A Ubiquitin-Protein Ligase Specific for Type III Protein Substrates*

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A previously studied species of ubiquitin-protein-ligase contains specific sites for the binding of basic (Type I) and bulky hydrophobic (Type II) NH2-terminal amino acid residues of protein substrates. We now describe another enzyme that ligates ubiquitin specifically to proteins that have NH2-terminal residues other than the above two categories (Type III substrates). The new species of ligase, that we call Eβ3, is separable from the formerly described ligase (termed Eα3) by affinity chromatography on protein substrate columns. Eβ3 was partially purified from extracts of rabbit reticulocytes and was shown to be required for the breakdown of Type III proteins. Apart from its different substrate specificity, it resembles Eα3 in some physical properties, in a requirement for ubiquitin carrier protein (E2) for conjugate formation, and in its action to ligate multiple ubiquitin units to the substrate protein. The denatured derivative of bovine pancreatic ribonuclease is a specific substrate for Eα3, while that of ribonuclease S-protein is a good substrate for Eβ3. Since S-protein is formed by the removal from ribonuclease NH2-terminal S-peptide, it is suggested that Eβ3 interacts with an NH2-terminal determinant exposed in ribonuclease S-protein.

Intracellular protein breakdown is a highly selective process. Some proteins are committed to degradation by ligation to the 76-amino acid polypeptide ubiquitin (for reviews, see Refs. 1–3). The rates of degradation of specific proteins are thus greatly influenced by their rates of ligation to ubiquitin. To understand the mechanisms of selectivity of intracellular protein breakdown, it is necessary to identify and characterize the enzymes involved in ubiquitin-protein ligation. A reason- able expectation is that several ubiquitin-protein ligases may exist, each of which recognizes certain structural features of proteins destined for degradation.

We have studied previously a species of ubiquitin-protein ligase, Eα3, that interacts mainly with certain NH2-terminal amino acid residues of substrate proteins (4–6). By the use of simple derivatives of amino acids that inhibit the binding of NH2-terminal residues of proteins to Eα3, three types of protein substrates that bind to distinct sites of Eα3 could be identified. Type I protein substrates have basic NH2-terminal amino acid residues, Type II substrates have bulky hydrophobic NH2-terminal residues, and Type III substrates have NH2-terminal residues other than the above two types (5). The two separate binding sites for basic and bulky hydrophobic NH2-terminal residues coexist on a single species of Eα3, as indicated by affinity chromatography on immobilized protein substrate columns (6). The same species of Eα3 also acts on some Type III proteins (6). We have observed, however, that another species of enzyme that ligates ubiquitin preferentially to Type III protein substrates does not bind to Type I or II protein substrate affinity columns (6).

In the present study we have followed up these observations. The new species of ligase (termed Eβ3) resembles the formerly described ligase (called Eα3) in some properties and physical characteristics, but is highly specific for certain Type III protein substrates.

Experimental Procedures

Materials

Bovine pancreatic ribonuclease, Ox-RNase,1 ribonuclease S-protein, hen egg white lysozyme, βLG, ribonuclease T1, ovalbumin, ubiquitin, and cytochrome c from Saccharomyces cerevisiae and from Candida krusei were purchased from Sigma, and H–αLA was obtained from Calbiochem. Reductively methylated ubiquitin was prepared as described (7). Ubiquitin-aldehyde was prepared by the procedure of Mayer and Wilkinson (8), except that salt formed in the last step was removed by dialysis against 20 mM Tris-Cl (pH 7.2), 0.1 mM EDTA. Reduction and carboxymethylation of RNase A and S-protein were carried out as described by Ferber and Ciechanover (9). Amino acid analysis of acid hydrolysates of these preparations showed essentially complete conversion of cysteine residues to carboxymethyl derivatives. All proteins were radiolabeled by the chloramine-T procedure, as described (10).

Preparations of Enzymes

Fraction II (a crude ubiquitin-free fraction) was prepared from lysates of rabbit reticulocytes by chromatography on DEAE-cellulose, as described (11). E1 and E2 (low molecular weight form) were purified from Fraction II by affinity chromatography on ubiquitin-Sepharose, followed by gel filtration chromatography on Ultrogel ACA-24 (11). Eα3 was purified by affinity chromatography on βLG-Sepharose and specific elution with Phe-Ala (6). The 26 S protease complex was purified as described (12) until the Sepharose 4B chromatography step. The leading one-half of the 26 S protease peak from the Sepharose 6B column was collected to ensure complete separation from Eα3 and Eβ3. Factor X was prepared as described (13).

Partial Purification of Eβ3

The separation of Eβ3 from Eα3 depended upon the removal of the latter by protein substrate affinity columns. For thorough removal of Eα3 we have used repeated application to H–αLA-Sepharose, since this immobilized protein binds Eα3 most tightly among the affinity columns tested (6). Fraction II was first applied to βLG-Sepharose for the isolation of Eα3 (6). The fraction not adsorbed to this column was concentrated by ultrafiltration on CF-20 cones (Amicon) to the

1 The abbreviations used are: Ox-RNase, bovine pancreatic ribonuclease oxidized with performic acid; H–αLA, human α-lactalbumin; βLG, bovine β-lactoglobulin; MeUb, reductively methylated ubiquitin; recm, reduced and carboxymethylated; DTT, dithiothreitol.

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shown in Fig. 1, a crude extract of reticulocytes was applied either Type I or II protein substrate affinity columns (6). The fraction not adsorbed to H-αLA-Sepharose was concentrated and incubated again with regenerated H-αLA-Sepharose under conditions similar to those described above. The affinity-unadsorbed fraction from the last treatment was collected. In this preparation, around 90% of E₃ resulted, as judged by the decrease in the rate of the degradation of 125I-H-αLA, a Type I substrate.

The affinity-unadsorbed fraction was precipitated with ammonium sulfate (0-38% saturation) under conditions similar to those described for the preparation of Fraction IIA (13). This preparation was concentrated by ultrafiltration to one-fifth of the initial volume of Fraction II and was subjected to gel filtration chromatography, as described under Fig. 2.

Determination of E₃ Activity

The following assays were used for the determination of activities of either E₃ or E₆.

Protein Breakdown Assay—E₃ activity was determined by the stimulation of protein degradation in an ATP/ubiquitin-dependent system reconstituted from purified components under conditions in which E₃ was rate-limiting. The reaction mixture contained in a volume of 50 μl: 50 mM Tris-HCl (pH 7.6), 3 mM DTT, 5 mM MgCl₂, 4 mM ATP, 3 μg of ubiquitin, 1.5 micromolars of E₃, 0.7 micromolars of E₆ (see Ref. 11 for the definition of a unit of activity), 5 μl of 26 S protease complex, 0.5 μl of Factor X and 125I-labeled protein substrate, and E₆ or E₆β as indicated in the legends to figures. Following incubation at 37 °C for 60 min, the release of labeled material soluble in 20% trichloroacetic acid was determined as described (11). A parallel incubation was conducted without E₃ and E₆-dependent activity was calculated by the difference. All results were taken from the range linear with E₃ concentration.

Ubiquitin Conjugation Assay by Gel Electrophoresis—Reaction mixtures contained in a volume of 20 μl: 50 mM Tris-HCl (pH 7.6), 5 mM MgCl₂, 2 mM DTT, 2 mM ATP, 1.5 micromolars of E₃, 0.7 micromolars of E₆, 15 μg of ovalbumin, 3 μg of MeUb, and 125I-labeled protein substrate and E₆ or E₆β as specified in the figure legends. MeUb was used instead of ubiquitin to prevent the formation of very large polyubiquitin derivatives (11, 14) that remain at the origin in polyacrylamide gel electrophoresis. Following incubation at 37 °C for 30 min, the samples were separated on 12.5% polyacrylamide sodium dodecyl sulfate gels, dried, and radioautographed.

Quantitative Ubiquitin Conjugation Assay—This was carried out by a previously described method in which the conjugation of 125I-ubiquitin to protein is followed by adsorption to a mixture of DE52 and CM52 resins (11). For the determination of the activities of E₆ and E₆β, the substrates were Ox-RNase or S-protein (0.2 mg/ml), respectively.

RESULTS AND DISCUSSION

In a previous study on the affinity purification of E₃ with immobilized protein substrates, we noted that an enzyme that ligates ubiquitin to some Type III proteins does not bind to either Type I or II protein substrate affinity columns (6). Several enzymes that ligate ubiquitin to proteins are apparently not involved in protein breakdown (15-18). We have therefore first examined whether this ligase is required for the degradation of Type III substrates. In the experiment therefore first examined whether this ligase is required for the degradation of Type III substrates. In the experiment described under Fig. 2.

The ligase specific for Type III proteins was partially purified from the affinity-unadsorbed fraction by fractionation with ammonium sulfate (see “Experimental Procedures”) and gel filtration chromatography (Fig. 2). Its activity was followed in purification by two assays: the ligation of S-protein to ubiquitin and the ATP/ubiquitin-dependent degradation of the same substrate in a proteolytic system reconstituted from purified components, the activity of which depends upon the supplementation of E₃ (see “Experimental Procedures”). As shown in Fig. 2A, a peak of enzyme activity that stimulated the breakdown of 125I-S-protein in the reconstituted proteolytic system eluted at an apparent molecular weight of approximately 350,000. There was little ATP-independent proteolytic activity in all column fractions (Fig. 2A), presumably due to the removal of most of ATP-independent multicatalytic protease by prior fractionation with ammonium sulfate (13, 19). To examine whether the enzyme that stimulates ATP-dependent degradation of 125I-S-protein is indeed identical to the ligase, fractions of the same gel filtration separation were assayed for the ligation of S-protein to ubiquitin. As shown in Fig. 2B, there was a strong ligase activity that eluted in exact coincidence with the activity that stimulated protein breakdown (center of peak at fractions 32-33 in both assays). We call this new species of ubiquitin-protein ligase E₆β, while the formerly characterized species of E₃ (that has binding sites for the NH₂-terminal residues of Type I and II protein substrates) is called E₆α.

E₆β resembles E₆α in several physical characteristics. Both enzymes have a similar apparent molecular weight of 350,000 in gel filtration chromatography (Fig. 2 and Ref. 11). Both are precipitated at similar concentrations of ammonium sulfate and are eluted at a similar ionic strength in hydrophobic chromatography on phenyl-Sepharose (data not shown). Sim-
jugates of 'Y-S-protein are formed with reductively methylated single lysine residue (21). With reductively methylated ubiquitin, a single low molecular weight conjugate was formed, while with native ubiquitin form polyubiquitin chains linked to the substrate protein. This was examined by the use of ribonuclase T1 that has a requirement for the formation of polyubiquitin derivatives (see below) and the observation that high molecular weight multiple conjugates of MeUb with 125I-S-protein. Samples of 5 µl of column fractions were assayed for the conjugation of 125I-S-protein (0.8 µg, 1.3 × 10⁶ cpm) by the gel electrophoresis assay described under “Experimental Procedures.” Prior to incubation, samples of column fractions were treated with ubiquitin-aldehyde (5 µM) for 10 min at 37 °C to inactivate ubiquitin-protein hydrolases (24). Fraction numbers are indicated at the top. Contam., a contamination in the preparation of 125I-S-protein.

The specificities of the two types of E2 enzymes include the formation of polyubiquitin derivatives (see below) and the requirement for E2 for conjugate formation (data not shown). E2β is thus clearly different from several types of E2 that transfer ubiquitin to certain proteins (15, 18, 20).

The specificities of the action of E2α and E2β in the ligation of ubiquitin to different proteins are compared in Fig. 3. While E2α acts strongly on the Type I and II protein substrates, lysozyme (NH₂ terminus: Lys) and βLG (NH₂ terminus: Leu), little if any activity of E2β on these proteins was observed. By contrast, E2β was much more active in the ligation of ubiquitin to some Type III proteins, such as ribonuclease S-protein and ribonuclease T1; (NH₂ termini: Ser and Ala, respectively). The two enzymes are similar in that they both ligate several molecules of ubiquitin to the substrate protein, as indicated by the observation that high molecular weight multiple conjugates of 125I-S-protein are formed with reductively methylated ubiquitin (Figs. 2B and 3). In addition E2β, like E2α, can form polyubiquitin chains linked to the substrate protein. This was examined by the use of ribonuclease T1, that has a single lysine residue (21). With reductively methylated ubiquitin, a single low molecular weight conjugate was formed, while with native ubiquitin E2β promoted the formation of multiple high molecular weight polyubiquitin derivatives of ribonuclease T1 (Fig. 3).

The specificities of the two types of E2 for different protein substrates were further examined by the quantitative protein breakdown assay (Table I). While E2β acts predominantly on Type III substrates, the action of E2α is not limited to Type I and II protein substrates. All Type III proteins tested are substrates for E2α as well, but the ratio of E2β/E2α activities is different for various Type III proteins. The activity of E2β (relative to E2α) is highest with S-protein and ribonuclease T1, lower with cytochrome c from S. cerevisiae and lowest with cytochrome c from C. krusei (Table I).

The question that arises is what specific features of Type III proteins are recognized by E2β. It is notable that Ox-RNase is a specific substrate for E2α (Table I), while ribonuclease T1, lower with cytochrome c from S. cerevisiae and lowest with cytochrome c from C. krusei (Table I).

**Fig. 2.** Gel filtration chromatography of a species of ligase that acts on ribonuclease S-protein. A sample of 1 ml of the 0-38% ammonium sulfate cut of the affinity-unadsorbed fraction (see “Experimental Procedures”) was applied to a column (1 × 48.5 cm) of Sepharose 6B equilibrated with 20 mM Tris-HCl (pH 7.2), 1 mM DTT, and 1 mg/ml ovalbumin. Elution was continued with the above buffer, and fractions of 0.66 ml were collected at 4 °C. A, stimulation of the degradation of 125I-S-protein. 10-µl samples of column fractions were assayed for the stimulation of the breakdown of 125I-S-protein (0.5 µg, 9 × 10⁶ cpm) with the reconstituted system described under “Experimental Procedures,” in the presence (O) or absence (X) of ATP. Marker proteins (arrows): AF, apoferritin, M, = 440,000; AM, β-amylase, M, = 200,000; ADH, yeast alcohol dehydrogenase, M, = 150,000. B, formation of conjugates of MeUb with 125I-S-protein. Samples of 5 µl of column fractions were assayed for the conjugation of 125I-S-protein (0.8 µg, 1.3 × 10⁶ cpm) by the gel electrophoresis assay described under “Experimental Procedures.” Prior to incubation, samples of column fractions were treated with ubiquitin-aldehyde (5 µM) for 10 min at 37 °C to inactivate ubiquitin-protein hydrolases (24). Fraction numbers are indicated at the top. Contam., a contamination in the preparation of 125I-S-protein.

**Fig. 3.** Ligation of different proteins to ubiquitin by E2α and E2β. Reaction conditions were as described under “Experimental Procedures” for the gel electrophoresis assay. 125I-Labeled proteins (indicated at the top) were supplemented at 1-1.8 × 10⁶ cpm and 1 µg, except for 125I-ribonuclease T1, that was 0.1 µg. Lanes 1, without E2, lanes 2, with 0.5 micromoles of E2α; lanes 3, with 0.12 micromoles of E2β. All incubations were carried out in the presence of MeUb (see “Experimental Procedures”), except for those with 125I-ribonuclease T1 (lanes i-3) that contained unmodified ubiquitin (5 µg). In lanes 4, 125I-ribonuclease T1 was incubated with MeUb and E2β. Preparations of E2β were treated with ubiquitin-aldehyde, as described for Fig. 2B. In lanes 1, all labeled protein bands of molecular sizes higher than unconjugated proteins are contaminants present in protein preparations. The arrow on the right indicates the monoubiquitin derivative of ribonuclease T1 with MeUb.
Table I

Specificities of E₆α and E₆β for the degradation of different types of protein substrates

The rates of degradation of different ¹²⁵I-labeled proteins were determined with the reconstituted proteolytic system (see "Experimental Procedures") in the presence of 1.5 micromolars of E₆α or 0.44 micromolars of E₆β.

| ¹²⁵I-Protein substrate | NH₂ terminus | Degraded | Ratio, E₆α/E₆β | % |
|------------------------|-------------|----------|----------------|---|
| Type I                 |             |          |                |   |
| Ox-RNase               | Lys         | 13.5     | 3.0            | 0.2 |
| H-αLA                  | Lys         | 58.8     | 0              | 0  |
| Type III               |             |          |                |   |
| S-protein              | Ser         | 2.8      | 31.9           | 11.4 |
| Ribonuclease T₁        | Ala         | 1.8      | 19.1           | 10.6 |
| Cytochrome c (S. cerevisiae) | Thr | 5.2     | 14.2           | 2.7 |
| Cytochrome c (C. krusei) | Pro | 4.3      | 3.6            | 0.8 |

FIG. 4. Inhibition of E₆β and E₆α by reduced carboxymethylated derivatives of ribonuclease. A, the degradation of ¹²⁵I-S-protein (0.4 µg, 5.5 × 10⁵ cpm) was determined with the reconstituted system rate-limited by ¹²⁵I-Ox-RNase. The nature of the NH₂-terminal signal in Type III proteins that is recognized by E₆β remains to be elucidated. Varshavsky and co-workers (23) have recently reported that the degradation in reticulocyte lysates of derivatives of β-galactosidase that have NH₂-terminal Ser, Ala, or Thr residues is specifically inhibited by dipeptides that have similar amino-terminal residues. These investigators proposed that a distinct "N-end recognizing activity" exists for the degradation of proteins with small and uncharged NH₂-terminal amino acid residues. Most of the NH₂-termini of the Type III protein substrates of E₆β used in the present study belong to this category (Table I). However, we could not detect any inhibition of E₆β by homologous dipeptides with the presently used Type III protein substrates (data not shown). Further study is required to define the NH₂-terminal signal and other recognition determinants that may exist in Type III proteins.

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