

ATP Serves Two Distinct Roles in Protein Degradation in Reticulocytes, One Requiring and One Independent of Ubiquitin

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ABSTRACT Protein degradation in rabbit reticulocytes is a nonlysosomal process requiring ATP. Recently, appreciable evidence has been presented that ATP is required for the covalent binding of the polypeptide ubiquitin to ϵ -amino groups on protein substrates. To test whether linkage of ubiquitin to substrates is required for ATP-dependent proteolysis, the amino groups of ^3H -methyl-casein and denatured ^{125}I -bovine serum albumin (BSA) were completely (93–99%) blocked by methylation, acetylation, carbamylation, or succinylation. In each case, the proteins lacking amino groups were still degraded by an ATP-stimulated process, although these various treatments altered absolute rates of proteolysis and reduced the magnitude of the ATP stimulation (two- to fourfold) below that seen measured with the unmodified substrates. When ubiquitin was removed by ion exchange chromatography, ATP still stimulated breakdown of casein and carbamylated casein twofold. The addition of ubiquitin in the presence of ATP caused a further twofold increase in the hydrolysis of unmodified casein but did not affect the degradation of casein lacking amino groups. Thus ubiquitin conjugation to substrates appears important in the breakdown of certain substrates (especially of BSA), but this reaction is not essential for ATP-stimulated proteolysis. The ATP-activated step that is independent of ubiquitin probably is also involved in the degradation of unblocked proteins, since both processes require Mg^{++} and ATP hydrolysis and are inhibited by hemin but not by protoporphyrin IX. These results suggest that ATP has distinct roles at different steps in the degradative pathway.

Protein degradation within mammalian and bacterial cells requires metabolic energy (1, 2). The finding that ATP can stimulate proteolysis in cell-free extracts (2–4) has made possible appreciable progress in elucidating the basis for this energy requirement. In rabbit reticulocyte extracts, Etlinger and Goldberg (3) described a soluble, alkaline proteolytic system that is dependent on ATP. This nonlysosomal system appears responsible for the selective degradation of abnormal proteins as well as for the elimination of many normal proteins during reticulocyte maturation (5, 6). Hershko, Rose, and co-workers (7–10) have presented extensive evidence that multiple protein components are necessary for the stimulatory effect of ATP. One of these is ubiquitin (10), an 8,500-dalton polypeptide (11), which in the presence of ATP can be ligated to ϵ -amino groups of lysine residues of various cellular proteins (12). In this process, ATP-Mg^{++} is required for the activation of the carboxyl glycine residue of ubiquitin to a form which can be linked by an isopeptide bond to lysines in proteins (13).

In support of this model, Chin et al. (14) showed that the degree of attachment of ubiquitin to denatured hemoglobins correlated with their rates of degradation when these proteins were microinjected into HeLa cells. Hershko et al. (2, 9) have argued that the formation of ubiquitin-protein conjugates is the explanation of the ATP requirement for protein breakdown. Thus ATP would be required not for protein hydrolysis but for an initial recognition reaction, in which the substrates undergo a modification that would enhance their susceptibility to degradation by cytosolic ATP-independent proteases.

One reason that this novel explanation of the ATP effect was attractive was that no precedent existed for proteolytic enzymes whose function required ATP. However, in the last two years, ATP-dependent proteases have been isolated from *Escherichia coli* (15–17) and mammalian mitochondria (19), and have been shown to be responsible for the energy-dependent degradation of abnormal proteins both in intact bacteria (15, 19–21) and in mitochondria (22). These novel enzymes, protease La from *E.*

coli (15, 16) and the similar protease from liver mitochondria (18, 23), contain an ATPase function which is essential for proteolysis and which is activated by protein substrates (17, 23, 24). It remains to be established whether similar enzymes exist in the mammalian cytosol. Rat liver (25), erythrocytes (26, 27), and other tissues (28) contain a high molecular weight soluble protease that is directly stimulated twofold by ATP without involvement of ubiquitin. Unlike protease La and the mitochondrial enzyme, this ATP-activated enzyme does not hydrolyze ATP (27). Nevertheless, a variety of observations suggest that it plays an essential role in the cytosolic ATP-dependent pathway (26).

We undertook this study to test whether in mammalian cells ubiquitin conjugation to substrates is essential for ATP-dependent hydrolysis, and whether ATP may play an additional role in the proteolytic process. One crucial feature of the ubiquitin model is that the amino groups on the proteins must be available for ligation to ubiquitin (7). To test this model, we investigated whether blocking these amino groups by various covalent modifications eliminated ATP-dependent proteolysis. Evidence is presented for multiple ATP-hydrolysing steps in this pathway.

MATERIALS AND METHODS

Materials: α -Casein, BSA, hemin, protoporphyrin IX, and ATP (disodium salt) were purchased from Sigma Chemical Co. (St. Louis, MO) and β , γ -methylene ATP (lithium salt) from P-L Biochemicals, Inc. (Milwaukee, WI). Na 125 I, [3 H]formaldehyde, and [3 H]NaBH $_4$ were obtained from New England Nuclear Corp. (Boston, MA).

Preparation of Reticulocyte Extracts, Fraction II, and Ubiquitin

Rabbit reticulocytes were obtained after phenylhydrazine treatment of male rabbits (8–10 lbs) as described previously (3). The washed cells were lysed by the addition of 1.5 vol of cold water containing 1 mM dithiothreitol (DTT). The lysate was centrifuged at 40,000 g for 90 min, and the supernatant was dialyzed overnight against 50 mM Tris-HCl buffer (pH 7.8) containing 8 mM KCl, 5 mM MgCl $_2$, 0.5 mM dithiothreitol, and 20% glycerol. These extracts were stable for several months when stored at -20°C .

Ubiquitin was removed from the extracts by DEAE-cellulose (Whatman Co., Clifton, NJ) chromatography as described by Ciechanover et al. (8) except that the extracts were prepared from reticulocytes without ATP depletion and the buffer contained 10 mM Tris-HCl (pH 7.0) and 20% glycerol. The ubiquitin (i.e., Fraction I) did not adsorb to the column. The bound material (i.e., Fraction II), which contained the proteolytic enzymes (8), was eluted with 0.5 M KCl in this buffer. This fraction was concentrated to 15 mg protein/ml by ultrafiltration with an Amicon PM-10 membrane and dialyzed against the same buffer. Ubiquitin was purified to homogeneity from rabbit erythrocytes as described by Ciechanover et al. (29), but the final purification step on CM-cellulose (Whatman Co.) was carried out according to Wilkinson et al. (10).

Assay of Proteolytic Activity: 50 μl of the reticulocyte extracts or 500 μg (30 μl) Fraction II were incubated in a total volume of 100 μl which contained 50 mM Tris-HCl (pH 8.0), 5.0 mM MgCl $_2$, 5 mM ATP, 1 mM DTT, and 20 μg $^3\text{H-CH}_3$ -casein (25,000 cpm) or 10 μg $^{125}\text{I-BSA}$ (50,000 cpm). $^3\text{H-Methyl-casein}$ was prepared by reductive methylation (30). BSA was iodinated with Na ^{125}I using Enzy-mo-Beads (Bio-Rad Laboratories, Richmond, CA) and denatured by reduction and carboxamidomethylation (31). After incubation for 3 h at 37°C , the reactions were terminated by addition of 0.7 ml of 10% trichloroacetic acid containing BSA (1 mg/ml) as carrier. The acid-soluble radioactivity was determined as described previously (15).

With either substrate, the production of acid-soluble radioactivity was linear for over 3 h. Our earlier studies using gel filtration have established that the appearance of acid-soluble radioactivity from $^3\text{H-casein}$ in these preparations is due to protein hydrolysis and the accumulation of $^3\text{H-amino acids}$ (27) both in the presence and in the absence of ATP. No evidence for demethylation or deiodination of these substrates was obtained in these preparations or in analogous studies with liver extracts (25).

In addition, since $^{125}\text{I-}$ or $^3\text{H-}$ labeled proteins behave similarly in these preparations (see below), it is unlikely that demethylation or deiodination contributes to these results.

Chemical Modification of Casein and BSA: Before derivatization of the amino groups, the sulphhydryl residues on $^3\text{H-CH}_3$ -casein and $^{125}\text{I-}$

BSA were reduced and carboxamidomethylated in 6 M guanidine hydrochloride (31). The alkylated proteins were then dialyzed against H $_2$ O at 4°C . About 2 mg of the unlabeled proteins and 0.2 mg of $^3\text{H-CH}_3$ -casein or $^{125}\text{I-BSA}$ were used in the various reactions.

SUCCINYLACTION: Solid urea was added to a final concentration of 6 M, and the solutions were buffered with 0.2 M NaHCO $_3$ (pH 8.5). Solid succinic anhydride was dissolved in dioxane (76 mg/ml) and was added in several 25- μl aliquots every 5 min at room temperature to give a 300-fold molar excess over the total number of amino groups on the protein. The pH was monitored by spotting 0.5 μl onto pH paper, and 10–20 μl of 1 M NaOH were added to maintain the pH between 7.5 and 8.5. After the final addition of reagent, the mixture was left overnight and then dialyzed against H $_2$ O.

ACETYLACTION: Proteins were acetylated in a similar way except that acetic anhydride was diluted 10-fold in dioxane before addition.

METHYLACTION: Proteins were prepared in 6 M urea and 0.1 M HEPES (pH 7.5). Formaldehyde was added to a final concentration of 12 mM and sodium cyanoborohydride at 16 mM (32). The reaction mixture was incubated overnight and then reagents were removed by dialysis.

CARBAMYLATION: Proteins were prepared in 6 M urea, 1 M Na borate (pH 8.6), and an equal volume of 2 M potassium cyanate dissolved in the same buffer was added. After 24 h at 37°C , the reagents were removed by dialysis.

Protein concentrations were generally estimated by the method of Bradford (33), with BSA as the standard. However, the concentration of protein in solutions of derivatized casein and BSA was determined spectrophotometrically by absorbance at 280 nm in the presence of 0.1% SDS and compared to that of standard solutions of unmodified proteins.

The degree of blockage of amino groups was estimated by reaction with fluorescamine (34). These treatments did not introduce cleavages or cross-linkages of the protein substrates, since after these modifications the proteins migrated similarly when examined by PAGE in the presence of SDS (35).

RESULTS

ATP Effect on Degradation of Proteins with Blocked Amino Groups

To examine whether ubiquitin conjugation to substrates is essential for ATP-dependent proteolysis, we used two substrates, $^3\text{H-CH}_3$ -casein and denatured $^{125}\text{I-BSA}$, and four different methods for blocking free amino groups on these proteins, including methylation, acetylation, carbamylation, and succinylation (36). After these treatments, 93–99% of the amino groups on casein and >99% of the amino groups on BSA were blocked (Table I), as shown by their inability to react with fluorescamine. Although these modifications altered the absolute rates of protein hydrolysis, in each case, ATP stimulated

TABLE I
Effect of Blocking Amino Groups of Protein on Their Degradation in Reticulocyte Extracts With or Without ATP

Chemical modification	% NH $_2$ groups blocked	Protein hydrolyzed (%/3 h)		ATP effect (+ATP/–ATP)
		–ATP	+ATP	
$^3\text{H-CH}_3$ -casein				
Control	—	11.4	51.3	4.5
Methylation	95	6.6	15.0	2.3
Acetylation	99	24.6	46.8	1.9
Carbamylation	98	9.6	20.1	2.1
Succinylation	93	8.4	17.7	2.1
Denatured $^{125}\text{I-BSA}$				
Control	—	0	35.7	∞
Methylation	99.4	0	2.1	∞
Acetylation	99.7	20.4	31.5	1.5
Carbamylation	98.9	2.7	12.9	4.3
Succinylation	99.7	0	3.6	∞

These data are the average of triplicate assays in two separate experiments. 1% hydrolysis of $^3\text{H-CH}_3$ -casein and $^{125}\text{I-BSA}$ correspond to 375 cpm and 750 cpm, respectively, generated in 3 h. 50 μl of reticulocyte extracts were used for these assays. ∞ , completely ATP-dependent.

degradation of the substrates lacking free amino groups (Fig. 1 and Table I). ATP promoted the hydrolysis of the unmodified casein 4.5-fold, and it increased the hydrolysis of casein with blocked amino residues about twofold (Table I). In the presence of ATP, the absolute rates of hydrolysis of the methylated, carbamylated, acetylated, and succinylated substrates were significantly less than that of unblocked proteins, while in the absence of ATP the various modifications had different effects on proteolysis (e.g., biphasic degradation rates). Whatever the explanation for these latter effects, the demonstration of ATP-stimulated breakdown of proteins lacking amino groups strongly suggests an ATP effect not involving ubiquitin conjugation.

Since the relative stimulation by ATP of casein degradation (2–4.5-fold) was much less than that for breakdown of BSA (Table I), analogous studies were carried out with the latter substrate. The breakdown of BSA to acid-soluble material was almost totally dependent on the presence of ATP, as found by others (29). Without this nucleotide, there was also no measurable breakdown of the methylated or succinylated BSA. ATP clearly enhanced breakdown of these blocked polypeptides, although proteolysis was much slower than that of the unmodified BSA in the presence of ATP. In addition, ATP promoted significantly the hydrolysis of acetylated and carbamylated BSA. (Interestingly, acetylation of BSA markedly increased its degradation in the absence of ATP, as was also found with casein.) Together, these findings are further evidence for an ATP effect on proteolysis independent of ubiquitin.

Effects of ATP and Ubiquitin on Proteolysis in Fraction II

These experiments do not, however, completely rule out the possibility that ubiquitin may still be essential for the ATP-stimulated degradation of the derivatized substrates. For example, the few amino residues that escaped being blocked (0.2–4% of the total) might possibly allow sufficient ubiquitin conjugation to activate proteolysis; alternatively, ubiquitin may stimulate proteolysis not by conjugation to substrates but by ligation to some other component (e.g., a key enzyme). To

eliminate these possibilities, the free ubiquitin was removed from the reticulocyte lysates by fractionation on DEAE-cellulose, and the ubiquitin-free material (Fraction II) was eluted from the column with high salt (8). (Unless this step was carried out, addition of ubiquitin to the extracts did not stimulate proteolysis, probably because of the presence of large amounts of free ubiquitin [7–10].)

In the absence of added ubiquitin (Table II), ATP stimulated degradation of casein at least twofold as well as of casein lacking amino groups. These findings further indicate an ATP effect independent of ubiquitin-conjugation, although they do not eliminate the possibility that some ubiquitin was generated during the experiment from cellular proteins and allowed the ATP-stimulation of proteolysis. However, when purified ubiquitin was added in the presence of ATP, it promoted twofold the degradation only of ^3H -casein with free amino groups and did not affect hydrolysis of carbamylated casein. The failure of ubiquitin to stimulate hydrolysis of the blocked substrate supports the earlier suggestion (12, 13) that this polypeptide acts by conjugation to amino groups on the substrate and our assumption that with the carbamylated proteins this linkage reaction is not possible.

The experiments in Tables I and II have dissociated the actions of ATP, one which does not require ubiquitin, and one which does. Furthermore, Table II shows that ATP by itself and ubiquitin (plus ATP) have additive effects on degradation of casein. These two effects together can account for the four- to fivefold increase in the extent of proteolysis by ATP seen in crude extracts.

It was also possible by this approach to dissociate two effects of ATP when ^{125}I -BSA was used as the substrate. By itself, ATP reproducibly caused a twofold increase in the extent of degradation of BSA in Fraction II (Table II). The addition of ubiquitin (along with ATP) caused a further fivefold rise in the amount of substrate hydrolyzed. Together, these responses can explain the 10-fold activation seen in the unfractionated extracts. It also is interesting that ubiquitin appears more important for maximal breakdown of BSA than of casein (Table II).

Properties of the Degradation of Blocked and Unblocked Proteins

Prior findings indicated that hydrolysis of ATP and Mg^{++} is required for ATP-dependent proteolysis in reticulocyte extracts (2). By contrast, an alkaline protease has been isolated from such cells that is activated twofold by nonmetabolizable ATP analogs and by ATP in the absence of Mg^{++} (26, 27). Therefore,

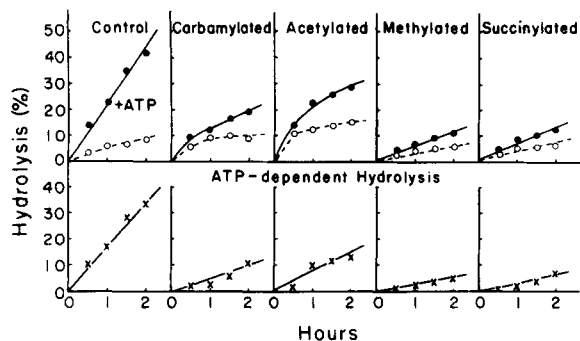


FIGURE 1 Effect of ATP on the hydrolysis of ^3H -CH₃-casein with free or blocked amino groups. Reaction mixtures contained 50 μl of reticulocyte extracts and the same amounts of substrate (20 μg) with (●) or without (○) ATP (5 mM). At the indicated times, the reaction was terminated, and the acid-soluble radioactivity was measured. ATP-dependent proteolysis (x) represents the net increase in substrate hydrolysis observed at each time point in the presence of the nucleotide (i.e., activity with ATP minus that without). Prior to assay, the labeled substrate was subjected to acetylation, methylation, carbamylation, or succinylation. After these modifications, nearly all the amino groups on the ^3H -casein were blocked, as shown with fluorescamine (see Table I).

TABLE II
Effects of ATP and Ubiquitin on the Degradation of Unmodified BSA, Casein, and Carbamylated Casein in Fraction II from Reticulocyte Extracts

Additions	Protein hydrolyzed (%/3 h)		
	^3H -CH ₃ -casein		^{125}I -BSA unmodified
	Unmodified	Carbamylated	
None	9.6	5.4	1.2
Ubiquitin	9.3	4.8	1.2
ATP	20.7	10.8	2.4
Ubiquitin + ATP	44.1	11.1	12.0

500 μg of Fraction II were used for the assay. These data are the average of duplicate assays from two separate experiments. Ubiquitin was added at 5 μg /tube and ATP at a final concentration of 5 mM.

we examined whether hydrolysis of ATP is necessary for the promotion of breakdown of proteins lacking amino groups. Table III shows that unlike ATP, the nonmetabolizable analog β,γ -methylene ATP did not stimulate the breakdown either of unmodified casein and BSA or of the proteins with blocked amino residues. Thus the terminal high-energy phosphate appears essential for the ubiquitin-independent ATP effect. In addition, Table III shows that removal of Mg^{++} prevented the ATP-stimulated degradation of proteins with and without amino groups.

Hemin has been shown to inhibit the energy-dependent degradation of abnormal and normal proteins in reticulocytes (37) as well as the breakdown of ubiquitin-conjugated proteins (38). As shown in Fig. 2, hemin at low concentrations caused a dramatic inhibition of the ATP-stimulated breakdown of 3H -casein ($K_I = 40 \mu M$) without reducing the ATP-independent process. This inhibitory effect appears highly specific, since it was not mimicked by the Fe-depleted derivative, protoporphyrin IX (Fig. 2). Furthermore, hemin markedly reduced the ATP-stimulated hydrolysis of casein and BSA even when these proteins lacked free amino groups (Table IV). The degree of inhibition by hemin was also quite similar after derivatization of these substrates, and in no case did hemin affect the basal proteolysis seen in the absence of ATP. In addition, protoporphyrin IX could not mimic this effect of hemin on the ATP-stimulated breakdown of derivatized casein (data not shown). Together, these various results suggest that degradation of blocked and unblocked proteins proceeds through the same, or at least very similar, steps, since both processes require ATP cleavage and Mg^{++} and are specifically inhibited by hemin.

Analogous experiments were also performed with Fraction

TABLE III

Effects of ATP, β,γ -Methylene ATP and Mg^{++} on the Degradation of Proteins with Free or Blocked Amino Groups

Chemical modification	Stimulation of hydrolysis (%/3 h)		
	ATP Mg^{++}	β,γ -Me ATP Mg^{++}	ATP No Mg^{++}
	3H -CH ₃ -Casein		
Control	39.9	9.3	7.2
Methylation	8.4	0	2.4
Acetylation	22.2	0.6	3.9
Carbamylation	10.5	3.0	0
Succinylation	9.3	0	2.4
	^{125}I -BSA		
Control	35.7	0	1.8
Methylation	2.1	0	0
Acetylation	11.1	0	0.9
Carbamylation	10.2	0	1.8
Succinylation	3.6	0.3	0

The results represent the hydrolysis seen with the nucleotides (5 mM) from which was subtracted the rate without added nucleotide. $MgCl_2$ was added in a final concentration of 5 mM. The basal rate of proteolysis observed in the absence of ATP did not differ with or without Mg^{++} . These data are the average of triplicate assays in two separate experiments.

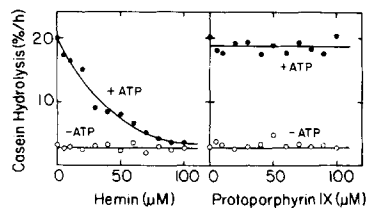


FIGURE 2 Effects of different concentrations of hemin and protoporphyrin IX on the hydrolysis of 3H -CH₃-casein with (●) or without (○) ATP (5 mM) in reticulocyte extracts.

TABLE IV

Effect of Hemin on ATP-dependent Degradation of Proteins with Free or Blocked Amino Groups

Chemical modification	ATP-stimulated Hydrolysis (%/3 h)			
	3H -CH ₃ -Casein		^{125}I -BSA	
	None	+Hemin	None	+Hemin
Control	39.9	11.7	35.7	6.9
Methylation	8.4	2.4	2.1	0
Acetylation	22.2	7.5	11.1	1.2
Carbamylation	10.5	5.4	10.2	3.6
Succinylation	9.3	0	3.6	0.6

The final concentration of hemin was 75 μM . ATP-stimulated proteolysis was measured as in Table III. Hemin had no effect on the proteolysis seen in the absence of ATP. These data are average of triplicate assays in two separate experiments.

TABLE V

Effects of ATP and Ubiquitin on Degradation of 3H -Casein in Fraction II

Additions	3H -CH ₃ -Casein Hydrolyzed (%/3h)		
	Control	+Hemin	No Mg^{++}
None	10.8	6.9	10.8
ATP	21.0	10.5	11.4
β,γ -Me ATP	10.2	—	—
Ubiquitin	9.0	7.8	—
ATP + Ubiquitin	40.2	19.8	11.7
β,γ -Me ATP + Ubiquitin	10.8	—	—

ATP or β,γ -methylene-ATP was added at a final concentration of 5 mM and hemin at 75 μM . Ubiquitin was added at 5 μg per assay. 500 μg protein of Fraction II were used for each assay.

II in order to compare more rigorously the two ATP-stimulated processes, one involving ubiquitin and one not. In the absence of ubiquitin, ATP increased the extent of hydrolysis of casein and BSA twofold in 3 h. This effect, which must be independent of ubiquitin ligation because it also occurred with proteins lacking amino groups (Table II), required Mg^{++} and ATP cleavage (Table V). Furthermore, the two ATP-activated processes were inhibited strongly by hemin. The simplest interpretation of Tables IV and V is that ubiquitin-dependent and -independent proteolyses proceed through a common ATP-activated, hemin-sensitive step.

DISCUSSION

Our study demonstrates that in reticulocyte extracts ATP can stimulate the breakdown of proteins, even when they lack free amino groups. This result, which was obtained with two substrates modified by four different methods, indicates that ATP plays a role in proteolysis that is independent of the ATP requirement for conjugation to ubiquitin.¹ Additional strong evidence for this conclusion was the finding that, after removal of ubiquitin, ATP caused a twofold increase in degradation of blocked and unblocked proteins. The addition of ubiquitin had a further stimulatory effect but only on the degradation of proteins with free amino groups. This latter observation thus

¹ We have recently learned that M. Rechsteiner (Salt Lake City) and R. Kulka (Hebrew University of Jerusalem) and their co-workers, using different approaches, have also obtained evidence that proteins with blocked amino groups require ATP for degradation (personal communications).

provides direct evidence that ubiquitin conjugation to amino groups on proteins promotes their degradation. At the same time, this result also indicates that the enhancement by ATP of breakdown of blocked proteins involves a ubiquitin-independent mechanism.

In these experiments ubiquitin conjugation appears important but not essential for the hydrolysis of proteins. It is noteworthy that ubiquitin enhanced the extent of hydrolysis of BSA fivefold but that of casein only twofold (Table II). Thus the magnitude of the ubiquitin effect may be specific to the substrate. Recently, Saus et al. (39) found that ubiquitin stimulated the breakdown of BSA but did not influence the degradation of various mitochondrial enzymes. It is thus possible that ubiquitin conjugation is required for the selective elimination of specific proteins (e.g., during reticulocyte maturation). In any case, the basis for the differences in ubiquitin's effect on different substrates is an important unanswered question. Most of the modifications of amino groups used in these studies reduced the absolute rates of proteolysis and the magnitude of the ATP stimulation (Table I). Although such effects may result from the prevention of ubiquitin conjugation, the blockage of amino residues probably also alters protein conformations and solubility and thereby may reduce susceptibility to proteolytic attack. In fact, these modifications were found to decrease casein and BSA hydrolysis by chymotrypsin, subtilisin, and the ATP-dependent protease La (L. Waxman, unpublished observations). Furthermore, with these different modifications, the rates of proteolysis varied widely, even though the degree of amino-group blockage was virtually complete in each case (Table I). Thus the differences in proteolytic rates are not simply related to the prevention of ubiquitin conjugation. In fact, after acetylation, casein, and BSA were degraded faster in the absence of ATP. The differing consequences of these various modifications emphasize the need for precise information on how conjugation to ubiquitin actually influences proteolytic susceptibility.

The ubiquitin-independent function for ATP probably involves hydrolysis of the nucleotide, since β,γ -methylene ATP did not enhance proteolysis, and since ATP was ineffective without Mg^{++} present. Further evidence for ATP cleavage is our recent finding that the breakdown of proteins in reticulocytes (even of ones lacking free amino groups) is sensitive to vanadate, a potent inhibitor of various ATPases (37) (K. Tanaka and A. L. Goldberg, in preparation). Since vanadate inhibits the ATP-dependent proteases from *E. coli* (7, 24) and liver mitochondria (18), it is attractive to suggest that the mammalian cytosol contains a similar enzyme which accounts for the ubiquitin-independent effect of ATP (see below).

One important property of this ubiquitin-independent process is that it is strongly inhibited by hemin (Table IV and V, Fig. 2). This effect appears highly specific, since it was not observed with protoporphyrin IX (Fig. 2). Hemin also reduces proteolysis in intact erythrocytes (37), and this effect may contribute to hemin's ability to promote net hemoglobin accumulation in vivo (37). It is noteworthy that hemin inhibited hydrolysis of both blocked and unblocked substrates to a similar extent. Thus, the ubiquitin-independent, ATP-activated process resembles closely the degradation of unblocked proteins in crude extracts (3). The simplest explanation of these similarities is that the ubiquitin-dependent and -independent processes involve common steps, one of which is inhibited by hemin (but not by protoporphyrin IX) and is activated by $ATP-Mg^{++}$, as suggested in Fig. 3. According to this model, the breakdown of all proteins necessarily proceeds through

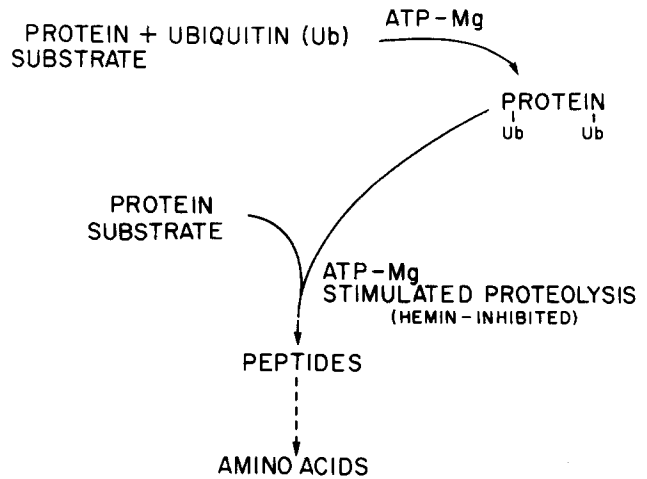


FIGURE 3 Suggested model to account for dual roles of ATP in proteolytic pathway. This scheme can account for similar effects of the hemin on degradation of proteins containing and lacking amino groups, and the findings in Tables II and V that the ubiquitin-independent and -dependent actions of ATP were additive. Additional evidence in support of this scheme will be published elsewhere (K. Tanaka and A. L. Goldberg, manuscript in preparation).

ATP-stimulated, hemin-sensitive step(s), and certain proteins enter this proteolytic pathway after ubiquitin conjugation, while others may bypass this step (e.g., those with blocked amino groups).

In support of this model is the finding (38) that hemin had little effect on the formation of ubiquitin conjugates but did inhibit the breakdown of the conjugated proteins. Furthermore, as shown in Table II, the ubiquitin-dependent and -independent effects of ATP are additive in promoting degradation of unblocked proteins. Although the present results can be explained most simply by a single degradative pathway involving multiple ATP-activated steps as in Fig. 3, we can not rule out the existence of two similar ATP-activated processes that digest the ubiquitin-conjugated and unconjugated proteins.

The novel aspect of the model in Fig. 3 is that it contains an ATP-requiring step in addition to that involved in ubiquitin conjugation. We recently obtained appreciable additional evidence for this scheme, including the observations that (a) vanadate, like hemin, can inhibit ATP-dependent proteolysis without reducing protein conjugation to ubiquitin (K. Tanaka and A. L. Goldberg, manuscript in preparation); (b) certain nucleoside triphosphates can promote protein breakdown but do not support ubiquitin-conjugation to substrates (K. Tanaka, L. Waxman, and A. L. Goldberg, manuscript in preparation). A key feature of the original proposal of Hershko et al. (7, 9) was that it did not postulate the direct involvement of ATP hydrolysis in the function of proteolytic enzymes. However, recently, the energy-dependent degradation of aberrant proteins in *E. coli* (19-21) and in mammalian mitochondria (22) has been shown to involve novel proteases that hydrolyze ATP and proteins in a linked fashion (23, 24). Although it is attractive to suggest that a similar enzyme plays a role in the breakdown of the blocked substrates, an enzyme with all these properties has not yet been demonstrated in the mammalian cytosol. Erythrocytes (26, 27) and other mammalian tissues (25, 28) do contain a soluble alkaline endoprotease that is directly stimulated by ATP (26). The inhibitor sensitivity pH optimum and the substrate specificity of this protease suggest that it plays an essential role in this pathway for protein breakdown (27). Of particular interest is the finding that this enzyme is

strongly inhibited by hemin (26). However, after purification, the protease does not hydrolyze ATP, which probably activates it by an allosteric mechanism (27). It remains to be proven whether a modified form of this protease or some unidentified enzyme is the ubiquitin-independent ATP-requiring component in this pathway.

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