Ubiquitin carboxyl-terminal hydrolase acts on ubiquitin carboxyl-terminal amides*

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Ubiquitin carboxyl-terminal hydrolase (formerly known as ubiquitin carboxyl-terminal esterase), from rabbit reticulocytes, has been shown to hydrolyze thiol esters formed between the ubiquitin carboxyl terminus and small thiols (e.g. glutathione), as well as free ubiquitin adenylate (Rose, I. A., and Warms, J. V. B. (1983) Biochemistry 22, 4234–4237). We now show that this enzyme hydrolyzes amide derivatives of the ubiquitin carboxyl terminus, including those of lysine (ε-amino), glycine methyl ester, and spermidine. It also hydrolyzes ubiquitin COOH-terminal hydroxamic acid, but is inactivated under the conditions for assaying ubiquitin-hydroxylamine adduct hydrolysis. Amide adducts formed between ubiquitin and ε-amino groups of protein lysine residues are much poorer substrates than is the ubiquitin amide of the ε-amino group of free lysine. The enzyme is thus a general hydrolase that recognizes the ubiquitin moiety, but is highly selective for small ubiquitin derivatives. It probably functions to regenerate ubiquitin from adventitiously formed ubiquitin amides and thiol esters. It also has the correct specificity to function in regenerating ubiquitin from small ubiquitin peptides that are probable end products of ubiquitin-dependent proteolysis. A simple, large-scale preparation of the enzyme from human erythrocytes is described.

Reticulocytes and other eukaryotic cells possess an ATP-dependent proteolytic pathway in which the small polypeptide ubiquitin serves as a cofactor (1). During ubiquitin-dependent protein breakdown, ubiquitin becomes covalently attached to target protein substrates, forming “conjugates” in which the linkage is through the ubiquitin carboxyl terminus and has the stability expected of an amide (2). Conjugate formation is a three-step process (3) in which ubiquitin is successively activated to a thiol ester of ubiquitin-activating enzyme (E1; Ref. 4), then transferred to a thiol group of a small ubiquitin (Ub) carrier protein (E2), and finally transferred to a protein amino group by a ligating enzyme (E3) (Equations 1–3).

\[
\begin{align*}
E1 + 2ATP + 2Ub & \rightarrow E1(\text{Ub})_{12} + AMP + 2PP, \\
E1-S-Ub + E2 & \rightarrow E2-S-Ub + E1 \\
E2-S-Ub + protein-NH_2 & \rightarrow Ub-NH\text{-protein} + E2
\end{align*}
\]

(Reticulocytes possess multiple E2s, only one of which functions in breakdown of serum albumin; Ref. 5.) Conjugation of ubiquitin to ε-amino groups of target protein lysines is known to occur (2); conjugation to the target protein NH₂ terminus may also occur (although it has not been reported), since a free target protein NH₂ terminus is required for ubiquitin-dependent proteolysis (6). The target protein portion of these conjugates is degraded by other enzymes in a ubiquitin-specific and ATP-dependent fashion (7). Ubiquitin acts catalytically in the degradative pathway (2, 8) and must therefore be regenerated. Enzymes that catalyze ubiquitin release from large (multiply ubiquitinated) conjugates are present in reticulocyte extracts (2, 7–9) and may function in ubiquitin regeneration during protein breakdown. (These “isopeptidases” could also reverse ubiquitination that serves a more general purpose of protein modification, by analogy with the case of the nuclear ubiquitin–H₂A conjugate (9, 10).) However, the cell must also be able to regenerate ubiquitin from small ubiquitin peptide or amino acid adducts (e.g. Lys-ubiquitin) that are probable end products of ubiquitin-dependent proteolysis. Small ubiquitin amides will also be formed adventitiously as a result of ubiquitin transfer to small amines (in particular, polyamines) catalyzed by E2-ubiquitin thiol esters (5).

Rose and Warms suggested (11) that small ubiquitin amides might be substrates for ubiquitin carboxyl-terminal esterase, an abundant enzyme from reticulocytes that hydrolyzes ubiquitin esters of small thiols. The observation that simple primary amines act as acceptors of ubiquitin from E2-ubiquitin thiol esters (5) has now made a test of this suggestion possible. Our finding that ubiquitin amides are substrates, reported here, has prompted us to change the name of the enzyme from “esterase” to “hydrolase.” It is referred to as ubiquitin carboxyl-terminal hydrolase throughout this paper.

**MATERIALS AND METHODS**

Most commercially available reagents and enzymes were obtained from previously described sources (5). Hydroxylamine hydrochloride (Aldrich) was crystallized twice from ethanol/water (7/1). The preparations of [γ-³²P]ATP, ubiquitin, [³¹P]-ubiquitin, ubiquitin-Sepharose affinity resin (7 mg of Ub/ml of resin), and fraction II (soluble reticulocyte lysate components retained on DEAE-cellulose) have been described (3, 5). [³¹P]-ubiquitin conjugates of native lysozyme, prepared as previously described (7), were a gift of Dr. Avram Herskko of the Technion, Haifa, Israel.

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1. The abbreviations used are: E1, ubiquitin activating enzyme; E2, ubiquitin carrier protein; E3, ubiquitin-protein ligase; DTT, dithiothreitol; Lys-ubiquitin, ubiquitin amide of the ε-amino group of lysine; GME-ubiquitin, ubiquitin amide of the ε-amino group of glycine methyl ester; DTT-ubiquitin, ubiquitin thiol ester of DTT; PFase, inorganic pyrophosphatase; SDS, sodium dodecyl sulfate; AMP-Ub, Ub adenylate.

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Preparation of E1 and E2

E1 (AMP/PPi eluate) and E2 (DTT eluate) were prepared, and their concentrations determined (by PPi burst), as described previously (5). Purified E2 protein 3 was obtained by Sephacryl 200 fractionation of the DTT eluate (5). Preparations of E1 and E2 were freed of traces of ubiquitin-carboxyl-terminal hydrolase by passage through a 0.3-ml ubiquitin-Sepharose column in the absence of ATP. Under these conditions, E1 and E2 break through the column (3), while hydrolase binds (11). The amounts of E1 and E2 obtained from Fraction II by affinity chromatography correspond to concentrations of 1 and 2 nmol, respectively, per 12 ml of packed reticulocytes.

Preparation of *[^1]I*-Ubiquitin Conjugates of Yeast Cytochrome c

These were prepared by *[^1]I*-ubiquitin transfer catalyzed by the ubiquitin thiol ester form of E2 protein 3, as described in Fig. 9 of Ref. 5 (reaction volume scaled up 8-fold). After 100 min of reaction, KCl (30 mM) was added, and the mixture was applied to a 0.2-ml carboxymethyl-cellulose column equilibrated with 50 mM Na acetate, pH 7, containing 30 mM KCl. The column was washed with 1 ml of the same buffer; the breakthrough fraction contained 90% of the unconjugated ubiquitin. Cytochrome c-ubiquitin was eluted with 2.5 ml of 50 mM Na acetate, pH 7, containing 0.2 M KCl. This eluate was dialyzed and concentrated (Amicon YM-5 membrane), and characterized by SDS-gel electrophoresis and autoradiography. *[^1]I*-ubiquitin was distributed among the species free ubiquitin (8%), apparent mono-ubiquitin conjugate of cytochrome c (84%), and apparent tri-ubiquitin conjugate (8%) (see Fig. 9 of Ref. 5). Recovery of conjugated ubiquitin was >90%. Conjugated ubiquitin was estimated to be 0.2 pmol, based on radioactivity; the preparation also contained free cytochrome c (estimated ~0.17 mM). The cytochrome c conjugates are expected to be lysine adducts, based on 1) the presence of 16 lysine residues and NH2-terminal threonine in yeast cytochrome c (12), and 2) the previous observation that E2-ubiquitin thiol esters transfer ubiquitin only to amino groups on primary carbons (5).

Small-scale Preparation of Ubiquitin Carboxyl-terminal Hydrolase

This enzyme was purified to ≥95% homogeneity (judged by SDS-gel electrophoresis and silver staining) by a procedure based on its known affinity for ubiquitin and its low molecular weight (11). Preparations of E2 catalyze ubiquitin transfer to small amines (5) 2) the previous observation that E2-ubiquitin thiol esters transfer ubiquitin only to amino groups on primary carbons (5).

Large-scale Preparation of Carboxyl-terminal Hydrolase from Human Erythrocytes

Buffers—Compositions were as follows: buffer 1, phosphate-buff- ered saline (0.154 M NaCl, 25 mM potassium phosphate, pH 7.4); buffer 2, 2.5 mM potassium phosphate, 1 mM DTT, pH 7.0; buffer 2A, buffer 2 with 20 mM KCl; buffer 3, 20 mM Tris-HCl (10% base), 0.5 M KCl, 1 mM DTT, pH 7.2 (25°C); buffer 4, 50 mM Tris-HCl (90% base), 0.1 mM EDTA, 5 mM DTT, pH 9 (25°C); standard buffer (“Materials and Methods”). The following steps, except affinity chromatography, were performed at 4°C.

Hemolysate—Outdated human blood was kindly provided by the American Red Cross. The packed cells (400 ml) were washed once with 5 volumes buffer 1 and lyed by addition of 3 volumes of 1 mM DTT. After 15 min, the lysate was centrifuged at 9000 × g for 40 min. The top 75% of the material in each bottle was taken (hemolysate, 930 ml).

DES2 Adsorption and Elution—To the hemolysate was added 47 ml of a 50% (w/v) suspension of DE-52 previously equilibrated with buffer 2, and the resulting suspension was stirred for 1 h. After an additional hour, the suspension was filtered through sintered glass. The cellulose was washed with buffer 2A (500 ml) to remove residual bound hemoglobin, and transferred to a beaker containing 100 ml of buffer 3 for elution of the enzyme. The suspension was stirred for 1 h before filtering through two pieces of Whatman No. 1 paper (Buchner funnel). The volume of the filtrate (DES2 KCl eluate) was 93 ml.

Trichloroacetic Acid Precipitation—Solid DTT was added to the KCl eluate to provide an additional 1 mM concentration. Trichloroacetic acid solution (50% w/v) was added, with stirring, to a final concentration of 5%. Protein was pelleted by centrifugation at 12,000 × g for 6 min. The pellet was suspended in 25 ml of buffer 4, using a
Specificity of Ubiquitin Carboxyl-terminal Hydrolysis

Assay of Ubiquitin

Small amounts (<100 pmol) of ubiquitin could be accurately determined by incubating samples in the presence of E1 under the conditions of the standard DTT-ubiquitin hydrolysis assay (except that hydrolyase was rigorously excluded). Under these conditions, ubiquitin is essentially quantitatively converted to DTT-ubiquitin, with stoichiometric production of \([^{32}\text{P}]\text{PP}\). The kinetics of \([^{32}\text{P}]\text{PP}\) production are independent of the concentration of dithiothreitol (20-50 mM) and show a very rapid burst and little or no subsequent rate (as expected, since ubiquitin cannot be regenerated from DTT-ubiquitin). Results obtained with samples were compared to a linear standard curve constructed using known amounts of ubiquitin. In constructing the standard curve, it was noted that the ubiquitin concentration obtained using this assay was only 40% of that calculated from 280 nm absorbance of the ubiquitin stock solution. (A similar discrepancy was noted in catalytic transfer of ubiquitin to amines.) Since the ubiquitin was homogeneous by SDS-gel electrophoresis and isoelectric focusing, we assume that 60% of the ubiquitin as isolated lacks its COOH-terminal glycylglycine. Removal of this dipeptide by cellular proteases (to produce ubiquitin-74) is known to be a problem in ubiquitin purification (15). However, the presence of a significant concentration ubiquitin-74 in hydrolyase assays is not expected to affect any results, since ubiquitin-74 binds at least 10 times more proteinase than does ubiquitin-70 (11). Ubiquitin concentrations reported in this paper are those obtained from the assay using E1 (i.e. ubiquitin-76 concentration).

Synthesis, Characterization, and Hydrolysis of Ubiquitin Hydroxamic Acid

Conditions were (pH 7.3, 37°C): 0.1 M Tris-HCl (20% base), 10 mM MgCl₂, 10 μM \([^{32}\text{P}]\text{ATP}\), 0.2 mM EDTA, 0.4 mM dithiothreitol, 1.5 units/ml PPase, 1.4 μM ubiquitin, 2.5 mg/ml bovine serum albumin, 50 mM NaCl, 150 mM KCl (95% base). After 60 min, assay of \([^{32}\text{P}]\text{PP}\), indicated that all ubiquitin had reacted with hydroxylamine. An equal volume of 1 M hydroxylamine (90% base) was then added together with 0.4 mM iodoacetamide. (This additional incubation was to ensure complete conversion of ubiquitin from O-acyl hydroxamate to O-acetyl hydroxamate.) After 120 min more, trichloroacetic acid (10% final) was added; the precipitate was collected by centrifugation, washed twice with 2% trichloroacetic acid, and resuspended in an appropriate volume of buffer containing 0.1 M Tris-HCl (20% base), 0.1 mM EDTA, and 0.2 mM dithiothreitol. This ubiquitin hydroxamic acid solution was assayed for ubiquitin (above), and was found to contain no detectable ubiquitin (relative to a control that lacked ATP in the initial incubation). Incubation of the ubiquitin hydroxamic acid at pH 13 and 37°C for 5 min did not result in appearance of detectable ubiquitin (the same incubation had no effect on ubiquitin-76 hydrolysis -ATP control); in contrast, the O-acyl hydroxylamine adduct of benzoic acid is instantaneously hydrolyzed by exposure to 10 mM NaOH at room temperature (16). These results indicate that the ubiquitin hydroxamic acid preparation contained no detectable O-acyl hydroxylamine adduct.

The ability of the hydrolyase to cleave ubiquitin (N-acyl) hydroxamic acid was tested in 25-μl incubations containing 20 mM Tris-HCl and 25 pmol of ubiquitin hydroxamic acid (pH 7.3, 37°C). After 30 min, hydrolyase was inactivated by treatment with iodoacetamide (1.8 mM final) for 20 min; dithiothreitol (20 mM final) was added to quench iodoacetamide, and the amount of free ubiquitin product was assayed by reaction with E1 (above).

Electrophoretic Methods

SDS-gel electrophoresis (acylamide/bisacylamide = 30/0.6) using 12.5% acrylamide gels was done by the discontinuous slab procedure of Laemmli (17). The procedure for silver staining was that of Wray et al. (18). Autoradiography of dried gels was as described (5).

RESULTS

Hydrolase Acts on the Ubiquitin Amide of Lysine—Hydrolase of Lys-ubiquitin was assayed by a modification of the ubiquitin "recycling" assay used previously for DTT-ubiquitin ("Materials and Methods"; Ref. 11). E2-ubiquitin thiols esters catalyze ubiquitin transfer to the ε-amino group of free lysine (5); incubation of a small amount of ubiquitin together with E1, E2, lysine, and Mg\[^{32}\text{P}\]ATP results in conversion of...
all the ubiquitin to Lys-ubiquitin (Scheme I), concomitant with a stoichiometric (b) burst.

**Scheme 1**

![Diagram of hydrolysis process](attachment:Scheme_1.png)

The observed hydrolysis of Lys ubiquitin (Fig. 2) is an activity of ubiquitin carboxyl-terminal hydrolyase, and not of a contaminating amidase, based on the following arguments. 1) The hydrolyase preparation is ≥95% homogeneous by the criterion of SDS-polyacrylamide gel electrophoresis (Fig. 1) and has a high specific activity (20 units/mg) in DTT-ubiquitin hydrolysis ("Materials and Methods"). DTT-ubiquitin is the standard thiol ester substrate for the hydrolyase (11). 2) Hydrolytic activities (kcat/Km) toward Lys-ubiquitin and DTT-ubiquitin are quantitatively similar (below). 3) Hydrolytic activities toward these two substrates are (a) stable to brief exposure of the enzyme to trichloroacetic acid, (b) sensitive to iodoacetamide treatment, and (c) protected by ubiquitin against iodoacetamide inactivation (Table I); the last three properties were reported previously for ubiquitin carboxyl-terminal hydrolyase (11).

**Hydrolyase acts on ubiquitin amides of other small amines**—Data summarized in Table II show that the hydrolyse acts on the ubiquitin amides of glycine methyl ester (GME-ubiquitin), and spermidine (spermidine-ubiquitin); the rates are similar to that obtained with the same concentration of Lys-ubiquitin. Very limited steady-state kinetic data suggest that the conditions of the standard assay of DTT-ubiquitin (0.11 μM) are those of kcat/Km, while those of the assay of Lys-ubiquitin (0.22 μM) are closer to kcat. (For these experiments, substrate concentration was varied by varying the ubiquitin concentration in the assay; not shown.) For DTT-ubiquitin, the hydrolyase rate increases approximately linearly with increasing substrate concentration in the range 0.056–

![Graph showing hydrolysis of Lys-ubiquitin by ubiquitin carboxyl-terminal hydrolyase](attachment:Graph.png)

**Fig. 2. Hydrolysis of Lys-ubiquitin by ubiquitin carboxyl-terminal hydrolyase, pH 7.3 and 37°C.** Reactions were carried out as described under "Materials and Methods." (Lys-ubiquitin hydrolysis assay) with 31 nM E1 and 188 nM E2, except that the incubations contained ubiquitin, lysine, and carboxyl-terminal hydrolyase as follows: ○, 65 pmol of ubiquitin (2.0 μM), no lysine (50 mM KCl), no hydrolyase; □, 65 pmol of ubiquitin, 50 mM lysine, no hydrolyase; •, 7.2 pmol of ubiquitin, (0.22 μM), 50 mM lysine, no hydrolyase; ▲, 7.2 pmol of ubiquitin, 50 mM lysine, 0.7 micromol of hydrolyase (0.038 nM); △, 7.2 pmol of ubiquitin, 50 mM lysine, 1.5 micromol of hydrolyase (0.084 nM); ●, 7.2 pmol of ubiquitin, 50 mM lysine, 17.5 micromol of hydrolyase (0.94 nM). Stimulation of steady-state PPi formation by hydrolyase required the presence of lysine (not shown).

**Table I**

<table>
<thead>
<tr>
<th>Loss of activity in hydrolysis of DTT-ubiquitin and Lys-ubiquitin</th>
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<tbody>
<tr>
<td><strong>Treatment</strong></td>
</tr>
<tr>
<td>----------------</td>
</tr>
<tr>
<td>Trichloroacetic acid&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Iodoacetamide&lt;sup&gt;b&lt;/sup&gt; (pH 7.3, 37°C, 0.5 mM)</td>
</tr>
<tr>
<td>Iodoacetamide + 3.2 μM ubiquitin&lt;sup&gt;c&lt;/sup&gt; (as above)</td>
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</table>

<sup>a</sup>Standard assay as described under "Materials and Methods." Control activity was 17.4 micromol/μl enzyme.

<sup>b</sup>Standard assay ("Materials and Methods"); control activity: 6.4 micromol/μl enzyme.

<sup>c</sup>Enzyme (8.7 micromol), in a volume of 50 μl (containing 34 μg of bovine serum albumin as carrier), was precipitated by addition of 100 μl of cold 16% trichloroacetic acid. The pellet was collected by centrifugation at 5°C, washed once with 900 μl of cold distilled water, and suspended in 50 μl of buffer containing 100 mM Tris-HCl, pH 7.6, 0.1 mM EDTA, and 0.2 mM dithiothreitol; aliquots were assayed for activity.

**Table II**

<table>
<thead>
<tr>
<th>Hydrolysis of ubiquitin derivatives by carboxyl-terminal hydrolyase, pH 7.3 (37°C)</th>
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</thead>
<tbody>
<tr>
<td><strong>Derivative</strong></td>
</tr>
<tr>
<td>----------------</td>
</tr>
<tr>
<td>DTT-ubiquitin</td>
</tr>
<tr>
<td>Lys-ubiquitin</td>
</tr>
<tr>
<td>GME-ubiquitin</td>
</tr>
<tr>
<td>Spermidine-ubiquitin</td>
</tr>
</tbody>
</table>

<sup>d</sup>Glycine methyl ester, 50 mM, inhibited DTT-ubiquitin hydrolysis by 35%; the rate shown for GME-ubiquitin has been corrected on the assumption that 50 mM glycine methyl ester also inhibits GME-ubiquitin hydrolysis by 35%. The basis for this inhibition is unclear at this time.
Thus, the values of $-6\, \text{m}^{-1}\text{s}^{-1}$; the slope of the line gives a value for $k_{\text{tot}}/K_{\text{M}}$ of $10^9\, \text{M}^{-1}\text{s}^{-1}$. In the case of Lys-ubiquitin, the hydrolase rate increases only slightly (20%) on increasing substrate concentration from 0.23 to 0.46 $\mu$M. The data are consistent with values of $-6\, \text{s}^{-1}$ for $k_{\text{cat}}$ and $2\times 10^7\, \text{m}^{-1}\text{s}^{-1}$ for $k_{\text{tot}}/K_{\text{M}}$. Thus, the values of $k_{\text{cat}}/K_{\text{M}}$ for thiol ester (DTT-ubiquitin) and amide (Lys-ubiquitin) substrates are close to diffusion-controlled and probably represent the rate of association of enzyme and substrate.

Hydrolase Has a Low Activity toward Ubiquitin Amidase of Proteins—Enzyme activity toward a ubiquitin conjugate of yeast cytochrome $c$ was also examined. (The ubiquitin in this adduct is almost certainly connected via an $\epsilon$-amino group of a cytochrome lysine residue; “Materials and Methods.”) Slow production of free ubiquitin, with a rate of $2.2\times 10^3\, \text{pmol/min}$, was observed in the presence of 0.16 $\mu$M cytochrome $c$-ubiquitin and 21 microunits of hydrolase (not shown; assay described under “Materials and Methods”). Results obtained with ubiquitin conjugates of native lysozyme were quantitatively similar. Hydrolase of Lys-ubiquitin under similar conditions of substrate concentration would occur with a rate of 3.9 pmol/min. These results show directly that the hydrolase is highly selective for ubiquitin amides of small amines. It is possible that the observed activity toward ubiquitin-protein conjugates reflects a low level (~0.05%) of contamination by an isopeptidase.

Hydrolase Acts on Ubiquitin Hydroxamic Acid—Hydroxylamine reacts with El- and E2-ubiquitin thiol esters (“Materials and Methods”). In many but not all cases (16), the reaction of hydroxylamine with acyl compounds occurs in two steps: an initially formed, unstable O-acyl adduct reacts with a second molecule of hydroxylamine to form the stable (N-acyl) hydroxamic acid (16, 19). Typically, the second step is much slower than the first (19). While the initial product of the reaction of hydroxylamine with the enzyme-ubiquitin thiol esters has not been chemically characterized, the product resulting from prolonged incubation with hydroxylamine is probably the N-acyl hydroxamic acid, as shown by the lack of a detectable free ubiquitin COOH terminus even after an incubation in alkali that should hydrolyze any O-acyl adduct present (“Materials and Methods”). Incubation of this alkali-stable COOH-terminal ubiquitin derivative (1 $\mu$M) with hydrolase (0.43 or 0.86 $\mu$M) for 30 min resulted in regeneration of the ubiquitin COOH terminus to the extent of ~95%, as assayed by restoration of its ability to react with El (not shown). This result suggests that the enzyme can hydrolyze ubiquitin (N-acyl) hydroxamic acid.

Inactivation during Hydroxylamine Adduct Cleavage—An attempt was made to quantitate activity toward ubiquitin-hydroxylamine adduct(s) with a steady-state assay similar to those used for the other ubiquitin derivatives. As shown in Fig. 3 (filled squares versus open circles), a large amount of hydroxylase allows extensive recycling of ubiquitin in the presence of 10 mM hydroxylamine (together with El, E2, and MgATP). The observed rate of PP, production is equal to the rate of hydroxylamine adduct formation seen in the presence of a high concentration of ubiquitin and no hydroxylase (Fig. 3, open squares), indicating that adduct cleavage does not contribute to rate limitation when a large amount of hydroxylase is present. The ability of a large amount of hydroxylase to recycle ubiquitin very rapidly (e.g., Fig. 3, filled squares) was independent of hydroxylamine concentration in the range 1–50 mM (not shown). This result suggests that if the reaction of hydroxylamine with enzyme-ubiquitin thiol esters proceeds through an O-acyl adduct, that adduct is a substrate for the hydroxylase. (At very low hydroxylamine concentration, the reaction of hydroxylamine with an O-acyl adduct should be very slow (19)).

Attempts to assay ubiquitin-hydroxylamine adduct hydrolysis in the presence of small amounts of hydroxylase resulted in apparent inactivation of the enzyme (Fig. 3, diamonds; Fig. 4, squares). Failure to restore activity by adding fresh ubiquitin (Fig. 4, inverted triangles) shows that the rate decrease does not reflect time-dependent accumulation of an unhydrolysable form of ubiquitin. Such a result would not be expected, since the hydroxylase cleaves (N-acyl) ubiquitin hydroxamic acid. The small burst observed on addition of fresh ubiquitin corresponds to transfer of this ubiquitin to hydroxylamine; observation of the burst excludes the trivial explanation of El inactivation for the rate decrease shown in Fig. 4 (squares). Addition of fresh hydroxylase after turnover has ceased brings about a further round of turnovers (Fig. 4, triangles). Thus the rate decrease (Fig. 3, diamonds; Fig. 4, squares) is actually caused by hydroxylase inactivation. The mechanism of this inactivation is currently being investigated; inactivation does
Specificity of Ubiquitin Carboxyl-terminal Hydrolase

Table III

Large-scale preparation of ubiquitin carboxyl-terminal hydrolase from erythrocytes

Table III: Large-scale preparation of ubiquitin carboxyl-terminal hydrolase from erythrocytes

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (ml)</th>
<th>mg/ml</th>
<th>Total (mg protein)</th>
<th>Hydrolase Activity</th>
<th>Units/mg</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemolysate*</td>
<td>930</td>
<td>10e</td>
<td>930</td>
<td>0.034</td>
<td>65</td>
<td>100</td>
</tr>
<tr>
<td>DE-52 KCl eluate</td>
<td>93</td>
<td>10f</td>
<td>93</td>
<td>0.034</td>
<td>65</td>
<td>65</td>
</tr>
<tr>
<td>Resuspended trichloroacetic acid pellet</td>
<td>28</td>
<td></td>
<td>-2 mg of protein containing ~1 mg of hydrolase</td>
<td>93 10'</td>
<td>336</td>
<td>31.3</td>
</tr>
<tr>
<td>Concentrated affinity column eluate</td>
<td>1.5</td>
<td>1.2g</td>
<td>Concentrated affinity column eluate</td>
<td>7590</td>
<td>11.4</td>
<td>9.5</td>
</tr>
<tr>
<td>Affinity column breakthrough</td>
<td>30</td>
<td></td>
<td>Affinity column breakthrough</td>
<td>30</td>
<td>2.4</td>
<td></td>
</tr>
</tbody>
</table>

* Standard assay of DTT-ubiquitin hydrolysis as described under "Materials and Methods," except KCl was not added.

**From 400-ml erythrocytes (source: outdated human blood).

**Estimated from absorbance at 280 nm.

**By the method of Lowry et al. (20).

Not occur on incubation of hydrolyase with hydroxylamine alone (50 mM, not shown).

Preparation of Hydrolase from Erythrocytes—A relatively simple preparation of hydrolyase from outdated human blood, which is available in large quantity, is described under "Materials and Methods." In addition to its ubiquitin affinity property, the purification scheme takes advantage of the enzyme's acidic pH (relative to hemoglobin) and acid stability. A volume of erythrocytes that was comfortably processed in a day yielded ~2 mg of protein containing ~1 mg of hydrolyase (Table III; calculated using a specific activity of 20 units/mg). Most of the remaining contaminating proteins are higher molecular weight than hydrolyase (not shown) and could be removed by adding a molecular weight fractionation step.

Discussion

Specificity of the Enzyme—Initial studies with ubiquitin carboxyl-terminal hydrolyase showed that this enzyme acts on small ubiquitin thiol esters and free ubiquitin adenylylate (11). The present work extends the known specificity of the enzyme to include more thermodynamically stable (small) COOH-terminal ubiquitin adducts, including the ubiquitin amides of lysine (a-amino), glycine methyl ester, and spermadine, as well as ubiquitin hydrazide. The observed activity of the enzyme toward the ubiquitin-hydroxylamine adduct formed at very low hydroxylamine concentration suggests that the enzyme can also hydrolyze an O-acetyl hydroxylamine adduct ("Results"). However, it remains to be shown experimentally that O-acetyl ubiquitin hydroxylamine is actually the predomi-
ubiquitin carboxyl-terminal hydrolase during isolation and storage in EDTA-containing buffers ("Materials and Methods"), and to treatment with strong acid (Table I; Ref. 11), argue against but do not exclude the possibility of a tightly bound metal ion that is required for catalysis. Studies on its mechanism of inactivation may shed light on the mechanism of the enzyme in normal catalysis. This reaction may also have practical value as a way to specifically inactivate hydrolase present in other enzyme preparations.

**Biological Role**—The question of the role of the hydrolase in vivo remains incompletely resolved. It almost certainly functions to "rescue" ubiquitin from various adducts that may result from adventitious reactions. A calculation based on the glutathione concentration in erythrocytes (3 mM; Ref. 26), the approximate second-order rate constant for reaction of dithiothreitol with the E1-ubiquitin thiol ester (380 M⁻¹ min⁻¹; Ref. 14), and the approximate concentration of E1 in rabbit reticulocytes, 80 nM, suggests that if all E1 were in the ubiquitin thiol ester form and were able to react with glutathione, all ubiquitin (~15 μM) in the cell would be converted to the glutathione thiol ester in less than 1 min. Similarly, if all E2 (~160 nM) were in the ubiquitin thiol ester form and were available to react with intracellular polyamines (1–2 mM; Ref. 27), all ubiquitin would be converted to polyamine amide adducts in a matter of minutes (kₚ ~18 M⁻¹ min⁻¹; Ref. 5). These calculations are very approximate but serve to show that the large amount of hydrolase in rabbit reticulocytes, ~30 milliunits/ml packed cells (11), is probably necessary to prevent accumulation of adventitious adducts of ubiquitin. In the absence of an enzyme with a broad specificity like that of the hydrolase, these reactions would result in entry of ubiquitin into metabolically inactive pools.

Based on its activity toward Lys-ubiquitin, the enzyme may be able to release ubiquitin from small lysine isopeptides that are expected to result when ubiquitin-protein conjugates are degraded (2). Studies of the specificity of the hydrolase have been limited, however, by the specificities of the enzymes used for synthesis of ubiquitin derivatives; at present, the only α-amino ubiquitin amide available is that of glycine (methyl ester). The finding that the hydrolase acts on the glycine methyl ester derivative suggests that other amino acid-ubiquitin amides may be substrates as well. This question is important in considering a role for the hydrolase in regenerating ubiquitin from possible ubiquitin conjugates of α-amino groups of proteins (6), once the size of such conjugates have been decreased by the ubiquitin- and ATP-dependent proteolytic enzyme.

Pending experimental demonstration of a dependence of protein breakdown on hydrolase activity, the hypothesis that most or all ubiquitin might be regenerated during breakdown by the action of isopeptidases on relatively large ubiquitin-protein adducts cannot be excluded. Because conjugate formation appears to be rate-limiting in protein breakdown (28), no kinetic evidence is available concerning the nature of the steps in ubiquitin-dependent proteolysis that follow ubiquitin-protein conjugate formation, except that conjugate degradation is ATP-dependent (7). Thus, we do not know how long ubiquitin must be retained on fragments of the original target protein molecule in order to ensure complete degradation of those fragments. We have been unable thus far to address experimentally the question of whether protein breakdown is hydrolase-dependent because of difficulty in totally depleting reconstituted lysates of hydrolase. This problem may perhaps be overcome in the future by treatment with hydroxylamine under appropriate conditions.

Özkaynak et al. (29) have recently reported that the ubiquitin gene in yeast consists of six ubiquitin-coding repeats in a head to tail arrangement. The sixth (COOH-terminal) repeat is distinct from the others in that it encodes a ubiquitin having an extra residue, asparagine, at the COOH terminus (*i.e.* COOH-terminal sequence -Gly 75-Gly 76-Aan 77). This gene arrangement raises the possibility that the ubiquitin monomer arises from processing of a poly-ubiquitin precursor protein. The authors suggest that ubiquitin carboxyl-terminal hydrolase might function in such processing, which would presumably involve removal of the COOH-terminal Asn residue, and cleavage at the internal Gly-Met junctions (not necessarily in this order). Hydrolysis of the ubiquitin COOH-terminal amide of Asn may be an activity of the hydrolase, based on observed activity toward the ubiquitin amide of glycine methyl ester. However, a role for hydrolase in cleavage at internal Gly-Met junctions seems inconsistent with results, discussed above, which suggest that the enzyme is highly specific for small (non-protein) ubiquitin amide adducts.

Further studies on this enzyme should be facilitated by a simpler purification scheme (Table III) using red cells, which are more easily obtained in large quantity than are reticulocytes. ATP-dependent proteolysis, a significant part of which is ubiquitin-dependent (30), decreases to negligible levels during maturation of reticulocytes to red cells (28, 31). It is not known which specific components of the ubiquitin system are lost during this developmental process. Speiser and Etlinger (31) reported that addition of a crude reticulocyte ubiquitin fraction to erythrocyte extracts restored ATP-dependent proteolysis to nearly the levels seen in reticulocyte extracts. Stimulation was tentatively attributed to ubiquitin itself or to an associated factor in the preparation. Added ubiquitin might stimulate if all red cell ubiquitin were tied up in small-molecule adducts as a result of a maturation loss of hydrolase activity. The finding that human red cell hydrolase activity, ~120 milliunits/ml packed cells, is higher than that reported in (rabbit) reticulocytes, ~30 milliunits/ml packed cells (11), argues against this explanation, which would also appear to be inconsistent with the observation that human red cells are an excellent source of ubiquitin (15). The observed stimulation thus seems most likely to be caused by some other factor.

**REFERENCES**

Specificity of Ubiquitin Carboxyl-terminal Hydrolase