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CHARACTERIZATION OF THE 30-kDa ENZYME FROM RED BLOOD CELLS THAT CLEAVES UBIQUITIN-PROTEIN CONJUGATES

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A 30-kDa enzyme from red blood cells responsible for the recycling of free Ubiquin has been characterized. This enzyme was previously known to have a Ubiquitin-C-terminal hydrolase activity on adducts of Ubiquitin to small compounds. In this study it was shown that the 30-kDa enzyme contained Ubiquitin-C-terminal hydrolase activity toward biosynthetic precursors of Ub as well as isopeptidase activity toward Ubiquitin histone conjugates. Detailed inhibition experiments with Ubiquitin aldehyde, iodoacetamide, and heat inactivation showed that the enzyme isopeptidase activity was affected differently from its hydrolase activity. © 1994 Academic Press, Inc.

The polypeptide ubiquitin (Ub) is involved in at least three types of cellular functions. One role is in intracellular protein breakdown in which conjugation with Ub marks proteins for degradation (1-3). The second function is non-proteolytic modification of protein structure as seems to be the case in the attachment of Ub to certain histones (4). The third process that requires the liberation of Ub is in the course of its biosynthesis, since Ub genes are either arranged in head-to-tail poly-Ub arrays or are fused to ribosomal proteins (5-7).

In all cases, Ub is linked in its C-terminus to an amino group in proteins. An essential feature of all the above processes is the necessity to generate free Ub. Enzymes which liberate Ub attached to the N-termini of the protein are denoted as "Ub-C-terminal hydrolases". Those which act on Ub-protein conjugates where the Ub is attached via its C-terminus to ε-amino groups of Lys residues of the protein, are called "Ub-C-terminal isopeptidases".

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ABBREVIATIONS: Ub, ubiquitin; Ubal, ubiquitin C-terminal aldehyde; HUbCEP-52, human ubiquitin carboxyl extension protein (CEP of 52 amino acids in length); HαLA, human α-lactalbumin; UbMT, ubiquitin metallothionein; 1.6 Ub, a fusion protein between Ub and 60% of its N-termini; A_{24}, histone H_2A conjugated to ubiquitin; $UbNH_2$, ubiquitin C-terminal amide; BSA, bovine serum albumin; DTT, dithiothreitol.

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Several Ub-C-terminal isopeptidases and hydrolases from reticulocytes and erythrocytes have been described (8-10), but have not been fully characterized. In the present study, the 30-kDa hydrolase (8) from erythrocytes was characterized in detail.

MATERIALS AND METHODS

Materials. Ub from bovine erythrocytes, HαLA, ovalbumin and molecular-mass marker proteins were obtained from Sigma (St. Louis MO 63178). Ubal was prepared as previously described (11).

Preparation of substrates.

(1) The expression of the HUbCEP-52 gene and the purification of the protein performed as previously described (12). The plasmid containing the HUbCEP-52 gene was generously provided by Dr. T.R. Butt (Dept. Molecular Pharmacology, Smith, Kline & French Laboratories, King of Prussia PA 19406-0939).

(2) A plasmid constructed for the expression of the UbMT gene in *Escherichia coli* was generously provided by Dr. T.R. Butt, and was expressed and purified as previously described (13).

(3) A plasmid constructed for the expression of the 1.6 Ub gene in *E. coli* was expressed and purified in the same way as the HUbCEP-52 protein (14).

(4) A_{24} was purified from calf thymus as previously described (15).

(5) $UbNH_2$ was made by transpeptidation as previously described (16), in a reaction mixture containing Ub, glycylglycine amide and trypsin.

(6) Conjugates of HαLA with Ub were prepared as previously described (10).

Determination of hydrolase and isopeptidase activities by the E1-end point assay. The release of free Ub from different substrates was monitored by an end-point assay that measures the formation of [^{3}H]AMP-Ub with E1 (17). E1 used for Ub assay was freed of contamination by Ub and Ub C-terminal hydrolase as follows. E1 isolated from extracts of human erythrocytes (18) was separated from residual Ub by chromatography on DE-52 (Whatman) as previously described for the removal of Ub from E3 (19). About 1 mg of protein of this preparation was applied to a 10 ml column of Ub-Sepharose equilibrated with 50 mM Tris-HCl (pH 7.2), 1 mm DTT, 0.1 mm EDTA, and 1 mg/ml ovalbumin. Under these conditions (in the absence of ATP), E1 does not bind to the Ub column, whereas Ub C-terminal hydrolase does. The fraction not adsorbed to the Ub column contained purified E1. Prior to the assay, samples were incubated with the appropriate substrate for 60 min at 37°C in the presence of 20 mM Tris-HCl (pH 7.6), 1 mm DTT, 1 mg/ml BSA, and 0.1 mm EDTA in a final volume of 10 μl. Unlabeled ATP was removed from HαLA-Ub conjugates by repeated precipitation with 12% trichloroacetic acid as described (20), except that following the second precipitation, the sediment was dissolved in 90 μl of 0.1 N NaOH and then neutralized with 1 N HCl in the presence of 10 mM Tris, pH 7.6. *A_{24} differs from A_{24} having a shorter histone moiety which is attached to the Ub. Following this incubation appropriate aliquots were mixed with 50 mM Tris-HCl (pH 7.6), 5 mM $MgCl_2$, 10 pmol of [2,8-^{3}H]ATP (ICN, Irvine CA 92713; 30 Ci/mmol), and 2 pmol of iodoacetamide-treated E1 in a final volume of 20 μl. Following incubation of 37°C for 20 min, [^{3}H]AMP-Ub was precipitated with 12% trichloroacetic acid and radioactivity in the precipitate was determined as described (20).

Preparation of the 30-kDa enzyme. The 30-kDa enzyme was purified according to the procedure described by Pickart *et al.* (8), with some modificatins. Fraction II (protein bound to DEAE-cellulose and eluted with 0.5 M KCl) was prepared from human erythrocytes as previously described (21). It was subjected to affinity chromatography on Ub-Sepharose in the absence of ATP as previously described for the purification of E3 from reticulocytes (22). Four milligrams of this column eluate at pH 9.0 ("pH 9 eluate") were subjected to gel filtration on an Ultrogel AcA-34 column (0.9 x 56 cm) (Pharmacia Biotech Inc., Piscataway NJ 08854), equilibrated with 20 mM Tris-HCl (pH 7.2), 1 mM DTT, and 1 mg/ml ovalbumin. Fractions of 1 ml were collected at 4°C. Fractions were assayed for isopeptidase activity on A_{24} and hydrolase activity on $UbNH_2$. Fractions containing the 30-kDa Ub-C-terminal hydrolase (8), were pooled. The enzyme was further purified by anion-exchange chromatography on an FPLC Mono-Q HR 5/5 column as previously described (10). The 30-kDa enzyme was purified by this step to ≥95% homogeneity as previously described (8).

RESULTS

Characterization of the 30-kDa enzyme. Different proteins from erythocytes eluted from a Ub-Sepharose affinity column at pH 9.0 were shown to possess either Ub-C-terminal isopeptidase activity or Ub-C-terminal hydrolase activity (8,10,23,). One is a 30-kDa enzyme that was previously known to have a Ub-C-terminal hydrolase activity on adducts of Ub to small compounds (8,23).

The specific activities of the purified 30-kDa using different substrates (Table 1) were determined. The enzyme possessed a high specific activity toward fusion proteins such as HUbCEP-52 and 1.6 Ub, and lower specific activity toward UbMT (Table 1). In addition the 30-kDa enzyme had an isopeptidase activity toward A_{24} and $*A_{24}$. Surprisingly, the specific activity of the enzyme toward $*A_{24}$ was 308 times higher than toward A_{24} (Table 1). When Ubal is incubated simultaneously with the substrate (Expt. II, Table 2), R value of HUbCEP-52 was 5-fold, and R value of $UbNH_2$ was 82-fold as observed in Expt. I. No isopeptidase activity toward branched poly-Ub-chains (HαLA-Ub) was observed. This activity was carried out exclusively by the 100-kDa isopeptidase (10; and data not shown).

Ubaldehyde treatment. Ubaldehyde (Ubal) strongly inhibits isopeptidases and hydrolases by binding to the active site of these enzymes (20,23). The effect of this inhibition on the activities of the 30-kDa, was determined. Two types of experiments were done. In the first, the enzymes were preincubated with Ubal prior to addition of the substrate, and in the second type, the effect of the inhibition was monitored after simultaneous addition of substrate and Ubal. The inhibition results are expressed by the value "R" which represents the molar ratio between Ubal and the enzyme, under conditions that cause 50% inhibition of enzyme activity (Table 2).

Following preincubation with Ubal (Expt. I, Table 2), the R values were between 0.5 and 2.0 toward the different substrates. The slightly high R value (R=2) obtained toward $UbNH_2$ could represent high affinity of this substrate to the active site while representing a low dissociation rate of the inhibitor from the enzyme. A 5-fold increase in R value was monitored with HUbCEP-52 and an 82-fold increase with $UbNH_2$ in Expt. II, relative to Expt. I (Table 2).

Table 1: Specific activity of the Ub recycling enzyme. The activity was assayed in the peak fractions of the enzyme eluted from the mono-Q HR5/5 column as described in *Methods*. The activity of the enzyme was linear with time and concentration. One μl of the 30-kDa enzyme contained 6.1×10^{-2} μg of enzyme protein.

Substrate	Specific Activity nmol Ub liberated/μg protein/hr
	30kDa
$UbNH_2$	533.00
HUbCEP-52	128.00
1.6Ub	87.00
UbMT	17.00
A24	2.87
A24*	885.00

Table 2: *Inhibition of the 30-kDa enzyme by Ubal.* Experiment I: Aliquots of the 30-kDa enzyme (in the linear range of enzyme concentration which released 1 pmol of Ub per hr from each substrate, as described in Table 1), were incubated with Ubal in the range of 5×10^{-5} to 50 pmol, in the absence of substrate. Following a 10-min incubation at 37°C in the presence of 20 mM Tris-HCl (pH 7.6), 1 mM DTT, and 0.1 mM EDTA, the appropriate substrate was added to each corresponding system and the incubation was continued for another 60 min. Activities were assayed as described in *Methods*. Experiment II: As experiment I, except that Ubal was added simultaneously with the substrate. The reaction was carried out for 60 min.

R (50% inhibition) = pmol Ubal / pmol enzyme

Substrate	Experiment No. I	II
	Following preincubation with Ubal	Without preincubation
	30 kDa	30 kDa
HUbCEP-52	1.0	5.0
$UbNH_2$	2.0	164.0
UbMT	0.5	0.5
1.6Ub	0.5	0.5
A24	0.5	0.5

These results are consistent with the assumption that $UbNH_2$ and to a lesser extent HUbCEP-52 have high affinity toward the 30-kDa enzyme. Therefore, under conditions of "free competition" which existed in Exp. II, these substrates were bound better to the enzyme then Ubal. On the other hand, other substrates like UbMT, 1.6 Ub, and A_{24} apparently have much lower affinity toward the enzyme and therefore their competition with Ubal on binding to the active site was insignificant.

Iodoacetamide treatment. The enzyme was treated with iodoacetamide which is known to inactivate isopeptidases and hydrolases by binding to their active sites (20,23). It was found that the 30-kDa enzyme activity toward 1.6 Ub and $UbNH_2$ was strongly inhibited by 5 mM iodoacetamide (95% inhibition) (Fig. 1). In contrast, iodoacetamide inhibited the activity toward A_{24} only by 50%.

Heat treatment. The activity of the 30-kDa enzyme toward A_{24} was very sensitive to heat treatment (70% activity inhibition after preincubation for 60 min at 37°C), as shown in Fig. 2. In contrast, its activity toward $UbNH_2$ and HUbCEP-52 was unchanged under the same conditions.

DISCUSSION

A monomeric Ub recycling enzyme from erythrocytes of molecular mass of 30-kDa was characterized. In previous studies it was shown that the purified 30-kDa enzyme possessed only hydrolase activity on adducts of Ub to small compounds (8,23). In this study the 30-kDa enzyme was also found to possess isopeptidase activity toward ubiquitinated histone H_2A and hydrolase activity toward biosynthetic precursors of Ub.

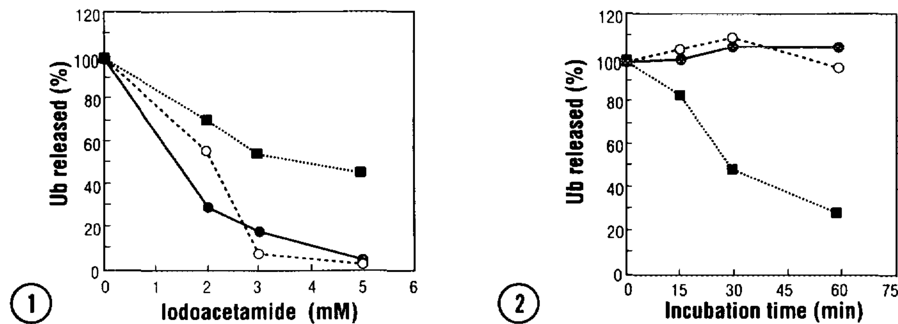

Fig. 1. **Effect of iodoacetamide on the activities of the 30-kDa enzyme.** Each enzyme (0.2 pmol) was incubated in 50 μl of a mixture consisting of 20 mM Tris-HCl (pH 7.6), 0.1 mM EDTA, 1 mM DTT, and 1 mg/ml BSA in the presence of increasing concentration of iodoacetamide for 10 min at 20°C. Following preincubation, iodoacetamide was neutralized by addition of 4 mM DTT. Activities of the enzyme were monitored as described in *Methods*. Enzyme activities of the 30-kDa enzyme were assayed using the following: A_{24} (■), 1.6 Ub (O), and $UbNH_2$ (●).

Fig. 2. **Effect of heat treatment on the activities of the 30-kDa enzyme.** The enzyme (0.2 pmol) was incubated in 50 μl of a mixture consisting of 20 mM Tris-HCl (pH 7.6), 0.1 mM EDTA, 1 mM DTT, and 1 mg/ml BSA for different periods of time. Following preincubation the activities were monitored as described in *Methods*. Enzyme activities of the 30-kDa enzyme were assayed using the following substrates: A_{24} (■), HUbCEP-52 (●), and $UbNH_2$ (O).

The activities of the purified enzyme were tested toward a number of fusion proteins and A_{24} (Table I). The relatively high specific activity of the 30-kDa enzyme toward 1.6 Ub, HUbCEP-52, and to some extent UbMT versus A_{24}, may reflect a major role of the 30-kDa protein in processing Ub precursors during its biosynthesis. Also, the Ubal inhibition experiments (Table 2) showed that under free competitive conditions between the inhibitor and a fusion protein substrate, only HUbCEP-52 could compete with Ubal on the binding to the enzyme (R=5 in Expt. II vs. R=0.5 in Expt. I). Since HUbCEP-52 was the only natural substrate tested, this observation may suggest a requirement for substrates of a specific structure.

The 30-kDa enzyme also acts on A_{24} in which histone H_2A is attached to mono-Ub by isopeptide bond (Table 1). A different isopeptidase containing this kind of activity has been described by Matsui *et al.* (25,26). Its role is probably in chromatin rearrangement during mitosis, since it is bound much better to the chromatin at this stage of the cell cycle. It may be that the 30-kDa enzyme participates in this process.

*A_{24} and $UbNH_2$, are substrates on which the enzyme has the highest specific activity (Table 1). A possible explanation for the activity toward $UbNH_2$ is that the enzyme is capable of recycling Ub from adducts of Ub to small compounds. This explanation has already been suggested by Pickart and Rose in regard to the 30-kDa hydrolase (8).

Moreover, the high competition of $UbNH_2$ with Ubal (Table 2) suggests high affinity of the enzyme to this substrate.

*A_{24} is a proteolytic product of A_{24} in which the histone portion is proteolyzed to a molecular mass of approximately 12-kDa (data not shown). This change resulted in a large induction in the specific activity of the 30-kDa enzyme toward *A_{24} compared to A_{24}, similar to

the value obtained toward $UbNH_2$ (Table 1). Thus, the 30-kDa Ub-C-terminal isopeptidase can play a role in removing the last Ub from the substrate destined for degradation.

A major difference observed between the 100 kDa isopeptidase (10) and the 30 kDa enzyme was the exclusive capability of the 100-kDa isopeptidase to liberate free Ub from conjugates of HαLA with poly-Ub chains (data not shown). The foregoing observations emphasize the difference between the characteristics of the 30-kDa enzyme and the 100-kDa isopeptidase regarding isopeptidase activity and may reflect different roles in the Ubiquitin system. For example, the 30-kDa enzyme can release Ub from products of the protein degradation system such as short peptides which have isopeptide bond to Ub, while the 100-kDa isopeptidase can reverse the modification of larger proteins by removing their Ub moiety.

Iodoacetamide (Fig. 1) inhibited the hydrolase activity of the 30-kDa protein more than its isopeptidase activity. Furthermore, heat treatment (Fig. 2) inhibited *only* the isopeptidase activity. These findings may indicate that the 30-kDa enzyme possesses two separate active sites, one for isopeptidase and one for hydrolase.

Obviously, further study is required to elucidate the integration of the 30-kDa activities with the Ubiquitin pathway. Also, it is important to investigate the mode of action of the enzyme toward other potential substrates under different physiological conditions.

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