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Skeletal muscle proteasome can degrade proteins in an ATP-dependent process that does not require ubiquitin

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ABSTRACT **The proteasome (the multicatalytic endoproteinase complex) in mammalian tissues hydrolyzes proteins and several types of peptides. When this structure was isolated rapidly from rabbit skeletal muscle in the presence of glycerol, its various peptidase and protease activities showed a large reversible activation by physiological concentrations of ATP (K_a = 0.3–0.5 mM). Hydrolysis of succinyl-Leu-Leu-Val-Tyr-(4-methylcoumaryl-7-amide) was stimulated up to 12-fold by ATP, whereas degradation of casein and bovine serum albumin increased 4- to 7-fold. Neither ADP nor AMP had any effect. CTP, GTP, UTP, and the nonhydrolyzable analogs adenosine 5′-[β,γ-imino]triphosphate (AMP*PP*[NH]*P*) and adenosine 5′-[α,β-methylene]triphosphate (AMP[CH_2]*PP*) increased peptide hydrolysis as well as ATP did. However, only ATP stimulated casein breakdown and only in the presence of Mg^{2+}. Thus, nucleotide binding allows activation of the peptidase functions, but ATP hydrolysis seems necessary for enhanced degradation of proteins. The ATP effect on proteolysis was reversible and did not require ubiquitin. Sensitivity to ATP was labile, and with storage at 4°C the enzyme became fully active in the absence of ATP or Mg^{2+}. The ATP-activated form closely resembles the proteasome complex described previously, which did not show ATP dependence: both have molecular masses of 650 kDa, contain the same 8–10 subunits, and are precipitated by the same antibodies. A similar ATP-activated form was found in rabbit liver but not in rabbit reticulocytes. The proteasome seems to represent a ubiquitin-independent, ATP-stimulated proteolytic activity within nucleated mammalian cells.**

The breakdown of most proteins in eukaryotic and bacterial cells requires metabolic energy (1, 2). In mammalian cells, there exist multiple pathways for protein breakdown (2, 3). One major route involves a soluble multicomponent system that requires ATP and ubiquitin (2, 4). This system appears responsible for the degradation of highly abnormal proteins (4, 5), certain short-lived normal proteins (6–8), and the bulk of proteins in growing fibroblasts (9) and maturing reticulocytes (10–13). This pathway has been best characterized in reticulocytes (2) but also has been demonstrated in yeast (14), fibroblasts (9), skeletal muscle, and liver (15). Proteins to be degraded by this pathway are ligated through their lysine amino groups to the polypeptide ubiquitin (2). This modification targets them for degradation by a very large ATP-dependent protease (1500 kDa) that is specific for ubiquitinated proteins (15–17). ATP is thus required for both the activation of ubiquitin prior to conjugation and also for the function of the ubiquitin-conjugate-degrading enzyme (called UCDEN) (15, 16).

ATP-hydrolyzing proteases that function independently of ubiquitin exist in bacteria (18, 19) and in mitochondria (7). Several observations have suggested that a ubiquitin-independent system may also exist in the mammalian cytosol (8, 20, 21). For example, certain proteins modified so that they cannot be ligated to ubiquitin are still degraded in an ATP-stimulated fashion (8, 20, 21). In extracts of murine erythroleukemia cells, Waxman *et al.* (22) failed to find a ubiquitin-dependent proteolytic process but demonstrated an ATP-requiring proteolytic activity of high molecular mass (>600 kDa) that functioned independently of ubiquitin.

The size and inhibitor profile of this enzyme resembles that of the 650-kDa protease complex present in extracts from various mammalian cells (23–31) and called by various names (e.g., multifunctional protease, macropain). This structure contains at least three distinct proteolytic activities that function against (*i*) proteins, (*ii*) basic peptides, and (*iii*) hydrophobic peptides (24, 27, 28, 30). These three activities can function in concert in the digestion of proteins to small peptides (3). The specific properties of this enzyme complex have varied considerably in earlier studies. Its proteolytic activity (23, 30) was reported by some workers to be stimulated up to 2-fold by ATP. This large enzyme can be isolated in a latent (inactive) form that is activated by exposure to heat, polyanions, fatty acids, or detergents (23, 28, 30). The physiological significance and biochemical basis of this activation are not understood. Recently, Arrigo *et al.* (32) and Falkenburg *et al.* (33) have shown that this complex corresponds to the 19S ring-shaped particles in nucleus and cytosol often called the "prosome" (34). Because of its proteolytic activities, this structure was renamed the proteasome (33). Although it constitutes the major neutral proteolytic activity in mammalian cells (24, 30), its precise role *in vivo* and its regulation are not understood. The present studies demonstrate that after rapid isolation, the proteasome has previously unrecognized biochemical properties, including a marked activation by ATP.

METHODS

Purification. The psoas muscles were excised from New Zealand White male rabbits (3 kg), and the crude homogenate was prepared using buffer A, containing 20 mM Tris·HCl (pH 7.6), 1 mM $MgCl_2$, 0.1 mM EDTA, 1 mM 2-mercaptoethanol, and 1% (vol/vol) glycerol, as described by Fagan *et al.* (16). After ultracentrifugation, the supernatant was applied to a 100-ml DE52 column equilibrated in 10 mM Tris·HCl/1 mM dithiothreitol/20% (vol/vol) glycerol, pH 7.0 (16). The column was washed until no protein was detected, and the bound protein was eluted with buffer A containing 0.5 M NaCl. The eluate contained about 10% of the total protein but over 90% of the activity. After concentration, the eluate (20 mg/ml) was dialyzed and applied to a Pharmacia Mono Q column equilibrated with buffer A containing 0.1 mM EDTA and 100 mM NaCl. Fractions of 2 ml were collected at a flow rate of 1.0 ml/min, and the bound protein was eluted using a linear gradient from 0.1–0.5 M NaCl. Active fractions were

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Abbreviations: MNA, methylnaphthylamine; MCA, 4-methylcoumaryl-7-amide; Cbz, benzyloxycarbonyl; Suc, succinyl; Ado*P*-*P*[NH]*P*, adenosine 5′-[β,γ-imino]triphosphate; Ado*P*[CH_2]*PP*, adenosine 5′-[α,β-methylene]triphosphate.

pooled, concentrated, and applied to a Pharmacia Superose 6 gel filtration column equilibrated in 20 mM Tris·HCl (pH 7.6)/1 mM dithiothreitol/200 mM NaCl/0.1 mM EDTA/20% glycerol. Fractions of 0.5 ml were collected at a flow rate of 0.1 ml/min. The active fractions from gel filtration were pooled, dialyzed, and applied to a 20-ml DEAE Affi-Gel Blue (Sigma) column equilibrated in buffer A. A linear gradient from 20–400 mM NaCl was used, and fractions of 2 ml were collected. Those with ATP-stimulated activity were pooled and used in all subsequent experiments.

Enzyme Assays. Hydrolysis of radiolabeled proteins was measured at 37°C for up to 2 hr in a buffer containing 50 mM Tris·HCl (pH 7.8), 10 mM $MgCl_2$, 1 mM dithiothreitol with or without neutralized ATP (2 mM). [^{14}C]Methylcasein (10 μg, 16,000 cpm) was prepared as described (16), and ^{125}I-labeled lysozyme–ubiquitin conjugates were prepared by the method of Hough *et al.* (17). The reaction was terminated by addition of 10% trichloracetic acid, and acid-soluble radioactivity was determined (15, 16). Hydrolysis of fluorogenic peptides (0.5 mM) was measured at 37°C in the same buffer. Hydrolysis of succinyl-Leu-Leu-Val-Tyr-(4-methylcoumaryl-7-amide) (Suc-Leu-Leu-Val-Tyr-MCA) was determined after 30 min by quenching with 1 ml of cold ethanol. The fluorescence was then measured at an excitation of 380 nm and emission of 440 nm. Incubations with benzyloxycarbonyl-Ala-Arg-Arg-methylnaphthylamine (Cbz-Ala-Arg-Arg-MNA) and succinyl-Phe-Leu-Phe-methylnaphthylamine (Suc-Phe-Leu-Phe-MNA) were terminated with 1 ml of $Na_2B_4O_7$ (pH 9.0) and read at an excitation of 335 nm and emission of 410 nm. Data shown are typical of results obtained in at least three experiments using different preparations of the proteasome.

Electrophoresis. PAGE was performed in the presence of sodium dodecyl sulfate according to the method of Laemmli (35).

RESULTS

The steps used for rapidly isolating the proteasome complex from skeletal muscle are summarized in Table 1. Little or no stimulation by ATP of peptide hydrolysis was observed in the crude extract or in the DEAE eluate (fraction II) (2). However, after Mono Q chromatography, a single peak with both peptide and casein-hydrolyzing activities was eluted that showed a large stimulation by ATP (Fig. 1*A*). This peak degraded several oligopeptide substrates including Cbz-Ala-Arg-Arg-MNA, Suc-Phe-Leu-Phe-MNA, and Suc-Leu-Leu-Val-Tyr-MCA, as well as casein in an ATP-dependent manner. In this and subsequent steps, all these activities eluted together, and all increased from 4- to 12-fold upon addition of ATP (2 mM). Previously, the proteasome from erythrocytes and liver was shown to degrade these substrates (24, 30); however, hydrolysis was not found to show a large activation by ATP.

The multifunctional complex was further purified using a Superose 6 gel filtration column and DEAE Affi-Gel Blue (Fig. 1*C*). The gel filtration step indicated a multimeric protein with molecular mass of 650 kDa (Fig. 1*B*). To assess its purity, the ATP-stimulated activity was subjected to PAGE under nondenaturing conditions. The enzyme migrated as a single band of ≈650 kDa on a 4.5% nondenaturing gel (data not shown). The ATP-activated form from muscle migrated to the same point as the proteasome purified from human erythrocyte and rat liver (24), although the latter two did not show activation by ATP.

Upon NaDodSO$_4$/PAGE analysis (35) 8–10 major subunit bands were seen (data not shown) that ranged in molecular mass from 22 to 34 kDa. Thus, the subunits of the ATP-stimulated enzyme from muscle resemble those previously reported for this complex in other cells (16, 24, 28), although the presence of larger polypeptides could be detected in the muscle preparations. Polyclonal antibodies have been prepared in rabbits against the human erythrocyte enzyme by T. Edmunds in this laboratory and rat liver enzyme by K. Tanaka (24). To test for cross-reactivity, samples of the ATP-activated proteasome were incubated at 4°C for 2 hr with either antibody. Protein A-Sepharose was then added for 2 hr before centrifugation. Both antibodies precipitated ≈85% of the ATP-dependent activity against Suc-Leu-Leu-Val-Tyr-MCA and 70% of the activity against casein.

Effect of ATP on Degradation of Different Substrates. As shown in Fig. 2, the hydrolysis of all three oligopeptides was stimulated by ATP, but the degree of stimulation varied with the peptide. Hydrolysis of Suc-Leu-Leu-Val-Tyr-MCA was stimulated most (to 12-fold), Suc-Phe-Leu-Phe-MNA was stimulated 4-fold, and Cbz-Ala-Arg-Arg-MNA was stimulated 2-fold. Degradation of casein (Fig. 2) displayed a 3- to 6-fold activation by ATP. Removal of the ATP by addition of hexokinase and glucose rapidly reduced protein and peptide hydrolysis to the levels seen without ATP (Fig. 3). Thus, this activation is readily reversible, unlike that seen with heat or detergents (24, 28).

Degradation of the proteins was not affected by addition of ubiquitin with the ATP. Because the proteasome was eluted from the gel filtration column with a molecular mass equal to 650 kDa, the proteasome should be free of ubiquitin and the conjugating enzymes required for ubiquitin-dependent proteolysis (2). Also, this enzyme did not digest ^{125}I-labeled lysozyme–ubiquitin conjugates in the presence of ATP, in contrast to the cruder preparations, which contain both the proteasome and a ubiquitin-conjugate-degrading activity (15). Interestingly, the proteasome with or without ATP exhibited very little activity against ^{125}I-labeled lysozyme, which is an excellent substrate for the ubiquitin-dependent pathway (data not shown).

Degradation of Suc-Leu-Leu-Val-Tyr-MCA and casein showed a similar dependence on ATP concentration (data not shown) with a half-maximal activation at 0.3–0.5 mM, which is well below intracellular concentrations. Other nucleotides did not significantly activate casein degradation (Table 2). GTP and UTP had little effect, whereas CTP (2 mM) caused

Table 1. Isolation of the proteasome from rabbit skeletal muscle

Step	Total protein, mg	Total activity, unit/mg		ATP stimulation, + ATP/− ATP	
		[^{14}C]Casein	Peptide	[^{14}C]Casein	Peptide
Cell extract	10,920	12	0.2	3.8	0.9
DE52 eluate	780	160	3.1	2.2	1.1
Mono Q	87	690	20	7.8	12.0
Superose 6	35	2000	68	4.0	8.0
Affi-Gel Blue	15	4800	260	4.0	9.0

One unit represents 1 ng of [^{14}C]methylcasein hydrolyzed in 2 hr, whereas one unit of peptide (Suc-Leu-Leu-Val-Tyr-MCA) represents 10 nmol of MCA produced in 30 min. Total activity equals the total units measured in the presence of ATP (2 mM). A single preparation involved four successive runs on the Mono Q column, with the active fractions from these runs pooled before gel filtration.

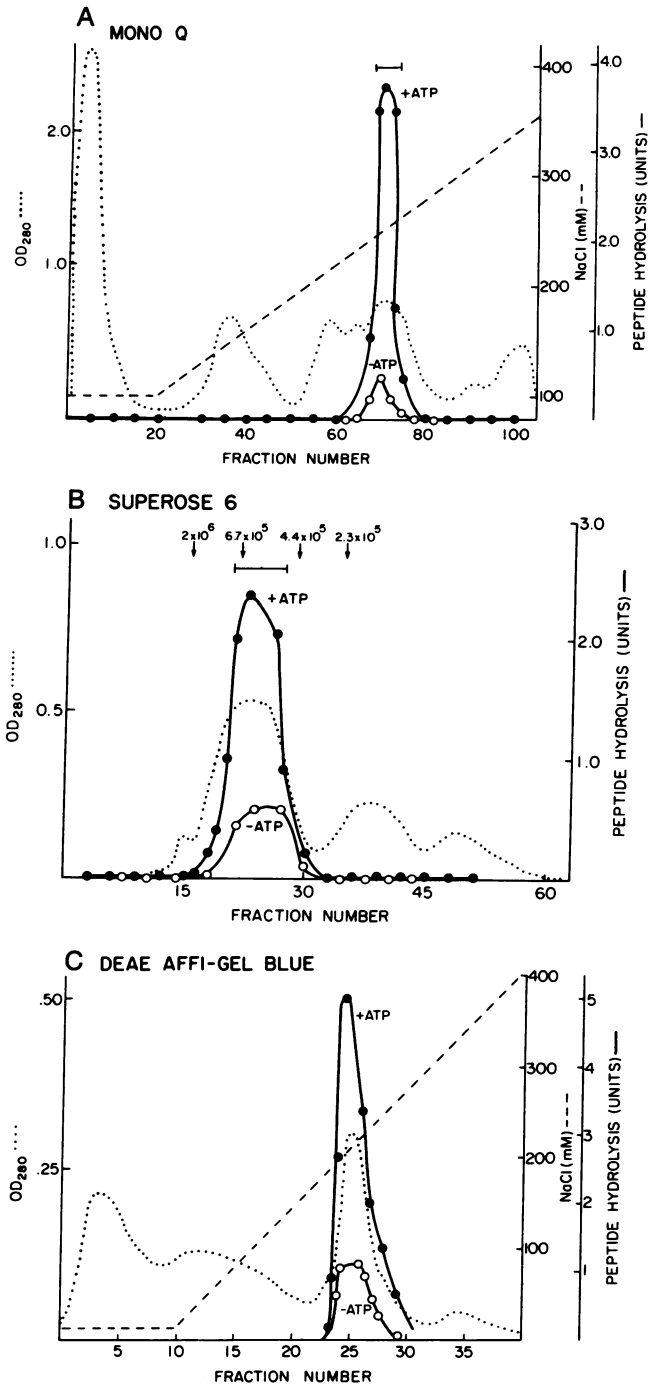

FIG. 1. (*A*) After DE52 chromatography the active fractions were subjected to Mono Q anion-exchange chromatography. Samples were assayed for Suc-Leu-Leu-Val-Tyr-MCA with (●) or without (○) 2 mM ATP. Active fractions were then chromatographed on Superose 6 gel filtration (*B*) and on DEAE-Affi-Gel Blue (*C*). Molecular mass markers used were blue dextran, thyroglobulin, ferritin, and catalase.

partial stimulation to that seen with ATP. However, all nucleotide triphosphates examined stimulated hydrolysis of the peptide Suc-Leu-Leu-Val-Tyr-MCA as well as did ATP. ADP did not activate the enzyme against proteins and enhanced only slightly the peptidase activity (Table 2), whereas AMP had no effect with either substrate. Of special interest was the finding that the nonhydrolyzed ATP analogs, Ado*PP*[NH]*P* and Ado*P*[CH_2]*PP*, also greatly stimulated cleavage of the peptide Suc-Leu-Leu-Val-Tyr-MCA, but they did not support casein hydrolysis (Table 2). Thus, ATP hydrolysis appears necessary for the accelerated degradation of proteins, but not of small peptides.

Furthermore, the enhancement of casein degradation by ATP also required Mg^{2+}. Only a small (2-fold) effect was seen in the presence of EDTA (Table 3), and Ca^{2+} (10 mM) could not replace the Mg^{2+} (data not shown). However, ATP stimulated the peptidase activities to a similar extent in the presence or absence of EDTA. Furthermore, at 1 mM Mg^{2+}, ATP (2 mM) stimulated peptide hydrolysis maximally, but caused only slight enhancement of casein hydrolysis (data not shown). Thus, the enhancement of protein and oligopeptide hydrolysis by ATP may involve different mechanisms and perhaps different binding sites on the proteasome.

As Fig. 4 indicates, the stimulatory effect of ATP on proteolysis was lost upon storage at 4°C, even in the presence of glycerol. The ATP-independent activity against either the peptide or casein progressively increased, whereas total hydrolytic activity remained constant. Under these conditions, the Mg^{2+} requirement for casein hydrolysis was also lost. This lability of the ATP activation probably accounts for the failure of others to demonstrate this effect. The rate of loss of the ATP effect varied in different preparations. Interestingly, the ATP-independent activity against casein seemed to increase more rapidly than it did against the peptide. These observations also suggest distinct mechanisms for the activation by ATP of peptide and protein hydrolysis.

A similar ATP-activated form of the proteasome was demonstrated in rabbit liver extracts by these same initial steps. However, when these procedures were used with reticulocyte extracts, the proteasome did not show a large stimulation by ATP at any stage (data not shown).

DISCUSSION

The discovery that the proteasome from skeletal muscle and liver functions in an ATP-dependent manner strongly suggests that this structure plays an important role in intracellular proteolysis. Furthermore, the ATP concentrations that fully activated the proteasome are lower than the concentrations present *in vivo* and necessary to support protein breakdown in cultured cells (10). Prior studies of this proteolytic complex failed to find a large stimulation by ATP, although earlier reports from this laboratory described a small activation by ATP and nonhydrolyzable analogs (23, 30). Most likely, the rapid purification method used here and the use of glycerol as a stabilizing agent enabled us to maintain this enzyme complex in a more native, ATP-responsive form. Various treatments (24, 28) or removal of the glycerol (30) result in irreversible activation of the enzyme. Also, with storage for several days, even in the presence of glycerol, the enzyme becomes fully active in the absence of ATP. (Presumably, glycerol helps maintain it in an inactive metastable conformation, resembling that seen *in vivo*.) Irreversible activation of the proteasome within cells could be very damaging. It seems likely that the ATP dependence somehow helps in controlling enzyme activation, as has been demonstrated for the ATP-hydrolyzing proteases from bacteria (18, 19). Possibly, the reversible activation of the proteasome by ATP involves a temporary conformational change similar to that occurring irreversibly upon activation by ammonium sulfate, heat, detergents, or storage. The ATP-stimulated and -independent forms of the proteasome appear very similar in both multimeric size and substrate specificity and cross-react immunologically, although they obviously must differ in some critical respects.

These studies demonstrate important mechanistic differences between the role of ATP in activating hydrolysis of oligopeptides and proteins. Because nonhydrolyzable ATP analogs stimulate peptide breakdown, this response must

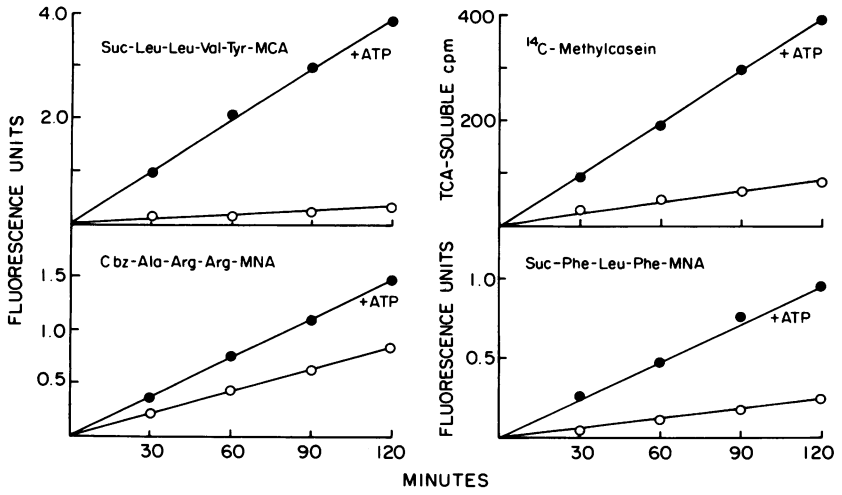

FIG. 2. Effect of ATP (2 mM) on degradation of different substrates by the muscle proteasome. TCA, trichloroacetic acid.

involve an allosteric effect of nucleotide binding, rather than an energy-requiring reaction. The binding reaction does not appear very specific, because a similar activation was seen with all nucleoside triphosphates and did not require a divalent cation as a cofactor. By contrast, the accelerated hydrolysis of large proteins is specific for ATP, requires Mg^{2+}, and seems to involve ATP hydrolysis (because nonhydrolyzable ATP analogs were ineffective). These differences suggest distinct ATP binding sites, as well as distinct mechanisms, for the activation of protein and peptide cleavage.

These observations appear quite similar to earlier findings on the mechanism of the ATP-dependent proteases (43, 44) from *Escherichia coli* and from mitochondria (38), where nucleotide binding is sufficient to support hydrolysis of tetrapeptides, but ATP hydrolysis is necessary for protein degradation. Nevertheless, we have thus far failed to demonstrate ATPase activity with the proteasome, as was found for the *E. coli* (18, 36, 37) and mitochondrial enzymes (38). Possibly, certain components are missing in the purified enzyme that are important for rapid ATP hydrolysis or protein phosphorylation. The existence of additional regulatory factors appears likely because the large activation of the peptidase by ATP was not seen until significant purification was achieved.

Most prior studies have argued that ubiquitin conjugation to protein substrates is essential for ATP-dependent proteolysis in eukaryotes (2). However, in reticulocyte extracts (8, 20) and intact hepatocytes (21) proteins modified so that they cannot be conjugated to ubiquitin are still degraded by an ATP-activated process; probably the proteasome catalyzes this process. In MEL cells Waxman *et al.* (22) failed to demonstrate ubiquitin-dependent proteolysis but did find a high-molecular weight ATP-dependent protease that functioned independently of ubiquitin; its properties resemble those of the proteasome purified here from muscle.

Once activated, the proteasome constitutes the major alkaline proteolytic activity in mammalian cell extracts (30). Waxman *et al.* (16) and Hough and co-workers (17) separated this structure from a larger ATP-dependent complex that

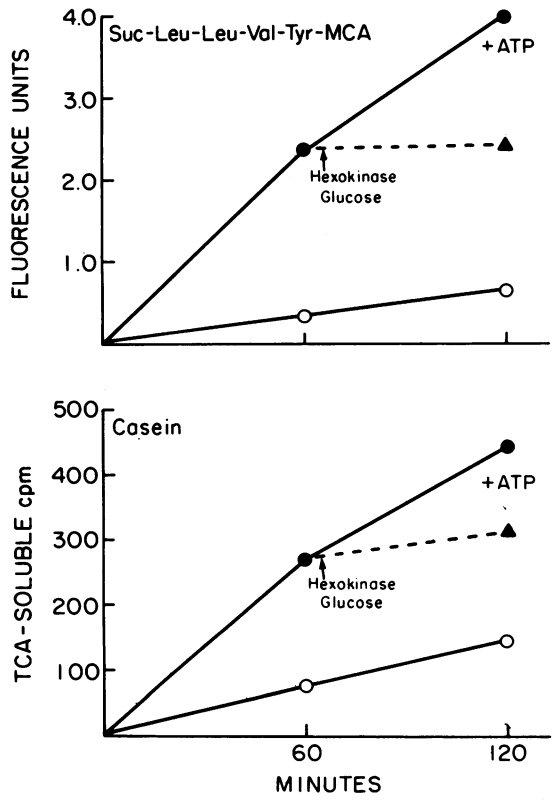

FIG. 3. Reversibility of the activation by ATP. At 60 min hexokinase (5 units/ml) and glucose (5 mM) were added to remove ATP. TCA, trichloroacetic acid.

Table 2. Effect of different nucleotides and ATP analogs on degradation of Suc-Leu-Leu-Val-Tyr-MCA amd [^{14}C]casein by the skeletal muscle proteasome

Addition, 2 mM	Relative activity, %	
	Suc-Leu-Leu-Val-Tyr-MCA	[^{14}C]Casein
Experiment 1		
None	100	100
ATP	820	488
CTP	890	252
GTP	840	195
UTP	820	196
Experiment 2		
ATP	788	448
ADP	176	117
AMP	71	89
Ado*P*[CH_2]*PP*	624	71
Ado*PP*[NH]*P*	588	100

Without any addition the enzyme hydrolyzed 10 nmol of peptide in 30 min or 50 ng of [^{14}C]methylcasein in 2 hr.

Table 3. Effect of EDTA and Mg^{2+} on ATP-dependent degradation of peptides and protein by muscle proteasome

Substrate	Relative Activity, %		
	Mg^{2+}	ATP + Mg^{2+}	ATP + EDTA
Suc-Leu-Leu-Val-Tyr-MCA	100	840	830
Suc-Phe-Leu-Phe-MNA	100	450	490
Cbz-Ala-Arg-Arg-MNA	100	286	281
[^{14}C]Methylcasein	100	686	209

EDTA and Mg^{2+} were each present at 10 mM, and ATP was present at 2 mM. Relative activities are the same as in Table 2.

degrades ubiquitin–protein conjugates. The latter activity (ubiquitin-conjugate-degrading enzyme) differs from the proteasome in molecular radius, preference for ubiquitinated substrates, and inhibitor sensitivity. Presumably, these ubiquitin-dependent and -independent structures can function *in vivo* in the hydrolysis of different types of proteins. However, recent studies suggest that the proteasome catalyzes certain steps in the critical ubiquitin-dependent pathway (39–41). Elsewhere we present evidence (41) that the proteasome is an essential component in the degradation of ubiquitin conjugates, even though this structure alone did not degrade ubiquitin conjugates.

We were not able to recover the proteasome in an ATP-dependent form from reticulocytes, where the ubiquitin-dependent pathway is easily demonstrated and has been most extensively studied (16, 17). This latter process appears very active in reticulocytes because they undergo extensive destruction of organelles and enzymes as they mature. By contrast, the ATP-dependent form of the proteasome was easily demonstrated in adult skeletal muscle and liver, which exhibit much less ubiquitin-conjugate-degrading enzyme activity (13). In these tissues, overall protein breakdown is carefully regulated by various physiological factors (42). These differences suggest that the ATP-dependent proteolytic system(s) vary in different cell types, in which proteolysis is regulated in distinct fashions.

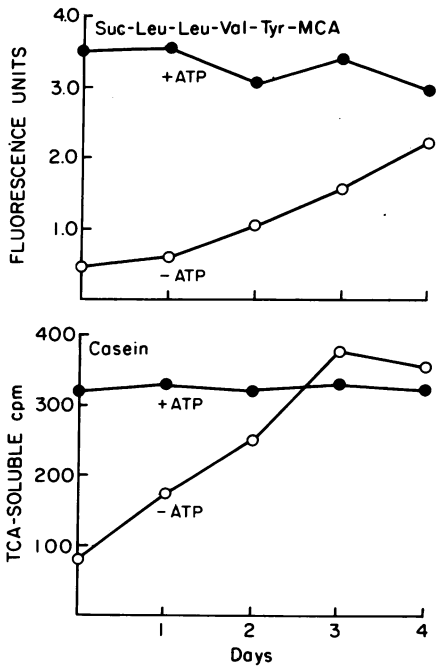

FIG. 4. Lability of the proteasome sensitivity to ATP. The isolated enzyme was stored at 4°C and assayed each day in the presence (●) of absence (○) of ATP (2 mM). TCA, trichloroacetic acid.

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