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RELEVANCE OF PROTEASE "INHIBITOR" TO THE ATP-UBIQUITIN PROTEOLYTIC SYSTEM

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Summary: ATP-dependent proteolysis in reticulocyte extracts is stimulated by ubiquitin, a polypeptide which is covalently conjugated to proteins. It has been proposed that ATP and ubiquitin act by repressing an inhibitor of an ATP-independent protease, rather than by conjugation to substrate proteins [Speiser, S. and Etlinger, J.D. (1983) Proc. Natl. Acad. Sci. U.S.A. 80, 3577-3580]. We find that the inhibitor preparation used by these authors contains a positively required factor of the ATP-ubiquitin proteolytic system, which can be separated from two types of protease inhibitors by gel filtration chromatography. The following observations indicate that the "inhibitors" are endogenous protease substrates which compete with the labeled substrate: (a) inhibition is competitive with exogenous substrate; (b) inhibition is abolished by a preincubation of "inhibitor" with protease prior to the addition of labeled substrate. These findings are not consistent with the notion that the inhibitors play a regulatory role in the ATP-ubiquitin proteolytic pathway.

Recent studies indicate that the conjugation of the polypeptide ubiquitin with proteins is involved in intracellular protein breakdown (for reviews, see refs. 1,2). The following lines of evidence support this conclusion: (a) Ubiquitin stimulates protein breakdown in an ATP-dependent cell-free system from reticulocytes (3,4). (b) The three enzymes involved in the conjugation of ubiquitin with proteins are all required for protein degradation (5). (c) In a mutant cell line which has a temperature-sensitive ubiquitin-activating enzyme, the degradation of short-lived proteins is arrested at the nonpermissive temperature (6,7). (d) There is a direct correlation between the rates of degradation of abnormal proteins and the levels of ubiquitin-protein conjugates in various cell types (8,9). (e) Reticulocyte extracts contain an enzyme system which degrades protein conjugated to ubiquitin, but not unconjugated protein (10). Evidence types a-c establish a relationship between ubiquitin conjugation and protein breakdown, but do not necessarily indicate that conjugation to the substrate protein is obligatory. On the other hand, evidence

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types d-e suggest that ubiquitin-protein conjugates are intermediates in protein breakdown. This is in accordance with an earlier proposal that conjugation with ubiquitin is the initial signal event in the degradation of the protein substrate (11).

In contrast to the above hypothesis, Speiser and Etlinger (12) have recently proposed that ATP and ubiquitin act by repressing an endogenous inhibitor of an ATP-independent protease. The present study examines the experimental evidence upon which this suggestion was based.

METHODS

Preparation of enzyme fractions. Fraction II (a ubiquitin-free, crude enzyme fraction) was prepared from lysates of rabbit reticulocytes by fractionation on DEAE-cellulose, as described (3,5). Fraction II was subjected to affinity chromatography on ubiquitin-Sepharose as described previously (5), except that the ubiquitin-conjugating enzymes E_1, E_2 and E_3 were eluted together with 6 column volumes of 50 mM Tris-HCl (pH 9.0) containing 5 mM dithiothreitol. The unadsorbed fraction of the affinity column was separated by ammonium sulfate fractionation to Fraction A (0-38% saturation) and Fraction B (42-75% saturation), as described earlier for Fraction II (13).

Partial purification of the ATP-independent protease. 45 mg protein of Fraction B were separated on a 1.5 x 85-cm column of Sepharose-6B in a buffer consisting of 20 mM Tris-HCl (pH 7.2) and 1 mM dithiothreitol. Fractions of 3 ml were collected, and samples of 5 µl of the column fractions were assayed for the breakdown of ^{125}I-lysozyme (see below). The protease eluted as a single peak at around 43-49 ml elution volume (molecular weight ≈500,000). The activity of this preparation was 56 ng of ^{125}I-lysozyme degraded/µg of protein/hour, under the assay conditions described below.

Assay of ATP-dependent protein breakdown. The reaction mixture contained in a final volume of 25 µl: 50 mM Tris-HCl (pH 7.6), 5 mM $MgCl_2$, 3 mM dithiothreitol, 1 mM ATP, 10 mM creatine phosphate, 5 µg creatine phosphokinase, 3 µg ubiquitin, 2.3 µg of the affinity-purified preparation containing E_1 + E_2+ E_3, 1 µg (∿ 10^5 cpm) of ^{125}I-labeled bovine serum albumin (^{125}I-albumin) or ^{125}I-labeled hen egg white lysozyme (^{125}I-lysozyme), and other enzyme fractions as specified. Following incubation at 37°C for 1 hr (^{125}I-lysozyme) or 2 hr (^{125}I-albumin), the release of material soluble in 5% trichloroacetic acid was determined as described earlier (5).

Assay of ATP-independent protease activity. The reaction mixture contained in a volume of 50 µl: 50 mM Tris-HCl (pH 7.6), 3 mM dithiothreitol, 5 mM $MgCl_2$, 1 µg ^{125}I-lysozyme (5-20 x 10^4 cpm), and enzyme as indicated. Following incubation at 37°C for 1 hr, the release of trichloroacetic acid-soluble products was determined.

RESULTS AND DISCUSSION

Examination of protease-inhibitor proposal with different substrates. The proposal that ATP and ubiquitin stimulate proteolysis by repressing an endogenous protease inhibitor was based on the following observations (12): Reticulo-

locyte extracts were fractionated with ammonium sulfate into two fractions. The fraction precipitated with high concentration of ammonium sulfate contained a protease which hydrolyzed ^{14}C-methyl-α-casein in the absence of ATP ("protease" fraction), and this was inhibited by the fraction precipitated with low concentration of ammonium sulfate ("inhibitor" fraction). ATP and ubiquitin stimulated proteolysis only when the two fractions were combined (12).

It should be noted that this fractionation procedure is essentially similar to that described in an earlier work from our laboratory (13), in which the results were interpreted that the ATP-dependent proteolytic system is composed of multiple, positively required components. A difference in experimental conditions is that we used ^{125}I-albumin as the substrate, which is attacked only slightly by ATP-independent protease(s) present in reticulocyte extracts. In the experiment shown in Table 1, the degradation of ^{125}I-albumin was compared to that of ^{125}I-lysozyme in fractions of reticulocyte extract. With ^{125}I-lysozyme, the pattern resembles that observed by Speiser and Etlinger (12), i.e., a high rate of ATP-independent proteolysis in Fraction B (40-75% saturation ammonium sulfate cut), its inhibition by Fraction A (0-38% satura-

Table 1. Activities of protease, inhibitor, and complementary factors of the ATP-dependent proteolytic system in fractions of reticulocyte extracts

Fraction	Acid-soluble (%)			
	^{125}I-lysozyme		^{125}I-albumin	
	-ATP	+ATP	-ATP	+ATP
Unfractionated	2.4	28.3	0.4	38.6
Fraction A ("inhibitor")	1.9	1.0	0.4	0.7
Fraction B ("protease")	19.5	25.4	2.4	4.5
Fraction A + Fraction B	2.9	23.0	0.2	22.0

Reaction conditions are described under "Methods." Where indicated, ATP was supplemented together with creatine phosphate, creatine kinase, ubiquitin, and the preparation containing E_1 + E_2 + E_3. The various fractions were supplemented at the following amounts (μg of protein): unfractionated affinity-unadsorbed fraction, 160; Fraction A, 84.3; Fraction B, 41.3.

tion ammonium sulfate cut), and requirement for both fractions for the restoration of ATP-dependent proteolysis. On the other hand, ^{125}I-albumin was not degraded much by the ATP-independent protease in Fraction B, but its ATP-dependent degradation still required the supplementation of both Fractions A and B. Since the rate of the ATP-dependent degradation of ^{125}I-albumin in the reconstituted system is much higher than that obtained with the "protease" fraction, it seems that in this case, at least, the complementation of activities is unrelated to the release of the protease from its inhibitor.

More recently, we have isolated three enzymes of the ATP-ubiquitin system by affinity chromatography on ubiquitin-Sepharose: a ubiquitin-activating enzyme (E_1) and two further enzymes, designated E_2 and E_3, which are required for ubiquitin conjugation and for protein breakdown (5,14). The fraction of reticulocyte extract not adsorbed to the ubiquitin column ("affinity-unadsorbed fraction") contains enzymes which degrade ubiquitin-protein conjugates (10). It is possible that ammonium sulfate fractionation separates the ubiquitin-conjugating enzymes (E_1, E_2, and E_3) from each other, which would result in the observed complementation. Therefore, we used the affinity-unadsorbed fraction to prepare Fractions A and B, and assayed ATP-dependent proteolysis with excess of affinity-purified E_1 + E_2 + E_3 (Table 1). Without E_1 + E_2 + E_3 or without ubiquitin, ATP did not stimulate the breakdown of either ^{125}I-albumin or ^{125}I-lysozyme to a significant extent (data not shown).

Separation of protease inhibitors from a factor of the ATP-dependent proteolytic system. We have shown earlier that Fraction A contains a high-molecular-weight factor which is stabilized by ATP against heat inactivation (13). To examine the possible relationship of this factor to the protease inhibitor, Fraction A was separated on a Sepharose-6B column, and activities to stimulate ATP-dependent protein breakdown and to inhibit the activity of the ATP-independent protease were determined in fractions of the same column (Fig. 1). The factor eluted at an apparent molecular weight of approximately 600,000 daltons. This was separated from two peaks of protease inhibitor: a sharp peak eluted at the void volume (>10^6 daltons) and a broad peak in the region

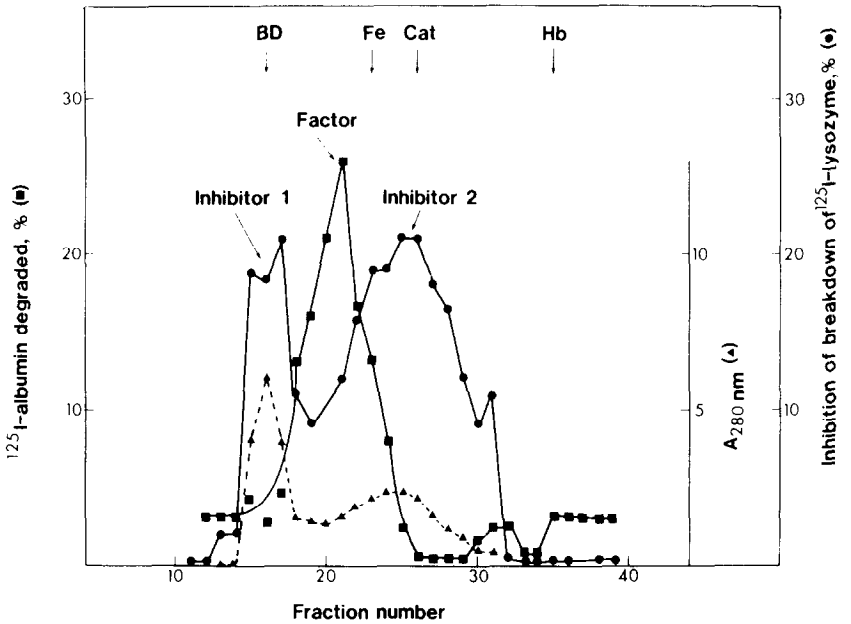

Fig. 1. Gel filtration chromatography of protease inhibitors and of a factor of the ATP-dependent system in Fraction A. Fraction A (99.8 mg of protein) was applied to a column (1.5 × 85 cm) of Sepharose-6B equilibrated with 20 mM Tris-HCl (pH 7.2), 1 mM dithiotreitol, and 1 mM ATP. Elution was with the above buffer, and fractions of 3.0 ml were collected at 4°C. The activity of the factor required for the ATP-dependent degradation of ^{125}I-albumin (■—■) was determined in fraction samples of 10 µl as described under "Methods," in the presence of 49.5 µg protein of Fraction B. The inhibition of the ATP-independent proteolysis of ^{125}I-lysozyme (●—●) was determined in fraction samples of 5 µl, in the presence of 5.5 µg protein of partially purified protease. The proteolysis of ^{125}I-lysozyme without inhibitor was 31%/hr, and the results are expressed as the decrease in the percentage of ^{125}I-lysozyme converted to acid-soluble material. Markers (arrows): BD, Blue Dextran (M_r > 2 × 10^6); Fe, ferritin (M_r = 480,000); Cat, catalase (M_r = 240,000); Hb, hemoglobin (M_r = 64,000).

of 150,000-500,000 daltons. The two peaks of protease inhibitor coincided with the bulk of total eluted protein.

Identification of "inhibitors" as endogenous protease substrates. The observation that the inhibitors eluted together with most of the total protein in the gel filtration column (Fig. 1) raised the question as to whether these are endogenous protein substrates which compete with the exogenous labeled protein substrate. To test this possibility, partially purified protease (prepared as described under "Methods") was incubated with increasing concentrations of ^{125}I-lysozyme, in the presence or absence of the two inhibitors (collected from the peak fractions shown in Fig. 1). As shown in Fig. 2, inhibition with both inhibitors was clearly competitive, since the extent of inhibition de-

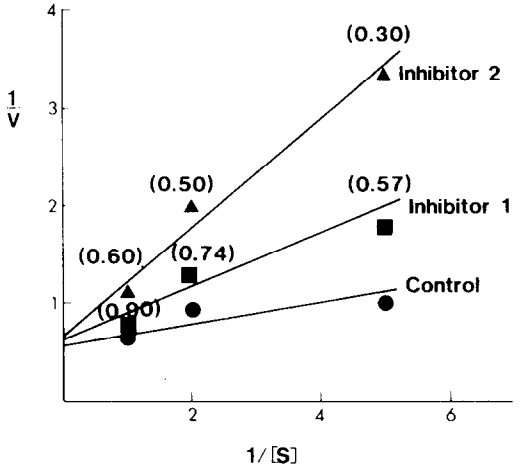

Fig. 2. Competition of inhibitors with substrate. ^{125}I-lysozyme at concentrations varying from 0.2 to 1 mg/ml was incubated with partially purified protease (5.5 µg of protein) without inhibitors ("Control," ●—●) or in the presence of 7.9 µg of inhibitor 1 (■—■) or 4.4 µg of inhibitor 2 (▲—▲) from the preparation described in Fig. 1. V is expressed as µg lysozyme degraded/hr under the reaction conditions employed, while S is the concentration of lysozyme (mg/ml). The numbers in parentheses are the fraction of activity with inhibitor relative to the activity without inhibitor at a similar concentration of lysozyme.

creased markedly with increasing concentrations of ^{125}I-lysozyme. Similarly, inhibition was abolished with increasing concentrations of [^{3}H]-globin (not shown).

The conclusion that the "inhibitors" are, in fact, endogenous proteins which are substrates for the ATP-independent protease was further corroborated by the following experiment: "Inhibitor" was preincubated at 37°C with partially purified protease for various periods of time before the addition of ^{125}I-lysozyme. As shown in Fig. 3, inhibition of the proteolysis of ^{125}I-lysozyme was progressively abolished with increasing times of preincubation. This was presumably due to the proteolysis of the "inhibitor" by the protease and not due to the lability of the inhibitor, since when the inhibitor was preincubated alone and then protease was added together with ^{125}I-lysozyme, inhibition was not abolished (Fig. 3). When the protease was preincubated without the inhibitor, proteolysis even decreased. This is due to the partial inactivation of the protease at 37°C, a property which resembles that of a neutral protease described in rat liver (15).

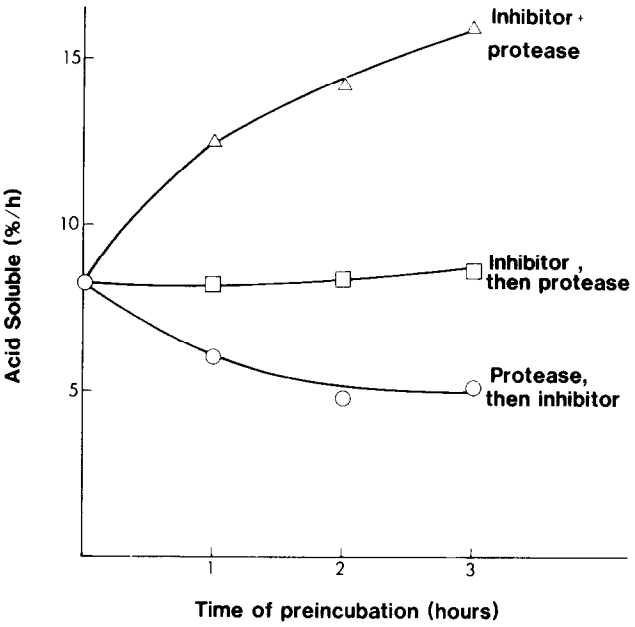

Fig. 3. Preincubation of inhibitor with protease abolishes inhibition. Inhibitor 2 (7.3 µg of protein) from the preparation described in Fig. 1 was preincubated with 5.5 µg of partially purified protease in a reaction volume of 50 µl containing 50 mM Tris-HCl (ph 7.6), 3 mM dithiotreitol, and 5 mM $MgCl_2$. Following preincubation at 37°C for the time periods indicated, ^{125}I-lysozyme (1 µg) was added, and the release of acid-soluble radioactivity was determined after a further incubation of 1 hr ("inhibitor + protease," Δ—Δ). In parallel incubations, inhibitor was preincubated without protease, and then ^{125}I-lysozyme was added with untreated protease ("inhibitor, then protease," □—□); or protease was preincubated without inhibitor, and then ^{125}I-lysozyme was added with untreated inhibitor ("protease, then inhibitor," o—o).

The above experiments do not support the suggestion (12) that the protease "inhibitor" has a regulatory function in ATP-dependent protein breakdown. It remains to be seen whether the protease has any role in the ubiquitin proteolytic pathway. In preliminary experiments, we have separated from Fraction B of the affinity-unadsorbed fraction three factors which are required for ATP, ubiquitin-dependent proteolysis. The three factors are also required, together with the ATP-stabilized factor from Fraction A, for the degradation of ^{125}I-lysozyme-ubiquitin conjugates (10). None of these factors co-purifies with the ATP-independent protease (unpublished observations).

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