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Crystal structure of a ubiquitin-dependent degradation substrate: A three-disulfide form of lysozyme

(proteolysis/ubiquitination/specificity determinant/stability)

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ABSTRACT **Covalent attachment of ubiquitin marks substrates for proteolysis, but features that identify ubiquitination targets such as chicken egg white lysozyme are poorly understood. Recognition of lysozyme first requires reduction of Cys-6 Cys-127, one of its four native disulfide bonds, and Cys-6,Cys-127-carboxymethylated (6,127-rcm) lysozyme can mimic this three-disulfide intermediate. The 6,127-rcm form of lysozyme is known to retain a substantially native-like conformation in solution, and we demonstrate that it is this folded structure that is recognized for ubiquitination. Because native lysozyme is not a substrate, differences between the native and three-disulfide structures must include features responsible for selective ubiquitination. The 1.9-Å resolution crystal structure of 6,127-rcm-lysozyme, reported here, affords a view of this ubiquitin-dependent degradation substrate. Two conformers of 6,127-rcm-lysozyme were obtained in the crystal. These differ uniquely from crystal forms of native lysozyme by displacement of the C-terminal residues. The structures suggest that localized unfolding at the C terminus of three-disulfide lysozyme allows the complex of E3α (ubiquitin–protein ligase) and E2 (ubiquitin–carrier protein) to bind to a surface that includes Lys-1 and the putative ubiquitination site Lys-13. From this we infer that the N-terminal and internal substrate recognition sites on the E3α·E2 complex are separated by ≈20 Å.**

Intracellular protein degradation is remarkable for its combination of extreme selectivity and the ability to accommodate an enormous variety of substrates. Protein half-lives *in vivo* span several orders of magnitude. Moreover, mutations, translational errors, mislocalization, and chemical damage all can lead to polypeptides that are rapidly degraded (1–5). How such proteins are distinguished from their long-lived counterparts is largely unknown. In eukaryotes, a major route for intracellular proteolysis involves covalent modification of protein lysine(s) with the protein ubiquitin (Ub) and subsequent degradation by the 26S Ub-dependent protease complex (6–8). Specificity resides, at least in part, with the Ub–protein ligase that marks a substrate for recognition by the protease. This process is best understood for the E3α ligase from rabbit reticulocytes and the related *UBR1*-encoded enzyme from yeast (9–13).

One important E3α recognition determinant is the substrate's N terminus, where only a subset of amino acids is permissive for ubiquitination (9–12). A permissive N terminus is not sufficient, however, and the relative orientations and distances that separate lysine ubiquitination sites from N-terminal and other, as yet undiscovered, determinants also are likely to be critical for recognition (14–17). Progress in this area has been hampered by the lack of Ub-dependent degradation substrates of defined structure. Indeed, to obtain competent *in vitro* substrates, proteins generally must be altered conformationally (16, 17). This generalization applies to chicken egg white lysozyme, where we have found that efficient ubiquitination requires prior reduction of one of the four native disulfide bonds, Cys-6 Cys-127. A reduced and carboxymethylated derivative, 6,127-rcm-lysozyme, can mimic this three-disulfide degradation intermediate (17). Three-disulfide lysozyme offers a unique opportunity to probe ubiquitination specificity because, unlike other E3α substrates described thus far, it retains a substantially structured, native-like conformation (17–19). We report here the x-ray crystal structure of 6,127-rcm-lysozyme determined to a resolution of 1.9 Å. An experiment that relates this structure to the substrate conformation recognized in solution by Ub–protein ligase is presented, and the results are discussed in terms of structural determinants for ubiquitination.‡

EXPERIMENTAL PROCEDURES

Protein Crystallization. The 6,127-rcm derivative of chicken egg white lysozyme (EC 3.2.1.17; Sigma, grade I) was prepared essentially as described (17), but with additional chromatography on a PolyCAT A (The Nest Group, Southboro, MA) cation-exchange column. Stock solutions were dialyzed against water and concentrated to 9 mg/ml in Centricon 10 microconcentrators (Amicon). Crystals were grown by the hanging drop method under conditions similar to those used to obtain tetragonal crystals of unmodified lysozyme. The reservoir solution (1 ml) was 7% NaCl with 50 mM sodium acetate at pH 3.8, and the hanging drops contained 10 μl of a 1:1 mixture of reservoir and protein stock solutions. Typically, crystals appeared after 2 days and grew to $0.3 \times 0.4 \times 0.5$ mm^3 within a week. The crystals belonged to orthorhombic space group $P2_12_12_1$ with cell dimensions a = 77.7 Å, b = 81.3 Å, and c = 38.0 Å.

Data Collection and Structure Determination. X-ray data extending to 1.9-Å resolution were collected from two crystals with a R-AXIS II imaging plate detector and a Rigaku RU-200 rotating anode source (CuKα radiation at 50 kV and 150 mA). Each frame, in which the crystal was oscillated by 1.5°, was acquired over 12 min; ≈35 frames were collected from each crystal before there was significant deterioration from radiation damage. Processing employed the MSC software package. The chicken egg white lysozyme structure of

Abbreviations: Ub, ubiquitin; 6,127-rcm, Cys-6,Cys-127-carboxymethylated; rcmA and rcmB, the two molecules per asymmetric unit in 6,127-rcm-lysozyme crystals; E3, ubiquitin–protein ligase; E2, ubiquitin carrier or conjugating protein; $(GlcNAc)_3$, N,N',N''-triacetylchitotriose.

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‡Coordinates and diffraction data have been deposited in the Protein Data Bank, Chemistry Department, Brookhaven National Laboratory, Upton, NY 11973 (reference 1RCM) (20, 21).

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