Nonhydrolyzable Diubiquitin Analogues Are Inhibitors of Ubiquitin Conjugation and Deconjugation†

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ABSTRACT: A series of nonhydrolyzable ubiquitin dimer analogues has been synthesized and evaluated as inhibitors of ubiquitin-dependent processes. Dimer analogues were synthesized by cross-linking ubiquitin containing a terminal cysteine (G76C) to ubiquitin containing cysteine at position 11 ($^{76-11}Ub_2$), 29 ($^{76-29}Ub_2$), 48 ($^{76-48}Ub_2$), or 63 ($^{76-63}Ub_2$). A head-to-head dimer of cysteine G76C ($^{76-76}Ub_2$) served as a control. These analogues are mimics of the different chain linkages observed in natural polyubiquitin chains. All analogues showed weak inhibition toward the catalytic domain of UCH-L3 and a UBP pseudogene. In the absence of ubiquitin, isopeptidase T was inhibited only by the dimer linked through residue 29. In the presence of 0.5 μM ubiquitin, isopeptidase T was inhibited by several of the dimer analogues, with the $^{76-29}Ub_2$ dimer exhibiting a K_i of 1.8 nM. However, USP14, the human homologue of yeast Ubp6, was not inhibited at the concentrations tested. Some analogues of ubiquitin dimer also acted as selective inhibitors of conjugation and deconjugation of ubiquitin catalyzed by reticulocyte fraction II. $^{76-76}Ub_2$ and $^{76-11}Ub_2$ did not inhibit the conjugation of ubiquitin, while $^{76-29}Ub_2$, $^{76-48}Ub_2$, and $^{76-63}Ub_2$ were potent inhibitors of conjugation. This specificity is consistent with the known ability of cells to form K29-, K48-, and K63-linked polyubiquitin chains. While $^{76-11}Ub_2$, $^{76-29}Ub_2$, and $^{76-63}Ub_2$ inhibited release of ubiquitin from a pool of total conjugates, $^{76-48}Ub_2$ and $^{76-76}Ub_2$ showed no significant inhibition. Isopeptidase T was shown to specifically disassemble two conjugates (assumed to be di- and triubiquitin with masses of 26 and 17 kDa) formed in the reticulocyte lysate system. This activity was inhibited differentially by all dimer analogues. The inhibitor selectivity for deconjugation of the 26 and 17 kDa conjugates was similar to that observed for isopeptidase T. The observations suggest that these two conjugated proteins of the reticulocyte lysate are specific substrates for isopeptidase T in lysates.

Ubiquitin is a small, highly conserved protein found in all eukaryotes. The best understood function of ubiquitin is its role as a targeting protein in a highly complex, intracellular protein degradation system. Ubiquitin is first attached to a target protein's lysine; then additional ubiquitin molecules[1] can be covalently joined to the ubiquitin on the target protein by the E1/E2/E3 pathway (*2*). The C-terminus of the incoming ubiquitin is conjugated using K48[2] on the proximal ubiquitin, i.e., the one closest to the target protein (*3*). As many as 14 ubiquitin monomers may be conjugated to a single lysine on the protein targeted for degradation (*4*). Once formed, this polyubiquitinated protein conjugate is targeted to the 26S multienzyme proteolytic complex that degrades the polyubiquitinated protein. This proteolytic complex does not degrade most nonubiquitinated proteins, and K48 linkages of polyubiquitin are sufficient to target proteins for proteasome-mediated protein degradation (*3, 5*). While the K48−G76 isopeptide linkage of polyUb appears to be the most common, polyubiquitin chains linked through K6, K11, K29, and K63 are also present in the cell (*6*−*9*). These alternative forms are poorly defined in terms of their structure, formation and degradation rates, steady-state level, or function. In vitro, E2 (25 kDa) catalyzes formation of K48-linked polyubiquitin chains with a free C-terminus (*10*). These are commonly referred to as unanchored chains as they are not attached to other proteins. The keratinocyte-specific enzyme E2-EPF autoubiquitinates itself with K11-linked chains, and Rad6 catalyzes an analogous autopolyubiquitination event forming K6-linked chains (*9*). Ellison's group demonstrated the presence of distinct polyubiquitin species in *S. cerevisiae* with isopeptide bonds between K29 and G76 as well as K63 and G76, and the latter may be

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[1] The terms polyubiquitin and polyubiquitin chains refer to oligomers of ubiquitin linked by an isopeptide bond between the C-terminus of the distal ubiquitin and a side chain lysine of the ubiquitin proximal to the target protein. Thus, the proximal ubiquitin in a chain has a free N- and C-termini (unanchored chains) or is the ubiquitin attached to the target protein in ubiquitinated proteins.

[2] Abbreviations: amino acids are specified using the standard one-letter code; ATP, adenosine 5′-triphosphate; DEAE, diethylaminoethyl; DTT, dithiothreitol; DUB, deubiquitinating enzyme; EDTA, ethylenediaminetetraacetic acid; H, nucleotides A, C, or T but not G; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TRIS, tris(hydroxymethyl)aminomethane; Ub, ubiquitin; Ub-AMC, ubiquitin C-terminal 7-amido-4-methylcoumarin; UBP, ubiquitin-specific processing protease; UCH, ubiquitin C-terminal hydrolase.

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involved in a stress response (*6*). Finley and co-workers further suggested that a K63-linked polyubiquitin species is related to DNA repair pathways in *S. cerevisiae* (*7*). These K63-linked chains are formed in a reaction requiring a presumed heterodimer of Ubc13p and an inactive Ubc-like protein, Mms2p (*11*). Recently, it has been shown that rabbit reticulocyte lysate contains enzymatic components that catalyze the assembly of unanchored K29-linked polyubiquitin chains. The partially purified E3 from this system also catalyzes the assembly of unanchored chains linked through K48. The kinetics of conjugation to K48 and K29 were similar, allowing the synthesis of both K48-linked and K29-linked heteropolymers (*12*).

After proteolysis, K48-linked polyubiquitin chains must be released from the proteasome and disassembled by the action of a deubiquitinating enzyme, isopeptidase T (*13, 14*). This enzyme contains multiple ubiquitin binding sites and processes free polyubiquitin chains by a proximal exo mechanism (*14*); that is, trimming each ubiquitin sequentially from the proximal end of the polyubiquitin chain. The degradation of the polyubiquitin signal is a significant concern for the cell, since it can inhibit the proteasome (*13, 15*) and monomeric levels of ubiquitin must continually be replenished in the cell in order to maintain the process of protein turnover. It is likely that other polyubiquitin chains linked through alternative lysines must also be disassembled. Little is known about the enzymes that synthesize or degrade these chains, and thus selective inhibitors would be useful in the dissection of these alternate pathways.

Known inhibitors of ubiquitin conjugation and deconjugation include the following: ubiquitin aldehyde, which is a general inhibitor of ubiquitin recycling and depletes the level of free ubiquitin (*16*); ubiquitin nitrile, which irreversibly modifies various deubiquitinating enzymes (*17*); methylated ubiquitin, which cannot form polyubiquitin chains and is an inhibitor of ubiquitin-dependent protein degradation (*18*); hemin, which interferes with the protease of the system (*19*); dipeptide esters, which inhibit the conjugation of ubiquitin to some proteins by inhibiting one E3 ligase (*20*); ribonuclease, which prevents the arginylation of the amino terminus of proteins containing acidic amino-terminal residues, a step that is necessary for the ubiquitination of these proteins (*21*); a synthetic peptide encompasing the C-terminus of ubiquitin, that could act as an inhibitor of E1 by virtue of the fact that it is efficiently adenylated (*22*); and adenosyl-phospho-ubiquitinol, a nonhydrolyzable ubiquitin adenylate analogue which is used as a specific inhibitor of the activation of ubiquitin (*23*).

In addition, a number of very selective inhibitors of the proteasome have been invaluable in defining the scope of this system (*24*). For instance, a variety of peptide aldehydes (*25*), boronic acids (*26*), and natural products (*27*−*30*) have been introduced and have been shown to be useful. Numerous studies using these inhibitors have demonstrated the role of the proteasome in important cellular processes. However, these inhibitors block all proteasomal degradation and are therefore not very specific for individual proteolytic pathways.

As useful as these inhibitors have been, there is still a need for stable and specific inhibitors to investigate the enzymatic specificity, binding sites, affinities, and catalytic mechanisms for the enzymes of the pathway and that will not have the pleiotropic effects of the known inhibitors currently available. Here we report a series of nonhydrolyzable analogues of different ubiquitin dimer linkages that show a specific pattern of inhibition of deubiquitinating enzymes, and that act as selective inhibitors of conjugation and deconjugation of ubiquitin. This series of inhibitors should serve as stable and selective inhibitors of enzymes involved in the metabolism of different chain linkages.

EXPERIMENTAL PROCEDURES

General. Buffer salts and bovine trypsin were purchased from Sigma Chemical Co. (St. Louis, MO). Ubiquitin C-terminal 7-amido-4-methylcoumarin (Ub-AMC) was prepared according to a modified method of Dang (*31*) and purified by the Emory University Microchemical Facility (Atlanta, GA).

Standard molecular biology techniques were applied in the following vector construction and cloning procedures. All restriction enzymes were purchased from either Promega Corp. (Madison, WI) or New England Biolabs (Cambridge, MA).

Ubiquitin Lysine to Cysteine Point Mutants. The ubiquitin protein expression vector (pRSUb) has been previously described (*32*). All cysteine point mutant constructs of ubiquitin were created by PCR-based site-directed mutagenesis of this vector. All primers were synthesized by Operon. PCR products were rescued in TA vector (Invitrogen). In the pRS^{G76C}Ub construct, the unique *Pst*I site contained in the template, pRSUb, was eliminated while introducing the G76C mutation (reverse degenerate mutagenic primer: 5′-TACCTGCACTTAAHAGCCACGC-3′). The rescued PCR fragment containing the pertinent G76C mutation was subcloned via the 5′ *Bgl*II and 3′ *Hin*dIII sites. For pRS^{K11C}Ub, PCR mutagenesis of pRSUB was carried out across the *Xba*I cassette with a reverse primer, 5′-CAAGACCTTAACCGGTTGCACCATAACTCTAGA-3′, which eliminated the unique *Hpa*I and a *Hin*cII site while mutating K11 to C. Creation of pRS^{K29C}Ub required PCR amplification with the following pair of primers: forward, 5′-TACATATGCAGATTTTCGTC-3′, and reverse, 5′-GGAATTCCTTCCTTGTCTTGAATGCAAGCC-3′. The forward primer eliminates the unique *Bgl*II site while the reverse primer mutates K29 to C. After rescuing this PCR fragment in the TA vector, it was subcloned into pRSUb using the *Nde*I and *Eco*RI sites. pRS^{K63C}Ub was created using the same forward primer described in the pRS^{K29C}Ub construct and the following reverse primer: 5′-GCAGGGTCGACTCGCACTGGAT-3′, likewise eliminating the unique *Bgl*II site. The rescued K63C-containing DNA fragment was subcloned into pRSUb using the unique 5′ *Nde*I and 3′ *Acc*I sites. An expression clone, pET-3a^{K48C}Ub, was obtained from C. M. Pickart, and the K48C-containing sequence was subcloned from that vector into pRSUb using the 5′ *Nde*I and 3′ *Afl*II to make pRS^{K48C}Ub. All mutants were verified by DNA sequencing.

Protein Purification: Ubiquitin Lysine to Cysteine Mutants. Cells expressing mutant ubiquitin were lysed from 4−12 L of induced culture as follows. Cell pellets were frozen, thawed on ice, and lysed in 50 mL of stock lysis buffer (30 mM TRIS-HCl, pH 7.5, 2.5 mM EDTA, 10 mM β-mercaptoethanol, 0.5 mM PMSF, 1 μg/mL pepstatin, 2 μg/mL antipain, 1 μg/mL chymostatin, aprotinin 2 μg/mL,

leupeptin 0.5 μg/mL, benzamidine 0.1 mM, and 100 μg/mL lysozyme) per liter of bacterial culture. Following suspension of the cells in the lysis buffer, the lysate was incubated at room temperature for 20 min. Bacterial DNA was sheared by 2 min of sonication, and the crude homogenate was centrifuged at 40000*g* in an Sorvall SS-34 rotor for 25 min. The supernatant was then filtered through 1 μm glass fiber filters to remove remaining debris prior to chromatography. The following DE batch buffer was used in subsequent steps: 30 mM TRIS-HCl, pH 7.5, 2.5 mM EDTA, and 10 mM β-mercaptoethanol. Approximately 50−100 g of DEAE-cellulose (Sigma) was preequilibrated in DE batch buffer in a Buchner funnel attached to a 1 L sidearm flask. The 40000*g* supernatant was then applied to the DEAE resin at room temperature and gentle suction was applied, creating a flow rate of 25 mL/min. The flowthrough contained the ubiquitin K to C mutant, except for the case of $^{K29C}Ub^{77D}$ where the additional aspartate allowed this mutant to be retained on DEAE. The latter mutant was eluted from DEAE with 100 mM NaCl in DE batch buffer in 50 mL fractions. These chromatography steps were all monitored analytically by HPLC (C8 silica, Biorad Laboratories) using 45% acetonitrile in 25 mM perchlorate buffer with 205 nm detection (*33*). Following DEAE chromatography, the protein was treated with 25 mM β-mercaptoethanol for 30 min to reduce oxidized disulfide dimers of the ubiquitin mutants, and then concentrated acetic acid was added until the pH dropped to 4.5. Precipitated contaminants were removed by centrifuging at 20000*g* for 20 min, and the supernatant was dialyzed versus 20 mM sodium acetate, pH 5.0. Fast Flow SP Sepharose cation-exchange resin (Amersham Pharmacia Biotech, Piscataway, NJ) was preequilibrated with 20 mM sodium acetate, pH 5.0, in a 50 mL column. The dialyzed protein was applied to the SP column, washed with 150 mL of dialysis buffer, and eluted with a 500 mL linear gradient of 0−350 mM NaCl in dialysis buffer at a flow rate of 4.0 mL/min with 280 nm detection. Fractions were judged greater than 99% pure by HPLC and SDS−PAGE. If disulfide formation occurred during chromatography, a second peak eluting at higher salt concentrations was observed. This protein could be recovered and reduced to monomeric ubiquitin by treatment with 25 mM β-mercaptoethanol at pH 7.5. In either case, reduced monomer was pooled and concentrated to 10 mg/mL and dialyzed against 3 changes of 10 mM HCl. Once the pH had been lowered, the protein was stable to thiol oxidation and was stored at −20 °C.

Synthesis of Nonhydrolyzable Diubiquitin Analogues. The lysine to cysteine mutants of ubiquitin were cross-linked via the unique sulfhydryl group with the bifunctional, thiol-specific reagent dichloroacetone (Aldrich Chemical Co., Milwaukee, WI). All reaction components were chilled on ice. The desired Ub K to C mutant (5 mL of 10 mg/mL protein in 10 mM HCl) was mixed on ice with 2 mL of ice-cold 250 mM sodium borate, pH 8.3. One to five equivalents of dichloroacetone (100 mM in dimethylformamide) was added, and the reaction was complete in less than 30 min as judged by HPLC. The resulting Ub−chloromethyl ketone adduct and an equimolar amount of ^{G76C}Ub were then mixed. The progress of the cross-linking reaction was monitored on HPLC, and when the reaction was judged complete (generally less than 30 min), the reaction was stopped by the addition of 50 mM β-mercaptoethanol. The excess thiol ensured the reduction of any dimer formed as a result of disulfide formation, and blocked any residual dichloroacetone or chloromethyl ketone−ubiquitin adducts. After 30 min of treatment with β-mercaptoethanol, the pH of the mixture was lowered to 5.0 with concentrated acetic acid, and the reaction mixture was dialyzed versus 20 mM ammonium acetate, pH 5.5. While the predominant species in the cross-linking reaction was $^{G76C}Ub-^{KnC}Ub$[3] (where $n = 11$, 29, 48, or 63), other cross-linked species existed (i.e., $^{G76C}Ub-^{G76C}Ub$ and $^{KnC}Ub-^{KnC}Ub$) as well as monomeric starting material. However, the three possible diubiquitin species were distinguishable by the charge differences imparted by the loss of a positive charge in the lysine to cysteine mutation. Cation exchange was then carried out on the dialyzed reaction mixture using two 5 mL HiTrap SP columns placed in series (Amersham Pharmacia Biotech, Piscataway, NJ). The cross-linked dialysate mixture was applied to the 10 mL SP column which had been preequilibrated with 20 mM ammonium acetate, pH 5.5, at a flow rate of 4 mL/min. Absorbance was monitored at 280 nm. The column was washed with 30 mL of ammonium acetate buffer and then eluted with a 250 mL linear gradient of 0−150 mM ammonium chloride at 4 mL/min. The cross-linked diubiquitin species had characteristic retention times on HPLC and subsequently were distinguished by this property through comparison to control samples of $^{G76C}Ub-^{G76C}Ub$ and other $^{KnC}Ub-^{KnC}Ub$ dimers synthesized by cross-linking the individual mutant proteins. In the $^{76-48}Ub_2$ reaction, for example, the three possible dimers elute from the salt gradient in the following order: first $^{48-48}Ub_2$, second $^{76-48}Ub_2$, and third $^{76-76}Ub_2$. This also represented the order in which these species were eluted upon C8 HPLC. Thus, after identifying the desired diubiquitin peak from cation exchange, the fractions were pooled, concentrated (2−4 mg/mL), dialyzed versus 10 mM TRIS-HCl, pH 7.5, 1 mM EDTA, 2 mM β-mercaptoethanol, aliquoted, and stored at −80 °C. In each case, the purified dimers gave a single band of the appropriate molecular weight on SDS−PAGE and a single peak on HPLC on a C-8 reverse phase column. Typical yields of dimer were about 25% based on the amount of starting ^{KnC}Ub. As dichloroacetone is a selective thiol reagent and no dimer products were obtained using ubiquitin that lacked cysteine, it is assumed that the cross-linking has occurred via cysteine.

Kinetic Methods. Deubiquitinating enzyme activity was measured using a fluorometric assay with ubiquitin-AMC as the substrate (*31*). Assay buffer for isopeptidase T, UCH-L3, and USP14 was 50 mM TRIS-HCl, pH 7.5, 0.5 mM EDTA, 50 μg/mL ovalbumin, and 2 mM DTT. Assay buffer for the product of the D38373 pseudogene also contained 10 mM NaCl. All assays were carried out at 37 °C.

In a typical kinetic run, 2.00 mL of assay buffer and 1−100 μL of the stock deubiquitinating enzyme solution were added to a 3 mL cuvette containing a magnetic stirring bar. The reaction was monitored in a Perkin-Elmer LS 50 B luminescence spectrometer. Reaction temperature was maintained at 37.0 ± 0.02 °C by a circulating water bath. After the reaction mixture had reached thermal equilibrium (∼2

[3] The following nomenclature is used to describe the nonhydrolyzable dimer analogues. Diubiquitin is abbreviated Ub_2, and the sites of cross-linking are indicated with a superscript prefix. Thus, the dimer formed by cross-linking G76C-Ub to K48C-Ub is designated as $^{76-48}Ub_2$.

min), 0.5−10 μL of Ub-AMC solution in DMSO was added to the cuvette to initiate the reaction. Reaction progress was monitored by the increase in fluorescence emission at 460 nm (λ_{ex} = 380 nm) or 440 nm (λ_{ex} = 340 nm) that resulted from the cleavage of AMC from Ub-AMC. For each kinetic run, 600−1200 data points were collected by a computer.

In these experiments, linear progress curves for the steady-state hydrolysis of Ub-AMC were first collected for a time sufficient to determine the control velocity in the absence of ubiquitin dimer analogues. After these data were collected, a small aliquot (<20 μL) of the ubiquitin dimer analogue was added to the cuvette containing the reaction solution, and then the progress curve for the inhibited reaction was monitored.

To determine the dissociation constants for the inhibitors, we conducted inhibitor studies using three or four concentrations of Ub-AMC. From the progress curves, we calculated the initial slope of the v vs S plot in the absence of inhibitor (V/K) using a nonlinear curve fitting. In presence of inhibitor, K_i was calculated from the observed V/K [$(V/K)_{obs}$] and the relationship:

$$K_i = \frac{[I]}{[(V/K)/(V/K)_{obs}] - 1}$$

Preparation of Enzymes. Reticulocyte fraction II was prepared according to Ciechanover's method (*34*). UCH-L3 and isopeptidase T enzymes were purified as described previously (*14*, *35*). The pseudogene product of D38373 was PCR-amplified from human genomic DNA, and USP14 (the human homologue to yeast Ubp6p) was amplified from a vector supplied by Dr. J. R. Katze (University of Tennessee, Memphis). Both PCR products were inserted into pRSetB using the *Nde*I and *Bam*HI sites introduced with the PCR primers. Expression of protein was induced with IPTG as we have described previously (*35*). USP14 was purified to homogeneity by gel filtration and ion exchange chromatography, and the pseudogene product was partially purified using gel filtration and ion exchange chromatography (Kui, Wesolowski, and Wilkinson, unpublished).

Iodination of Ubiquitin. The iodination was performed as described previously (*36*). Over 90% of the iodine present in the pooled fractions was bound to ubiquitin, and the pooled fractions had a specific activity of 8×10^5 cpm/μg of ubiquitin. The pooled fractions were divided into 100 μL aliquots and stored at −20 °C.

Reticulocyte Fraction II Assays for Conjugation and Deconjugation. An ATP energy mix was added to the conjugation reactions to maintain a constant supply of ATP in the reaction. After addition to a reaction, the concentrations of the components of the energy mix were 50 mM Tris-HCl (pH 7.6), 1 mM $MgCl_2$, 0.4 mM ATP, 0.4 mM DTT (dithiothreitol), and 2 mM phosphocreatine. In addition to the ATP mix, the reaction mixture contained 100 mM Tris-HCl (pH 7.6), 5 mM $MgCl_2$, 4 μM ATP, 3 mM DTT, and 0.3 unit of creatine phosphokinase. The final concentration of ^{125}I-Ub in each reaction was 1 μM.

When dimer analogues were being used in the conjugation reaction, they were preincubated with fraction II or reticulocyte lysate for 15 min at 37 °C before addition to the other components of the reaction. Fraction II was always added last to start the reaction. Reactions were incubated for 30 min at 37 °C. After incubation, SDS sample buffer was added to stop the reaction. The reaction samples were separated by SDS−PAGE. The gels were fixed in 10% glacial acetic acid, 20% methanol, and 70% water, rinsed in water, soaked in a 20% glycerol solution for an hour, and then dried on a gel dryer. After drying, the gels were exposed to film for 12−24 h at −80 °C.

For deconjugation experiments, a stock of conjugates was made by incubation for 30 min at 37 °C as described above. After the incubation, the stock was placed on ice, and *N*-ethylmaleimide, iodoacetic acid, and iodoacetamide were added to give a final concentration of 5 mM for each. The reaction was dialyzed overnight at 4 °C against 50 mM Tris-HCl (pH 7.6) to remove excess reagents.

To determine the inhibition constants for the inhibition of deconjugation of specific conjugates by ubiquitin dimer analogues, the radioactive samples were separated by SDS−PAGE. After gels were fixed and dried, the ubiquitinated proteins on the gels were detected by autoradiography. Then the films were scanned, and the intensity of specific bands was quantitated. From the progress curves, we calculated the k_{obs} using nonlinear curve fitting to an exponential decay. The K_i was obtained from plots of $1/k_{obs}$ vs [I].

RESULTS

Synthesis of Nonhydrolyzable Ubiquitin Dimer Analogues. Inhibitors of processes that utilize alternative polyubiquitin chain linkages (i.e., non-K48-linked) could be expected to be valuable tools in dissecting the role of these chains. To develop such inhibitors, we have synthesized a series of analogues that mimic the appropriate spatial orientation, but cannot be hydrolyzed by deubiquitinating enzymes. The strategy makes use of the fact that there are no cysteines in ubiquitin. The selective incorporation of cyteine allows facile and unique cross-linking via bifunctional thiol reagents. Thus, to make analogues of the isopeptide-linked chains, we mutated individual K residues to C and purified the resultant ubiquitin derivatives. To introduce a reactive site at the C-terminus, we separately mutated G76 to C and purified this protein also. Individual dimer analogues were then synthesized by cross-linking a single K to C derivative with the G76C mutant ubiquitin. A short cross-linker, dichloroacetone, was chosen because of its size and reactivity with thiol groups. When the reactions are monitored by HPLC, SDS−PAGE, and ion exchange chromatography, we observe that the cross-linking reaction leads to the desired dimers in high yield and with few side reactions (data not shown). The resultant linkages are shown in Figure 1. It can be seen that the synthetic linkage is one atom longer than the isopeptide linkage to be mimicked, but that the polarity of the linkage is largely maintained. These dimer analogues are stable to hydrolysis and have been found to be stable in crude extracts (data not shown). The most important feature of natural chains that is preserved in these analogues is the spatial orientation of the two monomers in the dimer analogue. As shown in Figure 1C, the normal position of isopeptide linkages we are concerned with can be represented by the vertexes of a trigonal bipyramid. Thus, K48-linked chains are assumed to have a very different geometry than K29-linked chains. If these chains have unique conformations,

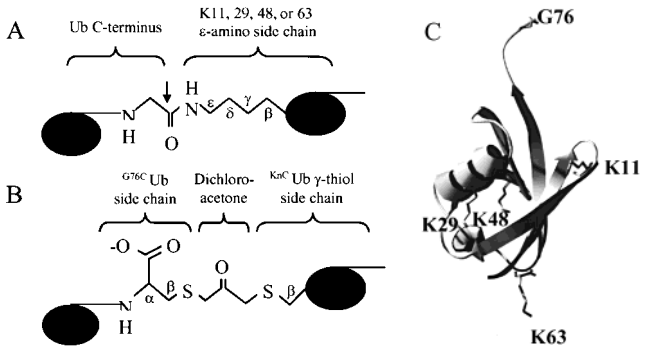

FIGURE 1: Structure of the native isopeptide bond (panel A) and the synthetic cross-link in the dimer analogues (panel B). An arrow marks the isopeptide bond acted upon by deubiquitinating enzymes (panel A). Panel C shows the 3-dimensional spatial relationships of the lysine residues known to form isopeptide linkages. Notice that these lysines are arranged at the vertexes of a trigonal bipyramid, with K11, K29, and K48 in equatorial positions and K63 and G76 in axial positions.

Table 1: Effects of Ubiquitin and Dimer Analogues on the Rate of Ubiquitin-AMC Hydrolysis by Purified Deubiquitinating Enzymes[a]

	K_i (nM)				
dimer analogue	UCH-L3	D38378 pseudogene product	USP14	Iso-T (−Ub)	Iso-T (+Ub)
ubiquitin	200	8300	>50000	−	3000
$^{76-11}Ub_2$	151	3300	>50000	>2000	25
$^{76-29}Ub_2$	199	11000	>50000	16	1.8
$^{76-48}Ub_2$	107	7700	>50000	>2000	5.6
$^{76-63}Ub_2$	38	11000	>50000	ND	13
$^{76-76}Ub_2$	13	3300	>50000	>2000	250

[a] All kinetic measurements used the fluorometric ubiquitin-AMC assay. Details are given in the text.

each physiological chain linkage will present a different face of the monomers to binding proteins, and this geometry will be retained in the analogues. Thus, we hypothesize that these ubiquitin dimer analogues will be faithful mimics of the physiological linkages.

Ubiquitin Dimer Analogues Are Selective Inhibitors of DUBs. To characterize the specificity of these inhibitors, we first examined their ability to inhibit purified deubiquitinating enzymes using Ub-AMC as the substrate. Table 1 shows that there is a great deal of selectivity to this inhibition. UCH-L3 is inhibited equally by ubiquitin and by dimer analogues linked through residues 11, 29, and 48 ($K_i \sim 200$ μM). The dimer linked through residue 63 and the head-to-head control dimer result in somewhat stronger inhibition. In the absence of ubiquitin, isopeptidase T is only significantly inhibited by the dimer linked through residue 29. In the presence of 0.5 μM ubiquitin, isopeptidase T is inhibited by several of the dimer analogues, with the dimer linked through residue 29 demonstrating the most inhibition ($K_i \sim 2$ nM). The order of effectiveness is 29 > 48 > 63 > 11. The catalytic domain encoded by the D38378 pseudogene is inhibited to a small degree equally by all the derivatives, but inhibition requires much higher concentrations. The human homologue to yeast Ubp6 is not inhibited at the concentrations tested. These results suggest that there is considerable selectivity to the potency of inhibition and that with each enzyme there is a clear pattern to the inhibition that varies with the enzyme used.

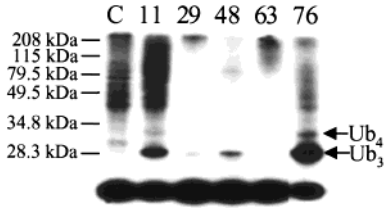

FIGURE 2: Effects of ubiquitin dimer analogues on ubiquitin conjugation catalyzed by reticulocyte lysate fraction II. Ubiquitin conjugation was assayed in incubations of 30 min duration. Besides standard assay components (see Experimental Procedures), the assay contained fraction II protein at 2 mg/mL, ^{125}I-ubiquitin at 1 μM, and the indicated dimers at 0.5 μM. Lanes labeled C, control without added inhibitors; 11, $^{76-11}Ub_2$; 29, $^{76-29}Ub_2$; 48, $^{76-48}Ub_2$; 63, $^{76-63}Ub_2$; and 76, $^{76-76}Ub_2$.

Effects of Ubiquitin Dimer Analogues on Ubiquitin Conjugation. The above results suggest that enzymes that normally recognize polyubiquitin (such as isopeptidase T) are also most effectively inhibited. Ubiquitin conjugation is often processive, suggesting that these dimer analogues may also inhibit binding of polyubiquitin by the conjugating enzymes. Thus, ubiquitin dimer analogues may also be effective inhibitors of the conjugation of ubiquitin to endogenous proteins in reticulocyte lysate fraction II. The steady-state level of conjugates formed in this reaction was determined by incubation of ^{125}I-ubiquitin with fraction II and ATP, followed by separation of the conjugates on SDS−PAGE and autoradiography. Figure 2 shows an autoradiograph from these reactions. Numerous conjugate bands can be seen in the positive control incubations containing no dimer analogues. Incubations containing $^{76-76}Ub_2$ and $^{76-11}Ub_2$ exhibit levels of conjugation roughly equal to the positive control. $^{76-29}Ub_2$, $^{76-48}Ub_2$, and $^{76-63}Ub_2$ exhibit significant inhibition of conjugation. Although levels of smaller polyubiquitin chains appear to be completely inhibited in the presence of $^{76-63}Ub_2$, levels of larger chains appear to be increased. $^{76-29}Ub_2$ almost totally inhibited conjugation except for a small increase at the top of the gel.

Another interesting aspect of these results was the large increase amount of labeled protein that comigrates with ubiquitin trimers and tetramers in certain incubations. The $^{76-76}Ub_2$ lane had a large increase in the amount of a conjugate comigrating with ubiquitin trimer. $^{76-11}Ub_2$ and $^{76-48}Ub_2$ lanes also had strong increases in a similar band while $^{76-29}Ub_2$ only showed a small increase. No trimers or tetramers were observed with $^{76-63}Ub_2$. These bands probably result from ubiquitination of the ubiquitin dimer analogues (see Discussion).

Preparation of Ubiquitin Conjugates. To examine the ability of these analogues to inhibit deconjugation catalyzed by deubiquitinating enzymes, a stock of stable ubiquitin conjugates was prepared. The conjugation reaction was allowed to proceed as described above, and *N*-ethylmaleimide, iodoacetic acid, and iodoacetamide were then added to inhibit deubiquitinating enzymes present in the lysates. Figure 3 (top panel) shows that the combination of *N*-ethylmaleimide, iodoacetic acid, and iodoacetamide completely destroyed the deubiquitinating enzyme activity, and stabilized the conjugates already formed in the conjugation reaction. Adding a fresh aliquot of fraction II to this conjugate stock after alkylating agents had been removed resulted in complete deubiquitination (Figure 3, bottom panel) and

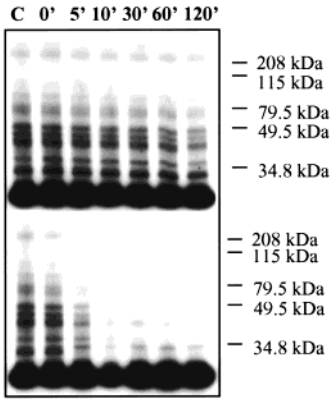

FIGURE 3: Stability of ubiquitin conjugates in the presence of boiled (upper panel) or fresh fraction II (lower panel). The reactions were incubated at 37 °C for the indicated times. The assays contained ^{125}I-ubiquitinated conjugate protein (total protein concentration 0.8 mg/mL) and 0.5 mg/mL fraction II protein.

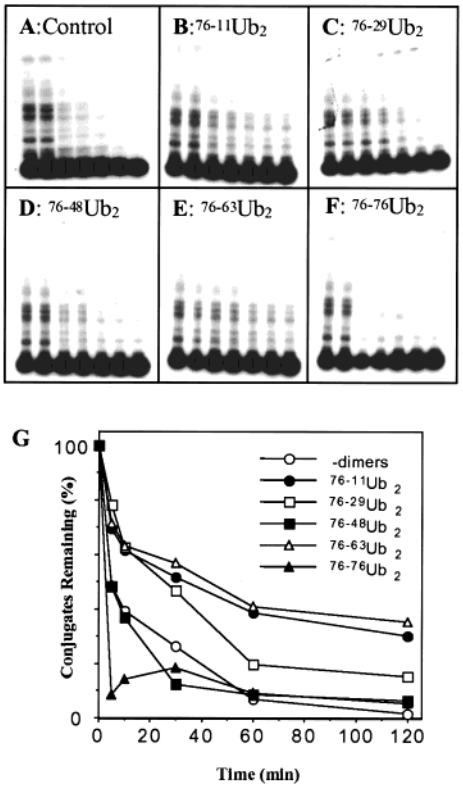

FIGURE 4: Inhibition of deconjugation by ubiquitin dimer analogues. The incubations contained fraction II protein at 0.4 mg/mL, ^{125}I-ubiquitinated conjugates (total protein concentration 0.5 mg/mL), and 0.5 μM of the indicated ubiquitin dimer analogues. In each panel, the lanes from left to right are: control with boiled fraction II, and 0, 5, 10, 30, 60, and 120 min after adding fresh fraction II. Panel G show the time course of deubiquitination observed in panels A−F.

allowed the analysis of the effects of the dimers on deconjugation to be examined.

Effects of Ubiquitin Dimer Analogues on Ubiquitin Deconjugation. The specificity of inhibition by ubiquitin dimer analogues was further examined by measuring the rates of deubiquitination catalyzed by reticulocyte lysate fraction II. ATP was absent in the deconjugation reaction, eliminating further ubquitin conjugation during deconjugation. Figure 4 shows the inhibition by 0.5 μM dimer analogues of deconjugation catalyzed by fraction II. Some of the analogues

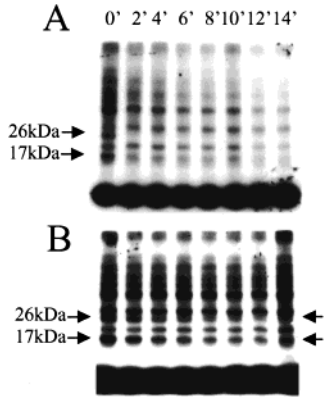

FIGURE 5: Deconjugation of labile ubiquitin conjugates without (panel A) and with the dimer analogue $^{76-29}$Ub$_2$ (panel B). Note that the concentration of fresh fraction II was 1/50 that in Figure 4. The 26 and 17 kDa conjugates showed a rapid rate of deconjugation catalyzed by low levels of fraction II while other conjugates were stable at this low level of fraction II.

showed significant inhibition of total deconjugation. In the $^{76-48}$Ub$_2$, $^{76-76}$Ub$_2$, and control incubations, conjugates were reduced about 80% in 30 min. In contrast, in the presence of $^{76-11}$Ub$_2$, $^{76-29}$Ub$_2$, or $^{76-63}$Ub$_2$, the conjugates were reduced about 50% in 30 min. At longer times, only $^{76-11}$Ub$_2$ and $^{76-63}$Ub$_2$ significantly inhibited deconjugation.

To evaluate the effects of analogues on deubiquitination by known DUBs, we used purified enzymes instead of reticulocyte lysate fraction II. UCH-L3, the product of the D38373 pseudo gene, and USP14 failed to deubiquitinate these conjugates under these conditions. However, isopeptidase T processed two conjugates with apparent masses of 26 and 17 kDa (data not shown). This activity is inhibited by $^{76-29}$Ub$_2$, suggesting that both conjugated proteins are the specific substrates for isopeptidase T. It is assumed that these bands correspond to unanchored chains of di- and triubiquitin (see Discussion).

We noticed that the same two bands were rapidly depleted in the reticulocyte extracts and that this deubiquitination was also inhibited by $^{76-29}$Ub$_2$ (Figure 5). If isopeptidase T in the reticulocyte extracts is responsible for deubiquitination of the 26 and 17 kDa conjugates, we would expect the inhibitor specificity to be similar for that reaction and for hydrolysis of substrates by purified isopeptidase T. Ubiquitin dimer analogues were used as inhibitors to investigate the inhibition patterns for processing the two specific bands by fraction II (data not shown). After scanning of the autoradiography film, the density of the bands was quantitated and analyzed. The K_i for dimer analogues was calculated from the initial rate of deconjugation and the concentration of inhibitors. Similar inhibition constants were also measured for the hydrolysis of ubiquitin-AMC by purified isopeptidase T. Table 2 shows that the inhibition constants of ubiquitin dimer analogues were similar for fraction II catalyzed deubiquitination of the 26 and 17 kDa proteins and for isopeptidase T catalyzed hydrolysis of ubiquitin-AMC.

DISCUSSION

K48-linked polyubiquitin chains act as a signal, targeting proteins for specific degradation by the proteasome. There is a degradative rate enhancement of 10-fold or more when a single ubiquitin is replaced by a K48-linked polyubiquitin

Table 2: Deconjugation of Two Labile Conjugates Catalyzed by Fraction II and Iso-T Show Similar Inhibitor Specificity[a]

	K_i (nM)		
dimer analogue	deconjugation of 26 kDa protein by fraction II	deconjugation of 17 kDa protein by fraction II	hydrolysis of Ub-AMC by isopeptidase T(+Ub)
$^{76-11}Ub_2$	33.5	24	25
$^{76-29}Ub_2$	2.2	0.7	1.8
$^{76-48}Ub_2$	10.3	2.8	5.6
$^{76-63}Ub_2$	7.5	5.3	13

[a] Each determination was done in triplicate, and the estimated error in K_i is ±20%.

chain (*3*). Further, tetraubiquitin chains bind to the proteasome more than 100-fold more tightly than diubiquitin chains (*37*). Polyubiquitin chains can also be linked by isopeptide bonds between G76 and either K6, K11, K29, or K63 (*6*−*9*, *11*, *12*). The significance of these linkages and their role is not yet known. Each of these polyubiquitin chains is synthesized by unique enzymes, and it seems likely that they are also disassembled by unique deubiquitinating enzymes. To test this hypothesis, we synthesized nonhydrolyzable ubiquitin dimer analogues of each of these linkages. These analogues showed a specific pattern of inhibition of deubiquitinating enzymes.

UCH-L3 is a small DUB with only one ubiquitin binding site (*38*−*40*). It is a member of the ubiquitin C-terminal hydrolase (UCH) family and shows specificity for small or disordered leaving groups. It has no activity in disassembling ubiquitin polymers, although it can hydrolyze small leaving groups from the proximal subunit in polyubiquitin chains. We find that this enzyme is inhibited equally by ubiquitin and by dimer analogues linked through residues C11, C29, and C48. The linkage through residue C63 marginally increases the effectiveness of inhibition, as does the nonphysiological cross-link through C76 of both subunits. These results are consistent with inhibition being due to binding of the proximal ubiquitin to the active site of UCH-L3 and variable degrees of interaction between the distal ubiquitin of different dimers and the body of the enzyme. Based on the available NMR (*41*, *42*) and crystalographic (*43*) data describing the complex between two UCH enzymes and ubiquitin, positions 11, 29, and 48 are accessible but close enough to the enzyme surface that the distal ubiquitin in a 11-, 29-, or 48-linked dimer may interact with the enzyme. Lysine 63 is well removed from possible interactions with the enzyme, and it is expected that attachment of ubiquitin at this position would have little or no effect on binding. Finally, extensive contacts between $^{76-76}Ub_2$ and the enzyme would be predicted. The results are consistent with $^{76-63}Ub_2$ binding such that the distal ubiquitin makes little or no contacts with the enzyme. The other dimer analogues bind more tightly, perhaps due to the contribution of nonspecific interactions between the body of UCH-L3 and the distal ubiquitin in the dimer analogue. Some interactions are possible with the distal ubiquitin of $^{76-11}Ub_2$, $^{76-29}Ub_2$, and $^{76-48}Ub_2$, and this may contribute to tighter binding. With $^{76-76}Ub_2$, extensive interactions with the distal ubiquitin may contribute significantly to the stability of the inhibitor complex. The best inhibitor binds with only 15-fold higher affinity than does ubiquitin, emphasizing that there cannot be a large number of strong interactions between the enzyme and the distal ubiquitin.

In contrast, isopeptidase T is known to have an extended binding site and is able to bind to multiple ubiquitin domains in polymeric ubiquitin (*14*, *31*). In the absence of ubiquitin, the activity of isopeptidase T is relatively low. We find that this form is only significantly inhibited by the dimer analogue linked through residue 29. In the presence of 0.5 μM ubiquitin, it is known that isopeptidase T undergoes a conformational change to generate a much more active form (*31*). This is thought to be the basis for the selectivity of this enzyme; i.e., the enzyme is maximally active only when the S1′ site[4] is occupied by a ubiquitin subunit containing a free C-terminus at the proximal end of a polyubiquitin chain. With small substrates such as Ub-AMC, the S1′ site can be occupied by ubiquitin, and the substrate-induced conformational change results in activation of the enzyme. We find that this activated enzyme is inhibited by several of the dimer analogues, but best by the dimer linked through residue 29.

With isopeptidase T, the best inhibitor is 1500-fold more effective than free ubiquitin as an inhibitor. This represents a significant increase in binding energy for the dimer compared to monomer ($\Delta\Delta G = 4.5$ kcal/mol), and suggests that specific interactions between the enzyme and both monomers in the inhibitor are responsible for the increased affinity. In contrast, the nonphysiological control ($^{76-76}Ub_2$) is bound by both enzymes about 15-fold tighter than is ubiquitin. This suggests that protein−protein interactions between the enzyme and the distal ubiquitin are relatively nonspecific and only contribute about 1.5 kcal/mol additional binding energy. We assume that these inhibitors act by binding to the S1 site on the enzyme, either by forming a ternary complex with Ub and Iso T (Ub in the S1′ site and $^{76-n}Ub_2$ spanning the S2-S1 sites) or by binding across the S1-S1′ sites and displacing Ub from its activating position. It should be noted that with real substrates such as polyubiquitin chains, the activating function of ubiquitin is furnished by the proximal ubiquitin in the chain. As the absolute values of the inhibition constants (Table 2) are similar for hydrolysis of both UbAMC (requiring Ub at the S1′ site) and di- and triubiquitin (requiring that the S1′ site be unoccupied), we assume that the observed inhibition is due to the ternary complex described above.

To further investigate the selectivity of this inhibition, we examined the effects of ubiquitin dimer analogues on the catalysis by a UBP with a very weak affinity for ubiquitin. The catalytic domain encoded by the human D38378 pseudogene product is most closely related to the mouse DUB-1 and DUB-2 enzymes (*44*). These have only a single intron and a large exon similar in sequence and length to a human ORF present in a 4.7 kb novel tandem repeat on chromosome 4p (*45*). Originally this ORF attracted our attention because it encoded a small UBP catalytic domain and thus was an example of a minimal core UBP. Several lines of evidence suggest this sequence is a pseudogene. First, the tandem repeat containing this ORF is present in 50 to

[4] The standard protease nomenclature of Schecter and Berger (*1*) has been adopted, except that a ubiquitin monomer is used in place of a single amino acid. For instance, isopeptidase t binds at least four ubiquitin subunits in the S3-S2-S1-S1′ sites with cleavage of the peptide bond between the monomers bound at the S1 and S1′ sites.

70 copies on chromosome 4p. Second, there is no evidence of any introns or other exons in this repeat. Finally, we have been unable to PCR amplify this ORF from a number of human cDNA libraries, but it is easily amplified from genomic DNA. This pseudogene product has a very high apparent K_m for ubiquitin (~8 μM) and is only weakly inhibited by all the analogues (K_i 3 to 10 μM). These results support the idea that specific binding of the ubiquitin domain is required for inhibition by these ubiquitin dimer analogues.

Finally, USP14, the human homologue of yeast Ubp6p, is a small UBP that contains an unusual N-terminal ubiquitin-like domain. The role of this domain is not known, although it is required for function in yeast (*46*). We have found that this enzyme has a very high K_m for ubiquitin (>50 μM, data not shown), suggesting that the enzyme does not bind monomeric ubiquitin and that the physiological substrate for this enzyme may be a ubiquitinated protein or a polyubiquitin chain. To test the latter hypothesis, we examined the effects of the ubiquitin dimer analogues to inhibit the hydrolysis of ubiquitin-AMC catalyzed by this enzyme. Table 1 demonstrates that USP14 is not inhibited at all by ubiquitin or the ubiquitin dimer analogues. These results would suggest that it is unlikely that this enzyme acts on polyubiquitin chains.

The mechanism of this inhibition by ubiquitin dimer analogues is likely to be competitive inhibition due to occupancy of the active site ubiquitin binding site. Consistent with this is the observation that inhibitor potency is similar in magnitude to the affinity of the target enzyme for ubiquitin. The inhibitors were designed to mimic the physiological linkages, although we do not have proof that they occupy the binding site in the same manner as the physiological chains. It is apparent, however, that they show high affinity and considerable selectivity for inhibition of some DUBs. In particular, these inhibitors show a preference for enzymes with multiple ubiquitin binding sites (compare inhibition of UCH-L3 with isopeptidase T). As such they should be useful probes of the structure and specificity of these enzymes. Below we discuss one example wherein isopeptidase T activity is shown to be responsible for the deubiquitination of two specific conjugates in reticulocyte lysate fraction II.

Selective Inhibition of Conjugation by Ubiquitin Dimer Analogues. In the ubiquitin conjugation system, at least three different classes of enzymes are involved in protein ubiquitination. These are known as E1, E2, and E3, and they ensure the required specificity and selectivity of ubiquitin conjugation. Often these reactions are processive, suggesting that the conjugating enzymes may have significant affinity for polyubiquitin. Little is known about the selectivity and regulation of these enzymes and their role in synthesizing different polyubiquitin chain linkages. To learn more about these enzymes, we utilized a rabbit reticulocyte lysate fraction II system and the various ubiquitin dimer analogues as inhibitors to determine their effects on ubiquitin conjugation. It has long been known that the reticulocyte fraction II system can catalyze the polymerization of ubiquitin through a number of linkages. Recent work by Pickart and co-workers demonstrated an E2/E3-mediated assembly of K29-linked and/or K48-linked polyubiquitin chains (*12*). Our results showed that the ubiquitin dimer analogues also act as selective inhibitors of conjugation. $^{76-11}Ub_2$ and $^{76-76}Ub_2$ showed no inhibition of the steady-state level of conjugates, although the patterns are significantly changed (see below). $^{76-29}Ub_2$, $^{76-48}Ub_2$, and $76^{-63}Ub_2$ showed stronger inhibition, almost completely inhibiting ubiquitin conjugation.

Also of interest is the appearance of trimer- and tetamer-sized ubiquitin conjugates in some of these incubations. Several enzymes have been shown to conjugate ubiquitin to polyubiquitin chains. It is likely that $^{76-11}Ub_2$ and $^{76-76}Ub_2$ both function as substrates for the conjugation of ubiquitin, in effect serving as alternative substrate inhibitors. This implies that they are not effective inhibitors of physiological conjugation. It should be noted here, that there is no evidence that these types of polyubiquitin chains accumulate in vivo, and thus there may be no conjugating enzymes that recognize this type of linkage. In contrast, K29-, K48-, and K63-linked polyubiquitin chains are well established in vivo, and conjugating enzymes for at least two of these linkages have been described in reticulocyte extracts. Thus, the inhibition of conjugation by the corresponding dimer analogues is expected, and indeed observed (Figure 2). Only a very small amount of ubiquitination of these dimer analogues is observed, consistent with the hypothesis that these inhibitors act as classical competitive inhibitors and not as alternative substrates. It is not clear if these effects derive from inhibition of E1or the conjugating enzymes, or if a single conjugating enzyme can be inhibited by all three dimer analogues.

Selective Inhibition of Deubiquitination by Ubiquitin Dimer Analogues. Dissassembly of polymeric ubiquitin conjugates is referred to as deconjugation and is catalyzed by processing proteases called deubiquitinating enzymes (*39*). These enzymes specifically hydrolyze peptide (or isopeptide) bonds after G76 of ubiquitin. As a group, deubiquitinating enzymes comprise the largest known family of enzymes in the ubiquitin system. There is significant deubiquitinating enzyme activity in the reticulocyte fraction II. To determine the effects of ubiquitin dimer analogues on the deubiquitinating enzymes in fraction II, we examined the effects of the analogues on the deconjugation of ubiquitin. A pool of stable ubiquitin conjugates was synthesized by quenching the conjugation reaction with high concentrations of thiol reagents. This serves to inhibit the endogenous deubiquitinating enzymes, all of which are thiol proteases. It is likely that the conjugates present in this preparation represent the most stable ones initially present, as DUBs are extremely active and difficult to fully inhibit. Nonetheless, the final preparation of ubiquitin conjugates is stable in the absence of added deubiquitinating enzymes (Figure 3A). Figure 4 demonstrates that ubiquitin dimer analogues are the selective inhibitors of deubiquitination catalyzed by reticulocyte lysate fraction II. $^{76-48}Ub_2$ and $^{76-76}Ub_2$ showed no significant inhibition, while $^{76-11}Ub_2$, $^{76-29}Ub_2$, and $^{76-63}Ub_2$ showed clear inhibition of deubiquitination. The lack of inhibition by $^{76-48}Ub_2$ is surprising since these are thought to comprise most of the ubiquitinated conjugates present in the steady state. However, it should be noted that deubiquitinating ezymes are very difficult to fully inhibit and it may be that the K48-linked chains are rapidly lost during the preparation of stable conjugates. Indeed, the 26 and 17 kDa proteins that are rapidly degraded by isopeptidase T (see below) are also the most labile in the presence of added fraction II, while most of the other conjugates are considerably more stable.

Since there are multiple deubiquitinating enzymes in fraction II, we examined the deubiquitination catalyzed by

several purified or partially purified deubiquitinating enzymes which include UCH-L3, the product of the D38373 pseudo gene, USP14, and isopeptidase T (Figure 5A). Only isopeptidase T showed the ability to dissemble the ubiquitin polymers. UCH-L3, the product of the D38373 pseudo gene, and USP14 did not catalyze deubiquitination in our system. Isopeptidase T disassembled at least two polyubiquitin chains corresponding to a 26 kDa protein and a 17 kDa protein. This reaction was inhibited by $^{76-29}Ub_2$ although we do not know the type of linkages present in these substrates. Both the 26 kDa and 17 kDa proteins are deubiquitinated by isopeptidase T as well as by reticulocyte fraction II (Figure 5). Isopeptidase T cannot disassemble the polyubiquitin chain attached to a target protein and only disassembles free polyubiquitin chains (*14*). Therefore, the 26 and 17 kDa proteins are believed to be a ubiquitin trimer and dimer, respectively. This suggested that both the 26 and 17 kDa proteins are degraded by the isopeptidase T present in reticulocyte lysate. To confirm this, we also determined the efficiency of ubiquitin dimer analogues as inhibitors of the deubiquitination of the two proteins catalyzed by reticulocyte fraction II (Table 2). The inhibition pattern for fraction II-catalyzed deconjugation of the 26 and 17 kDa proteins is similar to the pattern obtained from purified isopeptidase T with ubiquitin-AMC as substrate. This result supports the hypothesis that both bands of proteins are the specific substrates of isopeptidase T present in reticulocyte lysate.

Utility of the Ubiquitin Dimer Analogues. The results described above suggest that these analogues are effective inhibitors of enzymes that bind to and act upon polyubiquitin chains. Further, they are quite selective for such enzymes when they have extended binding sites that bind to more than one monomer. The different dimers also exhibit K_i values that vary by over 100-fold with a single enzyme. The pattern of this inhibition is expected to be enzyme-dependent and thus useful as a diagnostic tool. As shown here, this pattern can be used to identify the action of a single enzyme in a crude mixture.

It remains to be shown that the dimer analogues will show the expected specificity. They were designed to mimic physiological linkages, and as such, it is expected that inhibition by the individual analogues would be specific for the enzymes that bind to or act upon the analogous physiological chain. This specificity appears to pertain in the case of inhibition of ubiquitin conjugating enzymes. However, the finding that $^{76-29}Ub_2$ is a potent inhibitor of isopeptidase T was unexpected. We do not know if isopeptidase T can act upon K29-linked chains, but our observations suggest that this should be tested. Alternatively it is possible that $^{76-29}Ub_2$ binds to an inactive conformation of isopeptidase T. It is well-known that ubiquitin binding (probably at the S1′ site) activates isopeptidase T for the cleavage of ubiquitin-AMC. If $^{76-29}Ub_2$ binds to this inactive form, one would not expect it to hydrolyze K29-linked chains. Further studies are required to clarify the exact mechanisms involved, including a direct test of inhibition potency with defined polyubiquitin chains as substrate.

Finally, these dimers should be useful as affinity supports for the purification and characterization of enzymes of polyubiquitin metabolism. They could also be further modified to act as cross-linking reagents or spectrophotometric probes of polyubiquitin binding. Longer chains of these dimers may in fact be more selective, and we are currently exploring these possibilities.

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