Substrate Profiling of Deubiquitin Hydrolases with a Positional Scanning Library and Mass Spectrometry

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ABSTRACT: Deconjugation of ubiquitin from cellular proteins is catalyzed by the deubiquitin hydrolase (DUB) family of enzymes and is an important component of the ubiquitin regulatory system affecting cellular function beyond simple maintenance of monomeric pools of ubiquitin. Specific deconjugation of ubiquitinated substrates has been described, but substrate recognition is poorly understood. To determine whether specificity may be conferred by recognition of a primary cognate sequence, the substrate preferences of two DUBs, UCH-L3 and isopeptidase T (IsoT), were profiled using a positional scanning branched peptide library. The sequence of the library was based on K48-branched diubiquitin, RLXXXXK-(GGRLRLVL)QLEDGR, where X denotes a diversified position in the library (P1″−P4″ numbered from K48). Hydrolysis of the branched peptide was indicative of DUB activity and was detected and quantified by mass spectrometry. IsoT was active toward the library but demonstrated little preference for the diversified positions. In contrast, UCH-L3 exhibited minor amino acid preferences at P2″ and P4″ and a 10-fold preference for the basic residues Arg and Lys at P3″. Kinetic analysis of substrates with optimized and suboptimized sequences (as defined by the library profile) confirmed the preference at P3″. Substrate inhibition of UCH-L3 but not IsoT was noted for the optimized sequence at concentrations greater than 5 μM and with an IC_{50} of 12.2 μM; the inhibition was determined to be competition with Ub-AMC (ubiquitin C-terminal 7-amido-4-methylcoumarin).

Covalent modification of cellular proteins with ubiquitin, a highly conserved 76 amino acid protein, is an important regulatory mechanism that signals not only removal of damaged proteins from the cell but affects protein expression, gene silencing, cellular trafficking, and receptor internalization and downregulation (*1*; reviewed in refs *2*−*5*). Ubiquitin is conjugated to the ϵ-amine of the cognate lysine by an enzyme cascade involving ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2), and ubiquitin ligases (E3), and polyubiquitin chains are assembled by consecutive conjugation of the preceding ubiquitin moiety (*3*). Substrate specificity is conferred by a primary sequence recognized by the E3 ligase, and multiple enzymes within the cascade (E1, E2, and E3) enable several points of regulation (*3*). The fate of ubiquitinated proteins is dependent on the extent of ubiquitination (poly- versus monoubiquitination) and type of polyubiquitin chain formed (assembled from K48, K63, and K11) (*6*, *7*).

However, ubiquitin conjugation is not irreversible, and proteins marked for destruction may be rescued; deubiquitinating enzymes (DUB) catalyze the removal of ubiquitin from protein and peptide substrates. Deconjugation of cellular proteins from ubiquitin is an important regulatory mechanism, which antagonizes ubiquitin conjugation and is involved in many cellular processes including cell cycle progression (*8*, *9*), tissue development and differentiation (*10*, *11*), memory and learning (*12*), oncogenesis (*13*, *14*), viral infection (*15*), and neurodegenerative disorders (*16*−*19*), among others (see reviews in refs *20* and *21*). In addition, ubiquitin deconjugation by DUBs associated with the proteasome facilitates degradation of ubiquitinated proteins, and DUBs are responsible for maintaining pools of monomeric ubiquitin, disassembly of polymeric ubiquitin, and cleaving ubiquitin C-terminal fusions and adducts.

DUBs have been grouped into two classes on the basis of sequence similarity: ubiquitin C-terminal hydrolases (UCH) and ubiquitin-specific proteases (UBP). UCH enzymes are generally smaller in size (approximately 200 amino acids), are closely related in sequence, and efficiently cleave small C-terminal extensions and adducts from ubiquitin (*22*−*24*). UCHs are developmentally regulated and display tissue-specific expression (*21*, *25*). In contrast, UBPs range in size from 50 to 300 kDa and are more diverse in structure than UCHs. UBPs are purported to catalyze deconjugation of ubiquitin from larger protein substrates, and some members efficiently disassemble polyubiquitin chains (*21*, *26*). Nearly 20 DUBs have been identified in yeast, and the human genome is purported to contain more than 90 (*3*, *27*). The sheer number of DUBs contained in the genome suggests a potential for selective deconjugation of ubiquitin. Indeed, specific deubiquitination of p53, a tumor suppressor protein whose expression is regulated by ubiquitination, has been reported, thereby extending its half-life and potentially affecting cell cycle progression (*8*, *9*, *28*). Also, type 2 iodothyronine deiodinase is specifically deubiquitinated by von Hippel−Lindau protein-interacting deubiquitinating enzyme-1 affecting conversion of thyroxine to 3,5,3′-triiodothyronine (*29*). Additionally, inactivation of individual DUBs

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produces distinct phenotypes; the *Drosophila faf* gene is required for normal eye development (*10*), mutations in CYLD predispose individuals for cylindromatosis (*14*), and *cyk-3* mutants in *Caenorhabditis elegans* are defective in osmotic regulation and cytokinesis (*11*). However, the mechanism by which DUBs specifically recognize particular ubiquitinated substrates is not well understood. Substrate recognition may be conferred by binding a primary substrate sequence, facilitated by associating proteins, limited by compartmentalization or transcriptional regulation, or conferred by binding higher order motifs. Reports of sequence recognition have generally focused on the sequence of ubiquitin itself, and structural analyses of UCH group members have revealed ubiquitin-interacting residues that upon binding cause a conformational change, activating the enzyme for catalysis (*27*, *30*−*32*). However, these studies used ubiquitin or ubiquitin aldehyde, and information regarding the cognate substrate was speculative. In fact, the cognate specificities reported to date have been of limited scope (*22*, *24*, *33*). Therefore, a combinatorial chemistry approach was employed to query whether DUBs demonstrate primary sequence specificity for the ubiquitinated substrates. To this end, we synthesized a branched peptide library in order to simulate a variety of ubiquitinated substrates and evaluated the sequence specificity of cleavage for two DUBs, isopeptidase T (IsoT) from the UBP family and UCH-L3, a UCH family member.

EXPERIMENTAL PROCEDURES

Materials. UCH-L3 (rabbit), IsoT (rabbit), and Ub-AMC were purchased from Boston Biochem (Cambridge, MA). Fmoc-amino acids and Rink Amide AM resin were purchased from NovaBiochem (San Diego, CA), phenyl-$^{13}C_6$ isocyanate ($^{13}C_6$-PIC)[1] was purchased from Isotec (Miamisburg, OH), phenyl-$^{12}C_6$ isocyanate ($^{12}C_6$-PIC) was purchased from Sigma (St. Louis, MO), and EZ-Link NHS-LC-LC-biotin was purchased from Pierce (Rockford, IL). Unless otherwise noted, all reagents were used without further purification.

Peptide Synthesis. (A) Positional Scanning Branched Peptide Library. A 21 amino acid positional scanning branched peptide library (PSL) based on truncated ubiquitin dimer was synthesized on Rink Amide AM resin (0.67 mmol/g) (Figure 1). The final sequence of the peptide library was biotin-RLXXXXK(GGRLRLVL-$^{12}C_6$-PIC)QLEDGR, where X denotes a diversified position in the library. The base segment (biotin-RLXXXXK*QLEDGR, C-terminal amide) corresponds to R42 to R54 of ubiquitin (K* denotes K48) while the branched segment ($^{12}C_6$-PIC-LVLRLRGG) was coupled to the ϵ-amine of K48 (ubiquitin numbering) and corresponds to L69 to G76 of ubiquitin. The constant portion of the library was synthesized using FastMoc chemistry on an ABI 431A peptide synthesizer during one continuous run utilizing Dde-Lys(Fmoc)-OH, thus yielding Dde-K(GGRLRLVL-NH_2)QLEDGR-resin. The resin was then transferred to an Argonaut Quest 210 organic synthesizer and swelled with *N*,*N*-dimethylformamide (DMF) (5 mL/vessel) for a minimum of 30 min. The N-terminus of the branched segment was blocked by addition of a 5-fold excess of $^{12}C_6$-PIC in DMF for 2 h. The resin was washed with DMF (3 × 5 mL/vessel) and filtered prior to addition of 5 mL/vessel of 2% hydrazine in DMF for 1 h with agitation in order to remove the Dde protecting group. After being washed (3 × 5 mL/vessel DMF, 2 × 5 mL/vessel CH_2Cl_2) and dried under nitrogen, a small amount of resin was cleaved [95% trifluoroacetic acid (TFA), 2.5% water, 2.5% triisopropylsilane] and analyzed on a Voyager-DE MALDI-TOF mass spectrometer to verify that the peptide was ready for positional scanning construction. Radio-frequency tags and resin were loaded into 80 MicroKans (6.73 μmol of total peptide, 23 mg of resin/MicroKan) (Irori, San Diego, CA) using a resin loader plate (Irori). All MicroKans were sorted, and the diversified positions were assigned such that each MicroKan corresponded to a position in the library and consisted of exactly one fixed position and three randomized positions.

A fixed position consisted of 1 of the 20 natural amino acids, with the exception of cysteine and the inclusion of norleucine, while a randomized position consisted of an equimolar mixture of the 20 amino acids except cysteine and tryptophan. A fixed position was installed by adding Fmoc-amino acid monomer, 1-hydroxybenzotriazole (HOBt), and *N*,*N*′-diisopropylcarbodiimide (DICI) (1:1:1) in 2.5 mL of

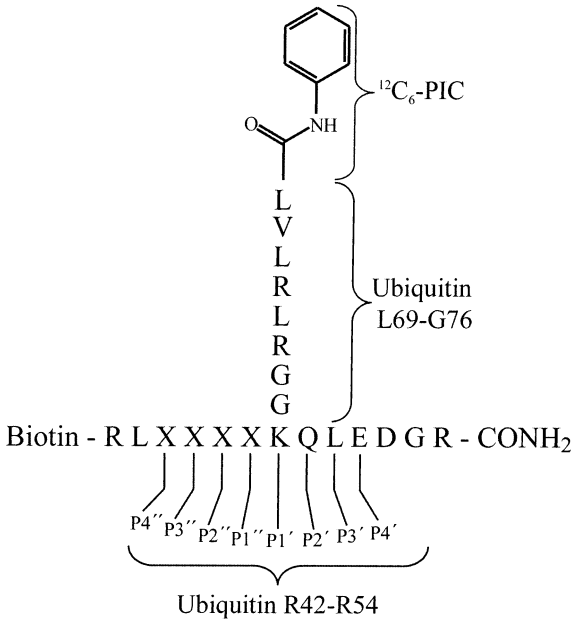

FIGURE 1: 21 amino acid PSL consisting of R42 to R54 of ubiquitin for the base segment with L69 to G76 ligated to the ϵ-amine of K48. The N-termini of the base and branched segments were blocked with biotin and $^{12}C_6$-PIC, respectively. X designates a diversified position in the library. Ubiquitin numbering is used.

[1] Abbreviations: ACN, acetonitrile; AMC, 7-amido-4-methylcoumarin; AUC, area under the curve; Boc, *tert*-butyloxycarbonyl; BSA, bovine serum albumin; Chaps, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; $^{13}C_6$-PIC, phenyl-$^{13}C_6$ isocyanate; CH_2Cl_2, dichloromethane; Dde-Lys(Fmoc)-OH, *N*-α-1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl-*N*-ϵ-Fmoc-L-lysine; DICI, *N*,*N*′-diisopropylcarbodiimide; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Fmoc, 9-fluorenylmethyloxycarbonyl; Hepes, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; HOAc, acetic acid; HOBt, 1-hydroxybenzotriazole; LC-MS, liquid chromatography−mass spectrometry; *m*/*z*, mass to charge ratio; Nle, norleucine; NMP, *N*-methyl-2-pyrrolidone; Pbf, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl; PSL, positional scanning library; SIC, single ion chromatogram; *t*-Bu, *tert*-butyl; TFA, trifluoroacetic acid; Trt, trityl; Ub-AMC, ubiquitin C-terminal 7-amido-4-methylcoumarin.

N-methyl-2-pyrrolidone (NMP) to an individual MicroKan in a 2 dram vial for a final amino acid concentration of 0.25 M. The solution was agitated overnight and then rinsed with NMP (3 × 2.5 mL) with agitation. The Fmoc protecting group was removed with 20% piperidine in NMP (2 × 2.5 mL, 1 h) prior to rinsing with NMP (4 × 2.5 mL) and CH_2Cl_2 (2 × 2.5 mL). A randomized position was installed by adding an isokinetic mixture of amino acids, HOBt, and DICI (1:1:1) in 100 mL of NMP to all MicroKans having a randomized position in a 500 mL round-bottom flask for a final amino acid concentration of 0.25 M (*34*). The isokinetic mixture consisted of the following mole percent of Fmoc-amino acids (*34*): Fmoc-Ala-OH, 3.58; Fmoc-Arg(Pbf)-OH, 6.87; Fmoc-Asn(Trt)-OH, 5.64; Fmoc-Asp(*O*-*t*-Bu)-OH, 3.7; Fmoc-Gln(Trt)-OH, 5.61; Fmoc-Glu(*O*-*t*-Bu)-OH, 3.84; Fmoc-Gly-OH, 3.04; Fmoc-His(Trt)-OH, 3.76; Fmoc-Ile-OH, 18.3; Fmoc-Leu-OH, 5.23; Fmoc-Lys(Boc)-OH, 6.56; Fmoc-Met-OH, 2.42; Fmoc-Phe-OH, 2.66; Fmoc-Pro-OH, 4.56; Fmoc-Ser(*O*-*t*-Bu)-OH, 2.93; Fmoc-Thr(*O*-*t*-Bu)-OH, 5.04; Fmoc-Tyr(*O*-*t*-Bu)-OH, 4.36; Fmoc-Val-OH, 11.9. The solution was stirred overnight and then rinsed with NMP (3 × 100 mL) with stirring. The Fmoc protecting group was removed with 20% piperidine in NMP (2 × 100 mL, 1 h) prior to rinsing with NMP (4 × 100 mL) and CH_2Cl_2 (2 × 100 mL).

After installation of the four diversified positions, all MicroKans were coupled with leucine followed by arginine using Fmoc chemistry, and the N-terminus was blocked with biotin (300 mg of NHS-LC-LC-biotin in 50 mL of DMF) and stirred overnight. The MicroKans were rinsed with NMP (4 × 100 mL) and CH_2Cl_2 (2 × 100 mL) and dried in vacuo.

The peptide library was sorted into a 96-well cleavage station (Irori) and cleaved from the resin with a TFA cleavage solution (1.8 mL/well, 95% TFA, 2.5% water, 2.5% triisopropylsilane) for 1.5 h. Cleavage solution was transferred to a 96-well plate, and peptides were concentrated in a GeneVac evaporator. The peptide library was resuspended in dimethyl sulfoxide (DMSO) to an approximate concentration of 500 μM total peptide.

(B) Synthesis of Single Branched Substrates. Single substrates were synthesized for the kinetic assay on the basis of the results of the PSL screening, with sequence Ac-RLGRVIK(GGRLRLVL-$^{12}C_6$-PIC)QLEDGR-NH_2, Ac-RLGDVIK(GGRLRLVL-$^{12}C_6$-PIC)QLEDGR-NH_2, and Ac-RLQDEYK(GGRLRLVL-$^{12}C_6$-PIC)QLEDGR-NH_2, and were designated as peptide-1, peptide-2, and peptide-3, respectively. The peptides were synthesized using FastMoc chemistry on an ABI 431A peptide synthesizer and the excess resin from the synthesis of the branched peptide library described previously. Peptides were acetylated at the N-terminus by adding acetic acid (HOAc) (0.3 M), HOBt (0.3 M), and DICI (0.3 M) to each peptide and agitating for 20 min. After being rinsed with NMP (3 × 5 mL) and CH_2Cl_2 (2 × 5 mL) and dried under nitrogen, the peptides were cleaved from the resin with a TFA cocktail as previously described. Peptides were precipitated in diethyl ether, then dissolved in acetonitrile (ACN):water (1:1), and lyophilized. Crude product was purified by reversed-phase HPLC (Phenomenex Luna 5 μm C18 column, 50 × 21.2 mm) and lyophilized, and the mass was verified on a Voyager-DE MALDI-TOF mass spectrometer.

(C) Synthesis of the $^{13}C_6$-PIC-LVLRLRGG-OH Standard Peptide. NH_2-LVLRLRGG-OH was synthesized from Fmoc-Gly-Wang resin using FastMoc chemistry on an ABI 431A peptide synthesizer, then transferred to an Argonaut Quest 210 organic synthesizer, and coupled to $^{13}C_6$-PIC as described previously. The peptide was HPLC purified and mass verified as described previously.

Hydrolase Assay with Positional Scanning Library. Purified enzymes (100 nM for UCH-L3 and 50 nM for IsoT) were incubated at 37 °C with 50 μM total peptide substrate (~8.5 nM/peptide) in 25 mM Hepes pH 7.5, 0.25 mM EDTA, 10 mM DTT, 0.1 mg/mL BSA, 10% DMSO, and 500 nM $^{13}C_6$-PIC-LVLRLRGG-OH peptide. After 24 h, the assay solution was quenched with 10 μL of 6% HOAc, filtered through a 0.22 μm filter (200*g* for 5 min; Multiscreen-GV, Millipore), and analyzed by LC-MS.

LC-MS Analysis of the Library. LC-MS analysis of samples was performed using an LCQ Deca XP Plus mass spectrometer modified with a home-built nanospray source configured for online desalting as described elsewhere (*35*). Samples were loaded onto a 100 μm i.d. precolumn packed with 2 cm of Monitor, 5 μm, C18 (Column Engineering, Ontario, Canada), and desalted for 5 min at 5 μL/min with 0.1 M HOAc. After desalting, the precolumn was placed inline with a 75 μm i.d. pulled tip (5 μm opening) packed with 10 cm of the same packing material, and the ACN concentration was increased from 0% to 70% over 5 min. The flow from the HPLC pump was passively split prior to the precolumn to achieve 250 nL/min. Prior to returning to starting conditions, the column was washed with 95% ACN, and the total cycle time was 25 min per sample.

Enzymatic cleavage of the library was assessed by plotting the single ion chromatogram (SIC) for the doubly charged ion of the expected product peptide ($^{12}C_6$-PIC-LVLRLRGG-OH, 502.3 ± 0.5). The SIC for the $^{13}C_6$-PIC peptide internal standard was also plotted (505.3 ± 0.5), and the areas under the curve (AUC) for both peptides were recorded. The identity of the product peptide was initially verified by tandem MS; however, to achieve good quantitation, only full scan spectra were recorded for the library analysis, and the peptide identity was confirmed during the library analyses by a combination of retention time and *m*/*z* (mass to charge ratio) information.

Single Substrate Kinetics with Branched Peptides. Kinetic analysis of UCH-L3 and IsoT with the three branched substrates, peptide-1, peptide-2, and peptide-3, was performed with substrate concentrations of 0.75, 2.5, 5, 10, 20, 30, 40, and 50 μM and enzyme concentrations of 100 and 50 nM, respectively. The assay buffer was 25 mM Hepes, pH 7.5, 0.25 mM EDTA, 0.05% Chaps, 10 mM DTT, and 10% DMSO and also contained $^{13}C_6$-PIC-LVLRLRGG-OH, the internal standard. The internal standard concentration in the buffer was varied on the basis of the substrate concentration and was 10 nM for 750 nM substrate, 50 nM for 2.5, 5, and 10 μM substrate, and 100 nM for 20, 30, 40, and 50 μM substrate. Samples were analyzed by LC-MS at 0 and 12 h after addition of substrate as described previously. The amount of cleaved product was calculated relative to the concentration of the peptide standard.

IC_{50} Assay for UCH-L3 and Branched Peptides. The inhibition of UCH-L3 in the presence of the three branched peptides, peptide-1, peptide-2, and peptide-3, was assayed by detecting the increase in fluorescence upon cleavage of Ub-AMC (*36*). Enzyme (25 pM final concentration) was

diluted in assay buffer (25 mM Hepes, pH 7.5, 0.25mM EDTA, 0.05% Chaps, 10% DMSO, and 10 mM DTT), mixed with branched peptide (0, 0.5, 2.5, 5, 10, 20, 35, and 50 μM), and incubated at room temperature for 10 min prior to addition of Ub-AMC (750 nM). The assay was performed at 37 °C, and fluorescence was detected on a Gemini EM microplate spectrofluorometer with excitation and emission wavelengths of 380 and 460 nm, respectively.

Competition Assay for UCH-L3. A competitive assay between peptide-1 (0.1, 0.25, 0.5, 1.0, 2.5, and 5 μM) and Ub-AMC (0, 2.5, 6, 12, 20, and 50 μM) for binding to UCH-L3 was performed with a final enzyme concentration of 25 pM. Fluorescent detection and assay conditions were similar to the IC_{50} assay described previously.

RESULTS

Library Design and Detection of Activity. It is well established that DUBs recognize ubiquitin, particularly the C-terminus, thus conferring specificity for ubiquitinated proteins and peptides (*27*, *30*−*32*). Our goal was to examine whether DUBs also recognize the sequence of the proteins and peptides to which ubiquitin is bound. To this end, a branched peptide library was synthesized in order to simulate a variety of ubiquitinated substrates, and the sequence specificity for DUB-mediated hydrolysis of the substrate library was measured. The library consisted of two segments joined by an isopeptide bond: The first, with sequence biotin-RLXXXXK*QLEDGR and designated the "base" segment, represents the sequence of ubiquitin from R42 to R54 (X denotes the diversified positions while K* represents K48 of ubiquitin). The second segment, with sequence $^{12}C_6$-PIC-LVLRLRGG and designated the "branched" segment, mimics the C-terminus of ubiquitin, with the C-terminal glycine ligated to the ϵ-amine of K48 of the base segment via an amide bond yielding the branched library (Figure 1). The length of the branch was chosen from sequences up to 10 amino acids long and maximized the rate of cleavage of a small C-terminal extension (data not shown). The library design was of a positional scanning format such that one diversified position was fixed per well of the library while the other diversified positions contained an equimolar mixture of 18 of the 20 natural amino acids (cysteine and tryptophan were excluded). The four positions adjacent and N-terminal to K48 on the base segment (P1″ to P4″; Figure 1) were diversified with the 20 natural amino acids, excluding cysteine but including norleucine, yielding an 80-well library with 5832 peptides per well. Therefore, each well of the library consisted of one fixed and three randomized positions, and the amount of activity per well was indicative of the enzyme specificity for the amino acid at the fixed position.

Deubiquitinating activity results in hydrolysis of the amide bond at the ϵ-amine of K48, yielding two product peptides, the base peptide containing the diversified positions and the branched peptide having the sequence $^{12}C_6$-PIC-LVLRL-RGG-OH. Regardless of the initial library sequence, the later peptide remains constant, and deubiquitinating activity can be determined by monitoring the amount of branched peptide produced over time. The library was analyzed by LC-MS, and the branched peptide was detected by plotting the SIC for its doubly charged ion. Because the branched peptide was modified at the N-terminus with $^{12}C_6$-PIC, the amount of cleaved product could be quantified relative to a stable isotope-labeled internal peptide standard, $^{13}C_6$-PIC-LVLR-LRGG-OH. Specificity profiles for each of the diversified positions were plotted for each fixed amino acid as the ratio of the AUC for the branched and the $^{13}C_6$ standard peptides. No chromatographic resolution of the branched and standard peptides was observed. Biotinylation of the library was originally performed to enable removal of uncleaved peptides and cleaved base peptides in order to simplify analysis, but sample cleanup was found to be unnecessary.

Profiling of UCH-L3 and Isopeptidase T. The branched library was used to profile the specificity of two DUBs, UCH-L3 and IsoT. To optimize the assay conditions for the profiling experiments, enzyme activity was monitored for up to 24 h with concentrations of DMSO from 0% to 20%. The enzymatic activity of both enzymes as monitored by V_{max} determination for Ub-AMC hydrolysis was stable under all conditions (data not shown). A concentration of 10% DMSO and a time point of 24 h were chosen for the profiling experiments. Cleavage of the library by IsoT was detected in all wells of the library, and the average standard deviation for two experiments was 9% (Figure 2). The profile of IsoT showed no apparent amino acid preference at any of the diversified positions tested in this library, thus indicating that IsoT cleaves branched substrates without specificity to the amino acids N-terminal to the branched site of the base segment.

Cleavage of the library by UCH-L3 was also detected in all wells of the library, and the average standard deviation for two experiments was 27% (Figure 3). UCH-L3 demonstrated no amino acid preference at the P1″ position, a 3−4-fold preference at the P2″ position for Arg, and a 2−3-fold preference for Arg, Lys, Gly, and Leu at the P4″ position. A 10-fold preference for Arg and Lys was observed at the P3″ position over other amino acids, thus indicating that UCH-L3 demonstrates significant substrate specificity toward amino acids N-terminal to the branched site in the base segment.

Kinetics with Single Branched Substrates. To confirm the preference of UCH-L3 for Arg and Lys at the P3″ position, the rates of UCH-L3-mediated hydrolysis were measured for three branched peptide substrates that differed only by the amino acids at the diversified positions. The first substrate (peptide-1) contained the preferred amino acid Arg at P3″. The sequence of peptide-2 was identical to peptide-1 except for the substitution at P3″ of the least active amino acid, Asp. Peptide-3 was composed of the least active amino acid at each of the four diversified positions including Asp at P3″. Measurements were taken at 0 and 12 h after addition of enzyme to establish the rates of hydrolysis. UCH-L3 cleaved all three substrates, but the rate of cleavage was highest for peptide-1 as predicted by the library (Figure 4 inset). The rate of cleavage for peptide-1 decreased in a concentration-dependent manner above 5 μM, which will later be discussed in further detail. k_{cat}/K_m for peptide-1, peptide-2, and peptide-3 were 8.127 (calculated using the linear region only), 1.781, and 0.232 M^{-1} s^{-1}. The single amino acid substitution at the P3″ position resulted in a 5-fold change in k_{cat}/K_m, thus confirming the preference for Arg at this position. Substitution of all four diversified positions with the least active amino acids, peptide-3, resulted in a further decrease in activity, also confirming the results of

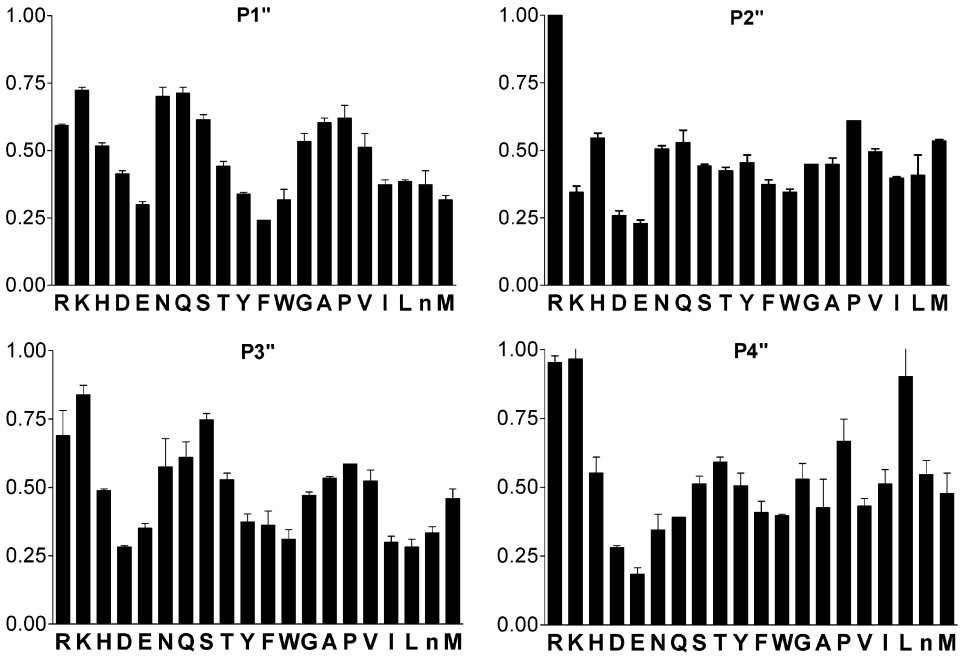

FIGURE 2: Activity profile of IsoT with PSL plotted as the ratio of the AUC for cleaved and internal standard peptides: 50 μM total peptide per well, 50 nM IsoT, and 500 nM $^{13}C_6$-peptide standard; average of two replicates.

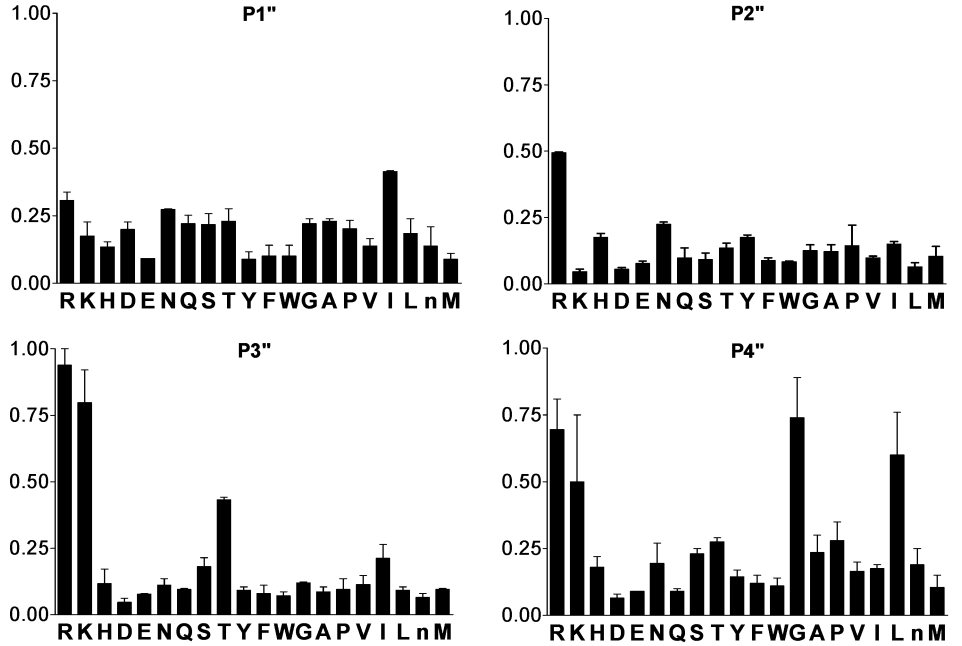

FIGURE 3: Activity profile of UCH-L3 with PSL plotted as the ratio of the AUC for cleaved and internal standard peptides: 50 μM peptide library per well, 100 nM UCH-L3, and 500 nM $^{13}C_6$-peptide standard; average of two replicates.

the library profile. In contrast to UCH-L3, no inhibition of IsoT was noted at higher concentrations of peptide-1, and k_{cat}/K_m was calculated to be 2.28 M^{-1} s^{-1} (Figure 4). k_{cat}/K_m for IsoT and for peptide-2 and peptide-3 were 0.561 and 0.1155 M^{-1} s^{-1}, respectively. Though little sequence preference was noted for IsoT, the variation in k_{cat}/K_m for the branched peptides does reflect the substrate profile reported in the library.

Because hydrolysis at sites other than the isopeptide bond may result in an underestimation of the hydrolysis kinetics, specifically progressive cleavage of the branched peptide, the raw LC-MS data from the single substrate kinetic experiments with UCH-L3 and IsoT were analyzed for alternate cleavage sites of the branched segment. The only alternative cleavage site detected was PIC-LVLRLR-OH with cleavage between R74 and G75 of the branched segment. The peptide was normalized relative to the $^{13}C_6$ peptide standard ($^{13}C_6$-PIC-LVLRLRGG), and a significant signal was only detected for UCH-L3-mediated hydrolysis of peptide-1. The trend for the rate of hydrolysis was similar to that reported for the branched peptide in Figure 4, a rapid increase in hydrolysis rate peaking at 5 μM substrate and then diminishing at higher substrate concentrations (data not shown). The complement to the alternatively cleaved peptide, the base segment with GG at the branched lysine, was also detected and normalized to the internal standard peptide. This peptide followed the same general trend as the alternative cleavage product, PIC-LVLRLR-OH.

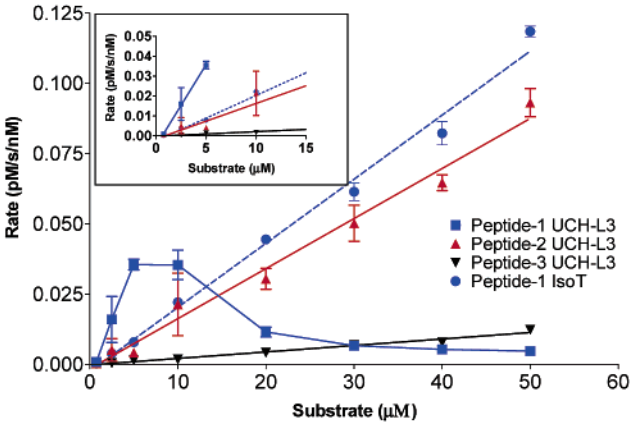

FIGURE 4: Single substrate kinetics of UCH-L3 with single synthetic branched substrates, peptide-1, peptide-2, and peptide-3, and IsoT with peptide-1. Detection by LC-MS with quantitation relative to $^{13}C_6$-PIC-LVLRLRGG-OH. The inset is the same plot constrained to the linear region of peptide-1. Normalized to enzyme concentration. Average of three replicates.

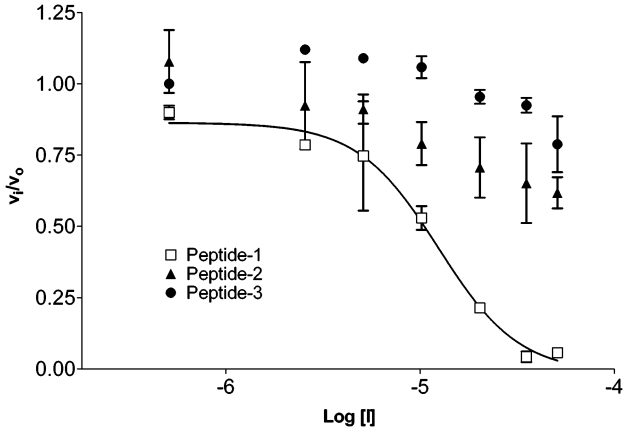

FIGURE 5: IC_{50} determination for UCH-L3 with peptide-1, peptide-2, and peptide-3. 750 nM Ub-AMC, 25 pM UCH-L3, and 0, 0.5, 2.5, 5, 10, 20, 35, and 50 μM branched peptides. Detection of the cleaved AMC fluorophore at 460 nm. Average of two replicates.

Characterization of Inhibition of UCH-L3 by Branched Peptides. UCH-L3 cleavage of Ub-AMC was measured in the presence of peptide-1, peptide-2, and peptide-3 in order to establish IC_{50} curves for the peptides. Peptide-1 prevented cleavage of Ub-AMC in a concentration-dependent manner with complete inhibition at 40 μM and an IC_{50} of 12.2 μM (Figure 5). Peptide-2 and peptide-3 produced only mild concentration-dependent inhibition and reduced Ub-AMC cleavage to 60% and 70%, respectively, of initial values at the highest peptide concentration tested. The inhibition of UCH-L3 by peptide-1 was shown to be competition with Ub-AMC hydrolysis (Figure 6).

DISCUSSION

Deconjugation of ubiquitin is an important component of the ubiquitin regulatory system affecting protein function and fate beyond the simple maintenance of monomeric ubiquitin stores. Multiple lines of evidence have been reported for selective deubiquitination of substrates, but the mechanism of substrate recognition is poorly understood. In this report, we have explored the primary sequence specificity of DUBs for the region adjacent and N-terminal to the branched site. Information regarding cognate specificity would be useful

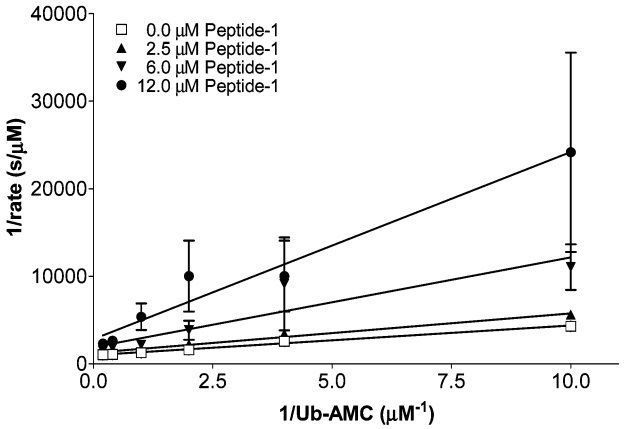

FIGURE 6: Lineweaver−Burk plot of competition assay for UCH-L3 with peptide-1 and Ub-AMC as substrate. 25 pM UCH-L3, 0.1, 0.25, 0.5, 1, 2.5, and 5 μM Ub-AMC, and, 0, 2.5, 6, and 12 μM peptide-1. Detection of the cleaved AMC fluorophore at 460 nm. Average of two replicates.

for several reasons. For example, highly discriminating DUBs may demonstrate a specific consensus sequence that could be used to predict macromolecular substrates and aid in the identification of physiological function. A profile of substrate specificity may also enable the functional characterization of subsite pockets for the design of specific inhibitors. Finally, current classification of DUBs is based on their sequence rather than their function: substrate profiles would enable functional comparisons of DUBs, potentially identifying subclasses or redundancy among DUBs. In this paper, a general method was reported for characterizing the primary sequence specificity of DUBs using a PSL and detection by MS.

Combinatorial peptide libraries are powerful tools that enable the generation of a nearly unlimited diversity of substrate sequences and thus are well suited for characterizing the primary sequence specificity of proteases. However, these libraries must be coupled to an analytical assay capable of both detecting protease activity and identifying the preferred substrates from a large excess of uncleaved peptides. Incorporation of fluorescent-quenched molecules into either a support-bound or solution phase library enables detection of protease activity and simultaneous characterization of preferred sequences for sites both N- and C-terminal to the scissile bond (*37*−*40*). However, the specific substrate and the actual site of cleavage are not immediately discerned by detection of fluorescence and therefore must be determined by subsequent analysis (*38*, *39*). In addition, the efficiency of fluorescent quenching and background fluorescence is related to the distance between the donor and quencher molecules, thus limiting the length of the substrate (*37*, *41*, *42*). Another technique uses a PSL with a latent fluorophore at the site of cleavage (*43*−*45*); the substrate preference is related to the intensity of fluorescence, and the sequence can be determined by the position in the library. However, because of the position of the fluorophore, simultaneous characterization of preferred sequences on both sides of the scissile bond is not possible. Screening of combinatorial libraries for enzyme specificity by mass spectrometry has also been reported (*46*−*48*). Because MS distinguishes library precursors and enzyme reaction products by changes in *m*/*z*, enzymes that catalyze a wide range of reactions can be characterized, including phosphatases, kinases, decar-

boxylases, and deformylases (*46*−*49*). However, as the library increases in size, so too does the number of isobaric components, and library members as well as their products become increasingly indistinguishable; Wang et al. reported that, even with high mass accuracy measurements, only 141 members of a 361-member library possessed unique masses, thus restricting the size of the library that can be analyzed (*47*).

Quantitation of reaction products is also essential for determining specificity since preferred substrates are cleaved at a higher rate than inferior substrates. Quenched fluorescence assays only provide semiquantitative information since the observed signal intensity may be attributed to disparate cleavage sites on the same substrate. A PSL with a latent fluorophore provides quantitative information regarding the level of protease activity toward a particular sequence, and this activity can directly be compared between library sequences. In a mass spectrum, the signal intensity observed is dependent on multiple factors including the sequence of the peptide itself as well as competition with other analytes for charge. Therefore, reliable MS quantitation is typically accomplished by the use of stable isotope-labeled standards, which differ in composition only by the mass of the isotopes. Synthesizing standards for each possible product from a large library can be unwieldy, although several approaches have been described (*47*, *48*).

Characterization of DUB specificity by screening combinatorial libraries poses a unique challenge since sequences on both sides of the scissile bond may be required for enzyme recognition, thus precluding methods that employ a cleavable latent fluorophore. The limitations of other techniques were overcome in the presented method by using a PSL designed to yield a constant hydrolysis product and its subsequent detection by MS.

A PSL allows for independent interrogation of multiple positions of a potential peptide substrate, and PSL have been used extensively to characterize enzyme substrate specificities (*44*, *45*, *50*). The main advantage of a PSL is that the sequence preferences of an enzyme can be characterized with a fraction of the experiments that would be required using individual substrates. However, a main assumption of a PSL approach is that the activity recorded for each position is independent of the amino acids at neighboring positions and no synergy or antagonism exists. Although neighboring amino acids can have nonadditive effects on enzyme activity, installation of a mixture of amino acids at positions adjoining the fixed position works to average the effects among the thousands of peptides per well. Nonadditive effects between nearby amino acids can be measured using a similar PSL approach by fixing two or more amino acids per well of the library and has previously been described (*44*, *45*, *50*). Installation of the randomized positions in the presented experiments was performed using an equikinetic mixture of amino acids as previously described by Ostresh et al. (*34*). Ostresh et al. demonstrated that reaction rates for protected amino acids in competitive couplings were primarily dependent on the free and not the resin-bound amino acid, and the concentrations of individual amino acids in the mixture were adjusted to produce peptides of approximate equimolarity (within 20%) (*34*). Because the randomized positions serve to minimize cooperation between adjoining positions, a small bias in the equimolar ratio of all peptides should not effect the specificity results and was not assayed for.

Cleavage of the branched library described in this paper yielded two peptides, a peptide with the diversified positions (base peptide) and a constant peptide (branched peptide). Because detection was based on the branched peptide that is the cumulative product of all diversified peptides cleaved per well, the assay is potentially more sensitive than detection of individual cleaved products. The limit of detection was estimated to be in the range of femtomoles per microliter based on dilution and detection of the standard (data not shown). In addition, the complexity of the starting mixture does not adversely affect the analysis since the branched peptide has different elution characteristics than the initial library members, thus enabling nearly unlimited diversity within the library, both in length and in number of diversified positions. Quantitation is accomplished by synthesis of a single stable isotope standard enabling absolute quantitation and comparisons of cleavage rates. The substrate profiles of two DUBs from different classes were reported using a PSL. Both enzymes cleaved a wide variety of substrates. However, the two profiles were distinct, and only UCH-L3 demonstrated a significant sequence preference for positions in the base peptide N-terminal to the site of cleavage. The contrasting results using the same starting library indicate that product detected in the assay was the result of enzymatic cleavage and not artifactual hydrolysis of the library. The good reproducibility of the assay is also consistent with this conclusion.

The substrate specificity of IsoT was profiled using a branched peptide library based on the ubiquitin sequence, and activity was observed. Previous reports described a distinct preference of IsoT for branched polyubiquitin chains but an inability to cleave monoubiquitinated proteins, thus suggesting discrimination for the sequence of ubiquitin (*26*, *33*, *51*); however, IsoT demonstrated little sequence preference for the positions N-terminal to the isopeptide bond including the sequence of ubiquitin. Wilkinson et al. noted that modification of the C-terminus of the proximal ubiquitin of di-, tri-, or tetraubiquitin derivatives greatly diminished the enzymes' capacity to hydrolyze the isopeptide bond, indicating that substrate discrimination is based on the status of the C-terminus of the proximal ubiquitin (*33*). Taken together, these data indicate that substrate discrimination by IsoT is not based on a primary sequence but may be due to recognition of higher order motifs. Specificity may be conferred by binding of the C-terminus to an alternate pocket of IsoT, thereby activating the enzyme or directing the docking of the isopeptide bond.

UCH-L3 efficiently cleaves small C-terminal amides of ubiquitin (*22*, *24*), and this proclivity suggests a role in processing proubiquitin with small C-terminal extensions, regeneration of C-terminally modified ubiquitin, or removal of ubiquitin from peptides following proteasomal degradation. UCHs specifically bind ubiquitin, and the side chain interactions have been predicted for UCH-L3 but recognition of C-terminal extensions remains uncertain (*31*, *32*). Previous reports indicated that UCH-L3 specificity was based chiefly on the size of the C-terminal extension rather than sequence or bond type (peptide versus isopeptide) (*22*, *24*, *52*). We profiled UCH-L3 using a small synthetic branched peptide library and observed hydrolysis of all library members. In contrast to previous reports, we noted a strong preference

for basic residues at the P3″ position with relatively little discrimination at other sites. These data are not necessarily counter to previous reports since cleavage was noted at all positions, indicating that UCH-L3 is capable of hydrolyzing a variety of substrates. However, the use of a PSL allowed a more comprehensive characterization of the specificity while previous reports only described a limited set of ubiquitin−peptide fusions. Interestingly, Wada et al. described cleavage of the C-terminus of a ubiquitin-like protein, NEDD8, by UCH-L3, and the linear C-terminal extension contained an arginine residue at the third amino acid from the P1′ position (*52*). UCH-L3 and NEDD8 demonstrate overlapping tissue localization and developmental expression, further suggesting that UCH-L3 may be involved in processing of NEDD8 (*52*), and the profiling data reported here is consistent with this assessment.

The average standard deviation of measurements in the library profile for UCH-L3 (27%) was approximately three times that of IsoT (9%) for two repeat measurements and is related to the method of quantitation of hydrolyzed product from the assays. Hydrolyzed branched peptide was quantified relative to the signal of a stable isotope-labeled standard peptide. As the ratio of the two peptides deviates from 1.0, the error increases due to the error inherent in measuring a small signal in the presence of a larger signal. Because IsoT was more active toward the library and less discriminating toward substrates than UCH-L3, that is, more substrates in the well were productive, resulting in hydrolyzed product, the average ratio of hydrolyzed product to the standard peptide was closer to 1.0 (0.43 for IsoT) than UCH-L3 (0.27), leading to a larger standard deviation for UCH-L3 than IsoT.

In addition to the profiling data with the PSL, individual branched substrates were synthesized in order to verify the preference of UCH-L3 observed in the library. Kinetics of UCH-L3 and the three branched peptides, peptide-1, peptide-2, and peptide-3, demonstrated initial rates of cleavage that were consistent with the order predicted by the PSL, thus confirming the potential of a PSL-based screen. Although the peptides were designed for validating UCH-L3 cleavage preferences, their rates of cleavage by IsoT were also determined. In contrast to UCH-L3, no substrate inhibition of IsoT was noted with peptide-1, but IsoT did cleave the substrates at different rates, which upon a closer inspection was consistent with the library profiling results of IsoT (Figure 2). As the substrate profile illustrates, IsoT cleaves a variety of substrates with little regard for sequence, but 2−3-fold differences in cleavage rate between pairs of amino acids were noted. Arg is cleaved nearly 3-fold faster than Asp at the P3″ position, and this is reflected in k_{cat}/K_m for peptide-1 and peptide-2. At the P2″ position, Glu is cleaved more than 2-fold slower than Val, further abrogating activity with peptide-3, which contains Asp at P3″ and Glu at P2″. The rates of cleavage of the single branched substrates by UCH-L3 and IsoT are consistent with substrate profiles reported with the library even though only minor preference in substrate specificity was noted for IsoT.

Because the kinetic information reported for UCH-L3 and IsoT was based on detection of the branched peptide, and progressive hydrolysis of the branched peptide would result in an underestimation of the hydrolysis kinetics, the raw LC-MS data were analyzed for alternative cleavage sites. A single alternative cleavage site was detected and was present in abundance only in the UCH-L3 kinetic experiment with peptide-1. The rate of hydrolysis for the alternate site diminished at the higher substrate concentrations, indicative of enzyme inhibition, and was similar to hydrolysis of peptide-1 yielding the branched peptide, thus indicating that the cleavage was due to UCH-L3 and not a contaminating proteolytic activity. The base peptide with GG attached at the branched lysine was also detected and followed a similar trend as the hydrolyzed peptides, further indicating that the alternatively cleaved peptide arises from the branched substrate and not progressive cleavage of the branched peptide. Because the amount of hydrolyzed products is small (<1%) relative to the original substrate concentration and the alternatively cleaved peptide arises from the branched substrate, the kinetic values reported for UCH-L3 and IsoT would not be affected. Whether the alternative cleavage site has any physiological significance is uncertain but appears to be dependent on the sequence of the base peptide.

The activity of both UCH-L3 and IsoT was considerably lower in the PSL as well as the single branched substrate assays compared to that reported with Ub-AMC (*36*). We attribute this lowered activity primarily to the use of truncated rather than full-length ubiquitin for the branched peptide. Unligated UCH-L3 assumes a closed and inactive conformation; however, upon binding ubiquitin, ionic interactions induce a conformational change activating the enzyme (*31*, *32*). The truncated form of ubiquitin employed in the library retains some of these required interacting residues, namely, R72 and R74, but does not produce the effect of full-length ubiquitin (*31*). Similar interactions may be required for IsoT, as truncated forms of Ub-AMC exhibit k_{cat}/K_m values 6 orders of magnitude less than full-length Ub-AMC (*36*, *53*). Although the truncated substrates are less active than their full-length counterparts, the observance of activity as well as sequence preferences indicates that the branched peptides are indeed substrates for these enzymes. The energy obtained upon binding full-length ubiquitin may be used for catalysis but is unavailable for cleavage of truncated substrates, thus lowering the activity but not obviating it.

Peptide-1 consisted of the optimized binding sequence for UCH-L3 as determined by the library and was observed to inhibit UCH-L3 at higher concentrations. Several lines of evidence indicate that this inhibition is due to the substrate and its binding at the active site. First, product was detected at lower concentrations of peptide-1 but decreased in a concentration-dependent manner, precluding inhibition by hydrolyzed peptide. Second, IsoT cleaved the substrate, and no inhibition was noted at higher substrate concentrations. Also, inhibition was noted in two different assays, the kinetic assay using LC-MS (Figure 4) and the IC_{50} assay using Ub-AMC as substrate (Figure 5), with similar results thus eliminating assay-dependent factors. Because the mechanism of inhibition was determined to be competitive with Ub-AMC, binding of peptide-1 at the higher substrate concentrations must also be to the active site. Given this, the mechanism by which peptide-1 inhibits product formation is unclear. Substrate inhibition has been described for other enzyme systems and was attributed to nonproductive binding or occlusion of the active site (*54*−*56*). Alternatively, it is likely that the higher, inhibitory concentrations of peptide-1 are above its K_m and the reaction favors the enzyme−substrate (ES) complex. Optimal binding of the enzyme

subsites could lead to stabilization of this complex, which may cause inhibition. Although inhibition was unexpected, optimized substrate sequences have previously been used for the design of selective enzyme inhibitors (*50*, *57*). In either case, the concentration of this substrate in the branched library was well below the measured IC_{50} and did not prevent substrate profiling.

In conclusion, this paper describes the first substrate profiling of members of the deubiquitinating family of enzymes using a novel PSL with detection and quantitation by LC-MS. Knowledge of the substrate specificity of these enzymes may enable functional profiles of the two classes, identification of novel substrates, and design of specific inhibitors. The synthesis of an unexpected substrate inhibitor provides an example of the potential usefulness of substrate profiling information. One shortcoming of the PSL used in this study is the lowered enzyme activity toward the truncated ubiquitin substrates, which may prevent substrate profiling of less active DUBs. This problem may only be overcome by use of a longer branched peptide in order to better simulate ubiquitinated substrates and maximize ubiquitin interactions, and those studies are currently underway. Also, this study reported on the substrate specificity for the residues N-terminal to the branched lysine (i.e., P″ positions), but residues C-terminal to this point (P′ positions) may also be important determiners of specificity. As discussed earlier, the complexity of the library is not a factor in its detection due to the use of mass spectrometry and its fundamental design. Therefore, the contribution of additional positions toward substrate specificity can be determined by this method.

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