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Biochemistry

An ATP-stabilized inhibitor of the proteasome is a component of the 1500-kDa ubiquitin conjugate-degrading complex

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ABSTRACT **Proteins conjugated to ubiquitin are degraded by a 26S (1500-kDa) proteolytic complex that, in reticulocyte extracts, can be formed by the association of three factors: CF-1, CF-2, and CF-3. One of these factors, CF-3, has been shown to be the proteasome, a 650-kDa multicatalytic protease complex. We have purified a 250-kDa inhibitor of the proteasome and shown that it corresponds to CF-2. In the presence or absence of ATP, this factor inhibited hydrolysis by the proteasome of both fluorogenic tetrapeptides and protein substrates. When the inhibitor, proteasome, and CF-1 were incubated together in the presence of ATP and Mg^{2+}, degradation of ubiquitin–^{125}I-lysozyme occurred. Both the inhibitory activity and the ability to reconstitute ubiquitin–^{125}I-lysozyme degradation were very labile at 42°C, but both activities were stabilized by ATP or a nonhydrolyzable ATP analog. SDS/PAGE indicated that the 250-kDa inhibitor fraction contained a major subunit of 40 kDa (plus some minor bands). The ^{125}I-labeled inhibitor and purified proteasome formed a complex. When CF-1, ATP, and Mg^{2+} were also present, the ^{125}I-labeled inhibitor along with the proteasome formed a complex of 1500 kDa. The inhibitor (CF-2) thus appears to be an ATP-binding component that regulates proteolysis within the 1500-kDa complex.**

In eukaryotes, the degradation of most cellular proteins is an energy-dependent process that requires a polypeptide cofactor, ubiquitin (1–3). The ligation of ubiquitin to cell proteins marks them for rapid hydrolysis (1, 2) by a very large proteolytic complex that has been the subject of many studies (3–10). This 26S (1500-kDa) complex selectively degrades proteins conjugated to ubiquitin by an ATP-dependent process (3–7). Although this large structure has been studied most extensively from reticulocyte extracts (5, 6), it was not detected following ATP depletion of the reticulocytes. However, such a complex could be reconstituted *in vitro* in an ATP-dependent reaction from three factors, referred to as CF-1, CF-2, and CF-3 (8). Eukaryotic cells also contain a 650-kDa proteolytic complex (11–15), referred to as the proteasome (3, 12). This structure contains 13–15 subunits of 20–33 kDa and multiple proteolytic sites, which cleave peptide bonds on the carboxyl side of hydrophobic, basic, or acidic amino acids (3, 11–18). Although the proteasome by itself does not degrade proteins conjugated to ubiquitin, immunoprecipitation or inactivation of the proteasome prevents hydrolysis of ubiquitinated proteins (17–19). The proteasome corresponds to one of the components, CF-3, of the 1500-kDa proteolytic complex (3, 9, 10). The regulatory or catalytic functions of the other two factors and their subunit composition have not been identified.

Incorporation of the proteasome into the ubiquitin conjugate-degrading complex clearly alters the properties of the proteasome. When a reticulocyte lysate is fractionated with ammonium sulfate, the proteasome (CF-3) is recovered in the 40–80% fraction, while CF-1 and CF-2 are precipitated with 0–38%. In the presence of ATP and the 0–38% fraction, the proteasome is incorporated into a large complex that degrades ubiquitin-conjugated proteins (9, 10). This structure contains proteasome subunits, as shown immunologically (10), and the characteristic peptidase activities (10, 11) of the proteasome. In the 1500-kDa complex, these proteolytic activities appear to be ATP-dependent (10), and the complex shows ATPase activity (20).

Understanding the functioning of the proteasome within the 1500-kDa complex and the mechanism for degrading ubiquitin-conjugated proteins will require purification and characterization of its other two components. In the present study, we have purified a 250-kDa ATP-stabilized factor that inhibits the proteolytic activities of the proteasome and corresponds to the "inhibitor of high molecular weight proteases" reported by Etlinger and coworkers (21, 22). We demonstrate that it is required to reconstitute degradation of ubiquitin-conjugated proteins and that it corresponds to CF-2 of the 1500-kDa complex (8).

MATERIALS AND METHODS

DEAE-cellulose (DE-52), CM-cellulose (CM-52), and phosphocellulose (P11) were obtained from Whatman. Ubiquitin (bovine erythrocyte), ammonium sulfate, succinylleucylleucylvalyltyrosine 7-amido-4-methylcoumarin (Suc-LLVY-MCA), and ATP were from Sigma. Lysozyme (chicken egg) was from Bachem. Lysozyme was radiolabeled with Na^{125}I by the chloramine-T method (23). Ubiquitin-conjugating enzymes (E1, E2, and E3) were isolated by ubiquitin-Sepharose affinity column chromatography (24) and were used to prepare ubiquitin–^{125}I-lysozyme conjugates (25). Mono Q anion-exchange (HR 10/10 or HR 5/5) and Superose 6 gel filtration (HR 10/30) columns were from Pharmacia. Protein concentration was determined with the Coomassie blue G-250 reagent (Pierce).

Purifications. Rabbit reticulocytes induced by phenylhydrazine injection were prepared as described previously (26) or were purchased from Green Hectares (Oregon, WI). They were depleted of ATP by incubation with 2,4-dinitrophenol and 2-deoxyglucose (26). Lysates were then prepared and subjected to DE-52 chromatography. The protein eluted with 0.5 M KCl (24) was concentrated by precipitation using ammonium sulfate at 80% saturation, centrifuged at 10,000 × *g* for 20 min, and suspended in 20 mM Tris·HCl, pH 7.6/1 mm dithiothreitol (buffer A). After extensive dialysis against the same buffer, the protein (fraction II) was either stored at −80°C in 0.5 mM ATP or fractionated further.

Fraction II (200 mg) was applied to a ubiquitin-Sepharose column, and the ubiquitin-conjugating enzymes were specifically eluted (24) and used in making ubiquitin–^{125}I-lysozyme (25). The unadsorbed fraction was brought to 38% saturation with ammonium sulfate and mixed for 20 min, as described

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Abbreviation: Suc-LLVY-MCA, succinylleucylleucylvalyltyrosine 7-amido-4-methylcoumarin.

(8). The precipitated proteins were collected by centrifugation at 10,000 × *g* for 15 min. The pellet was resuspended in buffer A and brought again to 38% saturation with ammonium sulfate. The precipitated material was collected as above and then suspended in buffer A containing 10% (vol/vol) glycerol. After dialysis against this buffer, the 0–38% pellet was chromatographed on a Mono Q anion-exchange column equilibrated with buffer A containing 10% glycerol. The protein was eluted with a 60-ml linear gradient of NaCl from 20 to 400 mM. Further purification was achieved by a second Mono Q chromatographic step using a more narrow gradient, from 150 to 400 mM NaCl. The second Mono Q step yielded a sharp peak of inhibitor activity. Fractions that inhibited the peptidase activity of the proteasome were pooled, concentrated, and then chromatographed on a Superose 6 (HR 10/30) gel filtration column equilibrated in buffer A containing 100 mM NaCl and 0.2 mM ATP. The column was run at a flow rate of 0.2 ml/min, and 1-ml fractions were collected. Fractions with inhibitory activity against the proteasome were pooled and dialyzed against 20 mM KH_2PO_4, pH 6.5/10% glycerol/1 mM dithiothreitol/1 mM ATP (buffer B). The sample was then applied to a 2-ml phosphocellulose column equilibrated in buffer B. The column was washed with 4 ml of this buffer, followed by 4 ml of buffer B containing either 20, 50, 100, 400, or 600 mM NaCl.

To partially purify CF-1, fractions from the first Mono Q step that were eluted from 100 to 240 mM NaCl were pooled, concentrated to 1 ml, and applied to a Superose 6 column equilibrated in buffer A containing 100 mM NaCl and 0.2 mM ATP. The fractions eluted at ≈600 kDa were used as the CF-1 fraction.

The proteasome was isolated from the supernatants of the two 38% ammonium sulfate precipitations. The supernatants were brought to 80% saturation with ammonium sulfate and mixed for 20 min. The precipitated protein was collected by centrifugation, resuspended in buffer A, and dialyzed extensively against this buffer. The proteasome was isolated by Mono Q anion-exchange chromatography followed by Superose 6 gel filtration (27). These preparations displayed only the characteristic subunits of 20–33 kDa.

Assays. Inhibition of the proteasome was measured by preincubating individual column fractions with the proteasome in the presence of 1 mM ATP at 37°C for 10 min. After preincubation, the reaction tubes were placed on ice, and either ^{125}I-lysozyme or Suc-LLVY-MCA was added. Reactions were carried out at 37°C for 60 min with ^{125}I-lysozyme or 10 min with Suc-LLVY-MCA. Protein hydrolysis was assayed by measuring production of radioactivity soluble in 10% (wt/vol) trichloroacetic acid, and peptide hydrolysis by the release of 7-amino-4-methylcoumarin (27). Degradation of ubiquitin-conjugated ^{125}I-lysozyme was assayed at 37°C for 60 min. Reactions contained either 5 mM EDTA or 2 mM ATP and 5 mM $MgCl_2$ and were terminated by adding 10% trichloroacetic acid.

Other Methods. Following the phosphocellulose step, the inhibitor (100 μg in buffer B) was labeled with Na^{125}I by incubation with Iodo-Beads (Pierce) for 15 min at 4°C, as recommended by the manufacturer. The radiolabeled inhibitor was then separated from free Na^{125}I by gel filtration twice on Sephadex G-25 columns and extensively dialyzed against buffer A prior to use. Denaturing polyacrylamide gel electrophoresis (SDS/PAGE) in 12% gels was performed as described by Laemmli (28). Nondenaturing PAGE (4% or 6%) was performed in the same buffer system but without the SDS.

RESULTS

Isolation of the Inhibitor. To understand how the proteasome is regulated *in vivo* and how it functions within the ubiquitin conjugate-degrading complex, we attempted to isolate factors that influence its activity. Fraction II from ATP-depleted reticulocytes was separated into fractions that precipitated with 0–38% or 40–80% ammonium sulfate. The latter fraction was used to isolate the proteasome. The proteasome obtained in this way showed appreciable activity against ^{125}I-lysozyme and Suc-LLVY-MCA that was independent of ATP (9, 10). Neither the proteasome nor the 0–38% fraction showed significant hydrolytic activity against ubiquitin-conjugated ^{125}I-lysozyme (9, 10). However, as reported previously, ATP-dependent degradation of the ubiquitinated lysozyme was observed after the proteasome and the 0–38% fraction were preincubated together in the presence of ATP (data not shown).

The 0–38% precipitated material then was fractionated by Mono Q anion exchange, and each fraction was assayed for its ability to influence proteasome activity against Suc-LLVY-MCA or ^{125}I-lysozyme. Column fractions were preincubated with the proteasome for 10 min and then either substrate was added. None of the column fractions by itself showed significant hydrolytic activity, and most did not affect proteasome activity (Fig. 1*A*). However, between 240 and 280 mM NaCl, a peak of inhibitory activity was eluted that decreased hydrolysis of ^{125}I-lysozyme (data not shown) and of the peptide to similar extents. The active fractions were further purified by a second Mono Q step using a more narrow NaCl gradient.

To purify the inhibitory activity further, the Mono Q active fractions were pooled and chromatographed by gel filtration. The inhibitor was eluted as a sharp peak with an apparent molecular mass of 250 kDa (Fig. 1*B*). The active fractions were then pooled and assayed for inhibitory activity. With increasing inhibitor concentration, proteasome activity decreased in a linear manner with both ^{125}I-lysozyme and Suc-LLVY-MCA as substrates, although the degree of the inhibition was highly variable between preparations (Fig. 2).

The Inhibitor Resembles Component CF-2 of the 1500-kDa Complex. One component of the 1500-kDa proteolytic complex, CF-2, has been reported also to have a molecular mass of 250 kDa (8). To test whether the inhibitor corresponded to CF-2, the inhibitor obtained by gel filtration was subjected to phosphocellulose chromatography (Fig. 1*C*). The inhibitory activity was recovered in the flowthrough and 20 mM NaCl eluate—i.e., in the region where CF-2 activity was reported by Eytan *et al.* (9). Individual phosphocellulose fractions were then assayed for their ability to reconstitute rapid degradation of ubiquitin–^{125}I-lysozyme (Fig. 1*C*) when combined with the proteasome and CF-1-containing fraction. By themselves the latter components did not support this process. However, when fractions with inhibitory activity were added, ubiquitin–^{125}I-lysozyme degradation increased sharply. Other phosphocellulose fractions did not stimulate this process.

These results suggest strongly that the inhibitor corresponds to CF-2 and thus is essential for hydrolysis of ubiquitin-conjugated proteins. One unusual property of CF-2 is that it is quite labile upon heating to 42°C but is stabilized by ATP (8). To test further whether the proteasome inhibitor corresponded to CF-2, the purified inhibitor (from the phosphocellulose column) was preincubated at 42°C with or without ATP. The proteasome was added, and after 10 min, peptidase activity was assayed. The degree of inhibition decreased rapidly during preincubation without any nucleotide added, but the presence of either ATP (Fig. 3) or a nonhydrolyzable ATP analog, adenosine 5′-[β,γ-imido]triphosphate (data not shown), prevented this loss of activity. Furthermore, the ability of this fraction to reconstitute ubiquitin-conjugate degradation also decreased rapidly during incubation at 42°C (Fig. 3), and the addition of ATP or adenosine 5′-[β,γ-imido]triphosphate (data not shown) blocked this inactivation. Since the inhibitory activity and

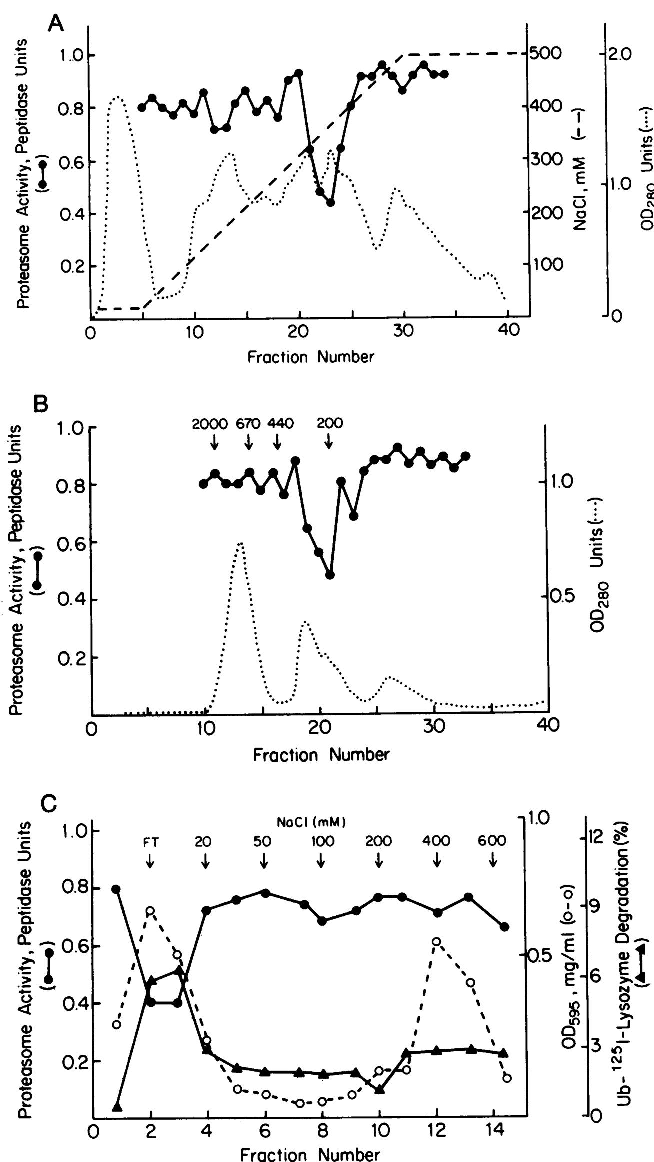

FIG. 1. (*A*) Influence of Mono Q fractions on the peptidase activity of the proteasome. The 0–38% ammonium sulfate fraction (150 mg) was separated using a Mono Q anion-exchange column with a linear gradient from 20 to 400 mM NaCl. The influence of individual column fractions on the peptidase activity of the proteasome was assayed. Incubations included 15 μl of column fraction and 1.5 μl of the proteasome (≈0.2 mg/ml). (*B*) Resolution of the inhibitory factor by gel filtration. Active Mono Q fractions (21–25) were pooled, rechromatographed by a second Mono Q step, concentrated, and applied to a Superose 6 column. Molecular size markers were dextran blue (≈2000 kDa), thyroglobulin (667 kDa), apoferritin (440 kDa), and β-amylase (200 kDa). Column fractions were assayed by using 20 μl of each fraction and 1.5 μl of the proteasome. (*C*) Phosphocellulose chromatography of the inhibitor. The active fractions from gel filtration (3 mg) were applied to a 1-ml column. Fractions were eluted with indicated NaCl concentrations and desalted by repeated 10-fold dilutions with buffer A followed by concentration in Centricon cones (Amicon). Fractions (5 μl of each) were then assayed for influence on proteasome activity and then for their ability to reconstitute degradation of ubiquitin (Ub)–^{125}I-lysozyme (≈7500 total cpm per assay) in the presence of the proteasome (1 μl), partially purified CF-1 (10 μl), and an ATP-regenerating system. FT, flowthrough.

ability to reconstitute ubiquitin-conjugate degradation were inactivated at similar rates and were stabilized similarly by ATP, these two activities probably reside in a single molecule that can bind ATP.

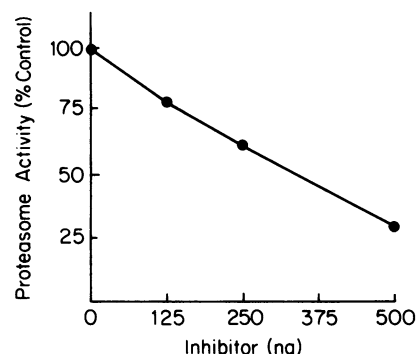

FIG. 2. Effect of increasing concentrations of the inhibitor on the hydrolysis of Suc-LLVY-MCA by the proteasome.

Properties of the Inhibitor. Upon freezing or upon storage at 4°C, the purified inhibitor was labile even in the presence of ATP and glycerol. Although ATP stabilizes the inhibitory factor, it is not essential for inhibition of the proteasome. When the inhibitor and proteasome were preincubated at 37°C for up to 20 min with or without ATP, a similar degree of inhibition was observed (data not shown). Nevertheless, because of the stabilization of the inhibitor by ATP, this nucleotide was routinely added to all incubations.

The fraction from the phosphocellulose column with inhibitory and CF-1 activities was then analyzed by PAGE. In nondenaturing gels, it migrated as a single species (Fig. 4*A*), similar in mobility to β-amylase (200 kDa) and urease (270 kDa). Upon SDS/PAGE, a heavily staining polypeptide of 40 kDa was evident consistently, as well as faint bands of about 100, 50, and 10 kDa, whose significance is unclear (Fig. 4*B*). The 250-kDa inhibitor thus contains a major subunit of 40 kDa that resembles in size a polypeptide of the 1500-kDa ubiquitin conjugate-degrading complex, as indicated by SDS/PAGE of the complex purified from reticulocyte extracts (ref. 9 and data not shown). Such a 40-kDa band was seen upon immunoprecipitation of this complex with anti-proteasome antibodies (data not shown). These observations are consistent with the conclusion that subunits of the inhibitor become associated with the proteasome within the 1500-kDa complex.

Incorporation of the Inhibitor into Larger Complexes. To test directly whether the 250-kDa inhibitor associates with the proteasome, the purified inhibitor was radiolabeled with Na^{125}I under gentle conditions. Upon nondenaturing 4% PAGE, the ^{125}I-labeled inhibitor and the unlabeled inhibitor migrated similarly (Fig. 4*C*). Incubation of the ^{125}I-labeled

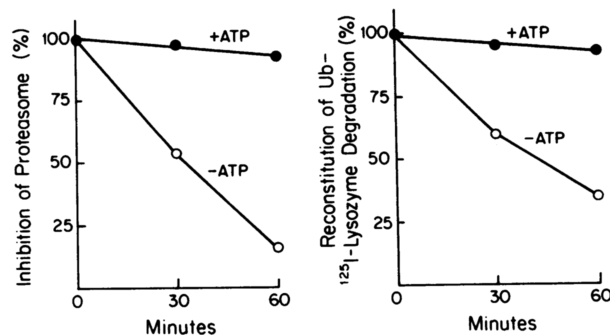

FIG. 3. (*A*) ATP stabilization of the inhibitor. Fractions (20 μl) from the phosphocellulose column were preincubated at 42°C in the presence or absence of ATP (1 mM) for the times indicated, and their ability to inhibit the proteasome (1.5 μl) was then determined. (*B*) Effect of these preincubations on the ability to reconstitute hydrolysis of ubiquitin (Ub)–^{125}I-lysozyme. Relative activity of 100% represents a 70% inhibition of the peptidase activity (*A*) or a 9% degradation of ubiquitin–^{125}I-lysozyme (*B*).

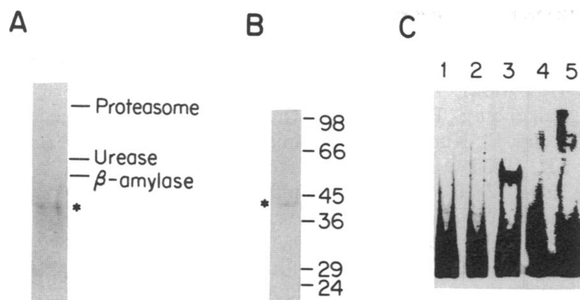

FIG. 4. (A) Nondenaturing PAGE of the inhibitor. The flowthrough fractions from the phosphocellulose columns were run in a nondenaturing 6% polyacrylamide gel and stained with Coomassie blue. (B) SDS/12% PAGE. Molecular size (kDa) standards are indicated. (C) Nondenaturing 4% PAGE of ^{125}I-labeled inhibitor (4 μg, 2×10^5 cpm) after incubation in a total volume of 50 μl of buffer A for 30 min at 37°C either alone (lane 1), with 60 μg of CF-1 (lane 2), 10 μg of proteasome (lane 3), or 60 μg of CF-1 and 10 μg of proteasome (lanes 4 and 5). For lanes 1–3, reaction mixtures contained ATP and Mg^{2+}, but similar results were obtained in their absence (see text). Lane 5 shows the formation of a higher molecular weight complex, not seen in the absence of ATP and Mg^{2+} (lane 4). Approximately 5% of added radioactivity was incorporated into the higher molecular weight complex in lane 5. Bands were detected by Phosphorimager radiography (Molecular Dynamics, Sunnyvale, CA).

inhibitor with CF-1 prior to electrophoresis did not alter its mobility (Fig. 4C); however, incubation of the inhibitor with the proteasome caused a decrease in its mobility so that the ^{125}I-labeled material migrated as a sharp peak whose mobility resembled that of the proteasome as identified by Western blot or Coomassie staining (10, 17). These findings suggest a stoichiometric association of the inhibitor and the proteasome. Formation of this complex occurred whether or not ATP and $MgCl_2$ were present.

Interestingly, when the CF-1 fraction was added to this mixture in the absence of ATP and Mg^{2+}, the inhibitor–proteasome complex was no longer evident. Thus, CF-1 seems to reduce the association of the inhibitor and proteasome. In contrast, when these three components were combined in the presence of ATP and Mg^{2+} to allow formation of the 1500-kDa complex, a small fraction of the ^{125}I-labeled material (Fig. 4C) appeared in the region of the gel where this complex is found (5, 10, 17). These results are consistent with the ATP-dependent incorporation of the inhibitor into the 1500-kDa complex.

Additional experiments tested more critically whether the inhibitor actually is a component of the larger complex. When subjected to gel filtration on Superose 6, the ^{125}I-labeled inhibitor was detected as a sharp 250-kDa peak. It also was eluted at this point after incubation with fraction II in the absence of ATP or $MgCl_2$ (Fig. 5A). When ATP and Mg^{2+} were added to the incubation, a major fraction (35%) of the ^{125}I-labeled inhibitor was found in a new peak at 1500 kDa (Fig. 5B). Since the ^{125}I-labeled inhibitor must be diluted by endogenous nonradioactive inhibitor, these findings suggest quantitative incorporation of the purified factor into the larger complex. Under these conditions, peptide-hydrolyzing activity also appeared in this 1500-kDa peak. These findings thus confirm the earlier conclusions (9, 10, 29) that the proteasome is a component of the 1500-kDa complex [which had recently been questioned (30)]. Moreover, they show that the inhibitor is incorporated along with the proteasome into this complex.

DISCUSSION

The 250-kDa inhibitor appears to be an essential component of the ATP/ubiquitin-dependent pathway and to function as

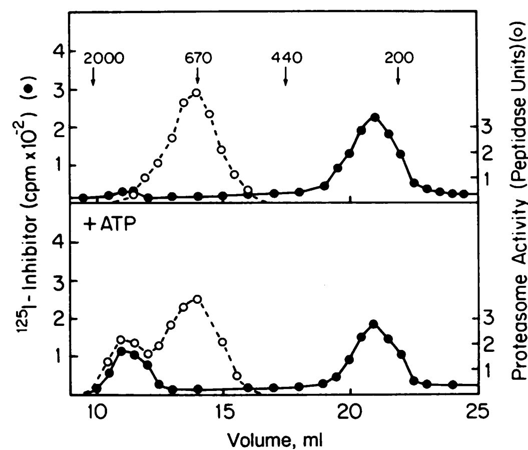

FIG. 5. ATP-dependent incorporation of the ^{125}I-labeled inhibitor into a 1500-kDa complex. The ^{125}I-labeled inhibitor (20 μg, 2×10^6 cpm in 200 μl) was preincubated for 30 min at 37°C with 0.5 ml of fraction II (15 mg/ml) in the absence (A) or presence (B) of ATP and $MgCl_2$. Samples were then applied to a Superose 6 column and chromatographed, and fractions of 0.5 ml were collected. Radioactivity and peptidase activity in each fraction were determined. Approximately 30–40% of added ^{125}I was detected in the 1500-kDa fraction-containing proteasome in B.

part of the 1500-kDa proteolytic complex. By several criteria, the inhibitor seems to be identical to component CF-2. (i) These two activities copurify from fraction II through ammonium sulfate fractionation, Mono Q, gel filtration, and phosphocellulose chromatography. (ii) The inhibitory activity and the ability to reconstitute ubiquitin–^{125}I-lysozyme degradation appear to be linked functions, since they show similar inactivation kinetics at 42°C and similar stabilization by nucleotide. (iii) In the presence of ATP, Mg^{2+}, and CF-1, the inhibitor becomes incorporated into the 1500-kDa complex along with the proteasome. (iv) The 250-kDa inhibitor contains a major subunit of 40 kDa (Fig. 4B), which resembles in size a subunit of the 1500-kDa proteolytic complex. Minor bands, however, were seen that may also be required for the inhibitory or CF-2 activity. Definitive information on its subunit composition has proven difficult to establish due to the instability of these activities upon purification.

The inhibitor purified here appears to correspond to the 250-kDa noncompetitive "endogenous inhibitor of high molecular weight proteases" reported by Etlinger and coworkers (21, 22), although we have not observed any inhibitory effect on calpains (data not shown) as Murakami and Etlinger had reported (22). The present studies support their proposal that the inhibitor plays a role in ubiquitin-dependent proteolysis (22, 31), but in addition, our results indicate that it is actually a part of the 1500-kDa ubiquitin-conjugate-degrading complex.

The marked stabilization of the purified inhibitor by ATP or a nonmetabolized ATP analog, even in the absence of Mg^{2+}, strongly suggests that this factor is a nucleotide-binding protein. ATP, however, was not necessary for the inhibitory activity or for the association of the ^{125}I-labeled inhibitor with the proteasome (Fig. 4C), as demonstrated by nondenaturing PAGE. Despite the ATP stabilization, we could not detect ATPase activity associated with the inhibitor [in accord with previous observations (20)], although it may be a latent ATPase that is activated upon formation of the 1500-kDa complex. This complex does show ATPase activity, unlike the three separate components (20). The ATP-binding site on the inhibitor may correspond to this ATPase and may be related to the ATP consumed during ubiquitin-conjugate degradation (1–3). Alternatively, the ATP-binding

site may be related to the ATP utilized in formation of the 1500-kDa complex (8, 9).

It is unclear to what extent the inhibitor and the proteasome exist *in vivo* as independent structures or associated with one another in larger complexes. In the cell, the multiple proteolytic activities of the proteasome require careful regulation (3), and an important function of the inhibitor may be to prevent inappropriate or excessive proteolysis. An attractive model would be that within the 1500-kDa complex, the inhibitory component normally maintains the proteasome in an inactive state and that interaction with ubiquitinated proteins and ATP hydrolysis temporarily release this inhibition to allow proteolysis. Moreover, we recently found that a 600-kDa ATP-binding component resembling CF-1 has the capacity to activate the proteasome many fold and that the inhibitor can block this activation (unpublished work). In addition, CF-1, in the absence of ATP, reduces the association of the inhibitor with the proteasome (Fig. 4*C*). Therefore, within the 1500-kDa complex, protein breakdown may involve cyclic, ATP-dependent alterations in the function of these opposing regulators of proteasome activity.

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