New de-ubiquitinating enzyme, ubiquitin C-terminal hydrolase 8, in chick skeletal muscle

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We have previously shown that chick muscle extracts contained at least 10 different ubiquitin C-terminal hydrolases (UCHs). Here we report the purification and characterization of one of the UCHs, called UCH-8, with ¹²⁵I-labelled ubiquitin- α -NH-MHISPPPESEEEEEHYC as a substrate. The purified UCH-8 behaved as a 240 kDa protein on a Superdex-200 column under non-denaturing conditions but as a 130 kDa polypeptide on analysis by PAGE under denaturing conditions, suggesting that the enzyme consists of two identical subunits. Thus this enzyme seems to be distinct in its dimeric nature from other purified UCHs that consist of a single polypeptide, except that UCH-6 is also a homodimer of 27 kDa subunits. UCH-8 was maximally active between pH 7.5 and 8, but showed little or no activity below pH 7 and above pH 9. Like other UCHs it was sensitive to

inhibition by thiol-blocking agents such as *N*-ethylmaleimide, and by ubiquitin aldehyde. The purified UCH-8 hydrolysed not only ubiquitin- α -NH-protein extensions, including ubiquitin- α -NH-carboxy extension protein of 80 amino acid residues and ubiquitin- α -NH-dihydrofolate reductase, but also branched poly-ubiquitin that are ligated to proteins through ϵ -NH-isopeptide bonds. However, it showed little or no activity against poly-His-tagged di-ubiquitin, suggesting that UCH-8 is not involved in the generation of free ubiquitin from the linear poly-ubiquitin precursors. These results suggest that UCH-8 might have an important role in the production of free ubiquitin and ribosomal proteins from their conjugates as well as in the recycling of ubiquitin molecules after the degradation of poly-ubiquitinated protein conjugates by the 26 S proteasome.

INTRODUCTION

Ubiquitin (Ub) is a highly conserved 76-residue polypeptide involved in a variety of cellular functions, including the regulation of intracellular protein breakdown, cell cycle regulation and stress response [1–6]. This small protein is covalently ligated to target proteins by a family of Ub-conjugating enzymes, called E2s [7,8], through an isopeptide linkage between the C-terminal Gly residue of Ub and the ϵ -amino group of Lys residue(s) of the proteins. Ubs by themselves or that have already been conjugated to proteins can also be ligated to additional Ub molecules to form branched poly-(Ub) by the linkage between the ϵ -amino group of Lys-48 of one Ub and the C-terminus of the other. Proteins ligated to multiple units of Ub are degraded by the ATP-dependent 26 S proteasome [1–3,9].

Ubs are encoded by two distinct classes of gene, neither of which encodes the monomeric form of Ub [10,11]. One is a poly-(Ub) gene encoding a linear polymer of Ubs that are linked through peptide bonds between the C-terminal Gly residue and the N-terminal Met residue of contiguous Ub molecules. The other encodes a fusion protein in which a single Ub is linked to a ribosomal protein consisting of 52 or 76–80 amino acid residues [12]. Thus the generation of free Ub from the linear poly-(Ub) and Ub fusion proteins and the recycling of Ub from the branched poly-(Ub) ligated to proteins should be essential for the Ub-requiring processes, such as intracellular ATP-dependent proteolysis.

A number of Ub C-terminal hydrolases (UCHs) that release Ub molecules conjugated to proteins by α -NH-peptide bonds and/or ϵ -NH-isopeptide linkages have been identified from different sources. *Saccharomyces cerevisiae* contains at least five different UCHs, including yeast ubiquitin hydrolase 1 (YUH1) and ubiquitin-specific protease (UBP)-1 to UBP-4 [13–16]. Of these, UBP-3 has recently been shown to regulate silencing by interaction with SIR4, which is required for the silencing of transcription at the silent mating type loci and at telomeres in yeast [17]. A family of UCHs, named L1, L2, L3 and H2, has been identified from bovine calf thymus [18–20]. Isopeptidase T in human red blood cells has been purified and its cDNA has been isolated [21,22]. In addition, the *fat facets* gene in *Drosophila*, which is required in eye development, and the *DUB-1* gene in mouse pro-B-cell line, which is specifically induced by interleukin-3 and involved in growth suppression, have recently been shown to encode a UCH [23,24]. We too have identified at least 10 different UCHs in chick skeletal muscle by using Ub- α -NH-MHISPPPESEEEEEHYC (referred to as Ub-PESTc) [25–27]. Because the Tyr residue next to the C-terminal Cys can be exclusively radioiodinated, we could assay the UCH activity by simple measurement of the radioactivity of the peptide portion that is released into acid-soluble products.

Although the number of UCHs identified from various organisms is rapidly growing, only a few have been purified and characterized *in vitro*. In the present study we have purified a new UCH, named UCH-8, from chick skeletal muscle and shown

Abbreviations used: CEP80, carboxy extension protein of 80 amino acid residues; DHFR, dihydrofolate reductase; Ub, ubiquitin; His-di-Ub, poly-(His)-tagged di-Ub; UBP, ubiquitin-specific protease; Ub-PESTc, ubiquitin- α -NH-MHISPPPESEEEEEHYC; UCH, ubiquitin C-terminal hydrolase; YUH1, yeast ubiquitin hydrolase 1.

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that the enzyme hydrolyses not only Ub- α -NH-protein extensions but also branched poly-(Ub) that are ligated to proteins through ϵ -NH-isopeptide bonds.

EXPERIMENTAL

Materials

Ub-PESTc, Ub- α -NH-carboxy extension protein of 80 amino acid residues (Ub-CEP80), Ub- α -NH-dihydrofolate reductase (Ub-DHFR) and poly-(His)-tagged di-Ub (His-di-Ub) were purified as described previously [26]. The purified Ub-PESTc was radiolabelled with Na¹²⁵I by using Iodo-Beads (Pierce) [28]. Ub-aldehyde was prepared by borohydride reduction of Ub in the presence of yeast YUH1 as described [29]. Reticulocytes were obtained by subcutaneous injections of phenylhydrazine to albino rabbits [30]. From the cells, fraction II containing the Ub-conjugating system was prepared as described [31]. For preparation of the anti-(UCH-8) serum, the purified UCH-8 was subjected to electrophoresis on a 10% (w/v) polyacrylamide gel under denaturing conditions [32]. After electrophoresis the 130 kDa protein band (see below) was cut out, minced and injected into albino rabbits. IgGs were then isolated from the antiserum by (NH₄)₂SO₄ fractionation.

Preparation of poly-ubiquitinated protein conjugates

To prepare ¹²⁵I-labelled poly-(Ub)- ϵ -NH-protein conjugates, 1 μ g of the ¹²⁵I-labelled Ub [(1–2) $\times$ 10⁶ c.p.m.] was incubated with 720 μ g of fraction II and an ATP-regenerating system consisting of 10 mM Tris/HCl, pH 7.8, 15 units/ml creatine kinase, 6.5 mM phosphocreatine, 1.5 mM ATP, 1 mM dithiothreitol, 0.5 mM MgCl₂ and 1 mM KCl in a final volume of 0.3 ml [31]. Incubations were performed for 2 h at 37 °C in the presence of 1 mM haemin to prevent proteolysis of the ubiquitinated protein conjugates by the 26 S proteasome. After incubation the samples were heated for 10 min at 55 °C to inactivate endogenous UCHs. The resulting samples were freed of excess ¹²⁵I-labelled Ub by treatment with 0.2 M NaOH and 100 mM dithiothreitol followed by gel filtration on a Sephadex G-75 column equilibrated with Tris/HCl (pH 7.8)/1 mM dithiothreitol/1 mM EDTA.

Assay of UCH activity

Reaction mixtures (0.1 ml) contained appropriate amounts of the purified UCH-8 or the chromatographic fractions, and 1 μ g of ¹²⁵I-labelled Ub-PESTc (10⁴ c.p.m./ μ g) in 100 mM Tris/HCl (pH 7.8)/1 mM dithiothreitol/1 mM EDTA/5% (v/v) glycerol. After incubation of the mixtures for various periods at 37 °C, the reaction was terminated by the addition of BSA and trichloroacetic acid to final concentrations of 0.3% and 10% (w/v) respectively. The samples were centrifuged and the resulting supernatants were counted for their radioactivity with a γ -counter. The enzyme activity was expressed as a percentage of ¹²⁵I-labelled Ub-PESTc hydrolysed into acid-soluble products.

When assaying the hydrolysis of Ub- α -NH-carboxy extension proteins, His-di-Ub or ¹²⁵I-labelled poly-(Ub)- ϵ -NH-protein conjugates, incubations were performed as above but in the presence of 5 μ g of the substrate. After incubation for appropriate periods, the samples were subjected to discontinuous PAGE (see below). Proteins in the gels were then detected by staining with Coomassie Blue R-250 or by exposure to X-ray films (Fuji) at –70 °C.

Preparation of muscle extracts

Chick pectoralis muscle tissues (2 kg) were minced and homogenized with a Waring blender in buffer A [25 mM Tris/HCl buffer (pH 7.8) containing 5 mM 2-mercaptoethanol, 1 mM EDTA and 10% (v/v) glycerol] in the presence of 100 mM NaCl. The homogenates were centrifuged at 10000 *g* for 1 h to remove cell debris; their supernatants were centrifuged again at 100000 *g* for 2 h. The resulting supernatants were titrated with 1 M Tris base to pH 7.8 and are referred to as the muscle extracts.

Electrophoresis

PAGE in the presence of SDS and 2-mercaptoethanol was performed as described by Laemmli [32] or by using Tris/Tricine buffer as described by Schagger and von Jagow [33]. The discontinuous slab gels contained 4%, 10% and 16% (w/v) polyacrylamide to improve the resolution of small proteins. The sample buffer contained 150 mM Tris/HCl (pH 6.8), 1.5% (w/v) SDS, 2% (v/v) 2-mercaptoethanol, 0.002% (w/v) Bromophenol Blue and 7% (v/v) glycerol. After electrophoresis the gels were stained with Coomassie Blue R-250 or autoradiographed.

RESULTS

Purification

To separate UCH-8 from the other UCHs, crude extracts (78 g of protein) were prepared from 2 kg of chick skeletal muscle [26]. The activity of UCH-8 in chromatographic fractions obtained during purification was monitored by determining its ability to hydrolyse ¹²⁵I-labelled Ub-PESTc. The extracts were applied to a DEAE-Sepharose column (5 cm $\times$ 30 cm) equilibrated with buffer A, pH 7.8, containing 0.1 M NaCl. After the column had been washed with the same buffer, the bound proteins were eluted with 0.3 M NaCl. The sample was diluted with an equal volume of buffer A and loaded on a heparin-Sepharose column (2.5 cm $\times$ 14 cm) equilibrated with buffer A containing 0.2 M NaCl. Proteins bound to the column were eluted with 0.5 M NaCl, dialysed against buffer A and loaded on a Mono-Q column (0.5 cm $\times$ 5 cm). After the column had been washed with

Figure 1 Separation of UCH-8 by a phenyl-Superose column

The UCH-8 preparation obtained from the Superose-6 column was subjected to a phenyl-Superose column as described in the text. Fractions of 0.5 ml were collected and aliquots (5 μ l) of them were assayed for their ability to hydrolyse ¹²⁵I-labelled Ub-PESTc (1 μ g) in the presence of 0.1 mg/ml poly-(L-Lys) (●) as described in the Experimental section. The broken line shows the reverse gradient of (NH₄)₂SO₄ and the dotted line indicates the protein profile.

Table 1 Summary of purification of UCH-8

One unit of enzyme activity is defined as 1 μ g of Ub-PESTc hydrolysed to acid-soluble products per hour. Abbreviation: n.d., not determined.

Step	Protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)
Crude extract	78 000	n.d.*	n.d.	n.d.
DEAE-Sepharose	1 410	n.d.	n.d.	n.d.
Heparin-Sepharose	39.2	41 600	1061	100
Mono-Q	6.5	17 300	2670	42
Superose-6	0.57	2700	4740	6.5
Phenyl-Superose	0.01	600	60 000	1.4

Figure 2 Estimation of the size of UCH-8

(A) Purified enzyme (7 μ g) was subjected to electrophoresis on a 10% (w/v) polyacrylamide slab gel containing SDS and 2-mercaptoethanol, as described by Laemmli [32]. Proteins in the gel were then revealed by staining with Coomassie Blue R-250. The arrow in lane a indicates the 130 kDa UCH-8 band. Lane m, protein size markers; their molecular masses are indicated at the left. (B) The enzyme was also subjected to chromatography on a Superdex-200 column (2.6 cm $\times$ 60 cm) equilibrated with buffer A containing 0.1 M NaCl. Fractions of 1 ml were collected and assayed for their ability to hydrolyse ^{125} I-labelled Ub-PESTc in the presence of 0.1 mg/ml poly-(L-Lys). The size markers used were: a, thyroglobulin (669 kDa); b, apoferritin (443 kDa); c, β -amylase (200 kDa); d, alcohol dehydrogenase (150 kDa); e, BSA (66 kDa).

buffer A containing 0.1 M NaCl, bound proteins were eluted with a linear gradient of 0.1–0.4 M NaCl. Fractions with high activity were pooled, dialysed against buffer A containing 0.1 M NaCl and loaded on a Superose-6 column (1 cm $\times$ 30 cm) equilibrated with the dialysis buffer. The active fractions were pooled, added to the same volume of buffer A containing 2.4 M $(\text{NH}_4)_2\text{SO}_4$ and applied to a phenyl-Superose column (0.5 cm $\times$ 5 cm). After the column had been washed with buffer A containing 0.9 M $(\text{NH}_4)_2\text{SO}_4$, bound proteins were eluted with a reverse gradient of $(\text{NH}_4)_2\text{SO}_4$ from 0.9 to 0.3 M. Fractions under the symmetrical peak of UCH-8 activity (Figure 1) were pooled, dialysed against buffer A, concentrated by ultrafiltration and stored at -70°C until use. A summary of the purification of UCH-8 is shown in Table 1. On comparing the capacity for degrading Ub-PESTc, the total activity of UCH-8 constituted approx. 1% of that in the soluble muscle extract (results not shown).

The size of the enzyme was estimated to be about 240 kDa by chromatography on a Superdex-200 column (2.6 cm $\times$ 60 cm)

Figure 3 Effect of the antibody against UCH-8 on the hydrolysis of Ub-PESTc

Aliquots (20 ng each) of the purified UCH-8 were incubated with increasing amounts of the anti-(UCH-8) IgG (●) or preimmune IgG (○) for 2 h at 4°C . The samples were then centrifuged in a Microfuge for 5 min to remove immunoprecipitates. After centrifugation the supernatants were assayed for their ability to hydrolyse ^{125} I-labelled Ub-PESTc in the presence of 0.1 mg/ml poly-(L-Lys). The enzyme activity in the absence of any IgG was taken as 100%; the others are expressed as values relative to that.

equilibrated with buffer A containing 0.1 M NaCl (Figure 2B). In contrast, it ran as a single protein of 130 kDa on analysis by PAGE in the presence of SDS and 2-mercaptoethanol (Figure 2A), suggesting that the protein was purified to apparent homogeneity. Thus it seems that the purified UCH-8 is a homodimer of 130 kDa subunits. To confirm that the purified enzyme preparation is indeed responsible for the Ub-PESTc-hydrolysing activity, immunoprecipitation analysis was performed by incubating the protein with increasing amounts of the antibody raised against the 130 kDa band or preimmune IgG for 2 h at 4°C . After centrifugation of the samples, the supernatants were assayed for their ability to hydrolyse ^{125} I-labelled Ub-PESTc. As shown in Figure 3, treatment of the anti-IgG, but not the preimmune IgG, prevented the enzyme activity in a dose-dependent fashion. These results indicate that the purified UCH-8 is responsible for the hydrolysis of Ub-PESTc.

Biochemical properties

We have previously shown that poly-(L-lysine) stimulates the Ub-PESTc-hydrolysing activity of all the UCHs, except UCH-1, that were partly purified from chick skeletal muscle [26]. To determine whether the activity of the purified UCH-8 is also

Figure 4 Effect of poly-(L-lysine) on the hydrolysis of Ub-PESTc by UCH-8

(A) Reaction mixtures containing 20 ng of UCH-8 and 1 µg of ^{125} I-labelled Ub-PESTc $[(1-2) \times 10^4$ c.p.m./µg] were incubated in the absence (○) or presence of 0.1 mg/ml of poly-(L-Lys) (●) for various periods at 37 °C. (B) The enzyme activity was also assayed as above but in the presence of increasing amounts of poly-(L-Lys). Incubations were performed for 30 min at 37 °C.

stimulated by poly-(L-lysine), the enzyme was incubated with ^{125} I-labelled Ub-PESTc in the presence and the absence of the reagent. As shown in Figure 4, poly-(L-lysine) markedly increased the rate of hydrolysis of ^{125} I-labelled Ub-PESTc; its stimulation occurred in a concentration-dependent fashion. Half-maximal stimulation was observed in the presence of 0.5 µg/ml poly-(L-lysine). The enzyme activity was also stimulated by poly-(L-arginine) to a similar extent, but not by other polycationic agents such as spermine, spermidine or putrescine (results not shown). Thus the stimulation of UCH-8 activity by poly-(L-lysine) seemed not to be due to a simple charge effect. To define more precisely the stimulatory effect of poly-(L-lysine), the rate of Ub-PESTc hydrolysis was determined in the presence of increasing concentrations of the substrate. By using double-reciprocal plots of the data, the K_m for Ub-PESTc was found to decrease from 17 to 0.2 µM on treatment with poly-(L-lysine). However, the V_{max} for Ub-PESTc hydrolysis was similar in the presence of poly-(L-

Figure 5 Effect of pH on the hydrolysis of Ub-PESTc by UCH-8

Effect of various pH values on the hydrolysis of ^{125} I-labelled Ub-PESTc by UCH-8 was determined with various buffers in the absence (○) or presence of 0.1 mg/ml poly-(L-Lys) (●). The buffers used were: sodium acetate (pH 4–5), sodium phosphate (pH 6–7), Tris/HCl (pH 7.5–8.5) and Na/glycine (pH 9–11). The reaction mixtures were incubated at 37 °C for 30 min.

Table 2 Effects of various protease inhibitors on the hydrolysis of Ub-PESTc by UCH-8

The purified UCH-8 was dialysed against buffer A that lacked EDTA. Aliquots (20 ng) of the dialysed enzyme were then incubated with the indicated reagents for 10 min at room temperature. After incubation the samples were assayed by further incubation with 1 µg of ^{125} I-labelled Ub-PESTc for 30 min at 37 °C. The activity without any addition was taken as 100%; the others are expressed as values relative to that.

Addition	Concentration	Relative activity (%)
None	—	100
Ub-aldehyde	10 nM	58
	50 nM	21
<i>N</i> -Ethylmaleimide	0.1 mM	17
	1 mM	4
PMSF	1 mM	102
EDTA	10 mM	98
<i>o</i> -Phenanthroline	1 mM	97

Table 3 Effects of cations on the hydrolysis of Ub-PESTc by UCH-8

Assays were performed as in Table 2 but in the presence of various monovalent and divalent cations. The activity without any addition was taken as 100%; the others are expressed as values relative to that.

Addition	Concentration (mM)	Relative activity (%)
None	—	100
CaCl ₂	2	89
	5	41
MgCl ₂	2	87
	5	39
MnCl ₂	2	24
	5	2
KCl	50	48
	200	8

lysine) (10 pmol/min) and in its absence (9.2 pmol/min). Thus it seems that poly-(L-lysine) increases the affinity of Ub-PESTc for UCH-8 without changing the V_{max} for the hydrolysis of the substrate. The enzyme was maximally active between pH 7.5 and 8, but was nearly inactive below pH 7 and above pH 9 (Figure 5), whether or not poly-(L-lysine) was present.

UCH-8 was highly sensitive to inhibition by thiol-blocking reagents such as *N*-ethylmaleimide (Table 2), suggesting that the enzyme contains a conserved Cys residue for catalysis, similar to other known UCHs [22]. Ub-aldehyde, which is known to inhibit the activity of certain mammalian UCHs including isopeptidase T [34,35], also strongly inhibited the hydrolysis of ^{125} I-labelled Ub-PESTc by UCH-8, whereas Ub did not show any effect. Inhibitors of serine proteases, such as PMSF, or metal-chelating agents, such as *o*-phenanthroline, showed little or no effect. Similarly to its effect on the mammalian UCH-L2 and UCH-H2 [18], Mn²⁺ at 5 mM strongly inhibited the hydrolysis of Ub-PESTc by UCH-8 (Table 3). The enzyme activity was also moderately inhibited by 5 mM Ca²⁺ and Mg²⁺ and much more strongly by 0.2 M KCl. Nearly identical results were obtained when the effects of the inhibitors were tested in the absence of poly-(L-lysine).

Substrate specificity

To determine the substrate specificity, the purified UCH-8 was incubated with various Ub- α -NH-protein extensions, such as

Figure 6 Hydrolysis of various Ub- α -NH-protein extensions by UCH-8

Aliquots (5 μ g each) of the purified Ub-PESTc (**A**), Ub-CEP80 (**B**), Ub-DHFR (**C**) and His-di-Ub (**D**) were incubated alone (lanes a) or with 20 ng of UCH-8 (lanes b) for 2 h at 37 °C. After incubation the samples were subjected to discontinuous PAGE under denaturing conditions as described in the Experimental section. Proteins in the gels were then revealed by staining with Coomassie Blue R-250. The arrowheads indicate the substrates and the cleavage products. The protein band seen just above His-di-Ub (in lane b of **D**) is an unknown protein contaminant introduced during preparation of the substrate. The positions of molecular mass markers are shown at the left.

Figure 7 Hydrolysis of the branched poly-(Ub) chains by UCH-8

Poly-(Ub) protein conjugates were prepared by incubating ^{125} I-labelled Ub and reticulocyte lysate fraction II as described in the Experimental section. The radiolabelled protein conjugates were then incubated alone (lane b) or with 20 ng of UCH-8 in the absence (lane c) or presence of 1 mM *N*-ethylmaleimide (lane d) or 0.1 μ M Ub-aldehyde (lane e) for 2 h at 37 °C. ^{125} I-labelled Ub alone is also shown (lane a). After incubation the samples were subjected to discontinuous PAGE under denaturing conditions followed by autoradiography. The positions of molecular mass markers are shown at the left.

Ub-DHFR, Ub-CEP80 and His-di-Ub. After incubation the samples were subjected to discontinuous PAGE under denaturing conditions followed by staining with Coomassie Blue R-250. Assays were also performed with Ub-PESTc as a control. As shown in Figure 6, UCH-8 could generate free Ub from Ub-DHFR and Ub-CEP80. However, it was not capable of hydrolysing His-di-Ub, nor could it release free Ub from the mono-Ub- ϵ -NH-conjugate of Ub(RGA)-P-ARRKWQKTGH-

AVRAIGRLSS, in which the C-terminal Gly residue of Ub was replaced by Ala, and a Pro residue was inserted at the N-terminus of the peptide of 20 residues for preventing the action of the enzyme preparation on the α -NH-peptide bond between Ub and the extension peptide (results not shown).

To determine whether the purified UCH-8 could act on branched poly-(Ub) chains, poly-(Ub)- ϵ -NH-protein conjugates were prepared by the incubation of ^{125} I-labelled Ub with reticulocyte lysate fraction II. The radiolabelled poly-(Ub)-protein conjugates were then incubated with the purified UCH-8. After incubation the amount of free Ub was increased significantly, with a concomitant decrease in the amount of the high-molecular-mass conjugates (Figure 7). Treatment with *N*-ethylmaleimide or Ub-aldehyde strongly inhibited the generation of free Ub from the conjugates. The antibody against the 130 kDa UCH-8 polypeptide also prevented the Ub-releasing activity (results not shown). Similar inhibitory effects were observed for the hydrolysis of Ub-DHFR and Ub-CEP80 by UCH-8. These results demonstrate that UCH-8 also has an isopeptidase activity against branched poly-(Ub) chains that are conjugated with proteins as well as against Ub- α -NH-protein extensions. However, poly-(L-lysine) showed little or no effect on the hydrolysis of these substrates (results not shown), unlike that of Ub-PESTc. Thus it remains obscure how poly-(L-lysine) can specifically stimulate the activity of UCH-8 against Ub-PESTc.

DISCUSSION

In the present study, a new UCH, called UCH-8, in chick skeletal muscle was purified to apparent homogeneity by using ^{125} I-labelled Ub-PESTc as a substrate. From 2 kg of chick skeletal muscle we obtained approx. 10 μ g of the purified enzyme. The purified UCH-8 behaves as a dimer of 130 kDa subunits under non-denaturing conditions. The other UCHs so far reported comprise a single polypeptide chain [13–16,35] except UCH-6 in chick skeletal muscle, which is also a homodimer of 27 kDa subunits [26]. UCH-8 is capable of hydrolysing not only Ub- α -NH-protein extensions, such as Ub-CEP80 and Ub-DHFR, but also branched poly-(Ub). However, it is unable to release free Ub from His-di-Ub or mono-Ub- ϵ -NH-protein conjugates. Thus UCH-8 in muscle might have a role in the generation of free Ub and the 52 and 80 kDa ribosomal proteins from Ub-ribosomal protein fusions, and in the recycling of Ub molecules after degradation of poly-(Ub)- ϵ -NH-protein conjugates by the 26 S proteasome.

It is noteworthy that poly-(L-lysine) markedly stimulates the Ub-PESTc-cleaving activity of UCH-8. It increases the affinity of the enzyme for Ub-PESTc approx. 80-fold but with relatively little effect on the $V_{\max}$ for the hydrolysis of the substrate. However, the same agent does not exhibit any stimulatory effect on the hydrolysis of Ub-CEP80, Ub-DHFR or branched poly-(Ub) (results not shown). We have previously shown that poly-(L-lysine) stimulates the Ub-PESTc-cleaving activity of all of the UCHs (i.e. UCH-2 to UCH-10) partly purified from chick skeletal muscle, except UCH-1. In addition, UCH-1 does not bind to a DEAE column at neutral or slightly alkaline pH (e.g. 7.8), whereas the others, including UCH-8, interact strongly with the ion-exchange matrix. These results suggest that the enzymes that are stimulated by poly-(L-lysine) have acidic pI values, whereas UCH-1 has a neutral or alkaline pI. It has recently been reported that the UCHs from yeast, from *Drosophila* and from human neuronal and B-cells have acidic pI values (approx. 5.0) [21,36]. Of these enzymes, we have shown that the activity of YUH1 against Ub-PESTc is also markedly stimulated by poly-(L-lysine). Therefore it seems that poly-(L-lysine) might facilitate

the interaction between the enzymes with acidic pI values and the Ub-PESTc substrate by neutralizing their negative charges under the assay conditions. However, it remains unclear why the other small polycationic reagents such as spermine, spermidine or putrescine are unable to stimulate the Ub-PESTc-cleaving activity of UCH-8 and the other UCHs with acidic pI values.

A large number of proteolytic enzymes, isolated from various tissues and organisms, have been shown to cleave specifically the carboxy side of the C-terminal Gly residue of Ubs that are linked to adjacent Ub molecules or proteins through an α -NH-peptide bond or an ϵ -NH-isopeptide bond. The proteases with this specificity have been named UCHs, UBPs or isopeptidases. Therefore Wilkinson and co-workers have grouped the proteases into two families [36]. The UCH family is a group of closely related thiol proteases, including the mammalian UCH-L1, UCH-L2 and UCH-L3 as well as YUH1, all of which have a relatively small size of 20–30 kDa and exhibit no apparent similarity to the UBP family. Because these enzymes prefer to cleave off small leaving groups and/or extended peptide chains from the C-terminus of Ub, it has been suggested that they are involved in the processing of the linear poly-(Ub) precursors and Ub- α -NH-extension proteins (i.e. Ub-CEP52 and Ub-CEP80), both of which are the Ub gene products [36].

The UBP family, which is also known as the UCH family 2, consists of several distantly related proteases of 50–300 kDa and shows similarities around the active site thiol and histidine residues. This family includes the mammalian isopeptidase T and the yeast UBP-1 to UBP-4. Because many of these enzymes are capable of processing both an α -NH-peptide bond and an ϵ -NH-isopeptide bond, it has been postulated that they are involved in the hydrolysis of various Ub-fusion proteins and/or the branched poly-(Ub) chains [36]. The purified UCH-8 from chick skeletal muscle has a subunit size of 130 kDa and cleaves both branched poly-(Ub) chains and certain Ub- α -NH extension proteins. Thus it seems that UCH-8 belongs to the UBP family. In this regard, a systematic nomenclature system might be required for the newly isolated enzymes that are involved in processing of various Ub-conjugates.

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