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An Enzyme with Ubiquitin Carboxy-Terminal Esterase Activity from Reticulocytes†

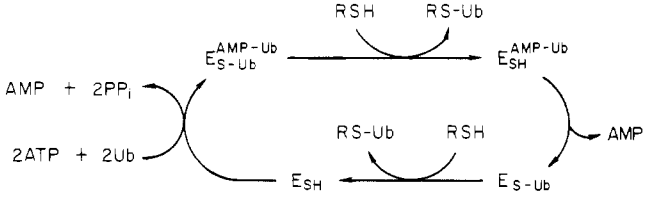
Irwin A. Rose* and Jessie V. B. Warms

ABSTRACT: Thiols such as dithiothreitol (DTT) are known to allow recycling of the ubiquitin activating enzyme presumably due to attack by thiol on E–ubiquitin forming E + DTT–ubiquitin. It is now reported that the resulting ubiquitin thiol ester is extremely susceptible to hydrolysis, giving rise to free ubiquitin that can then also recycle in the activating enzyme reaction. The instability of ubiquitin thiol esters in this system is caused by a ubiquitin carboxy-terminal thiolesterase activity found as a minor contaminant of the activating enzyme. This activity of rabbit reticulocytes has been extensively purified, and some of its properties are reported. The enzyme, which also cleaves carboxy-terminal adenosine 5′-phosphate–ubiquitin, is inhibited by free ubiquitin at micromolar concentrations. The ubiquitin-specific esterase probably functions to hydrolyze glutathione and other thiol esters of ubiquitin that would be formed spontaneously from activated ubiquitin in cells.

The ubiquitin activating enzyme reaction causes two kinds of activation of the 76 amino acid polypeptide ubiquitin (Ub):[1]

$$E + 2ATP + 2Ub \rightarrow E_{S-Ub}^{AMP-Ub} + 2PP_i + AMP$$

Covalent binding to the enzyme is through a thiol ester bond formed from one round of AMP–Ub formation and followed by a second round (Ciechanover et al., 1981, 1982; Haas et al., 1982). The linkage to ubiquitin in both chemical states is through ubiquitin's carboxyl-terminal glycine group (Hershko et al., 1981). Thiol ester activated ubiquitin is used most immediately for the formation of conjugates with target proteins (Haas et al., 1982). A major fate of such conjugates in reticulocytes is the complete degradation of the target protein with regeneration of the ubiquitin (Hershko et al., 1980; Haas & Rose, 1981). In the presence of pyrophosphatase, all of the activating enzyme is found in the E_{S-Ub}^{AMP-Ub} form, which is sufficiently stable to be isolated by gel filtration. When a thiol compound such as DTT is present, the rapid formation of 2 enzyme equiv of PP_i is followed by additional ATP breakdown (Haas et al., 1982). The thiol is presumed to cause the regeneration of free enzyme by a chemical transacylation:



As is reported here, under limiting concentrations of ubiquitin, the extent of ATP breakdown can exceed by many fold the total amount of ubiquitin present, an observation not explained by the above scheme. Three hypotheses may be considered to explain the catalytic role of ubiquitin: (1) the activating enzyme has an additional hydrolytic activity that is activated by –SH compounds; (2) thiol esters of ubiquitin are unstable; (3) a separate enzyme, a thiolesterase, is present in the preparation of activating enzyme previously believed to be pure. The present study reports a new thiolesterase reaction for ubiquitin carboxy-terminal thiol esters. Possible roles of the enzyme are considered.

Materials and Methods

Ubiquitin activating enzyme was prepared by the reaction affinity method of Ciechanover et al. (1982) with activated CH-Sepharose 4B to which Ub is bound. The enzyme forms a thiol ester linkage to the bound Ub in the presence of ATP and is eluted with AMP plus PP_i. The amount of functional enzyme was determined from the counts of [^{3}H]ATP made acid insoluble by formation of 1 enzyme equiv of [^{3}H]AMP–Ub. Treatment of the activating enzyme with iodoacetamide renders it unable to form E_{S-Ub} but has no effect on formation of E·AMP–Ub (Haas et al., 1982). This enzyme form was prepared by incubation for 15 min at 37 °C with 0.5 mM iodoacetamide in the presence of 50 mM Tris-HCl, pH 7.6, and 0.5 mg/mL BSA, followed by DTT.

Standard Assay for Thiolesterase Activity. A final volume of 50 μL contained 40 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, 0.2 mg/mL BSA, 5 pmol of ubiquitin, 20 mM DTT, 100–200

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[1] Abbreviations: Ub, ubiquitin; Ub_{74}, C-terminal des-Gly-Gly-ubiquitin; DTT, DL-dithiothreitol; DTT–Ub, thiol ester of DTT with the carboxyl-terminal glycine of ubiquitin; BSA, bovine serum albumin; Tris, tris(hydroxymethyl)aminomethane; TCA, trichloroacetic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetic acid.

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Table I: Requirements for Excess ATP Utilization

incubation components	$[^{32}P]P_i$ (pmol)
complete[a]	82
−ubiquitin	2
iodoacetamide-treated enzyme	1
−PPase	3
PPase added later[b]	77

[a] Standard assay conditions at 37 °C with 0.2 pmol of ubiquitin, quenched after 20 min. [b] Pyrophosphatase was omitted during the 20-min incubation but added to the acid-quenched and neutralized solution and incubated for 10 min to hydrolyze any PP_i that was formed in the first incubation.

pmol of [γ-^{32}P]ATP with a specific activity of ~500 cpm/pmol, 0.06 unit of pyrophosphatase, and 0.2 pmol of purified ubiquitin activating enzyme. Reactions were quenched after incubation at 37 °C by the addition of 200 μL of 1 N H_2SO_4 containing 0.8 mM P_i. The extent of hydrolysis of the ATP was determined by extracting $[^{32}P]P_i$ into 2-butanol as the acid phosphomolybdate complex (Berenblum & Chain, 1938). The unit of thiolesterase activity is defined in terms of the micromoles of [γ-^{32}P]ATP converted to $[^{32}P]P_i$ per min under standard conditions when enzyme is present in the range of proportionality, i.e., $<3 \times 10^{-6}$ unit, as seen in Figure 3.

Preparation of Ub and Trypsin-Modified Ub. ^{125}I-Ubiquitin was prepared by the chloramine-T method from the 76 amino acid polypeptide isolated from human erythrocytes (Ciechanover et al., 1980). C-Terminal des-Gly-Gly-ubiquitin, Ub_{74}, was prepared by brief treatment with trypsin followed by pancreatic trypsin inhibitor (Wilkinson & Audhya, 1981).

Preparation of AMP–Ub and DTT–Ub. [^{3}H]ATP and ^{125}I-ubiquitin were incubated with iodoacetamide-treated ubiquitin activating enzyme to form E·AMP–Ub, which was precipitated with TCA (Haas et al., 1982). The acid-washed precipitate (AMP–Ub) was dissolved in 40 mM Tris-HCl, pH 7.6, and incubated at 37 °C in the presence of 10 mM DTT. All of the tritium was made acid soluble within 5 min. Urea at 4 M was also included in some experiments of Figure 1 to prevent the action of any thiolesterase that might be carried over from the incubation in which the AMP–Ub was made.

Assay for DTT–^{125}I-Ubiquitin. Columns (0.5 × 7.5 cm) containing Bio-Rad Affi-Gel 501 organomercurial agarose were equilibrated with a solution containing 0.05 M Tris-HCl, pH 7.6, 0.1 M NaCl, 6 M urea, and 1 mg/mL ribonuclease A (product of Sigma). Ribonuclease was used to minimize nonspecific absorption of ^{125}I-ubiquitin to the column. After the sample that usually contained [^{35}S]cysteine as a marker was applied, the columns were washed with 5 column volumes of the column buffer. Bound thiols were released by further elution with the same medium containing DTT (5 mM). Isotope assays were done by γ counting (^{125}I) and liquid scintillation (^{35}S, ^{32}P, and ^{3}H).

Results

Ubiquitin Turnover in the Presence of Thiols. Addition of DTT, glutathione, cysteine, or β-mercaptoethanol to an incubation in which E_{S-Ub}^{AMP-Ub} is being formed caused conversion of much more [γ-^{32}P]ATP to $[^{32}P]P_i$ than expected from the amount of ubiquitin present. With 10 mM DTT, 0.2 pmol of activating enzyme, and 5 pmol of ubiquitin in the system, the activating enzyme turned over at a constant rate of 25 min^{-1}. Therefore, the whole pool of ubiquitin turned over every minute. With glutathione at 20 mM, a constant rate of 5 min^{-1} was maintained for 30 min. These results cannot be ascribed to a thiol-activated ATPase since as seen in Table I, there is complete dependence on the presence of ubiquitin, activating enzyme, and pyrophosphatase. Pyrophosphate must be the product of the thiol-activated reaction as shown by the need for pyrophosphatase to form $[^{32}P]P_i$ after the activating enzyme incubation is quenched. This shows that $[^{32}P]PP_i$ is the true product of the DTT-stimulated reaction since pyrophosphatase alone had no ATPase activity under the conditions used. The observation that treatment of activating enzyme with iodoacetamide blocks the stimulating effect of DTT was reported earlier (Haas et al., 1982). Iodoacetamide blocks formation of E_{S-Ub} but not formation of E·AMP–Ub, suggesting that the turnover of ubiquitin caused by DTT depends on formation of E_{S-Ub}.

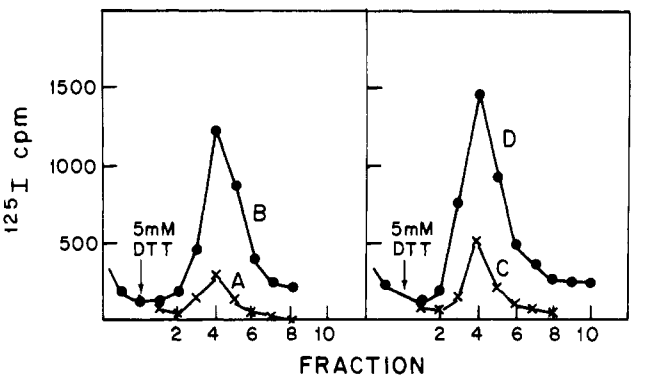


FIGURE 1: Demonstration of DTT–Ub and evidence of its enzymatic hydrolysis. DTT–Ub was prepared from AMP–^{125}I-Ub (0.39 pmol with 4200 cpm) that was generated from the ubiquitin activating enzyme (see Materials and Methods) and analyzed by determining counts recovered from an Affi-Gel 510–Hg^+ column (A) (600 cpm). For (B) (3000 cpm recovered), urea (4 M) was included when the TCA-precipitated AMP–Ub was dissolved in buffered DTT. (C and D) Repeats of (B) except that the urea was diluted 10-fold with buffer after the DTT–Ub was formed and incubated for 2 min in (C) (1100 cpm) or not diluted in (D) (4000 cpm).

Detection of DTT–Ub Thiol Ester. When the standard assay mixture showing the DTT effect was acidified and assayed for DTT–^{125}I-Ub on the basis of ^{125}I retention on a Hg^+ column, none was found. To test the possibility that DTT–Ub might be inherently unstable, an authentic sample was sought from AMP–^{125}I-Ub and DTT and tested for retention of ^{125}I on the Hg^+ column as detailed under Materials and Methods. As shown in Figure 1A, less than 20% of the expected ^{125}I-Ub was retained and eluted by DTT although [^{35}S]cysteine, added as an internal standard, was recovered quantitatively. This suggested that DTT–Ub once formed from the AMP–Ub is very unstable. Thiol esters of DTT such as DTT–acetate are not known to be unstable (Simpson, 1981). Therefore it seemed possible that a thiolesterase activity derived from the AMP–Ub preparation and able to survive TCA was responsible for hydrolysis of DTT–Ub once formed. The activating enzyme, a dimer of 200 000, seemed unlikely to survive TCA, but an enzyme contaminant of the activating enzyme or ubiquitin itself was considered. To examine these possibilities, 4 M urea was included during formation of DTT–Ub from the time the TCA-precipitated AMP–Ub was dissolved. Under these circumstances, 70% of the [^{3}H]AMP–Ub that disappeared, as measured by the loss of acid-insoluble tritium, was retained by the organomercury column, Figure 1B. Also shown is an experiment, Figure 1C, in which DTT–Ub formed in 4 M urea was diluted with 9 volumes of pH 7.6 buffer and further incubated for 2 min at 37 °C before analysis on the Hg column. In this case, the sample lost ~73% of the counts that would otherwise have been retained by the urea-equilibrated Hg column, Figure 1D. Thus, DTT–Ub is stable in the presence of urea. Its instability may be due either to the ability of ubiquitin itself to monitor its own C terminus for

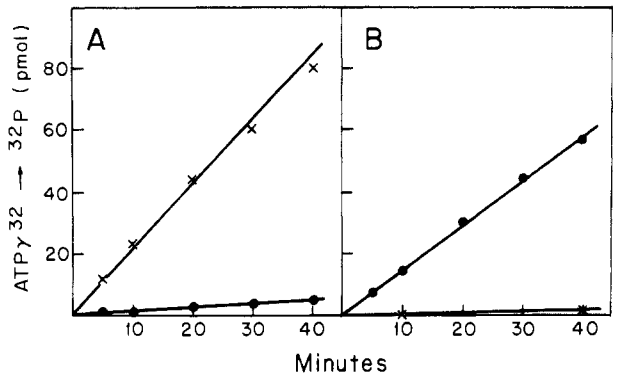


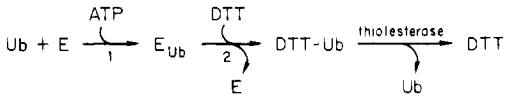
FIGURE 2: Ubiquitin activating enzyme recycled on Ub–Affi-Gel 10 is free of ATPase activity. The standard ubiquitin thiolesterase assay was done with Ub–Sepharose 4B purified enzyme (×) or with enzyme that was further purified on Ub–Affi-Gel 10 (●) according to Haas et al. (1982) with 0.12 pmol/50 μL for each time point (A). In (B), the less pure activating enzyme was precipitated with TCA and assayed for ATPase activity either alone (×) or in the presence of recycled activating enzyme (●).

thiol esters or to a distinct thiolesterase. The latter would have to arise as a TCA-stable contaminant of the activating enzyme.

Evidence for Acid-Stable DTT–Ub Thiolesterase Activity. The preparation of the ubiquitin activating enzyme from a Ub–Sepharose column depends on a "specific" elution with AMP plus PP_i. When the ubiquitin activating enzyme was recycled through a Ub–Affi-Gel-10 column (Haas et al., 1982), it was found to give a much lower rate of DTT-dependent "ATPase". The Ub–Affi-Gel-10 column contains a lower concentration of ubiquitin than the Ub–Sepharose column. As shown in Figure 2, the ATPase rate of this enzyme preparation was stimulated by addition of TCA-precipitated enzyme that had not been recycled. The TCA-treated enzyme was unable to act on ATP in the absence of the ubiquitin activating enzyme.

Purification of Thiolesterase Activity. The thiolesterase activity with a molecular weight of 200 000 can be separated completely from the ubiquitin activating enzyme on Sephadex G-75. Its elution position suggests a molecular weight of ~30 000. Elution of the thiolesterase activity together with the ubiquitin activating enzyme from a Ub–Sepharose column follows a 0.5 M KCl wash of the column, which suggests that the enzyme has affinity for ubiquitin. In that case, there is no reason to expect the enzyme to be coeluted with activating enzyme in the AMP plus PP_i fraction. In fact, an analysis of the other column fractions showed that the thiolesterase is mainly eluted in the pH 9 fraction after stepwise elutions with KCl, AMP plus PP_i, and DTT (Table II). The pH 9 fraction contained proteins of highest affinity for ubiquitin, about six in number on the basis of SDS–PAGE. Precipitation of this fraction with 10% TCA in the presence of BSA gave ~70% recovery of thiolesterase activity. If then cycled on Ub–Sepharose, only the thiolesterase was found in the pH 9, 5 mM DTT elution. Recovery was >80%. A single protein band of 30 000 daltons is seen on SDS gel electrophoresis. The specific activity of the purest enzyme was determined under standard assay conditions to be ~1 unit/mg.

Kinetic Properties of the ATPase Assay. The model favored to explain the catalytic utilization of Ub by activating enzyme, E, consists of three irreversible steps in steady state:



When Ub is below the K_m for the activating enzyme, the turnover of the first step will be controlled by step 3 up to the point that DTT–Ub is decreased to zero. Under the standard assay conditions (0.2 unit of E, 5 pmol of Ub, and 20 mM DTT in 50 μL), the thiolesterase loses control above ~3 × 10^{-6} unit/50 μL, Figure 3. The fact that at high esterase an increase in DTT from 20 to 40 mM increases the apparent ATPase rate indicates that part of the activating enzyme is tied up in product complexes in the steady state at 20 mM DTT. Since the rate increase is much less than 2-fold, the activating enzyme must be unable to maintain itself in a fully loaded state at the turnover rate of 60 pmol (20 min)$^{-1}$ (0.1 pmol of activating enzyme)$^{-1}$ or 0.5 s^{-1}. This rate is not far from the extrapolated maximum ATP–AMP exchange rate of 1.9 s^{-1} catalyzed by ubiquitin activating enzyme at optimal concentration of ubiquitin (Haas & Rose, 1982). When the steady-state rate is linearly dependent on the thiolesterase activity present, there is very little effect of increasing either activating enzyme or DTT presumably because the low steady-state concentrations of free ubiquitin that determine the rates are inversely affected by these changes.

Interaction of the C-Terminal Ubiquitin Esterase with Ub. The high affinity of the esterase for the Ub–Sepharose column suggests that the enzyme may be specific for esters of ubiquitin.

Table II: Fractionation on Ub–Sepharose of Extract from 1 mL of Rabbit Reticulocytes[a]

	analysis of first run		
elution fraction	Ub activating enzyme (pmol)	thiolesterase activity (units)	recycled thiolesterase (% recovered)
unadsorbed			8.5
1.0 M KCl		8.1×10^{-6}	6.6
2 mM AMP + 0.1 mM PP_i	45	5.9×10^{-6}	
10 mM DTT	~20	3.8×10^{-6}	
50 mM Tris-HCl + 5 mM DTT, pH 9.0	~3	2.7×10^{-2}	50.7

[a] Reticulocyte protein, absorbed to DEAE-cell ulose and eluted with 0.5 M KCl, was prepared, applied to a Ub-Sepharose column, eluted with 50 mM Tris-HCl, pH 7.2, containing the additions below, and concentrated in cones as previously described (Ciechanover et al., 1982). The activity of activating enzyme and thioesterase was determined as under Materials and Methods. Thiolesterase activity, derived from a TCA precipitate of the pH 9.0 fraction of the first run, was recycled on a smaller Ub-Sepharose column. In this case, ATP and $MgCl_2$ were omitted during application of the enzyme to the column. The AMP-PP_i and DTT elutions were also omitted.

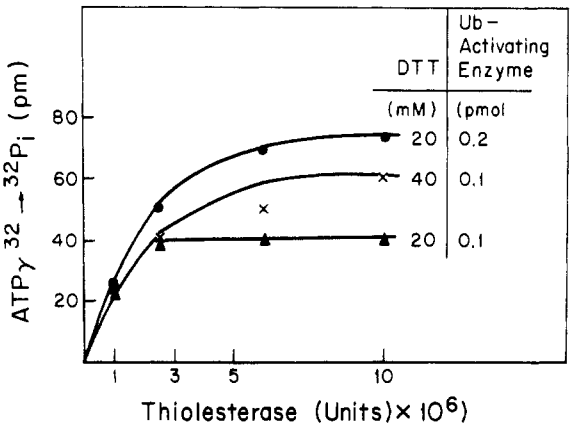


FIGURE 3: Effects of thiolesterase, activating enzyme, and DTT concentrations on ATPase rate. Conditions are as in the standard assay except as noted.

Table III: Interactions of Ubiquitin with C-Terminal Ubiquitin Esterase in Three Assays

additions	hydrolysis of DTT-Ub[a] (%)	inactivation by iodoacetamide[b] (%)	hydrolysis of AMP-Ub[c] (%)
nothing	80	70	38
Ub (0.8 μM)	68		
Ub (8 μM)	5	18	7
Ub_{74} (8 μM)	60	70	28

[a] DTT-^{125}I-Ub (1.5 pm) was incubated in 50 μL with 0.25 microunit of purified esterase (50 mM Tris-HCl, pH 7.6), and the noted additions. After 5 min at 37 °C, the sample was assayed for DTT-Ub remaining by retention of ^{125}I on the Affi-Gel 501-Hg^+ column. [b] Esterase (4 microunits) was incubated at 37 °C in 50 mM Tris-HCl, pH 7.6, with BSA (0.5 mg/mL), iodoacetamide (0.5 mM), and the noted additions. At 20 min, DTT (5 mM) was added and thiolesterase activity measured by the standard assay. [c] [^{3}H]AMP-Ub (0.25 pmol) and esterase (2 microunits according to the standard assay) were incubated for 20 min at 37 °C in 50 mM Tris-HCl, pH 7.6, and the noted additions in 50 μL. TCA to 12% was added at 5 min, and acid-soluble counts were determined.

Additional evidence for this comes from the observation that hydrolysis of DTT–Ub is strongly inhibited by Ub in the micromolar range, Table III. This effect is considerably diminished for Ub that lacks its C-terminal Gly-Gly, Ub_{74}.

It was found that ubiquitin esterase activity as measured by the standard ATPase assay is inactivated by iodoacetamide, $t_{1/2}$ = 15 min at pH 7.6, 37 °C, with 0.5 mM iodoacetamide. Ubiquitin but not Ub_{74} protects the enzyme from iodoacetamide, Table III. Glycylglycine at 1 mM had no effect on the standard assay with purified esterase.

During attempts to prepare and demonstrate DTT–Ub in the experiments reported in Figure 1, AMP–Ub prepared by acid precipitation of fully loaded ubiquitin activating enzyme not treated with iodoacetamide was observed to hydrolyze more rapidly than AMP–Ub prepared from the same enzyme complex isolated on Sephadex G-75 column. This hydrolysis that occurred in the absence of thiol compounds was shown to be catalyzed by the purest preparation containing thiolesterase activity, Table IV. Inhibition by ubiquitin but not by des-Gly-Gly-ubiquitin suggests that hydrolysis of DTT–Ub and AMP–Ub may be caused by the same iodoacetamide-sensitive enzyme.

Discussion

The present paper reaches the conclusion that simple thiols stimulate the formation of AMP plus PP_i from ATP by the ubiquitin activating enzyme by their action as good nucleophiles for the enzyme–ubiquitin thiol ester linkage. As evidence for this, incubations containing DTT and ^{125}I-ubiquitin gave rise to an ^{125}I-labeled compound that is retained on an organomercury column used for the adsorption of –SH compounds in the amount expected from DTT-dependent ATP cleavage. The adsorbed ^{125}I-ubiquitin derivative is eluted by DTT in the position at which [^{35}S]cysteine is eluted as a marker. The interpretation of this result is that the ubiquitin and DTT are linked through a single thiol ester bond, leaving the second –SH group to interact with the column. In the presence of the C-terminal ubiquitin esterase reported here, added thiols cause the activating enzyme to turn over more ATP than the ubiquitin that was present. The same enzyme preparation caused the cleavage of putative DTT–ubiquitin prepared by incubation of DTT with AMP–ubiquitin.

The affinity of the thiolesterase activity for ubiquitin thiol esters is surely due to the ubiquitin component of substrates as shown by the strong inhibition by ubiquitin of DTT–Ub hydrolysis and of iodoacetamide inactivation of the enzyme (Table III), as well as its high affinity for Ub–Sepharose (Table II). Specificity for the group linked to ubiquitin needs to be studied in greater detail. All simple thiols tested behaved like DTT in stimulating ATP utilization in excess of ubiquitin present. This nonspecificity suggests that there is little or no interaction between the hydrolyzing enzyme and the group bound to the C-terminal glycine of ubiquitin. A limitation to the size of the thiol residue is evident from the fact that the thiol ester linkage of ubiquitin with activating enzyme is not hydrolyzed. This bond was also not hydrolyzed after mild acid treatment, which denatures the activating enzyme though not ubiquitin.

In the ability of the presumed thiolesterase to hydrolyze AMP–Ub (Table III) is the suggestion that a wider variety of C-terminal derivatives of ubiquitin of suitable size may be acted upon. If the range of derivatives extends to amides made through the ϵ-NH_2 group of lysine, the hydrolyzing enzyme could function to regenerate ubiquitin that would be conjugated to small peptides in the last step of the ATP/ubiquitin system of protein degradation (Hershko et al., 1980).

The C-terminal ubiquitin esterase is probably important in overcoming the inevitable transfer of ubiquitin from the activating enzyme to glutathione. It would be important that any naturally occurring thiol compound able to carry out the transacylation of ubiquitin from the ubiquitin activating enzyme should be functional in the thiolesterase reaction. The thiolesterase activity of reticulocytes, 30 milliunits/mL of cell, is more than adequate to protect ubiquitin from reaction with the glutathione of the cell.

Registry No. Ubiquitin carboxy-terminal thiolesterase, 86480-67-3.

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