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The Co-crystal Structure of Ubiquitin Carboxy-terminal hydrolase L1 (UCHL1) with a tripeptide fluoromethyl ketone (Z-VAE(OMe)-FMK)

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Abstract

UCHL1 is a 223 amino acid member of the UCH family of deubiquitinating enzymes (DUBs), found abundantly and exclusively expressed in neurons and the testis in normal tissues. Two naturally occurring variants of UCHL1 are directly involved in Parkinson's disease (PD). Not only has UCHL1 been linked to PD, but it has oncogenic properties, having been found abnormally expressed in lung, pancreatic, and colorectal cancers. Although inhibitors of UCHL1 have been described previously the co-crystal structure of the enzyme bound to any inhibitor has not been reported. Herein, we report the X-ray structure of UCHL1 co-crystallized with a peptide-based fluoromethylketone inhibitor, Z-VAE(OMe)-FMK (VAEFMK), at 2.35Å resolution. The co-crystal structure reveals that the inhibitor binds in the active-site cleft, irreversibly modifying the active-site cysteine; however, the catalytic histidine is still misaligned as seen in the native structure, suggesting that the inhibitor binds to an inactive form of the enzyme. Our structure also reveals that the inhibitor approaches the active-site cleft from the opposite side of the crossover loop as compared to the direction of approach of ubiquitin's C-terminal tail, thereby occupying the P1' (leaving group) site, a binding site perhaps used by the unknown C-terminal extension of ubiquitin in the actual *in vivo* substrate(s) of UCHL1. This structure provides a view of molecular contacts at the active-site cleft between the inhibitor and the enzyme as well as furnishing structural information needed to facilitate further design of inhibitors targeted to UCHL1 with high selectivity and potency.

UCHL1, a protein normally expressed exclusively in the brain and testis, is a member of the ubiquitin carboxy-terminal hydrolase (UCH) family of enzymes, a subclass of a larger group of enzymes collectively called deubiquitinases (DUBs).^{1, 2} DUBs catalyze the hydrolysis of isopeptide or peptide bonds between ubiquitin and target proteins, or between monomers in

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polymeric chains of ubiquitin.^{3–6} Although the exact *in vivo* substrate(s) for this enzyme is not known, biochemical studies have shown that UCHL1 is active only towards ubiquitin conjugates with small leaving groups at the C-terminus of ubiquitin.^{7, 8} There are five classes of DUBs, four of which are cysteine proteases including the UCH family, the fifth class being zinc metalloproteases.^{4–6, 9} UCHL1, like its other family members, UCHL3, UCHL5, and BAP1, contains a catalytic triad consisting of a cysteine (Cys90), a histidine (His161), and an aspartate (Asp176). The X-ray crystal structure of the enzyme is known and reveals a misaligned catalytic triad with a Cys-His distance of ~8Å, much further than a catalytically competent distance of ~4Å for a cysteine protease.¹⁰ However, upon binding to ubiquitin, as revealed in the co-crystal structure of this enzyme with the ubiquitin-based suicide substrate, ubiquitin vinylmethylester (UbVME), the catalytic triad adopts a productive arrangement as seen in other active cysteine proteases.¹¹

The physiological function of UCHL1 is not known; however, abnormal expression of UCHL1 is observed in many forms of cancers, including colorectal, lung and pancreatic cancers.^{12–14} UCHL1 transgenic mice are prone to malignancy, primarily lymphomas and lung tumors,¹⁵ and the gad (gracile axonal dystrophy) mouse exhibits severe neurologic defects.¹⁶ UCHL1's role in disease makes it a possible target for design of therapeutics in the form of small-molecule inhibitors. Many other cysteine proteases have been so targeted, because elevated and uncontrolled levels of protease activity can result in physiological imbalance, leading to the onset of several diseases.^{17, 18} One of the most studied cysteine protease targets for inhibition is the caspase family of cysteine proteases, the members of which are covalently inactivated by peptide halomethyl ketones such as Z-VAD-FMK (benzyloxycarbonyl-Val-Ala-Asp fluoromethylketone).¹⁹ FMKs (fluoromethylketones) are irreversible inhibitors of cysteine proteases by virtue of their ability to alkylate the active-site thiol, leading to the displacement of the halide group by the catalytic cysteine to form a thioether bond between the cysteine and the inhibitor.^{18, 20}

Here we report the X-ray co-crystal structure of UCHL1 with a tripeptide fluoromethylketone, Z-VAE(OMe)-FMK (benzyloxycarbonyl-Val-Ala-Glu(γ -methoxy) fluoromethylketone),²¹ at 2.35Å resolution. The co-crystal structure reveals that the inhibitor binds in the active-site cleft, irreversibly modifying the active-site cysteine. However, the catalytic histidine is still misaligned as seen in the native structure,¹⁰ suggesting that the inhibitor binds to an inactive form of the enzyme. Our structure also reveals that the inhibitor approaches the active-site cleft from the opposite side of the crossover loop as compared to the direction of approach of ubiquitin's C-terminal tail thereby occupying the P1' site, a binding site perhaps used by the unknown C-terminal extension of ubiquitin in the actual *in vivo* substrate(s) of UCHL1. Our structure provides both the first view of molecular contacts at the active-site cleft between an inhibitor and this enzyme as well as the structural information needed to facilitate further design of inhibitors toward UCHL1 with high selectivity and potency.

Z-VAE(OMe)-FMK (hereafter referred to as simply VAEFMK) was synthesized as described previously.²¹ We identified VAEFMK as an inhibitor of UCHL1 during a screen of inhibitors for the viral DUB, UL36 (of herpes simplex virus type 1). During that screen, which included several CMK (chloromethyl ketone) and FMK based putative cysteine protease inhibitors, it was observed that some of these molecules inhibited UL36 both in lysates and in cell culture (unpublished data). The assay employed here relied on the ability of the DUB to react with HA (hemagglutinin)-tagged ubiquitin vinylmethyl ester (HA-UbVME). Curiously, we found one particular compound, VAEFMK, effectively inhibited UCHL1's reaction with HA-UbVME at the concentration of 100μM; however, there was no effect on UCHL3 and UCHL5 at that concentration. On the other hand, a related compound, Z-VAD-FMK, was unable to inhibit UCHL1 activity even at 440μM concentration

(Supplementary Figure 2). VAEFMK was co-crystallized with wild-type UCHL1 (upon covalent modification of the enzyme's thiol, the fluoride group is lost (see mechanism below), therefore, the fluoride group is not seen in the structure and the actual adduct we crystallized should be referred to as UCHL1-VAE thioether). The co-crystal structure was solved by molecular replacement (MR) using the structure of UCHL1 (Protein Data Bank (PDB) code: 2ETL) as the search model. After MR, the electron density map ($2F_O - F_C$) was inspected for presence of positive density that could correspond to bound inhibitor. Inspection of the map indeed revealed positive density corresponding to the inhibitor within the active-site cleft. Using ProDrug Server,²² we generated a model of the inhibitor and placed the molecule within the positive density and carried out rounds of restrained refinement combined with rounds of model building, which resulted in a final free R (R_{free}) and a crystallographic R (R_{cryst}) of 25.0 and 19.7 %, respectively (Table 1). The final refined model contains the complete 223 amino acids of the wild-type UCHL1 with a single molecule of bound inhibitor. The pentapeptide fragment carried over from the GST (glutathione S-transferase) expression tag after cleavage by PreScission Protease (GE Biosciences, USA) was disordered and therefore not modeled. Similarly to the native UCHL1 crystal, the model contains a dimer in the asymmetric unit, with each monomer in the asymmetric unit containing one copy of the inhibitor.

VAEFMK is a peptide-based inhibitor that resembles the well-known caspase inhibitor Z-VAD-FMK (Figure 1a). The usual mechanism of FMK inhibitors proceeds via a two-step addition followed by collapse/migration/displacement mechanism. Fluorine being a highly electron withdrawing group combined with the inductive effects of the carbonyl carbon oxygen makes the carbonyl carbon more electrophilic than the alpha carbon where the substitution ends up. Covalent modification proceeds by an initial nucleophilic attack on the carbonyl carbon by the reactive thiol from the enzyme, followed by the collapse of the carbonyl, migration of the thiol to the alpha carbon, and subsequent displacement of the fluorine (the overall reaction is shown in Figure 1b). Inspection of the electron density map ($2F_O - F_C$) immediately after the MR search showed electron density adjacent to the Cys90 sulfur atom, with continuous density emanating from Cys90. This allowed us to model a covalent bond between the cysteine and the carbon atom of the FMK group, as drawn in Figure 1. Thus, the co-crystal structure shows that VAEFMK binds to UCHL1 (the compound-bound structure is hereafter referred to as UCHL1-VAE thioether or simply as UCHL1-VAE) via a similar mechanism of action as that known for other FMK inhibitors that inactivate cysteine proteases, including caspases. The inhibitor sits within the solvent-exposed active-site cleft in an extended conformation. The benzyloxycarbonyl group of the inhibitor is exposed to solvent and is relatively disordered as indicated by lack of strong density for the benzylic group, although its position was clearly discernible in our structure (Figure 2). The Glu(OMe) end of the inhibitor is located next to a pocket adjacent to the catalytic cysteine in the active-site cleft.

Most of the interactions that stabilize the inhibitor within the active site of both the subunits of UCHL1 are between the groups that constitute the oxyanion hole of the enzyme with the backbone of VAEFMK (Figure 2 and Supplementary Figure 1). For example, the backbone NH groups of Ser89 and Cys90 hydrogen bond with the backbone carbonyl oxygen atom of the Glu(OMe) residue of the inhibitor. The carbonyl group of the Ala residue of the inhibitor is within hydrogen-bonding distance of the backbone NH groups of Asn88 and the side chain NH_2 group of the oxyanion-stabilizing residue, Gln84. Apart from these hydrogen-bonding interactions, van der Waals interactions also appear to play a role in stabilizing the bound inhibitor. For example, the $\text{C}\beta$ atom of the alanine residue in the inhibitor makes a contact with the side chain of Arg178, which seems to have turned around relative to its position in the native structure to accommodate the inhibitor (Figures 2 and 3). Also, the hydrophobic side chain of Val of VAEFMK is nestled against the side chain of Asn159

(Figure 2). The inhibitor in one of the subunits appears to be better defined within the active site, perhaps due to additional interactions with two protein-bound water molecules in the active site. Backbone NH of Met6 is hydrogen-bonded to a water molecule, which in turn, hydrogen bonds with the carbonyl group on the side chain of Glu(OMe) residue, and the other water molecule is held in place by hydrogen-bonding with the side chain of Asn159 (Figure 2). These water molecules were not visualized in the other subunit.

The overall architecture of inhibitor-bound UCHL1 has remained the same as in the native structure,¹⁰ with root mean square deviation (rmsd) of 0.67 Å² in Cα atoms of the two structures, suggesting a minimal conformational rearrangement upon binding of the inhibitor. The catalytic triad in the inhibitor-bound structure maintains the orientation seen in the native protein; His161 is still 8 Å away from Cys90, even though the inhibitor covalently modifies the catalytic cysteine (Figure 3). Since the misaligned active site represents an inactive form of the enzyme, nucleophilicity of the catalytic cysteine does not require the presence of the catalytic histidine in its vicinity (within hydrogen-bonding distance), somewhat surprising considering the widely-held view that the nucleophilicity of the catalytic cysteine in cysteine proteases is due to its existence as an ion-pair with the catalytic histidine (thiolate-imidazolium ion pair).²³

How does VAEFMK bind to UCHL1? We superimposed the structures of UCHL1-VAE with that of UbVME-bound UCHL1 (UCHL1-UbVME) (PDB code: 3KW5). The crossover loop comprising residues 150 to 160 can be thought of as a structural feature that divides the active-site cleft into two halves, on its right-hand side lies the P1 subsite (the way it is presented in Figure 4) and on the left, the P1' subsite. The C-terminal tail of ubiquitin in the UbVME-bound structure approaches the active-site thiol from the expected P1 side of the active-site cleft, whereas the inhibitor approaches from the other side (Figure 4). This binding mode may occur because of the bulky nature of the amino acids bearing the FMK moiety. The cleft on the P1 site is narrowest next to the catalytic cysteine, a feature that allows UCHL1 to position the scissile peptide bond right after the C-terminal Gly-Gly motif with exquisite precision. This feature, along with the crossover loop, might have prevented VAEFMK from approaching the catalytic thiol from the P1 side.

Abnormal expression of UCHL1, whose *in vivo* function remains to be established, has been associated with various forms of cancer, making UCHL1 an attractive target for therapeutics development. Our data may assist future structure-based efforts to design better inhibitors. The structure of UCHL1-VAE shows that VAEFMK targets the active site of UCHL1, as expected, resulting in covalent modification of the catalytic thiol, thereby irreversibly inactivating the enzyme. Although the inhibitor is tethered to the catalytic cysteine covalently, our structure shows additional non-covalent interactions between the inhibitor and atoms in the active-site cleft of the enzyme, mainly between the oxyanion hole groups with the peptide backbone of VAEFMK (Figure 2).

The co-crystal structure reported here presents a number of interesting observations. It shows intermolecular contacts between the inhibitor and the elements of active site structure of the enzyme. With very little induced fit (except for Arg178), VAEFMK binds to the active site with the Cys-His pair still in misaligned arrangement, as seen in the native structure, suggesting that the catalytic thiol is sufficiently nucleophilic even without forming an ion-pair with the catalytic histidine. Previous studies in papain indicated that the reactivity of the catalytic cysteine is due to formation of an ion-pair with the catalytic histidine, analogous to the activation of the catalytic serine in the eponymous serine proteases by histidine.²⁶ UCHL1 has been thought to be papain-like in mechanism of action, but our observation suggests that this may not necessarily be the case. The cysteine may be reactive enough without participation by the histidine, perhaps due to a lowered pK_a .

However, inspection of the contacts around the catalytic thiol reveals only two nearest neighbors, the backbone NH group of Phe162 and the side-chain NH₂ group of the oxyanion-stabilizing residue Gln84, which are at 4.6 Å and 5.0 Å from the S^γ atom, respectively. It is unclear if these groups, which are located significantly farther than hydrogen-bonding distances, could influence the *pK_a* of the catalytic thiol. Alternatively, the inhibitor may have been able to exploit proximity to form the covalent bond with a relatively unreactive thiol. It is also possible that fluoride being a good leaving group makes the histidine unnecessary for the reaction to proceed. The fact that the inhibitor seems to bind to a misaligned active site of UCHL1 may turn out to be advantageous from the point of view of specificity, because -among UCH members whose crystal structures have been solved- UCHL1 appears to be unique with its misaligned active-site triad. It may be that the very high intracellular concentration of UCHL1 makes it desirable to have the apoenzyme exist in a form that has little or no activity, and which is only activated by binding of specific substrates.

The co-crystal structure identifies a number of potential contacts that can be exploited for future inhibitor design. The methoxy group on the inhibitor is pointing toward a crevice in the active site that is lined by mostly hydrophobic side chains (Figure 5). Installation of a hydrophobic side chain in place of the methoxy group might yield tighter-binding analogs. The inhibitor side chain carbonyl group faces a surface that appears to be lined with mostly polar residues. We speculate that installation of a Glu -by removing the methoxy group- will improve the potency of the inhibitor, since Arg153 is poised to make a salt-bridge with the carboxylate group on the inhibitor. Lastly, by superimposing UCHL1-UbVME with UCHL1-VAE structures, we generated a composite view (Figure 4) that shows how UCHL1 might bind to a ubiquitin conjugate with a C-terminal extension. This composite picture should be viewed as a model representing how UCHL1 could bind to its yet-to-be-identified substrate for hydrolysis. The ubiquitin moiety would bind at the P1 site (as visualized in the UCHL1-UbVME structure). The leaving group attached to it will be threaded through the crossover loop, with part of it perhaps sitting in a similar position in the P1' site as seen occupied by VAEFMK. With the catalytic histidine in place, oriented correctly upon ubiquitin binding, hydrolysis of the ester or amide linkage between ubiquitin and the C-terminal extension would be executed, leading to the departure of the leaving group from the VAEFMK side of the crossover loop.

Our structural studies have provided the first inhibitor-bound X-ray structure of the deubiquitinase UCHL1 co-crystallized with Z-VAE(OMe)-FMK. Although the inhibitor is seen covalently tethered to the catalytic cysteine through reaction with the FMK group, this group alone is not sufficient. For example, a close structural analog of this compound, Z-VAD-FMK, a well-known caspase inhibitor, does not inhibit UCHL1's activity even at concentrations as high as 440 μM. This suggests that suitable FMK inhibitors can be developed with relatively high selectivity toward UCHL1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

Z-VAD-FMK	benzyloxycarbonyl-Val-Ala-Asp fluoromethylketone
Z-VAE(OMe)-FMK	benzyloxycarbonyl-Val-Ala-Glu(γ -methoxy) fluoromethylketone
BAP1	BRCA associated protein-1
CMK	chloromethyl ketones
DUBs	deubiquitinases
FMKs	fluoromethylketones
gad	gracile axonal dystrophy
MR	molecular replacement
UCHL1	ubiquitin carboxy-terminal hydrolase L1
UCHL3	ubiquitin carboxy-terminal hydrolase L3
UCHL5	ubiquitin carboxy-terminal hydrolase L5
UbVME	ubiquitin vinylmethyl ester

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Figure 1. VAEFMK

(a) Structure of Z-VAE(OMe)-FMK (VAEFMK). (b) UCHL1 is irreversibly modified by VAEFMK.

Figure 2. Interactions stabilizing VAEFMK within UCHL1's active site

Stereo view of the active-site residues involved in stabilizing VAEFMK within one subunit of UCHL1's active site. UCHL1 is shown in green sticks and VAEFMK in white sticks encapsulated by magenta mesh electron density map ($2F_O-F_C$) contoured at 0.8σ . The black line represents van der Waals interactions, while blue dotted lines are hydrogen-bonding interactions.

Figure 3. Superposition of wild-type UCHL1, UCHL1-UbVME, and UCHL1-VAE
Active site superposition of UCHL1, UCHL1-UbVME, and UCHL1-VAE showing differences in the spatial arrangement of active-site residues due to UbVME and VAEFMK binding compared to the wild type. Wild-type UCHL1 is shown in green sticks, UCHL1-UbVME in cyan sticks, and UCHL1-VAE in white sticks. Both UbVME and VAEFMK have been removed for clarity.

Figure 4. VAEFMK and ubiquitin binding sites within UCHL1

Superposition of UCHL1-UbVME and UCHL1-VAE reveals a novel binding site for VAEFMK, which approaches the active site from the opposite side of the crossover loop as compared to the C-terminal tail of ubiquitin. Ubiquitin is shown in magenta ribbon, VAEFMK in white sticks, and the crossover loop as blue ribbon.

Figure 5. Polar and non-polar pockets within UCHL1's active site

Polar (right) and non-polar (left) pockets within UCHL1's active-site cleft reveal potential interactions that could be exploited for further structural modification of VAEFMK for optimal binding to UCHL1. Residues 154-159 of the crossover loop have been deleted for clarity.

Table 1

Crystallographic Table of Statistics

	UCHL1 FMK
Residues	1–223
Data Collection	
Space Group	<i>P</i> 4 ₂ 2
Cell dimensions	
a, b, c (Å)	110.0, 110.0, 78.7
α, β, γ (°)	90, 90, 90
Resolution (Å)	50.0 – 2.35 (2.43 – 2.35)
*R _{sym}	5.1 (49.9)
I/σI	48.5 (2.1)
Unique reflections	19259
Completeness (%)	98.1 (89.6)
Redundancy	12.9 (7.8)
Refinement	
Resolution	34.9 – 2.35 (2.43– 2.35)
I _{Rcrys} /R _{free}	19.7/25.0
Number of atoms	3589
DRG Molecules	2
rmsd	
Bond lengths (Å)	0.005
Bond angles (°)	1.0
Ramachandran Plot	
Most Favored (%)	91.4
Additionally Allowed Regions (%)	7.3
Generously Allowed Regions (%)	0.3
Disallowed (%)	1.0
Bfactors (Å²)	
Protein	76.7
Solvent	59.4
DRG	93.6

* $R_{\text{sym}} = \sum_j |I(h)_j - \langle I(h) \rangle| / \sum_j I(h)_j$, where $I(h)_j$ is the scaled observed intensity of the j th observation of reflection h , and $\langle I(h) \rangle$ is the mean value of corresponding symmetry-related reflections.

$I_{\text{Rcrys}} = \sum \|F_{\text{obs}}| - |F_{\text{calc}}\| / \sum |F_{\text{obs}}|$ and $R_{\text{free}} = \sum \|F_{\text{obs}}| - |F_{\text{calc}}\| / \sum |F_{\text{obs}}|$, where R_{free} and R_{crys} are calculated using a randomly selected test set of 5% of the data and all reflections excluding the 5% test data, respectively. Numbers in parentheses are for the high-resolution shell.