A human de-ubiquitinating enzyme with both isopeptidase and peptidase activities in vitro

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Abstract Some enzymatic and physicochemical properties of a human ubiquitin-specific isopeptidase are reported. The enzyme was purified to homogeneity from red blood cells and its specificity towards polymeric ubiquitin substrates suggests a de-ubiquitinating activity capable of cleaving 'head-to-tail' polyUb chains as well as isoamide 'branched' Ub dimers. K_M values show a 10 fold preference for the cleavage of branched Ub dimers over head-to-tail Ub dimers. The enzymatic activity can be strongly inhibited by various peptides containing either of the cleavage site sequences found in Ub polymers, but not by unrelated peptides. The enzyme is monomeric under reducing conditions and exhibits a globular shape with an average diameter of 9 nm, an $S_{20,w}$ value of 5.2 S and a molar mass of 110 kDa ± 10%. Because the enzyme cleaves both peptide-linked and isopeptide-linked Ub moieties from substrates, we propose to name it de-ubiquitinase rather than isopeptidase.

Key words: De-ubiquitinating enzyme; Ubiquitin-specific isopeptidase; Isoamide bond; Multiubiquitin chain

1. Introduction

During ubiquitin-dependent protein degradation, proteins are marked for breakdown by covalent ligation to ubiquitin (Ub), forming 'conjugates' of multiubiquitin chains in which the carboxyl-terminal Gly-76 of one (or more) Ub molecules in the chain is joined to one (or more) internal Lys of targeted proteins via an isoamide bond. In the same way, each ubiquitin molecule can be ubiquitinated on its own Lys-48 to form 'branched' multiubiquitin chains [1,2]. Target proteins ligated to multiubiquitin chains are then programmed for degradation through the proteasome, an ATP-dependent 26 S protease complex [3,4].

Ubiquitin is expressed from polyubiquitin genes and ubiquitin fusion genes leading to 'head-to-tail' transient polyubiquitin and fusion proteins, which are processed to monomeric Ub. In yeast, various processing proteases cleave in vivo the peptide bonds Gly-76–Met-1 of head-to-tail polyubiquitins as well as Gly-76–X bonds in ubiquitin fusions that lack the head-to-tail Ub–Ub motif [5,6]. Two types of Ub C-terminal isopeptidase activities have been postulated [7,8]: (i) for the release of Ub units bond to a Lys residue of the protein substrate; and (ii) for the disassembling of multiUb chains during or after proteolysis of the targeted protein. Many Ub C-terminal isopeptidases and hydrolysases have been described [5,6,9–12] but their possible involvement in the above processes have not yet been fully elucidated.

We wish to describe here the functional properties of an 100 kDa 'isopeptidase' from human erythrocyte lysate which was purified to homogeneity and which, unexpectedly, cleaves in vitro both head-to-tail and branched polymeric Ub substrates, in contrast to the 30 kDa ubiquitin C-terminal hydrolysase.

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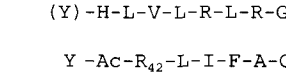
Abbreviations: Ub, ubiquitin; polyUb, 'head-to-tail' polyubiquitin; multiUb, 'branched' polyubiquitin; Ac, acetyl; (Y), tyrosine residue absent from Ub sequence but placed in the NH_2-terminal position of peptides to allow them to be coupled to protein carriers in further studies.

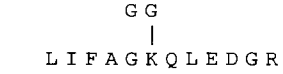
2. Materials and methods

2.1. Materials

Outdated human blood samples from the Central Hematology Laboratory of the Geneva Canton Hospital was the source of the 100 kDa de-ubiquitinating enzyme. Ubiquitin-derived peptides, i.e. the C-terminal decapeptide (Y)-H_{68}-L-V-L-R-L-R-G-G_{76}, the 20-residue long head-to-tail peptide, H_{68}-L-V-L-R-L-R-G-G_{76}-M_1-Q-I-F-V-K-T-L-T-G-K_{11}, and the isopeptide (G_{76}–K_{48}):



were synthesized according to standard techniques [13] and purified by reversed phase HPLC on a column (21 × 250 mm) of Nucleosil C8 (5 μm particle size), using as eluants increasing concentrations of acetonitrile in 0.1% (w/v) trifluoroacetic acid. Data (not shown) obtained from electrospray mass spectrometry, amino acid analysis and automated Edman degradation were consistent with the proposed structures. The analyses of peptides produced upon tryptic digestion of the branched peptide showed the presence of



thus confirming the isoamide linkage of Gly to Lys. Peptide concentrations were determined by amino acid analysis.

2.2. Enzyme purification from outdated human blood

The isopeptidase fraction (containing the 100 and 30 kDa proteins) was purified from an erythrocyte lysate as described by Pickart and Rose [14] with some modifications: fraction II eluted from DE-52 (Whatman) was diluted 1:1 with buffer D (20 mM Tris-HCl, pH 7.2, 0.2 mM DTT) and recycled overnight on an ubiquitin-Sepharose column previously equilibrated at 4°C in STD buffer (50 mM Tris-HCl, pH 7.2, 0.1 mM EDTA, 0.2 mM DTT, 5% glycerol). The column was successively washed with 3 vols. STD buffer, 3 vols. STD buffer + 0.5 M KCl, and 3 vols. STD buffer. The isopeptidase fraction was eluted with 5 vols. ISO buffer (50 mM Tris-HCl, pH 9.0, 0.1 mM EDTA, 10 mM DTT, 5% glycerol).

The isopeptidase fraction (30 ml) was concentrated on a small DE-52 column (0.3 ml) equilibrated in buffer C (20 mM Tris-HCl, pH 7.2), washed with buffer C and eluted with buffer C containing 300 mM

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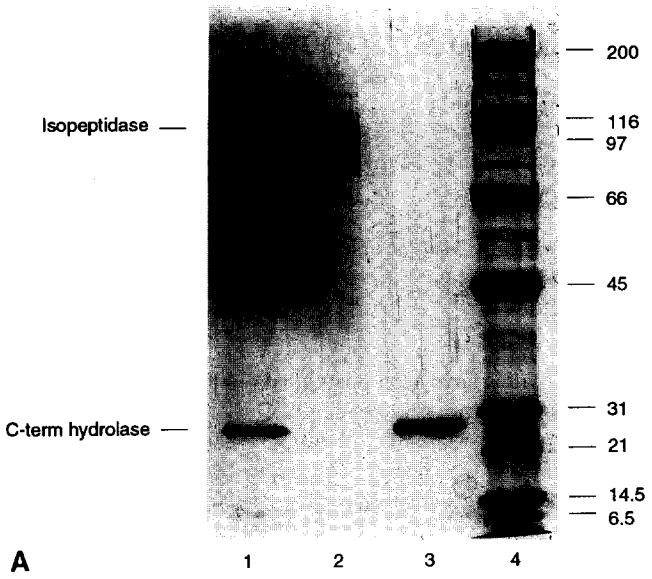
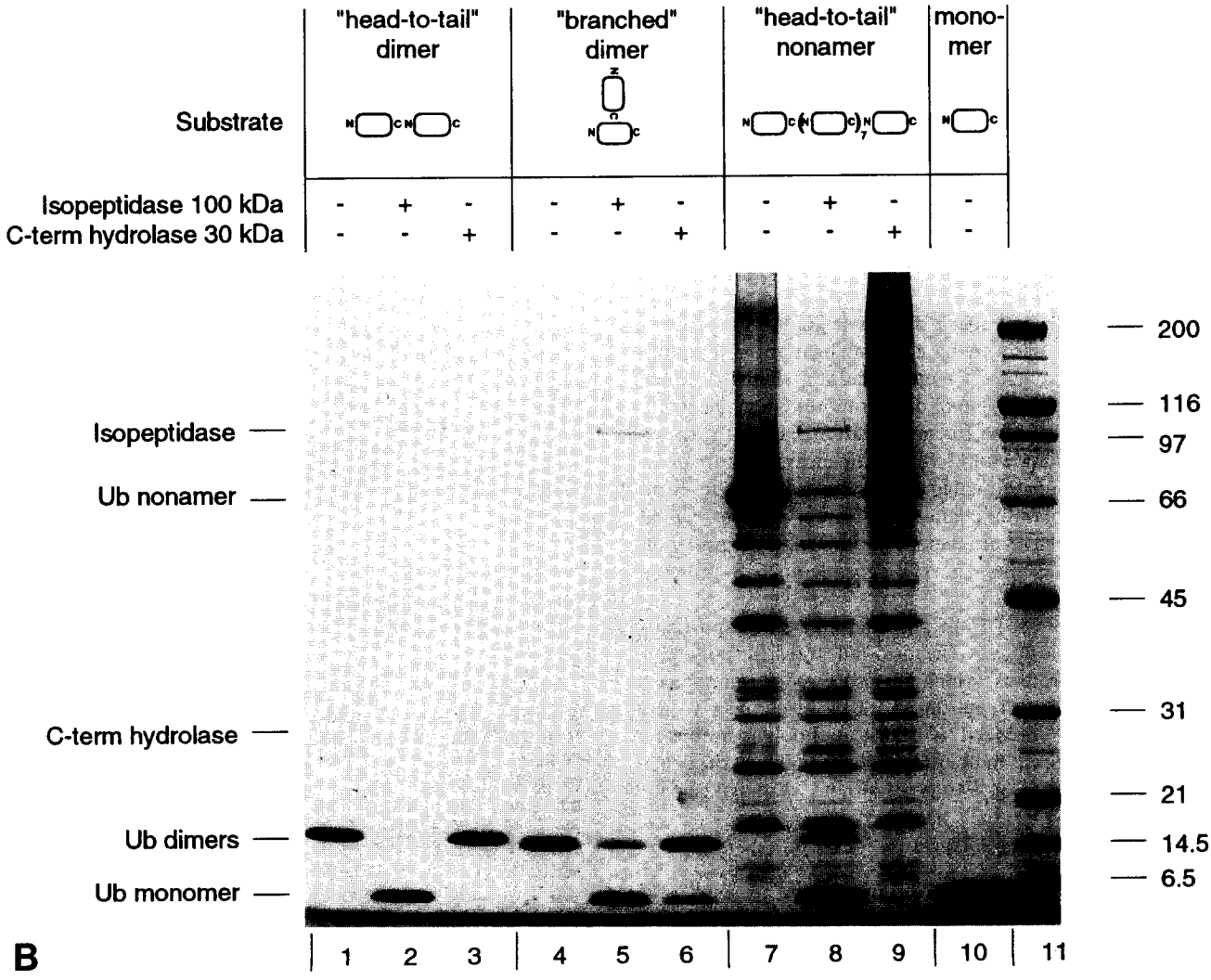


NaCl. Usually, 30 ml were concentrated to less than 2 ml. The concentrated protein solution was passed through a Superose 12 column equilibrated in PBS (pH 7.4) to separate the 100 kDa form from the 30 kDa C-terminal hydrolase. Both proteins were collected, diluted 1:1 with buffer C, and concentrated as described above. Glycerol was added to a final concentration of 30% and proteins were kept at −20°C.

2.3. Enzyme substrates

The *Manduca* polyubiquitin cDNA containing 9 repetitive coding units of the Ub gene (a generous gift from Dr. L.M. Schwartz, University of Massachusetts, USA) was inserted into pBluescript SK(−) vector (Stratagene); the dimeric form (Ub_2) was subcloned by digestion with *Bgl*II followed by direct religation, and recloning into pKK223-3 vector (Pharmacia). Transfection of *E. coli* SB1 cells and over-expression led to the accumulation of Ub_2 which was purified on DE-52 in 25 mM Tris-HCl, 1 mM EDTA buffer, pH 7.5, and Mono-S column (Pharmacia) in 50 mM Na-acetate buffer, pH 4.0. No contaminating proteins were detected by silver staining of an overloaded gel. The nonameric polyUb (Ub_9) was prepared as for the dimer and was found to be slightly contaminated by a series of polyUb proteins (octa-, hepta-, penta-, tetramers to monomer of Ub). The purified nonamer was cleaved by CNBr, and a single 8.5 kDa band was apparent on SDS-PAGE. Lys C digestion, in the presence of 8 M urea, followed by blotting over PVDF membrane and Edman degradation of selected peptides, confirmed the presence of the head to tail peptide, $E_{64}STLHLVLRLRGG_{76}M_1QIFVK_6$ (data not shown).



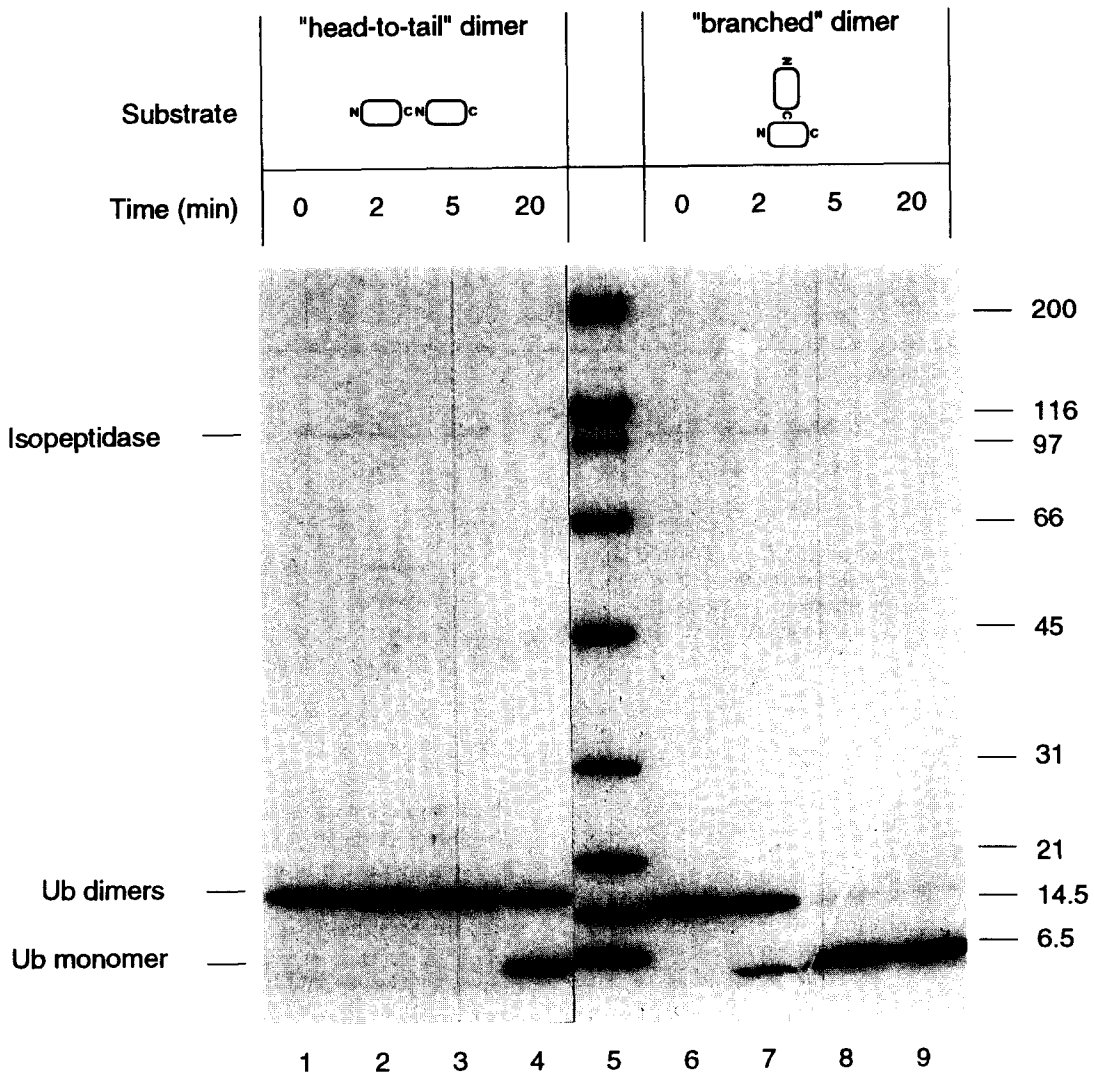


Fig. 2. Coomassie blue stained SDS-PAGE profiles of digestion mixtures (20 μl) at 37°C containing 2 μg (6 μM) of 'head-to-tail' (lanes 1–4) and 'branched' dimeric Ub (lanes 6–9) and 0.1 μg (0.05 μM) of 100 kDa isopeptidase for the period of time indicated. Lane 5, molecular weight markers, the sizes of which are shown at the right.

The branched di-ubiquitin was a generous gift from Dr. C.M. Pickart (University of Buffalo, USA); it was synthesized using recombinant E2-25 kDa, an enzyme which links Ub only by isopeptide bonds involving K-48 and G-76 [15].

Protein concentrations were determined by the micro BCA assay (Pierce).

2.4. Labelling of substrates

In order to quantitate substrates and products for K_M value determination, head-to-tail and branched Ub dimers (50 μg each) were labelled with 0.5 mCi of ^{125}I (Amersham) using the Iodogen kit from Pierce. After desalting, the specific activity was calculated to be 3.7×10^7 cpm/μg for the head-to-tail Ub dimer and 3.9×10^7 cpm/μg for the branched Ub dimer.

2.5. Enzymatic assays

The reaction mix (20 μl) contained 1 μg (3 μM) of head-to-tail Ub dimer and 0.2 μg (0.1 μM) of 100 kDa isopeptidase in 50 mM Tris-HCl buffer, pH 7.2, containing 1 mM DTT. The mixture was incubated for 1 h at 37°C and the reaction was stopped by adding 200 μl of 0.1% TFA. The sample was analyzed on a 7 μm RP300 column (2.1 × 30 mm; Brownlee Aquapore). The initial conditions were 0.1% TFA, 10% acetonitrile and the gradient was from 10% to 50% acetonitrile in 45 min with a flow rate of 300 μl/min.

2.5.1. SDS Tris–Tricine gel for K_M determination. Enzymatic reactions (20 μl) were carried out at substrate concentrations ranging from 0.5 to 4 μg (1.5–12 μM) and various digestion times from 0 to 30 min; digestion mixtures were analyzed by SDS Tris-Tricine gel [16]. In each case, an average of 2×10^5 cpm of labelled substrate was added per μg of unlabelled substrate. The reaction was stopped by adding 20 μl of SDS sample buffer and the sample was kept on ice. To facilitate gel band identification, unlabelled head-to-tail dimer and unlabelled monomer (1 μg each) were added to the samples prior to boiling and loading onto the gel. After Coomassie blue stain, bands were excised

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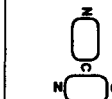
Fig. 1. (A) Silver stained SDS-PAGE of purified 100 kDa isopeptidase and 30 kDa C-terminal hydrolase. Lanes: (1) pH 9 eluate from Ub-Sepharose column; (2) purified 100 kDa isopeptidase; (3) purified 30 kDa C-terminal hydrolase; (4) broad molecular weight markers (kDa). (B) Coomassie blue stained SDS-PAGE profiles of digestion mixtures (10 μl) of polyUb chains with 100 kDa isopeptidase and C-terminal hydrolase. 2.5 μg of dimers (15 μM), 4 μg of nonamer (5 μM) were used in the presence and the absence of 0.1 μM of enzymes; digestion time 60 min at 37°C. Slots are numbered 1–11 at the bottom of the figure. For further details, see section 2.

Table 1
Inhibition of the 100 kDa isopeptidase with synthetic peptides

Substrate	ratio inhib: subs	Peptide inhibitors					
					$G_{75}G_{76}$	motilin	secretin
"head-to-tail" dimer 	1:1	-	-	-	n.d.	-	-
	10:1	++	++	+	n.d.	-	-
	40:1	++	++	++	-	-	-
"branched" dimer 	2:1	+	+	+	n.d.	n.d.	n.d.
	20:1	++	++	+	n.d.	n.d.	n.d.

++ = high inhibition (70-100 %)
+ = medium inhibition (20-70 %)
- = no inhibition (<20 %)
n.d. = not determined

 Ub "branched" peptide
 Ub "head-to-tail" peptide
 Ub "tail" peptide
$G_{75}G_{76}$ Glycylglycine

and ^{125}I radioactivity was counted on a Beckman Gamma 4000 apparatus. For each experiment, a control of substrate incubated without enzyme was loaded onto the gel and counted as described. Each concentration or time point was run in triplicate. Synthetic Ub-derived peptides described above were used as competitive inhibitors at molar ratios ranging from 1:1 to 40:1 relative to substrate concentration. K_M values were calculated using Lineweaver–Burk plots.

3. Results

No cross-contamination between the 100 kDa enzyme and the 30 kDa enzyme was visible, as revealed on the overloaded gel (Fig. 1A). Two minor fast migrating bands in the 100 kDa preparation are visible and reflect degradation products reactive with an antibody to the 100 kDa band. When subjected to analytical ultracentrifugation in 50 mM phosphate buffer, pH 7.2, the high M_r enzyme fraction exhibited a $S_{20,w}$ value of 5.2 S and a molar mass of 110 kDa ± 10%. Negative staining electron microscopy revealed small globules with an average diameter of 9 nm. For a spherical protein of M_r 100,000, a diameter of 7 nm is predicted [17], a figure in good agreement with experimental data. The enzyme fraction behaves as a monomer under reduced conditions (1 mM DTT), and spontaneously forms heavy aggregates upon reoxidation under the electron microscope (data not shown).

The results are illustrated in Fig. 1B; enzymes were added to substrates in a molar ratio of 1:140. The 30 kDa Ub specific C-terminal hydrolase was used as a control: it was unable to cleave peptide bonds in head-to-tail Ub dimers or nonamers (lanes 3, 9) and barely cleaved a branched dimeric Ub (lane 6). This is in agreement with earlier reports [18,19] which suggested that the C-terminal hydrolase was able to specifically split G-76–X bonds in Ub fusion proteins [18] but not in head-to-tail Ub dimers [20]. In contrast, the 100 kDa isopeptidase was able to cleave both isopeptidic bond G_{76}–K_{48} in dimeric branched Ub (lane 5) and G_{76}–M_1 peptide bonds in head-to-tail Ub_2 and Ub_9 polymers (lanes 2 and 8), leading to the accumulation of the monomeric Ub unit. Amino acid sequence determination of the monomeric Ub recovered from gels in both cases indicated the expected N-terminal sequence M-Q-I-F-V of Ub.

The timecourse study (Fig. 2) shows that the branched dimeric Ub was readily cleaved to the extent of about 80% after 5 min and 90% after 20 min, whereas no cleavage was apparent at 5 min and no more than 50% cleavage occurred after 20 min in the case of the head-to-tail dimeric Ub.

The K_M values for both substrates are markedly different, and were determined to be $2 \times 10^{-6} \pm 0.3 \times 10^{-6}$ M for the branched dimeric conjugate and $1.5 \times 10^{-5} \pm 0.5 \times 10^{-5}$ M in the case of the head-to-tail dimeric Ub.

The 100 kDa isopeptidase was unable to cleave small synthetic peptides encompassing the Ub sequence around the isoamide bond G_{76}–K_{48}, or around the head-to-tail bond G_{76}–M_1. The latter peptides and the C-terminal decapeptide (Ub tail) were then used as competitive inhibitors of the cleavage of dimeric Ub substrates (Table 1). Both branched and head-to-tail peptides were strong inhibitors, while the C-terminal decapeptide was less inhibitory. In contrast, the C-terminal glycylglycine dipeptide (G_{75}–G_{76}) was not inhibitory at all, as were two Ub unrelated peptides, motilin and secretin, used as controls. No significant differences in the degree of inhibition mediated by the branched peptide as compared to the head-to-tail peptide was apparent, as both peptides mimick a substrate of the enzyme.

4. Discussion

In 1992, Hadari et al. [11] described the isolation of an 100 kDa isopeptidase T from human red blood cells which acts as a Ub C-terminal enzyme that cleaves high M_r branched

multiUb chains; it, however, did not further disassemble medium-sized multiUb conjugates, nor did it cleave isoamide linkages between branched multiUb chains and target proteins.

Using characterized Ub substrates, we demonstrate here that the 100 kDa isopeptidase, isolated as described above from human red blood cells, is able to preferentially cleave the isoamide bond (G_{76}–K_{48}) in branched Ub dimers into the monomeric Ub moiety, and also that this enzyme cleaves head-to-tail (G_{76}–M_1) nonameric or dimeric polyUb chains into the monomeric Ub moiety, though at a reduced rate. This Ub specific enzyme does not seem to exhibit a highly restricted isopeptidase activity but rather behaves as a Ub specific de-ubiquitinase.

Both peptide analogs (G_{76}–M_1 head-to-tail and G_{76}–K_{48} branched peptides) are not cleaved at all by the isopeptidase but behave as efficient inhibitors (Table 1). It can be hypothesized that the small peptide analogs bind to the active center of the enzyme but that they are most likely too small for the enzyme to induce a distortion of the substrate that would force it toward a stabilized 'transition state'.

The 100 kDa de-ubiquitinase resembles the recently described yeast DOA4 gene product, and perhaps the human *tre*-2 oncogene product [12]. Indeed, DOA4 was reported to cleave both peptide-linked and isopeptide-linked Ub moieties from substrates. In yeast, several de-ubiquitinating enzymes are known, of which their functional diversity may reflect differences in their substrate specificity or in their role within the Ub pathway. De-ubiquitination reactions could be very important in (i) Ub precursor processing into the monomeric Ub moiety, (ii) in removing Ub from branched multiUb substrate conjugates before proteolysis, or (iii) in the proteolytic steps of the degradation pathway. It is not known whether the 100 kDa de-ubiquitinase described in this report may be involved in all three reactions. We show, however, that head-to-tail G_{76}–M_1 Ub polymers can be cleaved in vitro by the enzyme, in contrast to one Ub processing protease from yeast [6], which was active in vivo but inactive in vitro; it remains to be demonstrated whether the enzyme described here can also mediate such Ub precursor processing in vivo.

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