Synthesis and Characterization of Ubiquitin Ethyl Ester, a New Substrate for Ubiquitin Carboxyl-Terminal Hydrolase†

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ABSTRACT: A new substrate for ubiquitin carboxyl-terminal hydrolase, the carboxyl-terminal ethyl ester of ubiquitin, has been synthesized by a trypsin-catalyzed transpeptidation. In the presence of 1.6 M glycylglycine ethyl ester, trypsin removes the carboxyl-terminal glycylglycine of ubiquitin and replaces it with the dipeptide ester. The equilibrium mixture under these conditions contains 30% ubiquitin ethyl ester and 70% hydrolysis product, the 74-residue fragment of ubiquitin. Ubiquitin ethyl ester can be purified by gel filtration and ion-exchange chromatography. The structure of this product has been verified by identification of the products of base hydrolysis, tryptic cleavage in aqueous solution, and peptide mapping. When ubiquitin ethyl ester is incubated with purified ubiquitin carboxyl-terminal hydrolase, specific cleavage of the ester linkage is observed. A rapid, sensitive assay is described utilizing high-performance liquid chromatography. By use of this assay, it has been shown that ubiquitin carboxyl-terminal hydrolase is inactivated in the absence of thiols. Optimal protective effects are seen with 10 mM dithiothreitol. The rate of catalysis is maximal at pH 8.5, with evidence for catalytically important groups with pK values of 5.2, 7.6, and 9.5. These findings are consistent with the participation of a thiol group in the active site. Native ubiquitin is a competitive inhibitor of ubiquitin ethyl ester hydrolysis. Kinetic analysis of this reaction demonstrates that the K_m of the ester and the K_i of ubiquitin are similar, 5×10^{-7} M, and that the enzyme is specific for the ubiquitin portion of these carboxyl-terminal derivatives and exhibits activity toward oxygen esters as well as the previously demonstrated activity against thiol esters and amides. This substrate and assay should make it possible to examine the mechanism of action of this enzyme and others that exhibit ubiquitin carboxyl-terminal hydrolytic activity.

Intracellular proteolysis requires a continued supply of ATP (Goldberg & St. John, 1976). One of the systems that utilize ATP is the ubiquitin-dependent system (Ciechanover et al., 1984). In this system, the energy of ATP hydrolysis is used to covalently attach a highly conserved protein, ubiquitin, to a variety of cellular proteins (Hershko et al., 1980). The conjugates are linked by an amide bond between the carboxyl terminus of ubiquitin and the α- and ϵ-amino groups of the target protein (Hershko et al., 1984). It is thought that the attachment of ubiquitin serves to mark the target protein for proteases that recognize the ubiquitin portion of the conjugates and degrade the target protein (Hershko et al., 1980).

One of the predominant reactions of ubiquitin metabolism is the hydrolytic generation of free ubiquitin from carboxyl-terminal derivatives of ubiquitin. An enzyme that catalyzes this reaction has been purified to homogeneity from lysates of reticulocytes and erythrocytes (Rose & Warms, 1983; Pickart & Rose, 1985). This 30-kilodalton protein has been shown to catalyze cleavage of ubiquitin carboxyl-terminal thiol esters and amides with small leaving groups and has been termed the ubiquitin carboxyl-terminal hydrolase (UCH).[1] This enzyme has a low activity toward ubiquitin conjugates of lysozyme or cytochrome c. It has been suggested that this enzyme is responsible for cleaving residual peptides from the carboxyl terminus of ubiquitin and for reversing adventitious conjugates between activated ubiquitin and small molecular weight thiols and amines (Pickart & Rose, 1985). A protein with similar physical properties has been purified from calf thymus and catalyzes the cleavage of uH2a[1] to ubiquitin and histone H2a (Matsui et al., 1982; Kanada et al., 1984) and the cleavage of ubiquitin protein conjugates (Kanada et al., 1986). This enzymatic activity can be termed ubiquitin–protein lyase (UPL)[1] to distinguish it from the activity that releases small leaving groups. This enzyme has not been tested for activity on carboxyl-terminal derivatives on ubiquitin with small leaving groups. Finally, a poorly characterized activity with an apparent molecular mass of 200 000 daltons has been described that hydrolyzes the ubiquitin–lysozyme conjugates that are formed in reticulocyte extracts (Hershko et al., 1984b; Hough et al., 1986). The relationships between these enzymatic activities have not been adequately investigated.

Clarification of this field and definition of the role of these enzymatic activities require that substrates and initial rate assays be developed for enzymes that hydrolyze carboxyl-terminal derivatives of ubiquitin. Assays of this enzymatic activity have been previously described (Matsui et al., 1982;

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[1] Abbreviations: uH2a, the conjugate between histone H2a and ubiquitin formerly called protein A-24; UCH (ubiquitin carboxyl-terminal hydrolase), an enzyme that hydrolyzes carboxyl-terminal derivatives of ubiquitin with small leaving groups (Pickart & Rose, 1985) and has also been called ubiquitin carboxyl-terminal esterase (Rose & Warms, 1983); UPL (ubiquitin–protein lyase), the less well characterized enzymatic activity that hydrolyzes ubiquitin–protein conjugates and has variously been referred to as amidase (Hershko et al., 1980), protein A-24 lyase (Andersen et al., 1981), and isopeptidase (Matsui, et al., 1982; Kanada et al., 1986); ATP, adenosine 5′-triphosphate; HPLC, high-performance liquid chromatography; TPCK, N-tosyl-L-phenylalanine chloromethyl ketone; MES, 2-(N-morpholino)ethanesulfonic acid; HEPES, N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; PP_i, inorganic pyrophosphate; NADH, reduced nicotinamide adenine dinucleotide; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid.

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Rose & Warms, 1983), but they are cumbersome and it is difficult to quantitate or manipulate assay conditions. For these reasons, we thought it desirable to develop a substrate and assay conditions that could be used to gather more conventional kinetic and mechanistic data. This initial study has focused on the homogeneous UCH enzyme since it is the best characterized of this class of enzymatic activities. This paper reports the synthesis and characterization of a stable homogeneous substrate in milligram amounts, the carboxyl-terminal ethyl ester of ubiquitin, and the development of a rapid sensitive assay for UCH activity, using HPLC.

Materials and Methods

Ubiquitin was purified from erythrocytes (Haas & Wilkinson, 1985), fraction II from reticulocytes (Ciechanover et al., 1978), and ubiquitin carboxyl-terminal hydrolase from fraction II (Pickart & Rose, 1985). CH-activated Sepharose 4B, TPCK-treated trypsin, soybean trypsin inhibitor, and glycylglycine ethyl ester hydrochloride were purchased from Sigma Chemical Co., St. Louis, MO, and acetonitrile (HPLC grade) was purchased from Baker Chemical Co., Phillipsburg, NJ. All other chemicals were reagent grade or better from commercial sources. Sephadex G-50 was obtained from Pharmacia Fine Chemicals, Piscataway, NJ, and the C-8 HPLC column (5 μm, 4 mm × 250 mm) from Alltech Associates Inc., Deerfield, IL.

Ubiquitin and the various derivatives were separated analytically by HPLC on a C-8 reverse-phase column. The solvent contained 25 mM sodium perchlorate and 0.07% (w/w) perchloric acid in 41% (v/v) acetonitrile. The flow rate was 1 mL/min, and protein was detected by monitoring the 205-nm absorbance. Under these conditions, ubiquitin eluted at 12.5 min, the 74 amino acid tryptic hydrolysis product at 14.5 min, and ubiquitin ethyl ester at 18.5 min. Analysis times can be shortened by use of a faster flow rate or a shorter column.

Ubiquitin ethyl ester was synthesized in a final volume of 0.5 mL containing 7 mg/mL ubiquitin, 1.6 M glycylglycine ethyl ester hydrochloride, 0.35 M NaOH, and 0.7 mg/mL TPCK-treated trypsin. The reaction mixture was incubated at 37 °C for 12 h and quenched by the addition of soybean trypsin inhibitor to a final concentration of 0.7 mg/mL. The pH of the mixture was adjusted to 4.5 with glacial acetic acid and chromatographed on a Sephadex G-50 column (2.5 cm × 90 cm) equilibrated with 50 mM ammonium acetate, pH 4.5. The 280-nm absorbance of the collected fractions was determined, and selected fractions were subjected to HPLC as described above. Fractions containing ubiquitin ethyl ester were pooled and applied to a (carboxymethyl)cellulose column (Whatman CM-52, 0.5 cm × 10 cm) equilibrated with 50 mM ammonium acetate, pH 4.5. After the column was washed with 2 column volumes of starting buffer, the 74 amino acid tryptic hydrolysis fragment was eluted with 50 mM ammonium acetate, pH 5.5. The column was subsequently washed with 50 mM ammonium acetate, pH 7.0, to elute the pure ubiquitin ethyl ester. Fractions containing ubiquitin ethyl ester were pooled, dialyzed against 10 mM HCl, and stored frozen at −20 °C.

Ubiquitin ethyl ester was characterized by HPLC (Figure 3) after the following treatments: no treatment, incubation for 1 h at 37 °C after adjustment of the pH with HCl or NaOH to values ranging from pH 1 to 12, or incubation with 2% (w/w) TPCK-treated trypsin (Wilkinson & Audhya, 1981). In addition, tryptic peptide maps were obtained as described previously (Cox et al., 1986a).

Ubiquitin carboxyl-terminal hydrolase activity was determined by measuring the rate of conversion of ubiquitin ethyl ester to ubiquitin. Unless otherwise indicated, assays contained 10 mM dithiothreitol, 0.2 mM EDTA, 100 mM potassium phosphate, pH 7.2, and varying amounts of ubiquitin ethyl ester and enzyme. After the indicated time at 37 °C, aliquots of the reaction mixture containing from 1 to 10 μg of ubiquitin ethyl ester were subjected to HPLC as described above. The areas under the respective peaks were integrated manually by measuring peak heights and widths, and the concentration of substrate and product was calculated from the peak areas. Kinetic parameters were determined by use of an integrated rate expression for a uni–bi mechanism with product inhibition by one product (Orsi & Tipton, 1979). Separate experiments demonstrated that the ethanol released was not inhibitory. Initial experiments indicated that the K_m for ubiquitin ethyl ester and the K_i for ubiquitin were similar, and the equation was simplified to account for this (eq 1, Results).

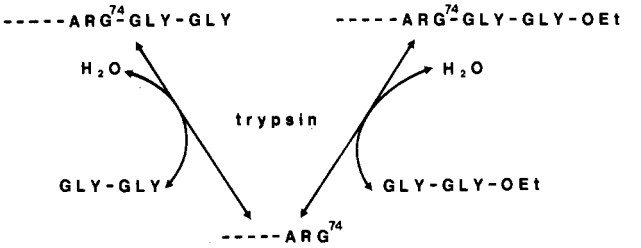

FIGURE 1: Synthesis of the carboxyl-terminal ethyl ester of ubiquitin by using trypsin-catalyzed transpeptidation.

The pH dependence of catalysis was examined in assays as described above, except that the substrate concentration was 200 μg/mL and the pH was varied. Buffers (100 mM at the indicated pH) were as follows: acetate (5.0–6.0), MES (6.0–6.8), HEPES (6.8–7.6), Tris (7.4–9.0), and borate (8.6–10.0).

Results

A synthetic route to carboxyl-terminal derivatives of ubiquitin (Figure 1) was suggested by the pronounced stability of the protein to tryptic cleavage (Wilkinson & Audhya, 1981). In aqueous solution, the only susceptible bond is that of arginine-74, resulting in the release of the carboxyl-terminal diglycine. When this tryptic digestion is carried out in the presence of 1.6 M glycylglycine ethyl ester, a new product is observed that elutes slightly later upon chromatography on HPLC. This product accumulates to about 30% of the total as judged by the areas under the peaks. When lower concentrations of ester are used, correspondingly less of the later eluting product is observed. In the absence of trypsin, no reaction is observed. These results are consistent with the trypsin-catalyzed cleavage of the dipeptide glycylglycine from native ubiquitin, followed by the trypsin-catalyzed synthesis of a new amide bond (Laskowski, 1978; Fruton, 1982) between the 74 amino acid fragment of ubiquitin and glycylglycine ethyl ester (Figure 1).

To characterize this later eluting product, it was purified by quenching the reaction with soybean trypsin inhibitor and chromatography on Sephadex G-50 and CM-52 (Figure 2). The upper panel of Figure 2 shows the 280-nm absorbance of the fractions from chromatography of the transpeptidation mixture on Sephadex G-50. The first peak eluted contains the trypsin–trypsin inhibitor complex, the second peak is excess trypsin inhibitor, and the third contains the ubiquitin-derived products. The elution position of these products is indistinguishable from that of native ubiquitin, indicating that the apparent molecular weights are similar to that of native ubiquitin. When the fractions containing these products were chromatographed on the cation-exchange resin CM-52 the

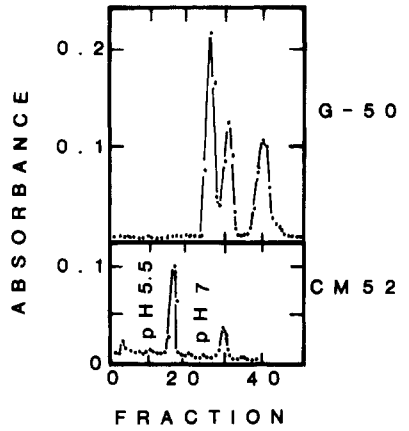

FIGURE 2: Purification of ubiquitin ethyl ester. (Top panel) The 280-nm absorbance of the fractions obtained upon chromatography of the transpeptidation reaction mixture on Sephadex G-50. The peak at fraction 40 contains the ubiquitin-derived products. (Bottom panel) The 280-nm absorbance of the fractions obtained upon chromatography of the ubiquitin-derived products (fractions 35–45 above) on (carboxymethyl)cellulose. The pH of the eluant is indicated. The peak eluting with pH 7 buffer contains ubiquitin ethyl ester.

results shown in the lower panel of Figure 2 are obtained. At pH 4.5, all of the protein is adsorbed to the column. When the pH of the eluant is raised to 5.5, a peak is eluted that contains approximately 70% of the absorbance applied. This protein coelutes with the 74 amino acid tryptic hydrolysis product of ubiquitin when chromatographed on HPLC. Native ubiquitin, if present, is also eluted under these conditions. Finally, when the column is washed with pH 7 buffer, the remainder of the absorbance is eluted. This peak contains the product of the transpeptidation reaction. The greater retention on this cation-exchange resin indicates that this product has more positive charges or fewer negative charges than native ubiquitin in this pH range.

To characterize this product, it was subjected to acid hydrolysis, base hydrolysis, and tryptic cleavage. Figure 3 shows the chromatographic behavior of these various reaction products. Panel A is a mixture of native ubiquitin (peak 1) and the 74 amino acid fragment generated by tryptic cleavage of the carboxyl-terminal glycylglycine of ubiquitin (peak 2). Panel B shows the chromatogram obtained with the purified transpeptidation product. In addition to small amounts of peaks 1 and 2, the major product elutes later and is indicated as peak 3. The product was stable from pH 1 to pH 9 but at basic pH was hydrolyzed to a compound that coelutes with native ubiquitin on HPLC (Figure 3, panel C). Treatment of the purified product with trypsin converted it to a product that coelutes with the 74 amino acid fragment of ubiquitin on HPLC (Figure 3, panel D). Finally, tryptic peptide mapping (Cox et al., 1986a) of this product confirmed that all of the expected peptides of ubiquitin are present in the product (data not shown). Thus, this product is identified as the carboxyl-terminal ethyl ester of ubiquitin.

When the purified ubiquitin ethyl ester is incubated with a crude preparation of the enzymes of the ubiquitin-dependent proteolysis system (reticulocyte fraction II) in the absence of ATP, there is a time-dependent conversion of the ester to native ubiquitin. No further reaction is detectable by HPLC. These results suggested that this compound is a substrate for the UCH activity present in fraction II (Rose & Warms, 1983). To demonstrate the specificity of this reaction, UCH was purified from fraction II by the procedures previously described (Pickart & Rose, 1985). Figure 4 shows that purified UCH also catalyzes the hydrolysis of ubiquitin ethyl ester to native

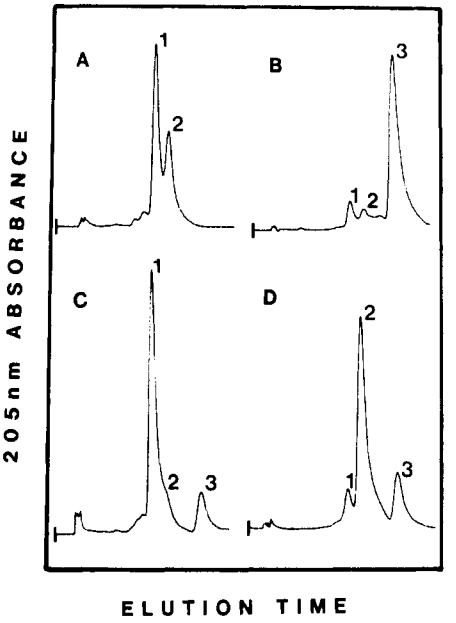

FIGURE 3: Characterization of ubiquitin ethyl ester and hydrolysis products by HPLC. Ubiquitin or the ethyl ester were treated as indicated and analyzed by HPLC as described under Materials and Methods. Peak 1 is ubiquitin, peak 2 is the 74 amino acid hydrolysis product resulting from the cleavage of the carboxyl-terminal glycylglycine, and peak 3 is ubiquitin ethyl ester. (Panel A) Ubiquitin in 50 mM Tris-HCl, pH 7.6, was treated with 0.5% (w/w) TPCK-treated trypsin for 2 min at 37 °C. (Panel B) Ubiquitin ethyl ester. (Panel C) Ubiquitin ethyl ester was incubated for 30 min at pH 12. (Panel D) Ubiquitin ethyl ester in 50 mM Tris-HCl, pH 7.6, was treated with 0.5% (w/w) trypsin for 10 min at 37 °C.

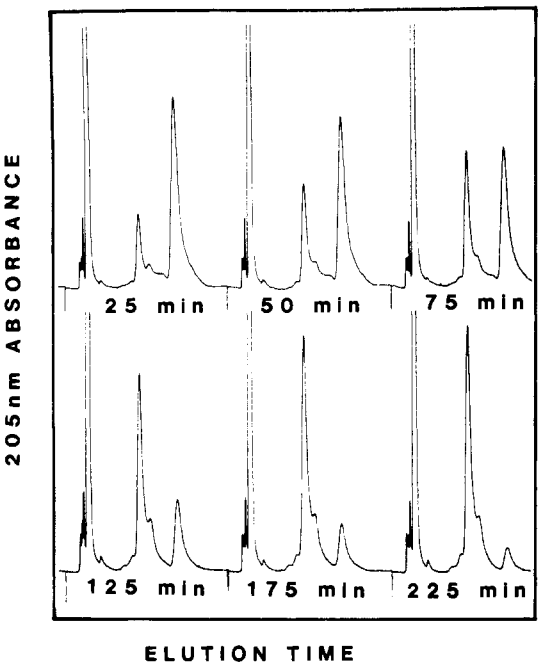

FIGURE 4: Conversion of ubiquitin ethyl ester to ubiquitin catalyzed by ubiquitin carboxyl-terminal hydrolase. Ubiquitin ethyl ester was incubated with enzyme under the standard assay conditions described under Materials and Methods. At the indicated times, aliquots were removed and analyzed by HPLC. The first peak eluted is due to buffer components, the second is ubiquitin, and the third is ubiquitin ethyl ester.

ubiquitin. There is no evidence for other reactions occurring during this time period.

Using purified ubiquitin ethyl ester and affinity-purified UCH, we began to examine the kinetic properties of this reaction. In the absence of thiols, there was a rapid loss of

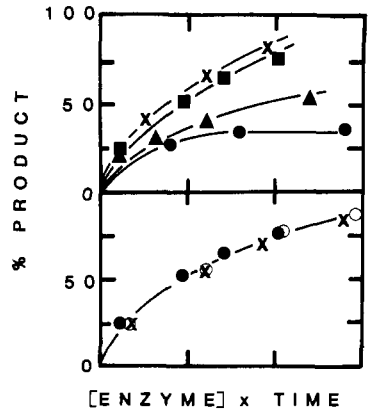

FIGURE 5: Time course for hydrolysis of ubiquitin ethyl ester by ubiquitin carboxyl-terminal hydrolase. Substrate and product concentrations were determined by HPLC. Percent conversion to product is plotted vs. the product of the enzyme concentration and the reaction time. All assays contained 0.1 M potassium phosphate, pH 7.2, 0.2 mM EDTA, 0.12 mg/mL ubiquitin ethyl ester, and 25 μL/mL purified UCH incubated at 37 °C for up to 1 h. (Top panel) UCH activity was measured in the presence of the indicated concentrations of DTT: 0 (●), 5 mM (▲), 10 mM (■), and 20 mM (×). (Bottom panel) Assays in the presence of 10 mM DTT as above except that the enzyme concentration was varied. UCH concentrations were 25 μL/mL (○), 50 μL/mL (●), and 75 μL/mL (×).

enzymatic activity, and the reaction stopped before the ester was consumed (Figure 5, upper panel, circles). Addition of more enzyme at this point resulted in another burst of ester consumption (not shown). In the presence of increasing concentrations of dithiothreitol, the inactivation was much less severe, with maximal protection being obtained with 10 mM thiol. However, the time courses were still markedly nonlinear (Figure 5). To determine if the curvature was due to enzyme inactivation we applied the method of Selwyn (1965). Plots of product formed vs. [E] × time described a single curve (Figure 5, lower panel), indicating that the curvature was due solely to substrate depletion and/or product inhibition. Thus, all subsequent assays were done in the presence of 10 mM dithiothreitol. Initial experiments indicated that the curvature seen in Figure 5 at high levels of thiol was due to product inhibition by ubiquitin. There was no evidence of inhibition by the ethanol produced in this reaction.

Having established the validity of these assay conditions, we next carried out experiments to determine the K_m for the ester and K_i for ubiquitin. Because of the need to integrate peak areas to quantitate product formation, it was most accurate to allow a significant fraction of the substrate to be consumed. This resulted in nonlinear time courses and the corresponding difficulties of determining a true initial rate. Therefore, we applied an analysis technique using the integrated rate expression for a uni–bi mechanism where one product may act as a competitive inhibitor (Orsi & Tipton, 1979). Plots of $(S_0 - S)/t$ vs. ln $(S_0/S)t$ indicated that the K_m for the ester and the K_i for the product (ubiquitin) were similar. When this is the case, the integrated rate expression can be simplified to give eq 1. When the data from a series

$$\ln [(S_0 + P_0)/S] = [V/(K + S_0 + P_0)]t + \ln (1 + P_0/S_0) \quad (1)$$

of time courses at different initial concentrations of ester are plotted according to eq 1, Figure 6 is obtained. In this experiment, approximately 10% of the substrate was present as free ubiquitin before addition of the enzyme, and the y intercept is therefore greater than 0. It can be seen that this rate equation accurately describes the data for over 70% hydrolysis of substrate. The inset shows a secondary plot of

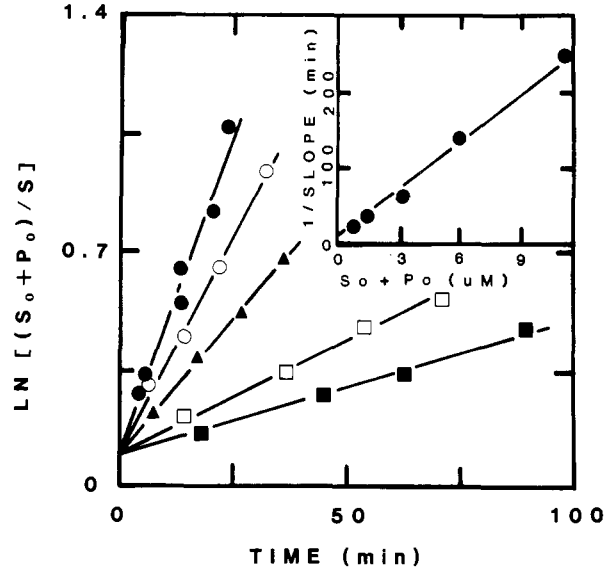

FIGURE 6: Determination of kinetic parameters for hydrolysis of ubiquitin ethyl ester by ubiquitin carboxyl-terminal hydrolase. S_0 is the initial substrate concentration, P_0 is the initial product concentration, and S is the substrate concentration at the indicated time. Data collected with different initial concentrations of substrate were plotted according to eq 1. Substrate concentrations (μM) were 0.6 (●), 1.2 (○), 2.9 (▲), 5.8 (□), and 10.5 (■). The inset shows a secondary plot of 1/slope vs. $S_0 + P_0$. The x-intercept of this plot yields $-K_m = -K_i$.

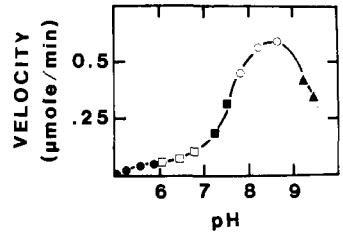

FIGURE 7: pH dependence of the catalytic rate. Assay conditions were as described under Materials and Methods. The buffers used were acetate (●), MES (□), HEPES (■), Tris (○), and borate (▲).

1/slope vs. $(S_0 + P_0)$. From this plot, we can estimate that the affinity ($K_m = K_i$) of the ubiquitin carboxyl-terminal hydrolase for ubiquitin is about 4 μg/mL (5×10^{-7} M).

Further mechanistic information was sought by examining the pH dependence of catalysis at saturating substrate concentration. Figure 7 shows the results obtained in this study. There are at least three distinct ionizations that affect the activity of this enzyme; increased activity is associated with the deprotonation of groups with pK values of 5.2 and 7.6 while decreased activity is observed upon deprotonation of a group with a pK of approximately 9.5. The optimum pH for this reaction is 8.5, while pH values above 9.5 lead to marked nonenzymatic hydrolysis of the substrate.

DISCUSSION

This paper describes the synthesis, purification, characterization, and use of the carboxyl-terminal ethyl ester of ubiquitin as a substrate for the enzyme, ubiquitin carboxyl-terminal hydrolase (Rose & Warms, 1983; Pickart & Rose, 1985).

The synthesis of the carboxyl-terminal ester of ubiquitin has been accomplished by a trypsin-catalyzed transpeptidation (Figure 2). It has been known for 30 years that hydrolytic enzymes such as trypsin will also synthesize the peptide bond under thermodynamic pressure. This microscopic reversibility has been utilized several times to synthesize large peptides

[reviewed by Fruton (1982)]. As in the case of trypsin inhibitor (Laskowski, 1978), no complicated blocking steps are necessary since the only bond cleaved by trypsin in aqueous solution is that of arginine-74 (Wilkinson & Audhya, 1981). In the presence of 1.6 M glycylglycine ethyl ester, transpeptidation occurs readily and to a final equilibrium value of 30% of the ubiquitin present. If we neglect the water term in the equilibrium (Homandberg et al., 1978), it can be calculated that the equilibrium constant for formation of the peptide bond is 0.27 M^{-1}. This can be compared to a value of 0.33 M^{-1} observed for the chymotrypsin-catalyzed synthesis of Cbz-tryptophanylglycinamide under similar conditions (Hommandberg et al., 1978). The similarity of these values indicates that the ester group is not significantly interacting with the rest of the protein molecule. This is in good agreement with the reported crystal structure of ubiquitin (Vijay-Kumar et al., 1985), which indicates that the carboxyl-terminus protrudes from the body of the protein into solution.

The product of the transpeptidation reaction has been purified by gel filtration and ion-exchange chromatography (Figure 2). The Stokes radius is similar to that of native ubiquitin, indicating a similar molecular weight and shape. Upon cation-exchange chromatography however, the product is more tightly retained, consistent with the loss of a negative charge from the carboxyl-terminus upon forming the ester. The product obtained by this purification procedure has the expected properties of the carboxyl-terminal ethyl ester. It is hydrolyzed under basic conditions to give the intact 76 amino acid ubiquitin (Figure 3) and by trypsin to give the 74 amino acid hydrolysis product (Figure 3). Further, tryptic peptide mapping (Cox et al., 1986a) gives the expected products (data not shown). The identity and usefulness of this transpeptidation product is further illustrated by the fact that the affinity-purified ubiquitin carboxyl-terminal hydrolase cleaves this compound to give native ubiquitin (Figure 4). Thus, the carboxyl-terminal ethyl ester of ubiquitin has been synthesized, purified, and characterized.

This ester has been found to be a tightly bound substrate for the ubiquitin carboxyl-terminal hydrolase (Figure 6), yielding native ubiquitin and ethanol. These results suggested that ubiquitin ethyl ester would be useful for assay of this enzymatic activity. The present method of assay for this activity suffers from a number of disadvantages. This assay indirectly measures the enzymatic activity by measuring the stimulation of $^{32}PP_i$ release in a system containing ubiquitin, activating enzyme, and a nucleophile (Rose & Warms, 1983). Adenylation of ubiquitin releases PP_i and the nucleophile traps the thiol ester intermediate. If UCH activity is present in the assay mixture, the carboxyl-terminal derivatives of ubiquitin (thiol esters or amides) will be hydrolyzed and additional PP_i will be released when the resulting ubiquitin is again adenylated. The rate of PP_i release in this coupled assay is expected to be proportional to the UCH activity, although the range of linear response is small in practice (Pickart & Rose, 1985). While this assay can be more rapidly performed and quantitated, it is not possible to specify the concentration of substrate present in the steady state nor to vary the assay conditions significantly from those that are optimal for activating and trapping the ubiquitin molecule.

The present method of assay, utilizing homogeneous ubiquitin ethyl ester, with detection and quantitation by HPLC, obviates the above difficulties. Sensitivity is good, with as little as 1 μg (0.1 nmol) of substrate being sufficient to quantitate product formation. Each determination requires approximately 10 min, and the assay mixture can be injected directly on HPLC, without any extractions or derivitization. In the absence of enzyme, there is no product formation observed between pH 1 and pH 9, and thus the base line rate is negligible. It should also be possible to determine the rate of reaction by measuring the ethanol released, by using alcohol dehydrogenase. The low K_m for ubiquitin ethyl ester, however, would necessitate the use of fluorescence to quantitate the NADH produced. In addition, a much larger assay volume would be necessary to measure product formation in this manner.

Using the HPLC assay, we have found that UCH is rapidly inactivated in the absence of thiols. Inclusion of 10 mM DTT in the assay prevents this inactivation (Figure 5). Addition of EDTA had no effect on the rate of ester hydrolysis or the inactivation, in agreement with earlier findings (Pickart & Rose, 1985). Addition of excess thiol to inactivated enzyme results in the complete recovery of activity (data not shown). These results suggest that UCH contains an easily oxidized thiol group that is required for activity. These results are consistent with the fact that UCH is strongly inhibited by alkylating agents such as iodoacetamide but not by chelating agents (Pickart & Rose, 1985). Pickart and Rose (1985) have reported that UCH has a high turnover number with thiol esters, and a similar activity toward ubiquitin amides. The latter measurements were made at low concentrations of thiol (0.2–0.3 mM), usually to avoid a high base line rate when the substrates were being generated in situ. Our results demonstrate that this concentration of DTT is insufficient to prevent inactivation. This suggests that the turnover number with amides may be higher than that previously observed.

Under the conditions used, the time course of ester hydrolysis is markedly nonlinear. This has been shown to be due solely to substrate depletion and/or product inhibition (Figure 5). In order to collect accurate kinetic data with this assay, it is most convenient to allow the enzyme to consume a significant portion of the substrate present. These two competing factors make it difficult to determine true initial rates. Therefore we have used an integrated form of the rate equation to fit the time courses and to determine kinetic parameters. Initial analysis of the time courses indicated that product inhibition of ubiquitin was significant and the K_m for the substrate was similar in magnitude to the K_i for the product. This situation leads to a simplification of the rate equation (eq 1, Results). When the data is analyzed according to this rate equation, the fit is excellent for over 70% of the reaction (Figure 6). It should be noted that deviations would be easily detected in this plot if the K_m and K_i differed by as little as 5-fold. The values of these kinetic parameters, 5×10^{-7} M, are similar to the earlier estimates [$(1–2) \times 10^{-6}$ M] calculated from the inhibition of hydrolysis by free ubiquitin (Pickart & Rose, 1985).

The agreement between K_i and K_m determined in this study indicates that the enzyme recognizes some feature of the ubiquitin portion of this substrate. This is consistent with the purification of this enzyme by affinity chromatography on immobilized ubiquitin. The presence or absence of the ester group makes little difference in the binding of ubiquitin to this enzyme. Previous workers have demonstrated that a variety of thiol esters and amides are cleaved by this enzyme, although kinetic constants could not be determined (Pickart & Rose, 1985).

Finally, we have exploited the flexibility of this assay to determine the pH dependence of catalysis. Under conditions of saturating ubiquitin, catalysis is optimal at pH 8.5. Deprotonation of a group with a pK of 9.5 results in the loss of

activity, as does the protonation of a group with a pK of 7.6. These results, and the finding of oxidative inactivation of the enzyme, support the previous suggestion that this enzyme may be a thiol protease (Pickart & Rose, 1986). One unusual feature of the pH dependence is the presence of a marked plateau of activity between pH 5.5 and pH 6.5 that is approximately 10% of the maximal activity. One possible explanation of this result is that there is a ionizable group in the vicinity of the active site that perturbs the ionization state of the active-site nucleophile. While it is not possible to define the nature of these ionizable groups from this limited data, it is clear that there are some unusual aspects to the active site of this enzyme.

The availability of this substrate in large amounts and the facile method of assay described here should make it possible to begin to define the mechanism and control of the UCH reaction. If ubiquitin ethyl ester is hydrolyzed by UPL activity, it should also prove useful in the comparative and mechanistic studies that will define the relationships of this important class of enzymes. The reaction catalyzed by this class of enzymes is required in ubiquitin-dependent proteolysis in order to regenerate ubiquitin from conjugates for another catalytic cycle (Hershko et al., 1980) and may play a role in determining the specificity of the system by hydrolyzing ubiquitin conjugates before proteolysis can take place (Hershko et al., 1980; Cox et al., 1986b). In addition, a similar activity is involved in the processing of the polyprotein precursor of ubiquitin (Ozkaynak, 1984; Dworkin-Rastl et al., 1984; Lund et al., 1985) and the cleavage of uH2a, a conjugate of ubiquitin and histone H2a (Andersen et al., 1981; Matsui et al., 1982; Kanada et al., 1984).

References

Andersen, M. W., Ballal, N. R., Goldknopf, I. L., & Busch, H. (1981) *Biochemistry 20*, 1100–1104.

Ciechanover, A., Hod, Y., & Hershko, A. (1978) *Biochem. Biophys. Res. Commun. 81*, 1100–1105.

Ciechanover, A., Finley, D., & Varshavsky, A. (1984) *J. Cell. Biochem. 24*, 27–53.

Cox, M. J., Shapira, R., & Wilkinson, K. D. (1986a) *Anal. Biochem. 154*, 345–352.

Cox, M. J., Haas, A. L., & Wilkinson, K. D. (1986b) *Arch. Biochem. Biophys.* (in press).

Dworkin-Rastl, E., Shrutkowski, A., & Dworkin, M. B. (1984) *Cell (Cambridge, Mass.) 39*, 321–325.

Fruton, J. S. (1982) *Adv. Enzymol. Relat. Areas Mol. Biol. 53*, 239–306.

Goldberg, A. L., & St. John, A. C. (1976) *Annu. Rev. Biochem. 45*, 747–803.

Haas, A. L., & Bright, P. M. (1985) *J. Biol. Chem. 260*, 12464–12473.

Haas, A. L., & Wilkinson, K. D. (1985) *Prep. Biochem. 15*, 49–60.

Hershko, A., Ciechanover, A., Heller, H., Haas, A. L., & Rose, I. A. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 1783–1786.

Hershko, A., Heller, H., Eytan, E., Kaklij, G., & Rose, I. A. (1984a) *Proc. Natl. Acad. Sci. U.S.A. 81*, 7021–7025.

Hershko, A., Leshinsky, E., Ganoth, D., & Heller, H. (1984b) *Proc. Natl. Acad. Sci. U.S.A. 81*, 1619–1623.

Hommandberg, G. A., Mattis, J. A., & Laskowski, M., Jr. (1978) *Biochemistry 17*, 5220–5227.

Hough, R., Pratt, G., & Rechsteiner, M. (1986) *J. Biol. Chem. 261*, 2400–2408.

Kanada, F., Matsui, S., Sykes, D. E., & Sandberg, A. A. (1984) *Biochem. Biophys. Res. Comm. 122*, 1296–1306.

Kanada, F., Sykes, D. E., Yasuda, H., Sandberg, A. A., & Matsui, S. (1986) *Biochim. Biophys. Acta 870*, 64–75.

Laskowski, M., Jr. (1978) in *Semisynthetic Peptides and Proteins* (Offord, R. E., & Di Bello, C., Eds.) pp 255–262, Academic, New York.

Lund, P. K., Moats-Staats, B. M., Simmons, J. G., Hoyt, E., D'Ercole, A. J., Martin, F., & Van Wyk, J. J. (1985) *J. Biol. Chem. 260*, 7609–7613.

Matsui, S., Sandberg, A. A., Negoro, S., Seon, B. K., & Goldstein, G. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 1535–1539.

Orsi, B. A., & Tipton, K. F. (1979) *Methods Enzymol. 63*, 159–183.

Ozkaynak, E., Finley, D., & Varshavsky, A. (1984) *Nature (London) 312*, 663–666.

Pickart, C. M., & Rose, I. A. (1985) *J. Biol. Chem. 260*, 7903–7910.

Rose, I. A., & Warms, J. V. B. (1983) *Biochemistry 22*, 4234–4237.

Selwyn, M. J. (1965) *Biochim. Biophys. Acta 105*, 193–195.

Vijay-Kumar, S., Bugg, C. E., Wilkinson, K. D., & Cook, W. J. (1985) *Proc. Natl. Acad Sci. U.S.A. 82*, 3582–3585.

Wilkinson, K. D., & Audhya, T. K. (1981) *J. Biol. Chem. 256*, 9235–9241.