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Role of the α-amino group of protein in ubiquitin-mediated protein breakdown

(protein turnover/conjugation/amino terminus/N^{α}-acetylation)

AVRAM HERSHKO*, HANNAH HELLER*, ETHY EYTAN*, GANGADHAR KAKLIJ†‡, AND IRWIN A. ROSE†

*Unit of Biochemistry, Faculty of Medicine, Technion-Israel Institute of Technology, Haifa, Israel; and †Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, PA 19111

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ABSTRACT **Previous studies suggest that the conjugation of ubiquitin to NH_2 groups of proteins is required for protein breakdown. We now show that the selective modification of NH_2-terminal α-NH_2 groups of globin and lysozyme prevents their degradation by the ubiquitin proteolytic system from reticulocytes. The conjugation by ubiquitin of ε-NH_2 groups of lysine residues, usually seen in multiples, was also inhibited in α-NH_2-blocked proteins. Naturally occurring N^{α}-acetylated proteins are not degraded by the ubiquitin system at a significant rate, while their nonacetylated counterparts from other species are good substrates. This suggests that one function of N^{α}-acetylation of cellular proteins is to prevent their degradation by the ubiquitin system. α-NH_2-blocked proteins can have their activity as substrates for degradation increased by incorporation of α-NH_2 groups through the introduction of polyalanine side chains. Proteins in which most ε-NH_2 groups are blocked but the α-NH_2 group is free are degraded by the ubiquitin system, but at a reduced rate. It is therefore suggested that the exposure of a free NH_2 terminus of proteins is required for degradation and probably initiates the formation of ubiquitin conjugates committed for degradation.**

Studies on the mode of action of an ATP-dependent proteolytic system from reticulocytes revealed a pathway for the degradation of intracellular proteins (for reviews see refs. 1 and 2). That system requires for activity the 8500-dalton polypeptide ubiquitin (Ub) (3, 4). Ub is covalently conjugated to proteins (5) by a sequence of reactions in which the COOH-terminal residue of the polypeptide is first activated by a specific Ub-activating enzyme, E_1 (6, 7), and activated Ub is transferred to protein by the action of two further enzymes, E_2 and E_3 (8). The structure of Ub-protein conjugates has not yet been characterized sufficiently, but at least some Ub molecules bind to ε-NH_2 groups of lysine residues by isopeptide linkages (5, 9). Proteins conjugated to multiple molecules of Ub are degraded by an ATP-dependent enzyme system that does not degrade unconjugated proteins (10). The metabolic function of the Ub proteolytic system was strongly supported by a recent study in which a mammalian cell line found to have a temperature-sensitive Ub-activating enzyme was found to be defective in degrading most of its rapidly turning over protein (11).

A central problem is what features of protein structure are recognized by the Ub conjugation system for commitment to proteolysis. Since most lysine residues are exposed at the surface of most proteins, the availability of any lysine does not seem to be sufficient for specific recognition. One approach is to study the influence of the modification of specific amino groups in proteins. Other investigators have used complete blocking of protein amino groups to distinguish between Ub-dependent and Ub-independent proteolytic systems (12, 13), and the requirement for free NH_2 groups has been confirmed for the Ub-dependent system. On the other hand, Hough and Rechsteiner (14) reported that guanidinated proteins are degraded in reticulocyte lysates by an ATP-dependent process. In the present study we have tried to use more selective methods, and we have found that a free α-NH_2 group has a special importance.

MATERIALS AND METHODS

Materials. Cytochrome *c* from horse heart (type III) or from yeast (*Saccharomyces cerevisiae*) (type VIII), enolase from baker's yeast (*S. cerevisiae*), and lysozyme from hen egg were purchased from Sigma, and enolase from rabbit muscle was obtained from Calbiochem. All the above proteins were found to be essentially homogeneous by PAGE. The sources of other proteins used in this study were as follows: bovine serum albumin, from Miles; aldolase (rabbit muscle), carbonic anhydrase (bovine erythrocyte), and glyceraldehyde-3-*P* dehydrogenase (rabbit muscle), from Boehringer Mannheim; actin (bovine muscle), lactate dehydrogenase (rabbit heart, H_4 isoenzyme) and ribonuclease S-protein (bovine pancreas), from Sigma; and ovalbumin (hen egg), from Worthington. Ubiquitin was purified from human erythrocytes as described earlier (4, 15). All proteins were radioiodinated by the chloramine-T procedure (5).

Protein Modification. Guanidination of proteins was performed according to Cupo *et al.* (16), except that the concentration of *O*-methylisourea was 1 M, and the reaction was carried out in 6 M urea at 4°C for 1 week. Acetylation was performed as described by Tanaka *et al.* (13). Reductive methylation (17) was carried out in 0.1 M Hepes–NaOH, pH 7.4/6 M urea, at 25°C for 1 hr; concentrations of formaldehyde and cyanoborohydride were varied as described in legends to figures. Carbamoylation of ^{125}I-labeled lysozyme (1 mg/ml) was carried out in 0.2 M potassium phosphate, pH 6.0/6 M urea/50 mM potassium cyanate (Aldrich). After incubation at 37°C for the time periods indicated in Fig. 1, the reaction was stopped with 150 mM glycylglycine. The pH was adjusted to 8.1 with 0.5 M K_2HPO_4 and KOH, and the samples were incubated at 37°C for 1 hr. This latter treatment releases carbamoyl groups bound to nonamine residues in protein (18, 19). For polyalanylation, *N*-carboxy-L-alanine anhydride was prepared according to ref. 20. The anhydride (100 mM) was added to the protein solution (0.3 mg/ml in water) and the samples were incubated at 25°C for 2 hr. After all modification reactions, reagents were removed by dialysis at 4°C for 48 hr, with at least six changes of water. The extent to which NH_2 groups were blocked was determined with fluorescamine (21).

To prepare N^{α}-carbamoylated [^{3}H]globin, rabbit reticulocytes were labeled with [^{3}H]leucine (500 μCi/ml, 2 hr, 1 Ci =

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Abbreviations: Ub, ubiquitin; Gdn, guanidine.

‡Present address: Biochemistry and Food Technology Division, Bhabha Atomic Research Center, Trombay 400 085, India.

37 GBq) and were lysed in water, as described (22). To remove most nonhemoglobin proteins, the lysate (5 ml) was passed through a 5-ml column of DEAE-Sephacel (Pharmacia) in 10 mM phosphate buffer (pH 7.0). The hemoglobin solution (36 mg/ml) was subjected to carbamoylation according to the procedure described by Manning (23), except that $K^{14}CNO$ (New England Nuclear, 50 μCi/ml) was added and the final concentration of KCNO was 20 mM. The amount of carbamoyl groups bound to protein was estimated by the incorporation of the ^{14}C label into material precipitated with 5% trichloroacetic acid. Both carbamoylated and unmodified hemoglobin preparations were extensively dialyzed against water, and globin was precipitated with acetone/HCl (24). The globin preparations were diluted to 2 mg/ml, brought to 0.01 M NaOH, and denatured by heating at 60°C for 1 hr (22). The extent of carbamoylation of α-NH_2 and ε-NH_2 residues in globin was estimated by the method of Manning *et al.* (25). In this procedure, globin is treated with hot acid to release valine hydantoin from carbamoylated α-NH_2 residues. The hydantoin is extracted into ethyl acetate, while carbamoylated lysine (homocitrulline) remains in the aqueous phase. Since the specific radioactivity of [^{14}C]carbamoyl groups is known, the amount of ^{14}C in the organic and aqueous phases is a measure of carbamoyl groups bound to α-NH_2 and ε-NH_2 residues, respectively.

Assay of Protein Breakdown. Unless otherwise stated, the complete reaction mixture contained, in a final volume of 50 μl: 50 mM Tris·HCl at pH 7.6, 5 mM $MgCl_2$, 3 mM dithiothreitol, 1 mM ATP, 10 mM phosphocreatine, 2.5 μg of creatine kinase, 3 μg of Ub, approximately 200 μg of protein of fraction II from reticulocytes, and 0.05–5 μg of labeled protein substrate. After incubation at 37°C for 1 hr, the release of radioactive material soluble in 5% trichloroacetic acid was determined as described (8). Fraction II (a Ub-free crude enzyme fraction) was prepared from lysates of rabbit reticulocytes as described previously (8). Preparations of fraction II used in this study contained ATP (0.5 mM), for stabilization in prolonged storage. Therefore, for incubations without ATP, hexokinase (3 μg, P-L Biochemicals, grade 300) was added together with 10 mM 2-deoxyglucose.

RESULTS

Influence of the Extent of Modification of Protein NH_2 Groups on Rates of Protein Breakdown. To examine the effects of various degrees of NH_2 group blocking on Ub-dependent protein breakdown, ^{125}I-labeled lysozyme (^{125}I-lysozyme) was subjected to increasing extents of reductive methylation or carbamoylation. Both types of reactions were carried out under denaturing conditions, to minimize effects of protein structure on the modification reactions. The rates of degradation of the various modified derivatives of lysozyme were examined with fraction II from reticulocytes, in which most ATP-dependent proteolysis requires the supplementation of Ub. With the reductively methylated derivatives (Fig. 1*A*), the decrease in rates of protein breakdown corresponded roughly to the proportion of NH_2 groups blocked: a decrease of 50% in degradation rates was obtained when 46% of the NH_2 groups were blocked, and protein degradation was abolished with a modification of 85% of NH_2 groups. On the other hand, when carbamoylation with cyanate at pH 6 was used for NH_2 group modification (Fig. 1*B*), protein breakdown decreased at markedly lower extents of NH_2 group blocking, and the slope of the plot increased by about 2-fold. In the latter case, a decrease of 50% in degradation rates was attained with 22% of NH_2 groups blocked, and a nearly complete inhibition of proteolysis was obtained with 45% of NH_2 groups blocked. Since lysozyme has seven NH_2 groups (six lysines and α-NH_2), these results indicate that the carbamoylation of about three of its NH_2 groups is sufficient to prevent its degradation.

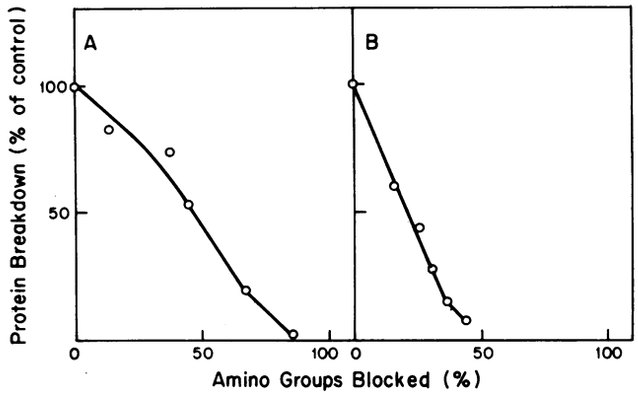

FIG. 1. Influence of partial blocking of lysozyme by different agents on its rate of degradation. (*A*) Reductive methylation. ^{125}I-lysozyme (1 mg/ml, 5×10^6 cpm/mg) was modified as described in the text. Maximal blocking was with 12 mM formaldehyde and 20 mM sodium cyanoborohydride, and for decreasing extents of modification, both reagents were diluted 5-, 10-, 20-, and 40-fold. The percentage of NH_2 groups blocked was determined with fluorescamine. The degradation of samples of the various derivatives (5 μg) was assayed in the absence of ATP and in the presence of ATP and Ub. ATP-independent proteolysis was subtracted and the results are expressed as the percentage of the value obtained with unmodified control lysozyme. Rates of degradation in the control were (%/hr): without ATP, 1.0; with ATP, 2.4; with ATP and Ub, 18.2. Rates of ATP-independent proteolysis were not affected by methylation. (*B*) Carbamoylation at pH 6.0. ^{125}I-lysozyme was subjected to carbamoylation as described in the text for the following time periods (hr): 0.5, 1, 2, 4, and 7. Protein breakdown dependent on ATP and Ub was assayed and the results were calculated as described for *A*. Rates of ATP-independent proteolysis were not affected by carbamoylation.

One possible explanation of these results is that whereas reductive methylation attacks the various NH_2 groups of the protein randomly, carbamoylation may selectively block some specific NH_2 group(s) essential for the action of the Ub system. It had been shown that when carbamoylation with cyanate is carried out at neutral pH or slightly below, the α-NH_2 groups of proteins react about 100-fold faster than the ε-NH_2 groups of lysine residues, due to the lower pK_a values of the former (18, 26). Thus, the question was raised whether a free α-NH_2 group of protein is necessary for its degradation by the Ub system.

Effects of Selective Modification of the α-NH_2 Group on Protein Breakdown and Conjugation with Ub. Selective carbamoylation of NH_2-terminal residues of hemoglobin has been extensively studied because of its possible use in the treatment of sickle cell anemia (27). In this case, too, cyanate reacts much more readily with the NH_2-terminal valines than with lysines when the pH is slightly acidic. Selectivity is further increased with deoxygenated hemoglobin, due to acceleration of reaction with the NH_2-terminal residue (23). To prepare selectively modified globin, hemoglobin was first labeled in rabbit reticulocytes with [^3H]leucine, and isolated deoxygenated hemoglobin was allowed to react with cyanate under conditions optimized for maximal blocking of α-NH_2 groups with minimal modification of lysines (23). Analysis of the product showed 1.1 carbamoyl groups per globin monomer, of which 0.68 were bound to NH_2-terminal valines, and 0.42 carbamoyl residues were linked (presumably randomly) to the 11 lysine residues of globin chains.

To examine the degradation of N^{α}-carbamoylated and unmodified globin chains, they were freed of heme and denatured by heat/alkali treatment (22). As shown in Fig. 2, in the absence of ATP the rates of degradation of unmodified and carbamoylated globin were essentially similar. A slight stimulation observed by the addition of ATP (without Ub)

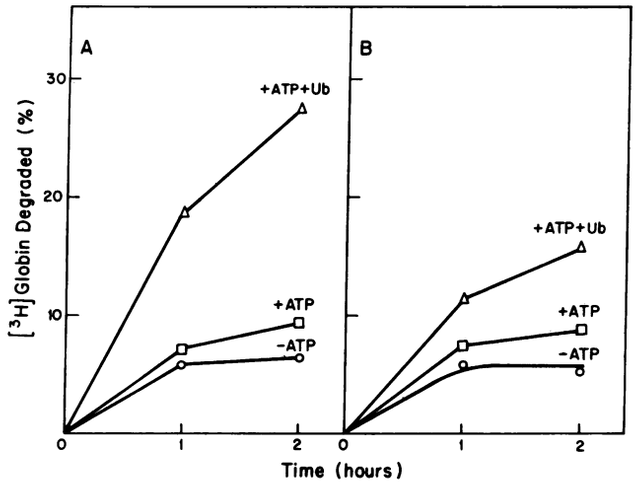

FIG. 2. Effect of selective carbamoylation of globin at the NH_2 terminus on its rate of degradation. Protein breakdown was assayed as described in *Materials and Methods*, except that [^{3}H]globin (*A*) or its carbamoylated derivative (*B*) was added at 20 μg (7000 cpm), and the reaction mixture was increased 4-fold. Where indicated, ATP was added together with phosphocreatine and creatine kinase.

was also similar. However, the further stimulation of degradation by the addition of Ub was reduced by about 60% in the N^{α}-carbamoylated derivative relative to unmodified globin. This compares well with the extent of the carbamoylation of α-NH_2 groups (68%) in this preparation.

We next asked whether the effect of blocked NH_2 termini on protein breakdown can be reversed by creating a new α-NH_2 groups on proteins. In its reaction with proteins, *N*-carboxyalanine anhydride reacts successively with protein amino groups to produce polyamino acid side chains (28). Thus, the ε-NH_2 groups of lysine residues are replaced by the α-NH_2 groups of the terminal amino acids of the side chains. As shown in Table 1, the poly-L-alanylated derivative of lysozyme is degraded by the Ub system somewhat more slowly than is unmodified lysozyme. By contrast, polyalanylation of lysozyme derivatives that had been previously carbamoylated at their α-NH_2 groups restored to a large extent their susceptibility to degradation in the presence of ATP and Ub. The low rate of ATP-independent proteolysis was not much affected by these treatments.

The question arose as to whether selective blocking of α-NH_2 groups interferes with the proteolytic system at the site of Ub-protein conjugation or at some later stage. As shown in Fig. 3, lanes 2–5, there was a marked reduction in the conjugation of Ub to N^{α}-carbamoylated globin, as compared to unmodified globin. The formation of all sizes of Ub-globin conjugates was equally diminished with α-NH_2-blocked globin. There was a similar reduction in the formation of high molecular weight conjugates with derivatives of lysozyme in which the α-NH_2 group was blocked to increasing degrees (Fig. 3, lanes 6–9). These data indicate that the conjugation of multiple molecules of Ub to the protein requires a free α-NH_2 group. It should be noted that while the formation of high molecular weight conjugates of lysozyme (M_r 45,000–100,000) is decreased with α-NH_2-blocked derivatives, the formation of some lower molecular weight derivatives (mainly a M_r 29,000 band) is actually increased (Fig. 3, lanes 6–9). It might be that this represents a different pathway for Ub-protein conjugation (on ε-NH_2 groups), which is not related to protein breakdown (see *Discussion*).

Table 1. Reversal of the effect of blocked NH_2 termini by poly-L-alanylation

Treatment of ^{125}I-lysozyme	Degradation rate, %/hr			
	Control		Poly-L-alanylated	
	−ATP	+ATP +Ub	−ATP	+ATP +Ub
None	2.0	24.8	2.5	15.5
Carbamoylated (2 hr)	2.1	7.8	3.2	14.4
Carbamoylated (7 hr)	2.1	3.4	2.3	11.1

Polyalanylation was performed either on untreated ^{125}I-lysozyme or on samples previously subjected to carbamoylation for 2 or 7 hr, which yields derivatives blocked in 30.9% or 44.3% of their amino groups, respectively (see Fig. 1*B*). Protein breakdown was assayed with 5-μg samples of the various derivatives.

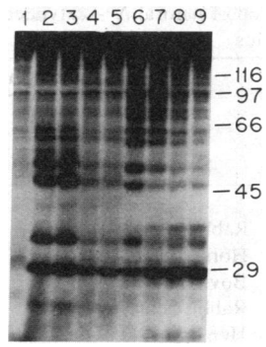

FIG. 3. Effects of α-NH_2 blocking of proteins on their conjugation with ubiquitin. Conjugation of ^{125}I-Ub (10^5 cpm, 3 μg) was assayed as described previously (5), in the presence of 4.5 nanounits of E_1, 1.1 nanounits of E_2, both purified by affinity chromatography and gel filtration chromatography (8), and 1.9 nanounits of E_3 (the pH 9 eluate of the Ub-affinity column) treated with iodoacetamide (5 mM) to inactivate the Ub-protein hydrolases present (8). The following protein substrates were added: lane 1, none; lanes 2 and 3, 10 and 20 μg of unmodified globin, respectively; lanes 4 and 5, 10 and 20 μg of N^{α}-carbamoylated globin, respectively; lane 6, 5 μg of unmodified lysozyme; lanes 7, 8, and 9, 5 μg of lysozyme carbamoylated for 2, 4, and 7 hr, respectively (see Fig. 1*B*). After incubation at 37°C for 30 min, the samples were subjected to PAGE, as described (5). Markers, in kDa: 116, β-galactosidase; 97, phosphorylase *b*; 66, bovine serum albumin; 45, ovalbumin; 29, carbonic anhydrase.

Naturally Occurring N^{α}-Acetylated Proteins are Not Degraded by the Ub Proteolytic System. Many cellular proteins have acetylated NH_2 termini, but the function of N^{α}-acetylation of proteins is not known (29, 30). It was noted that all previously known good substrates of the ATP/Ub proteolytic system from reticulocytes have free NH_2 termini (such as albumin, lysozyme, globin, α-lactalbumin, and α-casein), whereas ovalbumin, which was known to be inert for proteolysis and conjugation with Ub (8), has an acetylated NH_2 terminus. A more systematic study was therefore initiated to compare the degradation of N^{α}-acetylated and nonacetylated proteins. As shown in Table 2, enolase from muscle (N^{α}-acetylated) is not degraded by the Ub system, whereas yeast enolase (free α-NH_2) is degraded. Similarly, the degradation of heart cytochrome *c* (acetylated) is only slightly stimulated by ATP and Ub, whereas yeast cytochrome *c* (nonacetylated) is a good substrate. The degradation of other N^{α}-acetylated proteins tested (actin, lactate dehydrogenase, carbonic anhydrase, and ovalbumin) either is not stimulated by ATP and Ub or is only very slightly stimulated. Thus far we have found no exception to the rule that naturally N^{α}-acetylated proteins are not degraded by the ubiquitin system to a significant extent. On the other hand, not all proteins having free NH_2 termini are good substrates. For example, aldolase and glyceraldehyde-3-*P* dehydrogenase, which have very slow turnover rates *in vivo* (31), are not degraded significantly by the *in vitro* Ub proteolytic system (Table 2) and have free α-NH_2 termini.

Table 2. Degradation of natural N^{α}-acetylated and nonacetylated proteins

Protein	Source	Degradation rate, %/hr: −ATP (*a*)	+ATP +Ub (*b*)	ATP+Ub-dependent (*b* − *a*)
N^{α}-Acetylated				
Enolase	Rabbit muscle	10.7	9.9	−0.8
Cytochrome *c*	Horse heart	24.4	26.4	2.0
Actin	Bovine muscle	1.9	2.2	0.3
LDH	Rabbit heart	0.8	2.0	1.2
Ovalbumin	Hen egg	2.2	2.0	−0.2
Carbonic anhydrase	Bovine erythrocytes	2.4	1.6	−0.8
Nonacetylated				
Enolase	Yeast	24.2	37.7	13.5
Cytochrome *c*	Yeast	21.8	43.6	21.8
Aldolase	Rabbit muscle	0	0.9	0.9
GAPDH	Rabbit muscle	0.6	0.6	0

All proteins were radiolabeled with ^{125}I. Protein breakdown was assayed for periods of time ranging from 10 min to 2 hr. The results are expressed as percentage degradation per hour and were calculated from the linear parts of the time course. LDH, lactate dehydrogenase; GAPDH, glyceraldehyde-3-*P* dehydrogenase.

α-NH_2 Group in the Absence of ε-NH_2 Groups Is Sufficient for Protein Breakdown. The ATP-dependent degradation of guanidinated proteins has been reported by Hough and Rechsteiner (14). A survey of earlier literature shows that though most or all ε-NH_2 groups of proteins are guanidinated by *O*-methylisourea, the α-NH_2 group never reacts with this agent (32, 33). Analysis of guanidinated lysozyme (Gdn-lysozyme) prepared under conditions of strong guanidination shows 0.5 lysine residue remained *vs.* 1.4 amine residues (Table 3); the difference is accounted for by the free α-NH_2 group. Guanidinated proteins can therefore be used to examine the question whether the α-NH_2 group is not only necessary but also sufficient (in the absence of ε-NH_2 groups) for Ub-dependent proteolysis. As shown in Table 3, the degradation of Gdn-lysozyme by reticulocyte fraction II was significantly stimulated by ATP and Ub, though the Ub-dependent rate was about 25% that of unmodified lysozyme. With various preparations of fraction II, considerable variations were observed in the rate of Ub-dependent degradation of Gdn-lysozyme, ranging from 10% to 90% of that of unmodified lysozyme. When the remaining α-NH_2 group of Gdn-lysozyme was blocked by acetylation or methylation, Ub-dependent degradation was abolished. Similar results were obtained with the derivatives of bovine serum albumin (Table 3) and of ribonuclease S-protein (not shown).

It may be asked whether Ub conjugation is necessary for the degradation of proteins lacking free ε-NH_2 groups. It might be, for example, that free Ub stimulates the activity of an ATP-dependent protease that acts on Gdn-proteins. However, using a part of reticulocyte fraction II from which the Ub-conjugating enzymes had been removed by affinity chromatography (8), we found that the degradation of Gdn-lysozyme (like that of lysozyme) requires not only Ub and ATP but also the three Ub-conjugating enzymes E_1, E_2, and E_3 (data not shown). Furthermore, the genetic evidence already cited (11) establishes Ub activation as a requirement for protein degradation.

DISCUSSION

The present data show that a free α-NH_2 group of the NH_2 terminus of protein is required for its degradation by the ATP/Ub-dependent system of reticulocytes. Since many NH_2 termini are buried within protein structure, the exposure of an α-NH_2 group may serve as a recognition signal for protein breakdown. For example, free subunits of heteromeric proteins may become subject to degradation when present in excess of matching subunits necessary to stabilize their unblocked α-NH_2 groups, possibly a normal occurrence for excess globin chains of hemoglobin. An unblocked cytoplasmic protein may respond to metabolites or regulators particular to each protein, to modify the accessibility of its α-NH_2 residue and thus to control its breakdown by the Ub system. Denaturation may also expose a normally buried NH_2 terminus. The degradation of yeast enolase protein virtually ceased after about 30% of the ^{125}I label became acid solubilized. However, during this time none of the enolase enzymatic activity that survived the iodination procedure was lost, suggesting that only denatured forms were degraded. The fact that some proteins with free α-NH_2 residues, such as muscle aldolase and Ub, are not degraded, may reflect an inaccessibility of NH_2 termini; these proteins are also known to fold readily after denaturation.

The roles of Ub conjugation at the α- and ε-NH_2 groups are not yet clear. Preliminary evidence indicates that Ub can be conjugated to the α-NH_2 group of proteins (unpublished results). The present data show that the formation of high molecular weight Ub-protein conjugates (containing multiple molecules of Ub) is prevented when the α-NH_2 group is blocked (Fig. 3). This is compatible with the notion that the conjugation of Ub to the NH_2 terminus may precede ε-NH_2

Table 3. Degradation of proteins blocked in ε-NH_2 but not α-NH_2 groups

Protein and derivatives*	NH_2 groups remaining: %†	mol/mol‡	Lysine remaining, mol/mol§	Degradation rate, %/hr: −ATP	+ATP	+ATP +Ub
Lysozyme	100	7	6	2.8	5.0	34.2
Gdn-lysozyme	19.5	1.4	0.5	2.2	3.0	9.8
Ac,Gdn-lysozyme	1.0	0.07		2.7	2.9	1.6
Me,Gdn-lysozyme	0.2	0.01		2.3	2.2	2.2
Albumin	100	60	59	0.8	0.7	11.6
Gdn-albumin	11.6	7	8.4	0	0	6.2
Me-albumin	1.0	0.6		0.7	0.8	0.9
Me,Gdn-albumin	1.7	1.0		0.1	0.1	0.2

*Proteins were modified as described in *Materials and Methods*, except that reductive methylation was with 12 mM formaldehyde and 20 mM cyanoborohydride, for 20 hr at room temperature. Ac,Gdn- and Me,Gdn- indicate the protein was first guanidinated and then acetylated or reductively methylated, respectively.

†Assayed by ratio of fluorescamine reactivity to A_{280} relative to that of the unmodified protein.

‡Assuming that the various amines in the protein have identical fluorescamine reactivity.

§Determined by amino acid analysis after acid hydrolysis.

conjugation in the formation of high-molecular weight Ub-protein conjugates. It is clear, however, that other types of α-NH_2-independent conjugation reactions of Ub also occur. We find that with the α-NH_2-blocked derivative of lysozyme (though not with that of globin), the formation of low-molecular weight Ub conjugates is increased (Fig. 3). Furthermore, it is well known that a single Ub is conjugated to the ϵ-NH_2 group of a unique lysine residue of histone 2A, the α-NH_2 group of which is acetylated (9). This probably serves for protein modification rather than breakdown, since the turnover of the histone is much slower than that of its Ub moiety (1). Thus, a free α-NH_2 appears to be required for a pathway specific for the formation of high molecular weight Ub-protein conjugates committed for degradation. It may be mentioned that only the high molecular weight Ub-protein conjugates are substrates for degradation (10).

We find that guanidinated proteins, in which most ϵ-NH_2 (but not α-NH_2) groups are blocked, are degraded by the Ub system (Table 3). However, conjugation of Ub to lysine residues may still have a role, since the degradation of Gdn-proteins is generally slowed down in the presently employed experimental conditions. We observed that high concentrations of fraction II inhibit the breakdown of Gdn-lysozyme, without inhibiting that of unmodified lysozyme (unpublished results). This might be due to competition with endogenous protein substrates, which may indicate that conjugation of Ub to ϵ-NH_2 groups increases the affinity of the substrate to some component of the system.

The finding that natural N^{α}-acetylated proteins are not degraded by the Ub-dependent cell-free system (Table 2) raises the question as to whether α-NH_2-acetylation protects cellular proteins against degradation. N^{α}-acetylation of cellular proteins is a widespread process, and it has been reported that 80% of the soluble proteins of mammalian cells are N^{α}-acetylated (34). The idea that acetylation of NH_2-terminal amino groups may have a protective function against proteolysis was suggested by Jörnvall (29), in a theoretical study on the structural characteristics of 40 N^{α}-acetylated proteins. On the other hand, Brown (35) reported that N^{α}-acetylated and nonacetylated total cellular proteins in cultured L cells turned over at similar rates. In the latter study, it is not entirely clear how specific were the methods used for the analysis of acetyl groups bound to α-NH_2 residues. However, other reports also indicate that some proteins blocked in α-NH_2 residues can be degraded in intact cells. When totally methylated lysozyme or albumin (12) or α-NH_2-carbamoylated denatured hemoglobin (14, 36) was introduced into cultured cells by an erythrocyte-mediated microinjection technique, these proteins were degraded at relatively rapid rates. It is possible that some microinjected blocked proteins are subject to processes of unblocking, or even to a single endoproteolytic cleavage that would expose a new NH_2-terminal α-NH_2 group. It is also possible, however, that proteins blocked in NH_2 groups are degraded by an ATP-dependent pathway that differs from the Ub system, as suggested by Katznelson and Kulka (12). Tanaka *et al.* (13) observed that proteins with completely blocked NH_2 groups are still degraded in reticulocyte lysates by an ATP-dependent process, though rates of degradation were always reduced in comparison with unmodified proteins. In the present study, we find that the slight stimulation of the breakdown of globin caused by ATP (without Ub) is not affected by N^{α}-carbamoylation, while Ub-dependent degradation is drastically reduced (Fig. 2). This ATP-dependent degradation of NH_2-blocked proteins is even more noticeable in crude lysates of reticulocytes and seems to be partially lost in fraction II (unpublished results). Thus, some α-NH_2-blocked proteins may be degraded by non-Ub-dependent proteolytic systems. Other possible pathways for the degradation of N^{α}-acetylated proteins, such as selective deacetylation followed by Ub-dependent breakdown, remain to be investigated.

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