP was pre-incubated with myonase specific peptide, staining was reduced (Fig. 2, F and H), indicating that monoclonal anti-myonase specific peptide IgG conjugated with HRP specifically bound myonase. The muscle fibres of MDX-mouse and control mouse were both also recognized by FITC-aca-phe-leu-pheP$(OPh)_2$ (Fig. 2, I and K). This fluorescent derivative of an irreversible inhibitor of chymotrypsin (*37*) was shown to bind myonase. This inhibitor also inhibited myonase activity (data not shown). These results confirm that myonase is located inside the myocyte.

Endogenous Inhibitor of Myonase—The activity of myonase was extensively inhibited by an endogenous inhibitor within the myofibrils (Fig. 3A). However, this inhibition was substantially diminished at alkaline pH (pH 9.0; Fig. 3B). Furthermore, at pH 7.4, but not pH 9.0, inhibition was

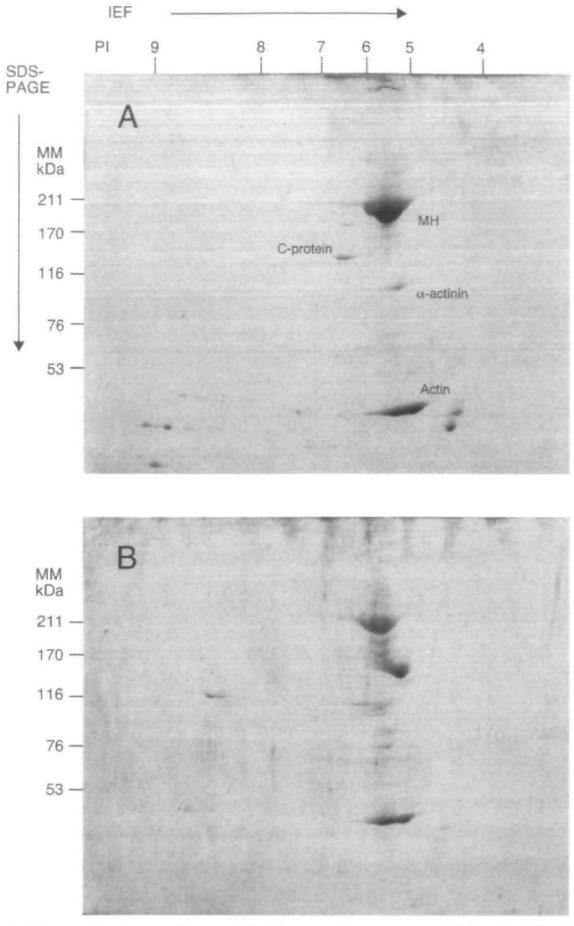

Fig. 6. **Degradation of myofibrils with myonase.** Myofibrils (0.93 mg protein) and purified myonase (56.5 milliunits) were incubated together in a final volume of 0.4 ml for 60 min. The digestion conditions were otherwise as described for the myonase assay in "MATERIALS AND METHODS." The reaction was stopped by the addition of 0.808 g of solid guanidine HCl. As a control, 0.808 g of solid guanidine HCl was added to an identical reaction mixture prior to incubation. Both samples were then dialyzed against urea solution (5 M urea/1 M thiourea/0.17% ß-mercaptoethanol/10 mM sodium pyrophosphate). A 90.6-μl aliquot of the dialyzate (1.22 ml) was subjected to TDGE. A, control; B, myofibril degraded with myonase.

dependent on the concentration of NaCl (Fig. 3B). Indeed, inhibition of myonase at pH 7.4 drastically changed at physiological concentrations of salt (0.1–0.3 M). Upon further investigation, the endogenous inhibitor was observed to inhibit myonase activity non-competitively with synthetic peptide substrate suc-leu-leu-val-phe-AMC (data not shown). Thus, the endogenous inhibitor does not function as a substrate of myonase.

Western Blotting of Myoblast—Using rabbit polyclonal anti-myonase IgG and monoclonal anti–myonase specific peptide IgG on Western blots of total cell extracts, myonase protein was observed to be present in both C_2C_{12} myoblasts and differentiated myotubes derived from myoblasts.

mRNA of Myonase in Myoblasts—Total poly(A)$^+$RNA from mouse myoblasts and differentiated myotubes derived from mouse myoblasts were extracted as described in "MATERIALS AND METHODS." Using myonase-specific primers for 3′RACE and 5′RACE, a cDNA fragment with the myonase sequence was generated from the poly(A)$^+$RNA. This data confirms that myoblasts express myonase protein. These results, together with the Western blot data, suggest that myonase accumulates within the myocyte.

Release of Myonase from Myofibrils—Myonase in myofibrils was gradually released at concentrations of NaCl greater than 0.6 M (Fig. 4, B and C). The increase in salt concentration also resulted in the solubilization of other myofibril proteins (Fig. 4A). However, even at 4 M NaCl, 80% or more of the myonase remained within the myofibrils (Fig. 4D). At alkaline pH (above pH 11), a considerable amount of the myonase in myofibrils was released (Fig. 5, B and C). Again, these conditions resulted in solubilization of other myofibril proteins (Fig. 5A). At pH 12, myonase and other components of the myofibril were almost exclusively found in the soluble fraction (Fig. 5, A, B, C, and D). Accordingly, high pH and high concentrations of salt were required in order to release myonase from myofibrils. However, such conditions are clearly not a physiological mechanism for myonase release in vivo.

Degradation of Myofibrils by Myonase—A number of the protein components of myofibrils were degraded after incubation with purified myonase *in vitro* (Fig. 6). Although actin proved to be relatively resistant to myonase activity, the C-protein was completely degraded and the myosin heavy chain was partially degraded.

DISCUSSION

The present study is concerned with a triggering proteinase for the cascade degradation of myofibrillar proteins during muscle atrophy or necrosis which develops in various pathological states of muscle including muscular dystrophy. For the three proteolytic pathways involving (i) Ca^{2+}-dependent proteinases, (ii) lysosomal proteinases, and (iii) ATP/ubiquitin-dependent proteolysis, many critical and fundamental questions arise if we try to relate them to the initial event in the cascade of reactions involved in myofibrillar degeneration.

Chymotrypsin-like serine proteinases have recently been purified from MDX-mouse skeletal muscle (myonase) (*28*) and from hamster skeletal muscle (mekratin) (*29*). These proteinases have different physicochemical properties. Mekratin could be solubilized with 0.47 M KCl at pH 6.8 (*29*), but myonase could not: it was only partially solubilized even with 2 M KCl (or NaCl) at pH 10 (*28*). Mekratin was not inhibited by 2.5% Triton X-100 (*29*), but myonase was completely inhibited by a concentration of more than 1% (unpublished). Mekratin recovered its activity after treatment with SDS (*29*), but myonase did not (unpublished). Mekratin did not require high salt concentration for its proteolytic activity (*29*), but myonase required a remarkably high salt concentration for its optimum activity [calculated salt concentration required for half of the maximum activity was 11.6 M NaCl or KCl (*28*)]. Mekratin was absent in myofibrils (*29*), but myonase present (Figs. 1 and 2). Myonase in myofibrils was inactive under physiological conditions (0.15 M KCl or NaCl, pH 7.4), but activity of purified myonase could be detected under physiological conditions (0.15 M KCl, pH 7.4), even though its activity was only 10–20% of that at optimal pH (pH 9.0) and relatively high salt concentration (2 M KCl or NaCl). Extracts of MDX-mouse skeletal muscle with 0.6 M NaCl/10 mM Tris-HCl, pH 7.8, contained activity similar to myonase,

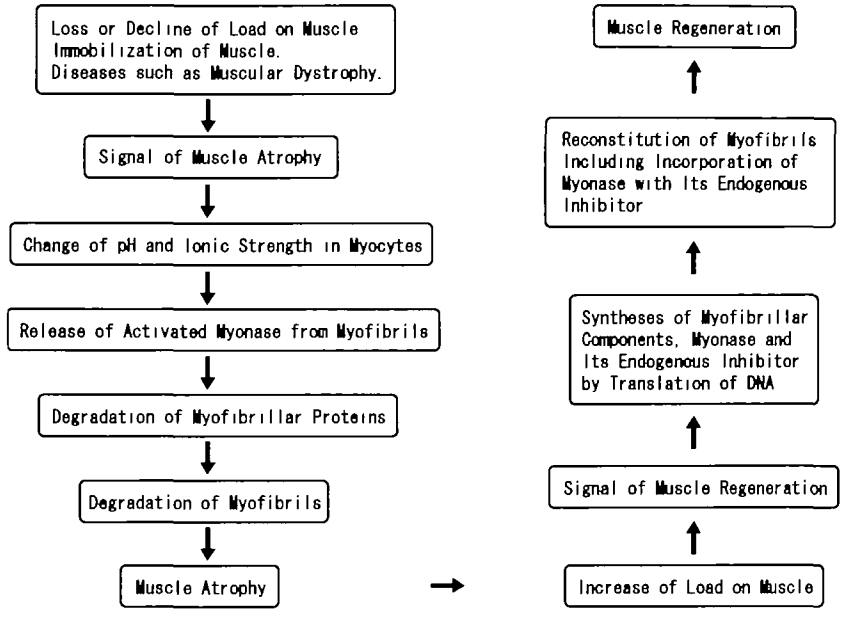

Fig. 7. **Hypothesis for degeneration and regeneration of myofibrils during muscle atrophy.** Myofibrils contain a large amount of proteinase, so-called myonase, stored in an inactive form by binding with the endogenous inhibitor. Thus, the myofibril is protected from this myonase activity. When the signal to degrade myofibrils in developing muscle atrophy arrives in the myocyte, myonase is released from the endogenous inhibitor and changed to its active form to degrade the myofibril. Muscle atrophy results. When the load on muscle is again increasing, the signal to regenerate myofibrils appears in the myocyte. The syntheses of myofibrillar components, myonase and its endogenous inhibitor result from translation of DNA. Reconstitution of myofibrils occurs, including the incorporation of myonase with its endogenous inhibitor. This mechanism is reasonable for the skeletal muscle to keep its structure by the dynamic equilibrium between degradation and reconstitution; unnecessary structures are rapidly degraded to remove them from the myocyte and provide space for new structures.

but washing of tissue with 0.6 M NaCl/10 mM Tris-HCl, pH 7.8, was necessary to attain high purification of myonase in our procedures. Chymotrypsin-like activity was detected in the extract with 0.6 M NaCl/10 mM Tris-HCl, pH 7.8, but Western blotting showed that the activity was due to a proteinase of lower molecular mass than myonase and that there was very little protein in the extract that had the same molecular mass as myonase. These results suggest that myonase released as an active form from myofibrils is labile or is rapidly metabolized and remove from the tissue.

The origin of chymotrypsin-like proteinases in muscle is still unclear. However, our observations (Figs. 1 and 2) show that myonase is present in myofibrils from normal mouse skeletal muscle. Myoblasts (C_2C_{12}) from mouse had developed mRNA of myonase. These results indicate that myonase is synthesized in myocytes by translation from DNA and locates in myofibrils. Furthermore, the amount of myonase in normal mouse skeletal muscle is the same as that in MDX-mouse skeletal muscle, although the recoverable amount of active myonase as a pure protein was 5–10-fold more when MDX-mouse was the source of the skeletal muscle. These results indicate that myonase in myofibrils of MDX-mouse skeletal muscle is more easily released than that in myofibrils of normal mouse skeletal muscle.

The release of myonase from myofibrils *in vitro* depended on the salt concentration (Fig. 4) and pH (Fig. 5). Even if ionic strength and pH are regulatory factors of myonase release *in vivo*, however, the high salt concentration and pH required *in vitro* would not arise *in vivo* in response to extracellular conditions. In addition, the extracellular pH does not affect the intracellular pH (*38*). Although these conditions of salt concentration and pH are unphysiological, conditions approaching them might arise in a local area or a specific intracellular compartment of myocytes in a pathological state. More likely, however, is that the regulatory factor(s) involved in release from the myofibril and activation of myonase in situ remain to be determined.

Myonase degraded structural proteins such as myosin heavy chain, especially proteins with molecular mass greater than 100 kDa, although the fast type C-protein seems to be the most preferred as substrate. Actin was resistant to the proteolysis by myonase (Fig. 6).

Our observations suggest a possible functional role for myonase (Fig. 7). Myonase is synthesized in myocytes by translation from its gene. The endogenous inhibitor and other myofibril proteins are also synthesized in myocytes. The contractile components of myofibrils, myonase and its endogenous inhibitor are constructed into the myofibrillar network. Under these conditions, myonase is inactive due the presence of to its endogenous inhibitor, but it is activated under pathological conditions. This active myonase triggers a cascade degradation of myofibril proteins associated with muscle atrophy. This mechanism may appear dangerous for the survival of muscle structure, but it might function for removal of unnecessary structures from muscle as quickly as possible to enable regeneration. If this mechanism can be shown to operate under physiological conditions in muscle, novel methods for therapy or prevention of muscle atrophy may suggest themselves.

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REFERENCES

1. Bulfield, G., Siller, W.G., Wight, P.A.L., and Moore, K.J. (1984) X chromosome –linked muscular dystrophy (mdx) in the mouse. *Proc. Natl. Acad. Sci. USA* **81**, 1189–1192
2. Tanabe, Y., Esaki, K., and Nomura, T. (1986) Skeletal muscle pathology in X chromosome-linked muscular dystrophy (mdx) mouse. *Acta Neuropathol.* **69**, 91–95
3. Bond, J.S. and Butler, P.E. (1987) Intracellular proteases. *Annu. Rev. Biochem.* **56**, 333–364
4. Katunuma, N. and Kominami, E. (1987) Abnormal expression of lysosomal cysteine proteinases in muscle wasting diseases. *Rev. Physiol. Biochem. Pharmacol.* **108**, 1–20
5. Orlowski, M. (1990) The multicatalytic proteinase complex, a major extralysosomal proteolytic system. *Biochemistry* **29**, 10289–10297
6. Demartino, G.N. and Ordway, G.A. (1998) Ubiquitin-proteasome pathway of intracellular protein degradation: Implica-

tions for muscle atrophy during unloading. *Exerc. Sport. Sci. Rev.* **26**, 219–252
7. Tidball, J.G. and Spencer, M.J. (2000) Calpains and muscular dystrophies. *IJBCB* **32**, 1–5
8. Sorimachi, H. (2000) Structure and function of calpain and its homologies (in Japanese). *Seikagaku* **72**, 1297–1315
9. Stracher, A. (1999) Calpain inhibitors as therapeutic agents in nerve and muscle degeneration. *Ann. N.Y. Acad. Sci.* **884**, 52–59
10. Mykles, D.L. (1998) Intracellular proteinases of invertebrates: Calcium-dependent and proteasome/ubiquitin-dependent systems. *Int. Rev. Cytol.* **184**, 157–289
11. Carafoli, E. and Molinari, M. (1998) Calpain: A protease in search of a function. *Biochem. Biophys. Res. Commun.* **247**, 193–203
12. Ikemoto, M., Nikawa, T., Takeda, S., Watanabe, C., Kitano, T., Baldwin, K.M., Izumi, R., Nonaka, I., Towatari, T., Teshima, S., Rokutan, K., and Kishi, K. (2001) Space shuttle flight (STS-90) enhances degradation of rat myosin heavy chain in association with activation of ubiquitin-proteasome pathway. *FASEB. J.* **15**, 1279–1281
13. Solomon, V. and Goldberg, A.L. (1996) Importance of the ATP-ubiquitin-proteasome pathway in the degradation of soluble and myofibrillar proteins in rabbit muscle extracts. *J. Biol. Chem.* **271**, 26690–26697
14. Solomon, V., Lecker, S.H., and Goldberg, A.L. (1998) The N-end rule pathway catalyze a major fraction of the protein degradation in skeletal muscle. *J. Biol. Chem.* **273**, 25216–25222
15. Solomon, V., Baracos, V., Sarraf, P., and Goldberg, A.L. (1998) Rates of ubiquitin conjugation increase when muscle atrophy, largely through activation of the N-end rule pathway. *Proc. Natl. Acad. Sci. USA* **95**, 12602–12607
16. Higuchi, H. and Ishiwata, S. (1985) Disassembly kinetics of thick filaments in rabbit skeletal muscle fibers. Effects of ionic strength, Ca^{2+} concentration, pH, temperature, and cross-bridges on the stability of thick filament structure. *Biophys. J.* **47**, 267–275
17. Andrews, M.A.W., Maughan, D.W., Nosek, T.M., and Godt, R.E. (1991) Ion-specific and general ionic effects on contraction of skinned fast-twich skeletal muscle from the rabbit. *J. Gen. Physiol.* **98**, 1105–1125
18. Belcastro, A.N., Scrubb, J., and Gilchrist, J.S. (1991) Regulation of ATP-stimulated releasable myofilaments from cardiac and skeletal muscle myofibrils. *Mol. Cell. Biochem.* **103**, 113–120
19. Woodbury, R.G., Everitt, M., Sanada, Y., Katunuma, N., Lagunoff, D., and Neurath, H. (1978) A major serine protease in rat skeletal muscle: Evidence for its mast cell origin. *Proc. Natl. Acad. Sci. USA* **75**, 5311–5313
20. Hartz, P.A. and Bird, J.W.C. (1988) The question of chymase in cultured muscle cells. *Archiv. Biochem. Biopys.* **263**, 293–298
21. Gorospe, J.R.M., Tharp, M.D., Hinckley, J., Kornegay, J.N., and Hoffman, E.P. (1994) A role for mast cells in the progression of Duchenne muscular dystrophy? Correlations in dystrophin-deficient humans, dogs, and mice. *J. Neurol. Sci.* **122**, 44–56
22. Stauber, W.T., Fritz, V.K., Dahlmann, B., Kay, J., Health, R., and Mayer, M. (1987) Alkaline proteinase localization in myoblasts. *J. Histochem. Cytochem.* **35**, 83–86
23. Heath, R., Kay, J., Kuehn, L., Dahlmann, B., Stauber, W.T., and Mayer, M. (1987) Immunochemical characterization of the myofibrillar proteinase from cultured rat myocytes as chymase. *Biochem. Int.* **14**, 675–683
24. Appell, H.J. (1990) Muscular atrophy following immobilization. *A review Sports Med.* **10**, 42–58
25. Nakagawa, Y., Morii, H., Totsuka, M., Sato, T., and Hirota, K. (1990) Changes in total fiber numbers of disused soleus muscle on rats. *Ann. Physiol. Anthropol.* **9**, 15–20
26. Martin, A.F., Rabinowitz, M., Blough, R., Prior, G., and Zak, R. (1977) Measurements of half-life of rat cardiac myosin heavy chain with leucyl-tRNA used as precursor pool. *J. Biol. Chem.* **252**, 3422–3429
27. Laurent, G.J., Sparrow, M.P., Bates, P.C., and Millward, D.J. (1978) Turnover of muscle protein in the fowl (Gallus domesticus). Rates of protein synthesis in fast and slow skeletal, cardiac and smooth muscle of the adult fowl. *Biochem. J.* **176**, 393–401
28. Hori, S., Ohtani, S., Hori, C., and Nokihara, K. (1998) Purification and characterization of myonase from X-chromosome linked muscular dystrophic mouse skeletal muscle. *J. Biochem.* **123**, 650–658
29. Holt, J.C., Hatcher, V.B., Caulfield, J.B., Norton, P., Umeda, P.K., Melendez, J.A., Martino, L., Mudzinsky, S.P., Blumenstock, F., Slayter, H.S., and Margossian, S.S. (1998) Cloning of the cDNA and nucleotide sequence of a skeletal muscle protease from myopathic hamsters. *Mol. Cell. Biochem.* **181**, 125–135
30. Meng, H., Leddy, J.J., Frank, J., Holland, P., and Tuana, B.S. (1996) The association of cardiac dystrophin with myofibrils/Z-disc regions in cardiac muscle suggests a novel role in the contractile apparatus. *J. Biol. Chem.* **271**, 12364–12371
31. Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254
32. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685
33. Stephano, J.L., Gould, M., and Rojas-Galicia, L. (1986) Advantages of picrate fixation for staining polypeptides in polyacrylamide gels. *Anal. Biochem.* **152**, 308–313.
34. Towbin, H., Staehin, T., and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**, 4350–4354
35. Hori, S., Sugiura, H., Shimizu, T., Hirabayashi, T., Ohtani, S., Yoshida, M., Miyamoto, K., and Tanabe, H. (1989) Detection of dystrophin on two-dimensional gel electrophoresis. *Biochem. Biophys. Res. Commun.* **161**, 726–731
36. Hirabayashi, T. (1981) Two-dimensional gel electrophoresis of chicken skeletal muscle proteins with agarose gels in the first dimension. *Anal. Biochem.* **117**, 443–451
37. Abuelyamen, A.S., Hudig, D., Woodard, S.L., and Powers, J.C. (1994) Fluorescent derivatives of diphenyl [1-(N-peptidylamino)alkyl]phosphonate esters: Synthesis and use in the inhibition and cellular localization of serine proteases. *Bioconjugate Chem.* **5**, 400–405
38. Bailey, J.L., England, B.K., Long, R.C.Jr., Weissman, J., and Mitch, W.E. (1995) Experimental acidemia and muscle cell pH in chronic acidosis and renal failure. *Am. J. Physiol.* **269**, C706–C712