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ATP-dependent degradation of ubiquitin-protein conjugates

(protein turnover/energy requirement)

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ABSTRACT **Previous studies have indicated that the ATP-requiring conjugation of ubiquitin with proteins plays a role in the energy-dependent degradation of intracellular proteins. To examine whether such conjugates are indeed intermediates in protein breakdown, conjugates of ^{125}I-labeled lysozyme with ubiquitin were isolated and incubated with a fraction of reticulocyte extract that lacks the enzymes that carry out ubiquitin-protein conjugation. ATP markedly stimulated degradation of the lysozyme moiety of ubiquitin conjugates to products soluble in trichloroacetic acid. By contrast, free ^{125}I-labeled lysozyme was not degraded under these conditions, unless ubiquitin and the three enzymes required for ubiquitin conjugation were supplemented. Mg^{2+} was absolutely required for conjugate breakdown. Of various nucleotides, only CTP replaced ATP. Nonhydrolyzable analogs of ATP were not effective. In the absence of ATP, free lysozyme is released from ubiquitin-lysozyme conjugates by isopeptidases present in the extract. Thus, ATP is involved in both the formation and the breakdown of ubiquitin-protein conjugates.**

A small polypeptide, ubiquitin (Ub), is involved in the energy-dependent degradation of intracellular proteins. It has been observed to be required for the action of an ATP-dependent proteolytic system from reticulocytes (1) and shown to form covalent conjugates with proteins in an ATP-requiring process (2, 3). It was proposed that conjugation with Ub may mark proteins for their subsequent breakdown (reviewed in ref. 4). This suggestion is supported by observations that the rapid degradation of abnormal proteins in intact cells is accompanied by a marked increase in the levels of Ub-protein conjugates (5, 6).

The enzymatic reactions responsible for the formation of Ub-protein conjugates have been partially characterized. Three enzymes are involved in Ub-protein conjugation. Ub is first activated by adenylylation at its COOH-terminal glycine, catalyzed by a specific Ub-activating enzyme, E_1 (7–9). The activated polypeptide is then transferred to a second enzyme (E_2) to form an E_2-Ub thiolester intermediate, which seems to be the donor for conjugate formation in the presence of the third enzyme, E_3 (10). All three enzymes of the Ub-protein ligase system have been purified by affinity chromatography on Ub-Sepharose (10, 11) and have been shown to be required for ATP-dependent proteolysis (10).

Although the results establish a relationship between Ub conjugation and protein breakdown, they do not necessarily indicate that conjugation with the protein substrate is an obligatory event. It is possible, for example, that a protease is activated by Ub conjugation or that an inhibitor of a protease is neutralized by the conjugation process (12). It seems important, therefore, to define the pathways of the degradation of Ub-protein conjugates. At present, there is no information available on the fates of Ub-protein conjugates, except for an isopeptidase that cleaves the linkage between Ub and histone 2A, with the release of undegraded histone (13, 14). We now report that reticulocyte extracts contain an enzymatic system that degrades the protein moiety of Ub-protein conjugates to acid-soluble products and that this system requires ATP for activity.

MATERIALS AND METHODS

Crystalline hen egg white lysozyme (Worthington) was radioiodinated by the chloramine-T method, as described (3). Ub was purified by a modification (11) of a previously described procedure (15). All nucleotides and ATP analogs were obtained from Sigma, except for adenosine 5′-[β,γ-methylene]triphosphate, which was from Miles.

Preparation of Enzyme Fractions. Fraction II (a Ub-free crude enzyme fraction) was prepared from rabbit reticulocytes as described (1, 10). To separate the three enzymes of the Ub-protein ligase system, fraction II was subjected to affinity chromatography as described (10), except that elution at pH 9 was with 6 column vol of buffer; this yielded a better recovery of E_3. The unadsorbed fraction of the affinity column was used as the source of conjugate-degrading enzymes for the present study. To ensure complete removal of Ub-conjugating enzymes from this fraction, it was again passed through the Ub-Sepharose column, under conditions identical to the first application. To remove ATP and Mg^{2+} from this fraction, it was precipitated twice with a large volume of ammonium sulfate (80% saturation). The pellet was dissolved in a minimal volume of Tris·HCl, pH 7.2/1 mM dithiothreitol and was passed through Sephadex G-25 equilibrated with the above buffer. The protein concentration of this "affinity-unadsorbed" preparation was around 20 mg/ml. The preparation was stored at −80°C in small samples and was thawed only once.

Preparation of ^{125}I-Labeled Lysozyme-Ub Conjugates. The reaction mixture contained (final vol, 400 μl) 50 mM Tris·HCl (pH 7.6), 5 mM $MgCl_2$, 2 mM ATP, 30 mM 2-mercaptoethanol, 120 μg of ^{125}I-labeled lysozyme (^{125}I-lysozyme; $\approx 4 \times 10^7$ cpm), 60 μg of Ub, 1 μg of inorganic pyrophosphatase (Sigma), 75 μg of protein from the AMP/PP_i affinity eluate (containing E_1), 160 μg of protein from the dithiothreitol-containing eluate (containing E_2 and residual E_1), and 2.5 mg of protein from the pH 9 eluate (containing E_3 and E_2; see ref. 10). After incubation at 37°C for 30 min, the reaction was stopped by the addition of 100 μl of concentrated formic acid. The high concentration of 2-mercaptoethanol was required for optimal conjugation of lysozyme with Ub. However, reduced lysozyme forms aggregates and treatment with acid was required to dissociate most aggregates. After further incubation at 37°C for 30 min, the sample was loaded on a column (1 × 50 cm) of Sephadex G-150 (medium) equilibrated with 5% (vol/vol) formic acid and bovine serum albumin at 0.1 mg/ml. Fractions of 0.8 ml were collected at room temperature. The elution profile of radioactivity is shown in Fig. 1. The void volume (fraction A) con-

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Abbreviations: Ub, ubiquitin; ^{125}I-lysozyme and ^{125}I-Ub, ^{125}I-labeled lysozyme and Ub, respectively.

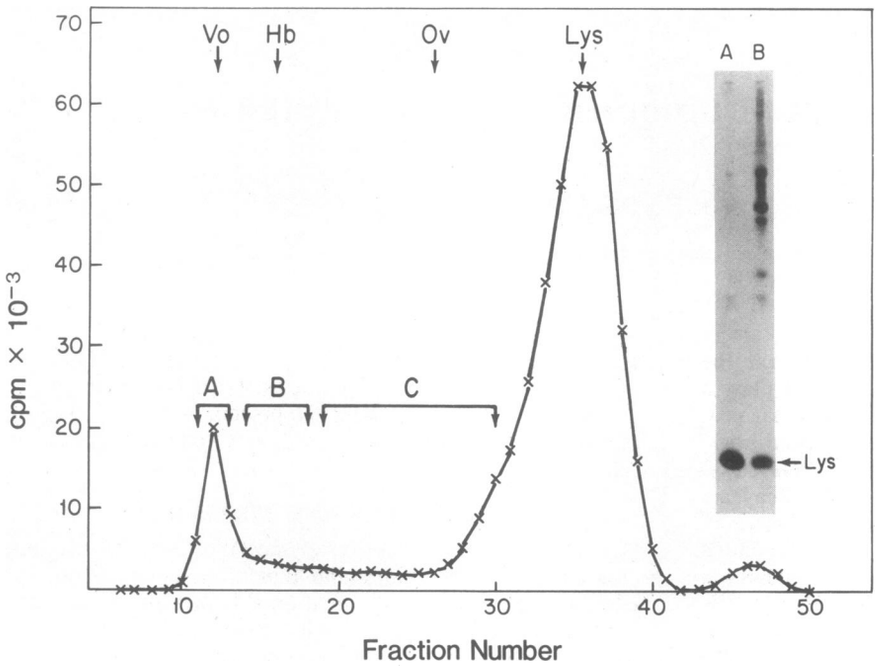

FIG. 1. Preparation of ^{125}I-lysozyme-Ub conjugates. Radioactivity was determined in 10-μl samples of column fractions. Markers were Hb (M_r = 64,000); Ov, ovalbumin (M_r = 43,000); Lys, lysozyme (M_r = 14,500). V_0, void volume. (*Inset*) Analysis of pooled fractions A and B. Electrophoresis was carried out on a 12.5% polyacrylamide/NaDodSO$_4$ gel as described (2). The samples were boiled for 5 min prior to electrophoresis in sample buffer containing 2% NaDodSO$_4$ and 3% 2-mercaptoethanol. Direction of migration is from top to bottom. For the estimation of apparent molecular weight of ^{125}I-lysozyme-Ub derivatives, see Fig. 2.

tained mainly aggregated lysozyme. The bulk of high molecular weight ^{125}I-lysozyme-Ub conjugates eluted in a shoulder that immediately followed the void volume (fraction B). The following fraction (C) contained low molecular weight (<32,000) conjugates and free lysozyme. These fractions were dialyzed overnight at +4°C against 2 liters of 50 mM Tris·HCl (pH 7.6). Dialysis was continued for a further 4–6 hr against 2 liters of water. The samples were lyophilized, dissolved in a small volume of water, and stored at +4°C in the presence of 0.02% NaN_3. The analysis of the conjugate preparation by NaDodSO$_4$/PAGE is shown in Fig. 1 *Inset*. Fraction A consisted mainly of free lysozyme and contained only a small amount of conjugate, while the contamination of free lysozyme in fraction B usually did not exceed 30% of total radioactivity. The majority of fraction B consisted of ^{125}I-lysozyme-Ub conjugates in the apparent molecular weight region of 32,000 to 65,000. In addition, numerous bands of ^{125}I-lysozyme-Ub conjugates with apparent molecular weights of >65,000 were present. These comprise about 20% of the total radioactivity of fraction B. The bands with apparent M_r >65,000 are observed when purified conjugate-forming enzymes are used for Ub conjugation and were not seen with a crude enzyme preparation (3). Since lysozyme has six lysine residues and the calculated M_r of Ub_6-lysozyme is 65,000, they might be due to anomalous migration of some classes of conjugates on PAGE. Alternatively, they may represent some as yet undefined chemical linkage, such as the conjugation of Ub to residues other than lysine, binding of Ub to another molecule of Ub already linked to lysozyme, or the linkage of both Ub and lysozyme to another protein. That all the high molecular weight derivatives of ^{125}I-lysozyme are conjugates with Ub is indicated by the observation that their formation required the presence of Ub conjugation enzymes, ATP, and Ub (data not shown). Unless otherwise indicated, fraction B from the Sephadex G-150 separation was used as the preparation of ^{125}I-lysozyme-Ub conjugates.

Assay of Breakdown of ^{125}I-Lysozyme-Ub Conjugates. Unless otherwise specified, the reaction mixture contained (final vol, 50 μl) 50 mM Tris·HCl (pH 7.6), 5 mM $MgCl_2$, 3 mM dithiothreitol, 4 mM ATP, ^{125}I-lysozyme-Ub conjugates (4–6 × 10^3 cpm), and 200 μg of protein from the affinity-unadsorbed fraction. When the degradation of free ^{125}I-lysozyme or of other fractions from the Sephadex G-150 separation (Fig. 1) was tested, these samples were supplemented at amounts similar to the lysozyme content of ^{125}I-lysozyme-Ub conjugates. After incubation at 37°C for 1 hr, the release of labeled material soluble in 5% trichloroacetic acid was estimated as described (10). The results are expressed as the percentage of total radioactive material converted to acid-soluble material.

RESULTS

The Breakdown of Ub-Protein Conjugates Is Stimulated by ATP. To examine the hypothesis that Ub-protein conjugates are intermediates in protein breakdown, conjugates of ^{125}I-lysozyme with unlabeled Ub were isolated (Fig. 1) and incubated with the part of reticulocyte Fraction II that does not bind to the Ub-Sepharose affinity column. This affinity-unadsorbed fraction is devoid of the three enzymes participating in Ub conjugation, but it is required for protein breakdown in the presence of the conjugate-forming enzymes (10). As shown in Table 1, ATP markedly stimulated the release of degradation products soluble in trichloroacetic acid from the fraction enriched with high molecular weight ^{125}I-lysozyme-Ub conjugates (fraction B). By contrast, there was much less ATP-dependent release of acid-soluble radioactivity from the fraction containing mainly aggregated lysozyme (fraction A) or from those enriched with low molecular weight ^{125}I-lysozyme-Ub conjugates (fraction C) and free lysozyme.

The Breakdown of ^{125}I-Lysozyme-Ub Conjugates Does Not Require Ub or Conjugate-Forming Enzymes. A trivial explanation for the influence of ATP on the breakdown of ^{125}I-lysozyme-Ub conjugates would be that ATP is required for Ub conjugation, assuming that significant amounts of conjugate-forming enzymes remained in the affinity-unadsorbed

Table 1. ATP-stimulated breakdown of Ub-protein conjugates

Fraction from Sephadex G-150 separation	Acid soluble (%/hr)		
	−ATP	+ATP	ATP-dependent
Aggregated lysozyme (A)	0.8	2.2	1.4
High molecular weight Ub-lysozyme conjugates (B)	5.2	20.7	15.5
Low molecular weight Ub-lysozyme conjugates (C)	5.9	7.8	1.9
Free lysozyme	4.8	5.1	0.3

Table 2. Different requirements for the degradation of free and Ub-conjugated lysozyme

Addition(s)	Acid soluble (%/hr)	
	^{125}I-lysozyme	^{125}I-lysozyme-Ub conjugates
None	4.5	7.2
ATP	6.1	20.7
ATP + Ub	6.2	19.6
ATP + E_1 + E_2 + E_3	8.9	22.0
ATP + Ub + E_1 + E_2 + E_3	18.8	27.1

Where indicated, additions were as follows: ATP, 4 mM; Ub, 1 μg; E_1 + E_2 + E_3, 4 μg of protein from the dithiothreitol-containing eluate combined with 10 μg of protein from the pH 9 eluate.

fraction. Free Ub, in turn, may be liberated in significant quantity by the action of isopeptidases on Ub-lysozyme conjugates (see below). In the experiment reported in Table 2, the requirements for the breakdown of ^{125}I-lysozyme-Ub conjugates were compared with those of free ^{125}I-lysozyme. The breakdown of conjugated lysozyme was nearly maximally stimulated by the addition of ATP alone, whereas the degradation of unconjugated ^{125}I-lysozyme also required Ub and the three conjugate-forming enzymes (E_1, E_2, and E_3). Without conjugate-forming enzymes (addition of ATP and Ub alone), the breakdown of free lysozyme was only slightly stimulated, indicating that the conjugate-forming enzymes are sufficiently removed from the affinity-unadsorbed fraction by the affinity chromatography procedure. Further control experiments showed that the breakdown of ^{125}I-lysozyme was not stimulated by the addition of the conjugate preparation, thus ruling out the unlikely possibility that the conjugate-forming enzymes, used for the preparation of conjugates, survived treatment by acid in the preparation procedure.

The conclusion that ATP stimulates the breakdown of Ub-protein conjugates by a mechanism unrelated to Ub conjugation was further borne out by experiments in which the effects of different inhibitors were compared (Table 3). The addition of a specific antibody directed against Ub markedly inhibited the breakdown of ^{125}I-lysozyme in the complete system but had no influence on the ATP-dependent degradation of ^{125}I-lysozyme-Ub conjugates. It should be noted that this antibody binds free Ub but not protein-conjugated Ub (5). Furthermore, the addition of a high concentration of unlabeled lysozyme decreased the release of acid-soluble radioactivity from ^{125}I-lysozyme (presumably by isotopic dilution) but had much less effect on the breakdown of ^{125}I-lysozyme-Ub conjugates. Some other inhibitors of ATP-dependent proteolysis such as hemin (16, 17), *o*-phenanthroline, and *N*-ethylmaleimide (18) inhibited the breakdown of both free and Ub-conjugated ^{125}I-lysozyme. These latter inhibitors may therefore inhibit sites common to both processes, though the possibility of their action at multiple sites should also be considered.

Table 4. Nucleotide specificity of the breakdown of ^{125}I-lysozyme-Ub conjugates

Addition(s)	Relative stimulation
ATP	1.00
ATP minus Mg^{2+}	0
ATP plus hexokinase* (1.5 μg) and deoxyglucose (10 mM)	0.02
Ado*P*[CH_2]*PP*	0.06
Ado*PP*[CH_2]*P*	0.13
Ado*PP*[NH]*P*	0.02
CTP	0.46
GTP	0.11
UTP	0.09

The degradation of ^{125}I-lysozyme-Ub conjugates was assayed as described in *Materials and Methods*, except that all nucleotides were supplemented at 2 mM. Results are expressed as fraction of ATP-stimulated breakdown; absolute values were (%/hr) no additions, 3.9; with ATP, 9.7. Ado*P*[CH_2]*PP*, Ado*PP*[CH_2]*P*, and Ado*PP*[NH]*P*; adenosine 5′-[α,β-methylene]triphosphate, 5′-[β,γ-methylene]triphosphate, and 5′-[β,γ-imido]triphosphate, respectively.

*From P-L Biochemicals (grade 300).

Specificity of Nucleotide Requirement. The effects of various nucleotides on the breakdown of ^{125}I-lysozyme-Ub conjugates are shown in Table 4. ATP was most effective, and Mg^{2+} was absolutely required. The conversion of ATP to ADP by hexokinase and 2-deoxyglucose completely abolished the stimulatory effect. Nonhydrolyzable ATP analogs, substituted at either the α-β or β-γ position, were not effective, suggesting that ATP hydrolysis may be required. Of the various nucleoside triphosphates tested, only CTP elicited a partial stimulation. HPLC analysis of the sample of CTP used showed no contamination by ATP, and the only detectable contaminant was CDP (data not shown). It should be noted that CTP does not promote the conjugation of Ub (7) or the breakdown of unconjugated proteins (19), indicating that CTP replaces ATP only at its second site of action.

Examination of the effect of ATP concentration showed a half-maximal stimulation at ≈0.2 mM, and maximal effect was obtained with 2–4 mM ATP. However, part of the ATP was hydrolyzed by the ATPases present in the crude affinity-unadsorbed fraction: in the presence of creatine phosphate (10 mM) and creatine phosphokinase (10 mg/ml), half-maxi-

Table 3. Effect of inhibitors on the breakdown of ^{125}I-lysozyme-Ub conjugates

Addition(s)	Acid soluble (%/hr)	
	^{125}I-lysozyme (+ Ub, E_1, E_2, and E_3)	^{125}I-lysozyme-Ub conjugates
None	1.8	6.1
ATP	19.7	27.9
ATP, plus anti-Ub	4.5	27.5
ATP, plus lysozyme (50 μg)	7.8	22.9
ATP, plus hemin (0.2 mM)	0	0.6
ATP, plus *o*-phenanthroline (5 mM)	0.9	5.8
ATP, plus *N*-ethylmaleimide (10 mM)	0	2.0

The degradation of ^{125}I-lysozyme-Ub conjugates was determined as described in *Materials and Methods* and that of free ^{125}I-lysozyme, under similar conditions, but with the addition of Ub, E_1, E_2, and E_3 as specified in Table 2. Anti-Ub is an antiserum raised against native Ub and purified by affinity chromatography as described in ref. 5. The antibody (160 μg) was incubated with the complete reaction mixture at 0°C for 15 min prior to the incubation at 37°C.

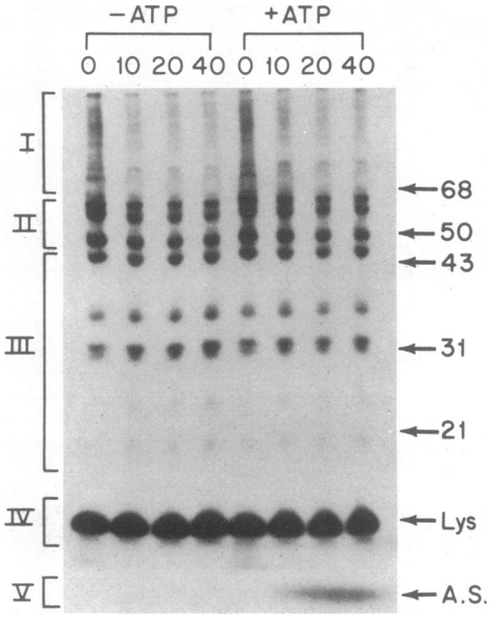

FIG. 2. Analysis of the breakdown of ^{125}I-lysozyme-Ub conjugates by NaDodSO$_4$/PAGE. Incubation conditions were similar to those described in *Materials and Methods*, except that the amount of labeled conjugates was increased 3-fold. The reaction was determined at the times indicated (min) by boiling with electrophoresis sample buffer. Electrophoresis on a 12.5% polyacrylamide/NaDodSO$_4$ gel was carried out as described (2), except that the gel was dried without fixation and staining prior to radioautography. Markers ($M_r \times 10^{-3}$) were 68, bovine serum albumin; 50, glutamate dehydrogenase; 43, ovalbumin; 31, carbonic anhydrase. Lys, lysozyme; A.S., acid soluble.

mal stimulation was attained by ≈30 μM ATP. Creatine phosphate alone had no significant effect (data not shown).

Alternative Pathways for the Breakdown of Ub-Protein Conjugates. Decay of Ub-protein conjugates [labeled with ^{125}I-labeled Ub (^{125}I-Ub)], with the release of free Ub, has been observed in reticulocyte extracts incubated in the absence of ATP (2, 3). To compare this process with the ATP-dependent breakdown of the protein moiety of the conjugates, ^{125}I-lysozyme-Ub conjugates were incubated with the affinity-unadsorbed fraction in the presence or absence of ATP, and the reaction products were analyzed by NaDodSO$_4$/PAGE (Fig. 2). The gel was radioautographed without staining or fixation, to prevent the loss of acid-soluble products. As shown, high molecular weight conjugates (>50,000) decayed rapidly both in the presence and in the absence of ATP. The decay of high molecular weight conjugates was accompanied by some increase in low molecular weight conjugates (20,000–46,000), which was more noticeable in the incubation without ATP. No degradation products could be observed that were not present in the original conjugate preparation, except for the acid-soluble small products accumulating in the presence of ATP. To analyze these changes more precisely, different regions of the gel were quantitated by γ counting (Fig. 3). The decline in the levels of high molecular weight conjugates (classes I and II) is rapid in the first 20 min and slows down afterward. This decay of high molecular weight conjugates seems to be somewhat faster in the absence of ATP than in its presence. The levels of low molecular weight conjugates (class III) increase significantly in the incubation without ATP. There was an even more pronounced increase in the region of free lysozyme, which was again more prominent in the absence of ATP. These results suggest that, in the absence of ATP, isopeptidase(s) strongly act on high molecular weight Ub-protein conjugates. This process releases undegraded lysozyme (and free Ub), presumably via the intermediary formation of low molecular weight conjugates. On the other hand, in the presence of ATP, the proteolytic system may act on the same high molecular weight conjugates and thus decreases the accumulation of isopeptidase products. That these two systems may compete on common Ub-protein conjugates is suggested by an experiment in which ^{125}I-lysozyme-Ub conjugates were first incubated with the affinity-unadsorbed fraction for 30 min and then with ATP. It was found that this first incubation in the absence of ATP nearly completely abolished the subsequent ATP-dependent release of acid-soluble radioactivity. This was presumably due to the breakdown of high molecular weight conjugates by isopeptidases and not to the inactivation of a necessary enzyme; when the extract was first incubated alone and then with ATP and ^{125}I-lysozyme-Ub conjugates together, there was no appreciable decrease of the release of acid-soluble products (data not shown).

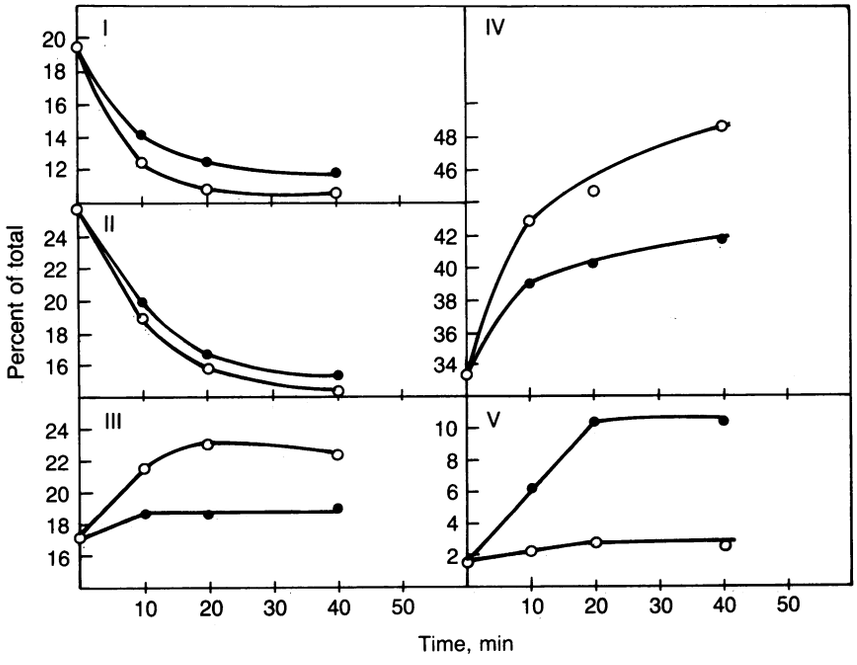

FIG. 3. Time course of breakdown of ^{125}I-lysozyme-Ub conjugates in the presence (●) and absence (○) of ATP. Each lane of the gel shown in Fig. 2 was cut into the zones indicated (*I*, M_r >65,000; *II*, M_r 50,000–65,000; *III*, M_r 20,000–46,000; *IV*, lysozyme; *V*, acid soluble) and radioactivity was estimated with a gamma counter. Radioactivity of each zone is expressed as percentage of total radioactivity in each lane. Recoveries of total radioactivities were essentially complete.

DISCUSSION

The present data indicate that there is a second site of action at which ATP is involved in the Ub proteolytic pathway. In addition to its previously described function in the formation of Ub-protein conjugates, we now find that ATP is also required for the breakdown of the protein moiety of these conjugates. We shall term this system the ATP-dependent conjugate-degrading system.

The ATP-dependent conjugate-degrading system from reticulocytes appears to be different from previously described ATP-stimulated proteases. It is not similar to an "ATP-stimulated" protease from liver cytosol (20), because the liver enzyme is also affected by anions such as creatine phosphate or citrate (21) and does not require Mg^{2+} (20). The conjugate-degrading system may bear more resemblance to an ATP-Mg dependent protease described in *Escherichia coli* (22) and in mammalian mitochondria (23). However, the *E. coli* protease degrades proteins to small peptides by itself whereas the reticulocyte ATP-dependent conjugate-degrading system appears to be composed of multiple components. Preliminary results indicate that the affinity-unadsorbed fraction of reticulocytes contains at least three separable protein factors, all of which are required for the conversion of ^{125}I-lysozyme-Ub conjugates to acid-soluble material in the presence of ATP (unpublished results). One of these appears to be identical to an ATP-stabilized factor described earlier (19). A further point of difference might be that, whereas the *E. coli* protease degrades free proteins, the conjugate-degrading system shows a preference for Ub conjugates, at least in the case of lysozyme. It remains to be seen whether the ATP-dependent conjugate-degrading system can also act on some proteins not conjugated to Ub. Goldberg and co-workers (24) have reported that, although the degradation of proteins fully blocked in their amino groups is diminished in rate, the residual degradation is still stimulated by MgATP. Those authors suggested that proteins lacking amino groups are broken down by a Ub-independent pathway, and that Ub-protein conjugates may be degraded by a similar enzyme. It should now be possible to examine whether proteins with blocked amino groups can be degraded by the conjugate-degrading system.

We find that, in the absence of ATP, Ub-lysozyme conjugates are rapidly cleaved by isopeptidases to yield free lysozyme (Fig. 3). The function of isopeptidases and the mode of partitioning of Ub-protein conjugates between the two competing pathways is not known. If isopeptidases have a correction function, as suggested earlier (3), it may be that some compartmentation or control is lost under these conditions. It seems reasonable to assume a control mechanism preventing a futile cycle due to isopeptidase action. It is possible, for example, that ATP stimulates conjugate breakdown by inhibiting a competing isopeptidase. Other questions raised by the present findings, such as the identification of classes of conjugates that are immediate precursors for conjugate-degrading action and the intermediary degradation products of this pathway, will necessitate the purification of its enzyme components.

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