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Endogenous inhibitor of nonlysosomal high molecular weight protease and calcium-dependent protease

(intracellular proteolysis/calpains/ubiquitin/energy requirement)

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ABSTRACT **An endogenous inhibitor of high molecular weight protease was purified from human erythrocytes and partially characterized. The inhibitor was isolated by DEAE-Sephacel ion-exchange chromatography followed by separation on a Bio-Gel A-0.5m column. The inhibitor displayed a native M_r of 240,000 and contained a single subunit of M_r 40,000 after NaDodSO$_4$/polyacrylamide gel electrophoresis. The M_r 240,000 hexamer inhibited high molecular weight protease noncompetitively ($K_i = 8.3 \times 10^{-8}$ M) and showed marked susceptibility to proteolytic digestion and heat treatment. The purified factor was also a potent inhibitor of calcium-dependent protease ($K_i = 2.8 \times 10^{-8}$ M), whereas it had no effect on trypsin, chymotrypsin, or papain. Heat treatment (50–70°C × 10 min) caused loss of inhibition against high molecular weight protease; however, inhibition of calcium-dependent protease was stable under the same conditions. This result is consistent with different domains on the inhibitor that interact with high molecular weight protease and calcium-dependent protease. Together with earlier studies in which repression of inhibitor by an ATP-ubiquitin-dependent process was proposed, the present results suggest a general mechanism for regulation of multiple nonlysosomal proteases that are complexed with endogenous inhibitors.**

Intracellular proteolysis occurs in the cytosol as well as in lysosomes by energy-requiring processes (1, 2). Several nonlysosomal proteolytic activities that are stimulated by ATP have been described (3–9). A system from *Escherichia coli* has been studied in detail and shown to involve a protease composed of four identical M_r 94,000 subunits that specifically requires ATP hydrolysis for protein degradation (8, 9). In contrast, ATP-dependent proteolysis in the soluble fraction of reticulocytes involves several distinct components, including the polypeptide ubiquitin (2, 10). It has been proposed that conjugation of ubiquitin by means of isopeptide bonds to ε-amino or possibly α-amino groups "tags" a protein for subsequent hydrolysis (11, 12). Several enzymes involved in the conjugation pathway have been isolated and, the first step, activation of the C-terminal residue of ubiquitin, requires ATP (13). ATP-ubiquitin-dependent proteolysis has been difficult to demonstrate in cells other than reticulocytes, although some evidence for ATP-ubiquitin-dependent proteolysis has been reported in liver (14) and muscle (15).

When proteases are extensively purified from the cytosol of various mammalian cells, including reticulocytes, they lack a specific ATP requirement. One class of such proteases displays unusually high molecular weights ranging from M_r 450,000 to 1,300,000 (16–21). These high molecular weight proteases (HMPs) have been isolated from reticulocytes (16), liver (17, 18), and muscle (19–21) and appear able to rapidly degrade a variety of proteins, including several that are dependent on ATP and ubiquitin for rapid degradation in reticulocyte extracts. Although the isolated HMPs are not dependent on ATP, they are sometimes stabilized by nucleotides and other phosphates (16–20). Several HMPs display sensitivity to the same agents—e.g., hemin, *N*-ethylmaleimide, and iodoacetamide—that inhibit ATP-dependent proteolysis in crude reticulocyte extracts or even in partially fractionated systems (2, 3). Thus, the HMPs may represent components and/or modified forms of the ATP-dependent system.

The other major class of nonlysosomal proteases includes the calcium-activated proteases (calpain I and II) that are present in many tissues (22–29). However, in most cells, calpain inhibitors are present that appear to block protease activity at calcium levels required to activate the isolated calpains (30, 31). Thus, it is puzzling how the calpains act in the presence of inhibitor, although there is evidence that such enzymes probably function in intracellular proteolysis (25–27).

Previous studies from our laboratory demonstrated that reticulocyte extracts could be separated into a fraction containing an inhibitor and another fraction containing protease activity that was not dependent on ATP (32). ATP together with ubiquitin stimulated protease activity only when both fractions were combined, suggesting a derepression of proteolysis by these factors (32). Here we demonstrate that HMP, in the absence of ATP, rapidly hydrolyzes bovine serum albumin, which absolutely requires ATP for its degradation in crude reticulocyte lysates. In addition, an endogenous inhibitor of this HMP has been purified and some of its properties have been described. This factor was found to also inhibit calpain, suggesting a general mechanism for ATP-ubiquitin-dependent regulation of multiple nonlysosomal proteases that interact with endogenous inhibitors.

METHODS

Purification of Inhibitor. Reticulocytes and erythrocytes from rabbit and human were prepared as described (32). In each case, cells were lysed with 3 vol of 4 mM Tris·HCl (pH 7.2) supplemented with 0.5 mM *p*-chloromercuribenzoate and 0.5 mM phenylmethylsulfonyl fluoride and centrifuged at 18,000 × *g* for 60 min. The supernatant was mixed with an equal volume of DEAE-Sephacel suspension (resin:buffer A = 1:2) for 30 min. Buffer A contained 0.5 mM dithiothreitol, 0.5 mM $MgCl_2$, 20 mM KCl, and 20 mM Tris·HCl (pH 7.2). The suspension was poured onto a Buchner funnel that contained another volume of fresh DEAE-Sephacel suspension, followed by washing with 3 vol of buffer A. Protein was then eluted with 0.3 M KCl in buffer A and precipitated with ammonium sulfate at 50% saturation. After dialysis, the protein was adsorbed on a DEAE-Sephacel column (2.5 × 7

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Abbreviation: HMP, high molecular weight protease.
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cm) and eluted with a gradient of 0–0.25 M KCl in 300 ml of buffer A. Fractions constituting the main peak of inhibitor activity were pooled and precipitated with ammonium sulfate at 50% saturation, followed by dialysis against buffer A containing 0.1 M KCl. The protein was then chromatographed at 5 ml/cm^2 per hr on a Bio-Gel A-0.5m column (1.5 × 100 cm) equilibrated with the same buffer. Fractions (2 ml) were collected and the combined peak fractions were concentrated to 1.5 mg/ml with an Amicon Centricon and stored in a −80°C freezer. The concentrated inhibitor was stable for several months.

Isolation of HMP. HMP was purified from rabbit erythrocyte lysates prepared as described above but without inclusion of phenylmethylsulfonyl fluoride and *p*-chloromercuribenzoate. The procedure was similar to that described for a similar enzyme isolated from liver (17). A protease fraction was obtained by batch elution with 0.4 M KCl from DEAE-Sephacel on a Buchner funnel, as described above, followed by elution from a DEAE-Sephacel column using a 50–400 mM KCl gradient. A single HMP assayed with bovine serum albumin (see below) eluted at ≈250 mM KCl, after the bulk protein. HMP was concentrated by precipitation with ammonium sulfate (75% saturation) and was chromatographed on Sepharose 4B. The HMP eluted after a ferritin standard (M_r 450,000) and was concentrated and stored at −80°C after dialysis against buffer A. The specific activity of the HMP preparation used in the present study was 180 units/mg of protein (see below for definition of units). The hydrolytic products of globin and bovine serum albumin with HMP were larger than M_r 2500, suggesting that the HMP preparation was free of peptidases. In addition, addition of EDTA or calcium had no effect on proteolysis, indicating the complete absence of any calcium-dependent proteases (calpain) in this preparation.

Preparation of Calcium-Dependent Protease (Calpain). Calpain was purified essentially according to the method of Waxman and Krebs (33). Rat leg muscle was homogenized with a Waring blender in 4 vol (vol/wt) of buffer containing 20 mM Tris·HCl (pH 7.2), 10 mM KCl, 0.5 mM dithiothreitol, and 5 mM EDTA. After centrifugation at 18,000 × *g* for 60 min, the supernatant was applied to a DEAE-Sephacel column (2.5 × 7 cm) equilibrated with the above buffer but containing 0.5 mM EDTA and the column was washed with 3 vol of buffer. Ca^{2+}-activated protease was recovered at 250 mM KCl and concentrated with 60% saturated ammonium sulfate. The precipitated protein was dialyzed against buffer containing 0.1 mM EDTA and stored at −80°C. The specific activity of the preparation used was 60 units/mg of protein (see units below) and the K_m for Ca^{2+} was ≈3 μM.

Preparation of [^{14}C]Methylated, *S*-Carboxymethylated Bovine Serum Albumin. Bovine serum albumin (10 mg/ml) was methylated with 20 mM sodium $CNBH_4$ and [^{14}C]HCHO (125 Ci/12.5 μmol; 1 Ci = 37 GBq) as described by Jentoft and Dearborn (34). After incubation for 1 hr at room temperature, the labeled bovine serum albumin was isolated after separation on Sephadex G-25 (0.7 × 15 cm). The specific activity was 1.0 × 10^4 cpm/μg of protein. [^{14}C]Methylated bovine serum albumin was then reduced and *S*-carboxymethylated by the method described by Crestfield *et al.* (35).

Protease and Inhibitor Assays. The proteolytic activity was measured as the acid-soluble radioactivity released from [^{14}C]methylated, *S*-carboxymethylated bovine serum albumin (labeled substrate). The reactions were carried out in a total volume of 50 μl containing 5 μmol of Tris·HCl (pH 8), 0.25 μmol of $MgCl_2$, 0.025 μmol of dithiothreitol, 5 μmol of KCl, 2 μg of labeled substrate (20,000 cpm), and enzyme fraction. Trichloroacetic acid (5%) and carrier bovine serum albumin (final concentration, 4 mg/ml) were added after 30 min of incubation at 37°C. Following centrifugation, radioactivity in aliquots of the supernatant was counted. One unit of enzyme was expressed as 1 μg of labeled substrate degraded after 30 min at 37°C (50% degradation in the above assay).

Ca^{2+}-activated protease activity was measured as above except that the reaction mixture contained 0.25 μmol of $CaCl_2$ replacing $MgCl_2$.

To measure inhibitory activity, aliquots of the protease (2.8 or 6.5 μg of protein, respectively, of HMP or Ca^{2+}-activated protease) and of inhibitor fraction were added in the assay mixture above but minus the labeled substrate. After 30 min in ice, substrate was added and the reaction was carried out as above. One unit of inhibitor was expressed as 50% inhibition of protease activities in the above assay.

Polyacrylamide Gel Electrophoresis (PAGE). PAGE was carried out basically as described by Laemmli (36). Native PAGE was performed at 4°C without $NaDodSO_4$. After electrophoresis for 7 hr at 20 mA, gels were cut into 2-mm slices, which were soaked in 200 μl of buffer A containing 0.1 M KCl overnight at 4°C to elute inhibitor from gel. The inhibitor, in aliquots of eluate, was measured as described above.

RESULTS

Purification of the Endogenous Inhibitor. Our early attempts to purify the inhibitor indicated that this factor was highly labile and heterogenous on ion-exchange or molecular sieving chromatography. It was found that inclusion of the protease inhibitors *p*-chloromercuribenzoate and phenylmethylsulfonyl fluoride in the initial lysate permitted purification of a stable inhibitor: The 100,000 × *g* supernatant fraction from human erythrocyte lysate was adsorbed to DEAE-Sephacel as described in *Methods*. Protein eluted at 0.3 M KCl and was again adsorbed on DEAE-Sephacel. Protein was eluted with a 0–0.25 M KCl gradient and inhibitor activity was assayed against HMP isolated from reticulocytes (Fig. 1). Although some inhibitor activity was detected across the gradient, the most prominent peak eluted at ≈0.2 M KCl.

We tested this crude inhibitor against several other proteases, including papain, trypsin, chymotrypsin, and calpain. Only inhibition of calpain was detected; thus, subsequent purification of the inhibitor to HMP involved parallel assay of inhibitor against calpain as well as HMP. Fractions (56–68) constituting the main peak from the DEAE- Sephacel column (Fig. 1) were pooled and chromatographed on a Bio-Gel A-0.5m column (Fig. 2). Inhibitor against HMP was recovered in a symmetrical peak corresponding to a M_r of

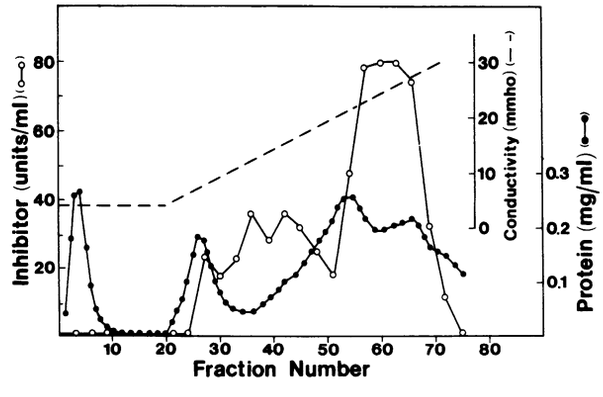

FIG. 1. DEAE-Sephacel chromatography of inhibitor. Batchwise (0.25 M KCl) eluate from DEAE-Sephacel obtained from the lysate of human erythrocytes (40 ml) was reapplied on the same column (2.5 × 7 cm) and eluted with a linear gradient of 0–0.25 M KCl in 300 ml of buffer A. The inhibitor activity was measured with 20 μl of each fraction against 0.5 unit of HMP.

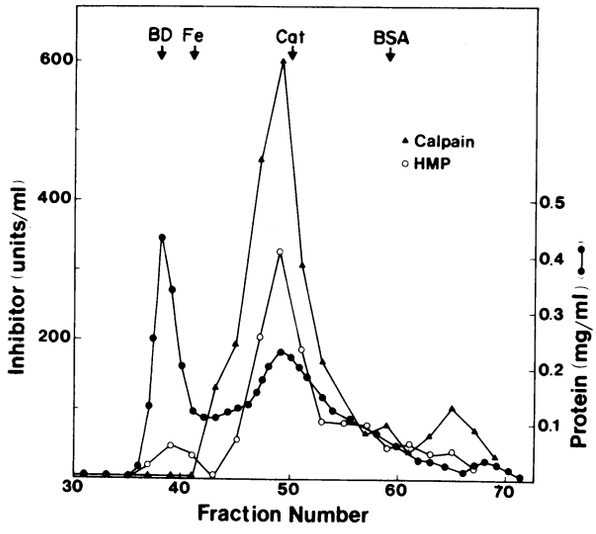

FIG. 2. Gel-filtration chromatography of inhibitor. Inhibitor fractions from the DEAE-Sephacel column were concentrated and chromatographed on a Bio-Gel A-0.5m column (1.5 × 100 cm) in buffer A containing 0.1 M KCl. Inhibitor activities were measured with 5- and 2.5-μl aliquots of fractions against 0.5 unit each of HMP (○) and calpain (▲), respectively. Arrows indicate elution positions of blue dextran (BD), ferritin, M_r 450,000 (Fe), catalase, M_r 240,000 (Cat), and bovine serum albumin, M_r 68,000 (BSA).

240,000. Furthermore, inhibition of calpain occurred in precisely the same peak (Fig. 2).

The active peak from the Bio-Gel column was analyzed by native PAGE (Fig. 3). The inhibitor preparation had a single major band that accounted for >90% of the protein that stained with Coomassie blue (Fig. 3*A*). This major band contained most of the inhibitory activity, as measured in the eluates from slices of an unstained gel run in parallel (Fig. 3*B*). Again, the peak of inhibitor activity against HMP coincided very closely with that for calpain (Fig. 3*B*).

To determine the subunit structure of the inhibitor, the major protein eluted from the native gel (Fig. 3) was analyzed

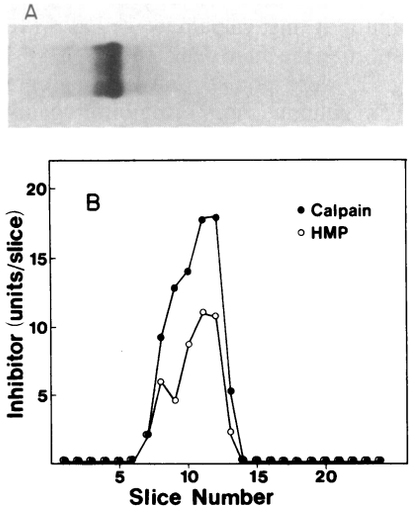

FIG. 3. Native PAGE of inhibitor. Inhibitor purified through a Bio-Gel A-0.5m column was analyzed on a 7.5% polyacrylamide gel without $NaDodSO_4$ according to the method of Laemmli (36). (*A*) Gel with 20 μg of protein stained with Coomassie blue. (*B*) Gel (unstained) with 45 μg of protein cut into 2-mm slices and soaked in 200 μl of buffer A containing 0.1 M KCl. Inhibitor activities were assayed against 0.5 unit each of HMP (○) and calpain (●) with 25 or 10 μl of eluate, respectively.

by $NaDodSO_4$/PAGE (Fig. 4). The inhibitor was homogenous, containing a single polypeptide of M_r 40,000 (Fig. 4). Thus, the native M_r 240,000 inhibitor appears to be a hexamer consisting of six M_r 40,000 subunits. In the presence of phenylmethylsulfonyl fluoride and *p*-chloromercuribenzoate, 1.9 mg of inhibitor was obtained from 100 ml of blood (40 ml of packed cells) with a recovery of 11%. Thus, it is estimated that erythrocytes contain 0.43 mg/ml, which is equivalent to 1.7 μM based on a M_r of 240,000.

Lysates from rabbit reticulocytes and erythrocytes contained an inhibitor that displayed the same properties on ionic-exchange and sizing columns as those described above (data not shown).

Mode of Inhibition. As was mentioned above, purified inhibitor was effective in inhibiting HMP and calpain. Therefore, we examined more carefully the sensitivity of HMP and calpain to the purified inhibitor. The M_r 240,000 inhibitor completely inhibited HMP and calpain (Fig. 5). Calpain was more sensitive to inhibitor since HMP showed a K_i of 8.3×10^{-8} M, approximately three times higher than that for calpain (2.8×10^{-8} M).

To ascertain the mechanism of inhibition, activity was measured at different concentrations (Fig. 6). Lineweaver–Burk plots indicate a noncompetitive mode of inhibition with a reduction in V_{max} and no change in K_m. (The K_m for bovine serum albumin as a substrate against HMP was 3 μM.)

Sensitivities of the Inhibitor to Various Factors. The sensitivities of the inhibitor to trypsin, RNase, and DNase were examined (Table 1). Loss of activity was only seen after tryptic digestion, indicating that the inhibitor is a protein and that no essential nucleic acid component is involved. Sensitivity to trypsin activity is not surprising since the presence of protease inhibitors during the initial isolation step of the inhibitor was important to prevent breakdown to smaller components as discussed above.

Sensitivity to heating was also examined (Table 1). Inhibitor activity against HMP was lost at 50°C for 10 min. In contrast, inhibitory activity assayed against calpain was relatively heat-stable, even at 70°C.

DISCUSSION

An endogenous inhibitor of HMP was purified from human erythrocytes and partially characterized. A similar inhibitor was also found in rabbit erythrocytes and reticulocytes (not shown). The inhibitor has a native M_r of 240,000 and is composed of a single subunit of M_r 40,000. It was also a potent inhibitor of calcium-dependent protease (calpain). Evidence that the same polypeptide is responsible for inhibition of both proteases is based on the parallel elution profile of active inhibitor against HMP and calpain after native PAGE. Furthermore, the major portion of the eluted peak displayed only one band on $NaDodSO_4$/PAGE. Although the same species of inhibitor appears to inhibit both proteases, heat treatment did not markedly affect its ability to inhibit calpain, whereas it caused a loss of inhibition against HMP. Thus, it is possible that different domains on the inhibitor are involved in its action against the two proteases.

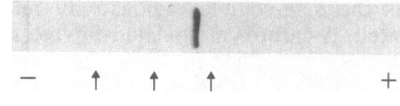

FIG. 4. $NaDodSO_4$/PAGE of inhibitor. Inhibitor was eluted from a native gel (20 μg of protein) run in parallel with the gels in Fig. 3. The major protein band was analyzed by 10% $NaDodSO_4$/PAGE as described (36). The arrows indicate mobilities of standard proteins in order (left to right): phosphorylase A (M_r 97,000), glutamate dehydrogenase (M_r 55,000), and lactic dehydrogenase (M_r 36,000).

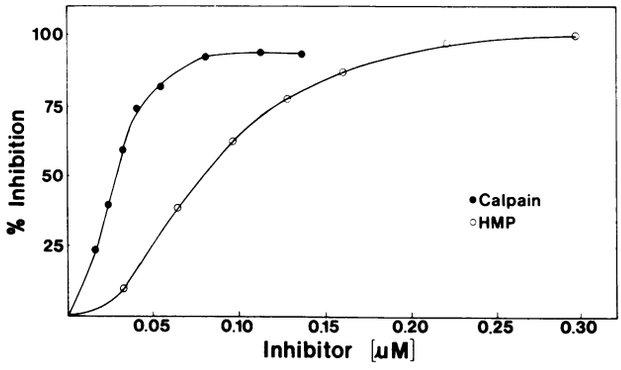

FIG. 5. Effect of inhibitor concentration on activity of HMP and calpain. Inhibitory activities against 0.5 unit each of HMP (○) and calpain (●) were measured as a function of increasing amounts of inhibitor.

Although the purified inhibitor reported here is able to completely block HMP and calpain, it had no effect on several other proteases, including trypsin, chymotrypsin, and papain (not shown). Although HMP as well as calpain appear to have essential thiol groups, the endogenous inhibitor does not simply act as a thiol protease inhibitor since papain was unaffected. Thus, the inhibitor appears to selectively inhibit the two major types of cytosolic proteases rather than a particular group of protease classified according to active site characteristics.

There have been many studies describing endogenous macromolecular inhibitors of the calpains. In general, these inhibitors display heat stability, sensitivity to proteolytic digestion, specificity for calpain, and noncompetitive kinetics (28, 29, 33, 37, 38). These properties resemble those seen in the present study. In contrast, the molecular weights reported previously show little or no agreement with each other or with the size estimates reported here. In general, multimeric inhibitors with subunits ranging from M_r 34,000 to 170,000 have been described (29, 37, 39, 40). Such discrepancies in molecular size could be due to the high susceptibility of the inhibitor(s) to proteolytic digestion (29, 33, 37). Despite the similarity in a number of properties, as discussed above, it remains to be determined conclusively if our inhibitor is related to a previously described calpain inhibitor.

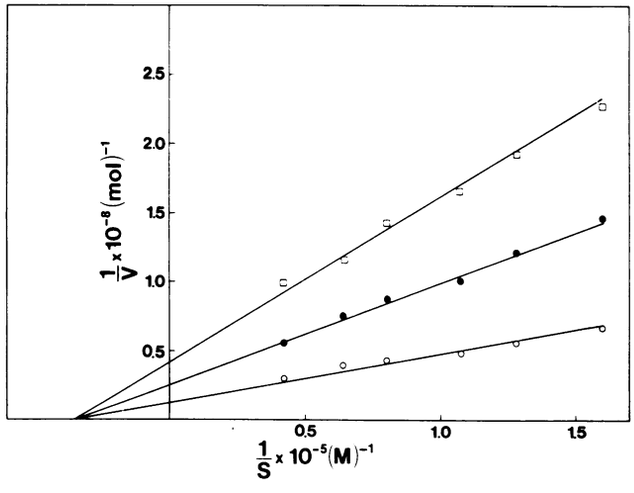

FIG. 6. Kinetics of inhibition of HMP by inhibitor. Hydrolysis was analyzed by a double reciprocal plot as a function of substrate concentration at different inhibitor levels. The reaction mixture (100 μl) contained 10 μmol of Tris·HCl (pH 8.0), 0.05 μmol of dithiothreitol, 1 unit of HMP, inhibitor at 0 μg (○), 1 μg (●), or 2 μg (□), and varying amounts of [^{14}C]methylated, S-carboxymethylated bovine serum albumin (50 cpm/pmol). The rate of hydrolysis was measured as mol of labeled substrate hydrolyzed during 20 min at 37°C.

Table 1. Effect of digestion and elevated temperature on inhibitor stability

Experiment	Treatment	Loss of inhibition: K_i, μg/ml
1 (HMP)	No addition	144
	Trypsin	448
	RNase	144
	DNase	152
2 (HMP)	0°C	20
	50°C	30
	70°C	50
3 (calpain)	0°C	6.6
	50°C	5.8
	70°C	4.6

For experiment 1, inhibitor was pretreated with trypsin, RNase, or DNase at 5 μg/ml for 2 hr at 25°C. Trypsin digestion was terminated by addition of trypsin inhibitor (10 μg/ml). For experiments 2 and 3, inhibitor was pretreated at the indicated temperature for 10 min. After treatment, the K_i for inhibition was determined by assaying [^{14}C]methylated, S-carboxymethylated bovine serum albumin proteolysis in the presence of varying inhibitor concentration. Assays contained 0.5 unit of HMP or calpain. Experiment 1 was carried out with an inhibitor preparation of lower specific activity.

Earlier studies from our laboratory reported the separation of the reticulocyte lysate into a crude inhibitor fraction precipitated by 30% ammonium sulfate and a fraction containing HMP (32). Both fractions, in addition to ubiquitin, were required for an ATP stimulation of proteolytic activity, suggesting that ATP and ubiquitin act to derepress protease activity. Eytan and Hershko questioned the significance of our earlier report since they claimed that only competitive substrate could account for the inhibition (41). However, the present study clearly demonstrates that the purified inhibitor is, in fact, a noncompetitive inhibitor. Thus, in the studies of Eytan and Hershko, it seems likely that the inhibitor was destroyed by proteolysis since they did not undertake measures to stabilize this factor. Furthermore, they argued that the ability of the 0–30% ammonium sulfate fraction to confer ATP dependency was due to the presence of a positive ATP-stabilized factor also present in this fraction (41). This latter conclusion was based on the inability of their HMP-containing fraction to significantly degrade bovine serum albumin without additional components of the ATP-ubiquitin system. However, this observation is not surprising since significant residual inhibitor to the bovine serum albumin-degrading HMP remains in the 30–75% ammonium sulfate fraction (K.M., unpublished observation). In contrast, the purified HMP used in the present study readily degraded bovine serum albumin in the absence of ATP and other factors.

Attempts to extensively purify proteases that could participate in the ATP-ubiquitin pathway have resulted in identification of enzymes that are generally active in the absence of ATP and ubiquitin (16–21). In contrast, partially purified activities are stimulated to varying degrees by ATP. Recently, limited purification of a protease activity that was stimulated by ATP was reported (42). However, this activity was labile and resisted further purification. It is of interest that when ATP-dependent protease was not detected, a large protease possessing other characteristics of ATP-dependent proteolysis was seen (42). Such observations are consistent with an ATP-dependent system composed of multiple components, including proteolytic and inhibitory factors. Modification or removal of components during fractionation or handling of cell extracts appears able to produce an active

protease capable of degrading substrates in the absence of ATP and ubiquitin.

Like other cellular proteases, including cathepsins B, H, and L and the calpains (37–40, 43–47), it is now clear that a macromolecular inhibitor against the HMP(s) is also present in cells. Crude inhibitor and protease fractions utilized in our previous studies also contained the various enzymes involved in ubiquitin conjugation, permitting the demonstration of ATP-dependent derepression of proteolysis in the presence of ubiquitin (32). In light of the present findings, it is proposed that both major classes of cytosolic proteases, HMP and calpains, which are complexed to endogenous inhibitors, are derepressed by an ATP and ubiquitin-dependent mechanism. However, the precise role of ubiquitin conjugation in this process remains to be clearly defined.

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