Structure of the Ubiquitin Hydrolase UCH-L3 Complexed with a Suicide Substrate*

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Ubiquitin C-terminal hydrolases (UCHs) comprise a family of small ubiquitin-specific proteases of uncertain function. Although no cellular substrates have been identified for UCHs, their highly tissue-specific expression patterns and the association of UCH-L1 mutations with human disease strongly suggest a critical role. The structure of the yeast UCH Yuh1-ubiquitin aldehyde complex identified an active site crossover loop predicted to limit the size of suitable substrates. We report the 1.45 Å resolution crystal structure of human UCH-L3 in complex with the inhibitor ubiquitin vinylmethyl-ester, an inhibitor that forms a covalent adduct with the active site cysteine of ubiquitin-specific proteases. This structure confirms the predicted mechanism of the inhibitor and allows the direct comparison of a UCH family enzyme in the free and ligand-bound state. We also show the efficient hydrolysis by human UCH-L3 of a 13-residue peptide in isopeptide linkage with ubiquitin, consistent with considerable flexibility in UCH substrate size. We propose a model for the catalytic cycle of UCH family members which accounts for the hydrolysis of larger ubiquitin conjugates.

A wide variety of cellular biochemical pathways are regulated by the post-translational addition of ubiquitin (Ub)† to protein substrates (1, 2). Although the enzymatic process of ubiquitin ligation has been studied extensively, that of ubiquitin deconjugation is less well understood. A group of enzymes collectively termed deubiquitinating enzymes (DUBs) catalyzes the hydrolysis of the isopeptide linkage that joins the C-terminal glycine of ubiquitin and a lysine side chain on the target polypeptide. The DUB family consists of four structurally distinct subfamilies: the ubiquitin C-terminal hydrolases (UCHs), ubiquitin-processing proteases (Ubps, USPs), OTU domain-containing enzymes (otubains) and the Jab/MPN domain-associated metalloisopeptidase domain-containing metalloproteases (3, 4). The first three enzyme classes all possess the sequence signature of cysteine proteases: a conserved catalytic triad of cysteine, histidine, and aspartic acid residues. Sequence analysis of the human genome predicts at least 100 DUBs, which begs the question of their physiological roles. Although restricted substrate specificity is predicted to underlie the requirement for such a large enzyme family, little is known about substrate specificity determinants.

The recently published structures for the Ubp family member USP7 (HAUSP) in the unliganded and liganded state (5) affords a unique opportunity to examine specificity determinants for this enzyme. Although the HAUSP catalytic residues are misaligned in the unliganded state, the catalytic core undergoes a dramatic conformational change when in a complex with the inhibitor ubiquitin aldehyde (Ubal), resulting in alignment of the catalytic residues with the C terminus of Ub. The open configuration of the HAUSP active site explains the ability of this class of enzyme to accommodate large ubiquitin conjugates as substrates (e.g. p53). In contrast, the UCH enzymes have an apparent preference for small C-terminal leaving groups, as typified by ubiquitin ethyl ester (6). The crystal structure of the yeast enzyme Yuh1 in a complex with Ubal provided some insights into the substrate specificity of UCHs (7). A 20-residue loop was observed to cover the active site cleft and is thus inferred to impose a limit on the size of the Ub-conjugated substrate that Yuh1 can accommodate. However, this model does not explain the apparent ability of some UCHs to hydrolyze much larger substrates (8–10), suggesting potential alternate conformations. This active site crossover loop could not be resolved in the crystal structure for unliganded human UCH-L3 (11), suggesting that this loop may have significant flexibility in the unliganded state. Although several ligand-induced conformational changes were inferred upon comparison of the structure for UCH-L3 with that of Yuh1-Ubal, the limited sequence identity between UCH-L3 and

linepropanesulfonic acid; Tricine, N-(2-hydroxy-1,1-bis(hydroxymethyl)ethylglycine; Ubal, ubiquitin aldehyde; Ubp, ubiquitin processing protease; UbVME, ubiquitin vinylmethyl ester; UCH, ubiquitin C-terminal hydrolase; USP, ubiquitin specific protease.

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* The abbreviations used are: Ub, ubiquitin; BP-Ub, branched peptide HA-ubiquitin; DTT, dithiothreitol; DUB, deubiquitinating enzyme; E1, ubiquitin-activating enzyme; E2, ubiquitin carrier protein; HA, hemagglutinin; HAUb, hemagglutinin-tagged ubiquitin; MOPS, 4-morpholinepropanesulfonic acid; Tricine, N-(2-hydroxy-1,1-bis(hydroxymethyl)ethylglycine; Ubal, ubiquitin aldehyde; Ubp, ubiquitin processing protease; UbVME, ubiquitin vinylmethyl ester; UCH, ubiquitin C-terminal hydrolase; USP, ubiquitin specific protease.
Yuh1 (30%) complicates a direct comparison.

To explain the properties and substrate preference of UCH-L3 we describe the 1.45 Å crystal structure of human UCH-L3 in

we describe the 1.45 Å crystal structure of human UCH-L3 in a complex with the mechanism-based inhibitor ubiquitin vinyl-

methylene thyl (UbVME). This structure allows the direct comparison of conformational changes that occur between the un-

liganded (11) and Ub-bound states of UCH-L3. We also report the synthesis of a branched peptide substrate that consists of an N-terminally biotinylated 13-residue peptide, with its sequence centered on ubiquitin lysine 48, in isopeptide linkage with HA-tagged Ub (HAUb). Although the configuration of the active site crossover loop observed in our structure precludes the accommodation of such a large isopeptide-linked substrate, we show that UCH-L3 can nonetheless hydrolyze the isopeptide bond in this branched peptide. Taking into consideration both the observed structure and substrate specificity, we propose a model for UCH-mediated Ub conjugate hydrolysis.

**EXPERIMENTAL PROCEDURES**

**Protein Expression and Purification**—UCH-L3 in pRSET vector was obtained from Dr. Keith Wilkinson. UCH-L3 was expressed in *E. coli* and purified as described previously (12). Expression and purification of ubiquitin (Ub), synthesis and ligation of the glycine VME moiety to ubiquitin, and purification of the UbVME complex were performed as described previously (13).

To prepare the UCH-L3-UbVME complex, the pH of the UbVME solution (in 50 mM sodium acetate, pH 4.5) was adjusted to 7.5 by the addition of 1 mM Tris, pH 7.5, then UCH-L3 (in 50 mM Tris, pH 7.5, 0.5 mM EDTA, 1 mM DTT, and 3% glycerol) was added so that the final UCH-L3-UbVME molar ratio was 2:1 (2 ml of UbVME at 0.3 mg/ml, 1.9 ml of UCH-L3 at 16 mg/ml). The mixture was incubated for 4–5 h at 4 °C, and the formation of the complex, which results in an 8-kDa shift in molecular mass of UCH-L3, was verified by SDS-PAGE. After dialysis, UCH-L3 and UCH-L3/H18528 were separated on a Mono Q column (1 ml, Amersham Biosciences), using a linear gradient from 0 to 500 mM NaCl in buffer A (50 mM Tris, pH 7.5, 1 mM EDTA, 3 mM DTT, and 5% glycerol). Fractions containing the UCH-L3-UbVME complex were dialyzed against 10 mM NaMOPS, pH 6.8, 10 mM DTT, 1 mM EDTA, and concentrated to 17 mg/ml.

**Synthesis and Subsequent Deubiquitination of Branched Ub Peptides**—1 μl of HAUb (0.1 mg/ml) was mixed with 1 μl of E1 (0.25 μM final concentration), 1 μl of E2–25K (2.5 μM final concentration), and 1 μl of buffer A (0.5 mM Tris, pH 7.4, 10 mM DTT, 20 mM ATP, and 25 mM MgCl2). Biotin-RLIFAGKQGED (2 μl of a 1 mg/ml solution) was added to the above mixture, and the reaction was incubated for 15 h at 37 °C to make the branched peptide HA-ubiquitin (PB-Ub). The products of this reaction were characterized by Western blot with the reaction mixture incubated successfully if bands confirmed the presence of a band of 10 kDa containing the HA and biotin epitopes. To perform the deubiquitination assay, 3 μl of buffer B (50 mM Tris, pH 7.4, 1 mM DTT, 2 mM ATP, 20 μM ZnCl2) and 1 μl of UCH-L3 (at 5 μg/ml, final concentration) were added to the above mix (final volume of 10 μl), and samples were incubated at 37 °C. Samples were quenched at five different time points (5, 10, 20, 30, and 45 min) by the addition of SDS-PAGE sample buffer and resolved on a Tris/Tricine peptide separation gel. The samples were transferred to a polyvinylidene difluoride membrane, and biotinylated products were detected with streptavidin-horseradish peroxidase. The membrane was then stripped and probed with 12CA5 α-HA antibody to confirm the continued presence of the HAUb moiety.

**Crystalization and Data Collection**—UCH-L3-UbVME crystals were grown by vapor diffusion at 4 °C from hanging drops containing 0.1 mM protein solution (17 mg/ml in 10 mM NaMOPS, pH 6.8, 10 mM DTT, 1 mM EDTA) and 0.5 mM of precipitant solution (100 mM Tris, pH 8.5, 23.5% PEG 4000, and 260 mM MgCl2). Rod-shaped crystals grew over 7–10 days. Crystals were soaked briefly in a cryoprotectant solution (100 mM Tris, pH 8.5, 22% PEG 4000, 20% PEG 400, and 260 mM MgCl2) and flash cooled in liquid nitrogen. X-ray data were collected at 1.45 Å with an ADSC Q315 charge-coupled device detector at beamline 8-BM at the Advanced Photon Source of Argonne National Laboratory (Argonne, IL). The crystals belong to space group P1, with unit cell dimensions a = 46.11 Å, b = 49.29 Å, c = 67.62 Å, α = 86.12°, β = 75.03°, γ = 76.78°, with two UCH-L3-UbVME complexes/asymmetric unit. Diffraction data were processed using HKL2000 (HKL Research, Charlotteville, VA). Data statistics are listed in Table 1.

**Structure Determination and Refinement**—The structure was deter-
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biotin group ensures that the central lysine contains the only free amine atom in this peptide. The C-terminal glycine 76 of HAUb was then covalently attached to the ε-amino group of the peptide using purified E1 and E2 ubiquitin ligases to form a branched peptide-HAUb complex (see "Experimental Procedures"). The adduct carries both an HA epitope tag (Ub) and a biotin moiety (peptide) (Fig. 2A). We then tested the ability of UCH-L3 to hydrolyze the isopeptide bond of the BP-Ub conjugate. The substrate was incubated in the presence or absence of UCH-L3, and aliquots were removed at various time points. UCH-L3 inactivated by pretreatment with N-ethylmaleimide served as a negative control (Fig. 2B). The samples were resolved by gel electrophoresis, transferred to a polyvinylidene difluoride membrane, and probed with streptavidin-horseradish peroxidase to monitor the presence of biotinylated branched peptide. Fig. 2B shows that UCH-L3 rapidly hydrolizes the BP-Ub isopeptide bond, as indicated by the loss of streptavidin reactivity. An anti-HA blot shows the continued presence of HAUb as expected. Although the preparation of BP-Ub in small scale prevents an accurate determination of substrate concentration and subsequently catalytic efficiency, we estimate a substrate:enzyme ratio of at least 5:1. Because we observe more than 90% substrate conversion in 5 min, we conclude that BP-Ub is a suitable substrate for UCH-L3. Efforts for preparation and purification of BP-Ub on a preparative scale, to allow accurate kinetic measurements, are currently ongoing.

Structure Determination—The UCH-L3-UbVME complex was crystallized, and its structure was determined to 1.45 Å resolution (see "Experimental Procedures"). Table I shows data collection, processing, and refinement statistics. The UCH-L3-UbVME model includes all ubiquitin and UCH-L3 residues except for the UCH-L3 first residue, for which no electron density was observed. The UCH-L3 structure consists of a six-stranded anti-parallel β-sheet surrounded by eight α-helices, including an additional helix and loop that were disordered in the unliganded UCH-L3 structure (11). We refer to this helix as H6’ because it immediately follows the previously named H6 helix (Fig. 3). It is also notable that residues Ile154, His158, and Ala154 form a conserved hydrophobic face on helix H6, anchoring the helix to the rest of the protein. These residues are conserved among all human UCHs: UCH-L1, UCH-L3, UCH37, and BAP1, but not Yuh1, which lacks an α-helix in this region.

There are two UCH-L3-UbVME complexes per asymmetric unit. These two complexes are nearly identical with a root mean square deviation of 0.350 Å on Cα atoms for UCH-L3 and 0.354 Å for UbVME. However, by superimposing the UCH-L3 structures we observe a rigid body rotation of UbVME between the two complexes (Fig. 4). This movement encompasses a 10° rotation that hinges around residues 73 and 9 of ubiquitin, leaving the C-terminal Ub residues 73-76 and loop residues 8-9 unchanged. Although this rotational motion of ubiquitin is likely a crystallographic artifact resulting from crystal packing interactions, it highlights that the C terminus and the first N-terminal loop of the ubiquitin comprise the major interactions with UCH-L3 (see below). In contrast, the interactions of Gly77 and His88 of UbVME with Leu41 and Met44 located at the H2 helix of UCH-L3, which are present only in one UCH-L3-UbVME complex, are likely minor determinants of UCH-L3-UbVME specificity because they can be disrupted by the generally weak crystal packing force without disassembling the UCH-L3-UbVME complex.

UbVME Targets the Active Site Cysteine of UCH-L3—Ubiquitin derivatives with C-terminal electrophilic traps are specific inhibitors of DUBs, including UCH-L3 (13, 19). Despite the specificity of these inhibitors, a precise mode of inhibition has not been determined conclusively, for example, through isolation of an inhibitor-modified, active site-derived peptide. It has been argued that inhibition of DUBs by these ubiquitin derivatives may occur through covalent modification of the active site cysteine or possibly through nonspecific alkylation of the active site histidine residue. The 1.45 Å crystal structure of UCH-L3-UbVME reported here allows the unambiguous identification of the residue modified by this inhibitor. UCH-L3 reacts with UbVME in a 1,4-Michael addition to yield a thioether bond. Inspection of the electron density in the UCH-L3 active site indicates formation of a covalent bond between the side chain sulfur atom of cysteine 96 and the former vinyl group of the inhibitor (Fig. 5) as expected. The location of the intermolecular thioether bond corresponds to the carbonyl carbon of the ubiquitin glycine 76, which is the
natural cleavage site of ubiquitin from substrates of DUBs. The density distribution of ubiquitin, from the methyl ester portion of inhibitor suggests alternate conformations for this moiety, implying free rotation of the methylester group (Fig. 5). A high resolution structure of the UCH-L3-UbVME complex detailed here allows the description of the conformational changes observed when UCH-L3 interacts with Ub. Overall, our structure of UCH-L3 in complex with UbVME is similar to that of free UCH-L3 (root mean square deviation = 1.18 Å over 206 Cα atoms present in both structures; 0.42 Å over 174 Cα atoms when regions of apparent conformational change are omitted from analysis). When superimposed, unliganded UCH-L3 and UbVME-liganded UCH-L3 display significant conformational differences in regions adjacent to the active site (Fig. 6). The largest structural change is the stabilization of a large, previously unstructured loop of UCH-L3 (residues 146–167) into a helix (H6'; 142–154) followed by an S-shaped loop (155–167) that crosses over the active site of the enzyme. Residues 142–146 switch from an extended conformation in the unliganded structure to form the first turn of the H6' helix in the UCH-L3-UbVME complex. The N-terminal 12 residues of UCH-L3 also undergo a drastic conformational change, forming an elongated strand that opens the active site cleft for docking of the C terminus of ubiquitin and avoids steric clash with the newly structured helix H6'. This N-terminal elongated strand forms parallel β-sheet hydrogen bonds with the C terminus of Ub. We also observe an ~2 Å shift of helix H2 and its flanking loop (residues 34–44) as well as the loop preceding helix H4 (residues 89–94) in UCH-L3 to accommodate docking of ubiquitin. With the exception of the S-shaped loop, all of these changes are necessary to render the active site of UCH-L3 accessible to its ubiquitin conjugate substrates. The corresponding regions of Yuh1 have similar conformations in the Yuh1-Ubal complex (7). Based on the homology of Yuh1 with UCH-L3 it was proposed that UCH-L3 would use a similar mechanism. That suggestion is now confirmed experimentally and suggests a conserved substrate docking mechanism among the UCH family members. Importantly, the conformation of the crossover loop is remarkably similar for UCH-L3 and Yuh1, especially considering the poor sequence conservation between them in that region (Fig. 3).

**DISCUSSION**

Although the UCHs are among the best characterized DUBs, their precise catalytic role in Ub biochemistry has yet to be explained fully. Here we describe a high resolution crystal structure for UCH-L3 in a complex with the DUB inhibitor UbVME. The availability of the UCH-L3 structure in the unliganded state (11) now allows a precise definition of conformational changes that occur when Ub binds. Despite the low sequence identity between UCH-L3 and Yuh1 (30%), we observe a striking similarity between our structure and that of Yuh1 in complex with Ubal. This high degree of structural conservation suggests that the findings described by Johnston et al. (7, 11) and in this report may well be common across the UCH family.
Surprisingly, we observe a variation in the approach angle of Ub into the UCH-L3 active site cleft for the two complexes present in the asymmetric unit. The 10° rotation observed leads to the disruption of two sites of molecular contact between UCH-L3 and Ub. We therefore infer that these interactions are of lesser importance in substrate recognition by UCH-L3. These interactions were not observed in the Yuh1-Ubal structure, were not predicted based on NMR studies with UCH-L3 (20), nor are these residues conserved among UCHs (Fig. 3), thus supporting this conclusion. In contrast, the importance of contacts between UCH-L3 and both the C terminus and the first N-terminal loop of Ub is underscored by the observation that these contact points remain fixed between both complexes. Consistent with previous biochemical and
We have synthesized Ub derivatives with a variety of electrophilic traps that can react differentially with at least 35 DUBs, including all human UCHs, many Ubps, and the OTU domain containing enzymes otubain 1 and A20 (13, 19, 24, 29). An important reason for understanding the structural analysis of the UCH-L3 was to determine unambiguously the mechanism of action for this class of inhibitors. The electron density shown for reference. The electron density figures were generated in O (16).

state. This comparison enables the clear demonstration of the dramatic changes in conformation which occur in the N terminus of UCH-L3 to accommodate the C terminus of Ub into the active site cleft of UCH-L3. Additionally, the UCH-L3-UbVME structure differs from that of Yuh-1-Ubal, in that UCH-L3 contains an additional α-helix (H6') that forms at the N-terminal segment of the active site crossover loop. This helix also was observed to interact extensively with the C terminus of Ub at Arg^{74}, thus further stabilizing the position of the Ub C terminus in the active site cleft. The structure described here also shows a rigid body movement of Ub, not apparent in the Yuh-1-Ubal structure. This suggests that the last 4–5 C-terminal residues and the first N-terminal loop of Ub are critical contact points, as these contacts are maintained in the rotated conformation. Finally, our structure shows that UbVME and by extension other electrophilic traps C-terminally linked to Ub present a new class of inhibitors that, like ubiquitin-aldehyde, covalently modify the active site cysteine of DUBs but do so irreversibly.

We have synthesized Ub derivatives with a variety of electrophilic traps that can react differentially with at least 35 DUBs, including all human UCHs, many Ubps, and the OTU domain containing enzymes otubain 1 and A20 (13, 19, 24, 29). An important reason for understanding the structural analysis of the UCH-L3 was to determine unambiguously the mechanism of action for this class of inhibitors. The electron density
maps we obtained demonstrate conclusively that UbVME inactivates UCH-L3 by forming a covalent bond with the active site cysteine residue. We observe broad reactivity with these inhibitors, which suggests that members of the Ubp family of DUBs likely share the active site chemistry observed in this crystal structure. This mechanism-based inhibitor allows us to determine and compare relative enzyme activities for individual DUBs in cell lysates (19). We have applied this concept to obtain DUB activity profiles from a panel of human cell lines and observed clear tissue-specific DUB activities.

Although members of the UCH family of DUBs have been linked to disease states as well as long term facilitation in Aplysia (26) the identification of physiologic substrates for these enzymes has proven difficult. Given their apparent preference for cleaving small C-terminal leaving groups from Ub, UCHs have been proposed to cleave small and/or unstructured extensions of amino acids that are attached to the C terminus of ubiquitin which is then released free of the ubiquitin (6, 18, 27). In this context, ubiquitin molecules that accidentally react with intracellular nucleophiles or ubiquitin precursors with short extensions may constitute physiologically relevant substrates for UCH-L3 (18). Indeed, cells that lack UCH-L1 contain reduced amounts of free ubiquitin (28). The disordered active site crossover loop in human UCH-L3 (11) or its ordered counterpart observed in the Yuh1-Ubal structure (7) and this study may be involved in restricting access of a broader range of substrates to the UCH-L3 active site. One possible interpretation is that any extension attached to the C terminus of ubiquitin would have to be threaded through the UCH-L3 active site crossover loop until the glycine 76 residue of Ub is situated properly at the UCH-L3 active site.

Threading of C-terminal extensions of Ub through the UCH-L3 active site crossover loop would be thermodynamically and kinetically unfavorable, unless these extensions are very small, thereby limiting the size of the Ub conjugate substrate. However, there is no biochemical or structural evidence that suggests UCH-L3 substrates are threaded through the active site crossover loop.

Several recent reports, in addition to our observations, indicate that UCH-L3 and its homologs can process a subset of extensions attached to the C terminus of ubiquitin which is larger and more diverse than anticipated previously. A ubiquitin precursor Ub-CEP52, consisting of ubiquitin in linear fusion with a 52-residue ribosomal protein fusion has been shown to be a substrate for UCH-L3, Yuh1, and Drosophila UCH-D (6, 10). Ubiquitinates IκBα, an adduct even larger than Ub-CEP52, is also an in vitro substrate for Drosophila UCH-D (8). Finally, chicken UCH-6, with 86% overall and 95% active site crossover loop identity to human UCH-L3, can cleave ubiquitin from a Ub-β-galactosidase fusion protein both in vivo and in vitro (9). Based on the highly conserved structures of Yuh1-Ubal and UCH-L3-UbVME, it is likely that there are no significant structural differences between these enzymes, which are between 30 and 60% identical with UCH-L3.

Recently, the substrate specificity of rabbit UCH-L3 was probed using a positional scanning library of peptides whose sequence is based on Lys$_{48}$-branched diubiquitin (25). A branched peptide substrate was constructed composed of the C-terminal 7 residues of Ub in isopeptide linkage to the central lysine of a 13-residue peptide. Using the sequence of Ub flanking Lys$_{48}$ as the anchor point, a positional scanning peptide library was constructed in which the 4 residues between Leu$_{43}$...
lysine(s) to which ubiquitin is attached. Some, or a combination of both, on the residues flanking the truncated branched peptides, BP-Ub should be considered unimportant for the substrate specificity of UCHs, and unlike the substrate for UCH-L3. Ubiquitin provides the interactions important for the substrate specificity of UCHs, and unlike the truncated branched peptides, BP-Ub should be considered incapable of retrograde threading. Physiologically, a BP-Ub substrate may be formed by the activity of endoproteases, proteasomes, or a combination of both, on the residues flanking the lysine(s) to which ubiquitin is attached.

The most striking structural feature relating to substrate specificity is the presence of a 20-residue loop that traverses the active site cleft both in Yuh1 and UCH-L3. This loop must be considered in any model that accounts for the size of substrates hydrolyzed by UCHs. As mentioned above, in one scenario substrates would enter the active site cleft by passing underneath the arch created by the active site crossover loop. This model would readily explain the ability of UCHs to hydrolyze small Ub C-terminal extensions, as the loop itself would likely impose significant size restrictions on potential substrates. However, the recent reports of larger substrates hydrolyzed by UCH enzymes conflict with the model of substrate threading through the crossover loop. The maximum theoretical internal diameter of the active site crossover loop in its most relaxed and open conformation is 12–15 Å. It seems highly improbable that the 52-residue extension of Ub-CEP52, or a branched polypeptide such as BP-Ub, would thread with ease through such a small opening. Furthermore, polyubiquitinated IXBo and Ub-β-galactosidase, large structured substrates, would be impossible to accommodate underneath even the most relaxed loop. An alternate model for UCH hydrolysis is thus required to account for the observed action of these enzymes.

To accommodate larger substrates in the active site of UCHs one must relieve steric constraints by peeling away the active site crossover loop from the active site cleft (11). Fig. 7 illustrates the proposed catalytic cycle of UCH-L3 in the hydrolysis of a branched peptide substrate into ubiquitin and a free peptide (note that this model could also be applied to other, even larger UCH-L3 substrates). First, UCH-L3 recognizes and binds a ubiquitin adduct, with the active site crossover loop away from the active site. Docking of the C terminus of ubiquitin into the active site displaces the UCH-L3 N terminus, causing it to form an elongated strand with parallel β-sheet hydrogen bonds with the C terminus of Ub. Other conformational changes involving helix H2 and the loop preceding helix H4 allow proper docking of the ubiquitin adduct substrate. In this respect, the active site cleft serves as a saddle on which the C terminus of ubiquitin binds, whereas ubiquitin and the branched peptide are each located on opposite faces of the enzyme. Next, the active site Cys95 executes a nucleophilic attack on the carbonyl group of the Gly76 of ubiquitin, hydrolyzing the isopeptide bond between ubiquitin and the branched peptide and forming a covalent intermediate with ubiquitin. The branched peptide would then be released from the active site cleft, allowing the active site crossover loop to fold back over the active site cleft in the conformation observed in our structure. The crossover loop in this conformation forms multiple interactions with ubiquitin residues, stabilizing the C terminus of ubiquitin. The active site residues His169 and Asp184 then deprotonate a water molecule, which hydrolyzes the covalent bond between Cys95 of UCH-L3 and Gly76 of ubiquitin via nucleophilic attack on the carbonyl group of Gly76 of ubiquitin. The final step involves the release of regenerated ubiquitin with the concomitant return of the N terminus of UCH-L3 to its unliganded conformation. The key elements of this model are that 1) the flexible nature of the active site crossover loop prior to substrate binding allows its positioning away from the active site and beneath large substrates bound and hydrolyzed by UCHs; 2) sequence variability or length of the crossover loop within the UCH family may play a role in dictating substrate specificity through steric constraints and/or specific interactions of substrates with the loop; and 3) the crystallographic structures of Yuh1-Ubal and UCH-L3-UbVME represent the conformation of the crossover loop in the covalent intermediate state rather than the substrate binding conformation. A possible role of the locked conformation of active site crossover loop is to stabilize the active site residues in their most active conformation. Additionally, the active site crossover loop, in the locked conformation, may act to restrict access of all but the smallest nucleophiles (e.g. water) into the active site cleft, thus preventing the generation of nonproductive reactive intermediates that may result from the entry of larger nucleophilic species. Structures of true Ub-isopeptide substrate-bound UCH enzymes will be necessary to determine the position and role of the UCH active site crossover loop in substrate binding and selection.

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