Specific Disulfide Cleavage Is Required for Ubiquitin Conjugation and Degradation of Lysozyme*

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Both ubiquitin conjugation and ubiquitin-dependent degradation of chicken egg white lysozyme in a reticulocyte lysate depend on the presence of a reducing agent. We present evidence that the reduction of a specific disulfide bond, namely that at Cys²-Cys¹⁸⁷, facilitates ubiquitination and is a prerequisite to the formation of a multiubiquitin chain on one of at least four chain initiation sites on lysozyme. The Cys²-Cys¹⁸⁷ disulfide bond in lysozyme can be specifically reduced, and the modified protein can be isolated after carboxymethylation of the 2 resulting cysteines. This modified lysozyme no longer requires the presence of a reducing agent for ubiquitin conjugation and degradation. Inhibition of ubiquitination by the dipetide Lys-Ala revealed that this modified lysozyme, like the unmodified protein, is recognized via the binding of the ubiquitin protein ligase, E3, to the substrate’s N-terminal lysyl residue. Both the rate and the extent of ubiquitin-lysozyme conjugation, however, are significantly higher with this modified substrate. Likewise, ubiquitin-dependent degradation of 6,127-reduced-carboxymethylated lysozyme was 2–4-fold faster than degradation of the unmodified counterpart. These results are consistent with an interpretation that the modified lysozyme mimics an intermediate formed at the rate-limiting step of the degradation of lysozyme in the reticulocyte lysate. Reduction of the Cys²-Cys¹⁸⁷ disulfide bond is expected to unhinge the N-terminal region of lysozyme, and we propose that the recognition of this otherwise stable protein by the ubiquitin pathway is due to facilitated binding of E3 that results from such a conformational transition.

Genetic and biochemical studies have shown that, in eukaryotes, cellular abnormal and damaged proteins are degraded by a ubiquitin-mediated pathway. In this pathway, ubiquitin, a 76-amino acid polypeptide, is linked through its C terminus to form an amide (formally, an isopeptide) bond with a lysyl ε-amino group on the substrate protein (reviewed in Refs. 1 and 2). How proteins are targeted for this covalent modification is fundamental to the issue of selective intracellular protein turnover. Moreover, the ubiquitin system further discriminates among potential substrates in that not all ubiquitin conjugation targets are destined for degradation. Understanding the substrate selection process will require a description of those structural features recognized by the ubiquitination enzymes.

For one pathway of ubiquitin-dependent degradation, several studies have pointed to the importance of the N-terminal region of proteins in the substrate recognition step. Bachmair et al. (3), by the use of Escherichia coli β-galactosidase derivatives containing specific N-terminal extensions, showed that the N-terminal residue in these proteins provided a signal for their recognition by the ubiquitin degradation pathway in the yeast Saccharomyces cerevisiae. By variation of the N-terminal residue, the efficacy of each of the 20 common amino acids as the recognition signal was ranked in yeast and, more recently, in rabbit reticulocyte lysate (3, 4). The same N-terminal extensions have been shown to confer recognition when fused with the otherwise stable dihydrofolate reductase (5). Involvement of the N-terminus as a recognition signal also has been established in work by Hershko and his colleagues (6, 7), who demonstrated an N-terminal amino acid dependence for the binding of substrates to the ubiquitin protein ligase (also called E3). E3, together with ubiquitin carrier proteins (also called E2 or ubiquitin conjugation enzymes), was required for ubiquitination in vitro of certain proteins such as α-lactalbumin, β-lactoglobulin, and ribonuclease A derivatives.

Notwithstanding the substantial evidence implicating the N-terminal amino acid as a recognition determinant, other features also must be involved in substrate selection. Whereas native ribonuclease A is not ubiquitinated despite having a permissive or “destabilizing” N-terminal residue, a variety of chemically modified forms of the protein are excellent substrates (8, 9). Recognition of these derivatives is due to specific interactions with the damaged or modified amino acids but is a consequence of the unfolding that accompanies the covalent modifications (9). Similarly, in addition to a permissive N-terminal amino acid, ubiquitination of β-galactosidase and dihydrofolate reductase requires a 33–45-residue N-terminal extension that is thought to be unstructured and relatively flexible (5, 10).

Hen (chicken) egg white lysozyme has been used extensively as a substrate to study the ubiquitin-dependent proteolytic pathway in vitro. The recognition of this substrate protein has been shown to require the binding of the ubiquitin protein ligase, E3, to the N-terminal lysine residue in lysozyme (6). Thus, lysozyme appears to be ubiquitinated via the same pathway as the ribonuclease derivatives and fusion protein...
substrates discussed above. However, whereas some aspect of "unfoldedness" is involved in the selection of these latter proteins for ubiquitination, native lysozyme has been employed routinely as an effective substrate. In the present study, we provide evidence that native lysozyme in fact is not ubiquitinated, but that, under conventional ubiquitin conjugation and degradation assay conditions, a small fraction of the protein is specifically reduced at one of the four disulfide bonds (Cys5-Cys127). This is the first step in the recognition of lysozyme for degradation by the ubiquitin pathway in reticulocyte extracts in a process that leads to the formation of a multiquitin chain. The ubiquitin moieties in this chain are joined to each other via an isopeptide bond that is formed between the C-terminal Gly of one ubiquitin and Lys of an adjoining ubiquitin. This structure previously was found to target the degradation of β-galactosidase (10), presumably by serving as a docking site for the ubiquitin-dependent protease. Our observations regarding lysozyme ubiquitination and the properties of a three-disulfide lysozyme derivative offer, for one class of E3-dependent substrates, the opportunity to explore the reaction pathway of ubiquitin-mediated proteolysis with a structurally defined protein.

**EXPERIMENTAL PROCEDURES**

**Materials**—[35S]Labeled proteins were prepared by radioiodination with chloramine T (11) and carrier-free Na[35S]I from Amersham Radiochemicals. The diphtheria toxin A chain, lysozyme, chicken (chicken egg white lysozyme (3 × crystallized, grade I) were purchased from Sigma. Ubiquitin was purified further by cation-exchange chromatography using a Pharmacia LKB Biotechnology Inc. Mono S column and fast protein liquid chromatography system. [leucine-3H]Ubiquitin was produced in E. coli harboring a plasmid encoding yeast ubiquitin and grew in [3H]leucine medium (provided by J. Setsuda, UCLA). Ubiquitin-depleted reticulocyte lysate (Fraction II) was prepared as described previously (12), from washed rabbit reticulocytes purchased from Green Hectares (Oregon, WI) except that lysates contained 0.1 mM DTT.2 Comparable activity and stability of the lysates were found whether 0.1 mM or the more usual 1 mM DTT was used for storage. Purified ring-necked pheasant lysozyme was generously provided by Drs. E. Prager and A. C. Wilson of the University of California at Berkeley. Prestained molecular weight markers were from Bethesda Research Laboratories.

**Synthesis of Ubiquitin Variants**—The chemical alterations in the ubiquitin variants are shown in Fig. 1. Ub-C48 differs from wild-type ubiquitin in having a cysteine at position 48 instead of a lysine. This variant protein was obtained by expression of a mutated ubiquitin gene in E. coli AR58 cells and was isolated as described previously (13). Reductive methylation and S-aminoethylthiolation of Ub-C48 were done, as reported previously (13), and are described in more detail below. Purified and lyophilized Ub-C48 was dissolved to 1 mg/ml in 8 M urea, 1 M DTT, and 0.1 M Na+-Hepes, pH 7.0. Sodium cyanoborohydride and formamidhydrazide were then added sequentially to final concentrations of 20 and 12 mM, respectively. After gentle stirring for 18 h at room temperature, further additions of the two reagents were made to the solution and the reaction was continued for an additional hour. The solution was then dialyzed against six changes of 20 mM DTT and lyophilized. The fraction of unblocked amino groups in the MeUb-C48 preparation was less than 4%, as judged by its reaction with fluorescamine (14). To convert MeUb-C48 to MeUb-AEtC48 (12), 100 mg of lyophilized MeUb-C48 was dissolved in 30 ml of water, and the sulfhydryl content of this protein solution was determined by its reaction with 5,5'-dithiobis(2-nitrobenzoic acid) (15). The solution was then adjusted to 100 ml by the addition of N-ethylylmethacrylate acid, pH 8.5, to a final concentration of 0.25 M. A 25-fold molar excess of N-(3-iodoethyl)trifluoroacetamide (Aminoethyl-8TM, Pierce Chemical Co.) in 2 ml of methanol was added in two portions, 1 h apart, and the reaction was stirred gently at 50 °C. Progress of the reaction was monitored with the sulfhydryl group assay. In general, the reaction was complete within 4 h, and the sample was then dialyzed against water and lyophilized. The products were separated from unreacted protein by cation-exchange chromatography as follows. The proteins were desalted using a column of 100 mM ammonium acetate, pH 4.5, and then loaded onto a 2 cm × 20 cm column of carboxymethylsepharose (CM52, from Whatman) equilibrated in the same buffer. After washing with 200 ml of the pH 4.5 buffer, proteins were eluted with a 500-ml gradient of 25 mM ammonium acetate, pH 4.5-7.0. MeUb-C48 eluted at pH 5.2 and MeUb-AEtC48 at pH 5.7, and they were identified by their distinctive conjugation patterns with a β-galactosidase derivative (10). Each peak was pooled and electrophorographed on an LKB SP-5PW column, using the same pH gradient, to obtain the pure proteins. A yield of 20 mg of Ub-AEtC48 was obtained from 100 mg of Ub-C48. The sample was dialyzed against water and stored as a lyophilized powder. MeUb-MeAETC48 was obtained by reductive methylation of MeUb-AEtC48 using the same procedure for the derivatization of Ub-C48.

**Conjugation and Degradation Assays**—The conjugation of ubiquitin or its variants to [35S]lysozyme or to [35S]125I-6,127-rcm-lysozyme in the ubiquitin-depleted reticulocyte lysate was done at 37 °C for 30 min in a 25-μl reaction containing 50 mM Tris-HCl, pH 7.5, 2 mM DTT, 5 mM MgCl2, 2 mM ATP, and an ATP-regenerating system (10 mM creatine phosphate and 10 mM creatine phosphokinase) (16). Either no ubiquitin or 50 μM ubiquitin (or one of its variants) was also added, and reactions were stopped by the addition of SDS-gel sample buffer. Alternatively, assays with [35S]ubiquitin (2.5 μM, 5 × 104 cpm in 20 μl) and in a 50 mM Na+-Hepes buffer, pH 7.2, were done as described (9) and yielded similar results. Products were analyzed by gel electrophoresis (17) and autoradiography. The mobility of lysozyme for degradation by the ubiquitin pathway in reticulocyte extracts in a process that leads to the formation of a multiquitin chain. The ubiquitin moieties in this chain are joined to each other via an isopeptide bond that is formed between the C-terminal Gly of one ubiquitin and Lys of an adjoining ubiquitin. This structure previously was found to target the degradation of β-galactosidase (10), presumably by serving as a docking site for the ubiquitin-dependent protease. Our observations regarding lysozyme ubiquitination and the properties of a three-disulfide lysozyme derivative offer, for one class of E3-dependent substrates, the opportunity to explore the reaction pathway of ubiquitin-mediated proteolysis with a structurally defined protein.

1. L.-K. Bruhn, J. Setsuda, and R. E. Cohen, unpublished procedure.
2. The abbreviations used are: DTT, dithiothreitol; Heps, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; rcn, reduced and carboxymethylated; SDS, sodium dodecyl sulfate; HPLC, high performance liquid chromatography; Me, methyl; AET, aminoethyl.
as a rcm/three-disulfide lysozyme derivative was calculated from the relative peak areas.

**Analytical Methods**—Protein concentrations were determined spectrophotometrically with extinction coefficients at 280 nm of 0.16 ml mg⁻¹ cm⁻¹ for ubiquitin (19) and 2.64 ml mg⁻¹ cm⁻¹ for lysozyme (20). The lysozyme extinction coefficient was found to apply well to 6,127-rcm-lysozyme. This was established by determination of 6,127-rcm-lysozyme stock solution concentrations with the trinitrobenzene sulfonate assay (21) using lysozyme as a standard.

Amino acid compositions were determined with the o-phthalaldehyde precolumn derivatization method (22) as described (9). Protein sequencing was done at the UCLA Protein Microsequencing Laboratory with an Applied Biosystems model 470A gas-phase sequenator equipped with a 120A phenylthiohydantoin analyzer for on-line HPLC detection. Prior to sequencing, some samples were fully reduced and S-pyridylethylated with 4-vinylpyridine (Aldrich) (23).

Circular dichroism spectra were acquired with a Jasco 5-600 spectropolarimeter. The instrument was calibrated with (+)-10-camphorsulfonic acid (24), and protein solutions of 0.3–0.5 mg/ml were used in a 0.02-cm path length quartz cell (Hellma Cells, Inc.). Typically, a 10 nm/min scan rate with a 4-s time constant was used, and 12 scans were averaged for each spectrum.

Gel filtration analyses were done with a Tosohaas G2000-SWXL column (0.78 × 30 cm) eluted at 0.4 ml/min with 0.2 M NaP₀₇, pH 7.0. An HPLC system equipped with Waters 510 pumps and a Shimadzu SPD-6AV detector was employed.

**RESULTS**

**Conjugation of Hen (Chicken) Egg White Lysozyme with Ubiquitin Variants in Reticulocyte Lysate**—Previous studies have shown that ubiquitin-lysozyme conjugates, containing multiple ubiquitin moieties, are formed prior to the degradation of lysozyme in reticulocyte lysates (16, 25). At least a portion of the ubiquitin moieties are in the form of ubiquitin-ubiquitin linkages, given that the number of ubiquitins can far exceed the total of 6 lysine residues in lysozyme (26, 27). Furthermore, the number of ubiquitin moieties in these conjugates was reduced when ubiquitin was replaced with an N-methylated ubiquitin derivative (27); we have previously described a set of ubiquitin variants that can be used to test for the presence of specific ubiquitin-ubiquitin linkages in protein conjugates (13). Fig. 1 depicts the alterations that were introduced into ubiquitin to generate the variants used in this study. The proteins all were formed by chemical modification of the site-specific mutant protein, Ub-C₄₈, in which Lys⁴₈ of wild-type ubiquitin has been replaced by a cysteine. Neither MeUb-C₄₈ nor MeUb-MeAEtC₄₈ can form ubiquitin-ubiquitin linkages due to the lack of free amino groups. Ub-C₄₈ has the potential of forming wild-type ubiquitin-ubiquitin linkages except at residue position 48, whereas linkage to MeUb-AEtC₄₈ is restricted to the S-aminoethylcysteine at position 48. Native or variant ubiquitin was added to a ubiquitin-depleted reticulocyte lysate to test for the elaboration of specific multiubiquitin chain structures onto lysozyme, and the results are described below.

Fig. 2A shows the autoradiograph of an SDS-polyacrylamide gel of the ubiquitin-lysozyme conjugates that were formed between [¹²⁵I]-lysozyme and ubiquitin or one of the ubiquitin variants. With native ubiquitin, as had been shown previously by others (16), distinct ubiquitin-lysozyme conjugates can be resolved according to their molecular sizes (Fig. 2A, left panel, lane 2). When the ubiquitin-depleted reticulocyte lysate was supplemented with either MeUb-C₄₈ or MeUb-MeAEtC₄₈, neither of which is capable of ubiquitin-ubiquitin linkage, four conjugates were detected (Fig. 2A, left panel, lanes 3 and 4). These species migrated with apparent molecular masses of 23, 29, 38, and 41 kDa, consistent with conjugates having one to four ubiquitin moieties, respectively. This result suggested that at least 4 lysines on lysozyme can be linked with ubiquitin. With native ubiquitin, conjugates of greater molecular weights than were found with the N-methylated variants indicate formation of multiubiquitin chains. That the ubiquitin-ubiquitin linkages in the lysozyme conjugates are formed exclusively through an isopeptide bond between the C-terminal carboxyl group of one ubiquitin and the ϵ-amino group of Lys⁴₈ in another ubiquitin is suggested by two lines of evidence. First, the conjugates obtained with either Ub-C₄₈ or MeUb-C₄₈ were indistinguishable on SDS-polyacrylamide gels (data not shown), indicating that ubiquitin-lysines other than Lys⁴₈ did not contribute to ubiquitin-ubiquitin linkages. Second, conjugates with the MeUb-AEtC₄₈ variant included a number of high molecular weight species found with wild-type ubiquitin but not with the other derivatives.

Ubiquitin-dependent degradation of lysozyme is shown in Fig. 2B. Only MeUb-AEtC₄₈ was comparable (within 10%) to native ubiquitin in its ability to stimulate lysozyme degradation in the ubiquitin-depleted reticulocyte lysate, whereas the other three ubiquitin variants stimulated degradation by no more than 30% (data not shown). This result is in agreement with previous studies (10) that suggested that a specific multiubiquitin chain is responsible for the proteolytic targeting of a ubiquitin-protein conjugate.

The formation of ubiquitin-lysozyme conjugates exhibited a strong dependence on the presence of the reducing reagent, DTT. Conjugate formation decreased greatly when 0.01 rather than 2 mM DTT was used (compare left and right panels, Fig. 2A); a similar DTT dependence was observed when conjugation assays employed unlabeled lysozyme and either [¹²⁵I]- or [leucyl-¹³C]ubiquitin (data not shown). Likewise, DTT was essential for ubiquitin-dependent proteolysis of [¹²⁵I]-lysozyme (Fig. 2B). Although ubiquitin conjugation enzymes and the protease component in the reticulocyte lysate possess critical cysteines that must be preserved for their activity, two lines of evidence suggested that the primary effect of the reducing agent in these experiments is to convert lysozyme from an inactive to an active substrate. First, DTT-depleted and 2 mM DTT-supplemented reticulocyte extracts were equally capable...
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Fig. 2. Ubiquitin conjugation and degradation of hen (chicken) egg white lysozyme. A, conjugation was performed with 6 μM 125I-lysozyme (2 x 10⁶ cpm/μg) and 50 μM ubiquitin or its variant in a ubiquitin-depleted reticulocyte lysate in the presence of ATP for 10 min at 37 °C. The reaction mixtures contained 0.01 (right panel) or 2 mM DTT (left panel). The reactions were terminated by the addition of SDS sample buffer, and the products were separated by electrophoresis on a 12% SDS-polyacrylamide gel and visualized by autoradiography. The migration positions of 125I-lysozyme and a contaminant are marked at the top. The addition of SDS sample buffer, and the products were separated by electrophoresis on a 12% SDS-polyacrylamide gel and visualized by autoradiography. The migration positions of 125I-lysozyme and a contaminant are marked at the left as HEWL and cont., respectively. Arrows on the right indicate the positions (from top to bottom) of the prestained molecular mass markers ovalbumin (43 kDa), carbonic anhydrase (29 kDa), β-lactoglobulin (18.4 kDa), and lysozyme (14.3 kDa). Lane 1, no addition of ubiquitin; lane 2, ubiquitin; lane 3, MeUb-C48; lane 4, MeUb-AEtC48; lane 5, MeUb-MeAEC48. B, degradation of 125I-lysozyme was done under the same conditions as in A, except that the reactions were terminated by the addition of 10% trichloroacetic acid at the specified times (see “Experimental Procedures”). The acid-soluble counts at each time point were determined and are expressed as a percentage of 125I-lysozyme degraded. Solid lines, reactions with 50 μM MeUb-AEtC48 and 0.01 (B) or 2 mM DTT (C) without added ubiquitin.

of ubiquitin conjugation to several ribonuclease A derivatives as well as numerous endogenous proteins in the lysate (9). Similarly, ubiquitin conjugation and ubiquitin-mediated degradation of calmodulin were equally efficient with either 0.01 or 2 mM DTT (not shown). Second, increasing concentrations of DTT lead to concomitant increases in the formation of ubiquitin-lysozyme conjugates. Experiments that demonstrate this are described below.

Specific Reduction of the Cys8-Cys127 Disulfide Bond in Lysozyme—To examine the effect of DTT on lysozyme, we first treated the protein with 2 mM DTT, a concentration found to be effective for the ubiquitin-dependent degradation of lysozyme, under conditions of pH and ionic strength which mimicked those of conjugation and degradation assays. The

Fig. 3. Partial reduction of lysozyme by 2 mM DTT under conjugation assay conditions. A, disulfide linkages in hen (chicken) egg white lysozyme (29). B, HPLC fractionation of partially reduced lysozyme after trapping with iodoacetic acid. Lysozyme (0.4 mg/ml) was incubated at 37 °C in 90 mM NaCl plus 50 mM NaHepes, pH 7.2, either with no DTT or with 2 (inset (b)) or 4 mM (inset (a)) DTT. After 1 h, the reactions were quenched with 0.5 M K²-iodoacetate and proteins were processed and separated by cation-exchange HPLC as described under “Experimental Procedures.” The elution positions of the carboxymethylated three-disulfide lysozyme (3 (S-S)), a contaminant of the commercial lysozyme (cont.), and native lysozyme are indicated. The ordinate represents a full scale of 0.05 absorbance units.
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sequenced through eight Edman degradation cycles. Whereas S-pyridylethylcysteine was detected at position 6 of the control lysozyme derivative, carboxymethylcysteine was found exclusively, with no trace of the pyridylethyl compound, at position 6 of the three-disulfide lysozyme sample. Thus, the small amount of reduced lysozyme formed under conjugation assay conditions is cleaved specifically at the Cys127-Cys127 disulfide, and this partially reduced protein could be trapped to yield 6,127-rcm-lysozyme.

The iodoacetate trapping/HPLC method was used to quantify the fraction of three-disulfide lysozyme formed at different concentrations of DTT, and the results are shown in Fig. 4. As expected, the reduced lysozyme increased linearly with up to 8 mM DTT. Beyond 10 mM DTT, however, the yield of the three-disulfide species began to plateau (not shown), a result we attribute to increasing aggregation of proteins in the reaction. If the three-disulfide derivative, but not native lysozyme, is the true ubiquitination substrate, then conjugate yields should show a similar dependence upon DTT concentration. Conjugation assays with 125I-ubiquitin and lysozyme were performed with various levels of DTT, and the conjugates were quantified by excision and γ-counting of the appropriate band from an SDS-polyacrylamide gel. The result, shown for tetraubiquitinated lysozyme, is that conjugation parallels generation of the three-disulfide form of lysozyme (Fig. 4) and supports our conclusion that cleavage of the Cys5-Cys127 disulfide is the obligatory step for conversion of lysozyme into a ubiquitination substrate.

When 6,127-rcm-lysozyme was tested as a substrate instead of native lysozyme, we found that both its ubiquitin conjugation and its degradation in reticulocyte lysate did not require DTT (Fig. 5). Moreover, the rate and extent of both ubiquitin conjugation and degradation of the modified lysozyme were considerably higher than those of its native counterpart in the presence of 2 mM DTT (Fig. 6). Thus, prior reduction of the Cys5-Cys127 disulfide bond not only circumvented the requirement for a reducing agent in the assay, but the modified lysozyme also behaved as a kinetically competent intermediate. These results suggest that the rate-limiting step in the degradation of lysozyme in reticulocyte lysate is the reduction of the Cys5-Cys127 disulfide bond. To validate this conclusion, it was necessary to demonstrate that the reduction and the carboxymethylation of Cys5 and Cys127 did not lead to an alternative pathway for ubiquitination of the modified lysozyme. Experimental support for this point is described below.

Previous studies had shown that an early event in the recognition of lysozyme by the ubiquitin pathway is the specific binding of the ubiquitin-protein ligase, E3, to the N-terminal lysine on lysozyme (6). The importance of this pathway for lysozyme ubiquitination in our experiments was apparent from comparative studies with ring-necked pheasant lysozyme. This lysozyme, which has a 93% sequence identity with the chicken protein, has Gly (rather than Lys) as its first amino acid (30). As expected for E3-dependent ubiquitination and a protein with a nonpermissive N-terminus (4), virtually no conjugation to the pheasant lysozyme was observed in the reticulocyte lysate, with or without DTT (data not shown). The conclusion, that lysozyme ubiquitination in these reactions was predominantly E3-dependent, was extended to

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Footnote: It should be noted that ubiquitin conjugation to 6,127-rcm-lysozyme decreased with time in the lysate, even without added ATP, as determined from lowered conjugate yields following preincubation of the substrate in the lysate prior to ubiquitin and ATP addition. This inactivation was much faster when 2 mM DTT was included. Whether this effect is due to further reduction of disulfide bonds in the 6,127-rcm-lysozyme, nonspecific proteolysis, or other causes remains to be determined.

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same specific radioactivity was performed in the reticulocyte lysate containing 50 μM of either native or modified lysozyme radioiodinated to the same specific radioactivity (1.5 \times 10^4 \text{cpm/μg}). The reactions were terminated at the times indicated (min) by the addition of SDS sample buffer, separated by electrophoresis on a 12% SDS-polyacrylamide gel, and autoradiographed. Arrows on the right indicate the migration positions of molecular weight markers as in Fig. 2. HEWL, 125I-lysozyme.

6,127-rcm-lysozyme by the use of a Lys-Ala dipeptide to inhibit E3 binding to the substrate. Fig. 7 shows that this dipeptide inhibits both the conjugation and the degradation of 6,127-rcm-lysozyme, as had been reported previously for native lysozyme (6). Thus, reduction of the Cys^4-Cys^{127} disulfide bond and carboxymethylation of the cysteines did not alter the initial E3 recognition step in the reaction pathway. We note that, without DTT, even a 20-fold excess of native lysozyme did not inhibit ubiquitin conjugation to the 6,127-rcm derivative (data not shown). The results presented here, therefore, strongly suggest that the modified lysozyme mimics an intermediate that is generated during the degradation of lysozyme in reticulocyte lysates.

The 6,127-rcm-lysozyme Derivative Has a Native-like Structure—The conformation of 6,127-rcm-lysozyme was evaluated by comparison of its circular dichroism spectrum with that of native lysozyme. As can be seen in Fig. 8, the two proteins appear nearly identical with respect to their overall secondary structures. Coelution upon gel filtration on a G2000-SWXL HPLC column (0.78 \times 30 cm) showed both proteins to have similarly compact structures (data not shown). In these respects, lysozyme offers a very different system than was found in lysozyme converts this protein from an inactive to an active substrate for ubiquitination and its subsequent degradation in a reticulocyte lysate.

The use of a crude reticulocyte lysate to study substrate selection was convenient in that it allowed for the evaluation of proteins with respect to both ubiquitination and degradation in the same reaction mixture. However, a major concern regarding the use of such a crude assay system arises from the inevitable presence of multiple ubiquitin conjugation enzymes, many of which are believed to conjugate ubiquitin in a manner that does not lead to the degradation of the acceptor proteins (2, 31). The possibility that a degradation substrate protein can be ubiquitinated at multiple sites by different conjugation enzymes and that not all ubiquitination products are necessarily relevant to proteolysis must be considered. Because of these concerns, we have first established in this study the relevance of the ubiquitin-lysozyme conjugates that

\[ \frac{5}{R. L. Dunten and R. E. Cohen, unpublished observations.}\]
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we obtained in the crude reticulocyte lysate. We have demonstrated here that the proteolytic targeting of lysozyme by ubiquitin conjugation, like the previously studied β-galactosidase proteins (10), requires the formation of a multiubiquitin chain. Our studies indicated that, although at least four lysine sites on lysozyme can be ubiquitinated, the attachment of ubiquitin to any of these sites is dependent on the binding of the ubiquitin-protein ligase, E3. Because E3-dependent ubiquitination of proteins thus far is characteristic of ubiquitin-mediated proteolysis (2, 31), our results suggest that multiple lysine sites on lysozyme may be used in the degradation of lysozyme. The role of these conjugation sites in serving as initiation points for multiubiquitin chain synthesis and proteolytic targeting is being evaluated in a separate study. It is important to point out here, however, that the reduction and carboxymethylation of Cys6-Cys127 did not alter the ubiquitination sites in lysozyme.

The observation that ubiquitin conjugation to lysozyme and the ubiquitin-dependent degradation of this substrate protein were both greatly decreased in a DTT-depleted reticulocyte lysate suggested that the reducing agent may act to convert lysozyme from a largely, if not entirely, inactive substrate (see discussion below) to an active substrate. The alternative explanation, that the enzyme components had been rendered inactive due to the absence of DTT, can be ruled out since ubiquitin conjugation to several proteins, including ribonuclease A derivatives and calmodulin, was not affected. Because four disulfide bonds are present in native lysozyme, a likely possibility is that the effect of DTT is due to reduction of one or more of these disulfide bonds. The results obtained in this study are all consistent with this hypothesis. That the reduction of the Cys6-Cys127 disulfide is the relevant intermediate in the degradation of lysozyme is suggested by our finding that this disulfide bond is reduced preferentially under the conditions normally employed for degradation assays in reticulocyte lysate. Further support is provided by the demonstration that the 6,127-rcm-lysozyme behaves as a kinetically competent intermediate for both conjugation and degradation. A more rigorous proof of this hypothesis will require direct demonstration of the specific reduction of this disulfide bond in the ubiquitin-lysozyme conjugates. Nevertheless, the present results do indicate that the reduction of this most susceptible disulfide bond in lysozyme is sufficient to convert this protein to a highly active form for ubiquitin-mediated proteolysis.

By kinetic criteria, the 6,127-rcm-lysozyme is a far superior substrate as compared to native lysozyme. This is expected if the reduction by DTT of the same disulfide bond in native lysozyme is rate-limiting in the assay. The higher yield of ubiquitin conjugates with this modified lysozyme is also expected, as only a small fraction of native lysozyme is in the three-disulfide form at any time during the assay. Given the location of this disulfide bond, it is reasonable to assume that its reduction, by decreasing the conformational constraints on the N-terminal region of lysozyme, may facilitate E3 binding to the substrate N-terminus. The observation that native lysozyme, unlike the Lys-Aia dipeptide, did not inhibit ubiquitin conjugation to the 6,127-rcm-lysozyme suggests that the native protein, with this disulfide bond intact, either cannot be bound by E3 or is bound with reduced affinity.

It should be noted that the depletion of DTT from 2 to 0.01 mM did not abolish completely the formation of ubiquitin-lysozyme conjugates, although ubiquitin-dependent degradation is virtually undetectable. Several possibilities can account for the residual ubiquitination observed. We cannot rule out that some of the disulfide bonds in lysozyme are either already broken or that their reduction is catalyzed by residual DTT or by thiol groups on protein present in the reticulocyte lysate. In addition, an E3-independent pathway may be responsible for a minor amount of ubiquitin conjugation to fully disulfide-bonded, native lysozyme. Equally difficult to exclude is the possibility that E3 can bind to native lysozyme but with greatly reduced affinity. None of these uncertainties, however, detract from the basic conclusion that the specific reduction of the Cys6-Cys127 disulfide bond converts lysozyme from a largely inactive into an active substrate.

It is uncertain to what extent reduction of the Cys6-Cys127 disulfide bond actually leads to unfolding and enhanced accessibility of the N-terminal region of lysozyme. If such limited unfolding were the case, then the explanation for the much higher reactivity of the 6,127-rcm derivative is likely to apply as well to the examples of β-galactosidase and dihydrofolate reductase, where addition of a presumably unstructured N-terminal extension was required to convert these proteins from inactive to active substrates (5, 10). An intriguing possibility is that, although reduction of the Cys6-Cys127 disulfide bond may remove a structural constraint within lysozyme to permit conformational transitions at the N-terminal region of the protein, the lowest energy folded structure of the protein need not have been altered. In such a model, the affinity of E3 for its substrate would be determined in part by the energetics of conformational transitions between native and E3-accessible states.

Our demonstration that 6,127-rcm-lysozyme is an effective substrate for E3-dependent ubiquitination offers a unique opportunity to better understand the determinants for recognition. Whereas the structures of other proteins known to be substrates for this pathway are, at best, poorly defined, the lysozyme derivative used here has a substantially native-like conformation. We have demonstrated this at the level of secondary structure via circular dichroism spectroscopy, and gel filtration chromatography also suggests a folded conformation similar to that of native lysozyme. Independently, from detailed physical studies and comparisons of enzyme activities, Denton and Scheraga (32) have also concluded that the 6,127-rcm derivative has a structure approximating the native enzyme. Earlier, Acharya and Taniuchi (32) had generated a partially reduced and carboxymethylated lysozyme preparation (LH1) that was largely, if not entirely, the 6,127-rcm derivative described here. Because of the dramatic difference between native and 6,127-rcm-lysozyme with respect to ubi-

\[^{a}\text{L. Gregori and V. Chau, unpublished results.}\]

\[^{7}\text{M. Denton and H. A. Scheraga, submitted for publication.}\]
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ubiquitination, a detailed comparison of their three-dimensional structures and structural dynamics is of particular interest, and experiments along these lines are in progress. In this context, determination of the sites of ubiquitin attachment to lysozyme is of obvious importance, and the results of such a study will be described elsewhere.

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