Differential Effects of Ubiquitin Aldehyde on Ubiquitin and ATP-Dependent Protein Degradation†

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ABSTRACT: ATP-dependent proteolysis of ^{125}I-labeled human α-globin, bovine α-lactalbumin, bovine serum albumin, or chicken lysozyme was assessed in a rabbit reticulocyte extract supplemented with ATP, excess ubiquitin, and variable amounts of ubiquitin aldehyde (Ubal), an inhibitor of many ubiquitin−protein isopeptidases. Low concentrations (0.8 μM) of Ubal increased the ATP-dependent degradation of ^{125}I-α-globin by ∼30% after 2 h at 37 °C, had little effect on ^{125}I-lysozyme turnover, and decreased ^{125}I-α-lactalbumin or ^{125}I-albumin degradation by ∼20%. The ATP-dependent degradation of all substrates was inhibited by high concentrations (>3 μM) of Ubal throughout the incubation (15 min to 2 h); after 2 h, this inhibition ranged from 15% for ^{125}I-α-globin to ∼85% for ^{125}I-α-lactalbumin and ^{125}I-albumin. Levels of ubiquitin−^{125}I-protein conjugates were increased significantly with Ubal; with ≥8.0 μM Ubal, high molecular mass multiubiquitinated conjugates were particularly evident for ^{125}I-α-globin and ^{125}I-α-lactalbumin. These mixtures also accumulated ubiquitin conjugates with sizes expected for di- through pentaubiquitin oligomers. The results are consistent with the following proposed events: The ATP-dependent degradation of ^{125}I-α-lactalbumin or ^{125}I-albumin is probably mediated almost exclusively through polyubiquitinated intermediates. High Ubal concentrations inhibit an isopeptidase(s) which normally disassembles "unanchored" polyubiquitin chains that remain after substrate degradation by the 26S proteasome; these chains accumulate to inhibit further conjugate degradation. Much of the ATP-dependent degradation of ^{125}I-α-globin and, to a lesser degree, ^{125}I-lysozyme may occur through alternative structures where ubiquitin monomers or short oligomers are ligated to one or more substrate lysines. For ^{125}I-α-globin, even low concentrations of Ubal effectively inhibit deubiquitination of these conjugates to enhance α-globin degradation.

In the ATP- and ubiquitin-dependent pathway for the proteolysis of intracellular proteins, the carboxyl terminus of the monomeric protein ubiquitin (M_r = 8565) is covalently linked to the ϵ-amino group of a lysine residue of the protein destined for degradation (for recent reviews, see Ciechanover, 1994; Hochstrasser, 1995). In some cases, ubiquitins are linked by this "isopeptide" bond to multiple lysine residues of the protein substrate (Shaeffer, 1994a). In other situations, additional Ub[1] molecules extend from the first to form a branched-chain polyubiquitin moiety linked by isopeptide bonds from the C-terminus of one Ub to an ϵ-amino group (generally on Lys48) of another (Chau *et al.*, 1989). Regardless of which form of ubiquitination is used to prepare the substrate protein for proteolysis, the Ub "tag" of the conjugate is then recognized by a large, 26S multisubunit protease or "proteasome" present in the cytoplasm (reviewed by Rubin & Finley, 1995). The protein substrate moiety is degraded by this 26S complex, whereas the Ub components are returned to the soluble pool of free Ub monomers.

Levels of free Ub are maintained in part by Ub C-terminal hydrolases which release Ub from various C-terminal adducts (Rose, 1988). Some of these hydrolases are isopeptidases which may be involved in the proteolysis scheme outlined above. For example, enzymes which deubiquitinate newly-formed Ub−protein conjugates (Hershko *et al.*, 1984; Hough & Rechsteiner, 1986; Hershko & Rose, 1987) cleave the isopeptide bond(s) between the Ub (or polyubiquitin) adduct and the substrate ϵ-amino group during protein degradation by the 26S proteasome (Eytan *et al.*, 1993), or disassemble polyubiquitin chains (*e.g.*, isopeptidase T; Hadari *et al.*, 1992) have been reported. The role that each of these isopeptidases has in the proteolysis of a particular protein substrate is likely to depend on the pattern of its ubiquitination and the mechanism of its recognition and degradation by the 26S proteasome. Selective inhibition of these enzymes might reveal their functions in Ub-dependent proteolysis and, in turn, differentiate protein substrates with respect to their patterns of ubiquitination and modes of degradation.

In their studies of a 30 kDa Ub C-terminal hydrolase from rabbit reticulocytes, Pickart and Rose (1986) found that ubiquitin aldehyde (Ubal), *i.e.*, Ub modified to have an aldehyde instead of a carboxyl group at its C-terminus, was a potent inhibitor. Subsequently, Ubal was found to inhibit many, although not all, Ub−protein isopeptidases. For example, among the isopeptidases mentioned above, Ubal inhibited isopeptidase T (Hadari *et al.*, 1992) and the as yet uncharacterized isopeptidase(s) which disassembles newly-formed conjugates (Hershko *et al.*, 1984; Hough & Rechsteiner, 1986; Hershko & Rose, 1987), but not an ATP-stimulated isopeptidase associated with the 26S proteasome (Eytan *et al.*, 1993). Thus, Ubal potentially could be used to elucidate roles of isopeptidases in protein ubiquitination

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[1] Abbreviations: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; Ub, ubiquitin; Ubal, ubiquitin aldehyde.

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and degradation.

Addition of Ubal severely inhibited the ATP-dependent degradation of ^{125}I-lysozyme in a crude reticulocyte extract (fraction II), but only when Ub was present in limiting amounts (Hershko & Rose, 1987). Accordingly, the pool of endogenous free Ub was progressively diminished during the reaction in the presence, but not in the absence, of Ubal. The inhibition of ^{125}I-lysozyme degradation was almost abolished by the addition of excess Ub. These results suggested that Ubal inhibited Ub C-terminal hydrolases responsible for recycling Ub in the cell extract. Much less is known about the effect of Ubal on the various stages of the proteolytic pathway in the presence of excess Ub. The condition of excess Ub presumably will be necessary to study selective inhibition of the various isopeptidases directly involved in protein degradation without the complications caused by Ub depletion. In the investigation discussed above, the amounts of Ub−protein conjugate intermediates were increased substantially when Ubal was added to the proteolysis reaction in the presence of adequate supplementary Ub. This effect of Ubal was used by us to isolate Ub conjugates of yeast cytochromes *c* (Sokolik & Cohen, 1991, 1992) and of human α-globin (Shaeffer, 1994a) in amounts sufficient for structural studies.

The objective of the present work was to compare the effects of Ubal on the ATP-dependent degradation of several ^{125}I-labeled proteins in an unfractionated rabbit reticulocyte extract. The effect of this isopeptidase inhibitor on degradation varied with its concentration, the time of incubation, and, most notably, the protein substrate. These variations are consistent with proposed patterns of ubiquitination and subsequent degradation that differ among the substrates. The results provide the first experimental evidence in support of an "editing" role of Ub−protein isopeptidase(s) in which disassembly of ubiquitinated proteins competes with their degradation by the 26S proteasome.

MATERIALS AND METHODS

Preparation of Rabbit Reticulocyte Extract and ^{125}I-Labeled Protein Substrates. Rabbits were made anemic with phenylhydrazine, and the red blood cells (approximately 80% reticulocytes) were harvested and washed (Allen & Schweet, 1962). A cell extract was prepared, dialyzed against hemolysate buffer, and concentrated to 150 mg/mL (hemoglobin A_{540}) as described previously for human blood (Shaeffer, 1988), except that the crude lysate was centrifuged (80000*g*) for 2 h at 4 °C to remove polyribosomes and residual stroma before dialysis. Human α-globin was prepared from the α chains of adult human hemoglobin A, labeled with Na^{125}I using Iodo-Beads (Pierce Chemical Co.), purified by SDS−PAGE to remove contaminating aggregates, treated with triethylamine to remove SDS, and dissolved in 10 mM Na^+ formate, pH 4.0, as described previously (Shaeffer, 1994b). About 1.0 mg each of bovine α-lactalbumin (Sigma, L5385), chicken lysozyme (Sigma, L6876), bovine ubiquitin (Sigma, U6253), or bovine serum albumin (Sigma, A7030) was labeled with 1.0 mCi of Na^{125}I using chloramine T (Ciechanover *et al.*, 1980), and the iodinated proteins were desalted by gel filtration (Pharmacia NP10 columns) in H_2O. ^{125}I-Labeled proteins (except the ^{125}I-Ub) were concentrated to 1−2 mg of protein/mL (determined with the Bio-Rad dye-binding protein microassay) by centrifugation at 4 °C in Amicon Centricon-10 concentrators. Aliquots of the ^{125}I-proteins were stored at −20 °C.

Construction and Incubation of Proteolysis Reaction Mixtures. Each proteolysis reaction mixture in a total volume of 25 μL contained the following: 50 mM Tris·HCl, pH 7.5 (23 °C), 3 mM dithiothreitol, 5 mM $MgCl_2$, 1.25 mg of dialyzed rabbit reticulocyte extract, and 3.75 μg (∼2 × 10^6 cpm) of an ^{125}I-labeled protein as the proteolysis substrate. Some reaction mixtures (designated "+ATP") also contained 0.5 mM ATP (Na^+), 10 mM creatine phosphate, 24 units/mL creatine phosphokinase (Sigma, C3755), and 2 units/mL inorganic pyrophosphatase (Sigma, I1643). The +ATP mixtures usually were supplemented with either 141 or 282 μM Ub. Some mixtures also contained Ub aldehyde (Ubal), which was prepared by a modification (manuscript in preparation) of the procedure described previously (Dunten & Cohen, 1989). For experiments that employed a range of Ubal concentrations, from 1.25 to 10 μL of a stock solution of Ubal in H_2O was transferred to a microcentrifuge tube and evaporated to dryness *in vacuo*. The residue was redissolved with the reticulocyte extract, and this mixture was preincubated at 37 °C for 5 min before addition of the other components. For all other reactions, a batch mixture containing the reticulocyte supernatant and Ubal at about twice its final concentration (or H_2O as a control) was preincubated at 37 °C for 5 min. After cooling on ice, the ^{125}I-labeled protein substrate was added, and identical aliquots of this batch mixture were transferred to tubes containing the other reagents. Each tube was sealed and then incubated at 37 °C for the time specified.

Assay for Degradation of ^{125}I-Labeled Protein Substrates. Proteolysis reactions were stopped by chilling on ice, and 2 μL (∼1.5 × 10^5 cpm) was transferred to a 1.5 mL microcentrifuge tube containing 7.7 μL of a human erythrocyte lysate (150 mg/mL) as carrier protein and 15.3 μL of H_2O. Proteins were precipitated by the addition of 0.5 mL of 10% trichloroacetic acid, incubated at 4 °C for 1 h, and centrifuged at 14000*g* for 5 min, and the soluble phase was transferred to a 3 mL plastic tube. The pellet (acid-insoluble phase) was dissolved in 0.5 mL of 1 N NaOH and transferred to a separate tube. The ^{125}I-radioactivities of the acid-soluble and acid-insoluble phases were determined in a Packard Multi-Prias γ-counter. Protein degradation was calculated as the fraction (%) of the total ^{125}I-radioactivity recovered in the acid-soluble phase corrected for that (≤3.0%) obtained from an unincubated control reaction.

Detection and Quantification of Ubiquitin−^{125}I-Labeled Protein Conjugates by SDS−PAGE. Aliquots (4 μL) from the proteolysis reactions were mixed with 36 μL of SDS−PAGE sample buffer (Laemmli, 1970), heated at 95 °C for 7 min, and then subjected to SDS−PAGE on 1.5 mm thick slab gels (13% acrylamide) essentially as described previously (Shaeffer, 1994b). Molecular weight standards from 14.2 to 66 kDa (Sigma, SDS-7) were used. After electrophoresis, the separating gel was fixed in 10% acetic acid/37% methanol for 1 h and stained with 0.05% Coomassie Brilliant Blue R250 in 10% acetic acid/50% methanol for 2 h. The stained gel was rinsed briefly in 10% acetic acid/25% methanol and then destained with 25% methanol. The gel was dried *in vacuo* at 70 °C between two sheets of cellophane and counted for 10 h in a Packard InstantImager to determine the distribution of ^{125}I-radioactivity in each

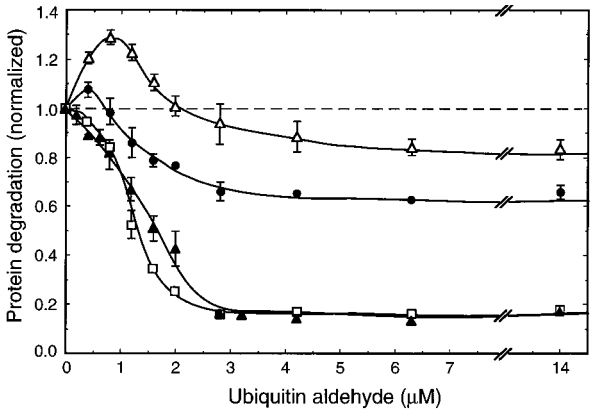

FIGURE 1: Effect of Ubal on the ATP-dependent proteolysis of ^{125}I-labeled substrates by a rabbit reticulocyte extract. Each reaction mixture was incubated at 37 °C for 2 h with either ^{125}I-human α-globin (unfilled triangles), ^{125}I-chicken lysozyme (filled circles), ^{125}I-bovine α-lactalbumin (unfilled squares), or ^{125}I-bovine serum albumin (filled triangles) with or without ATP and an ATP-regenerating system. The +ATP mixtures were supplemented with 282 μM Ub or, in reactions with ^{125}I-bovine serum albumin, 141 μM Ub. Protein degradation was determined as described in Materials and Methods. The values obtained in the absence of ATP (5.3%, 8.9%, 24.6%, and 1.7% for ^{125}I-labeled α-globin, lysozyme, α-lactalbumin, and albumin, respectively) remained essentially constant with the different Ubal concentrations and were subtracted from the corresponding +ATP values. For each Ubal concentration, ATP-dependent proteolysis values are plotted as the ratio to the ATP-dependent proteolysis *without* added Ubal (39.0%, 28.7%, 39.5%, and 17.4% for ^{125}I-labeled α-globin, lysozyme, α-lactalbumin, and albumin, respectively). Most data points shown are averages of 2−4 experiments; standard errors are indicated by the error bars (some are obscured by the point symbols).

sample lane. The ^{125}I-containing bands in each lane that represented undegraded substrate and its conjugates with either one (Ub_1), two (Ub_2), from three to six (Ub_{3-6}), or more than six ($Ub_{>6}$) molecules of Ub were identified by comparison with the positions of the stained molecular weight standards.

RESULTS

Protein Degradation Is Modulated by Ubiquitin Aldehyde in a Substrate-Dependent Manner. Initial studies showed that the proteolysis (conversion to acid-soluble ^{125}I-radioactivity) of the four substrates by the reticulocyte extract *in the absence of added Ub* ranged from 16% for ^{125}I-bovine serum albumin to 67% for ^{125}I-bovine α-lactalbumin after incubation at 37 °C for 2 h (data not shown). Most of this degradation was ATP-dependent. Addition of Ubal at a moderate concentration (2 μM) to a similar reaction mixture inhibited the ATP-dependent degradation by 43−80%. These results confirm and extend that observed previously with ^{125}I-lysozyme (Hershko & Rose, 1987) and occur presumably because of limiting amounts of endogenous Ub in the reticulocyte extract. Therefore, the reaction mixtures used in our subsequent studies to determine the effect of Ubal on the ATP- and Ub-dependent pathway were always supplemented with ≥100 μM Ub (see Materials and Methods); under these conditions, degradation rates were not limited by the Ub concentration (see below).

Proteolysis reaction mixtures containing each of the ^{125}I-labeled protein substrates were incubated with increasing concentrations of Ubal at 37 °C for 2 h. Figure 1 shows that the effect of Ubal on protein degradation was substrate dependent. With low Ubal (<2 μM), the ATP-dependent proteolysis of ^{125}I-bovine α-lactalbumin or of ^{125}I-bovine serum albumin was severely inhibited, whereas that of ^{125}I-human α-globin was increased. With more than 3 μM Ubal, the ATP-dependent proteolysis of each of the substrates was decreased below the levels observed in the absence of the inhibitor. However, there was still substantial ATP-dependent (and presumably Ub-dependent) degradation of ^{125}I-human α-globin and of ^{125}I-chicken lysozyme at the highest concentrations of Ubal used. The results of Figure 1 suggest a difference in Ubal-sensitive isopeptidase involvement, and possibly a difference in mechanism or rate-limiting step, in the degradation of at least some of the ^{125}I-α-globin and ^{125}I-lysozyme[2] substrates compared to ^{125}I-α-lactalbumin and ^{125}I-albumin.

Table 1: Supplementation with Ub Does Not Reverse the Inhibition or Enhancement of Proteolysis by Ubal[a]

^{125}I-protein substrate	Ubal (μM)	Ub (mM)[b]	proteolysis (%)
bovine α-lactalbumin	0	0.28	66.3
		0.70	64.1
	8.0	0.14	34.0
		0.28	32.8
		0.70	34.3
		1.41	33.6
chicken lysozyme	0	0.05	37.5
	7.0	0.05	25.4
		0.14	26.1
		0.28	29.7
		0.75	27.2
		1.22	27.3
bovine serum albumin	0	0.05	17.7
		0.14	16.6
		0.28	13.9
		0.70	10.3
	8.0	0.05	4.7
		0.14	5.0
		0.28	4.9
		0.70	4.5
human α-globin	0	0.05	37.9
		0.14	37.6
	0.8	0.05	48.5
		0.14	49.6
		0.28	49.9
		0.75	48.2
	3.2	0.05	36.4
		0.14	36.6
		0.28	36.9
		0.75	36.5

[a] Reaction mixtures containing ATP and an ATP-regenerating system were incubated at 37 °C for 2 h, and the amount of proteolysis was determined as described in Materials and Methods. The values shown were not corrected for ATP-independent proteolysis. [b] Endogenous Ub (<10 μM; Shaeffer, 1988) is not included.

The data in Figure 1 do not represent initial rates, and this point is elaborated in the section below. To ensure that the inhibition of proteolysis observed in Figure 1 with high Ubal concentrations was not caused by Ub depletion, otherwise identical mixtures were incubated with additional free Ub. Table 1 shows that supplementary Ub even in excess of 1 mM did not reverse the inhibition of proteolysis by high concentrations (3.2−8.0 μM) of Ubal. Moreover,

[2] Reduction of a specific disulfide bond in lysozyme precedes its ubiquitination and can be rate-limiting for lysozyme's degradation (Dunten *et al*., 1991). This slow step might contribute to the relatively weak inhibition by Ubal seen for the degradation of ^{125}I-lysozyme. We thank one of the reviewers for bringing this possibility to our attention.

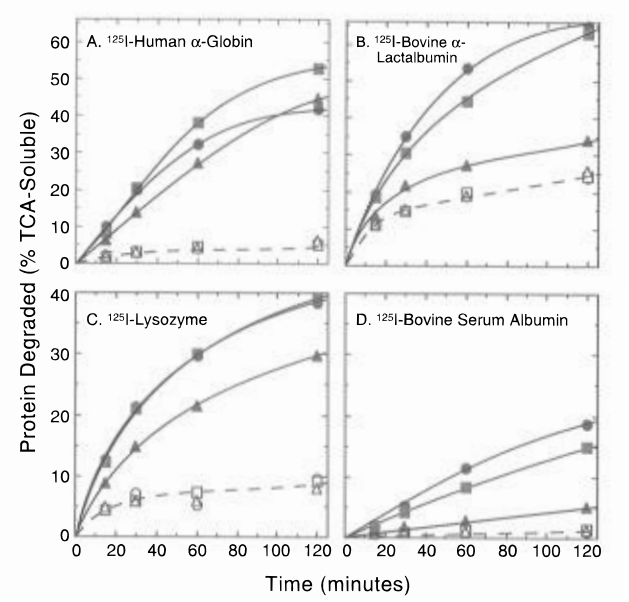

FIGURE 2: Degradation of ^{125}I-labeled proteins with zero, low (0.8 μM), or high (8.0 μM) concentrations of Ubal. Reaction mixtures were prepared with or without ATP (and an ATP-regenerating system) and incubated at 37 °C for either 0, 15, 30, 60, or 120 min as described in Materials and Methods. The amount of proteolysis in each reaction mixture was determined after incubation with one of the following substrates: ^{125}I-human α-globin (panel A), ^{125}I-bovine α-lactalbumin (panel B), ^{125}I-chicken lysozyme (panel C), or ^{125}I-bovine serum albumin (panel D). The results are plotted for mixtures with ATP and no (filled circles), 0.8 μM (filled squares), or 8.0 μM (filled triangles) Ubal; the unfilled symbols represent data from the corresponding mixtures without added ATP. Note that most of the nonlinearity of the curves in panels B, C, and D can be accounted for by the ATP-independent components of the degradation reactions. The data shown are from single experiments.

the extra Ub did not affect the ~30% increase in proteolysis of ^{125}I-human α-globin observed with 0.8 μM Ubal. The addition of supplementary Ub to reaction mixtures without Ubal increased the proteolysis of each ^{125}I-labeled protein substrate only slightly, if at all, beyond that catalyzed by the Ub endogenous to the unfractionated reticulocyte lysate (data not shown). Table 1 shows, however, that the degradation of ^{125}I-bovine serum albumin in the absence of Ubal was significantly inhibited with >141 μM of added Ub. The reason for this inhibition remains unknown. Nevertheless, in the experiments described in Figure 1 and below, proteolysis reactions with ^{125}I-albumin were supplemented with 141 μM Ub, whereas 282 μM Ub was added with the other substrates.

Time Course of Ubal's Effect on Protein Turnover. Proteolysis reaction mixtures with each of the four ^{125}I-labeled protein substrates containing either zero, low (0.8 μM), or high (8.0 μM) Ubal were incubated from 15 min to 2 h at 37 °C. The unique enhancement of ATP-dependent proteolysis observed for ^{125}I-α globin at low Ubal concentrations was apparent only after 30 min (Figure 2A). This time dependence, which was not observed with the other substrates, could be due to a lag in the accumulation of Ub−protein conjugates or, more directly, to slow-binding inhibition by Ubal of an isopeptidase. By either mechanism, the result further suggests that, for at least some fraction of the ^{125}I-α-globin, there is a different ubiquitination pattern or rate-limiting step for Ub-catalyzed degradation compared to that of the other substrates. On the other hand, the inhibition of ATP-dependent proteolysis with high Ubal concentrations was readily observed for all substrates as early as 15 min (Figure 2) and continued throughout the incubation period. Together with the finding that inhibition was not further increased with Ubal concentrations greater than ~3 μM (Figure 1), this result indicates that the degradation of all of the substrates was promoted, directly or indirectly, by a common Ubal-sensitive activity, presumably a Ub−protein isopeptidase(s). Figure 2 also shows that, as expected, increasing concentrations of Ubal had no effect on the background level of ATP-independent proteolysis for each substrate.

Ubal Enhances Levels of Ubiquitin−Protein Conjugates. Samples of selected reaction mixtures from the experiments of Figure 2, panels A−C, were denatured with SDS−PAGE sample buffer, and the patterns of the Ub−^{125}I-protein conjugates were analyzed by SDS−PAGE. Figure 3A shows the Coomassie Blue-stained proteins in the reaction mixtures with ^{125}I-human α-globin, and Figure 3B shows the ^{125}I-labeled species. Quantitative analyses of the data from this gel and two similar gels with samples from reactions with ^{125}I-α-lactalbumin or ^{125}I-lysozyme are presented in Table 2. The concentrations of all Ub−protein conjugates for each of the three ^{125}I-labeled protein substrates were increased by addition of Ubal to the +ATP reaction mixtures. These results confirm those obtained previously from ubiquitination reactions catalyzed by reticulocyte extracts containing as substrates either oxidized RNase A (Hershko & Rose, 1987; Dunten & Cohen, 1989), lysozyme (Hershko & Rose, 1987), cytochrome *c* (Sokolik & Cohen, 1991), or α-globin (Shaeffer, 1994b). Increased conjugate levels were evident particularly at the later incubation times with high (8.0 μM) Ubal for the multiubiquitinated ($Ub_{>6}$) conjugates of ^{125}I-human α-globin (Figure 3B, lane 7, and Table 2, line 7) and of ^{125}I-bovine α-lactalbumin (Table 2, line 13). A similar finding, although less pronounced, was observed for the $Ub_{>6}$−^{125}I-lysozyme conjugates.

Inhibition of Degradation Correlates with Polyubiquitin Chain Accumulation. The accumulation of Ub−protein conjugates together with the inhibition in the same mixtures of total ATP-dependent proteolysis of the ^{125}I-proteins (Figure 2) suggests that Ubal, either directly or indirectly, inhibits degradation of polyubiquitinated conjugates by the 26S proteasome. Indirect inhibition could occur by the binding of "unanchored" polyubiquitin chains, *i.e.*, polyubiquitin chains not ligated to a protein substrate, at the 26S proteasome site normally reserved for polyubiquitinated degradation intermediates (Deveraux *et al.*, 1994; Beal *et al.*, 1996). Excess unanchored polyubiquitin chains might accumulate in a reaction mixture with Ubal due to inhibition of isopeptidases which normally disassemble them. Coomassie Blue-stained proteins from the reaction mixtures with ^{125}I-α-globin (Figure 3A) in the presence of Ubal showed bands at positions expected for diubiquitin (Ub_2), triubiquitin (Ub_3), and tetraubiquitin (Ub_4). Similar patterns of these putative unanchored polyubiquitin chains in the reaction mixtures containing Ubal and ^{125}I-α-lactalbumin or ^{125}I-lysozyme were observed (not shown). Stained bands representing polyubiquitin chains of molecular masses higher than Ub_4 were not seen, possibly because of the overlapping array of intensely-stained reticulocyte proteins.

Evidence for the accumulation of polyubiquitin chains also was obtained from experiments that employed ^{125}I-labeled

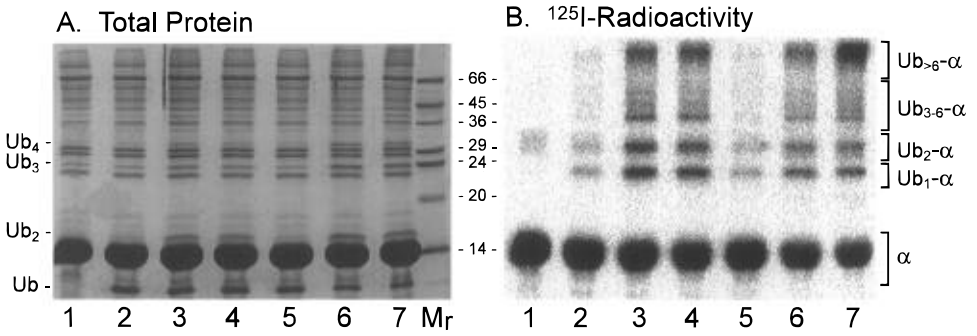

FIGURE 3: Total and ^{125}I-labeled proteins of proteolysis reaction mixtures with ^{125}I-human α-globin. Samples of reaction mixtures selected from those in the experiment of Figure 2A were analyzed by SDS−PAGE as described in Materials and Methods. The samples were from mixtures which received no incubation (lane 1) or were incubated with ATP (and an ATP-regenerating system) at 37 °C for 15 min with no (lane 2), 0.8 μM (lane 3), or 8.0 μM (lane 4) Ubal, or at 37 °C for 60 min with no (lane 5), 0.8 μM (lane 6), or 8.0 μM (lane 7) Ubal. The gels were stained with Coomassie Blue and dried (panel A), and ^{125}I-radioactivity was detected by counting in a Packard InstantImager (panel B). Stained protein bands representing monomeric Ub (Ub) and putative unanchored chains (see text) of diubiquitin (Ub_2), triubiquitin (Ub_3), and tetraubiquitin (Ub_4) are identified in panel A. The positions and molecular masses (in kDa) of marker proteins, shown in the border lane (M_r) of panel A, also are indicated on the left side of panel B. The ^{125}I-radioactivity in the regions of each gel lane denoted by the brackets corresponds to undegraded substrate (α) or conjugates of ^{125}I-α-globin with either one (Ub_1-α), two (Ub_2-α), three to six (Ub_{3-6}-α), or more than six ($Ub_{>6}$-α) molecules of Ub; these results are quantified in Table 2.

Table 2: Fates of ^{125}I-Labeled Proteins after Incubation in a Reticulocyte Lysate with Ub, ATP, and Various Amounts of Ubal[a]

reaction conditions		distribution of ^{125}I-radioactivity (%)					
time (min)	Ubal (μM)	TCA-soluble	intact substrate	conjugate species[b] Ub_1	Ub_2	Ub_{3-6}	$Ub_{>6}$
		^{125}I-Human α-Globin					
15	0	9.2	82.8	3.1	1.1	2.4	1.5
	0.8	7.3	49.1	10.6	8.0	13.9	11.1
	8.0	5.7	47.6	10.4	8.2	14.1	14.1
60	0	31.5	59.9	2.9	1.7	2.6	1.4
	0.8	36.9	32.5	6.1	5.8	9.7	9.1
	8.0	28.2	25.9	5.6	5.5	12.7	22.1
		^{125}I-Bovine α-Lactalbumin					
15	0	19.2	65.9	5.7	2.7	3.9	2.7
	0.8	18.4	47.1	13.1	6.6	10.0	4.8
	8.0	13.5	43.9	16.8	7.4	11.9	6.4
120	0	65.1	21.5	1.8	1.4	4.9	5.3
	8.0	33.9	6.1	1.0	2.2	23.7	33.0
		^{125}I-Chicken Lysozyme					
15	0	12.7	82.7	1.1	0.2	1.9	1.4
	0.8	12.3	77.3	2.8	0.9	3.0	3.8
	8.0	8.8	77.0	2.8	1.6	3.8	6.0
120	0	38.3	58.2	1.3	0.2	1.4	0.6
	0.8	38.9	53.9	2.1	1.0	2.4	1.8
	8.0	29.6	57.7	2.4	1.8	3.7	4.9

[a] Samples selected from the reaction mixtures described in Figure 2 were analyzed for conjugate formation by SDS−PAGE and assayed for total protein degradation (TCA-soluble radioactivity). The total ^{125}I-radioactivity in each lane was assumed to represent the TCA-insoluble fraction, and values for the conjugate species were corrected for radioactivity in the corresponding gel zone of a control (unincubated) reaction mixture. [b] Ub_n−substrate conjugates were resolved by SDS−PAGE, and the extent of ubiquitination (n) was determined by comparison to molecular mass standards (see Figure 3).

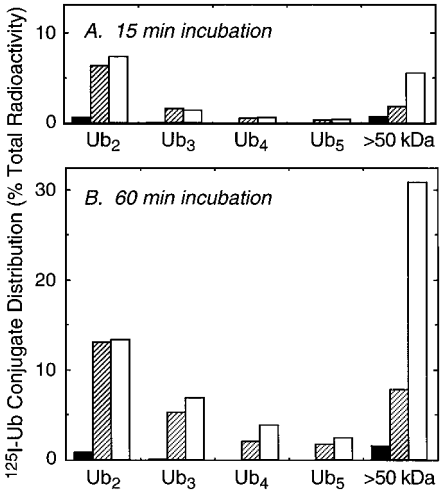

FIGURE 4: Ubal promotes the accumulation of polyubiquitin conjugates. Reactions were as in Figure 3, except that unlabeled α-globin (0.15 μg/μL) and ^{125}I-labeled Ub (282 μM; 10^4 cpm/μg) were used. Samples from 15 min (panel A) and 60 min (panel B) incubations were analyzed by SDS−PAGE and the distributions of ^{125}I-radioactivity measured with the Packard InstantImager. Discrete ^{125}I-labeled bands corresponding in size to Ub and polyubiquitin chains (Ub_n, $n = 2-5$) were observed; higher M_r material (>50 kDa) was an indistinct smear at the top of each lane. These species are shown as the fraction (%) of the total ^{125}I-radioactivity for reaction mixtures with zero (solid bars), 0.8 (hatched bars), or 8.0 (unfilled bars) μM Ubal. In each lane, free ^{125}I-Ub accounted for the balance of the radioactivity. When α-globin was omitted from otherwise identical reaction mixtures with 8.0 μM Ubal, virtually the same results were obtained as shown in panel B.

Ub (Figure 4).[3] In the presence of 0.8 or 8.0 μM Ubal, distinct radiolabeled species formed whose sizes correlated with Ub_2, Ub_3, Ub_4, and Ub_5. The concentrations of these oligomers were only slightly higher with 8.0 μM as compared to 0.8 μM Ubal, and in either condition, approximately 18 μM Ub_2, 6 μM Ub_3, 2 μM Ub_4, and 1 μM Ub_5 had accumulated during the 60 min incubation (Figure 4B). These concentrations are 2- to 6-fold higher than those observed after only 15 min (Figure 4A). Additionally, ^{125}I-Ub was incorporated into high molecular mass conjugates of *ca.* >50 kDa; in this size range, the higher Ubal concentration yielded significantly more labeled product. To what extent this material included polyubiquitin conjugated to other proteins in addition to "unanchored"[4] chains is not known.

Low concentrations (0.8 μM) of Ubal promoted the disappearance of ^{125}I-human α-globin or of ^{125}I-bovine

[3] Previously, the accumulation of $Ub_{\leq 4}$ oligomers in Ubal-supplemented reticulocyte fraction II had been noted for Ub−cytochrome *c* conjugation reaction mixtures (Sokolik & Cohen, 1991).

[4] "Unanchored" is used here to include polyubiquitin chains which may have an attached substrate peptide remnant as well as those with a free Ub carboxyl terminus.

α-lactalbumin from the substrate pool (Table 2). This phenomenon was readily observed after only 15 min of incubation and was accompanied by an increase of conjugates, particularly those containing one or two ubiquitins (Ub_1 and Ub_2 in Table 2). These results are consistent with the hypothesis that Ubal inhibits "disassembly" of the low molecular mass conjugates of these substrates, thereby preventing return of the unconjugated protein to the substrate pool. Inhibition by Ubal of the disassembly of conjugates of ^{125}I-lysozyme ligated to methylated Ub molecules has been reported (Hershko & Rose, 1987). However, with unmodified Ub, we did not observe a similar Ubal-promoted disappearance of ^{125}I-lysozyme from the substrate pool (Table 2). Perhaps the Ub_1− and Ub_2−^{125}I-lysozyme conjugates are less susceptible to disassembly by isopeptidases in the reaction mixture (see the Discussion).

DISCUSSION

Several isopeptidases probably are directly involved in the ATP- and Ub-dependent pathway for proteolysis of intracellular proteins. Among these are isopeptidases which disassemble Ub−protein conjugates, remove Ub (or polyubiquitin) at the 26S proteasome during degradation of the substrate protein, hydrolyze peptide remnants from (poly)-ubiquitin chains, and recycle Ub by polyubiquitin chain disassembly. We have studied the effect of Ubal, an inhibitor of many isopeptidases, on this pathway in an attempt to understand better the mechanism of degradation of various protein substrates. The pattern of ubiquitination and subsequent ATP-dependent degradation of a protein substrate may depend on both its primary and higher-order structures (Dunten & Cohen, 1989; Sokolik & Cohen, 1992; Varshavsky, 1992; Hill *et al.*, 1993). For example, the Ub conjugates of chicken lysozyme (Hershko *et al.*, 1984; Hough & Rechsteiner, 1986), bovine α-lactalbumin, and bovine serum albumin probably have a branched-chain polyubiquitin moiety attached to one (or a few) substrate lysine residues, whereas the conjugates of human α-globin may consist primarily of monomeric Ub molecules attached to several α-globin lysines (Shaeffer, 1994a). With the former proteins, which generally are not native to the cytoplasm and possess "destabilizing" N-terminal amino acid residues, E3α or E3β Ub−protein ligases catalyze rapid and probably processive polyubiquitin chain assembly (Varshavsky, 1992; Ciechanover, 1994). These N-end recognizing Ub−protein ligases are not likely to be involved in the degradation of α-globin, which has a "stabilizing" residue (valine) at its N-terminus; the ligase primarily responsible for α-globin ubiquitination has not yet been identified.

In the current work, the ATP-dependent proteolysis of each of the four ^{125}I-labeled protein substrates was inhibited significantly by >3 μM Ubal throughout the period of analysis (15−120 min) of the reactions. A progressive accumulation of multiubiquitinated ($Ub_{>6}$) conjugates of the ^{125}I-labeled substrates was observed, and there was a concomitant appearance of putatively "unanchored" polyubiquitin chains. We suggest that the unanchored polyubiquitin chains are produced during the ATP-dependent degradation of polyubiquitin−protein conjugates formed with either the exogenous protein substrates or endogenous proteins in the reticulocyte extract. In the absence of Ubal, these unanchored chains are degraded rapidly by isopeptidases such as isopeptidase T (Hadari *et al.*, 1992; Wilkinson *et al.*, 1995; Beal *et al.*, 1996). High concentrations of Ubal strongly inhibit these enzymes. The undegraded polyubiquitin chains bind to the site(s) on the 26S proteasome normally reserved for polyubiquitin conjugates of protein substrates, thus inhibiting their degradation and resulting in their observed accumulation.

Deveraux *et al.* (1994) showed that branched Ub oligomers (linked via Lys48) inhibited the binding of polyubiquitinated ^{125}I-lysozyme to the S5 subunit of the 26S proteasome, and that S5 separated in a polyacrylamide gel had greatest affinity for polyubiquitin chains of $n \geq 4$. The addition of such exogenously-synthesized polyubiquitin chains to an isopeptidase-depleted reticulocyte extract inhibited substantially the Ub-dependent degradation of ^{125}I-α-lactalbumin (Beal *et al.*, 1996). Our results support those of Beal *et al.*, but because the polyubiquitin chains in our experiments were introduced by synthesis *in situ*, the nature of the Ub−Ub linkages was not defined. However, in a recent comparison of polyubiquitin chains linked via Lys6, Lys11, or Lys48, Baboshina and Haas (1996) have shown that, for any of these Ub−Ub linkages, chains of $n > 4$ bind similarly to purified S5 subunit with $K_d \leq 18$ nM. Thus, it is quite reasonable that Ub-dependent degradation in cell extracts is inhibited by the 10^{-6}−10^{-7} M levels of Ub_4 and Ub_5 that we see accumulate in the presence of Ubal (see Figure 4). Interestingly, the major difference between 0.8 and 8.0 μM Ubal with respect to ubiquitinated species that accumulate is in the high mass (>50 kDa) region of the gel. We speculate that polyubiquitin chains of $n \geq 5$ are enriched by high [Ubal] and that these higher-order chains are responsible for the progressively greater inhibition of protein degradation seen in Figure 1. All of these studies make clear that, in addition to recycling of Ub from conjugates, isopeptidases are required to prevent the accumulation of polyubiquitin chains which otherwise would inhibit conjugate degradation by the 26S proteasome.

That substantial proteolysis of ^{125}I-α-globin and of ^{125}I-lysozyme occurred even in the presence of high Ubal concentrations (Figures 1 and 2) suggests a mechanism for their ATP-dependent degradation in addition to that involving polyubiquitin chains. Monoubiquitinated ^{125}I-α-globin is the predominant conjugate species observed after a 15 min incubation of ^{125}I-human α-globin in a reaction mixture without Ubal (Shaeffer, 1994b). This monoubiquitinated species probably is processed to a polyubiquitinated conjugate very slowly compared to that of ^{125}I-bovine α-lactalbumin (Table 2). Moreover, structural analysis of monoubiquitin−^{125}I-α globin showed that it was a mixture of molecules in which a Ub monomer could be attached to either of at least two lysine residues of α-globin (Shaeffer, 1994a). Low concentrations of Ubal not only partially inhibit the isopeptidase(s) which degrades unanchored polyubiquitin chains but also inhibit deubiquitination of the newly-formed ubiquitin−^{125}I-α globin conjugates. The latter action promotes an increase of ubiquitin−^{125}I-α globin conjugates that contain Ub monomers ligated to several lysine residues of ^{125}I-α globin with a concomitant decrease in the pool of the free ^{125}I-α globin substrate (Shaeffer, 1994a). These conjugates of ^{125}I-α globin are recognized and degraded by the 26S proteasome (Shaeffer & Kania, 1995), although probably with lower efficiency than ^{125}I-α-globin conjugates of the type with a branched-chain polyubiquitin moiety.

Others (Hershko & Heller, 1985; Hershko & Rose, 1987) have shown that ^{125}I-lysozymes, each ligated to several

molecules of N-methylated Ub (conjugates which presumably are similar to those proposed for α-globin ligated to several Ub monomers), were degraded by the Ub-dependent protease, but less readily than polyubiquitinated ^{125}I-lysozyme. In the reaction mixtures with ^{125}I-α-globin and low concentrations (<2 μM) of Ubal, the level of ^{125}I-α-globin conjugates with multiple Ub moieties gradually increases so that, after incubation for 30 min, the total ATP-dependent degradation of ^{125}I-α-globin is higher than that observed in the absence of Ubal (Figures 1 and 2A). When the reaction mixtures contain sufficiently high concentrations (>3 μM) of Ubal, the degradation of polyubiquitin−^{125}I-α-globin conjugates is blocked, and hence, the total ATP-dependent degradation of ^{125}I-α-globin is less than that observed without Ubal.

Although low concentrations of Ubal result also in an increase of Ub−^{125}I-lysozyme conjugates, there appears to be little, if any, concomitant decrease in the pool of ^{125}I-lysozyme substrate (Table 2). The newly-formed monoubiquitin−^{125}I-lysozyme conjugates may be protected from isopeptidases which disassemble them, perhaps because of the rapid extension of a nascent polyubiquitin chain with additional Ub monomers by the concerted action of a Ub−protein ligase (E3) and Ub conjugating enzyme (E2), a mechanism presumably absent in the processing of monoubiquitin−^{125}I-α globin. Others have suggested that, in proteolysis reactions with a crude reticulocyte enzyme fraction, Ub−lysozyme conjugates were less susceptible to "Ub-releasing" enzymes than conjugates of RNase A derivatives (Hershko & Rose, 1987). Substantial accumulation of polyubiquitinated ^{125}I-lysozyme concomitant with a severe inhibition of its proteolysis was not observed with high Ubal concentrations, at least to the extent found here with ^{125}I-α-lactalbumin (Figure 1 and Table 2). Possibly, ^{125}I-lysozyme conjugates with relatively short polyubiquitin chains can be readily degraded by the 26S proteasomes, whereas the conjugates of ^{125}I-α-lactalbumin require a longer polyubiquitin extension to be recognized efficiently.

In addition to Ub recycling and polyubiquitin chain disassembly, our results support the idea of an "editing" role for Ub−protein isopeptidases. A minimal model to explain our data in the context of such a scheme is shown in Figure 5. This model elaborates upon an early proposal of Hershko *et al.* (1980) in which competition between conjugate disassembly and protein degradation may be used to enhance the overall fidelity of degradation. We base our scheme on the idea that *poly*ubiquitination commits a "good" degradation substrate to rapid proteolysis by the 26S proteasome, whereas the relatively inefficient degradation of conjugates with just one or a few attached ubiquitins allows for conjugate disassembly by isopeptidase(s) and regeneration of the substrate. By this mechanism, the specificity of Ub-dependent degradation principally relies upon Ub−protein ligases to polyubiquitinate good substrates such as bovine α-lactalbumin; such polyubiquitination most likely is processive or highly cooperative. Although a "poor" substrate such as α-globin can be ubiquitinated, the elaboration of polyubiquitin chains on these substrates is relatively slow. Thus, conjugate disassembly by isopeptidases might selectively decrease the overall degradation of poorly (and, perhaps, erroneously) ubiquitinated substrates.

We propose that an "editing" isopeptidase need not exhibit any specificity for either the protein target or the length of the (poly)ubiquitin moiety. Rather, by restricting this isopeptidase to the removal of single Ub units from the "growing" end of a (poly)ubiquitin chain, conjugates with single-Ub adducts can be disassembled preferentially, as indicated by the relative reaction rates in Figure 5. The turnover of such proteins would be enhanced selectively by inhibition of this "editing" isopeptidase with Ubal. A second and separate effect of Ubal is the general inhibition of protein degradation that correlates with the accumulation of unanchored polyubiquitin chains and polyubiquitinated protein conjugates. However, although different Ubal concentrations were observed to promote enhancement and inhibition of ^{125}I-α-globin degradation in the reticulocyte extract, it is not yet known if distinct isopeptidases are responsible for these opposite effects.

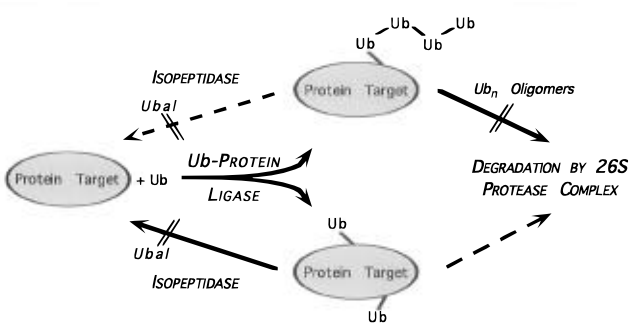

FIGURE 5: Editing within the Ub-dependent protein degradation pathway by competition between conjugate disassembly and substrate proteolysis. In this model, two classes of ubiquitinated degradation intermediates can be generated by Ub−protein ligase, those with polyubiquitin chains (upper path) or single Ub adducts (lower path). Both conjugate types are subject to either of two fates: disassembly to regenerate substrate, or degradation by the 26S protease. The solid and dashed arrows represent, respectively, relatively rapid and slow processing steps, and steps susceptible to inhibition by Ubal or polyubiquitin chains are indicated. Note that ligase/isopeptidase-catalyzed interconversions of these two classes of Ub−protein conjugates, though not shown, may occur.

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