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# Detection, Resolution, and Nomenclature of Multiple Ubiquitin Carboxyl-Terminal Esterases from Bovine Calf Thymus†

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ABSTRACT: In vivo, ubiquitin exists both free and conjugated through its carboxyl terminus to the $\alpha$- and $\epsilon$-amino groups of a wide variety of cellular proteins. Ubiquitin carboxyl-terminal hydrolytic activity is likely a necessary step in the regeneration of the ubiquitin cofactor from ubiquitin–protein conjugates. In addition, this type of activity is required to generate the active, monomeric ubiquitin from the only known gene products: the polyprotein precursor and various ubiquitin fusion proteins. Thus, this activity is of vital importance to systems that utilize ubiquitin as a cofactor. A generic substrate, ubiquitin ethyl ester, was previously developed [Wilkinson, K. D., Cox, M. J., Mayer, A. N., & Frey, T. (1986) *Biochemistry 25*, 6644-6649] and utilized here to monitor the fractionation of these activities from calf thymus. By use of a rapid HPLC assay, four distinct, ubiquitin-specific esterases were identified and separated. A previously undescribed activity has been resolved and characterized, in addition to the bovine homologue of ubiquitin carboxyl-terminal hydrolase purified from rabbit reticulocytes. Two other activities resemble deconjugating activities previously detected in crude extracts but not previously purified. These activities appear to form a family of mechanistically related hydrolases. All four activities are inhibited by iodoacetamide, indicating the presence of an essential thiol group, and are inhibited to various extents by manganese. All have specific ubiquitin binding sites as judged by the low observed $K_m$ values (0.6-30 $\mu$M). The carboxyl-terminal aldehyde of ubiquitin is a potent inhibitor of these enzyme activities, with $K_i$ values approximately 1000-fold lower than the respective $K_m$ values. The use of ubiquitin ethyl ester provides a rapid, quantitative assay for purification of these enzyme activities and for the comparison of the known and postulated differences between the members of this important group of enzymes. A systematic nomenclature for these enzyme activities and their substrates is proposed in order to facilitate comparison of studies by various laboratories. This nomenclature clearly indicates the nature of the reactions catalyzed and the substrates used to detect the various activities and should avoid the idiosyncrasies of the multiple nomenclatures which have arisen previously. These results should greatly facilitate the purification and characterization of the proteins and the genes of this important family of enzymes from a single mammalian source.

Ubiquitin is a 76 amino acid polypeptide found in all eukaryotic organisms studied. It exists as the free monomer and covalently attached to a wide variety of cellular proteins. The dynamic nature of the cellular ubiquitin–protein conjugate pool has been demonstrated in vivo by microinjection (Carlson et al., 1987; Carlson & Rechsteiner, 1987) and immunochemical techniques (Haas & Bright, 1985). These studies have demonstrated that the reversible ubiquitination of proteins is a controlled phenomenon that responds to external stimuli such as heat shock and nutritional status. The enzymes which catalyze the conjugation of ubiquitin have been partially characterized (Hershko et al., 1983; Haas et al., 1982). In contrast, the enzymes which proteolytically process the poly-

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protein gene products and/or remove ubiquitin from ubiquitin–protein conjugates have not been characterized. These enzymes are of obvious importance in maintaining the steady-state levels of ubiquitin–protein conjugates in vivo, and therefore the cellular functions of ubiquitin. In addition, there has been much interest in these enzymes as a tool to study the effect of the amino-terminal amino acid on the half-life of protein (Bachmair et al., 1986) and in the expression of various recombinant proteins as ubiquitin-fusion proteins in eukaryotic cells.

The proteolytic processing of ubiquitin fusion proteins and removal of ubiquitin from ubiquitin–protein conjugates are attributable to enzymes which possess ubiquitin carboxyl-terminal hydrolytic activity, i.e., the cleavage of carboxyl-terminal derivatives of ubiquitin. Three enzyme preparations which catalyze this chemistry have been reported previously. A deconjugating activity present in reticulocytes has been observed to compete with ubiquitin-dependent proteolysis (Hershko et al., 1980; Hough et al., 1986). A second enzyme from reticulocytes, ubiquitin carboxyl-terminal hydrolase (UCH),[1] has been purified to homogeneity, and its mechanism and enzymatic specificity are well characterized (Pickert & Rose, 1986, 1985). In calf thymus and liver, the disappearance of the ubiquitin–H2a conjugate has been attributed to a third enzymatic activity (Matsui et al., 1979; Mueller et al., 1985; Goldknopf et al., 1980). Finally, the observation that the known ubiquitin gene products are head to tail repeats of the native sequence, or ubiquitin–fusion proteins (Ozkaynak et al., 1987; Lund et al., 1985), implies the existence of an uncharacterized polyprotein processing enzyme similar to the reported deconjugating enzymes. It is not clear whether there are other ubiquitin carboxyl-terminal hydrolytic activities in some tissues nor if any single enzyme can catalyze more than one of the above reactions.

Only one of these activities has been purified, partly due to the difficulty and nonlinearity of the available assays. A direct comparison of these enzyme activities has been precluded by the variation in the source and in assay methods employed by different groups (Hershko et al., 1980; Hough et al., 1986; Pickert & Rose, 1986; Wilkinson et al., 1986a). In the present work, the recently synthesized carboxyl-terminal ethyl ester of ubiquitin (UbOEt) (Wilkinson et al., 1986a) was employed as a generic substrate to detect these activities and to monitor the purification of four distinct ubiquitin carboxyl-terminal esterases from a single source, bovine calf thymus.

## Experimental Procedures

*Materials.* Ubiquitin was isolated from bovine erythrocytes as described previously (Haas & Wilkinson, 1985). TPCK-treated trypsin, soybean trypsin inhibitor, phenylmethanesulfonyl fluoride (PMSF), glycylglycine ethyl ester, and dithiothreitol (DTT) were purchased from Sigma Chemical Co., St. Louis, MO. The ion-exchange resins CM-52 and DE-52 were from Whatman Ltd., Maidstone, Kent, England. The C-8 HPLC column (5 μm, 4 mm × 250 mm) was from Alltech Associates, Deerfield, IL, and acetonitrile (HPLC grade) was from Baker Chemical Co., Phillipsburg, NJ. Sephacryl S-300 was from Pharmacia Fine Chemicals, Piscataway, NJ. Bovine calf thymus was purchased from Pel-Freeze, Rogers, AK. Carrier-free $Na^{125}I$ was from ICN Radiochemicals, Irvine, CA. All electrophoresis reagents were purchased from Bio-Rad, Richmond, CA. All other chemicals were of reagent grade from commercial sources.

*Assay for Hydrolysis of Ubiquitin Ethyl Ester (UbOEt).* Unless otherwise indicated, the standard assay contained (total volume 0.02 mL) 50 mM Tris, pH 8.0 (37 °C), 1 mM EDTA, 10 mM DTT, 11.6 μM UbOEt, and the indicated enzyme. Standard activities were measured with a final concentration of 0.5 milliunit/mL. Aliquots were withdrawn at 10-min intervals and immediately injected onto the C-8 column. Chromatography, detection, and quantitation of substrate and product and analysis of kinetic data were as described previously (Wilkinson et al., 1986a). One unit of activity catalyzes the hydrolysis of 1 μmol/min UbOEt to ubiquitin.

*Separation of Esterase Activities from Calf Thymus.* Frozen calf thymus (22 g) was pulverized and homogenized in 2 volumes (v/w) of 10 mM Tris-HCl, pH 7.0 (4 °C), 1 mM EDTA, 10 mM DTT, 2.5 mM magnesium chloride, 1 mM PMSF, and 10% glycerol (v/v) in a Waring blender. In some experiments the following additions were made to the homogenization buffer: 10 μg/mL aprotinin, 10 μg/mL pepstatin, 5 μg/mL α-macroglobulin, 5 μg/mL leupeptin, 10 μg/mL chymostatin, and 10 μg/mL antipain. The homogenate was centrifuged at 100000*g* for 1 h, and the supernatant was aspirated through glass wool to remove suspended fats. The supernatant was adsorbed onto a DE-52 column (2.5 cm × 30 cm) equilibrated with 50 mM Tris-HCl, pH 7.5 (4 °C), 1 mM EDTA, 10 mM DTT, 0.1 mM PMSF, and 10% glycerol (v/v). The column was washed with 2 column volumes of equilibration buffer, and the bound proteins were eluted with a linear KCl gradient from 0 to 0.5 M KCl in equilibration buffer (7 column volumes). The conductivity and 280-nm absorbance of the eluate were measured, and assays for UbOEt hydrolytic activities were performed as described above. Pooled fractions (as indicated in Figure 1) were concentrated by ultrafiltration, and individually chromatographed on a calibrated Sephacryl S-300 column (1.5 cm × 90 cm) equilibrated with 50 mM Tris-HCl, pH 7.5 (4 °C), 1 mM EDTA, 10 mM DTT, and 10% glycerol (v/v).

*Synthesis of the Ubiquitin Carboxyl-Terminal Aldehyde.* The ethyl ester of des-glycine$^{76}$-ubiquitin was synthesized as described for the 76 amino acid derivative (Wilkinson et al., 1986a), except that glycine ethyl ester was substituted for glycylglycine ethyl ester. The resulting equilibrium concentration and purification yield for the 75 amino acid ubiquitin ester was the same as those for the 76 amino acid ester. To activate the carboxyl terminus, the ester was incubated with 1 M hydrazine hydrochloride, pH 8.0, at 37 °C for 90 min, and the resulting acyl hydrazide was dialyzed against water (4 °C) for 18 h to remove the excess reagent. The acyl azide was generated by incubation with 0.5 M sodium nitrite and 0.5 M HCl at −4 °C for 10 min (Klausner & Bodanszky, 1974) and reacted in situ with 1.0 M aminoacetaldehyde diethyl acetal for 35 min. After dialysis against water, ubiquitin aldehyde was generated from the diethyl acetal by treatment with 1 M HCl at 23 °C for 30 min followed by neutralization with sodium carbonate. To obtain the ubiquitin carboxyl-terminal alcohol, the aldehyde was incubated with 1 M sodium borohydride for 15 min and then diluted 10-fold into 0.1 M HCl to discharge the remaining borohydride. HPLC of tryptic digests of this product (Cox et al., 1986) confirms that the modification has been limited to the carboxyl

[1] Abbreviations: UCH, ubiquitin carboxyl-terminal hydrolase, the enzyme or its homologue originally described by Pickart and Rose (1985); UbOEt, carboxyl-terminal ethyl ester of ubiquitin (Wilkinson et al., 1986a); SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; IU, international unit (also referred to as unit), the amount of enzyme which hydrolyzed UbOEt to ubiquitin at a rate of 1 μmol/min.

terminus of ubiquitin. No evidence of lysine modification was observed.

*Measurement of Deconjugating Activity.* $^{125}$I-Ubiquitin was synthesized by the chloramine-T method described previously (Hershko et al., 1984). $^{125}$I-Ubiquitin was conjugated to the proteins of reticulocyte fraction II by the method of Hough et al. (1986). After incubation for 30 min at 37 °C, iodoacetamide was added to a final concentration of 50 mM and allowed to react for 30 min on ice. The mixture was chromatographed on a Sephadex G-50 column (1.5 cm × 60 cm) equilibrated with 0.1 M ammonium acetate, pH 8.0. Fractions containing $^{125}$I-ubiquitin–protein conjugates eluted at the exclusion volume and were pooled and used as substrate for the deconjugation assays. Approximately 40% of the $^{125}$I-ubiquitin was incorporated into high molecular weight complexes.

To measure deconjugation, $^{125}$I-ubiquitin conjugates ($3 \times 10^4$ cpm) were incubated at 37 °C with $1 \times 10^{-4}$ unit of the esterases in 0.3 mL of 50 mM Tris-HCl pH 8.0, containing 0.1 mM EDTA and 10 mM DTT. At the indicated times the reaction was terminated by the addition of 0.05 mL of reaction mixture to 0.025 mL of 9% SDS, 15% glycerol, 0.2 M Tris-HCl, and 3 mM EDTA, pH 6.8 (SDS–PAGE sample buffer). The samples were then subjected to SDS–PAGE according to the method of Laemmli (1970). The resulting gel was stained with Coomassie blue 250-R, dried, and subjected to radioautography.

*Other Methods.* Protein concentrations were determined by the method of Lowry (Lowry et al., 1951) or by a modification of the Bradford method (Bearden, 1978). Bovine serum albumin was used as the standard for both assays.

## Results and Discussion

Current studies of ubiquitin–protein conjugate metabolism have identified two major pathways of conjugate metabolism: deconjugation and proteolysis of the attached protein (Hershko et al., 1980; Hough et al., 1986; Hershko & Rose, 1987). In both cases, the regeneration of free ubiquitin appears to be effected by a family of ubiquitin carboxyl-terminal hydrolases which possess a general specificity for ubiquitin. The leaving group specificity of these enzyme activities may relate to the physiologic role of a particular enzyme. Only one of these enzymes has been purified, ubiquitin carboxyl-terminal hydrolase from reticulocytes. The similarity of this enzyme to others with specificity for ubiquitin has not been investigated, nor has the tissue distribution of this class of enzymes. Our approach to the study of the ubiquitin carboxyl-terminal hydrolases was to utilize UbOEt as a generic substrate that could circumvent the leaving group specificities of the hydrolases while utilizing the ubiquitin specificity requirement.

*Assay of Esterase Activities.* The assay of the reticulocyte enzyme ubiquitin carboxyl-terminal hydrolase (UCH), using ubiquitin ethyl ester as the substrate, was reported previously (Wilkinson et al., 1986a). In the present work, this assay was extended to monitor the separation of four distinct ubiquitin-specific esterases from the cytosolic fraction of bovine calf thymus. By use of a reverse-phase C-8 column (Wilkinson et al., 1986a), it was determined that intact ubiquitin was the only product formed upon incubating UbOEt with the crude extracts or the individual enzyme preparations characterized below (data not shown). The HPLC assay used here has several advantages over other assays used to detect this activity. First, it is rapid and can be carried out at saturating substrate concentrations. Quantitative results are easily obtained, and it is therefore well-suited for use in monitoring enzyme purification. It is a general assay, in that any enzyme exhibiting binding specificity for ubiquitin and capable of hydrolyzing

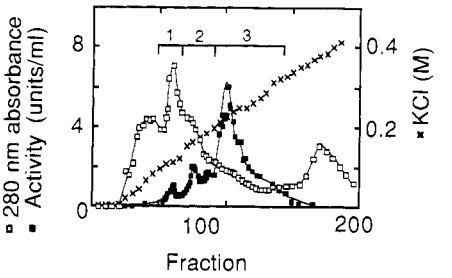


FIGURE 1: Ion exchange chromatography of UbOEt hydrolytic activity from calf thymus. A total of 100 g of bovine calf thymus was homogenized and centrifuged, and the resulting supernatant was chromatographed on a DE-52 anion exchange column as described under Experimental Procedures. Shown is the 280-nm absorbance (□), the ubiquitin esterase activity (■), and the salt concentration (×). Horizontal bars labeled 1–3 indicate the fractions pooled for further purification.

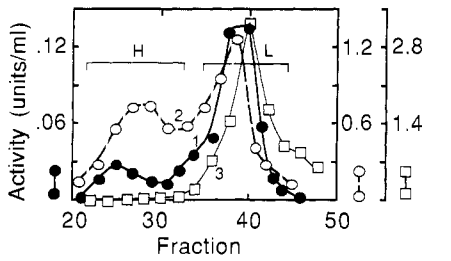


FIGURE 2: Gel filtration chromatography of UbOEt hydrolytic activities. The indicated pooled fractions from the DE-52 column (Figure 1) were separately concentrated and chromatographed on a Sephacryl S-300 column as described under Experimental Procedures. Curve 1 (●), 1 unit applied from DE-52 peak 1; curve 2 (○), 10 units applied from DE-52 peak 2; curve 3 (□), 30 units applied from DE-52 peak 3. The high and low molecular mass ranges are indicated with horizontal bars.

carboxyl-terminal derivatives of ubiquitin should be detected. Finally, it is quite specific, since the HPLC separation easily distinguishes between ubiquitin, the ester, and the tryptic cleavage product, des-glycylglycine-ubiquitin. Assays based on migration in an SDS–PAGE system cannot distinguish these products. The identification and comparison of four ubiquitin esterases from a single tissue extract was made possible by this assay method.

*Resolution of Esterase Activities.* The first separation step employed was a DE-52 anion exchange column (see Experimental Procedures). The bound proteins were eluted with a linear salt gradient, yielding at least three peaks of esterase activity at potassium chloride concentrations of 0.11, 0.15, and 0.20 M (labeled peaks 1–3, Figure 1). Each activity peak was concentrated and applied to a Sephacryl S-300 gel filtration column (Figure 2). This separation revealed that each peak of activity obtained from the ion exchange column contained at least one enzyme possessing a molecular mass of approximately 30 000 Da. The second ion exchange peak contained, in addition, a larger molecular mass activity (approximately 100–200 kDa). Rechromatography of this high molecular mass activity on the Sephacryl S-300 column resulted in a single peak of activity eluting at the original position (not shown), indicating that it is either a single polypeptide or a tightly associated oligomer, but not simply a loosely aggregated form of the lower molecular mass activities. Esterases L1, L2, and L3 refer to the low molecular mass activities from peaks 1–3 from the DEAE separation, and H2 refers to the high molecular mass activity from peak 2 of the DEAE separation.

In order to test the possibility that the multiple activity peaks observed were artifacts generated by proteolysis, the homo-

Table I: Isolation of Ubiquitin Carboxyl-Terminal Esterases[a]

| step | total protein (mg) | total activity (units) | specific activity (units/mg) |
|---|---|---|---|
| supernatant | 560 | 39.4 | 0.070 |
| DE-52 | | | |
| peak 1 | 66.7 | 1.3 | 0.019 |
| peak 2 | 21.5 | 3.9 | 0.181 |
| peak 3 | 37.2 | 25.9 | 0.696 |
| S-300 | | | |
| L1 | 20.1 | 0.66 | 0.033 |
| H2 | 5.7 | 0.92 | 0.161 |
| L2 | 7.4 | 1.58 | 0.214 |
| L3 | 5.9 | 15.3 | 2.50 |

[a] Calf thymus (22 g) was homogenized and fractionated as described under Experimental Procedures. One unit is defined as the activity of enzyme required to hydrolyze 1 μmol of UbOEt/min under the assay conditions described under Experimental Procedures. The fractions listed correspond to the activity peaks designated in Figures 1 and 2.

genization step was performed in the presence of several protease inhibitors (see Experimental Procedures) in addition to the PMSF and EDTA present in the original experiments. The resulting activity profiles were essentially identical with those shown in Figures 1 and 2, making it unlikely that the multiple activities were generated by proteolysis.

A quantitation of the separation procedures is shown in Table I. Due to the problem of quantitating the individual activities prior to their resolution, the fold purification and recovery for the individual enzymes in the crude homogenate is not readily obtained. The pertinent information offered by these data is an estimate of the relative contribution made by each enzyme to the total UbOEt hydrolytic activity in thymus extract. Under $V/K$ conditions the activity peak labeled L3 constitutes about 95% of the total UbOEt hydrolytic activity in the tissue supernatant. The activities designated L1, L2, and H2 individually comprised 1–2% of the total UbOEt hydrolytic activity, suggesting that in vivo hydrolysis of small molecular mass ubiquitin conjugates is dominated by esterase L3.

*Ubiquitin Binding Properties.* The affinity of each enzyme for UbOEt and ubiquitin was estimated by analyzing the kinetic behavior of the esterases from thymus. By use of the integrated Michaelis–Menten equation, modified to account for a uni-bi reaction in which one product is a competitive inhibitor (Wilkinson et al., 1986a), it was found that, for each enzyme studied, the $K_m$ for UbOEt was equal to the $K_i$ for ubiquitin (Table II). All four of the enzymes identified here possess a binding site for ubiquitin as judged by the low observed $K_i$ values (0.6–30 μM, 5–250 μg/mL). The fact that the $K_i$ and $K_m$ values were identical for each enzyme indicates that, with the ester substrate, the primary interactions with the enzymes are determined by the ubiquitin portion of the substrate.

In light of these relatively low dissociation constants for ubiquitin, it was of interest to examine the binding of these activities to a ubiquitin–Sepharose affinity column (Hershko et al., 1983). Only the most anionic activity (peak 3, Figure 1) bound appreciably to the immobilized ubiquitin. Despite the relatively high affinities for ubiquitin exhibited by these enzymes, three activities did not bind appreciably to the ubiquitin–Sepharose column. The simplest explanation is that the region(s) on the ubiquitin molecule required for L1, L2, and H2 binding are not accessible on this solid support. Preliminary studies using ubiquitin immobilized via arginine residues (Duerksen & Wilkinson, 1987) support this hypothesis, since the reversible binding of L1, L2, and H2 to this new support has been observed (P. Duerksen-Hughes and K. D. Wilkinson, unpublished results). This may indicate that these enzymes recognize regions or conformations of the ubiquitin molecule that are not available on the ubiquitin–Sepharose column.

Table II: Properties of the Isolated Esterases

| | L1 | L2 | H2 | L3 |
|---|---|---|---|---|
| $K_m = K_i$ (μM)[a] | 1.2 | 30 | 8.0 | 0.6 |
| % bound to ubiquitin–Sepharose[b] | <5 | <5 | <5 | 85 |
| % inhibition by | | | | |
| Ubal (50 nM)[c] | 80 | 43 | 73 | 91 |
| $IAcNH_2$ (10 mM)[d] | >95 | >95 | >95 | >95 |
| EDTA (10 mM) | <5 | <5 | <5 | <5 |
| PMSF (1 mM) | <5 | <5 | <5 | <5 |
| $Mn^{2+}$ (5 mM) | 17 | 47 | 69 | 26 |

[a] Analysis of kinetic data was performed as described previously (Wilkinson et al., 1986a). [b] Each enzyme preparation (1 milliunit) was applied to a 1-mL uniquitin–Sepharose column (11 mg/mL ubiquitin) equilibrated with 50 mM Tris-HCl, pH 7.6, 0.1 mM EDTA, and 0.1 mM DTT, followed by the application of 0.1 mL of equilibration buffer. After 10 min of incubation at room temperature, the column was eluted with 2 mL of equilibration buffer, and the activity eluted was determined as described above. [c] Assay of esterases in the presence of ubiquitin aldehyde was performed as described under Experimental Procedures except that 50 mM sodium bicarbonate, pH 8.0, was used due to the apparent "inactivation" of the aldehyde by Schiff base formation with the primary amine of Tris base. [d] The indicated enzyme was incubated at 23 °C for 15 min in the presence of 10 mM iodoacetamide, after which DTT was added to a final concentration of 50 mM to discharge the alkylating agent.

*Sensitivity to Inhibitors.* To characterize these enzymes in terms of mechanistic class, the enzyme preparations were tested for inhibition of UbOEt hydrolytic activity by iodoacetamide and manganese (inhibitors of thiol hydrolases), as well as the serine protease inhibitor PMSF, and an inhibitor of metallo proteases, EDTA. The results shown in Table II provide evidence that the enzymes contain essential thiol groups. Iodoacetamide fully inactivated all four enzymes, whereas EDTA and PMSF had no measurable effect on the activities. The effect of 5 mM manganese was variable, ranging from 17 to 69% inhibition with the different enzymes. Both reticulocyte UCH (Pickert & Rose, 1986; Pickert & Rose, 1985) and uH2a isopeptidase (Kanda et al., 1986) have been reported to be inactivated by iodoacetamide, insensitive to metal chelators, and inhibited by manganese.

*Mechanism-Based Inhibition by Ubiquitin Aldehyde.* Further evidence for ubiquitin binding and the formation of a tetrahedryl intermediate is provided by the marked inhibition of all four activities by ubiquitin aldehyde (Hershko & Rose, 1987) shown in Table II. The concentration of inhibitor used (50 nM) is about 2 orders of magnitude below the ubiquitin dissociation constants of these enzymes. When ubiquitin aldehyde was pretreated with sodium borohydride to generate the C-terminal alcohol, no inhibition was observed under the same conditions. This suggests that the potent inhibition observed requires the aldehyde functionality at the carboxyl terminus of ubiquitin aldehyde. It is noteworthy that the percent inhibition due to the aldehyde is inversely related to the respective dissociation constants for ubiquitin. The inhibition constants for this compound calculated from the data given in Table II are about 1000-fold lower than the respective $K_m$ values in each case. The extra increment of binding interaction is probably the result of the formation of a tetrahedral adduct between the active site nucleophile and the aldehyde moiety at the carboxyl terminus of this inhibitor. This is to be expected for an esterase which attacks the carbonyl group of glycine-76 of ubiquitin conjugates and carboxyl-terminal derivatives (Hershko & Rose, 1987). Similar binding interactions between UCH from erythrocytes and ubiquitin aldehyde have been reported recently (Hershko & Rose, 1987),

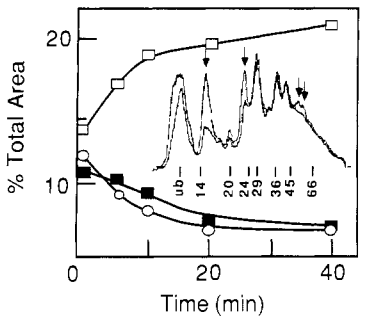


FIGURE 3: Deconjugation of $^{125}$I-ubiquitin–fraction II conjugates by esterase H2. $^{125}$I-Ubiquitin–protein conjugates were incubated with 0.1 milliunit/mL H2 at 37 °C for the indicated times. The inset shows the densitometric scans of radioautograms from SDS–PAGE of the 40-min reaction in the absence and presence of H2. Arrows mark the peaks which diminish in intensity, while free ubiquitin increases in intensity upon incubation. The time courses show the intensities of three radiolabeled bands: ubiquitin (□), a 15-kDa conjugate (○), and a 23-kDa conjugate (■).

but not with any other of the hydrolases.

*Leaving Group Specificity.* Having identified and characterized these enzyme activities with a generic substrate, it was of interest to investigate the leaving group specificity exhibited by these enzymes. To this end, each enzyme (present at $3 \times 10^{-4}$ unit/mL) was tested for its ability to hydrolyze endogenous $^{125}$I-ubiquitin–protein conjugates formed by reticulocyte fraction II (Ciechanover et al., 1980; Hough et al., 1986; Kanda et al., 1986). The approach of using an equal number of esterase units of each enzyme in this experiment should allow an estimation of the *relative activity* of each enzyme toward the conjugates (normalized to the activity toward ubiquitin ethyl ester, a substrate containing a "nonspecific" leaving group). It does not mimic the conditions in the whole extract since these activities are present in quite different amounts (Table I). Only H2 exhibits measurable activity in the deconjugation of ubiquitin–protein conjugates under the conditions used. Further, as shown in the inset to Figure 3, four conjugate bands (approximately 15, 23, 50, and 60 kDa) appear to be preferred substrates. Also shown is a time course for the specific deubiquitination of the 15- and 23-kDa bands and the appearance of free ubiquitin catalyzed by H2. At higher concentrations and longer times of incubation, other conjugates were also metabolized, albeit at a slower rate. The rapid disappearance of ubiquitin conjugates seen in reticulocyte extracts is due to both deconjugation and proteolysis. The deconjugation component may be due to high levels of one or more of these activities which are present in extracts. However, with the substrates used, H2 is the most active deconjugating enzyme when normalized to the activity observed with the nonspecific ester leaving group. Thus, at least one of these enzymes appears to differ from the others in the specificity for the leaving group attached to ubiquitin.

The preparation of conjugates used in the above experiments was formed in a 30-min labeling reaction and thus is probably most representative of slowly degraded ubiquitin–protein conjugates. Shorter labeling periods would be expected to preferentially label rapidly degraded conjugates. It should be noted that this preparation of conjugates contains a few high molecular mass conjugates. We have observed that the presence of high molecular mass conjugates in such reaction mixtures is variable and depends on the integrity of both the fraction II preparation and the $^{125}$I-ubiquitin [compare Figure 2 of Wilkinson et al. (1980) and Figure 7 of Evans and Wilkinson (1985)]. When the conjugation reaction mixture used above was quenched directly into SDS buffers (instead of iodoacetamide) and subjected to SDS–PAGE, a similar distribution of conjugates was obtained. Thus, the concentration of iodoacetamide used to quench the reaction for preparation of these conjugates was effective in inhibiting the endogenous "deconjugating" enzyme activity present in fraction II.

An apparent discrepancy exists between this result and that reported by Kanda et al. (1986), in which a similar conjugate preparation was completely deubiquitinated by the action of a large amount of a 150-fold purified preparation of the 38-kDa isopeptidase. The amount of enzyme activity used in these latter studies can be calculated as 0.06 unit of esterase activity on the basis of the data given (Kanda et al., 1986) and our results shown in Table II. This is the amount of activity present in the homogenate from 3 g of tissue. In contrast, we have used $1 \times 10^{-4}$ unit of esterase activity, and this corresponds to the amount of activity present in the homogenate of 6 mg of tissue. One possible explanation for the apparent discrepancy is that the previous preparation was contaminated with a small amount of H2. Another is that at very high concentrations the isopeptidase may display a weak activity in removing ubiquitin from many other conjugates. Additional studies to resolve this matter are under way.

*Relationships between the Four Activities and Previously Studied Hydrolases.* One of the enzyme activities (L3) resembles reticulocyte UCH, an enzyme with specificity for small molecular mass leaving groups. The similarities between the reticulocyte and the thymus enzyme include size, charge, kinetic constants with UbOEt as substrate, affinity for ubiquitin–Sepharose, inability to cleave ubiquitin–protein conjugates, and inhibition by thiol reagents and ubiquitin aldehyde. The activity profile resulting from salt gradient elution of the ion exchange column (Figure 1) revealed L3 to be the predominant UbOEt hydrolytic activity in thymus extract. Survey studies showed L3 to comprise a similar fraction of the total activity in rat liver and rabbit reticulocytes (not shown). Thus, the widespread abundance of this enzyme activity is consistent with its postulated function as the "housekeeper" of the ubiquitin pathway, liberating ubiquitin from adventitiously formed adducts and small peptide fragments (Pickert & Rose, 1985, 1986). In this context, the relatively minor amounts of L1, L2, and H2 may suggest more specific functions for these enzymes, since in vivo small adduct hydrolysis should be dominated by UCH.

In previous studies, the ion exchange fractionation of thymus extract was monitored by ubiquitin–H2a cleavage, resulting in the detection of one peak of activity, whereas three peaks of the appropriate size were detected with UbOEt as the substrate (Figure 2). Taken together, these results suggest that only one of the four esterases shown here to be present in thymus extract cleaves this conjugate. The evidence suggests that either esterase L1 or esterase L2 resembles the isopeptidase; i.e., they exhibit similar elution positions from the DE-52 column [0.1–0.15 M KCl at pH 7.5, Figure 1 and Kanda et al. (1986)], similar size [~30 kDa, Figure 2 and Kanda et al. (1986) and Matsui et al. (1982)], and a similar pattern of inhibition by thiol reagents and metal ions (Table II). A direct comparison of UbOEt hydrolysis with ubiquitin–H2a cleavage is necessary to definatively establish the relationship between these enzyme activities.

A different deconjugating activity from rabbit reticulocyte fraction II was previously identified with ubiquitin–lysozyme conjugates as the substrate (Hough et al., 1986). This activity eluted from a DEAE-Fractogel column at about 0.15 M KCl and exhibited a molecular mass of ~200 kDa. This enzyme

may possess some homology with esterase H2 on the basis of similar molecular mass and chromatographic behavior. Additionally, H2 was the only esterase found to exhibit significant activity toward ubiquitin–Fraction II protein conjugates (Figure 3) when present at $3 \times 10^{-4}$ unit/mL. This experiment was designed to detect the enzyme(s) which deconjugate(s) ubiquitin most efficiently compared to the rate at which they hydrolyze ubiquitin ethyl ester. With larger amounts of enzyme activity (0.5 unit/mL) all four enzyme preparations catalyze the cleavage of this and other conjugates (data not shown), and these or other enzymes may contribute to the rapid deconjugation seen in most tissues. At the current state of purity, it is not possible to eliminate the possibility that the low molecular mass preparations are contaminated with H2. In at least one case (L3) this appears likely since it has been previously demonstrated that ubiquitin–protein conjugates are not substrates for homogeneous UCH (Pickert & Rose, 1986, 1985).

The fourth activity, similar in many respects to the other three, has not been detected before, and its physiological role is unknown. A *potential* role for this activity may be suggested by the fact that several ubiquitin fusion proteins have been shown to be processed to monomeric ubiquitin. These include the polyprotein gene product (Ozkaynak et al., 1987), the ubiquitin fusion proteins coded for in the eukaryotic genome (Lund et al., 1985; Finley et al., 1987), and artificial fusion proteins between ubiquitin and $\beta$-galactosidase (Bachmair et al., 1986). The chemistry of this reaction is similar except that the leaving group is attached via an $\alpha$-amino group rather than an $\epsilon$-amino group as in the cases above. It can be anticipated that this difference in the sites of attachment might also play a role in determining the specificity of the enzymatic cleavage of such substrates.

It should be emphasized that the above assignments are circumstantial and direct studies will need to be done to identify the substrate specificity of each of these activities. We have not ruled out the occurrence of other enzymes which might metabolize ubiquitin conjugates and derivatives. In particular, serine proteases would not have been detected in these studies since PMSF was included in the early steps of the isolation procedures. Also, the use of ubiquitin ethyl ester as the substrate would probably not detect enzymes with primary specificity for the leaving group attached to ubiquitin.

*Nomenclature.* With the recent recognition that several types of ubiquitin conjugates exist and multiple enzymes are capable of removing ubiquitin from them, it has become apparent that a systematic nomenclature is required. We propose the following system for these substrates and enzyme activities.

First, ub is used to designate ubiquitin ligated to another molecule via its carboxyl-terminal glycine residue. The prefixes to this designation should indicate the type of bond involved, and the suffix indicates the identity of the molecule attached. For example, $N\epsilon^{119}$-ubH2a would describe the structure of the conjugate between the carboxyl terminus of ubiquitin and the $\epsilon$-amino group of lysine 119 in histone H2a. If the exact site of conjugation is unknown, the superscript is omitted. If the identity of the attached protein is unknown, the suffix P is used to indicate protein. Thus, unidentified conjugates between ubiquitin and cellular proteins can be referred to by the designation N$\epsilon$-ubP. Ubiquitin fusion proteins are described similarly; i.e., N$\alpha$-ub$\beta$gal specifies the ubiquitin–$\beta$-galactosidase fusion protein (Bachmair et al., 1986) and N$\alpha$-ub$_n$ the polyprotein precursor of ubiquitin (Ozkaynak et al., 1987; Finley et al., 1987). Carboxyl-terminal derivatives with small molecules attached would be indicated in the same manner [S-ubDTT for the thiol ester between the carboxyl terminus of ubiquitin and DTT (Pickert & Rose, 1986); N$\epsilon$-ubLys for the amide between ubiquitin and the $\epsilon$-amino group of lysine] except where the prefix would be redundant (ubOEt for the carboxyl-terminal ethyl ester of ubiquitin).

The enzyme activities which hydrolyze these bonds will be referred to by the principle substrates used to detect and quantitate these activities. The enzyme activity which hydrolyzes small molecule adducts of ubiquitin but not larger conjugates has been termed ubiquitin carboxyl-terminal hydrolase (UCH) and should continue to be so designated. Isoforms may be designated, such as UCH-L1, UCH-L2, and UCH-L3 as in the present work. Enzymes that can release the protein portion from ubiquitin–protein conjugates should be referred to as amidases (Hershko et al., 1980). The enzyme activity which deconjugates ubiquitin from $N\epsilon^{119}$-H2a has been called isopeptidase but this is not appropriate since the specificity appears to be limited to the isopeptide bond with ubiquitin (Kanda et al., 1986). Similarly, it has been termed a lyase (Andersen et al., 1981), but this term is normally reserved for enzymes that make or break double bonds. Thus, the name ubiquitin isoamidase or its systematic equivalent ($N\epsilon^{119}$-ubH2a amidase) is to be preferred. Activities which show less specificity and act on general conjugates would be indicated by the designation N$\epsilon$-ubP amidase (N$\epsilon$-ubiquitinyl protein amidase, abbreviated N$\epsilon$-UPA). Thus, esterase H2 could also be designated N$\epsilon$-UPA-H2 because it appears to hydrolyze at least some ubiquitin–protein conjugates. The activities which are responsible for processing ubiquitin fusion proteins could be designated N$\alpha$-ubP amidases, or with a more descriptive suffix if the specificity is more limited (N$\alpha$-ub$_n$ amidase for the enzyme which processes the ubiquitin polyprotein to the monomer).

In summary, the present work is the logical extension of previous studies which led to the development of a substrate and assay method for carboxyl-terminal hydrolases and represents a step toward the methodical study of the enzymes that exhibit ubiquitin carboxyl-terminal hydrolytic activity. Potential roles for these enzymes include regulation of the specificity of the ubiquitin-dependent proteolysis system (Hershko et al., 1980; Hough et al., 1986; Pickert & Rose, 1986, 1985), control of gene transcription and the cell cycle (Ciechanover et al., 1980; Matsui et al., 1979; Mueller et al., 1985; Goldknopf et al., 1980), regulation of the stress response (Carlson et al., 1987; Carlson & Rechsteiner, 1987; Finley et al., 1987), and posttranslational processing of newly synthesized polyubiquitin and ubiquitin fusion proteins (Ozkaynak et al., 1987; Lund et al., 1985; Finley et al., 1987). Future work must focus on purifying these enzymes and defining the leaving group specificities of the purified preparations by semisynthetic and genetically engineered conjugates as the substrates.

**Registry No.** UCH, 86480-67-3; ubiquitin, 60267-61-0.

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# Tertiary Structure of Human Complement Component C5a in Solution from Nuclear Magnetic Resonance Data

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ABSTRACT: The tertiary structure for the region 1-63 of the 74 amino acid human complement protein C5a in solution was calculated from a large number of distance constraints derived from nuclear Overhauser effects with an angular distance geometry algorithm. The protein consists of four helices juxtaposed in an approximately antiparallel topology connected by peptide loops located at the surface of the molecule. The structures obtained for the helices are compatible with $\alpha$-helical hydrogen-bonding patterns, which provides an explanation for the observed slow solvent exchange kinetics of the amide protons in these peptide regions. In contrast to the peptide region 1-63, no defined structure could be assigned to the C-terminal region 64-74, which increasingly acquires dynamic random coil characteristics as the end of the peptide chain is approached. An average root-mean-square deviation of 1.6 Å was obtained for the $\alpha$-carbons of the first 63 residues in the calculated ensemble of C5a structures, while the $\alpha$-helices were determined with an average root-mean-square deviation of 0.8 Å for the $\alpha$-carbons. A comparison between the solution structure of C5a and the crystal structure of the functionally related C3a protein, as well as inferences for the interaction of C5a with its receptor on polymorphonuclear leukocytes, is discussed.

The complement system consists of a set of regulatory factors and proteolytic enzymes (C1-C9) which aid in the recognition and elimination of foreign substances. In classical activation, a cascade of proteolytic cleavages of complement proteins C1-C5 is triggered by the formation of immune complexes. This ultimately results in the formation of a molecular lytic complex between C5b, C6, C7, C8, and C9 which assists in the lysis of bacterial cells [reviewed in Mayer (1979)]. The

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