Importance of the ATP-Ubiquitin-Proteasome Pathway in the Degradation of Soluble and Myofibrillar Proteins in Rabbit Muscle Extracts

(Received for publication, February 26, 1996, and in revised form, July 12, 1996)

Vered Solomon and Alfred L. Goldberg

From the Department of Cell Biology, Harvard Medical School, Boston, Massachusetts 02115

Recent studies have suggested that activation of the ubiquitin-proteasome pathway is primarily responsible for the rapid loss of muscle proteins in various types of atrophy. The present studies were undertaken to test if different classes of muscle proteins are degraded by this pathway. In extracts of rabbit psoas muscle, the complete degradation of soluble proteins to amino acids was stimulated up to 6-fold by ATP. Peptide aldehyde inhibitors of the proteasome or the removal of proteasomes markedly inhibited only the ATP-dependent process. Addition of purified myosin, actin, troponin, or tropomyosin to these extracts showed that these proteins served as substrates for the ubiquitin-proteasome pathway. By contrast, degradation of myoglobin did not require ATP, proteasomes, or any known proteases in muscles.

When myosin, actin, and tropoerin were added as actomyosin complexes or as intact myofibrils to these extracts, they were not hydrolyzed at a significant rate, probably because in these multicomponent complexes, these proteins are protected from degradation. Accordingly, actin (but not albumin or troponin) inhibited the degradation of 125I-myosin, and actin was found to selectively inhibit ubiquitin conjugation to 125I-myosin. Also, the presence of tropomyosin inhibited the degradation of 125I-troponin. However, neither actin nor tropomyosin inhibited the degradation of 125I-tropomyosin. Therefore, specific interactions between the myofibrillar proteins appear to protect them from ubiquitin-dependent degradation, and the rate-limiting step in their degradation is probably their dissociation from the myofibril.

In both eukaryotic and prokaryotic cells, the degradation of most proteins requires ATP (1, 2). In eukaryotes, this energy requirement is primarily due to the ATP-requiring proteolytic system, involving the polypeptide cofactor ubiquitin (Ub), and the proteasome (3–5). In this pathway, protein substrates are initially conjugated covalently to Ub by an ATP-requiring process (4, 5). This modification marks the proteins for rapid degradation by the ATP-dependent 26 S (2000 kDa) proteasome complex (6, 7). The proteolytic core of this large structure is the 20 S proteasome, which contains multiple peptidase activities. Ub conjugation is believed to be the rate-limiting step in this pathway (3, 5), in which most substrates are completely hydrolyzed to free amino acids. It has generally been believed that the primary role of this pathway is to degrade abnormal proteins and short-lived regulatory components (8, 9). However, recent studies using proteasome inhibitors (10) and earlier ones involving ATP depletions suggest that this pathway also catalyzes the breakdown of most long-lived proteins in cultured mammalian cells. In addition, various studies of isolated skeletal muscles suggest that the rapid loss of muscle protein in fasting (11–13), denervation atrophy (11, 13), sepsis (15), and metabolic acidosis (16), and cancer cachexia (17, 18) is primarily due to activation of the Ub-proteasome pathway.

In addition to the Ub-proteasome system, skeletal muscles contain at least three other proteolytic systems that can contribute to intracellular proteolysis. 1) Lysosomal proteases are responsible for degradation of endocytosed proteins and many membrane proteins (19–21). 2) A still poorly defined nonlysosomal degradative system that functions independently of ATP (1, 22). In red blood cells, this system appears responsible for the rapid degradation of oxidatively damaged hemoglobin (23, 24). However, there is conflicting evidence whether this process involves the proteasome (25) or a distinct proteolytic system (26), such as the insulin-degrading enzyme (27). 3) The cytosol also contains two Ca2+-activated proteases, calpain-μ and -m, whose in vivo function in normal cells remains unclear (28). The precise roles of all these degradative systems in the breakdown of different muscle proteins are yet to be determined.

Knowledge about the physiological importance of the Ub-proteasome pathway in mammals has advanced slowly largely because of the lack of effective inhibitors. Recently, competitive inhibitors of the 20 S proteasome that block protein degradation in intact cells have been identified (6, 10). For example, certain peptide aldehydes (e.g., Cbz-Leu-Leu-leucinal, MG132) inhibit the chymotryptic and peptidylglutamylpeptidase activities (10), and thereby reduce the degradation of Ub-conjugated proteins. In lymphoblasts, these agents reduce the degradation of the bulk of cell proteins and similarly inhibit the breakdown of short- and long-lived proteins (10). The system that degrades the long-lived contractile proteins of the myofibrils had long been unclear. Recently, proteasome inhibitors have been shown to decrease the overall proteolysis in incubated rat muscles and especially the enhanced degradation characteristic of atrophying muscles, in which breakdown of myofibrillar proteins is accelerated (29).
These studies suggesting an important role of the Ub-proteasome pathway in muscle have all been indirect and have not utilized specific muscle proteins as substrates. Of particular interest is the proteolytic system that degrades myofibrillar proteins, which comprise the majority of muscle proteins. In the sarcomere, such proteins are in an insoluble, highly ordered structure. The rate-limiting steps in their degradation and the responsible proteases are unknown. These studies were undertaken to investigate the capacity of the ATP-proteasome pathway in skeletal muscle to degrade soluble muscle proteins and the major myofibrillar components, and to explore the conditions that may favor their degradation.

EXPERIMENTAL PROCEDURES

Reagents—Protein substrates and reagents were purchased from Sigma. The monoclonal antibody against the insulin-degrading protease was kindly provided by Dr. R. A. Roth (Stanford University, Stanford, California). The proteasome inhibitors MG101 (N-acetyl-Leu-Leu-norleucinal) and MG132 (Cbz-Leu-Leu-leucinal) were kindly provided by ProScript, Inc. (formerly MyoGenics, Inc., Cambridge, MA). Myofibrils were isolated in bulk from fresh rabbit psoas muscles and were purified as described previously (31). Myosin, actin, and troponin were labeled with \(^{32}P\) using the chloramine-T procedure (32), and myoglobin was labeled with \(^{14}C\)formaldehyde (33).

Preparation of Muscle Extracts—Male New Zealand White rabbits (3–4 kg) were killed by lethal injection of sodium pentobarbital, and the extracted psoas muscles were prepared as described earlier (34). Homogenates were centrifuged at 30,000 \(g\) for 30 min to remove myofibrils. “Crude extracts” were prepared by centrifuging the supernatants at 100,000 \(g\) for 1 h and were either studied directly or fractionated on DEAE-cellulose (34) into Fraction II, the resin-bound material, which contains the proteasomes and most of the enzymes required for Ub conjugation, and Fraction I, the flow-through, which contains Ub and certain enzymes required for ubiquitination and degradation of N-\(\alpha\)-acylated proteins (35, 36). Both crude extracts and Fraction II were then dialyzed against buffer containing 20 mM Tris (\(pH\) 7.6), 2 mM DTT, 10 mM magnesium acetate, 20 mM potassium chloride, and 10% glycerol and stored at \(-70^\circ C\) until use.

Assays

All assays of proteolysis were linear with time for up to 2 h. The data in a specific figure or table were obtained in a single experiment and are the averages of triplicate determinations, which agreed within 10%. All experiments were repeated at least three times with similar results. However, the specific activity of the degradative system varied from preparation to preparation.

Protein Content—Protein content was measured using BSA as the standard by the Bradford method (37).

Measurement of Peptidase Activity of Proteasome in Extracts—The proteasome’s peptidase activity was measured by following the hydrolysis of fluorometric substrate, Suc-Leu-Leu-Val-Tyr-MCA, as described previously (38).

Degradation of Soluble Proteins—Degradation of soluble proteins in crude extracts and Fraction II was measured by assaying the free tyrosine in the trichloroacetic acid-soluble supernatant (34). Since muscles neither synthesize nor degrade this amino acid, its accumulation reflects the net degradation of proteins (39). In experiments with Fraction II, the degradation of endogenous proteins was measured upon addition of Ub (100 \(\mu\)g/ml reaction mixture), since removal of Fraction I from the crude extracts eliminates most of the Ub (34).

Degradation of Exogenous Proteins—Breakdown of \(^{15}N\)-labeled proteins or \(^{14}C\)myoglobin in the crude extracts was measured by following the release of trichloroacetic acid-soluble radioactivity using a \(y\) or liquid-scintillation counter. Alternatively, 50–75 \(\mu\)g of the non-labeled protein substrate was added to the reaction mixture containing I–2 mg of soluble crude extracts in 0.1 ml of buffer (20 mM Tris (\(pH\) 7.6), 2 mM DTT, 10 mM MgCl\(_2\), 100 mM KCl, 5% glycerol), with or without 2 mM ATP. Reaction mixtures were incubated at 37 °C for 90 min, and to terminate reactions at the end of the incubation period, trichloroacetic acid (10% final concentration) was added to all reaction mixtures. The total amount of tyrosine released from proteins was measured fluorometrically (39). The amount of tyrosine generated by breakdown of the exogenous protein substrate was then calculated by subtracting from the total amount of tyrosine generated (i.e. total proteolysis) the amount of tyrosine released by breakdown of the endogenous soluble proteins measured in a parallel incubation, in which no exogenous substrate was added to the extracts.

RESULTS

Ubiquitin and Proteasomes Are Necessary for ATP-stimulated Proteolysis—As reported previously (34), the soluble extracts of muscles after dialysis (to remove free amino acids) degraded endogenous proteins completely to amino acids, as shown by the appearance of free tyrosine. This process was linear for 2 h at 37 °C and was stimulated 3–6-fold by ATP (Fig. 1, top). To test if proteasomes are required for the ATP-dependent process, two approaches were used. First, most of the proteasomes were removed from the crude extracts by ultracentrifugation at 100,000 \(g\) for 6 h (40). To confirm that the proteasome content was in fact reduced, we assayed the hydrolysis of Suc-Leu-Leu-Val-Tyr-MCA, a preferred substrate of the proteasome (41). This activity was 60–70% lower after ultracentrifugation than in the whole extracts before centrifugation. This loss of proteasomes did not alter the proteolysis seen in the absence of ATP, but reduced the ATP-stimulated breakdown of soluble proteins by 70% (Table I).

In addition, we tested the effects of the peptide aldehyde inhibitors of the proteasome, MG101 (N-acetyl-Leu-Leu-norleucinal) and MG132 (Cbz-Leu-Leu-leucinal). Both these inhibitors competitively inhibit the peptidase activities of the 20 S proteasome (10) and reduce proteolysis in isolated rat muscle (29).\(^\text{a}\) Addition of either agent to the crude extracts inhibited the ATP-stimulated breakdown of endogenous proteins, while the much lower ATP-independent activity was largely unchanged (Fig. 2). MG132 is a much more potent inhibitor of the 20 S and 26 S proteasomes than MG101 (10). Accordingly, 50% inhibition of the ATP-stimulated proteolysis was observed with MG132 at 40 \(\mu\)M and at >100 \(\mu\)M for MG101. Although both
agents can also inhibit lysosomal proteases and calpains, under these experimental conditions (pH 7.4 in the absence of Ca\(^{2+}\)), these enzymes should be inactive. Thus, the proteasome is necessary for the ATP-dependent degradation of soluble muscle proteins.

To test whether this ATP-proteasome-dependent process also requires Ub, DEAE-cellulose chromatography was performed. The fraction that did not bind to the column (Fraction I) contained Ub (34), while the bound fraction (Fraction II) contained proteasomes and certain Ub-conjugating enzymes (3, 42). Both fractions were essential for reconstitution of the ATP-dependent process. Addition of ATP alone to Fraction II did not stimulate tyrosine production, but Fraction I and ATP restored the protein degradation (data not shown). The active component in Fraction I appeared to be Ub, since addition of purified Ub to ATP-supplemented Fraction II stimulated up to 6-fold the degradation of endogenous proteins (Fig. 1, F-II), which occurred at a linear rate for 2 h. Thus, degradation of soluble muscle proteins involves the Ub-proteasome pathway.

**ATP Stimulates Breakdown of Individual Myofibrillar Proteins**—Subsequent studies tested whether the ATP-proteasome pathway also catalyzes the degradation of individual myofibrillar proteins added to these soluble extracts (from which myofibrils had been removed). Addition of purified myosin had little effect on tyrosine production in the absence of ATP, but in its presence caused up to a 3-fold increase in tyrosine production (Fig. 3). Thus, native myosin appeared to be a substrate for this degradative pathway. In order to verify that this effect of myosin is, in fact, due to its degradation (rather than to a stimulation of the breakdown of some protein(s) in the extract), 125I-myosin was used as a substrate. In the presence of ATP, 125I-myosin was hydrolyzed 2–3 times faster than in its absence (Fig. 3). This result was observed consistently, although the rate of myosin degradation varied in different preparations of extracts. The degradation of native as well as 125I-myosin was linear for up to 2 h. By contrast, at 25 °C, no degradation of either myosin was observed in 2 h, even in the presence of ATP (data not shown). Thus, the increase in tyrosine production is due to myosin degradation.

Similarly, addition of native actin, troponin, or tropomyosin individually to the crude extracts increased production of tyrosine, especially in the presence of ATP, apparently because these proteins were degraded in an ATP-stimulated manner (Table II). Accordingly, 125I-actin and 125I-tropomyosin were also hydrolyzed, and ATP stimulated this process up to 6-fold. Moreover, the increase in tyrosine production was proportional to the added amount of actin or troponin or tropomyosin (up to 1 mg/ml) (data not shown). Therefore, the extent of hydrolysis of the non-labeled exogenous substrate added (i.e. the amount of tyrosine generated by its hydrolysis) could be determined simply by subtracting from the total free tyrosine production, the amount produced by breakdown of endogenous proteins (i.e. in the absence of an exogenous protein). This non-labeled approach could also be used to follow the degradation of non-myofibrillar exogenous substrates, such as lysozyme, which was hydrolyzed in an ATP-stimulated fashion similar to 125I-lysozyme (data not shown).

To follow the degradation of native myosin, the amount of myosin added to the extracts was critical. At low concentrations (<0.3 mg/ml), no significant increase in tyrosine production could be measured, while at high concentrations (>1 mg/ml), myosin aggregates and precipitates in the low ionic strength reaction buffer. Addition of myosin at concentrations above 1 mg/ml inhibited total proteolysis, probably due to the coprecipitation of some components of the degradative pathway with the myosin (unfortunately, the high-salt concentrations that maintain myosin in solution inhibit the ATP-dependent proteolytic activity). Unlike myosin and actin, which were degraded efficiently only in the presence of ATP, troponin and tropomyosin were degraded significantly in the absence of ATP, although this process was stimulated further when ATP was added (Table II).

To confirm that proteasomes are essential for the ATP-dependent degradation of these myofibrillar proteins, we examined the effects of proteasome depletion by prolonged ultracentrifugation, as described above. This treatment did not alter the ATP-independent activity against these various substrates; however, the ATP-stimulated degradation of myosin, actin, and troponin was reduced by 70%. The high molecular weight component(s) necessary for this ATP-stimulated degradation process are probably proteasomes (Table I). Further evidence for

---

**TABLE I**

| Protein substrates | Crude extract | Extract depleted of proteasomes | Inhibition |
|--------------------|---------------|--------------------------------|------------|
|                    | pmol of tyrosine | %                              |            |
| Endogenous proteins| 550           | 90                             | 83         |
| Myosin             | 395           | 0                              | 100        |
| Actin              | 470           | 70                             | 85         |
| Tropomyosin        | 385           | 30                             | 92         |
| Troponin           | 430           | 40                             | 90         |

---

**Fig. 2.** Proteasome inhibitors reduced the ATP-stimulated breakdown of proteins in muscle extracts. Crude extracts (5 mg/ml) were preincubated for 5 min at 25 °C with MG101 or MG132 at the indicated concentrations; 2 mM ATP was added to half of the reaction, and the incubations continued at 37 °C for 90 min. The amount of tyrosine generated was then measured. 100% is the amount of proteolysis (i.e. tyrosine production) in the absence of inhibitors, which equaled 700 pmol with ATP present and 120 pmol without ATP.
The involvement of proteasomes in the breakdown of \(^{125}\text{I}\)-labeled myosin, actin, and troponin was obtained by studying the effects of inhibitors of various proteases (Table III). Addition of proteasome inhibitors MG101 and MG132 significantly reduced the ATP-stimulated degradation of these myofibrillar proteins, while the ATP-independent activity remained unchanged. By contrast, E64 (which covalently inactivates several cysteine proteases present in muscle, e.g. cathepsins B, H, and L, as well as the calpains), or leupeptin (a reversible inhibitor of these enzymes) had negligible effect on the degradation of myofibrillar proteins (Table III). The insulin-degrading metalloprotease had been proposed to play a role in later steps in an ATP-dependent proteolytic pathway (43). However, complete removal of this metalloprotease from the crude extracts by immunoprecipitation did not affect either the ATP-independent or -dependent degradation of myofibrillar proteins to amino acids (data not shown). Thus, the proteasome (and no other known cell proteases) appears to catalyze the ATP-stimulated degradation of myofibrillar proteins.

To test if Ub is also required for this process, degradation of myosin, actin, troponin, and tropomyosin was compared in crude extracts and in Fraction II (Table IV). After removal of Fraction I by DEAE-chromatography, no degradation of these proteins was observed, unless Fraction I was added back to the Fraction II. Addition of purified Ub and ATP to Fraction II did not stimulate the degradation of any of these myofibrillar proteins. Some factor(s) in Fraction I, in addition to Ub, are therefore required specifically for degradation of these myofibrillar proteins. As shown below, Ub could be conjugated to myosin in an ATP-dependent manner in crude extracts, but not in Fraction II alone (see below). Furthermore, recent studies (35, 36) have shown that conjugation of Ub and other N-acetylated proteins to reticulocyte lysate requires a specific Ub-conjugating enzyme (E2-F1) and a specific factor (Factor II) present in Fraction I. Since myosin, troponin, and tropomyosin are N-acetylated (44), it is not surprising that addition of Ub alone did not support their degradation in ATP-supplemented Fraction II.

**Actin Inhibits the ATP-stimulated Degradation of Myosin**—In these extracts, the isolated myosin, actin, troponin, and tropomyosin were degraded surprisingly rapidly. Based on the

---

**Table II**

Myofibrillar proteins are degraded rapidly in an ATP-dependent manner when isolated, but not when associated in myofibrils

| Substrates | Proteolysis | 
|------------|-------------|
|            | ---         | +ATP | -ATP +ATP |
|            | pmol of tyrosine |        |
| Myosin     | 150         | 415  | 2.75 |
| Actin      | 110         | 630  | 5.7  |
| Tropomyosin| 220         | 680  | 3.1  |
| Troponin   | 415         | 845  | 2.0  |
| Actomyosin | 75          | 90   | 1.2  |
| Myofibrils | 24          | 40   | 1.5  |

**Table III**

Proteasome inhibitors decrease the ATP-stimulated degradation of \(^{125}\text{I}\)-labeled myofibrillar proteins

| Substrates | Proteasomes | Other proteases |
|------------|-------------|----------------|
|            | +MG132      | +MG101         |
|            | -E64        | -Leupeptin     |
| % inhibition | 60 | 25 | 10 | 0 |
| \(^{125}\text{I}\)-Myosin | 70 | 40 | 16 | 5 |
| \(^{125}\text{I}\)-Troponin | 70 | 50 | 12 | 2 |
rates of tyrosine release, up to 6% of the proteins were completely degraded in an hour, yet these proteins in vivo are quite long-lived, having half-lives of many days. However, in vitro, the majority of these proteins exist primarily as components of the myofibrils and not as free soluble molecules. In fact, when these proteins were added in the form of actomyosin complexes or as intact purified myofibrils, they were degraded very slowly even in the presence of ATP (Table II). One likely explanation for this discrepancy is that the association of these proteins with each other in the myofibril reduces or prevents their rapid degradation. To check this possibility, we tested whether the addition of actin could decrease specifically the degradation of myosin. 125I-Myosin was preincubated at 25°C with nonlabeled actin at two different molar ratios for 5 min, ATP and extracts were added, and 125I-myosin degradation was measured at 37°C. When actin was present in a molar ratio of 2 actins/myosin, up to 40% inhibition of 125I-myosin degradation was observed. Under these conditions, some myosin was probably associated with actin, while some remained free. However, when the ratio was increased to 4 actin/myosin to enhance actomyosin formation, ATP-dependent degradation of 125I-myosin was inhibited almost totally (Table V).

To test whether this inhibition was a specific effect of actin, we measured 125I-myosin degradation in the presence of BSA or troponin, a myofibrillar protein that is a substrate of the Ub-proteasome pathway, but does not bind to myosin. Unlike actin, neither BSA nor troponin protected the 125I-myosin from degradation. Actin associates with myosin through its S-1 proteolytic fragment. To reduce the capacity of actin to form a complex with 125I-myosin, we added actin to the proteolytic system together with some S-1 fragment of myosin. As a result, the capacity of actin to inhibit 125I-myosin degradation was less than with actin alone. These findings suggest that in myofibrils, myosin is protected from proteolysis by its association with actin.

An alternative explanation of these results could be that the actomyosin complex somehow inhibited the ATP-Ub proteasome system. Therefore, we tested whether these concentrations of actin and myosin reduced the degradation of 125I-lysozyme, a widely studied substrate of the Ub-proteasome pathway. The degradation of 125I-lysozyme was not inhibited by the addition of either actin and myosin together or by actin or myosin separately. These findings indicate that actin inhibits myosin degradation specifically. It is also possible that under these conditions, actin may have decreased the hydrolysis of 125I-myosin by causing the precipitation of actin-125I-myosin complex. To evaluate this possibility, we measured the fraction of 125I-myosin that was still in the solution at the end of incubation. In all reaction mixtures, all of the myosin remained in the solution after centrifugation at 14,000 rpm for 10 min, even when myosin degradation was inhibited by 90%. Thus, this inhibition was not due to the precipitation of the substrate.
addition of 125I-myosin resulted in the formation of very high molecular weight Ub-myosin conjugates, which remained at the origin of the gel. Formation of these conjugates was totally dependent on the presence of ATP and of GST-Ub. However, these large Ub-myosin conjugates were not formed when actin was included in the reaction mixture, at concentrations where actin inhibits markedly myosin degradation, apparently by associating with the myosin (Table V). Myosin-Ub conjugates were formed normally upon addition of BSA, which does not reduce myosin degradation (see Fig. 4). Thus, the association with actin protects myosin from ubiquitination and presumably, therefore, from ATP-dependent degradation. These findings suggest that the rate-limiting step in the ubiquitination of myosin in vivo is dissociation from actin.

Degradation of Myoglobin Is Independent of ATP and Proteasomes—Significant ATP-independent proteolysis occurs in cultured cells (1) and in incubated rat muscles (48), although this process is not activated in atrophying muscle (18, 22). Accordingly, the ATP-UB pathway does not appear to be responsible for the degradation of all proteins in the muscle extracts. Addition of [14C]myoglobin or native myoglobin to the crude extracts resulted in their rapid degradation even in the absence of ATP (Table VII), and this process was stimulated only slightly by the addition of ATP. Furthermore, removal of proteasomes by ultracentrifugation or inhibition of proteasomes with MG132 abolished the fraction of degradation that was ATP-stimulated, but most myoglobin breakdown was not affected (Table VII). To characterize the enzymes responsible for this ATP-independent process, we studied the effects of various protease inhibitors on [14C]myoglobin degradation (Table VII). Although EDTA or bestatin did not inhibit, o-phenanthroline at the same concentration inhibited ATP-independent degradation of [14C]myoglobin by about 40%, suggesting that Zn2+ or Co2+-metalloendoprotease is likely to be involved. The cytosolic insulin-degrading enzyme is a metalloprotease sensitive to o-phenanthroline (43), which has been suggested to degrade oxidatively damaged hemoglobin (a homologue of myoglobin) in red blood cells (27). However, when this enzyme was removed completely from the extracts by immunoprecipitation (as confirmed on Western blots), there was no reduction in the degradation of myoglobin, indicating that insulin-degrading enzyme is not involved in the degradation of myoglobin (data not shown). Chymostatin, an inhibitor of mast cells which contaminate rat muscle extracts (49), and PMSF, an inhibitor of serine proteases, caused 30–40% inhibition. Since the effects of chymostatin and o-phenanthroline appeared additive, these data suggest the involvement of multiple proteases in myoglobin degradation, including a serine protease (possibly chymase) and an unidentified metalloprotease.

**TABLE VI**

Degradation of 125I-troponin (1 μM) was measured in the crude extracts in the presence of actin (3 μM), troponymosin (3 μM), bovine serum albumin (3 μM), or myoglobin (3 μM) as in Table V. The degradation of 125I-lysozyme (1 μM) was assayed under similar conditions as for troponin, i.e. in the presence of nonlabeled troponin (1 μM) and in each of the listed proteins. 3.7% of 125I-troponin and 5% of 125I-lysozyme were degraded in the extracts in the absence of added proteins.

| Additions | 125I-Troponin | 125I-Lysozyme |
|----------|--------------|---------------|
| None     | 100          | 100           |
| + Actin  | 79           | 87            |
| + Tropomyosin | 25    | 96            |
| + Bovine serum albumin | 83    | 80            |
| + Myoglobin | 83    | 80            |

The present findings that both soluble (cytoplasmic) proteins and the major myofibrillar proteins are degraded by the Ub-proteasome pathway provide strong support for recent physiological studies, suggesting that most of the proteolysis in incubated rat muscles is by an ATP-dependent nonlysosomal pathway and that the rapid loss of muscle protein, especially of myofibrillar components in various muscle atrophy, is primarily due to activation of the Ub-proteasome pathway (11–13, 15, 17, 18). Muscle extracts contain many other proteolytic enzymes, including lysosomal proteases, calpains, and proteases derived from mast cells (50), and all these enzymes at times have been proposed to be important in degrading myofibrillar proteins. However, recent studies on incubated muscles (51–53) and a number of the presented observations indicate that these proteases do not contribute in an important way to overall protein degradation in muscles. Only the removal or inhibition of proteases (10, 40) severely decreased the degradation of soluble and myofibrillar proteins. Although peptide aldehyde inhibitors MG101 and MG132 can also inhibit calpains and lysosomal cysteine proteases, had any effect on the ATP-stimulated process. These findings argue strongly that the proteasome complex is responsible for most of the degradation of soluble as well as isolated myofibrillar proteins in crude muscle extracts.

In mammalian cells, certain proteins can be degraded by the 26 S proteasome in an ATP-dependent, Ub-independent fashion (6, 38, 54–56) in Fraction II of reticulocyte lysate completely lacking Ub. In contrast, Fraction I was absolutely essential for the ATP-dependent degradation of the soluble muscle proteins as well as the purified myofibrillar proteins. One component of Fraction I necessary for this process is Ub, but additional components of Fraction I are also necessary for...
the breakdown of myosin, actin, troponin and tropomyosin. Accordingly, Ub is conjugated to \(^{125}\text{I}\)-myosin in crude extracts, but not in Fraction II supplemented with Ub. In reticulocytes, actin has been shown to be degraded in an Ub-dependent process (57) that requires two components from Fraction I, E2-F1 (36) and Factor II (35). Possibly, these same components are necessary also for ubiquitination of myosin, tropomyosin, and troponin. These findings and related ones (10) indicate that the Ub-proteasome system does not just degrade short-lived or abnormal proteins, as had been widely believed (5). Instead, the proteasome seems to be the primary site in mammalian muscle cells for degradation of most cytosolic and nuclear proteins, including short- and long-lived components (2) and most muscle proteins, which tend to be long-lived.

Although the degradation of most muscle proteins is by this pathway, some proteins in the extracts are degraded by a system not requiring either ATP or the proteasome. Significant ATP-independent proteolysis has also been observed in cultured cells, red cells, and incubated intact rat muscles (1, 48). The proteolytic enzymes responsible for this process and most of their intracellular substrates are still unidentified. Native troponin and tropomyosin were both degraded at a significant rate in the absence of ATP, although ATP addition stimulated their breakdown severalfold. In contrast, myoglobin was degraded primarily by an ATP-independent fashion, and our inhibitor experiments indicated that myoglobin breakdown is not by any of the well-characterized proteolytic enzymes (insulin-degrading enzyme, calpains, or lysosomal activities) and suggested that more than one enzyme, probably a metalloprotease and a serine protease (perhaps chymase present in muscle extracts derived from mast cells), are involved in this ATP-independent degradation of myoglobin.

Myofibrillar proteins constitute 50–70% of the total proteins in striated muscle and comprise the major protein reserve in the body. However, little information is available on the mechanisms of disassembly and turnover of these proteins. Although purified myosin, actin, troponin, and tropomyosin are hydrolyzed rapidly by the Ub-proteasome pathway, these proteins were much more stable when present in myofibrils or as soluble actomyosin complexes. These experiments provide strong evidence that the specific associations between these proteins in the contractile apparatus protect them from degradation. The presence of actin in the extracts prevented both ubiquitination and degradation of \(^{125}\text{I}\)-myosin, and the tropomyosin protected \(^{125}\text{I}\)-troponin from ATP-dependent degradation. This inhibition was not due to nonspecific inhibition of the degradative pathway or due to precipitation of these labeled substrates, and was not seen upon addition of other proteins that do not specifically associate with the myosin or troponin. Therefore it seems most likely that actin, by associating with myosin to form the actomyosin complex, prevents the ubiquitinating enzymes from modifying myosin, and thus blocks degradation. Accordingly, the soluble S-1 fragment of myosin, which should compete with myosin for actin molecules, reduced the stabilizing effect of actin.

These findings suggest that the dissociation of free myosin, troponin, and other myofibrillar proteins from the contractile filaments is the rate-limiting step in their degradation. In one proposed model for turnover of myofibrillar proteins, the contractile proteins involve exchange of proteins between myofilaments, and that proteins at the periphery of the myofibrils may dissociate and interact with a soluble degradative system (58). Mammalian muscles contain an easily dissociating pool of myofilaments (59). Once dissociated from myofibrils, the isolated myofibrillar proteins can be readily ubiquitinated and degraded by proteasomes. Possibly, certain Ub-conjugating enzymes recognize the conformations of these dissociated proteins as “abnormal” ones, not exposed when these proteins are in the myofibrils. The dissociation of individual proteins from the myofibrils must occur at some basal rate, which could be the rate-limiting step in proteolysis normally. In catabolic states, loss of myofibrils is rapid and may involve additional factor(s) that promote myofibrillar disassembly, perhaps by severing the filaments, like gelsolin (30), or by acting like a molecular chaperone (14), or by covalently modifying or cleaving a key component, leading to disruption of the myofibrils, and therefore accelerating Ub-dependent proteolysis.

Acknowledgment—We thank Aurora Scott for assistance in the preparation of this manuscript.

REFERENCES
1. Gronostajski, R., Pardee, A. B., and Goldberg, A. L. (1985) J. Biol. Chem. 260, 3344–3349
2. Goldberg, A. L. (1992) Eur. J. Biochem. 203, 9–23
3. Ciechanover, A. (1994) Cell 79, 13–21
4. Finley, D., and Chau, V. (1991) Annu. Rev. Cell Biol. 7, 25–69
5. Hershko, A., and Ciechanover, A. (1992) Annu. Rev. Biochem. 61, 761–807
6. Coux, O., Tanaka, K., and Goldberg, A. L. (1995) Annu. Rev. Biochem., in press
7. Goldberg, A. L., Ross, S., and Adams, J. (1995) Chem. Biol. 2, 503–508
8. Elingher, J., and Goldberg, A. L. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 54–58
9. Hershko, A., Eytan, E., Ciechanover, A., and Haas, A. L. (1982) J. Biol. Chem. 257, 13964–13970
10. Rock, K. I., Grimm, C., Rothstein, L., Clark, K., Stein, R., Dick, L., Hwang, D., and Goldberg, A. L. (1994) Cell 78, 761–771
11. Medina, R., Wing, S. S., and Goldberg, A. L. (1995) Biochem J. 307, 631–637
12. Medina, R., Wing, S. S., Haas, A., and Goldberg, A. L. (1991) Biomed. Biochim. Acta 50, 347–356
13. Wing, S. S., Haas, A. L., and Goldberg, A. L. (1995) Biochem J. 307, 639–645
14. Hartl, F. U., and Martin, J. (1995) Curr. Opin. Struct. Biol. 5, 92–102
15. Tso, G., Fagan, J. M., Samuels, N., James, J. H., Hudson, K., Lieberman, M., Fischer, J. E., and Hasselgreven, P. O. (1994) J. Clin. Invest. 94, 2255–2264
16. Mitch, W. E., Medina, R., Greber, S., May, R. C., England, B. K., Price, S. R., Bailey, J. L., and Goldberg, A. L. (1994) J. Clin. Invest. 93, 2127–2133

TABLE VII

\(^{14}\text{C}\)Myoglobin is degraded primarily by an ATP-independent system not requiring the proteasome

The extracts (5 mg/ml) were preincubated for 5 min at 37 °C with various inhibitors prior to addition of \(^{14}\text{C}\)myoglobin (60 µg/ml). Incubation was then continued for 90 min at 37 °C, and degradation of \(^{14}\text{C}\)myoglobin was measured. Concentration of ATP is 2 mM. Shown is the percent of labeled protein degraded of total added.

| Exports                              | Additions          | % Protein degraded | % Inhibition |
|--------------------------------------|--------------------|-------------------|--------------|
| Crude extract                        | + MG132 (0.1 mM)   | 14.5              |              |
| Crude extract + ATP                  |                    | 20                |              |
| Proteasome-depleted extract          | + MG132 (0.1 mM)   | 16.5              |              |
| Proteasome-depleted extract + ATP    | + MG132 (0.1 mM)   | 14                |              |
|                                      | + EDTA (0.1 mM)    | 14                | 0            |
|                                      | + 0-Phenanthroline (0.1 mM) | 8.5             | 40           |
|                                      | + Bestatin (0.2 mg/ml) | 14              | 0            |
|                                      | + PMSF (0.1 mM)    | 10                | 30           |
|                                      | + Chymostatin (0.2 mg/ml) | 8               | 40           |
|                                      | + Chymostatin + bestatin + 0-phenanthroline | 5.2          | 60           |
17. Temparisi, S., Asensi, M., Taillandier, D., Aurousseau, E., Larbaud, D., Obled, A., Bechet, D., Ferrara, M., Estrela, J. M., and Attia, D. (1994) Cancer Res. 54, 5568–5573
18. Baracos, V. E., De Vivo, C. Hoyle, D. H., and Goldberg, A. L. (1995) Am. J. Physiol. 268, E996–1006
19. Furuno, K., and Goldberg, A. L. (1986) Biochem. J. 237, 859–864
20. Dice J. F. (1987) FASEB J. 1, 349–357
21. Lardeux, B. R., and Mortimore, G. F. (1987) J. Biol. Chem. 262, 14514–14519
22. Wing, S. S., and Goldberg, A. L. (1993) Am. J. Physiol. 265, E695–E676
23. Davies, K. J. A., and Goldberg, A. L. (1987) J. Biol. Chem. 262, 8227–8234
24. Fagan, J. M., Waxman, L., and Goldberg, A. L. (1986) J. Biol. Chem. 261, 5705–5713
25. Giulivi, C., Pacifici, R. E., and Davies, K. J. (1994) Arch. Biochem. Biophys. 311, 329–341
26. Matthews, W., Tanaka, K., Driscoll, J., Ichihara, A., and Goldberg, A. L. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 2597–2601
27. Fagan, J. M., and Waxman, L. (1992) J. Biol. Chem. 267, 23015–23022
28. Demartino, G. N., and Goldberg, A. L. (1981) Enzyme 26, 1–7
29. Bailey, J. L., Wang, X., England, B. K., Price, S. R., Ding, X., and Mitch, W. E. (1996) J. Clin. Invest., in press
30. D'Haese, J., Rutschmann, M., Dahlman, B., and Hinssen, H. (1987) Biochem. J. 248, 397–402
31. Perry, S. V., and Corsi, A. (1958) Biochem. J. 68, 5–13
32. Ciechanover, A., Heller, H., Elias, S., Haas, A. L., and Hershko, A. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 1365–1368
33. Dottavio-Martin, D., and Ravel, J. M. (1978) Anal. Biochem. 87, 562–565
34. Fagan, J. M., Waxman, L., and Goldberg, A. L. (1987) Biochem. J. 243, 335–343
35. Gonen, H., Schwartz, A. L., and Ciechanover, A. (1991) J. Biol. Chem. 266, 19211–19213
36. Gonen, H., Smith, C. E., Siegel, N. R., Kahana, C., Merrick, W. C., Chakraburtty, K., Schwartz, A. L., and Ciechanover, A. (1984) Proc. Natl. Acad. Sci. U. S. A. 91, 7648–7652
37. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
38. Driscoll, J., and Goldberg, A. L. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 787–791
39. Fulks, R. M., Li, J. B., and Goldberg, A. L. (1975) J. Biol. Chem. 250, 290–298
40. Hegde, A. N., Goldberg, A. L., and Schwartz, J. H. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 7436–7440
41. Fisher, M. Hilt, W., Richter-Russ, B., Gonen, H., Ciechanover, A., and Wolf, D. H. (1994) FEBS Lett. 355, 69–75
42. Hershko, A., and Ciechanover, A. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 19–56
43. Kirschner, R. J., and Goldberg, A. L. (1983) J. Biol. Chem. 258, 967–976
44. Brown, J. L., and Roberts, W. K. (1976) J. Biol. Chem. 251, 1009–1014
45. Funatsu, T., Anazawa, T., and Ishiwata, S. (1994) J. Muscle Res. Cell. Motil. 15, 158–171
46. Scheffner, M., Werness, B. A., Huijbregtsge, J. M., Levine, A. J., and Howley, P. (1990) Cell 63, 1129–1136
47. Scheffner, M., Huijbregtsge, J. M., Vierstra, R. D., and Howley, P. (1993) Cell 75, 495–505
48. Kettelhut, I. C., Wing, S. S., and Goldberg, A. L. (1988) Diabetes Metab. Rev. 4, 751–772
49. Libby, P., and Goldberg, A. L. (1980) Biochem. J. 188, 213–220
50. Goll, D. E. Kleese, W. C., and Scpacenko, A. (1989) in Animal Growth Regulation (Campion, D. R., Hausman, G. J., and Martin, R. J., eds) pp. 141–182, Plenum Publishing Corp., New York
51. Lowell, B. B., Ruderman, N. B., and Goodman, M. N. (1986) Biochem. J. 234, 237–40
52. Goodman, M. N. (1987) Biochem. J. 241, 121–127
53. Furuno, K., Goodman, M. N., and Goldberg, A. L. (1990) J. Biol. Chem. 265, 8550–8557
54. McGuire, M. J., Reckelhoff, J. F., Croull, D. E., and Demartino, G. N. (1988) Biochim. Biophys. Acta 967, 195–203
55. Murakami, Y., Matsuuf, S., Kameji, T., Hayashi, S., Igarashi, K., Tamura, T., Tanaka, K., and Ichihara, A. (1992) Nature 360, 597–599
56. Jariel-Encontre, I., Pariat, M., Martin, F., Carillo, S., Salvat, C., and Piechaczyk, M. (1995) J. Biol. Chem. 270, 11623–11627
57. Meyer, A., Siegel, N. R., Schwartz, A. L., and Ciechanover, A. (1989) Science 244, 1480–1483
58. Millward, D. J., and Bates, P. C. (1981) Acta Biol. Med. Germ. 40, 1309–1315
59. van der Westhuyzen, D. R., Natsumoto, R., and Etinger, J. D. (1981) J. Biol. Chem. 256, 11791–11797
Importance of the ATP-Ubiquitin-Proteasome Pathway in the Degradation of Soluble and Myofibrillar Proteins in Rabbit Muscle Extracts
Vered Solomon and Alfred L. Goldberg

J. Biol. Chem. 1996, 271:26690-26697.
doi: 10.1074/jbc.271.43.26690

Access the most updated version of this article at http://www.jbc.org/content/271/43/26690

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 56 references, 30 of which can be accessed free at http://www.jbc.org/content/271/43/26690.full.html#ref-list-1