Degradation of Monoubiquitinated α-Globin by 26S Proteasomes†

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ABSTRACT: Ubiquitin-^{125}I-α-globin conjugate fractions containing either one (Ub_1-α), two (Ub_2-α), or a mixture of three and four ($Ub_{3,4}$-α) molecules of ubiquitin (Ub), covalently linked to one ^{125}I-α-globin molecule were isolated after incubation of a proteolysis reaction mixture containing ATP, ubiquitin aldehyde-treated reticulocyte lysate, and human ^{125}I-α-globin. Each of the purified conjugate fractions or an identically-purified control sample of unconjugated ^{125}I-α-globin was incubated as a substrate in companion proteolysis reaction mixtures containing either purified 26S or 20S rabbit reticulocyte proteasomes. The initial rate of ATP-dependent degradation of the Ub_1-α conjugate by the 26S proteasomes was ~0.44% (1.1 fmol)/min while that of the free ^{125}I-α-globin was undetectable. The initial rates of ATP-dependent degradation by the 26S proteasomes of the Ub_2-α and $Ub_{3,4}$-α conjugates were 2- to-3-fold that of the Ub_1-α species. Conversely, the degradation of free ^{125}I-α-globin and its ubiquitinated conjugates by the 20S proteasomes was not dependent on ATP, nor did it increase with the size of the Ub adduct. Analysis of the products of a reaction mixture with 26S proteasomes by sodium dodecyl sulfate−polyacrylamide gel electrophoresis showed no conversion of the Ub_1-α conjugate substrate to higher-molecular-mass conjugates. These results suggest that monoubiquitinated α-globin can be degraded significantly and specifically by interaction directly with the 26S proteasomes. This finding is consistent with the hypothesis that a substantial fraction of the Ub_1-α conjugate intermediate in the ATP-dependent proteolysis of ^{125}I-α-globin in whole reticulocyte lysate [Shaeffer, J. R. (1994) *J. Biol. Chem. 269*, 22205−22210] is degraded by this interaction.

A major pathway for the intracellular degradation of proteins, which uses ATP and ubiquitin, is present in the cytoplasm of all eukaryotes (reviewed in Hershko & Ciechanover, 1992). In this pathway, ubiquitin, a heat stable polypeptide of 8565 Da, is activated in an ATP-dependent reaction, and one or more ubiquitin molecules are subsequently linked covalently to the protein destined for proteolysis. The protein substrate moiety of these ubiquitin−protein conjugates is then degraded by the 26S proteasome, a multisubunit protease complex, in a process which also utilizes ATP (reviewed in Rechsteiner *et al.*, 1993). The 26S proteasome presumably recognizes the putative substrate for degradation by means of the catalytic ubiquitin "tag" or signal. Several investigations have shown that the structure preferred as a recognition signal for the degradation of abnormal, foreign, or short-lived natural proteins is a polyubiquitin moiety of several ubiquitin monomers (Hershko & Heller, 1985; Chau *et al.*, 1989; Hershko *et al.*, 1991). However, conjugates in which one (or a few) ubiquitin monomer(s) is attached to a long-lived natural protein have also been isolated (Goldknopf & Busch, 1977; Matsui *et al.*, 1982; Gregori *et al.*, 1985; Sokolik & Cohen, 1991; Shimogawara & Muto, 1992; Parag *et al.*, 1993); the role of these conjugates remains unknown. Recently, one of us showed that only monoubiquitinated ^{125}I-α-globin (and to a much lesser extent some diubiquitinated molecules) was observed when ^{125}I-α-globin, the protein moiety of the α chains of human hemoglobin, was incubated with unfractionated reticulocyte lysate in a proteolysis reaction mixture (Shaeffer, 1994a). Subsequent kinetic and other studies with isolated monoubiquitinated ^{125}I-α-globin during this investigation suggested that it was an intermediate in the ATP-dependent degradation of ^{125}I-α-globin. The question remained whether monoubiquitinated ^{125}I-α-globin could be recognized by the 26S proteasome or whether prior conversion to polyubiquitinated conjugates, a step undetected in the lysate reaction mixtures, was obligatory in this proteolysis. The results of the present work suggest that monoubiquitinated α-globin, but not free α-globin, can be degraded directly by purified 26S proteasomes.

MATERIALS AND METHODS

Isolation and Purification of 26S and 20S Proteasomes. The 26S proteasomes were prepared from washed rabbit reticulocytes (Green Hectares Farms, Oregon, WI). The cells were lysed with 1.5 volumes of 1 mM dithiothreitol (DTT)[1] and gentle shaking at 4 °C for 10 min. The crude lysate was made 1 mM in ATP and 5 mM in $MgCl_2$ and clarified by centrifugation at 100000*g* for 60 min. The supernatant was recentrifuged at 100000*g* for 5 h. The pellet containing

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[1] Abbreviations: DTT = dithiothreitol; SDS = sodium dodecyl sulfate; PAGE = polyacrylamide gel electrophoresis; Ub = ubiquitin; Ubal = ubiquitin aldehyde; Ub_1-α, Ub_2-α, and $Ub_{3,4}$-α = ubiquitin$_1$-^{125}I-α-globin, ubiquitin$_2$-^{125}I-α-globin, and ubiquitin$_{3,4}$-^{125}I-α-globin, i.e., the conjugates of one, two, and a mixture of three and four molecules of ubiquitin, respectively, with one molecule of ^{125}I-labeled α-globin. In this work, the conjugates can only be detected because of their ^{125}I-label, and hence, the theoretical M_r given for each (see text) includes the weight of 1 mol of ^{125}I.

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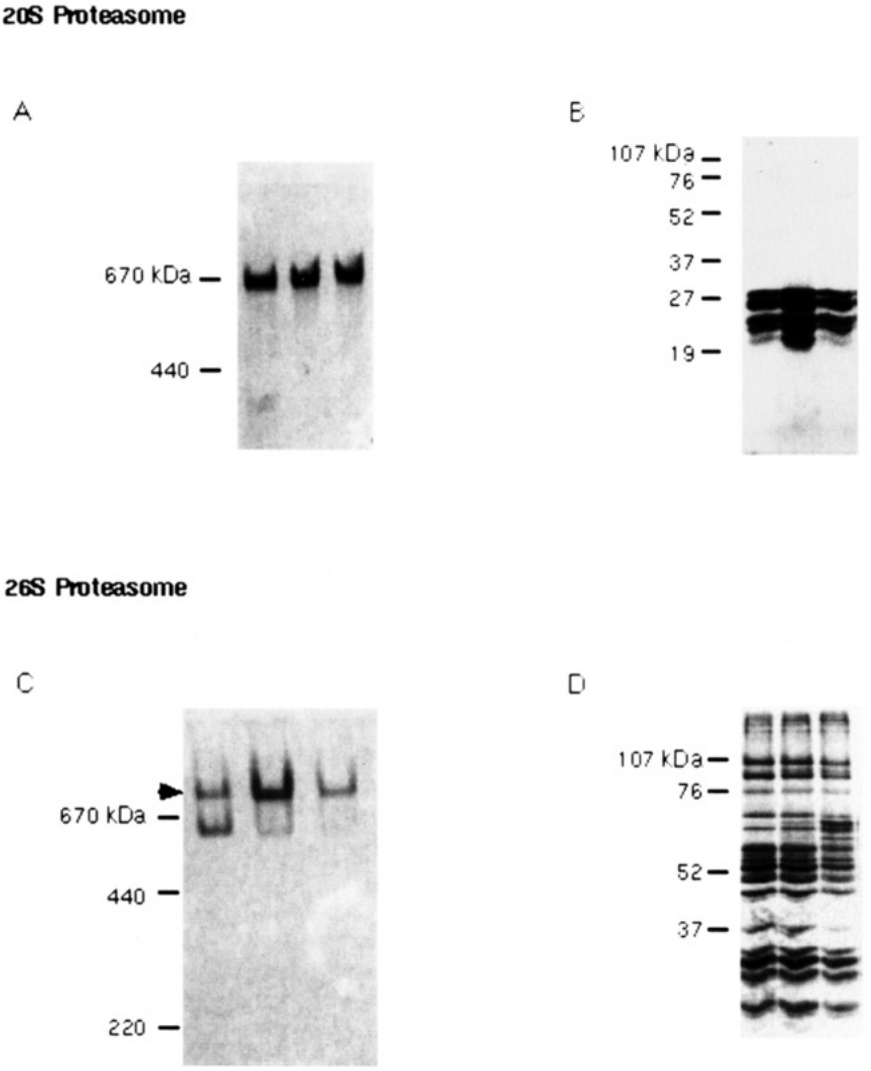

FIGURE 1: Polyacrylamide gel electrophoresis of the purified 20S and 26S proteasomes. The peak fractions of sucrose density gradients containing 20S (panels A and B) or 26S (panels C and D) proteasomes (see Materials and Methods) were resolved by 4% acrylamide nondenaturing gel electrophoresis with 50 mM Tris·HCl, pH 8.8, and 50 mM glycine buffer (A, C) or by SDS−PAGE in gels with 12.5% acrylamide (B, D) as described by Laemmli (1970). The fractions shown on the gels were pooled for use in the proteolysis reaction mixtures with ubiquitin-^{125}I-α-globin conjugates. The nondenaturing gels were stained with Coomassie Brilliant Blue, and the denaturing gels were stained with silver. An arrowhead (panel C) indicates the position of the 26S proteasomes in the nondenaturing gel. The position of the following molecular mass marker proteins, run in gel reference lanes, are shown in the figure: thyroglobulin (670 kDa), apoferritin (440 kDa), catalase (220 kDa), phosphorylase B (107 kDa), bovine serum albumin (76 kDa), ovalbumin (52 kDa), carbonic anhydrase (37 kDa), soybean trypsin inhibitor (27 kDa), and lysozyme (19 kDa); the SDS−PAGE marker proteins were prelabeled with stain and obtained from Bio-Rad Laboratories.

impure 26S proteasomes was suspended in 20 mM Tris·HCl, pH 7.6, 1 mM DTT, 5 mM $MgCl_2$, 1 mM ATP, and 10% glycerol (26S buffer) and separated by chromatography on a HiLoad 16/10 Q-Sepharose high performance column (Pharmacia No. 17-1064-01, bed volume ~20 mL) at 1 mL/min with a linear gradient of 0−500 mM NaCl in the 26S buffer. Eluted fractions which had an ATP-stimulated peptidase activity, measured with the peptide succinyl-Leu-Leu-Val-Tyr-4-methylcoumaryl-7-amide as described by Tanaka *et al.* (1986), were pooled, concentrated with centrifugation filters (Amicon), and applied to a 15-mL linear gradient of 25−50% (w/v) sucrose (Peters *et al.*, 1991) in the 26S buffer without glycerol. After centrifugation in a TH641 rotor (Sorvall) at 34 000 rpm (200000g) for ~18 h, fractions separated in the sucrose gradient which had the ATP-stimulated peptidase activity were assayed for purity by nondenaturing gel electrophoresis and by SDS−PAGE (Figure 1), pooled, and frozen in aliquots at −70 °C.

The 20S proteasomes were prepared from a crude "Fraction II", precipitated with $(NH_4)_2SO_4$ as described previously (Driscoll & Goldberg, 1990), of ATP-depleted rabbit reticulocytes. The $(NH_4)_2SO_4$ pellet was suspended in and dialyzed against 20 mM Tris·HCl, pH 7.6, 1 mM DTT, and 10% glycerol (20S buffer). The dialyzed solution was separated by chromatography, as described above for the 26S particle, with a NaCl gradient in the 20S buffer. Fractions eluting at ~400 mM NaCl and containing peptidase activity (without ATP stimulation) were pooled, concentrated, and separated by centrifugation, as described above, in a 15−40% (w/v) sucrose gradient in the 20S buffer without glycerol. A gradient zone with maximal peptidase activity was harvested, assayed for purity (see Figure 1) as described for the 26S proteasomes, and frozen in aliquots at −70 °C.

Figure 1 shows protein profiles similar to those of Hough *et al.* (1987) who purified 26S and 20S proteasomes from

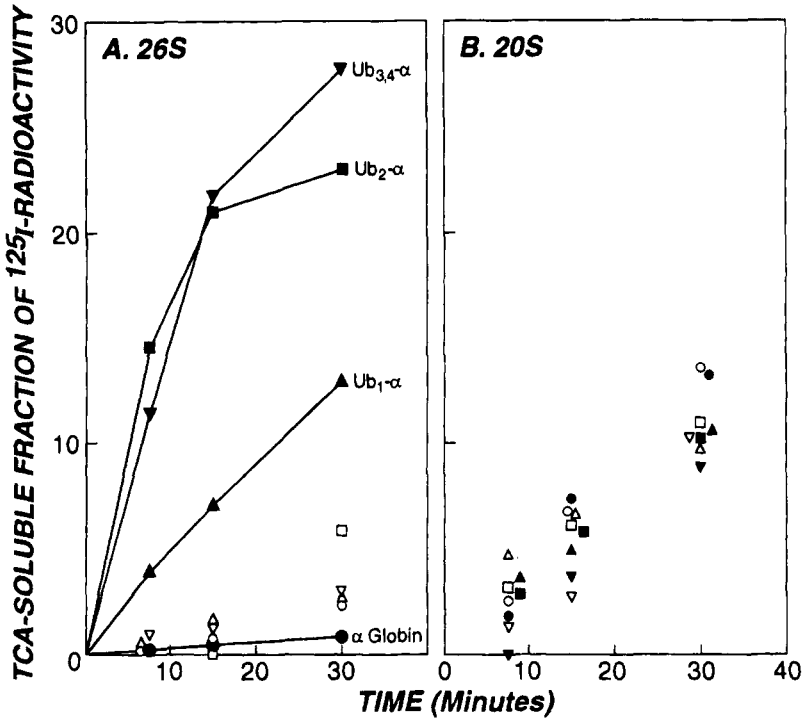

FIGURE 2: Rates of degradation of ubiquitin-^{125}I-α-globin conjugates and of free ^{125}I-α-globin in proteolysis reaction mixtures with rabbit reticulocyte 26S (panel A) or 20S (panel B) proteasomes. Replicate mixtures were prepared, incubated at 37 °C for 7.5, 15, or 30 min, and analyzed as described in Materials and Methods. The amount of proteolysis, represented by the acid-soluble fraction of the total ^{125}I-radioactivity corrected for that (≤3.0%) obtained after incubation of an identical mixture without proteasomes, is shown for the +ATP reactions by the solid symbols, as follows: Ub_1-α, ▲; Ub_2-α, ■; $Ub_{3,4}$-α, ▼; and α-globin, ●. The results from the companion −ATP reactions are shown by the corresponding open symbols.

rabbit reticulocytes by procedures employing more chromatographic steps than used by us. These profiles suggest that our preparations have a purity comparable to that of those investigators.

Construction, Incubation, and Analysis of the Proteolysis Reaction Mixture. Each mixture was prepared in a 1.5-mL plastic microcentrifuge tube and contained 50 mM Tris·HCl, pH 7.5 (23 °C), 5 mM DTT, 5 mM $MgCl_2$, 2 mM ATP *or* 0.15 unit of apyrase (Sigma No. A6410), 2.0 μg of purified 26S or 20S proteasomes, ~15% (w/v) sucrose (from the proteasome preparation), and ~800 cpm of one of the ^{125}I-labeled substrates in a total volume of 30 μL. The samples of the purified 26S or 20S proteasomes were incubated initially with 0.20 μM ubiquitin aldehyde (Ubal; Dunten & Cohen, 1989) at 37 °C for 5 min before inclusion in the reaction mixture. The ubiquitin-^{125}I-α-globin conjugate substrates (Ub_1-α, Ub_2-α, and $Ub_{3,4}$-α) were isolated by SDS−PAGE and subsequent purification procedures from a proteolysis reaction mixture containing Ubal-treated human reticulocyte lysate and human ^{125}I-α-globin, as described previously (Shaeffer, 1994a); the unconjugated ^{125}I-α-globin substrate was separately but identically exposed to SDS−PAGE and the other purification procedures. Each tube containing a proteolysis reaction mixture was capped and incubated at 37 °C for 7.5, 15, or 30 min. The reaction was stopped by chilling in ice and the addition of 200 μg (2 μL) of bovine serum albumin and 0.50 mL of 10% trichloroacetic acid (TCA). The mixtures were incubated at 4 °C for 1 h, and the acid-soluble and acid-insoluble phases were separated by centrifugation and counted in a γ-scintillation counter.

In one experiment, to examine the products of the proteolysis reaction, a 30-μL mixture containing ATP, 26S proteasomes, and 1400 cpm of purified Ub_1-α conjugate was prepared as described above. After incubation at 37 °C for 30 min, 60 μL of SDS−PAGE sample buffer (Shaeffer, 1994a) and 90 μg (0.6 μL) of human erythrocyte lysate (as carrier protein) were added, and this mixture was heated at 95 °C for 5 min. In a companion experiment, a proteolysis reaction mixture containing Ubal-treated human reticulocyte lysate, ATP, supplementary ubiquitin, and a sample of the same Ub_1-α conjugate fraction was prepared, incubated at 37 °C for 30 min, diluted with SDS−PAGE sample buffer, and heated at 95 °C, as described previously (Shaeffer, 1994a). Samples of these two diluted reaction mixtures and of duplicate mixtures not incubated at 37 °C were analyzed by SDS−PAGE (see Figure 3 legend).

RESULTS

Replicate proteolysis reaction mixtures were constructed to contain purified rabbit reticulocyte 26S proteasomes and, as a substrate, an isolated fraction of a ubiquitin-^{125}I-α-globin conjugate containing either one (Ub_1-α), two (Ub_2-α), or a mixture of three and four ($Ub_{3,4}$-α) molecules of ubiquitin covalently linked to one human ^{125}I-α-globin molecule (see Materials and Methods). Control mixtures with unconjugated ^{125}I-α-globin substrate, the precursor of the isolated conjugate fractions, were also constructed. Both conjugated and free ^{125}I-α-globin substrates had been purified identically by SDS−PAGE and ancillary procedures. The reaction mixtures were incubated at 37 °C for either 7.5, 15, or 30 min with (+) or without (−) ATP; the −ATP mixtures contained apyrase, an ATPase added to hydrolyze ATP present in the buffer of the proteasome preparations. Figure 2A shows that

the initial rate of ATP-dependent degradation, as measured by the conversion to acid-soluble ^{125}I-cpm after 7.5 min, of the Ub_1-α conjugate (▲) was ~0.44% (1.1 fmol)/min while that of the unconjugated ^{125}I-α-globin (●) was undetectable. In one experiment, the nonradioactive α-globin used as a carrier protein during the preparation of the unconjugated ^{125}I-α-globin (Shaeffer, 1994a) was replaced by ovalbumin, and the initial rate of degradation by the 26S proteasomes was still negligible; thus, the lack of proteolysis of the free ^{125}I-α-globin in Figure 2A was not due to isotope dilution. The initial rates of ATP-dependent degradation of the Ub_2-α (■) and $Ub_{3,4}$-α (▼) conjugates were 2- to 3-fold that of the Ub_1-α species (Figure 2A).

The values shown in Figure 2 have been corrected for the amounts of acid-soluble ^{125}I-cpm present in companion reaction mixtures after incubation without proteasomes. Most of this latter acid-soluble ^{125}I-cpm, which ranged from 0.5% to 3.0% of the total ^{125}I-cpm, was initially present in the unincubated substrate preparations (data not shown). Thus, the SDS−PAGE-purified ^{125}I-labeled substrates contained little apparent endogenous protease activity. The finding of an increase in the amount of ATP-stimulated proteolysis with increase in the size of the ubiquitin adduct in the substrates (Figure 2A) is consistent with the ubiquitin recognition signal or "tag" hypothesis. To investigate an alternative possibility that the increase in proteolysis observed with the higher-molecular-mass conjugate fractions was mediated by factors other than the ubiquitin moieties, e.g., non-conjugate protein contaminants copurified from the reticulocyte lysate during conjugate isolation (Shaeffer, 1994a), additional experiments were done with 20S proteasomes. The 20S proteasome is another multisubunit protease complex in the cytoplasm, which, unlike the 26S proteasome, does not prefer ubiquitinated substrates (Rechsteiner *et al.*, 1993; Waxman *et al.*, 1987; Orlowski, 1990). The degradation of free ^{125}I-α-globin and its ubiquitinated conjugates by purified rabbit reticulocyte 20S proteasomes (Figure 2B), although significant, was not dependent on ATP, nor did it increase with the amount of conjugated ubiquitin. The failure to find a pattern of substrate and energy specificity by the 20S similar to that of the 26S proteasomes is consistent with the hypothesis that the increase in ATP-dependent degradation by the latter particles of the higher-molecular-mass substrates occurs because of the ubiquitin component and its increase in size.

To explore the possibility that the Ub_1-α conjugate substrate in the experiments of Figure 2A was first converted to a polyubiquitinated conjugate by enzymes possibly contaminating the reactant preparations, a reaction mixture containing 26S proteasomes, ATP, and this substrate was incubated for 30 min, and the products were analyzed by SDS−PAGE. Little protein ^{125}I-cpm was detected in the region of the gel (Figure 3A, ●, fractions 1−28) corresponding to a range of molecular masses higher than that (23.8 kDa) of the Ub_1-α conjugate substrate. The small heterodisperse peak of protein ^{125}I-cpm at gel fractions 13−21 was also present in companion mixtures that were unincubated (Figure 3A, - - -, right-hand scale) or incubated without proteasomes (data not shown) and may represent covalently linked, nonspecific aggregates of the ^{125}I-labeled substrate. Conversely, incubation of another sample of the same Ub_1-α conjugate preparation in a reaction mixture with whole Ubal-treated reticulocyte lysate instead of purified 26S proteasomes resulted in the appearance of 31% of the initial protein ^{125}I-cpm in the higher-molecular-mass region of the gel (Figrue 3B, ●, fractions 1−28). The finding of peaks representing the Ub_2-α (32.3 kDa) and Ub_3-α (40.9 kDa) conjugate species as well as a heterodisperse zone of protein ^{125}I-cpm near the top of the gel (fractions 1−6) representing polyubiquitinated-^{125}I-α-globin (>10^5 kDa) in this control experiment was expected because of the presence of free ubiquitin and its activating and conjugating enzymes (reviewed in Hershko & Ciechanover, 1992; Jentsch, 1992) in the whole lysate. These results suggest that, in the experiments of Figures 2A and 3A, the isolated ubiquitin$_1$-^{125}I-α-globin conjugate was degraded by interaction directly with the 26S proteasomes without prior conversion to higher-molecular-mass conjugates. The peak of product ^{125}I-radioactivity migrating at ~15 kDa (Figure 3A, ●, fractions 47−55) may represent unconjugated ^{125}I-α-globin which occurred because of Ubal-insensitive ubiquitin C-terminal hydrolase activity associated with the 26S proteasomes (Eytan *et al.*, 1993). The role, if any, of this unconjugated ^{125}I-α-globin in the pathway of the degradation of the Ub_1-α conjugate substrate by the 26S particles remains to be explored.

The relative initial rates of conversion of protein to acid-soluble ^{125}I-radioactivity of each conjugate fraction in Figure 2A presumably reflect the relative rates of degradation of the respective molecular species, because only the α-globin component is ^{125}I-labeled and each conjugate species would be expected to have the same molar specific ^{125}I-radioactivity. The specific ^{125}I-radioactivity (2.2×10^5 cpm/μg) of the unconjugated ^{125}I-α-globin progenitor of the conjugate fractions was used to estimate that each 30-μL reaction mixture initially contained ~0.25 pmol of a purified conjugate. The finding that the fraction of protein ^{125}I-radioactivity initially degraded per minute (Figure 2A) remained the same when only half of the amount of each ^{125}I-labeled substrate was included in the reaction mixture (data not shown) suggests that the initial rate of proteolysis was limited by the substrate concentration. As a precaution, in these experiments the proteasome preparations were pretreated with ubiquitin aldehyde (see Materials and Methods) to block ubiquitin−protein hydrolase activity (Hershko & Rose, 1987), a potential contaminant which might prematurely disassemble (deubiquitinate) the conjugate substrates and result in lower apparent degradation rates; however, omission of the Ubal in one experiment with Ub_1-α conjugate substrate resulted in no change in the rate of degradation. A recent report (Deveraux *et al.*, 1994) shows that short polymeric chains of ubiquitin, e.g., dimers and trimers, inhibit the binding of ubiquitin-^{125}I-lysozyme conjugates to the regulatory complex component of the 26S proteasomes. The possibility that the rates of ubiquitin-^{125}I-α-globin conjugate degradation observed in Figure 2A were influenced by the putative presence of short Ub polymers copurified during conjugate fraction isolation cannot be eliminated.

DISCUSSION

The denaturation of the ubiquitin-^{125}I-α-globin conjugate substrates by SDS during their preparation does not preclude the possibility that their specific proteolysis by purified 26S proteasomes (Figure 2A) is relevant to the degradation of similar α-globin conjugates in intact cells. The investigators (Henderson *et al.*, 1979) who developed the method of quantitative removal of SDS by treatment with triethylamine, used by us (Shaeffer, 1994a) to purify the conjugates after

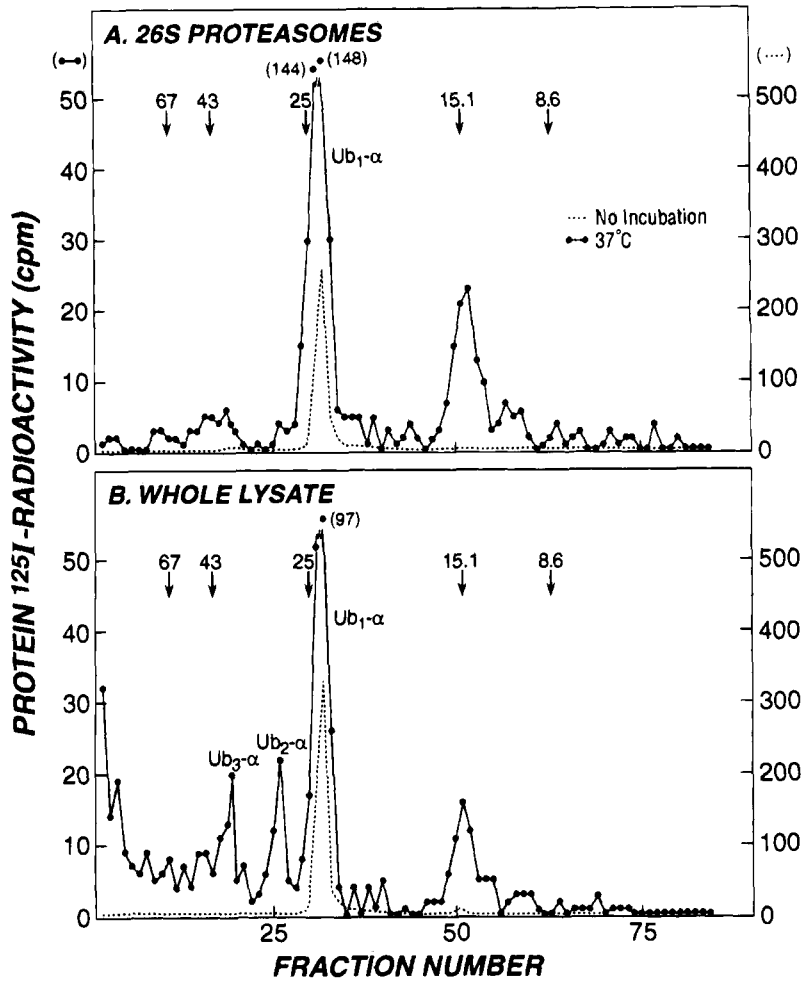

FIGURE 3: Absence of higher-molecular-mass conjugates during proteolysis of a Ub$_1$-α conjugate substrate by rabbit reticulocyte 26S proteasomes. Companion proteolysis reaction mixtures containing ATP, purified Ub$_1$-α conjugate, and either 26S proteasomes or whole reticulocyte lysate were incubated at 37 °C for 30 min and prepared for product analysis (see Materials and Methods). The figure shows the distribution (●, left-hand scale) of protein ^{125}I-radioactivity (^{125}I-cpm) after SDS−PAGE (15% acrylamide gel; Shaeffer, 1994a) of an aliquot (750 cpm) of the mixture with 26S proteasomes (panel A) and of an aliquot (840 cpm) of the mixture with whole lysate (panel B). Each panel also shows the protein ^{125}I-cpm distribution (- - -, right-hand scale) after SDS−PAGE of corresponding aliquots of duplicate reaction mixtures that were not incubated at 37 °C. The positions of molecular mass marker proteins (m = 8.6, 15.1, 25, 43, and 67 kDa; Shaeffer, 1994a) are shown in each panel. The peaks of protein ^{125}I-cpm that represent the Ub$_2$-α and Ub$_3$-α conjugate products in the reaction with whole lysate (panel B) and the Ub$_1$-α conjugate substrate in each mixture are denoted. The recovery of ^{125}I-cpm in the fractions separated by electrophoresis for each unincubated mixture was essentially 100% of that applied to the gel. The corresponding recoveries for the mixtures incubated at 37 °C were 83% and 68% for the reactions with 26S proteasomes or whole lysate, respectively, and were probably less than 100% because of the conversion during the proteolysis of some of the protein ^{125}I-cpm to soluble ^{125}I-cpm.

their isolation by SDS−PAGE, recovered reverse transcriptase and thromboplastin enzyme activities after exposure to this denaturant. Weber and Kuter (1971), using an entirely different method of SDS extraction, renatured a substantial fraction of aspartate transcarbamylase, aldolase, and other enzymes after SDS−PAGE. Thus, it is possible that the isolated, SDS-free conjugate substrates in this work were in a "renatured" form.

Moreover, isolated human α-globin, trace-labeled with Na^{125}I and incubated as a proteolysis substrate in a hemolysate of the erythroid blood cells from a β-thalassemic individual to produce the ubiquitin-^{125}I-α-globin conjugates (Shaeffer, 1994a), is probably similar in structure to a natural substrate in intact β-thalassemic reticulocytes. α-Globin, the apoprotein which results from removal of heme groups from hemoglobin α chains, is not usually found in the developing red cells of normal individuals. However, in β-thalassemia, a severe anemia characterized by a deficiency in the synthesis of hemoglobin β chains, a substantial excess of hemoglobin α chains accumulates in the maturing erythroid cells (reviewed in Bunn & Forget, 1986). These excess α chains are relatively unstable (Rachmilewitz *et al.*, 1971) compared to normal tetrameric ($\alpha_2\beta_2$) hemoglobin and undergo spontaneous oxidation to a methemoglobin form, some of which further denatures by heme group loss (Bunn & Jandl, 1968) to form α-globin. α-Globin has a relatively disordered secondary structure (Waks et al., 1973) compared to the α chains or tetrameric hemoglobin, and thus this "partially denatured" protein is probably a good candidate for proteolysis even without exposure to SDS.

The significant ATP-independent degradation of free ^{125}I-α-globin by purified reticulocyte 20S proteasomes (Figure 2B) but not by 26S proteasomes (Figure 2A) could suggest that nonubiquitinated proteins are degraded in intact cells primarily by the former particles. Other studies showed that casein (Tanaka et al., 1988; Driscoll & Goldberg, 1990), several oxidized, but not native, enzymes (Rivett, 1985), and lens α_2-crystallins (Ray & Harris, 1985) were degraded in

cell-free systems by 20S proteasomes from various mammalian sources. A common feature which likely renders these proteins and also α-globin in the present work particularly susceptible to cell-free proteolysis by 20S proteasomes and perhaps other proteases may be a relative lack of ordered secondary structure, as mentioned earlier. Also, the possibility that our reticulocyte 20S proteasomes, despite excellent purity (Figure 1), contained a minor contaminating protease, which spuriously degraded both ubiquitinated and free ^{125}I-α-globin, cannot be eliminated.

The above observations notwithstanding, even partially unfolded nonubiquitinated α-globin molecules may not necessarily be degraded in intact cells by free-standing 20S proteasomes. Past studies demonstrated that hemoglobin α subunits were degraded largely by the ATP- and ubiquitin-dependent proteolysis pathway in both intact β-thalassemic reticulocytes and their unfractionated lysates (Shaeffer, 1983, 1988). The present finding (Figure 2A) that purified reticulocyte 26S proteasomes selectively degrade ubiquitin-^{125}I-α-globin conjugates, shown recently (Shaeffer, 1994a) to be intermediates in the proteolysis of ^{125}I-α-globin by β-thalassemic lysates, is consistent with the earlier work and also with the hypothesis that 26S and not 20S proteasomes are involved in α-globin turnover in intact cells. Several workers showed that the 20S particle is a component of and can be incorporated into the 26S proteasome in cell-free mixtures (reviewed in Rechsteiner *et al.*, 1993). It is probable that in intact cells a large proportion of the total 20S proteasome population is present in the 26S particle (Orino *et al.*, 1991; Peters *et al.*, 1991). *In vitro* experiments in several laboratories showed that various artificial substances, e.g., SDS or polylysine, stimulate dramatically the degradation of protein or fluorogenic peptide substrates by 20S proteasomes, leading some investigators to suggest that these particles exist normally in an inactive or "latent" form (reviewed in Rivett, 1993). If so, perhaps the "activation" of the dormant 20S proteasomes in intact β-thalassemic reticulocytes occurs by the ATP-dependent assembly of the 26S complex from the 20S core particles and other protein components, a process known to occur in rabbit reticulocyte extracts (Driscoll & Goldberg, 1990; Armon et al., 1990). A determination of whether the 20S proteasome entity is directly involved in the degradation of unconjugated α-globin in intact β-thalassemic reticulocytes must await the development of a satisfactory analysis of the cellular concentration and proteolytic activity of these particles.

Rapid degradation of ubiquitin-^{125}I-lysozyme conjugates by a crude reticulocyte enzyme fraction requires the presence of a polyubiquitin moiety of several ubiquitin monomers (Hershko et al., 1984; Hough & Rechsteiner, 1986). However, conjugates of ^{125}I-lysozyme in which each of several lysine residues is ligated to a single molecule of ubiquitin modified by reductive methylation of the amino groups are also degraded at a substantial rate (Hershko & Heller, 1985). Thus, formation of polyubiquitin chains is not obligatory for the breakdown of ^{125}I-lysozyme, a relatively "foreign" protein. Other investigators (Haas *et al.*, 1990) showed that ~1% of the conjugates of ^{125}I-histone H3 ligated to reductively methylated ubiquitin could be degraded by purified 26S proteasomes during a 20-min incubation. The relevance of this observation to the proteolysis of histone, predominantly a nuclear protein, in an intact cell containing unmodified ubiquitin remains unknown. Conversely, the present results (Figure 2A) show that ~13% of an ^{125}I-α-globin substrate ligated to a single molecule of unmodified ubiquitin is degraded by the 26S proteasomes during a 30-min incubation.

In the past one of us showed that the α chains of human hemoglobin, a long-lived cytoplasmic protein, were degraded largely by the ATP- and ubiquitin-dependent proteolysis pathway in both intact reticulocytes (Shaeffer, 1983) and their unfractionated lysates (Shaeffer, 1988). This finding is consistent with those of Rock *et al.* (1994) who showed by use of peptide aldehyde inhibitors of proteasome function that most long-lived proteins inside mouse lymphoblasts were degraded by this pathway. A search for the putative conjugates of the hemoglobin α subunits when the unfractionated reticulocyte lysates were incubated with ^{125}I-α-globin (in the absence of the artificial inhibitor Ubal) revealed the presence of only the Ub_1-α (and to a much lesser extent the Ub_2-α) species (Shaeffer, 1994a). Subsequent studies showed that the Ub_1-α conjugate preparation consisted of a mixture of molecules in which 57% had Ub attached to the amino-terminal two-thirds and 43% had Ub attached to the carboxyl-terminal one-third of the ^{125}I-α-globin monomer (Shaeffer, 1994b). Both types of monoubiquitin-^{125}I-α-globin molecules were found to be intermediates in the proteolysis of nonubiquitinated ^{125}I-α-globin by unfractionated lysates. The present finding that monoubiquitinated, but not unconjugated, α-globin can be degraded at a significant rate by the 26S proteasomes suggests that polyubiquitination of free α-globin is not an obligate step in its ATP-dependent proteolysis by unfractionated lysates. These observations are consistent with the hypothesis that a monoubiquitinated molecule may be the predominant form of the conjugated intermediates in the proteolysis of hemoglobin α chains, and perhaps of other long-lived cytoplasmic proteins, in intact cells.

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