Structural basis for the specificity of ubiquitin C-terminal hydrolases

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The release of ubiquitin from attachment to other proteins and adducts is critical for ubiquitin biosynthesis, proteasomal degradation and other cellular processes. De-ubiquitination is accomplished in part by members of the UCH (ubiquitin C-terminal hydrolase) family of enzymes. We have determined the 2.25 Å resolution crystal structure of the yeast UCH, Yuh1, in a complex with the inhibitor ubiquitin aldehyde (Ubal). The structure mimics the tetrahedral intermediate in the reaction pathway and explains the very high enzyme specificity. Comparison with a related, unliganded UCH structure indicates that ubiquitin binding is coupled to rearrangements which block the active-site cleft in the absence of authentic substrate. Remarkably, a 21-residue loop that becomes ordered upon binding Ubal lies directly over the active site. Efficiently processed substrates apparently pass through this loop, and constraints on the loop conformation probably function to control UCH specificity.

Keywords: crystal structure/cysteine protease/de-ubiquitinating enzyme/enzyme specificity/ubiquitin

Introduction

The highly conserved 76 amino acid protein ubiquitin is best known for its role in targeting proteins for degradation by the 26S proteasome (Ciechanover and Schwartz, 1998). Ubiquitin has been implicated in numerous cellular processes which include cell-cycle control, oncoprotein degradation, receptor function, regulation of transcription, stress responses, signaling pathways, antigen presentation and the degradation of abnormal proteins. Ubiquitin functions through ligation of its C-terminus to the lysine sidechains of acceptor proteins, a process that is catalyzed by a series of E1, E2 and E3 enzymes. Efficient targeting for degradation by the 26S proteasome appears to require polyubiquitination, in which a lysine sidechain of the first ubiquitin is ligated to another ubiquitin, a process that is repeated to build a chain of polyubiquitin from the target protein (Chau *et al.*, 1989; Pickart, 1997). In addition to the isopeptide linkages made to lysines, the ubiquitin C-terminus can also form peptide bonds to α -amino groups. For example, all known ubiquitin genes encode fusion proteins of either α -linked polyubiquitin or ubiquitin

followed by a C-terminal peptide extension (Özkaynak *et al.*, 1987).

Proteolytic processing at the ubiquitin C-terminus is catalyzed by de-ubiquitinating enzymes whose functions include: liberation of monomeric ubiquitin from the fusion-protein precursors, release of polyubiquitin from the remnants of 26S proteasome substrates, disassembly of polyubiquitin to recycle monomeric ubiquitin, reversal of regulatory ubiquitination, editing of inappropriately ubiquitinated proteins, and regeneration of active ubiquitin from adducts with small nucleophiles (such as glutathione) that may be produced by side reactions (Wilkinson and Hochstrasser, 1998). In light of the many different substrates and the extensive biological consequences of ubiquitination, it is not surprising that numerous de-ubiquitinating enzymes have been identified. These enzymes fall into two families of cysteine proteases, UBPs (ubiquitin-specific proteases) (Tobias and Varshavsky, 1991) and UCHs (ubiquitin C-terminal hydrolases) (Pickart and Rose, 1985). Both classes of enzymes can hydrolyze the peptide bond (either α - or ϵ -linked) at the C-terminus of ubiquitin.

UCH and UBP substrate specificities probably overlap since disruption or deletion of *YUH1*, the only UCH gene in *Saccharomyces cerevisiae*, confers no discernible phenotype (Miller *et al.*, 1989; Baker *et al.*, 1992). However, some UCH enzymes are tissue-specific in higher organisms and are likely to target distinct substrates (Wilkinson *et al.*, 1992). A defect in the neuronal enzyme, UCH-L1, which is highly abundant in mammalian brain (Wilkinson *et al.*, 1989), has been implicated in some cases of Parkinson's disease (Leroy *et al.*, 1998). A UCH has also been implicated in long-term nerve potentiation in *Aplysia* (Hegde *et al.*, 1997). A nuclear UCH, BAP1, has been implicated in the function of the breast/ovarian cancer susceptibility gene product, BRCA1 (Jensen *et al.*, 1998). Finally, a more general role in the regulation of ubiquitin-dependent proteolysis has been indicated for a UCH found within the PA700 (19S) regulatory complex of mammalian 26S proteasomes (Lam *et al.*, 1997a,b; W.Xu, G.N.DeMartino and R.E.Cohen, unpublished data). Although different UCHs are expected to possess distinct specificities, a common feature appears to be very high specificity for the ubiquitin part of the substrate.

Four UCH active site residues, Gln84, Cys90, His166 and Asp181 (Yuh1 numbering), were identified previously through mutagenesis of UCH-L1 (Larsen *et al.*, 1996) and determination of the crystal structure of UCH-L3 (Johnston *et al.*, 1997). Despite disparate amino acid sequences, structural similarities with the papain-like cysteine proteases provided insight to the likely catalytic mechanism of the UCH enzymes. Thus, it appears that His166 and Asp181 function to activate the sidechain of Cys90 for nucleophilic attack on the carbonyl carbon atom of the

scissile bond, while Gln84 and the main chain amide of Cys90 form an 'oxanion hole' which accommodates the negative potential on the substrate carbonyl oxygen during the first step in catalysis.

In an effort to understand the specificity and mechanism of de-ubiquitinating enzymes, we have determined the crystal structure of Yuh1 bound to the tight-binding inhibitor ubiquitin aldehyde (Ubal). Ubal, which is identical to ubiquitin except for conversion of the C-terminal carboxylate to an aldehyde, is an efficient inhibitor of many de-ubiquitinating enzymes (Hershko and Rose, 1987) and displays a $K_i < 10^{-12}$ M for at least some UCHs (Pickart and Rose, 1986; Dang *et al.*, 1998). Consistent with the expected catalytic mechanism, we find that the Yuh1–Ubal complex is a thiohemiacetal analogue of the tetrahedral reaction intermediate. The structure and accompanying kinetic data provide the first view of ubiquitin bound to another protein, and reveal how the very high specificity of UCHs is achieved through a combination of extensive interactions, an unusual loop structure at the active site, and conformational changes triggered by binding of ubiquitin.

Results and discussion

Structure determination

The Yuh1–Ubal crystal structure was determined by the method of multiwavelength anomalous diffraction (MAD) and refined against 2.25 Å data to an R -factor (R_{free}) of 24.8% (28.5%). The two complexes in the asymmetric unit (Yuh1-1/Ubal-1 and Yuh1-2/Ubal-2) display different degrees of order. Three regions of Yuh1-1 (residues 1–5, 63–77, 235–236) and Yuh1-2 (residues 1–6, 61–78, 235–236) lack defined electron density and have been omitted from the model. Some sidechains also lack defined density and have been included with an occupancy of zero. The structures of Yuh1-1 and Yuh1-2 are essentially identical, with a root mean square (r.m.s.) deviation of 0.3 Å on C_α atoms (0.8 Å for all atoms).

The Ubal-1 molecule is well-defined throughout. In contrast, the only well-defined regions of Ubal-2 are found at the contacts with Yuh1-2 (Ubal residues 6–10, 40–43 and 69–76). The ordered segments of Ubal-2 are essentially identical to the equivalent residues in Ubal-1. Regions neighboring the resolved Ubal-2 residues show diffuse and broken electron density, and further away the density is completely absent. It is unlikely that Ubal-2 is unfolded in the crystal since the compact ubiquitin structure is known to be very stable (Wintrod *et al.*, 1994). Rather, it appears that Ubal-2 is able to pivot about the Yuh1-2 contacts as a rigid body, resulting in the diffuse density away from the Yuh1 surface.

Structures of Yuh1 and Ubal

The Yuh1 structure consists of a six-membered antiparallel β -sheet surrounded by α -helices (Figure 1). As expected from the 33% sequence identity, Yuh1 resembles the previously determined UCH-L3 structure (Johnston *et al.*, 1997), with least squares overlap yielding an r.m.s. deviation of 1.61 Å for 146 matching C_α atoms. Yuh1 secondary structural elements have been named according to their counterparts in UCH-L3. Thus, Yuh1 has three additional short strands, S0, S2¹ and S2², and lacks the

helix H3 (Figure 2). A disordered loop in UCH-L3 becomes ordered in Yuh1; we call this segment (residues 144–164 of Yuh1; yellow in Figure 1) the 'active-site-crossover loop', since it adopts a functionally suggestive conformation which wraps over the catalytic center (see below).

As expected, the structure of Ubal in the complex with Yuh1 closely resembles that of ubiquitin (Vijay-Kumar *et al.*, 1987). However, unlike ubiquitin, the Ubal C-terminal residues are ordered in the Yuh1 complex where they provide a major determinant of substrate recognition (see below).

Implications for catalysis

Catalysis by UCHs is thought to parallel the mechanism established for the papain family of cysteine proteases (Storer and Ménard, 1994; Dang *et al.*, 1998). In this scheme (Figure 3), the cysteine nucleophile (Cys90) attacks the carbon atom of the scissile bond to form a tetrahedral intermediate, which collapses to the more stable acyl intermediate through expulsion of the C-terminal leaving group. Attack by a water molecule then generates a carboxylate on the ubiquitin product and regenerates a free thiol on the enzyme. Nucleophilicity of the catalytic cysteine is enhanced by an adjacent histidine residue (His166), and a catalytic triad is completed by hydrogen bonding between the histidine and an asparagine or aspartate sidechain (Asp181). The second major structural feature for catalysis is a binding site for the oxygen atom of the scissile peptide bond, known as the oxanion hole, where negative potential on the oxygen atom is dissipated through hydrogen bonding with the main chain amide of the catalytic cysteine residue and the sidechain amide of a glutamine residue (Gln84). Consistent with this mechanism, the Yuh1 active-site geometry closely resembles that seen for papain-like enzymes, with the four catalytic residues located in a cleft on the enzyme surface (Figure 1A), in positions essentially identical to those seen in the unliganded UCH-L3 structure (Johnston *et al.*, 1997).

Inspection of electron density maps indicated that a covalent bond is formed between the S_γ atom of Yuh1 Cys90 and the C-terminal carbon atom of Ubal Gly76. Furthermore, the Gly76 carbon atom clearly has tetrahedral as opposed to planar geometry. The X-PLOR topology and parameter files were therefore modified to include restraints toward a 1.8 Å S–C bond length and tetrahedral bond angles. The refinement then satisfied these target values for both complexes, with only small deviation from the target geometry and good agreement to both $F_o - F_c$ and simulated annealing omit electron density. This geometry indicates that the structure is trapped as a thiohemiacetal analogue of the tetrahedral reaction intermediates (TI-1 and TI-2 of Figure 3). The thiohemiacetal is stabilized through its optimal interactions with residues in the active site, and because forward reaction progress is blocked due to the poor leaving ability of hydrogen. Thus, the Yuh1–Ubal crystal structure reveals details of the catalytic interactions and strongly supports the view that the UCH and papain enzymes share a common enzymatic mechanism.

Fig. 1. Stereoview ribbon representation of the Yuh1-Ubal complex. (A) Ubal is not shown in this panel. Sidechains of the active-site residues Gln84, Cys90, His166 and Asp181 (red) are labeled Q, C, H and D. N- and C-termini are labeled. The disordered segment (residues 63–77) is indicated with the adjacent ordered residues labeled in magenta. The active-site-crossover loop is colored yellow. Secondary structures were as defined by PROMOTIF (Hutchinson and Thornton, 1996). Strands are colored green and the helices blue. Helix 4, which contains the active-site nucleophile Cys90, is colored cyan. This helix undergoes a severe kink, indeed PROMOTIF defines residues 90–100 and 103–105 as separate helices. We describe this as one continuous helix in order to maintain consistency of nomenclature with UCH-L3, which also has a severe kink in the corresponding part of helix 4. The following are labeled on the figure: strand 0 (S0, residues 11–12), strand 1 (S1, 31–36), strand 2 (S2, 54–60), strand 2¹ (S2¹, 81–82), strand 2² (S2², 128–129), strand 3 (S3, 165–172), strand 4 (S4, 176–180), strand 5 (S5, 189–193), strand 6 (S6, 227–233); helix 1 (H1, residues 15–25), helix 2 (H2, 44–46), helix 4 (H4, 90–105), helix 5 (H5, 111–122), helix 6 (H6, 132–143), helix 7 (H7, 205–221). Helices are alpha-type, except for helix 2 and residues 102–105 of helix 4, which adopt the 3_{10} conformation. There is a turn of alpha helix (residues 146–150) within the active-site-crossover loop. (B) Same as (A), but including the Ubal shown in magenta. The sidechains of Ubal residues discussed in the text are shown in orange and labeled. Figures 1, 4, 5 and 6 were produced with the programs MOLSCRIPT (Kraulis, 1991) and RASTER 3D (Bacon and Anderson, 1988).

Specific interactions between ubiquitin and Yuh1

The contacts between Yuh1 and Ubal are extensive (Figure 1B), apparently burying a total of 2500 Å² of solvent-accessible surface area. They include the covalent bond between the Ubal C-terminal carbon atom and the Yuh1 Cys90 sidechain, 20 hydrogen bonds (including 1 salt bridge) and numerous van der Waals contacts. Most of the specificity determinants lie within the last five residues of Ubal, which extend along the active-site cleft bounded on one side by residues in the N-terminus of

Yuh1 and on the other side by elements of the central β -sheet (Figure 4). The Ubal C-terminal residues adopt an extended β -strand-like conformation, with many main chain groups forming hydrogen bonds with Yuh1, including a small anti-parallel β -sheet motif involving Yuh1 residues 11–12 and Ubal residues 74–75. The importance of some contacts seen in the crystal structure has been investigated by steady-state kinetic analyses with substrates (Table I) and inhibitors (Table II).

The only salt bridge between the two proteins is formed

Fig. 2. Alignment of UCH sequences. Secondary structures are shown above for Yuh1 (upper, large) and UCH-L3 (lower, small), with the same color code as for Figure 1. Every tenth Yuh1 residue is indicated by a gap. Disordered residues are indicated with an open bar. Residues which are structurally equivalent between Yuh1 and UCH-L3 are shown boxed on a blue background. Structural equivalence is defined by an LSQMAN (Kleywegt and Jones, 1994b) optimization starting from an overlap on the four active-site residues, and requiring a minimum fragment length of four residues. The maximum C_{α} - C_{α} separation following this alignment is 3.28 Å. Although Yuh1 Ala46 and UCH-L3 Ser43 are separated by 3.6 Å following LSQMAN alignment, we consider these residues to be structurally equivalent on the basis of visual inspection. Alignment of the other sequences has been adjusted in light of the Yuh1 and UCH-L3 structures. Invariant residues are shown on a yellow background (active-site residues red). Residues that define specificity for the Ubal Gly75–Gly76 residues are indicated with a red triangle. SwissProt Database entries shown are: YUH1 (*S.cerevisiae*; SW:P35127); UCH-L3 (Human; SW:P15374); UCH-L1 (Human; SW:P09936); UBL-DROME (*Drosophila melanogaster*; SW:P35122); SCHPO (*Schizosaccharomyces pombe*; SW:Q10171). The partial BAP1 sequence is from Jensen *et al.* (1998).

Fig. 3. Schematic of the UCH/cysteine protease reaction cycle. TI-1 and TI-2 denote the high energy tetrahedral intermediates; AI, the acyl intermediate; S, a cysteine; and Im, a histidine. Adapted from Storer and Ménard (1994).

between Ubal Arg72 and the invariant Yuh1 Asp35. We have confirmed the importance of this contact by inhibition studies with the ubiquitin-like protein NEDD8, which has essentially the same structure and Yuh1-contacting residues as ubiquitin, except that residue 72 is an alanine in NEDD8 (Whitby *et al.*, 1998). The K_i of NEDD8 with Yuh1 is increased 20- and 50-fold compared with that of ubiquitin and NEDD8(A72R), respectively (Table I). This confirms that Arg72 contributes to UCH specificity by providing a significant favorable contribution to binding affinity. The slightly (2.7-fold) greater inhibition shown by NEDD8(A72R) compared with ubiquitin may result

from an unfavorable interaction between ubiquitin Asp39 and Yuh1 Glu55; Asp39 is the only other ubiquitin residue that approaches Yuh1 and is not conserved in NEDD8, where it is a glutamine. A functional consequence of the Asp35–Arg72 interaction may be to discriminate against NEDD8 and its yeast homolog Rub1.

Other sidechains near the Ubal C-terminus also appear to make significant interactions. Ubal Val70 and Leu71 make some hydrophobic contacts with Yuh1, and the Leu73 sidechain is packed against a hydrophobic surface that includes the Yuh1 sidechains of Leu58, Leu165 and Asn225. The hydrogen-bonding potential of the Ubal

Fig. 4. Contacts between Yuh1 and the C-terminal residues of Ubal. Stereoview of Ubal C-terminus (magenta) and surrounding Yuh1 residues (yellow). Hydrogen-bonding interactions are shown in green. Note that, for clarity, Gln153 is truncated to Ala in this figure, as are residues Glu155–Asp162 in the active-site-crossover loop. A covalent bond is shown between Yuh1 Cys90 S_γ and the terminal carbon atom of Ubal.

Table I. Kinetic parameters for Yuh1 with various ubiquitin–amide and ubiquitin–protein substrates

Substrate ^a	K_m (μM)	k_{cat} (s ^{−1})	k_{cat}/K_m (M ^{−1} ·s ^{−1})
Ub–AMC	0.020 ± 0.005	4.49 ± 0.52	(2.23 ± 0.63) × 10 ⁸
Ub–D77	3.2 ± 1.1	10.0 ± 1.0	(3.1 ± 1.1) × 10 ⁶
Ub(L8A)–D77	65 ± 14	4.9 ± 0.3	(7.5 ± 1.7) × 10 ⁴
Ub–OMCHI1 ^b	–	0	–
Ub–rOMCHI1	4.2 ± 1.7	0.105 ± 0.013	(2.5 ± 0.4) × 10 ⁴
Ub–rcamOMCHI1	1.8 ± 1.0	0.066 ± 0.009	(3.7 ± 2.1) × 10 ⁴

^aUb–AMC, ubiquitin followed by 7-amido-4-methylcoumarin; Ub–D77, ubiquitin followed by an aspartate residue; Ub–OMCHI1, ubiquitin followed by a seven-residue linker (GLQVPSF) and the first domain of chicken ovomucoid; Ub–rOMCHI1, Ub–OMCHI1 with the three OMCHI1 disulfide bonds reduced by preincubation for 30 min at 37°C with 5 mM DTT; Ub–rcamOMCHI1, Ub–OMCHI1 with the OMCHI1 disulfides reduced and carboxyamidomethylated.

^bReaction mixtures contained 0.05 mM DTT, 5.7 μM substrate and 3 μM Yuh1; no products were observed after 4 min.

Arg74 guanidinium is saturated, with five hydrogen bonds from N_{η1} and N_{η2} groups to main chain carbonyl oxygen atoms of Yuh1. Additionally, the hydroxyl of Yuh1 Ser154 receives a hydrogen bond from Ubal Arg74 guanidinium N_ε and mediates a hydrogen-bond network to the carboxylate sidechain of the invariant Yuh1 Glu12.

The Yuh1–Ubal structure explains the high UCH specificity observed for the two C-terminal residues Gly76 and Gly75 (Rose and Warms, 1983). This specificity is not defined by main chain conformation, since both glycines adopt phi, psi angles that are accessible by other residues. Rather, in both cases, specificity is defined by the proximity of conserved sidechains which block access for residues larger than glycine (Figure 5). Addition of a C_β to the sidechain of Ubal Gly75 would, in the absence of conformational changes, clash sterically with the highly conserved Yuh1 Leu58 and Tyr167 sidechains. Likewise, addition of a C_β onto Ubal Gly76 would result in steric clash with the invariant Yuh1 sidechain of Asn88. Compensatory changes to avoid these potential clashes appear to be ruled out by the extensive Yuh1–Ubal interface and by tight packing constraints on conserved residues. The observation that inhibition by *des*-GlyGly ubiquitin (Ub⁷⁴) is only 1.5-fold weaker than inhibition by ubiquitin (Table

Table II. Inhibition of Yuh1 by derivatives of ubiquitin and NEDD8

Inhibitor ^a	K_i (μM)
ubiquitin	1.70 ± 0.38
Ub ⁷⁴	2.56 ± 0.50
Ub(L8A)	65.8 ± 13.6
Ub(L8W)	137 ± 26
Ub(I44A)	2.36 ± 0.30
Ubdiol	0.154 ± 0.035
NEDD8	32.1 ± 9.8
NEDD8(A72R)	0.64 ± 0.16

^aUb⁷⁴, residues 1–74 of ubiquitin, i.e., lacking the two terminal glycine residues; Ub(L8A), Ub(L8W), Ub(I44A), ubiquitin point mutants; Ubdiol, ubiquitin with the C-terminal glycine replaced by 3-amido-1,2-propanediol; NEDD8(A72R), NEDD8 point mutant.

II) is consistent with the view that these residues define specificity primarily by their ability to adopt a productive binding geometry that cannot be accommodated by other residues, rather than by simply providing a favorable interaction.

In addition to the C-terminal residues, a second major contact to Yuh1 is provided by the type I β-turn of Ubal residues 7–10 (Figure 1B). This turn is sandwiched between the Yuh1 helix7–turn–strand7 motif on one side, and Yuh1 residues 36–39 on the other. The sidechain of Ubal Leu8 is oriented deeply into a crevice on the surface of Yuh1 where it is surrounded by main chain atoms at the ends of strands 1 and 7 and by the sidechains of Ile36, Leu39, Leu57, Val211, Met215, Phe226 and Met228. The importance of ubiquitin Leu8 for substrate recognition is confirmed by the 20-fold increase in K_m for hydrolysis of Ub(L8A)–D77 compared with Ub–D77 (Table I). Inhibition studies also indicate that Leu8 functions in substrate binding, since the Ub(L8A) and Ub(L8W) proteins showed, respectively, 40- and 80-fold weaker inhibition than ubiquitin (Table II).

Because most of the Ubal interactions are with invariant or conserved groups on Yuh1, all UCHs are expected to bind their substrate ubiquitin moieties with the same geometry. Consistent with this, interaction of substrate with the PA700-associated UCH is mediated to a significant extent by Leu8 and, to a lesser degree, by Ile44 of

Fig. 5. Specificity at the P1 and P2 positions is defined by conserved sidechains of Yuh1. Yuh1 (yellow), Ubal Gly75 and Gly76 (magenta). Black lines indicate C_{α} - C_{β} bonds if the Ubal glycine residues are mutated to alanine without a change in conformation. Expected steric clashes with Yuh1 residues are indicated with black dashed lines (typical van der Waals contact distances are ~ 3.9 Å). Hydrogen bond interactions of the Yuh1 Asn88 sidechain are shown as dashed green lines.

ubiquitin (Lam *et al.*, 1997a); Ile44 makes a peripheral interaction with Yuh1 Tyr37 and provides only marginal contributions to binding as judged by inhibition studies (Table II). The UCH-binding determinants of ubiquitin also overlap with those for the 26S proteasome S5a subunit and the ubiquitin-activating E1 enzyme. Solution studies show that, like Yuh1, S5a contacts the ubiquitin sidechains of Leu8, Ile44 and Val70 (Beal *et al.*, 1996, 1998), and E1 contacts ubiquitin Arg72 (Burch and Haas, 1994). A functional consequence is that, while bound to E1, S5a, and possibly other ubiquitin-pathway proteins, ubiquitin conjugates are likely to be protected from UCHs.

Conformational changes upon binding Ub_{al}

In addition to the specific interactions described above, it appears that UCH specificity is further enhanced by maintenance of an inaccessible active site in the absence of bound ubiquitin. The active-site cleft of the unliganded UCH-L3 structure is obstructed by residues near the N-terminus of the enzyme, especially by the sidechain of UCH-L3 Leu9 (equivalent to Yuh1 Ile11) which completely buries the catalytic cysteine nucleophile (Johnston *et al.*, 1997). These residues apparently move to open the active-site cleft upon substrate binding, with a displacement between the UCH-L3 Leu9 and Yuh1 Ile11 C_{α} atoms of ~ 4 Å (Figure 6A).

The oxyanion hole is also obstructed in the unliganded UCH-L3 structure. The oxyanion hole-forming glutamine and cysteine residues adopt the same conformations in the UCH-L3 and Yuh1-Ubal structures, but the binding site for the substrate oxygen is occupied by the carbonyl oxygen atom of UCH-L3 Ser92 (equivalent to Lys87 of Yuh1). This oxygen atom undergoes an apparent ~ 5 Å displacement in the Yuh1-Ubal structure as a result of an $\sim 180^{\circ}$ rotation of the Lys87 peptide bond to a new conformation which is stabilized, in part, by a hydrogen bond with the backbone nitrogen of Yuh1 Val8 (Figure 6B). In addition to the altered peptide bond orientation, the

conformational change at the oxyanion hole can be described as a hinging motion of residues 85–87, which form a turn just before the start of helix 4. Interactions seen between these residues and the N-terminal residues in both the Yuh1-Ubal complex and the unliganded UCH-L3 structures provide a mechanism to couple opening of the oxyanion hole to the binding of ubiquitin. The requirement of ubiquitin binding for the generation of an accessible conformation at the substrate-binding cleft and oxyanion hole probably provides important selection against inappropriate substrates for the intracellular UCH proteases.

Another significant conformational change is seen for the active-site-crossover loop (Yuh1 residues 144–164), which is disordered in UCH-L3 but ordered in the complex, where it contacts Ub_{al} and other regions of Yuh1. Because this loop passes directly over the active site, it appears that productive binding requires that the ubiquitin C-terminal adduct of a substrate must pass through this loop (Figure 7). Even if the active-site-crossover loop is modeled in an alternative maximally open conformation, the internal diameter measured from the polypeptide backbone would be only ~ 15 Å (Johnston *et al.*, 1997) which is too small to allow passage of folded substrates larger than a single helix.

Some of the interactions between the active-site-crossover loop and Ub_{al} have been described above. In addition, two invariant Yuh1 sidechains, Glu12 and Asn88, are notable in that they appear to stabilize the loop conformation by making hydrogen bonds with main chain groups of the loop. The Yuh1 Glu12 carboxylate hydrogen-bonds NH groups of Gln153 and Ser154 at a point where the loop makes a sharp turn (Figure 4). Yuh1 Asn88 N_{H2} is within hydrogen bonding distance of Ala159 and Ala161 carbonyl oxygen atoms (Figure 5), thereby defining the loop conformation close to the catalytic center. The participation of invariant residues in these hydrogen bonding interactions supports the view that the active-site-

Fig. 6. Apparent changes in UCH conformation upon binding of Ubal. (A) Yuh1 (yellow), Ubal (magenta) and UCH-L3 (blue) structures are shown after superimposition on the C_{α} atoms of the four active-site residues. UCH-L3 Leu9 occupies the space taken by Ubal Gly75 in the Yuh1 complex crystal structure. The equivalent Yuh1 Ile11 residue is apparently displaced ~ 4 Å upon binding of Ubal. (B) The carbonyl oxygen of UCH-L3 Ser92 blocks the oxyanion hole in the unliganded structure. Hydrogen bonds observed from Yuh1 Gln84 N_{ϵ} and Cys90 N to Ubal Gly76 O are shown in green. Hydrogen bonds observed from the equivalent amide groups to UCH-L3 Ser92 O are shown in black. In the Yuh1–Ubal complex, the Lys87 peptide (equivalent to UCH-L3 Ser92) is rotated by $\sim 180^{\circ}$ to allow formation of a hydrogen bond between Lys87 O and Val8 N.

Fig. 7. Space-filling representation showing Ubal C-terminus through the Yuh1 active-site-crossover loop. (A) Yuh1 is in the same representation as in Figure 1. Ubal is in CPK representation. This view direction is the same as Figure 1. (B) Same as (A) following a 90° rotation about the vertical axis. (C) Space-filling representation of Ubal (magenta) and Yuh1 (yellow). View direction is into the opening of the active-site-crossover loop, i.e. the image has been rotated $\sim 70^{\circ}$ about the horizontal axis and $\sim 20^{\circ}$ about the vertical axis with respect to (A).

crossover loop conformation seen in the Yuh1–Ubal crystal structure is of functional importance and that, despite the diverse sequences for this loop, the conformation seen here will closely resemble that for active complexes of other UCHs.

Role of the active-site-crossover loop

In order to investigate the roles of the adduct following ubiquitin and of the active-site-crossover loop in substrate selection, we have performed kinetic analysis on a variety of model substrates (Table I). Hydrolysis of Ub–AMC

and Ub-D77 (i.e. ubiquitin followed by 7-amido-4-methylcoumarin or by an aspartate residue) shows that the more hydrophobic AMC leaving group confers a 138-fold lower K_m , but that k_{cat} values are similar for both substrates. This is consistent with previous studies on UCH-L3 (Dang *et al.*, 1998) and with the suggestion that the substrate residue following ubiquitin is in a generally hydrophobic environment under the active-site-crossover loop. This environment may also provide the basis for the relatively high inhibition shown by Ubdiol, a 76-residue ubiquitin derivative with an uncharged C-terminus (Table II).

The activity of Yuh1 against larger folded substrates has been investigated using fusion constructs of ubiquitin with a domain of chicken ovomucoid (OMCHI1), green fluorescent protein (GFP), or OMCHI1 followed directly by GFP (OMCHI1-GFP). In each construct, ubiquitin was joined to the C-terminal protein extension by the same flexible seven-residue linker. An advantage of these constructs is that the OMCHI1 domain can be either stably folded, or unfolded via reduction ('rOMCHI1') or reduction/alkylation ('rcamOMCHI1').

Whereas Ub-OMCHI1 could not be cleaved by Yuh1, the unfolded forms of OMCHI1 were released, although with k_{cat} reduced dramatically compared with other substrates (Table I). Consistent with the view that UCH enzymes bind predominantly to the ubiquitin domain of substrates, the K_m values for Ub-rOMCHI1 and Ub-rcamOMCHI1 were similar to that for Ub-D77. Proof that the unfolded Ub-OMCHI1 substrates do not thread through the active-site-crossover loop was obtained by comparisons of Ub-GFP, Ub-OMCHI1-GFP, and Ub-rcamOMCHI1-GFP as substrates. Yuh1 could release ubiquitin only from the last of these three fusion proteins, although the characteristic GFP fluorescence indicated that the GFP moiety was folded in every case (data not shown). Thus, flexibility at the ubiquitin-protein linkage region appears to be a key requirement for productive binding of large substrates. One interpretation of these observations is that the active-site-crossover loop makes a relatively small contribution to the energetics of substrate binding, and that larger substrates can be accommodated by displacement of the loop from its preferred conformation. This displacement may reduce k_{cat} if the precise geometry of critical residues at the catalytic center is altered (perhaps via contacts with Asn88). Alternatively, Yuh1 may only rarely be in a conformation able to bind the larger substrates; whereas non-productive binding would not have been detected as an altered K_m , a low abundance for this form of the enzyme could explain the apparent decrease in k_{cat} . By either model, efficient catalysis requires that substrates pass through the active-site-crossover loop.

Physiological substrates

A major unanswered question for UCH enzymes is the identity of their physiological substrates. Many of the potential substrates are relatively large, such as the products of the yeast *UBI1-4* genes, polyubiquitin chains that target substrates to the 26S proteasome, and proteins whose functions are modified by reversible ubiquitination. However, the structural and biochemical data presented here and elsewhere (Rose and Warms, 1983; Pickart and Rose, 1985; Larsen *et al.*, 1998) suggest that the authentic substrates for UCHs are small or unstructured. The Yuh1-

Ubal structure and the kinetic data suggest that preferred substrates may pass through the active-site-crossover loop, consistent with the possibility that authentic UCH substrates include small adducts formed by chance reaction with intracellular nucleophiles such as glutathione (Pickart and Rose, 1985). Other possible physiological substrates for UCHs are the short extensions on ubiquitin precursor proteins, and perhaps the similar extensions found on some ubiquitin-like proteins. Although Yuh1, and UCHs in general, cannot hydrolyze within either ϵ - or α -linked polyubiquitin, Yuh1 can release polyubiquitin chains intact from small adducts such as aspartic acid (Piotrowski *et al.*, 1997) or any of several different unfolded ϵ -linked conjugates (C.Sokolik, R.Dunten, W.Xu, T.Yao and R.E.Cohen, unpublished data). This is consistent with the Yuh1-Ubal crystal structure, in which the Ubal Lys48 sidechain is highly exposed and able to accommodate additional ubiquitin moieties.

Several observations argue that UCH enzymes process a wider range of substrates *in vivo* than obviously suggested by the biochemical data. For example, the presence of specific substrates is suggested by the tissue specificity of some UCH isozymes (Wilkinson *et al.*, 1992), and by the implication of some UCHs in specific diseases (Jensen *et al.*, 1998; Leroy *et al.*, 1998) and long-term nerve potentiation (Hegde *et al.*, 1997). Important insight may be provided by the UCH associated with PA700, which is thought to function in the editing of 26S proteasome substrates by removal of the most distal ubiquitin moiety in Lys48-linked polyubiquitin chains (Lam *et al.*, 1997a,b). Remarkably, the isolated recombinant PA700-associated UCH has a substrate specificity different from the intact PA700 complex and more like that of Yuh1 and other UCHs, which cannot disassemble polyubiquitin (W.Xu, G.N.DeMartino and R.E.Cohen, unpublished data). This change in specificity upon incorporation into a multiprotein complex may be a general property of UCH enzymes. Thus, the active-site-crossover loop may function to restrict access to the active site until the enzyme is incorporated into a complex that can effect a conformational change to increase the activity against larger substrates. At least one other UCH, the BRCA1-associated protein BAP1, appears to exist as part of a larger complex (Jensen *et al.*, 1998).

Materials and methods

Purification and crystallization

Recombinant Yuh1 was expressed in *Escherichia coli* MM294 under control of the *TRP* promoter from plasmid p-1A2/TrpYuh-1 (Miller *et al.*, 1989; kindly provided by R.Vandlen, Genentech Corp.). Cells were grown under ampicillin selection (50 μ g/ml) at 37°C; 20 ml of an overnight culture in Luria-Bertani medium was diluted into 1 l of M9 minimal medium enriched with 1% glucose and 0.1% casamino acids. Cells were induced at OD₆₀₀ 0.6 with 50 mg/l of 3- β -indole acrylic acid and harvested after 5 h by centrifugation. The cells were suspended in buffer A (50 mM Tris-HCl pH 8.0, 1 mM EDTA, 10 mM β -mercaptoethanol, 10% v/v glycerol) plus 0.5 mM phenylmethylsulfonyl fluoride and 0.02 mM lysozyme; lysis was by French press and sonication.

The lysate was filtered (0.45 μ m), applied to a 100 ml DEAE Sepharose Fast Flow anion exchange column and eluted with a 500 ml linear gradient of 0–0.5 M NaCl in buffer A; Yuh1 eluted at ~0.4 M NaCl. Yuh1-containing fractions, identified by SDS-PAGE, were dialyzed against buffer A, adjusted to 11% (w/v) (NH₄)₂SO₄, and loaded onto a Phenyl Sepharose Hi-Load 26/10 column equilibrated in buffer B [= buffer A + 11% (NH₄)₂SO₄]. A 400 ml linear gradient of buffer B to buffer A eluted Yuh1 as a broad peak starting at ~8% (NH₄)₂SO₄.

Table III. Data processing statistics

	Native ^a	$\lambda 1^b$	$\lambda 2^b$	$\lambda 3^b$
Wavelength (Å)	1.080	0.9789	0.9786	0.930
Resolution limit (Å)	2.25	2.20	2.20	2.20
No. of unique reflections	26101	26503	26372	26010
Completeness (%)	99 (96)	96 (81)	95 (79)	94 (81)
$\langle I/I \rangle$	14.1 (4.3)	11.9 (2.0)	12.7 (2.0)	13.6 (2.5)
Redundancy	4.3 (2.5)	4.1 (4.0)	4.0 (3.9)	3.6 (3.5)
R_{sym} (%)	3.7 (20.2)	4.4 (19.2)	4.7 (21.3)	5.2 (16.8)
Mosaicity (°)	0.34	0.66	0.67	0.71

Numbers in parentheses refer to data from the highest resolution shell. Data were processed with DENZO and SCALEPACK (Otwinowski, 1993).

^aNative data were collected on a MAR imaging plate detector at SSRL 7–1.

^bMAD data were collected on a Brandeis CCD detector at NSLS X12C.

^c $R_{\text{sym}} = 100 * \sum_i |I_i - \langle I \rangle| / \sum_i I_i$

Yuh1 fractions were dialyzed into buffer C (buffer A at pH 7.45), and applied to a 1 ml MonoQ anion exchange column; Yuh1 was eluted at ~0.3 M NaCl with a 40 ml gradient of 0–1 M NaCl in buffer C. Yuh1 appeared as a single band on Coomassie Blue-stained SDS–PAGE gel, and activity was demonstrated by cleavage of Ub–MEFMHISPPESHSS (a gift from M.Rechsteiner). Yuh1 concentrations were determined from a calculated (Gill and vonHippel, 1989) extinction coefficient of 27310 M⁻¹·cm⁻¹ at 280 nm. For kinetic studies, active enzyme concentrations were checked by active-site titrations with Ubal (Lam *et al.*, 1997b; Dang *et al.*, 1998).

Ubal was synthesized as described previously (Dunten and Cohen, 1989; Lam *et al.*, 1997a). Yuh1–Ubal complex, prepared by addition of Ubal to excess Yuh1 in buffer C, was resolved from free Yuh1 on a MonoQ column using the conditions described above. Purified complex was dialyzed into crystallization buffer [10 mM Na–MOPS pH 6.85, 10 mM dithiothreitol (DTT), 1 mM EDTA] and concentrated to 20 mg/ml (Centricon-10; Amicon Corp.).

Crystallization was performed at 21°C in sitting drops. The reservoir solution was 16% polyethyleneglycol 6000, 0.1 M sodium acetate pH 4.4. Most drops did not give usable crystals, although occasionally large single crystals (0.4–0.6 mm × 0.4–0.6 mm × 0.6–1.0 mm) grew within 5–10 days. The space group is R3 with cell dimensions $a = 199.3$ Å, $c = 36.8$ Å. There are two Yuh1–Ubal complexes in the asymmetric unit with a solvent content of 40%.

Selenomethionine-substituted Yuh1 (SeYuh1) was expressed from p-1A2/TrpYuh-1 in *E. coli* (Studier and Moffatt, 1986). Single colonies from LB–ampicillin plates were used to inoculate 100 ml of LB media containing 100 µg/ml ampicillin. After overnight growth at 37°C, cells were pelleted and resuspended in selenomethionine-modified M9 media (M9 salts, vitamins, MgSO₄, FeSO₄·7H₂O, glucose, ampicillin and individual L-amino acids at 40 µg/l, with methionine replaced by seleno-L-methionine and without tryptophan); 25 ml of cell suspension was used to inoculate each liter of modified M9 media. Subsequent steps in growth, induction, purification and crystallization of SeYuh1–Ubal were as for wild-type Yuh1.

Structure determination

Prior to data collection at 100 K, crystals were transferred, in 2% glycerol increments, to a solution identical to the crystallization reservoir with 20% glycerol. Crystals were suspended in a rayon loop (Hampton) and cooled in liquid nitrogen. Multiwavelength data were collected from a single SeYuh1–Ubal crystal at beamline X12C of the National Synchrotron Light Source, Brookhaven National Laboratory. The three wavelengths used were selected from the fluorescence spectrum: $\lambda 1$ (0.9789 Å; inflection; minimum f''); $\lambda 2$ (0.9786 Å; peak; maximum f''); $\lambda 3$ (0.9300 Å; remote; maximum f'). The three data sets were scaled together over the resolution range 5.0–2.2 Å (Otwinowski and Minor, 1997), and resulting scale factors were applied separately to each individual data set over the resolution range 30–2.2 Å. Native data were collected to 2.25 Å resolution at beamline 7–1 of the Stanford Synchrotron Radiation Laboratory, Palo Alto, CA. See Table III for data processing statistics.

Crystallographic computing used programs from the CCP4 suite (CCP4, 1994) unless otherwise noted. The four Se sites, corresponding

Table IV. Refinement statistics

Resolution range (Å)	30.0–2.25
High resolution shell (Å)	(2.35–2.25)
R_{value} (%) ^a	24.8 (34.9)
R_{free} (%) ^b	28.5 (43.0)
R.m.s.d. (bonds) (Å) ^c	0.015
R.m.s.d. (angles) (°) ^c	1.79
No. of residues included (total)	520 (624)
No. of atoms with occupancy = 0.0 ^d	50
No. of water molecules	75
$\langle B \rangle$ (Å ²) protein/water/Wilson ^e	55/50/52
No. of ϕ/ψ angles (%): most favored ^d	88.4
additional	10.5
generous	0.9
forbidden	0.2

^a $R_{\text{value}} = 100 * \sum |F_p(\text{obs})| - |F_p(\text{calc})| / \sum |F_p(\text{obs})|$

^b $R_{\text{free}} = R_{\text{value}}$ for a randomly selected subset (5%) of the data that were not used for minimization of the crystallographic residual (Brünger, 1992).

^cStereochemistry was analyzed with PROCHECK (Laskowski *et al.*, 1993).

^dNon-hydrogen atoms only. Fifty atoms were assigned an occupancy of zero because they lack defined electron density.

^eWilson plot in resolution range 3.5–2.5 Å.

to Yuh1 Met215 and Met228 for the two complexes in the asymmetric unit, were identified from difference Patterson and Fourier functions, and refined with MLPHARE (Otwinowski, 1991) treating $\lambda 1$ as the native data of a conventional multiple isomorphous phase determination (Ramakrishnan and Biou, 1997) to give a mean figure-of-merit of 0.26 for 30–2.5 Å data.

The map was improved by two-fold averaging using the symmetry operator derived from the Se positions (Kleywegt and Jones, 1994a), although the absence of a molecular mask prevented refinement of the phases at this stage. Map interpretation (Jones *et al.*, 1991) was assisted by knowledge of the UCH–L3 structure (Johnston *et al.*, 1997). Construction of an approximate molecular mask for the Yuh1 molecules allowed phase refinement by averaging, solvent flattening, and histogram shifting with DM (Cowtan, 1994) to give a mean figure-of-merit of 0.684 and a readily interpretable density map.

Refinement calculations with X-PLOR 3.851 (Brünger, 1996) were interspersed with model building with program O (Jones *et al.*, 1991). Refinement against the SeMet data was not satisfactory, probably because of systematic error in the data. Refinement was therefore continued against native data. Molecular masks and non-crystallographic symmetry operators were updated during refinement to allow calculation of improved MAD/DM maps. The R_{factor} (R_{free}) is 24.8% (28.5%), using all data from 30 to 2.25 Å resolution and a bulk solvent correction. Statistics are given in Table IV. The relatively high residuals probably result from the presence of partially ordered segments, including most of one Ubal molecule, that are not represented in the atomic model. Coordinates (1cmx) and diffraction data (r1cmxsf) have been deposited at the Protein Data Bank

Yuh1 substrates and steady-state kinetics

Ubiquitin–Asp⁷⁷ (Ub–D77) was expressed from pRSUbD in *E. coli* BL21(DE3)pLysS and was purified as described (Piotrowski *et al.*, 1997). Wild-type (bovine) ubiquitin was from Sigma and recombinant Ub(L8A), Ub(L8W) and Ub(I44A) proteins were from a previous study (Lam *et al.*, 1997a). Purified NEDD8 and NEDD8(A72R) proteins were gifts from G.Xia and C.Pickart (Johns Hopkins University); Ub(L8A)-D77 and des-GlyGly-Ub (Ub⁷⁴), encoded by plasmids pET3a-L8A,D77-Ub and pET3a-Ub74 (provided by C.Pickart), were expressed and purified essentially as for the other ubiquitin variants. Protein concentrations were determined spectrophotometrically at 280 nm for the ubiquitin derivatives (Lam *et al.*, 1997a); for the NEDD8 proteins, a calculated extinction coefficient at 280 nm (Gill and vonHippel, 1989) of 2600 M⁻¹·cm⁻¹ was used.

Plasmid pRS-Ub–OMCHI1, which encodes a fusion of N-terminal ubiquitin to chicken ovomucoid first domain followed by a C-terminal His₆ tag, was constructed from pRS-Ub (provided by K.Wilkinson, Emory University) and pOMCHI1-C (provided by A.Robertson, University of Iowa; DeKoster and Robertson, 1997). Plasmid pRS-Ub–pstI, in which the ubiquitin stop codon was changed to GGA (glycine), was derived from

pRS-Ub by PCR amplification with (5'-TAATACGACTCACTATAGGG-3') as the 5' primer and (5'-TGGTACCTGCAGTCCGCCGCCACG-CAG-3') as the 3' primer. The PCR product was cloned into the *Nde*I and *Pst*I sites of pRSUb to generate pRSUb-pstI. The OMCH1 gene was amplified from pOMCH1-C by PCR using the 5' primer (5'-GCAAGCAAGCTTCATGGCAGAAGTTGACTGCAGCA-3') and the His₆-encoding 3' primer (5'-TACGATAAGCTTTTAATGGTGATGGT-GATGGTGCATCGGAACAGTTTCTTTGCATTCGCC-3'). Each primer introduced a *Hind*III site, and the PCR product was cloned into the *Hind*III site immediately downstream of the ubiquitin gene in pRSUb-pstI. The resulting plasmid, pRSUb-OMCH1, encodes a fusion of ubiquitin followed by GLQVPSF, then 69 amino acids from OMCH1, and ending with a C-terminal His₆ tag. Ub-OMCH1 was expressed from pRSUb-OMCH1 in BL21(DE3)pLysS essentially as described for OMCH1 (DeKoster and Robertson, 1997). The cell pellet from a 2 l culture was frozen, thawed and suspended in lysis buffer (50 mM NaP, pH 8.0, 0.3 M NaCl and 10 mM imidazole). Lysozyme was added (1 mg/ml) and the cell suspension was stirred on ice for 30 min, sonicated and centrifuged at 10 000 g for 20 min. The clarified lysate was mixed with 4 ml of NiNTA resin (Qiagen) for 45 min at 4°C, loaded into a column and, after washing with 180 ml of lysis buffer, bound proteins were eluted with a 60 ml gradient of 10–500 mM imidazole in lysis buffer. Fractions containing Ub-OMCH1, identified by SDS-PAGE, were pooled, adjusted to 0.18 M EDTA pH 8.0, and dialyzed against 20 mM Tris-HCl pH 8.0. The protein was then passed through a 1 ml MonoQ column in 20 mM Tris-HCl pH 8.0, concentrated (Centricon-10), and applied to a Superdex 75 column eluted with 0.1 M NaCl in the same buffer. Fractions with pure Ub-OMCH1 were pooled and concentrated by ultrafiltration (Microcon-3; Amicon Corp.).

To unfold irreversibly the OMCH1 domain of Ub-OMCH1, the three disulfide bonds of the native protein were reduced and carboxamidomethylated. Ub-OMCH1 (0.4 mg/ml) was incubated at 25°C in 5.1 M guanidinium-HCl, 0.17 M Tris-HCl pH 8.6, 8.5 mM DTT and 8.5 mM EDTA for 1 h. Iodoacetamide then was added to 25 mM and the incubation continued in the dark. After 30 min, β -mercaptoethanol was added to 40 mM and the mixture was dialyzed against 50 mM Na-HEPES pH 7.5. The reaction was judged to be complete as the product, Ub-rcamOMCH1, had a slower mobility than Ub-OMCH1 upon native gel electrophoresis (10% polyacrylamide; 50 mM Tris-HCl pH 8.6), and no unreacted Ub-OMCH1 was detected.

Yuh1 activity assays were carried out at 25°C in 50 mM Na-HEPES pH 7.5, with 0.1 mg/ml ovalbumin, 1 mM DTT (unless specified otherwise) and 0.5 mM EDTA. With the Ub-D77 substrate, 60 pM Yuh1 was used, whereas with Ub(L8A)-D77 the enzyme was increased to 1.8 nM. Aliquots taken at various times were mixed with equal volumes of 1 M acetic acid to stop the reaction, and the products were resolved by cation exchange HPLC (TosoHaas TSK SP-NPR column eluted at 1 ml/min over 15 min with a linear gradient of 0–0.4 M NaCl in 25 mM ammonium acetate pH 4.5). The proteins eluted were monitored and quantified by their absorbance at 235 nm. Fluorimetric assays employed the substrate ubiquitin-7-amido-4-methylcoumarin (Ub-AMC), which was a gift from R.Stein (Proscript, Inc.). Initial velocities for Ub-AMC hydrolysis were determined as described (Dang *et al.*, 1998) with slight modifications. The reaction buffer was as described above, and 2 pM Yuh1 was used. Product formation was monitored continuously by fluorescence (SLM 4800C spectrofluorometer; λ_{ex} = 355 nm, λ_{em} = 430 nm) and quantified by reference to a standard solution of AMC. For hydrolysis of disulfide-reduced Ub-OMCH1 (Ub-rcOMCH1) and Ub-rcamOMCH1, 15 nM Yuh1 and 0.01 mg/ml ovalbumin were used, and the reactions were stopped by the addition of ≥ 4 volumes of 9% CH₃CN in 0.05% trifluoroacetic acid. Products and substrate were resolved by reverse-phase HPLC (Phenomenex Jupiter™ C5 column, 30×0.4 cm, eluted at 1 ml/min with a gradient of 27–36% CH₃CN in 0.05% trifluoroacetic acid) and detected by absorbance at 205 or 235 nm. Concentrations were determined from peak integrations with ubiquitin as a standard. Cleavage by Yuh1 of native Ub-OMCH1, Ub-GFP or Ub-rcOMCH1-GFP was evaluated by SDS-PAGE; the GFP fusion proteins were available from a separate study (S.M.Riddle and R.E.Cohen, unpublished). For tests of competitive inhibition by ubiquitin or NEDD8 derivatives, Yuh1 was preincubated 5 min at 25°C with inhibitor before substrate was added to initiate the reaction. Initial velocity data were used to determine values for K_m , K_i and k_{cat} from non-linear fits of the Michaelis-Menten equation, modified where appropriate for competitive inhibition, with the program Ultrafit (Biosoft, Cambridge, UK).

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References

- Bacon, D. and Anderson, W.F. (1988) A fast algorithm for rendering space-filling molecule pictures. *J. Mol. Graph.*, **6**, 219–220.
- Baker, R.T., Tobias, J.W. and Varshavsky, A. (1992) Ubiquitin-specific proteases of *Saccharomyces cerevisiae*: cloning of UBP2 and UBP3 and functional analysis of the UBP gene family. *J. Biol. Chem.*, **267**, 23364–23375.
- Beal, R., Deveraux, Q., Xia, G., Rechsteiner, M. and Pickart, C. (1996) Surface hydrophobic residues of multiubiquitin chains essential for proteolytic targeting. *Proc. Natl Acad. Sci. USA*, **93**, 861–866.
- Beal, R.E., Toscano-Cantaffa, D., Young, P., Rechsteiner, M. and Pickart, C.M. (1998) The hydrophobic effect contributes to polyubiquitin chain recognition. *Biochemistry*, **37**, 2925–2934.
- Brünger, A.T. (1992) Free R value: a novel statistical quantity for assessing the accuracy of crystal structures. *Nature*, **355**, 472–475.
- Brünger, A.T. (1996) *X-PLOR Version 3.843, A System for X-ray Crystallography and NMR*. Yale University, New Haven, CT.
- Burch, T. and Haas, A. (1994) Site-directed mutagenesis of ubiquitin. Differential roles for arginine in the interaction with ubiquitin-activating enzyme. *Biochemistry*, **33**, 7300–7308.
- CCP4 (1994) The CCP4 suite: programs for protein crystallography. *Acta Cryst.*, **D50**, 760–763.
- Chau, V., Tobias, J.W., Bachmair, A., Marriotti, D., Ecker, D.J., Gonda, D.K. and Varshavsky, A. (1989) A multiubiquitin chain is confined to specific lysine in a targeted short-lived protein. *Science*, **243**, 1576–1583.
- Ciechanover, A. and Schwartz, A.L. (1998) The ubiquitin-proteasome pathway: the complexity and myriad functions of proteins death. *Proc. Natl Acad. Sci. USA*, **95**, 2727–2730.
- Cowtan, K.D. (1994) 'dm': an automated procedure for phase improvement by density modification. *Joint CCP4 and ESF-EACBM Newsletter on Protein Crystallography*, **31**, 34–38.
- Dang, L.C., Melandri, F.D. and Stein, R.L. (1998) Kinetic and mechanistic studies on the hydrolysis of ubiquitin C-terminal 7-amido-4-methylcoumarin by deubiquitinating enzymes. *Biochemistry*, **37**, 1868–1879.
- DeKoster, G.T. and Robertson, A.D. (1997) Thermodynamics of unfolding for Kazal-type serine protease inhibitors: entropic stabilization of ovomucoid first domain by glycosylation. *Biochemistry*, **36**, 2323–2331.
- Dunten, R.L. and Cohen, R.E. (1989) Recognition of modified forms of ribonuclease A by the ubiquitin system. *J. Biol. Chem.*, **264**, 16739–16747.
- Gill, S.C. and von Hippel, P.H. (1989) Calculation of protein extinction coefficients from amino acid sequence data. *Anal. Biochem.*, **182**, 319–326.
- Hegde, A.N., Inokuchi, K., Pei, W., Casadio, A., Ghirardi, M., Chain, D.G., Martin, K.C., Kandel, E.R. and Schwartz, J.H. (1997) Ubiquitin C-terminal hydrolase is an immediate-early gene essential for long-term facilitation in *Aplysia*. *Cell*, **89**, 115–126.
- Hershko, A. and Rose, I.A. (1987) Ubiquitin-aldehyde: a general inhibitor of ubiquitin-recycling processes. *Proc. Natl Acad. Sci. USA*, **84**, 1829–1833.
- Hutchinson, E.G. and Thornton, J.M. (1996) PROMOTIF – a program to identify and analyze structural motifs in proteins. *Protein Sci.*, **5**, 212–220.
- Jensen, D.E. *et al.* (1998) BAP1: a novel ubiquitin hydrolase which binds to the BRCA1 RING finger and enhances BRCA1-mediated cell growth suppression. *Oncogene*, **16**, 1097–1112.
- Johnston, S.C., Larsen, C.N., Cook, W., Wilkinson, K.D. and Hill, C.P. (1997) Crystal structure of a deubiquitinating enzyme (human UCH-L3) at 1.8 Å resolution. *EMBO J.*, **16**, 3787–3796.
- Jones, T.A., Zou, J.-Y., Cowan, S.W. and Kjeldgaard, M. (1991) Improved methods for building protein models in electron density maps and location of errors in these models. *Acta Crystallogr.*, **A47**, 110–119.

- Kleywegt, G.J. and Jones, T.A. (1994a) Halloween . . . masks and bones. In Bailey, S., Hubbard, R. and Waller, D. (eds), *From First Map to Final Model*. Daresbury Laboratory, Warrington, UK, pp. 59–66.
- Kleywegt, G.J. and Jones, T.A. (1994b) A super position. *Joint CCP4 and ESF-EACBM Newsletter on Protein Crystallography*, **31**, 9–14.
- Kraulis, P.J. (1991) Molscript: a program to produce both detailed and schematic plots of protein structures. *J. Appl. Crystallogr.*, **24**, 946–950.
- Lam, Y.A., DeMartino, G.N., Pickart, C.M. and Cohen, R.E. (1997a) Specificity of the ubiquitin isopeptidase in the PA700 regulatory complex of 26 S proteasomes. *J. Biol. Chem.*, **272**, 28438–28446.
- Lam, Y.A., Xu, W., DeMartino, G.N. and Cohen, R.E. (1997b) Editing of ubiquitin conjugates by an isopeptidase in the 26S proteasome. *Nature*, **385**, 737–740.
- Larsen, C.N., Price, J.S. and Wilkinson, K.D. (1996) Substrate binding and catalysis by ubiquitin C-terminal hydrolases: identification of two active site residues. *Biochemistry*, **35**, 6735–6744.
- Larsen, C.N., Krantz, B.A. and Wilkinson, K.D. (1998) Substrate specificity of deubiquitinating enzymes: ubiquitin C-terminal hydrolases. *Biochemistry*, **37**, 3358–3368.
- Laskowski, R.A., MacArthur, M.W., Moss, D.S. and Thornton, J.M. (1993) PROCHECK: a program to check the stereochemical quality of protein structures. *J. Appl. Crystallogr.*, **26**, 283–291.
- Leroy, E. et al. (1998) The ubiquitin pathway in Parkinson's disease. *Nature*, **395**, 450–451.
- Miller, H.I., Henzel, W.J., Ridgway, J.B., Kuang, W.-J., Chisholm, V. and Liu, C.-C. (1989) Cloning and expression of a yeast ubiquitin–protein cleaving activity in *Escherichia coli*. *Biotechnology*, **7**, 698–704.
- Otwinowski, Z. (1991) Maximum likelihood refinement of heavy atom parameters. In Wolf, W., Evans, P.R. and Leslie, A.G.W. (eds), *Isomorphous Replacement and Anomalous Scattering*. Daresbury Laboratory, Warrington, UK, pp. 80–86.
- Otwinowski, Z. (1993) Oscillation data reduction program. In Sawyer, L., Isaacs, N. and Bailey, S. (eds), *Data Collection and Processing*. SERC Daresbury Laboratory, Warrington, UK, pp. 56–62.
- Otwinowski, Z. and Minor, W. (1997) Processing of X-ray diffraction data collected in oscillation mode. *Methods Enzymol.*, **176**, 307–326.
- Özkaynak, E., Finley, D., Solomon, M.J. and Varshavsky, A. (1987) The yeast ubiquitin genes: a family of natural gene fusions. *EMBO J.*, **6**, 1429–1439.
- Pickart, C.M. (1997) Targeting of substrates to the 26S proteasome. *FASEB J.*, **11**, 1055–1066.
- Pickart, C.M. and Rose, I.A. (1985) Ubiquitin carboxyl-terminal hydrolase acts on ubiquitin carboxyl-terminal amides. *J. Biol. Chem.*, **260**, 7903–7910.
- Pickart, C.M. and Rose, I.A. (1986) Mechanism of ubiquitin carboxyl-terminal hydrolase: borohydride and hydroxylamine inactivate in the presence of ubiquitin. *J. Biol. Chem.*, **261**, 10210–10217.
- Piotrowski, J., Beal, R., Hoffman, L., Wilkinson, K.D., Cohen, R.E. and Pickart, C.M. (1997) Inhibition of the 26 S proteasome by polyubiquitin chains synthesized to have defined lengths. *J. Biol. Chem.*, **272**, 23712–23721.
- Ramakrishnan, V. and Biou, V. (1997) Treatment of multiwavelength anomalous diffraction as a special case of multiple isomorphous replacement. *Methods Enzymol.*, **276**, 538–557.
- Rose, I.A. and Warms, J.V.M. (1983) An enzyme with ubiquitin carboxy-terminal esterase activity from reticulocytes. *Biochemistry*, **22**, 4234–4237.
- Storer, A.C. and Ménard, R. (1994) Catalytic mechanism in papain family of cysteine peptidases. *Methods Enzymol.*, **244**, 486–500.
- Studier, F.W. and Moffatt, B.A. (1986) Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J. Mol. Biol.*, **189**, 113–130.
- Tobias, J.W. and Varshavsky, A. (1991) Cloning and functional analysis of the ubiquitin-specific protease gene Ubp1 of *Saccharomyces cerevisiae*. *J. Biol. Chem.*, **266**, 12021–12028.
- Vijay-Kumar, S., Bugg, C.E. and Cook, W.J. (1987) Structure of ubiquitin refined at 1.8 Å resolution. *J. Mol. Biol.*, **194**, 531–544.
- Whitby, F.G., Xia, G., Pickart, C.M. and Hill, C.P. (1998) Crystal structure of the human ubiquitin-like protein NEDD8 and interactions with ubiquitin pathway enzymes. *J. Biol. Chem.*, **273**, 34983–34991.
- Wilkinson, K.D. and Hochstrasser, M. (1998) The deubiquitinating enzymes. In Peters, J.M., Harris, J.R. and Finley, D. (eds), *Ubiquitin and the Biology of the Cell*. Plenum Press, New York, NY, pp. 99–125.
- Wilkinson, K.D., Lee, K., Deshpande, S., Duerksen-Hughes, P., Boss, J.M. and Pohl, J. (1989) The neuron-specific protein PGP9.5 is a ubiquitin carboxyl-terminal hydrolase. *Science*, **246**, 670–673.
- Wilkinson, K.D., Deshpande, S. and Larsen, C.N. (1992) Comparisons of neuronal (PGP 9.5) and non-neuronal ubiquitin C-terminal hydrolases. *Biochem. Soc. Trans.*, **20**, 631–637.
- Wintrod, P.L., Makhataadze, G.I. and Privalov, P.L. (1994) Thermodynamics of ubiquitin unfolding. *Proteins*, **18**, 246–253.

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