Studies on the activation by ATP of the 26 S proteasome complex from rat skeletal muscle

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The 26 S proteasome complex is thought to catalyse the breakdown of ubiquitinated proteins within eukaryotic cells. In addition it has been found that the complex also degrades short-lived proteins such as ornithine decarboxylase in a ubiquitin-independent manner. Both proteolytic processes are paralleled by the hydrolysis of ATP. Here we show that ATP also affects the hydrolytic activity towards fluorigenic peptide substrates by the 26 S proteasome complex from rat skeletal muscle tissue. Low concentrations of ATP (about 25 μM) optimally activate the so-called chymotryptic and tryptic activity by increasing the rate of peptide hydrolysis but not peptidylglutamylpeptide hydrolysis. Activation of the enzyme by ATP is transient but this effect can be enhanced and prolonged by including in the assay an ATP-regenerating system, indicating that ATP is hydrolysed by the 26 S proteasome complex. Although ATP cannot be substituted for by adenosine 5′-[β,γ-methylene]triphosphate or AMP, hydrolysis of the phosphoanhydride bond of ATP seems not to be necessary for the activation process of the proteasome complex, a conclusion drawn from the findings that ATP analogues such as adenosine 5′-[β,γ-imido]triphosphate, adenosine 5′-*O*-[γ-thio]triphosphate, adenosine 5′-*O*-[β-thio]-diphosphate and adenosine 5′-[α,β-methylene]triphosphate give the same effect as ATP, and vanadate does not prevent ATP activation. These effects are independent of the presence of Mg^{2+}. Thus, ATP and other nucleotides may act as allosteric activators of peptide-hydrolysing activities of the 26 S proteasome complex as has also been found with the lon protease from *Escherichia coli*.

INTRODUCTION

Hydrolysis of peptide bonds is an exergonic process. Nevertheless, a considerable part of intracellular proteolysis is energy dependent, a surprising and enigmatic observation made several decades ago [1,2]. In 1971, Umaña [3] demonstrated that in liver extracts a neutral endopeptidase activity is increased by ATP, and subsequently Etlinger and Goldberg [4] identified a non-lysosomal, ATP-activated proteolytic system in reticulocyte extracts. These findings led to the idea that ATP is directly involved in regulating the activity of intracellular proteolytic enzymes. Moreover, other researchers [5,6] presented experimental evidence showing that ATP is also required for modification of proteins to render them susceptible to that proteolytically active system. Since then, numerous studies have provided evidence that a wide range of cellular proteins is tagged by multi-ubiquitin chains in an ATP-consuming process (for recent reviews see [7,8]) and that these proteins are destined for degradation in an ATP-dependent manner by the 26 S proteasome complex [9–11]. During the last few years the 26 S proteasome complex has been isolated from several tissues, e.g. rabbit reticulocytes [12], frog oocytes [13], human kidney [14], fly embryos [15], rat liver [16] and chicken skeletal muscle [17]. It has been shown that the 26 S proteasome complex is a heteromultimeric complex containing the multicatalytic proteinase, 20 S proteasome, as a core enzyme [13,18–23]. Although it is generally accepted that the 26 S proteasome complex is responsible for the energy-dependent degradation of protein–ubiquitin conjugates [7], it has also been shown that the complex is able to degrade non-ubiquitinated substrates such as ornithine decarboxylase in an ATP-dependent manner [24]. To date, the exact role of ATP in the degradative reaction of both ubiquitinated and non-ubiquitinated substrates is still unclear. Similarly, although this phenomenon was first observed several years ago [12], it is currently not understood how ATP stimulates the hydrolysis of synthetic peptide substrates by the 26 S proteasome complex. To obtain better insight into the mechanism of the latter process, we have assessed the nucleotide-stimulated hydrolysis of fluorigenic peptide substrates by the 26 S proteasome complex.

MATERIALS AND METHODS

Materials

Wistar rats (200–300 g body wt.) were obtained from Winkelmann (Borchen, Germany), Sepharose 6B, Mono Q and arginine–Sepharose were from Pharmacia (Freiburg, Germany) and TSK-Fractogel DEAE 650 S was purchased from Merck (Darmstadt, Germany). Succinyl-LLVY-4-methyl-7-coumarylamide (Suc-LLVY-NMec) was from Calbiochem-Novabiochem (Bad Soden, Germany), benzyloxycarbonyl-LLE-β-naphthylamide (Z-LLE-βNA) from Bachem (Bubendorf, Switzerland) and all other peptide substrates from Serva (Heidelberg, Germany). The various nucleotides were purchased from Boehringer Mannheim (Mannheim, Germany) or from Sigma Chemie (Deisenhofen, Germany). Reagents for acrylamide gel electrophoresis were obtained from Serva (Heidelberg,

Abbreviations used: p[NH]ppA, adenosine 5′-[β,γ-imido]triphosphate; p[CH_2]ppA, adenosine 5′-[β,γ-methylene]triphosphate; pp[CH_2]pA, adenosine 5′-[α,β-methylene]triphosphate; ATP[S], adenosine 5′-[γ-thio]triphosphate; ADP[S], adenosine 5′-[β-thio]diphosphate; Bz, benzoyl; βNA, β-naphthylamide; DTT, dithiothreitol; NEpHGE, non-equilibrium pH-gradient electrophoresis; NMec, 4-methyl-7-coumarylamide; Suc, succinyl; Z, benzyloxycarbonyl.

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Germany), polyvinyl difluoride (PVDF) blotting membrane from Millipore (Eschborn, Germany), and SDS/PAGE molecular mass standards were from Boehringer Mannheim.

Purification of the 26 S proteasome complex

Preparation of crude extract

To purify the 26 S proteasome complex from rat muscle tissue we used essentially the methodology described by Hough et al. [12], where 20% (v/v) glycerol is included in all buffers (which do not contain ATP).

Pooled skeletal muscle tissue (200 g) was suspended in 400 ml of TSG buffer [10 mM Tris/HCl, 1 mM dithiothreitol (DTT) and 20% glycerol, pH 7.0] and was homogenized in a Waring Blendor for 2 min. The homogenate was centrifuged at 20000 *g* for 2 h and the supernatant was filtered through a layer of glass wool to obtain the crude extract.

Chromatography on DEAE-Fractogel

The muscle extract was loaded on to a column (2 cm × 15 cm) of Fractogel TSK DEAE 650 S equilibrated with TSDG buffer [10 mM Tris/HCl, 25 mM KCl, 10 mM NaCl, 1.1 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT and 20% glycerol (v/v), pH 7.0]. After a further wash with 125 mM KCl dissolved in TSDG buffer, bound proteins were eluted with a linear gradient (400 ml) of 125–300 mM KCl in TSDG buffer. Fractions of 1 ml were collected.

Chromatography on Sepharose 6B

Enzyme solution obtained from anion-exchange chromatography was concentrated in an Amicon cell (YM-100 membrane) and then subjected to gel filtration on Sepharose 6B (5 cm × 95 cm) equilibrated with TSDG buffer. Fractions (10 ml) were collected.

Chromatography on arginine–Sepharose

Arginine–Sepharose was equilibrated with TSDG buffer. Enzyme solution was charged on to the column (1 cm × 4 cm) and the column was washed with TSDG buffer until the A_{280} of the effluent returned to baseline. The column was then washed with 1 bed-volume of 100 mM KCl/TSDG/buffer followed by elution with a linear gradient (200 ml) of 100–200 mM KCl/TSDG buffer. Fractions (2 ml) were collected.

Proteinase assay

Peptide-4-methyl-7-coumarylamide and peptide-β-naphthylamide substrates were incubated with the enzymes at 37 °C and the rate of their hydrolysis was determined as described in [25], except that 30 mM Tris/HCl, 10 mM KCl, 5 mM $MgCl_2$ and 0.5 mM DTT, pH 7.8, was used as substrate buffer. Unless stated otherwise, the concentration of substrate used in screening tests during the purification procedure was 50 μM and 100 μM in all other tests. ATP (0.5 mM in screening tests) and other nucleotides were also dissolved in substrate buffer.

Electrophoretic techniques

For electrophoresis under non-denaturing conditions polyacrylamide slab gels [2.5% (w/v) stacking gel, 4.5% (w/v) separating gel] of two sizes were used: standard gels of 180 mm × 100 mm × 1 mm and midget gels of 100 mm × 80 mm × 0.75 mm. The gels were run under conditions as described by Hough et al. [12] and Hoffman et al. [19], respectively. In two-dimensional gel electrophoresis the first dimension was performed under non-equilibrium pH-gradient electrophoresis (NEpHGE) conditions [26] using pH gradients from 3 to 10. The second dimension was carried out in SDS/polyacrylamide slab gels of 12.5% (w/v) polyacrylamide concentration [27].

Staining

For detection of proteins exhibiting peptide-hydrolysing activity in non-denaturing gels, the gels were incubated in substrate buffer containing 200 μM Suc-LLVY-NMec, 2 mM ATP, 10 mM creatine phosphate, and creatine kinase (10 μg/ml), pH 7.8. Coomassie Blue or silver staining of proteins in polyacrylamide gels, semi-dry Western blotting of proteins after PAGE and detection of antigen–antibody complexes were performed as described elsewhere [28,29].

RESULTS

Purification of the 26 S proteasome complex

Anion-exchange chromatography on Fractogel TSK DEAE 650 S of crude muscle extract resulted in the separation of proteasome activity into three peaks, one containing Suc-LLVY-NMec-hydrolysing activity not stimulated by added ATP and eluting ahead of two peaks which contained an activity that was significantly enhanced in the presence of ATP (Figure 1). By analysis of the respective peak material on non-denaturing PAGE and with incubation *in situ* with Suc-LLVY-NMec it became apparent that the peptide-splitting activity in peak I (fractions 75–100) was due to the presence of the 20 S proteasome, that in peak III (fraction 176–215) was due to the 26 S proteasome complex (see Figures 2 and 3), while peak II (fractions 101–175) contained a mixture of both forms of proteasome complexes as

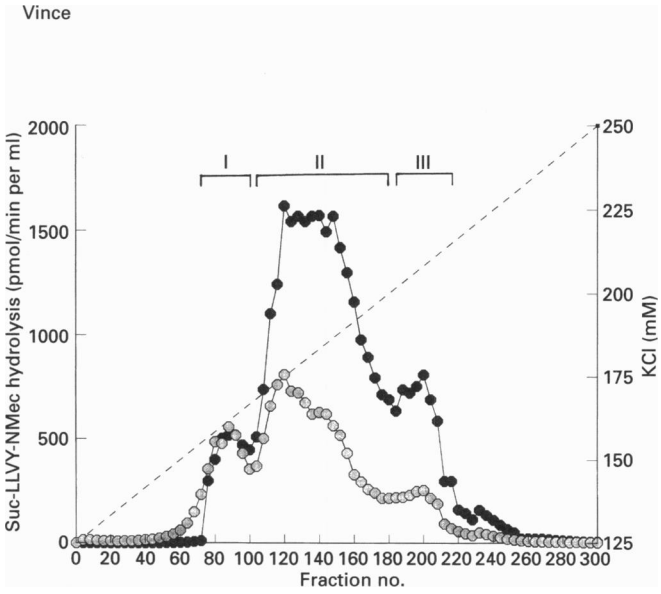

Figure 1 Chromatography of rat skeletal muscle extract on Fractogel TSK DEAE 650 S

Resin was equilibrated in TSDG buffer before muscle extract was loaded on to the column. Bound proteins were eluted from the column by a linear gradient of KCl in TSDG buffer (– – –). Fractions were tested for their Suc-LLVY-NMec-hydrolysing activity in the absence (⦸) or presence (●) of ATP. The fractions of the three proteolytically active peaks were pooled as indicated by the bars.

Table 1 Purification of 26 S proteasome from rat skeletal muscle

Data represent means ± S.D. from three preparations starting from 200 g of hindlimb skeletal muscle tissue. Total activity is expressed per total volume.

		Suc-LLVY-NMec hydrolysis			
		−ATP		+ATP	
	Total protein (mg)	total activity (nmol/min)	Specific activity (nmol/min per mg of protein)	Total activity (nmol/min)	Specific activity (nmol/min per mg of protein)
Crude extract	10455 ± 3423	4653 ± 3271	0.41 ± 0.17	6055 ± 5574	0.51 ± 0.36
TSK-DEAE Peak III	86 ± 52	34.7 ± 20.6	0.43 ± 0.13	133 ± 116.8	1.75 ± 1.07
Sepharose-6B	8.6 ± 4.5	9.4 ± 8.0	0.96 ± 0.54	41.1 ± 32.3	3.62 ± 2.82
Arginine–Sepharose	1.46 ± 0.23	2.3 ± 0.2	1.44 ± 0.16	4.3 ± 1.0	2.68 ± 0.67

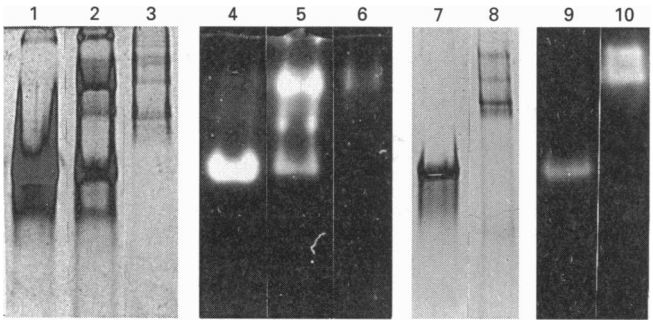

Figure 2 Non-denaturing PAGE of the 20 S and 26 S proteasome preparations

The following samples were applied to midget gels: aliquots of peak I (100 μg, lanes 1, 4), peak II (100 μg, lanes 2, 5) and peak III (30 μg, lanes 3, 6) respectively obtained from chromatography on TSK-Fractogel DEAE and 70 μg each of purified 20 S proteasome (lanes 7, 9) and 26 S proteasome (lanes 8, 10). The Suc-LLVY-NMec-hydrolysing activity of the samples was detected by the overlay technique (lanes 4–6, 9, 10) before proteins were stained with Coomassie Blue (lanes 1–3, 7, 8).

well as a third Suc-LLVY-NMec-hydrolysing protein and many other proteins not active towards this substrate. At present, the reason why the 20 S and 26 S proteasomes of this peak do not fractionate into peak I and peak III respectively is not known. All further experiments described here were performed with the material eluting in peak I (20 S proteasome) and peak III (26 S proteasome complex) only.

Further purification of the 26 S proteasome complex was achieved by gel filtration on Sepharose 6B and finally by chromatography on arginine–Sepharose from which the enzyme was eluted at 130–160 mM KCl. From both columns the enzyme eluted as a single symmetrical activity peak, only the first half of which was pooled in each case to separate out the maximum number of contaminating proteins (Table 1). The 20 S proteasome was purified by FPLC on Mono Q by a procedure similar to that described elsewhere [25], except that TSDG buffer was used for chromatography.

After these purification procedures each enzyme preparation revealed a single protein band, when electrophoresed on non-denaturing polyacrylamide gels of standard size, containing Suc-LLVY-NMec-hydrolysing activity (results not shown). Since Hoffman et al. [19] have demonstrated that proteasomes occur in multiple forms when subjected to electrophoresis in polyacrylamide midget gels, we analysed our proteasome preparations by this technique also. The 20 S proteasome again migrated as a single proteolytically active protein band (Figure 2). However, the 26 S proteasome split up into slow- and fast-migrating proteolytically active forms. A third protein running ahead of the proteasomes was proteolytically inactive. When this protein was cut out of the non-denaturing polyacrylamide gel and then subjected to SDS/PAGE it dissociated into multiple protein bands, the pattern of which was identical with that of the regulatory 'ball' complex [19,22] (results not shown).

In order to demonstrate the presence of 20 S proteasome subunits in the 26 S complex, both proteasomes were subjected to two-dimensional PAGE, transblotted and probed with a polyclonal antibody raised against purified 20 S proteasome [25]. As shown in Figure 3, the antibody detected the same set of subunits in the 20 S proteasome and in the 26 S proteasome complex preparations, confirming that the 20 S proteasome is a constituent of the 26 S complex, as has been found by other investigators [9–23].

Peptide-hydrolysing activities of the 20 S proteasome and 26 S proteasome complex

When comparing the peptide-splitting activities of the two enzymes, we found that they shared a number of features. Thus, hydrolysis of Suc-LLVY-NMec was inhibited at supra-optimal substrate concentrations, a phenomenon not observed with benzoyl (Bz)-VGR-NMec as substrate. Also, the sigmoidal curve of hydrolytic activity typically obtained with increasing concentrations of Z-LLE-βNA as a substrate was observed with both the 20 S and 26 S proteasomes (Figure 4). K_m values of the three substrates for both types of enzymes were almost identical. As the hydrolysis rates in Figure 4 are expressed per μg of protein, one reason for the 2–3-fold lower activities of the 26 S proteasome complex towards the substrates Suc-LLVY-NMec and Bz-VGR-NMec compared with those of the 20 S proteasome may be the presence of proteolytically inactive 'ball' complexes within the 26 S proteasome preparation.

Effect of ATP on the activities of the 26 S proteasome complex

Measurement of the effect of increasing concentrations of ATP on the hydrolytic rate of various peptide substrates showed that the optimum concentration of ATP is about 25 μM, irrespective of the substrate examined. While the hydrolysis of Suc-LLVY-

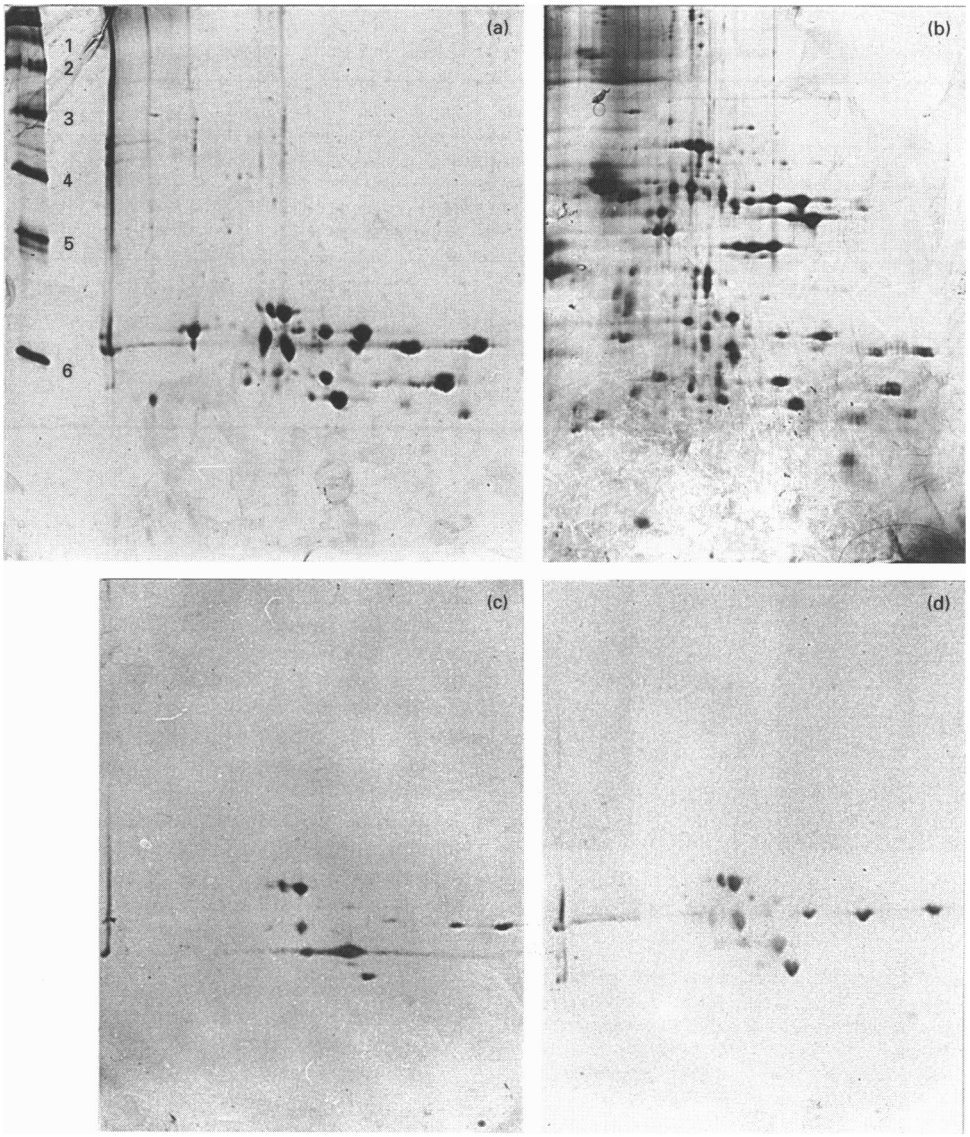

Figure 3 Two-dimensional PAGE of the 20 S and 26 S proteasome preparations

About 200 μg of each purified 20 S (**a**, **c**) and 26 S (**b**, **d**) proteasomes were subjected to two-dimensional PAGE. During the first dimension the anode was on the left and the cathode was on the right side. Proteins were either silver stained (**a**, **b**) or Western blotted on to Immobilone membrane and then incubated with a polyclonal antibody raised against rat 20 S proteasome. Antigen–antibody complexes were detected with alkaline phosphatase—conjugated second antibody (**c**, **d**). Molecular mass standards (1–6) are α_2-macroglobulin (170 kDa), β-galactosidase (116.4 kDa), fructose-6-phosphate kinase (85.2 kDa), glutamate dehydrogenase (55.6 kDa), aldolase (39.2 kDa) and triosephosphate isomerase (26.6 kDa) respectively.

NMec was enhanced about 3-fold, the stimulating effect on hydrolysis of other substrates tested was only small (Figure 5), and Z-LLE-βNA hydrolysis was not affected by ATP. With regard to the peptide-hydrolysing activities of the 20 S proteasome, ATP showed no stimulatory effect (results not shown).

As shown in Table 2, the effect of ATP was independent of the presence of Mg^{2+}, since a 15–20% stimulation by 1 mM $MgCl_2$ was measured irrespective of the absence or presence of ATP. To study the process of activation of the 26 S proteasome by ATP in more detail, we next analysed the time course of Suc-LLVY-NMec hydrolysis in the presence or absence of ATP. As shown in Figure 6 the activity measured in the presence of ATP deviated from linearity, indicating that the activation process is a time-dependent process. Further, activation was accompanied by ATP consumption. This became apparent from the experiment shown in Figure 6 where an ATP-regenerating system was included and the activation by ATP was further enhanced. Furthermore, the extent of activation by ATP was dependent on the incubation time of the enzyme with the nucleotide before starting the hydrolytic reaction. Thus, as shown in Figure 7, maximum activation (7–8-fold) of the enzyme was observed with 60–90 min of preincubation. In experiments using higher concentrations of 26 S proteasome, permitting assay times of 5 min only, maximum activation was already achieved with 30–60 min (results not shown). When preincubation was continued beyond the time necessary to reach maximal activation, the enzyme returned to the de-activated state. The de-activation process could be prevented, or at least retarded, by including an ATP-regenerating system, indicating both that ATP is hydrolysed by

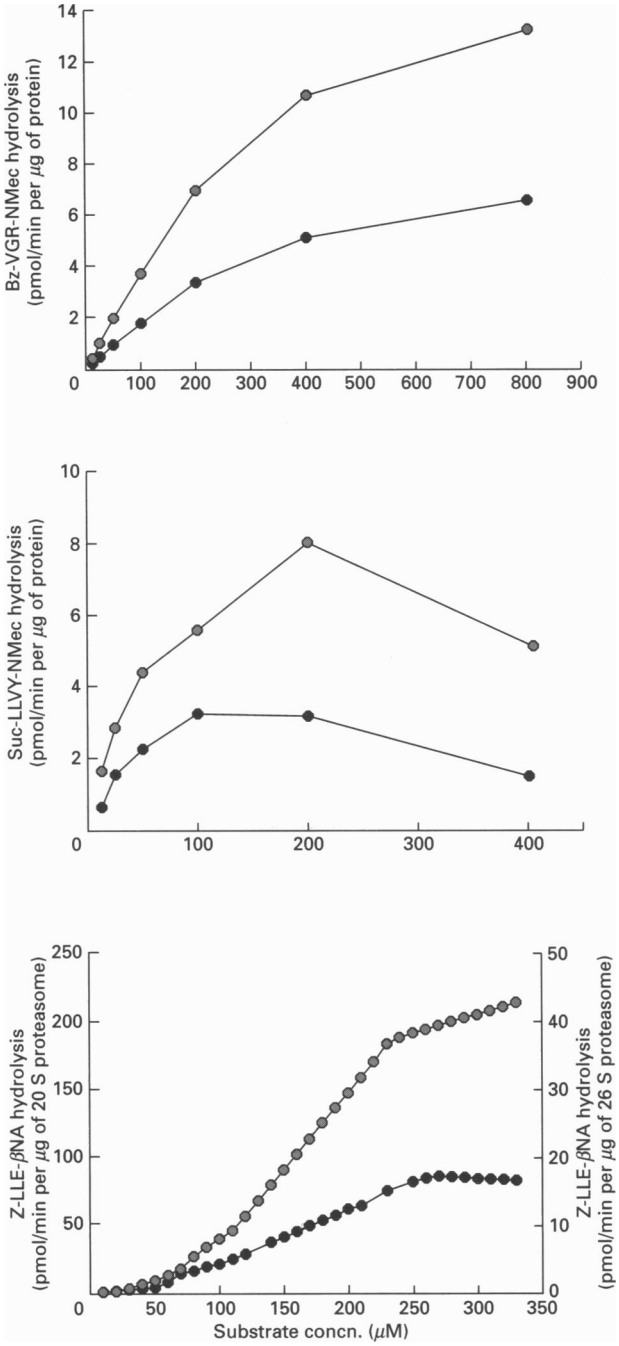

Figure 4 Rates of hydrolysis of peptide substrate by 20 S and 26 S proteasomes

Rates of hydrolysis of increasing concentrations of peptide substrates by the purified 20 S (◎) and 26 S (●) proteasomes were determined. Enzymes were dissolved in TSDG buffer. Substrates were dissolved in 30 mM Tris/HCl, 5 mM $MgCl_2$, 10 mM KCl and 0.5 mM DTT, pH 7.8. Activities towards the three substrates were measured with two to three different concentrations (1.2–10.7 μg) of the enzymes at substrate concentrations as indicated. All activities measured were calculated per μg of protein, and are given as mean values of the measurements.

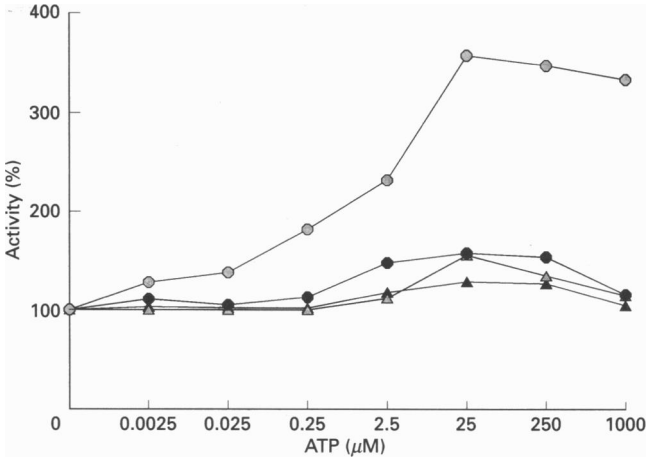

Figure 5 Effect of increasing ATP concentrations on the hydrolytic activities of the 26 S proteasome

Peptide-substrate hydrolysis was determined by mixing 50 μl aliquots containing 1.5 μg of purified 26 S proteasome in TSDG buffer, 50 μl of increasing concentrations of ATP dissolved in 30 mM Tris/HCl, 5 mM $MgCl_2$, 10 mM KCl and 0.5 mM DTT, pH 7.8, and 100 μl of substrate solutions. The hydrolytic reaction was terminated after incubation for 60 min at 37 °C. Data are mean values of two experiments (variation of less than 5%). ◎, Suc-LLVY-NMec; ●, Suc-AAF-NMec; △, Z-GGL-NMec; ▲, Bz-VGR-NMec.

Table 2 Effect of Mg^{2+} on the Suc-LLVY-NMec-hydrolysing activity of the 26 S proteasome complex in the presence and absence of ATP

Substrate hydrolysis by the purified 26 S proteasome complex [1.5 μg dissolved in 10 mM Tris/HCl, 25 mM KCl, 10 mM NaCl, 1 mM DTT and 20% (v/v) glycerol, pH 7.0] was determined in the presence of increasing concentrations of $MgCl_2$ dissolved in the same buffer in the absence or presence of 25 μM ATP (dissolved in 30 mM Tris/HCl, 10 mM KCl and 0.5 mM DTT, pH 7.8).

$MgCl_2$ (mM)	Suc-LLVY-NMec hydrolysis (pmol/min per ml)		Stimulation by ATP (fold)
	−ATP	+ATP	
0	43	94	2.2
0.2	49	96	1.9
0.4	50	103	2.0
0.8	53	107	2.0
1.0	52	107	2.0
1.5	41	101	2.4
2.0	43	104	2.4
5.0	42	102	2.4
10.0	36	90	2.5

the proteasome and that non-hydrolysed ATP is required also, to maintain the enzyme in an activated state.

From these observations the question arises as to whether the hydrolysis of ATP, which obviously takes place in the presence of the 26 S proteasome complex, is a prerequisite for the activation process itself, or whether an association of the nucleoside triphosphate and the proteasome (without hydrolysis of one of the phosphoanhydride bonds) suffices for protease activation. To address this question we examined the ability of hydrolysable and non-hydrolysable ATP analogues to activate the 26 S proteasome complex (Table 3). Neither adenosine 5′-[β,γ-methylene]triphosphate (p[CH_2]ppA) nor AMP nor cyclic AMP measurably affected the activity of the enzyme, whereas the extent of activation by adenosine 5′-[α,β-methylene]triphosphate (pp[CH_2]pA) was similar to that by ATP. These results would be compatible with the idea that the β,γ-phosphoanhydride bond of ATP has to be hydrolysed to achieve the shift from the basal to

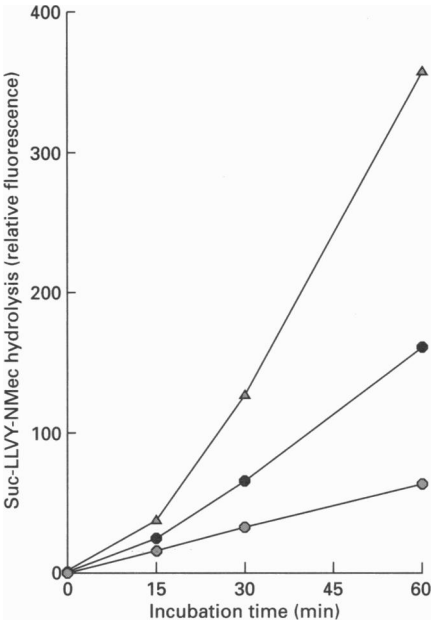

Figure 6 Activation of the hydrolytic activity of the 26 S proteasomes by ATP and an ATP-regenerating system

26 S proteasome (72 μg/ml in TSDG buffer) was mixed with Suc-LLVY-NMec substrate solution (●), with substrate solution containing 10 μM (final concn.) ATP (●), or with substrate solution containing ATP plus regenerating system (△, 10 mM creatine phosphate and 10 μg/ml creatine kinase) at 37 °C. At the times indicated, the reaction was terminated and substrate hydrolysis was determined. Data are mean values from two experiments (variation of less than 5%).

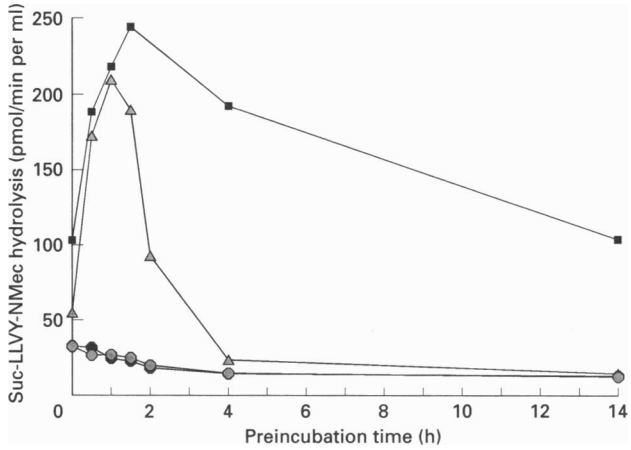

Figure 7 Effect of ATP on activation of the peptide-hydrolysing activity by the 26 S proteasome complex as a function of preincubation time

26 S proteasome (72 μg/ml) dissolved in TSDG buffer, pH 7.0, was mixed with an equal volume of 10 μM (final concn.) ATP (△) or with 10 μM ATP plus regenerating system (■, see the legend to Figure 6) and was preincubated at 37 °C. At the times indicated, the hydrolytic activity of the proteasome was tested by incubating 100 μl of the preincubation mixture with an equal volume of Suc-LLVY-NMec solution for 30 min at 37 °C. Controls contained 26 S proteasome only (●), or 26 S proteasome plus ATP-regenerating system (●).

the activated state with regard to the peptide-splitting activity of the 26 S proteasome complex. However, not consistent with this interpretation is the finding that substances like adenosine

Table 3 Effect of ATP and ATP analogues on Suc-LLVY-NMec-hydrolysing activity of the 26 S proteasome complex

ATP or its analogues at increasing concentrations (dissolved in 30 mM Tris/HCl, 5 mM $MgCl_2$, 10 mM KCl and 0.5 mM DTT, pH 7.8) were mixed with purified 26 S proteasome complex (1.5 μg dissolved in TSDG buffer) and incubated for 5 min at 21 °C before starting hydrolysis by addition of substrate, then for 60 min at 37 °C. Data are mean values from two experiments (variation of less than 5%). Abbreviation: nd, not determined.

	26 S Proteasome activity (%)				
ATP analogue concn. (μM)...	0	250	500	750	1000
ATP	100	475	452	411	380
ADP	100	187	185	198	199
ADP[S]	100	299	321	nd	346
AMP	100	107	113	118	108
Cyclic AMP	100	109	102	100	99
p[CH_2]ppA	100	109	112	126	128
pp[CH_2]pA	100	318	337	328	295
p[NH]ppA	100	450	477	488	475
ATP[S]	100	1083	1112	1056	983

Table 4 Effect of the ATPase inhibitor, vanadate, on ATP-stimulated Suc-LLVY-NMec hydrolysis by the 26 S proteasome complex

A constant amount of 26 S proteasome complex (3.5 μg dissolved in TSDG buffer) was incubated for 60 min at 21 °C with increasing amounts of ATPase inhibitor in the absence or presence of 10 μM ATP before substrate hydrolysis was tested.

	Suc-LLVY-NMec hydrolysis (pmol/min per ml)	
Vanadate (μM)	−ATP	+ATP
0	32	150
62	31	131
125	28	127
250	28	124

5′-[β,γ-imido]triphosphate (p[NH]ppA) and adenosine 5′-[β-thio]diphosphate (ADP[S]) do stimulate the Suc-LLVY-NMec-hydrolysing activity to an extent similar to that seen with ATP, and that adenosine 5′-[γ-thio]triphosphate ATP[S] was the most effective among all nucleotides tested. In another experiment we tested whether vanadate, a known inhibitor of the ATPase of the 26 S proteasome complex [14,16], affected the ATP stimulation of Suc-LLVY-NMec hydrolysis by the 26 S proteasome. As shown in Table 4, vanadate did not significantly reduce the peptide-splitting activity of the 26 S proteasome and did not prevent the 4–5-fold activation of this activity by ATP. Similar data were obtained with quercetine, another ATPase inhibitor (results not shown).

From these cumulative data we conclude that ATP hydrolysis is not necessary to stimulate the peptide-degrading activity of the 26 S proteasome complex.

Effect of ATP on kinetic parameters of peptide hydrolysis by the 26 S proteasome complex

As it is of interest to determine which kinetic parameters are affected during ATP activation of the 26 S proteasome complex, the Suc-LLVY-NMec-hydrolysing activity of the enzyme was measured at various substrate concentrations and ATP

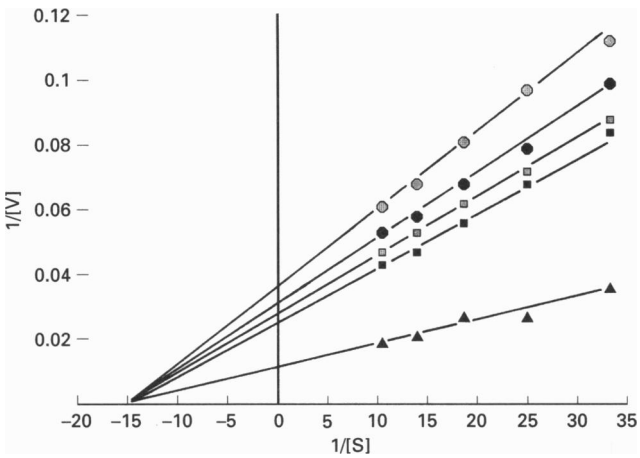

Figure 8 Effect of ATP on kinetic parameters of Suc-LLVY-NMec hydrolysis by the 26 S proteasome complex

Hydrolysis of various concentrations (30–95 μM) of Suc-LLVY-NMec was tested with 3.5 μg of 26 S proteasome complex in the absence (◎) or presence of 0.002 mM (●), 0.004 mM (▨), 0.006 mM (■) and 0.025 mM (▲) ATP for 60 min at 37 °C. Values are means ± S.D. of three determinations (variation of less than 5%).

concentrations. From Lineweaver–Burk plots it became clear that, in the presence of ATP, the velocity of the hydrolytic reaction was increased, whereas enzyme–substrate affinity remained unchanged (Figure 8).

DISCUSSION

By following the protocol of Hough et al. [12] that has since been extended by workers in the same laboratory [19], we have been successful in identifying and isolating the 26 S proteasome complex from various rat tissues. Similarly to the results obtained by Rechsteiner and co-workers [12,19], our enzyme preparation obtained from rat skeletal muscle tissue ran as a single band in non-denaturing polyacrylamide gels of standard size but split up into slow- and fast-migrating forms in midget gels. An additional proteolytically inactive protein component in the 26 S proteasome preparation could be identified as the 'ball' complex. The presence of free 'balls' is most likely due to a release of 'balls' from the 26 S proteasome containing two of the regulatory 'balls'. As already shown by Sawada et al. [22] this process results in the formation of proteasomes complexed with one 'ball' only, giving rise to the occurrence of slow- and fast-migrating forms of the 26 S proteasome. A further dissociation into 20 S proteasomes and free 'balls' seems not to occur under the conditions used in our experiments as no 'free' 20 S proteasomes were detected in our 26 S proteasome preparations. When resolved by two-dimensional PAGE, the 26 S proteasome complex was found to contain the subunits typical of the multicatalytic proteinase as well as of the 'ball' components that have also been found in the 26 S proteasome complex from rat liver tissue (see, for example, Figure 4 in [21]). In accordance with these results and in contrast to our previous conclusions [30,31] we have reason to believe that the 20 S proteasome is a genuine constituent of the 26 S proteasome complex.

Although the structure and function of the 26 S proteasome complex are not yet understood in full detail, several of its features have been determined during recent years. Electron-microscopic investigations have shown that the regulatory components ('balls' [19], conjugate-degrading factors [32], μ particles [15]) are associated with the outer discs of the cylindrical 20 S proteasome particle [13,18,21]. Formation of this 26 S complex has been found to be ATP/Mg^{2+}-dependent and failure of p[NH]ppA to substitute for ATP indicates that ATP serves as a free-energy donor in this reaction [9,10,32]. Further, degradation of ubiquitin–protein conjugates by 'Fraction II' of reticulocytes has been found to have an absolute requirement for ATP/Mg^{2+} which cannot be substituted for by non-cleavable ATP analogues, indicating that free energy of phosphodiester-bond hydrolysis is necessary for driving this proteolytic reaction [10,33–36]. This feature has been confirmed for the purified 26 S proteasome complex, since the ATP- and ubiquitin-conjugate-hydrolysing activities are closely linked reactions [14,16,37]. Three subunits in the regulatory 'balls' contain highly conserved sequences with a putative ATP–binding site that is common to all members of a recently discovered family of eukaryotic ATPases [38]. Thus it is very likely that these are subunits of an oligomeric ATPase complex that is an essential constituent of the regulatory 'ball' of the 26 S proteasome complex. It is still not known whether this ATPase complex is responsible for delivering the free energy of ATP hydrolysis for 26 S complex formation, for peptide-bond hydrolysis of ubiquitinated proteins or for both.

In addition to these ATP-consuming reactions it was found that the Suc-LLVY-NMec-hydrolysing activity was stimulated by about 30% in the presence of 1 mM ATP and that ATP could be substituted for by CTP and GTP but less well by UTP. p[CH_2]ppA and ADP had only a small effect, whereas AMP and PP showed no activating effect. On the other hand, an ATP-regenerating system further enhanced the activating effect of ATP [12]. Similar results were obtained when the enzyme was treated with various nucleotides before being subjected to non-denaturing PAGE and then analysed by the substrate overlay technique [19]. These data clearly show that the hydrolysis of peptide bonds by the 26 S proteasome complex can be activated by ATP. As shown here, ATP primarily stimulates Suc-LLVY-NMec hydrolysis; little activation is measured with Suc-AAF-NMec, Z-GGL-NMec or Bz-VGR-NMec as a substrate (Figure 5). When measuring ATP-stimulated peptide-hydrolysing activity by the 26 S proteasome complex most investigators have used 1–2 mM concentrations of ATP [12,17,19]. In the present study we have shown that maximal stimulation is already achieved at a concentration of 25 μM ATP. As this activation of the peptide-splitting activity of the 26 S proteasome is not dependent on Mg^{2+} [or Ca^{2+} or Mn^{2+} (results not shown)], the question arose as to whether the stimulatory process requires hydrolysis of the nucleotide. That ATP is hydrolysed by the purified 26 S complex can be concluded from our finding that activation is transient when the 26 S proteasome complex is incubated with ATP and that addition of an ATP-regenerating system maintains the proteinase in the activated state. Thus, non-hydrolysed ATP is necessary for both the activating step itself and for keeping the enzyme in its activated form. According to our result, ATP can be substituted for by non-hydrolysable ATP analogues such as ATP[S], p[NH]ppA, pp[CH_2]pA, ADP and ADP[S], to achieve the same effect. The activation of peptide-splitting activity by ATP and its analogues cannot result from a reassociation of free 'balls' to the 20 S proteasome, as it has been reported that p[NH]ppA and ADP[S] are unable to substitute for ATP in the context of 20 S proteasome activation by PA700, a process resembling 26 S complex formation [39,40]. Similarly, ATP-dependent breakdown of ubiquitin–lysozyme conjugates as well as of antizyme–ornithine decarboxylase complexes by the 26 S proteasome is strictly ATP-dependent: ATP cannot be replaced by pp[CH_2]pA, p[CH_2]ppA or p[NH]ppA [24,34]. Thus, the intrinsic ATPase activity of the 26 S proteasome seems unable to

hydrolyse these types of ATP analogues. Since, with the exception of p[CH_2]ppA, these analogues are able to activate the peptide-hydrolysing activity of the 26 S proteasome complex, we suggest that in this step ATP does not serve as a donor of free energy. This conclusion is supported by the finding that ATPase inhibitors such as vanadate and quercetin do not affect the stimulation by ATP of Suc-LLVY-NMec hydrolysis. The ATP-binding site seems to be located in one of the regulatory 'ball' components as these nucleotides do not affect the activities of free 20 S proteasome. Binding of ATP or its analogues may induce conformational changes and thus may act as an allosteric activator of the 20 S proteasome, the core enzyme of the 26 S proteasome complex.

This type of allosteric function of ATP has also been found in protease La (lon protease) from *Escherichia coli*. The peptide-hydrolysing activity of this proteinase, which contains ATP and protein-hydrolytic sites within the same subunit, is activated by ATP[S], p[CH_2]ppA and p[NH]ppA almost as effectively as by ATP, and vanadate does not reduce this activation [41,42]. A different situation is found with protease Ti (Clp protease) from *E. coli*, where protein- and ATP-hydrolytic sites are located on different subunits. Here, breakdown of small peptide substrates is not stimulated by ATP [43,44]. Thus, for all three proteinases ATP hydrolysis is not required for peptide-bond cleavage. Rather, the free energy of phosphoanhydride-bond cleavage seems to be necessary for unfolding large protein substrates, thus rendering them acceptable for the proteolytically active sites of the three proteinases [45].

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