A Specific Inhibitor of the Ubiquitin Activating Enzyme: Synthesis and Characterization of Adenosyl-Phospho-Ubiquitinol, a Nonhydrolyzable Ubiquitin Adenylate Analogue†

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ABSTRACT: A nonhydrolyzable analogue of ubiquitin adenylate has been synthesized for use as a specific inhibitor of the ubiquitination of proteins. Ubiquitin adenylate is a tightly bound intermediate formed by the ubiquitin activating enzyme. The inhibitor adenosyl-phospho-ubiquitinol (APU) is the phosphodiester of adenosine and the C-terminal alcohol derived from ubiquitin. APU is isosteric with the normal reaction intermediate, the mixed anhydride of ubiquitin and AMP, but results from the replacement of the carbonyl oxygen of Gly^{76} with a methylene group. This stable analogue would be expected to bind to both ubiquitin and adenosine subsites and result in a tightly bound competitive inhibitor of ubiquitin activation. APU inhibits the ATP–PP_i exchange reaction catalyzed by the purified ubiquitin activating enzyme in a manner competitive with ATP (K_i = 50 nM) and noncompetitive with ubiquitin (K_i = 35 nM). AMP has no effect on the inhibition, confirming that the inhibitor binds to the free form of the enzyme and not the thiol ester form. This inhibition constant is 10-fold lower than the dissociation constants for each substrate and 30–1000-fold lower than the respective K_m values for ubiquitin and ATP. APU also effectively inhibits conjugation of ubiquitin to endogenous proteins catalyzed by reticulocyte fraction II with an apparent K_i of 0.75 μM. This weaker inhibition is consistent with the fact that activation of ubiquitin is not rate limiting in the conjugation reactions catalyzed by fraction II. APU is similarly effective as an inhibitor of the ubiquitin-dependent proteolysis of β-lactoglobulin. Degradation of reduced carboxymethylated bovine serum albumin is not inhibited however, presumably due to the fact that arginylation is the rate-limiting step with this substrate. The tight binding and observed specificity of this inhibitor suggest that this class of inhibitors, with optimized geometry and/or different spacer lengths, may be useful as inhibitors of protein ubiquitination in vitro and in the design of in vivo inhibitors.

Ubiquitin is an extremely conserved, universally distributed protein which becomes posttranslationally attached to a variety of cellular proteins (Hershko, 1983). This conjugation is thought to serve a signaling function which targets the attached protein for intracellular proteolysis or modulates its function in some way (Wilkinson, 1987; Rechsteiner, 1987). Current evidence suggests that ubiquitination may be involved in the metabolism of proteins in the nucleus (Wu et al., 1981), in the cytoplasm (Rechsteiner, 1987; Ciechanover et al., 1984a), and on the cell surface (Gallatin et al., 1986).

In the nucleus, ubiquitination of histones H2a and H2b is thought to be involved in chromatin condensation (Matsui et al., 1979) or transcriptional regulation (Levinger & Varshavsky, 1982) by modifying the structure and function of the core octamer structure. In the cytoplasm, attachment of ubiquitin to soluble proteins can target them for intracellular proteolysis, especially if the proteins have become damaged or denatured. Thus, this system is thought to be responsible for the degradation of abnormal or damaged proteins which are formed during stress such as heat shock, oxidative damage, or premature termination of translation (Hershko & Ciechanover, 1986). Immunochemical studies suggest that ubiquitination of microtubules (Murti et al., 1988) and proteins present in neurofibrillary plaques (Perry et al., 1987; Mori et al., 1987) can occur. The role of ubiquitination in the metabolism of membrane-bound proteins is unclear, but it is known that antibodies to ubiquitin and ubiquitin–protein conjugates are able to bind to the cell surface and to inhibit high-affinity choline uptake by synaptosomic vesicles (Meyer et al., 1986). Additionally, it has been shown that the T-cell homing receptor (Siegelman et al., 1986) and the PDGF receptor (Yarden et al., 1986) are ubiquitinated. These modifications may be involved in receptor downregulation, receptor affinity, or signal transduction.

In all cases, the chemistry of ubiquitination is thought to be similar (Hershko et al., 1983). A ubiquitin activating enzyme (E1) first adenylates the carboxyl terminus of ubiquitin and then forms an intramolecular thiol ester. This thiol ester undergoes transesterification with a thiol group on one of a family of ubiquitin carrier proteins (E2) transfering the activated ubiquitin to these acyl carrier proteins. Finally, this thiol ester is subjected to nucleophilic attack by ϵ-amino groups of the target protein, in some cases catalyzed by one of a family of separate ligases (E3). It is presumed that the specificity

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of ubiquitination is controlled by the E2 and/or E3 component of this pathway.

To clarify the diverse array of postulated functions, it is necessary to be able to interfere with specific pathways of ubiquitination by genetic or chemical means. A temperature-sensitive ubiquitin activating enzyme has been identified in a cell cycle mutant derived from a mouse mammary carcinoma (Matsumoto et al., 1983; Finley et al., 1984). At the nonpermissive temperature, this cell line (ts85) fails to catalyze conjugation of ubiquitin (Finley et al., 1984) or degradation of damaged proteins (Ciechanover et al., 1984b), and it is arrested in early G2 phase of the cell cycle. A second cell cycle mutant in yeast, CDC34 (Goebl et al., 1988), has been shown to result from a mutation in the structural gene for one of the E2 family proteins. Another mutation in the yeast E2 family, RAD6 (Jentsch et al., 1987), results in cells that are sensitive to radiation damage and fail to repair such lesions.

Known inhibitors of the ubiquitin pathway include the following: ubiquitin aldehyde, which is a general inhibitor of ubiquitin recycling and depletes the levels of free ubiquitin (Hershko & Rose, 1987); hemin, which interferes with the proteases of the system (Haas & Rose, 1981); dipeptide esters, which inhibit the conjugation of ubiquitin to some proteins by inhibiting the ligases (Reiss et al., 1988); ribonuclease, which prevents the arginylation of the amino terminus of proteins containing acidic amino-terminal residues, a step that is necessary for the ubiquitination of these proteins (Ferber & Ciechanover, 1987); a synthetic peptide encompasing the carboxyl terminus of ubiquitin, which could possibly act as an inhibitor of the activating enzyme by virtue of the fact that it is efficiently adenylated (Jonnalagadda et al., 1988).

As useful as these inhibitors and mutants have been, there is still a need for stable and specific inhibitors that can be introduced into cells in acute experiments and that will not have the pleiotropic effects of the known perturbations currently available. We have explored the use of ubiquitin derivatives as such inhibitors. Ubiquitin is quite stable in cells with a half-life of 10–20 h in Hela cells (Carlson & Rechsteiner, 1987). It would be expected to impart a great deal of specificity by delivering an attached inhibitor to specific ubiquitin binding proteins in the cell. As a test of this approach, we have synthesized a stable analogue of the tightly bound intermediate in the action of the ubiquitin activating enzyme, ubiquitin adenylate. In vitro studies confirm that this analogue is stable and an inhibitor of the ubiquitin activating enzyme with no apparent interactions with any of the other enzymes of ubiquitin-dependent protein degradation.

Experimental Procedures

Preparative and Analytical Chromatography. Nucleotide derivatives were purified by standard open-column chromatography on silica gel or Diaion HP-20 resin (Mitsubishi Chemical Inc.) or by preparative HPLC[1] on a Whatman Magnum 20 ODS-3 column (50 cm) using a Beckman Model 332 chromatograph fitted with preparative pump heads and with detection at 254 nm. HPLC analyses were done on the same instrument with an Altex Ultrasphere 5-mm reversed-phase column. The structures of nucleotide intermediates and products were confirmed by ^{1}H NMR on a Jeol FX90Q spectrometer. Fast atom bombardment exact mass measurements were performed on a VG ZAB-HF spectrometer by MCA voltage scanning at a resolution of 10000; poly(ethylene glycol) was the reference.

Ubiquitin derivatives were purified either on (carboxymethyl)cellulose (CM-52, Whatman) or by FPLC on a Mono S column (Pharmacia). They were analyzed by HPLC on a C-8 reverse-phase column (Alltech Associates) with buffers A [25 mM $NaClO_4$, 0.07% (w/w) $HClO_4$] and B (buffer A in 75% acetonitrile), monitoring at either 205 or 260 nm.

5′-Adenylic Acid Mono(2-aminoethyl ester) (I). The preparation was based on a procedure reported for 5′-adenylic acid mono(2-amino-2-phenethyl ester) (Krauss et al., 1978) rather than the published methods of Hecht et al. (1974) and Sandrin and Boissonnas (1966), which gave less satisfactory results in our hands. N^6,$O^{2'}$,$O^{3'}$-Triacetyl-5′-adenylic acid (Krauss et al., 1978) (6.0 g, 12.7 mmol) and 2-[(benzyloxycarbonyl)amino]ethanol (Rose, 1974) (7.42 g, 38.0 mmol) were dissolved in 75 mL of dry pyridine. The pyridine was then removed in vacuo at 30 °C, and this procedure was repeated twice more. The dry residue was dissolved in 36 mL of dry pyridine, 2,4,6-triisopropylbenzenesulfonyl chloride (5.75 g, 19.0 mmol) was added, and the resulting solution was stirred at room temperature in the dark and under argon for 4 days. The reaction solution was stripped in vacuo to a viscous gum, which was triturated twice with 150 mL of petroleum ether and then once with 190 mL of diethyl ether. The resulting gum was chromatographed on silica gel in a mixture of toluene, acetone, and water (2:8:1) to give 3.96 g of N^6,$O^{2'}$,$O^{3'}$-triacetyl-5′-adenylic acid mono[2-[(benzyloxycarbonyl)amino]ethyl ester], which was then deacetylated by stirring for 24 h at room temperature in a mixture of 52 mL of pyridine and 87 mL of concentrated ammonium hydroxide. The solvents were removed in vacuo, and the residue was twice dissolved in 200 mL of water and stripped to dryness. Acetamide was removed from the product by chromatography on Diaion HP-20 resin using a gradient from water to 3:1 methanol–water; lyophilization gave 2.78 g (83%) of 5′-adenylic acid mono(ammonium salt) mono[2-[(benzyloxycarbonyl)amino]ethyl ester] as a hemihydrate. Anal. Calcd for $C_{26}H_{28}N_7O_9P\cdot0.5H_2O$: C, 43.64; H, 5.31; N, 17.81. Found: C, 43.73; H, 5.52; N, 17.88.

Hydrogenolysis of the benzyloxycarbonyl-protected derivative (700 mg, 1.27 mmol) was carried out over 18 h with 450 mg of 5% palladium on carbon in 165 mL of water in a Paar apparatus under 50 psi of hydrogen. After filtration to remove the catalyst, lyophilization gave 436 mg (80%) of 5′-adenylic acid mono(2-aminoethyl ester) (I) as a hydrate. HPLC analysis using 50% aqueous methanol at a flow rate of 0.2 mL/min and detection at 254 nm showed the product to be >99% pure. Anal. Calcd for $C_{12}H_{19}N_6O_7P\cdot2.25H_2O$: C, 33.45; H, 5.50; N, 19.51. Found: C, 33.23; H, 5.04; N, 19.56.

5′-Adenylic Acid Mono(2-glycinamidoethyl ester) (II). To an ice-cooled suspension of 156 mg (0.362 mmol) of 5′-adenylic acid mono(2-aminoethyl ester) hydrate in 10 mL of dry dimethylformamide containing 112 mL (0.800 mmol) of triethylamine was added *N*-(*tert*-butoxycarbonyl)glycine *p*-nitrophenyl ester (118 mg, 0.400 mmol). After stirring in an ice bath for 1 h, the reaction was allowed to stir at room temperature overnight. The residue left after thorough removal of the solvent in vacuo was dissolved in 1 mL of water and separated by preparative HPLC in two runs, elution being with 35% methanol in water at a flow rate of 5.6 mL/min to give after lyophilization 96 mg of 5′-adenylic acid mono[2-[*N*-(*tert*-butoxycarbonyl)glycinamido]ethyl ester].

[1] Abbreviations: HPLC, high-pressure liquid chromatography; FPLC, fast protein liquid chromatography; APU, 5′-adenosyl-phospho-ubiquitinol (VII); PP_i, pyrophosphate; ATP, adenosine 5′-triphosphate; AMP, adenosine 5′-monophosphate; GTP, guanosine 5′-triphosphate; BSA, bovine serum albumin; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

The *N*-(*tert*-butoxycarbonyl) group was cleaved by stirring this product (90 mg) in 2 mL of ice-cold 9:1 trifluoroacetic acid–water for 45 min. After the solvent had been stripped off in vacuo, the resulting syrup was taken up in 0.5 mL of water and separated by preparative HPLC using a gradient from water to 15% methanol in water at a flow rate of 5.6 mL/min to give after lyophilization 26.4 mg of 5′-adenylic acid mono(2-glycinamidoethyl ester) (II) as a hydrate (18%). Anal. Calcd for $C_{14}H_{22}N_7O_8P\cdot 3H_2O$: C, 33.54; H, 5.63; N, 19.56. Found: C, 33.12; H, 4.97; N, 19.53. The composition of the product was confirmed by fast atom bombardment high-resolution mass measurement: calcd, 448.135 for $C_{14}H_{22}N_7O_8P$ (M + H); obsd, 448.134.

Des-Gly^{76}*-Ubiquitin Carboxyl-Terminal Acyl Azide (VI).* The carboxyl-terminal ethyl ester of des-Gly^{76}-ubiquitin (IV) was synthesized and purified as described for the ethyl ester of ubiquitin (Wilkinson et al., 1986) except that glycine ethyl ester was used in the place of glycylglycine ethyl ester in the transpeptidation reaction. Retention times on HPLC and the yield of product formed are similar to those reported with glycylglycine ethyl ester. Treatment of IV with 1 M hydrazine hydrate, pH 8.0, for 90 min at 37 °C resulted in a quantitative conversion to the carboxyl-terminal hydrazide (V) as judged by analytical HPLC (Wilkinson et al., 1986). After dialysis against water, V (200 μL, 2 mg/mL) was treated with 0.5 M nitrous acid at −5 °C for 2 min to generate VI in situ (Bodansky, 1985).

5′-Adenosyl-Phospho-Ubiquitinol (VII). The acyl azide (VI) generated above was mixed immediately with 17 mg of I and 32 μL of triethylamine, and after 2 min at −5 °C excess reagents were removed by dialysis against 50 mM NH_4OAc, pH 4.8 at 4 °C. APU (VII) was further purified by FPLC on a Mono S column with a linear gradient of 50–500 mM NH_4OAc, pH 4.8. Under these conditions, APU was eluted at approximately 0.24 M salt, shortly before ubiquitin. The concentration of APU was determined by integration of the HPLC profiles, by assuming that the extinction coefficient was the sum of that for ubiquitin and equimolar I. Aliquots were stored at −80 °C until used.

Assays. Ubiquitin activating enzyme was purified as previously described (Haas et al., 1982) and ATP–PP_i exchange was measured by the method previously described (Haas & Rose, 1982). Ubiquitin-dependent proteolysis was assayed with ^{125}I-reduced carboxymethylated serum albumin or ^{125}I-β-lactoglobulin as substrate (Evans & Wilkinson, 1985) with the following modifications. ATP was present in these incubations at 10 μM, a concentration sufficient to saturate the activating enzyme, and GTP was present at 2 mM to stabilize the proteases. These conditions were chosen so that the degree of inhibition of ATP-PP_i exchange and catalysis could be measured under comparable conditions. The rate of proteolysis measured under these conditions was at least 75% that of the rate measured under standard assay conditions. Four different ubiquitin carboxyl-terminal esterase isozymes were fractionated and assayed as described by Mayer and Wilkinson (1989). Deconjugation of ubiquitin–protein conjugates catalyzed by reticulocyte fraction II was determined as previously described (Hershko et al., 1980).

Results

Synthesis and Characterization of APU. The nucleotide intermediate 5′-adenylic acid mono(2-aminoethyl ester) (I) was synthesized by a modification of published procedures (Krauss et al., 1978) in a final yield of 67%. The structure and purity of I were confirmed by HPLC, elemental analysis, mass spectroscopy, and NMR analysis. Additionally, the

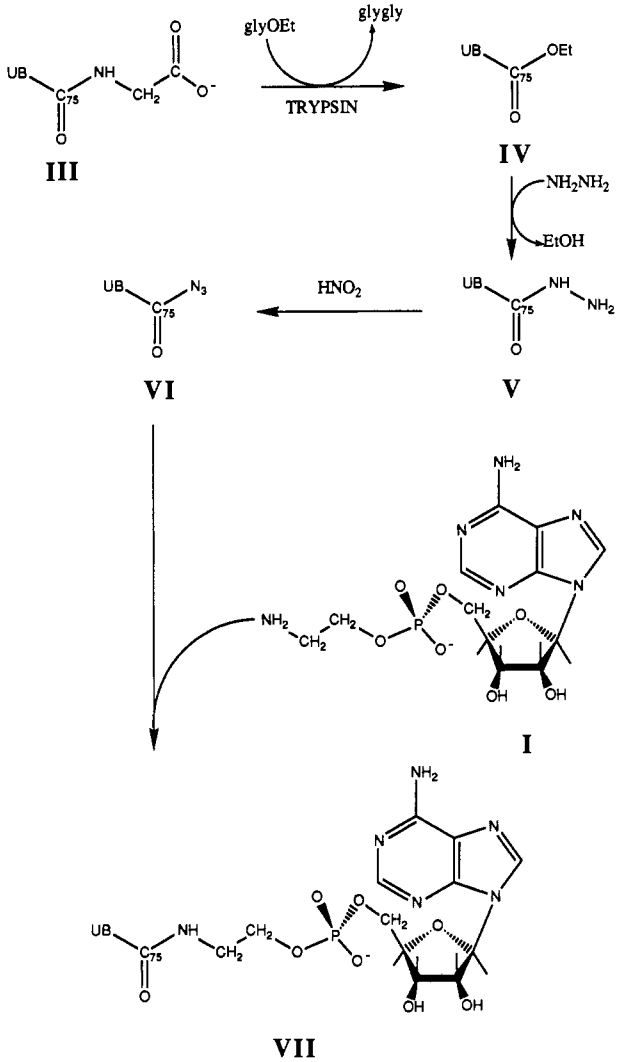

FIGURE 1: Synthesis of APU (VII). Details are given in the text.

glycyl derivative of this intermediate, 5′-adenylic acid mono-(2-glycinamidoethyl ester) (II), was synthesized as a standard to assist in the determination of the structure of the ubiquitin adenylate analogue.

Figure 1 shows the synthetic scheme utilized to couple I to the carboxyl terminus of des-Gly^{76}-ubiquitin. This procedure takes advantage of our previous finding that trypsin-catalyzed transpeptidation of ubiquitin in the presence of peptide esters introduces an ester group to the carboxyl terminus of ubiquitin (Wilkinson et al., 1986). This reaction is limited to the peptide bond between Arg^{74} and Gly^{75} because of the inability of trypsin to cleave at any of the other Arg or Lys residues in the native ubiquitin structure. With glycine ethyl ester in the transpeptidation reaction, the product is the carboxyl-terminal ethyl ester of des-Gly^{76}-ubiquitin (IV). Further activation of the carboxyl group of Gly^{75} was accomplished by hydrazinolysis to form the acyl hydrazide (V). Hydrazide formation proceeded smoothly at 37 °C with the product eluting near ubiquitin on HPLC (data not shown). Control experiments showed that the treatment of ubiquitin under these conditions had no effect on the retention time of ubiquitin in this system or on the ability of ubiquitin to stimulate proteolysis catalyzed by reticulocyte fraction II. The hydrazide can be stored for several months at −20 °C with no apparent degradation.

The acyl azide (VI) was prepared in situ immediately before coupling to adenylate derivative I to give APU (VII). This reaction was carried out at low temperatures in order to minimize the Curtis rearrangement. Analytical HPLC of the

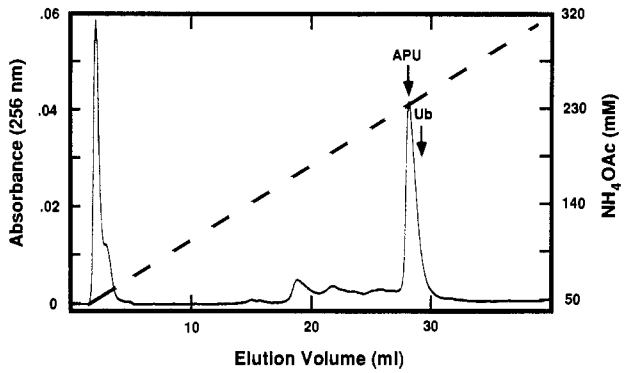

FIGURE 2: Purification of APU by ion exchange chromatography. FPLC of the dialyzed crude product (VII) on Mono Q (0.5 × 10 cm) utilized a linear gradient from 50 to 500 mM NH_4OAc, pH 4.8, with a volume of 60 mL. Absorbance was monitored at 256 nm. The elution position of APU is indicated by an arrow, as well as the elution position of ubiquitin. The latter compound is not detected under these conditions due to its low extinction at this wavelength. HPLC analysis showed that the trailing edge of the APU peak was contaminated with ubiquitin, and these fractions were excluded from the pool of fractions containing APU.

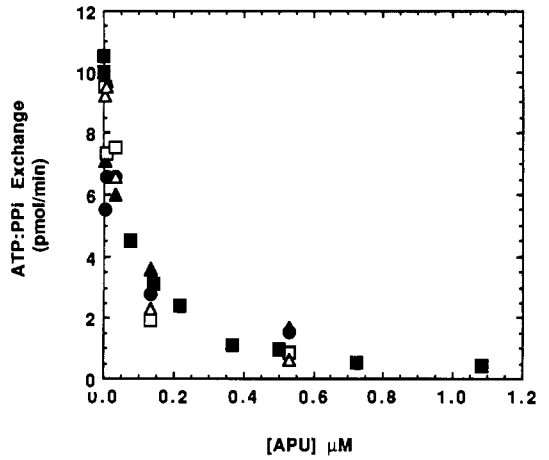

FIGURE 3: Inhibition of E1-catalyzed ATP–PP_i exchange by APU. The velocity of the exchange reaction was measured in the presence of 10 μM ATP, 0.1 mM PP_i, and the indicated concentrations of APU (closed symbols, three experiments) or as above but in the presence of 100 μM AMP (open symbols, two experiments).

dialyzed reaction mixture under the conditions described previously (Wilkinson et al., 1986) showed two major peaks when monitored at 205 nm, one at 7.3 min coeluting with ubiquitin and one at 6 mins. When the chromatograms were monitored at 260 nm, only the 6-min peak contained significant absorbance, consistent with the presence of the nucleotide derivative in that species. None of the earlier eluting peak was formed in the absence of I or in control incubations where the ubiquitin hydrazide was omitted. APU was further purified by FPLC on a Mono S column (Pharmacia) with a linear gradient of 50–500 mM NH_4OAc, pH 4.8 (Figure 2). Under these conditions, APU eluted at approximately 0.24 M salt, shortly before ubiquitin. HPLC of the fractions containing APU showed that the trailing edge of this peak was contaminated with an ubiquitin-like product, probably Ub^{75} resulting from the hydrolysis of VI during coupling. Fractions were pooled to exclude this contaminant. The concentration of APU was determined by integration of the HPLC profiles by assuming that the extinction coefficient was the sum of that for ubiquitin and equimolar I.

The structure of APU was confirmed by limited tryptic digestion. Cleavage with trypsin is expected to yield the 74 amino acid fragment of ubiquitin and II due to cleavage at Arg^{74} (Wilkinson & Audhya, 1981). When APU was treated with trypsin (2.5% w/w) and the mixture was analyzed by HPLC using 2% solvent B, a product was observed that had strong absorbance at 260 nm and eluted at 5.5 min. This product coeluted with authentic II. Integration of the area of this peak, monitored at either 205 or 260 nm, demonstrated that stoichiometric II was released from APU under these conditions.

Inhibition by APU of ATP–PP_i Exchange Catalyzed by Ubiquitin Activating Enzyme. To determine if APU binds to the active site of the ubiquitin activating enzyme, we measured its inhibition of the ATP–PP_i exchange reaction catalyzed by this enzyme. It was observed that the rate of exchange was greatly reduced in the presence of APU (solid symbols, Figure 3). There are two forms of the activating enzyme which could bind the inhibitor in order to give the observed inhibition (see below): the free enzyme or the intermediate form with ubiquitin bound as a thiol ester (Haas & Rose, 1982). It is known that only the latter form is capable of tightly binding AMP. Therefore, we repeated these experiments in the presence of 100 μM AMP, which should relieve the inhibition if the inhibitor is binding to the thiol ester form of the enzyme. AMP had no effect on the exchange rate observed in the absence of inhibitor, nor did it prevent inhibition (open symbols, Figure 3). Therefore, APU most likely exerts its inhibitory effect by binding to the free form of the enzyme.

The time course of inhibition was examined to assure that the measurements were made under steady-state conditions and that APU was not a slow binding inhibitor. When the activating enzyme was preincubated with 50 nM APU for 2–60 min before the ATP–PP_i exchange reaction was started, there was no significant difference in the degree of inhibition observed (50% inhibition, data not shown). Thus, the binding and inhibition are complete on a time scale that is short compared to the time required for assay.

The inhibition patterns were determined to characterize the nature of this inhibition (data not shown). The inhibition is competitive vs ATP, and the secondary plot of slope vs inhibitor concentration is linear, indicating a K_i of 50 nM. The inhibition is noncompetitive vs ubiquitin, and the secondary plots of slope vs inhibitor concentration are linear. Both K_{ii} and K_{is} are approximately 35 nM. Thus, the inhibition patterns are consistent with the ordered kinetic mechanism previously determined with ATP binding first, followed by ubiquitin.

Inhibition by APU of Conjugate Formation Catalyzed by Reticulocyte Fraction II. If APU is a specific inhibitor of the activation of ubiquitin, it should also be an effective inhibitor of the conjugation of ubiquitin to endogenous proteins of the reticulocyte fraction II. The steady-state level of conjugates formed was measured by incubation of ^{125}I-ubiquitin with fraction II and ATP, followed by separation of the conjugates on SDS–PAGE, radioautography, and densitometric quantitation. Figure 4 (open squares) shows that APU is a good inhibitor of the accumulation of ubiquitin–protein conjugates at 10 μM ATP, a subsaturating level of ATP. The *apparent* K_i can be estimated from the half-maximal inhibition and is 750 nM (see Discussion). ATP is able to completely relieve this inhibition at concentrations greater than 0.2 mM (Figure 4, solid squares). Control experiments showed that neither AMP nor 5′-deoxyadenosine had any effect on the levels of ubiquitin conjugates (data not shown), suggesting that the inhibition observed in Figure 4 requires both the adenosine and the ubiquitin portions of APU.

To ensure that APU was not degraded during the incubation with fraction II, aliquots were withdrawn from the reaction

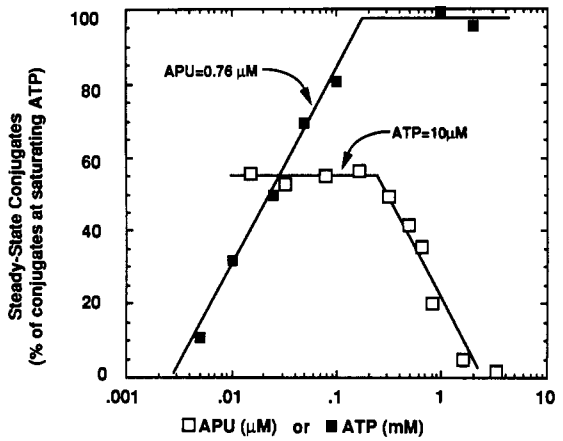

FIGURE 4: Dependence of the levels of ubiquitin conjugates on the concentration of ATP and APU. The steady-state levels of conjugates formed by reticulocyte fraction II were quantitated as described under Experimental Procedures. Results are expressed as a percentage of the level of conjugates observed in control experiments (saturating ATP and no inhibitor). The levels of steady-state ubiquitin conjugates present are plotted vs the concentration of ATP in the presence of 0.75 μM APU (closed squares) or vs the concentration of APU in the presence of 10 μM ATP (open squares). Note that APU completely abolishes conjugate formation at high concentrations and that high levels of ATP are able to completely overcome the inhibitory effects of APU.

mixtures above for determination of APU. Treatment of the aliquots with 5% perchloric acid precipitated most of the endogenous fraction II proteins, leaving ubiquitin and APU in the supernatent. HPLC of the supernatants, monitored at 205 and 260 nm, demonstrated that APU was not significantly degraded during the incubations described above.

APU could inhibit the accumulation of ubiquitin–protein conjugates in the steady state by interfering with ubiquitin conjugation or by enhancing conjugate degradation. The effect of APU on conjugate degradation was examined by measuring the rate at which preformed conjugates are deconjugated (i.e., the rate of ubiquitin release from conjugates). ^{125}I-Ubiquitin–protein conjugates were formed as described above in the absence of APU, and at time zero the conjugation reaction was stopped by the addition of hexokinase, 2-deoxyglucose (to consume the remaining ATP), and APU. At various times, samples were removed, and the levels of conjugates remaining were determined as described above. Under these conditions APU had no effect on the net rate of disappearance of ubiquitin conjugates (Table I). Separate experiments confirmed that these concentrations of APU had no effect on the ubiquitin esterase activity of the four known ubiquitin carboxyl-terminal hydrolases from bovine thymus (Table I). Together these results demonstrate that APU is a potent inhibitor of the net conjugation reaction but has little or no effect on the rates of ubiquitin conjugate degradation.

Effects of APU on Protein Degradation. The specificity of APU inhibition was further examined by measuring the rates of ubiquitin-dependent protein degradation catalyzed by reticulocyte fraction II. In order to test this inhibitor under conditions similar to those used in the ATP–PP_i exchange and the conjugation assays (i.e., 10 μM ATP), it was necessary to modify the standard assay conditions. The standard assays routinely contain 2 mM ATP in order to stabilize the proteases. This concentration of ATP would make it difficult to observe inhibition by APU due to the competitive nature of the inhibition. Therefore, we used a modified assay mixture containing 10 μM ATP to support ubiquitin conjugation and 2 mM GTP to stabilize the proteases. Control experiments showed that this modification of the assay conditions supported proteolysis to an extent of at least 75% that observed under standard conditions and exhibited a normal ubiquitin saturation behavior.

Table I: Effects of APU on Other Enzymes of Ubiquitin-Dependent Proteolysis[a]

activity measured	% inhibition by APU
degradation of Ub–protein conjugates[b]	<5
UCH-L1	<5
UCH-L2	<5
UCH-L3	<5
UCH-H2	<5
proteolysis of β-lactoglobulin[d]	90
proteolysis of BSA[d]	<5

[a] When present, the concentration of APU was 1 μM except for measurements of the proteolysis of BSA, where it was 2.5 μM. The estimated accuracy of these assays is ±5%. [b] Degradation of Ub–protein conjugates catalyzed by reticulocyte fraction II was assayed exactly as described previously (Hershko et al., 1980), except for the addition of APU. Levels of conjugates were quqntitated by densitometric scanning of radioautograms obtained from SDS–PAGE of the reaction mixtures. [c] The four ubiquitin carboxyl-terminal hydrolases were partially purified and assayed exactly as described previously (Mayer & Wilkinson, 1989), except for the addition of APU. [d] Ubiquitin-dependent proteolysis catalyzed by reticulocyte fraction II was measured as described under Experimental Procedures.

With ^{125}I-β-lactoglobulin as the substrate, it was found that APU effectively inhibited ubiquitin-dependent proteolysis at 1 μM (Table I). This is consistent with the effects of APU on the formation of ubiquitin conjugates as shown in Figure 4. When reduced carboxymethylated BSA was used as a substrate however, there was no demonstrable inhibition at concentrations of APU up to 2.5 μM. These results are consistent with the known properties of these two substrates (see Dicussion). Serum albumin substrates require the arginylation of the amino terminus in order to be efficiently degraded while β-lactoglobulin degradation is completely independent of arginylation (Ferber & Ciechanover, 1987). These results also demonstrate that APU has little or no effect on the arginyl-tRNA transferase involved in the rate-limiting arginylation of protein substrates with acidic amino termini.

Discussion

The ubiquitin pathway plays multiple roles in cellular physiology. These studies were designed to synthesize and evaluate a specific inhibitor of the cellular ubiquitination. The ubiquitin activating enzyme was chosen as a target, since its mechanism of action is well understood and inhibition of this enzyme would be expected to prevent all ubiquitination reactions.

The synthesis described in this work points to the general utility of the carboxyl-terminal activation strategy previously used to synthesize ubiquitin ethyl ester (Wilkinson et al., 1986) and ubiquitin–protein conjugates (Wilkinson, 1987). The trypsin-catalyzed transpeptidation is sufficiently nonspecific with respect to the nucleophile to allow the addition of most nucleophiles to the carboxyl terminus of ubiquitin, including primary amines, peptide esters, and even cystamine (unpublished results). Thus, it should be possible to tailor various linkers for the synthesis of a wide variety of carboxyl-terminal derivatives of ubiquitin. The subsequent formation of the acyl azide (Figure 1) proceeds smoothly with reasonable yields of peptide bond formation upon addition of the amine nucleophile. We estimate that at least half of the acyl azide generated in situ is able to be coupled under the reported conditions.

APU was designed to bind tightly to the ubiquitin activating enzyme by virtue of its similarity to tightly bound ubiquitin adenylate, the intermediate in the catalytic cycle of this enzyme

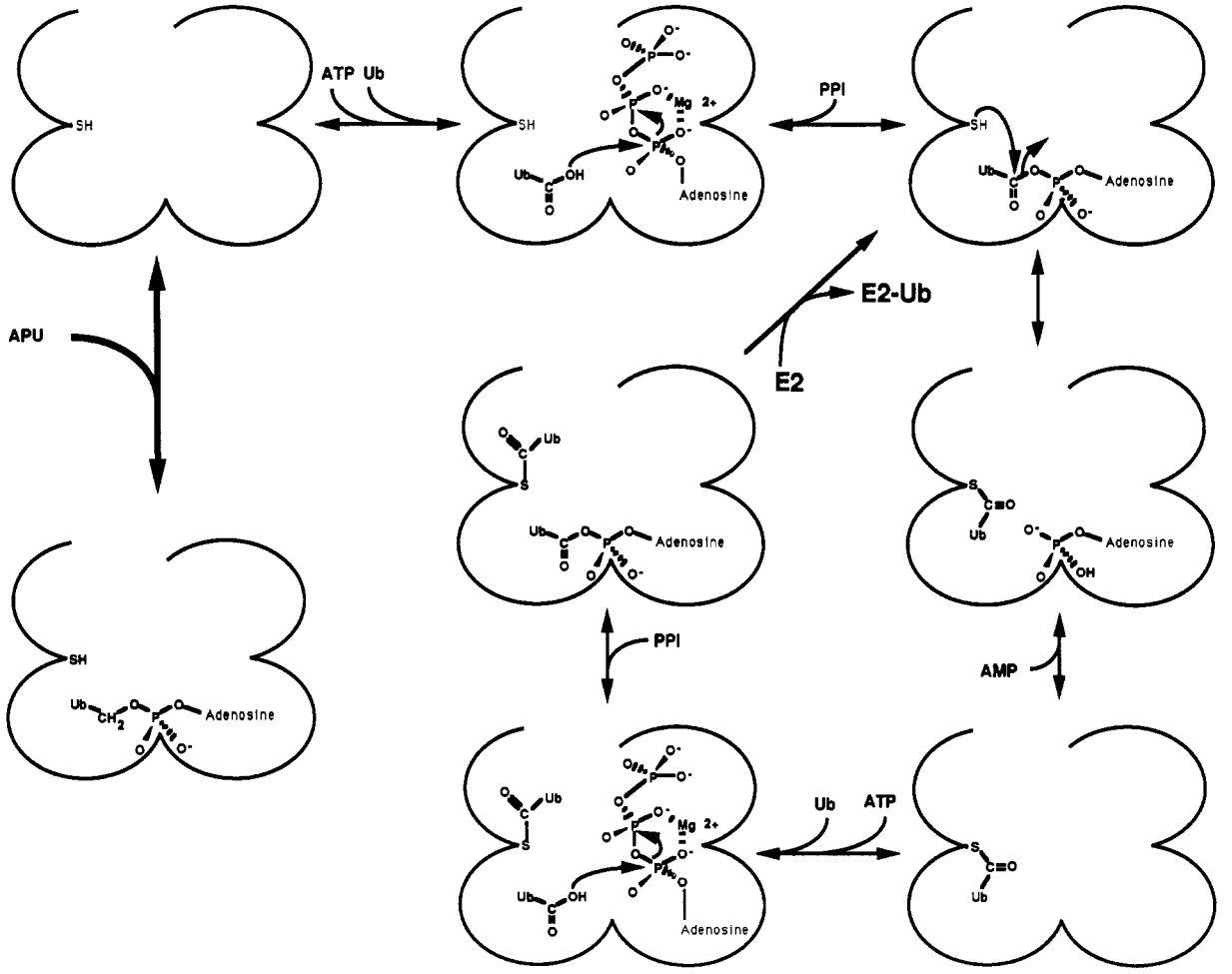

FIGURE 5: Model of the active site and catalytic intermediates of the ubiquitin activating enzyme. The inhibition by APU is shown with a bold arrow, while the rest of the enzyme forms participate in the catalytic cycle.

(Haas et al., 1983). Ubiquitin activating enzyme is known to have a binding site for ATP and other nucleotides. There are two sites for bound ubiquitin, a noncovalent catalytic binding site and a second site for the thiol ester of ubiquitin with the activating enzyme (Haas & Rose, 1982). The latter two sites are apparently nonoverlapping since ATP–PP_i and ATP–AMP exchange reactions can be catalyzed by the thiol ester form of ubiquitin activating enzyme and the fully charged enzyme is able to pass ubiquitin to the ubiquitin carrier proteins (E2). Figure 5 summarizes the catalytic model inferred from these and earlier studies (Haas et al., 1982, 1983; Haas & Rose, 1982). The catalytic ubiquitin binding site has at least some affinity for the C-terminal peptide of ubiquitin, since peptides of six to eight residues with the same sequence as this region of ubiquitin can stimulate ATP–PP_i exchange. This implies that they are adenylated (Jonnalagadda et al., 1988). However, these peptide adenylates do not undergo transfer to the thiol group of the activating enzyme. The affinity of the activating enzyme for these peptides is at least 3 orders of magnitude lower than that for ubiquitin on the basis of concentrations required for half-maximal stimulation of the exchange reactions (Jonnalagadda et al., 1988). The specificity of the adenosine binding site is less clear. Optimal binding and catalysis are observed with ATP, but studies with other nucleotides suggest that the exocyclic amino group and, to a lesser extent, the 2′-hydroxyl group of ATP are important in binding and catalysis. There is little specificity for the polyphosphate backbone as several phosphorothioates and nonhydrolyzable analogues at the α–β bridge oxygen position are substrates (Haas et al., 1983).

The ubiquitin adenylate analogue APU appears to bind to a form of the enzyme prior to release of the first molecule of AMP. This is demonstrated by the fact that AMP has no effect on the inhibition of the ATP–PP_i exchange reaction (Figure 3). If a significant portion of the inhibition were due to binding to the thiol ester form of the activating enzyme, AMP should relieve this inhibition. In the presence of AMP, exchange can still occur due to the reversal of the adenylation reaction (Figure 5). The rate of this reaction is at least 75% that of the total rate in the absence of AMP. The inhibition patterns observed (competitive vs ATP and noncompetitive vs ubiquitin) suggest that inhibition is exerted by binding to the free form of the enzyme. If the binary E–ATP complex were able to bind the analogue, the inhibition patterns should be noncompetitive vs ATP and competitive vs ubiquitin. If both forms could bind the inhibitor, the patterns would be noncompetitive in both cases.

The most likely explanation of this selectivity in binding of APU to the different forms of the activating enzyme is that thiol-esterified ubiquitin and the ubiquitin portion of APU compete for the same ubiquitin binding site on the enzyme. This explanation is also attractive in explaining the fact that ubiquitin adenylate cannot participate in thiol ester formation with the acyl carrier proteins (E2's). As drawn, Figure 5 suggests that the ubiquitin portion of the E1–ubiquitin thiol ester must be displaced from the specific binding site by the formation of another molecule of ubiquitin adenylate. Only after translocation to another site does ubiquitin become available for reaction with E2.

To determine the effects of APU on the other enzymes of

ubiquitin-dependent proteolysis, we examined the effects of the inhibitor on the conjugation of ubiquitin to proteins, the deconjugation of these proteins, and the overall degradation of proteins. Figure 4 demonstrates that APU is an effective inhibitor of conjugate formation catalyzed by reticulocyte fraction II. The *apparent* K_i under these conditions is 0.75 μM, some 20-fold higher than the extrapolated K_i for inhibiting ATP–PP_i exchange. This was not due to degradation of the inhibitor since it was stable in these incubations (data not shown). Most of this difference can be explained if activation of ubiquitin is not the rate-limiting step for protein ubiquitination. Two lines of evidence suggest that this is the case: during these incubations the thiol esters between ubiquitin and the acyl carrier proteins (E2) are observed to accumulate, showing that their formation (ubiquitin activation) occurs faster than their consumption (ubiquitination of proteins); and the addition of purified E1 to the reaction mixtures does not increase the rate of protein degradation or increase the level of ubiquitin conjugates (Dr. Arthur Haas, private communication). Thus, the activity of the ubiquitin activating enzyme must be decreased many fold by the inhibitor (i.e., more inhibitor is required) before it becomes the slowest step in the pathway.

To determine if the inhibition of conjugate formation led to a corresponding decrease in protein degradation, we examined the effects of APU on protein degradation catalyzed by fraction II (Table I). The concentration of ATP present in these assays was 10 μM, the same as that used in the other assays. Under these conditions, proteolysis is severely inhibited due to the instability of the proteases at low ATP concentrations. To stabilize the proteases, GTP was added to 2 mM. This nucleotide has been shown to stabilize or stimulate the proteases (Hough et al., 1987) while having little effect on the activating enzyme (Haas et al., 1983). Rates of protein degradation under these conditions were at least 75% that observed in the presence of ATP, and the ubiquitin concentration dependence was unchanged. Under these conditions, APU was observed to be an inhibitor of the degradation of β-lactoglobulin (Table I), with an efficiency consistent with its ability to inhibit the accumulation of ubiquitin–protein conjugates. The results suggest that conjugation of ubiquitin to β-lactoglobulin to generate the proteolytic substrate occurs at a rate similar to the rate of ubiquitination of endogenous fraction II proteins.

In contrast, the degradation of reduced carboxymethylated BSA was not significantly inhibited by APU at concentrations as high as 2.5 μM (Table I). This result suggests that a step other than conjugate formation is rate limiting in the degradation of BSA. It is known that the acidic amino terminus of BSA must first be arginylated in an Arg-tRNA-dependent reaction before it is efficiently ubiquitinated (Ciechanover et al., 1988). Addition of ribonuclease to assay mixtures decreases the rate of BSA degradation, demonstrating that the arginylation of this substrate is at least partially rate limiting in its degradation. From data presented by Ferber and Ciechanover (1987), it can be calculated that BSA is degraded only about 10% as fast as amino-terminal arginylated BSA by reticulocyte fraction II.[2] This demonstrates that the arginylation of BSA is the rate-limiting step in its degradation and that the reactions leading to ubiquitination and degradation of this intermediate are much faster. This is probably the reason it has been so difficult to reproducibly demonstrate the ubiquitination of BSA in crude extracts. In order for APU to inhibit the degradation of BSA, the rate of conjugation of ubiquitin must be reduced more than 10-fold until it becomes slower than the arginylation reaction. Thus, one would predict that it would take as least 10 μM APU to inhibit the degradation of BSA to the same extent as observed with the degradation of β-lactoglobulin inhibited by 1 μM APU. It has not been possible to decrease this rate sufficiently to interfere with the degradation of BSA. These results also demonstrate that APU has no significant effect on the enzymes which catalyze the arginylation of proteins with acidic amino termini.

We also examined the effect of APU on the deconjugation of ubiquitin from ubiquitin–protein conjugates. These enzymes are known to bind ubiquitin tightly, and it was possible that the analogue may also inhibit these enzymes. APU at 2.5 μM had no effect on the rate of disappearance of ubiquitin–protein conjugates after depletion of ATP (Table I). Under these conditions, the disappearance of ubiquitin–protein conjugates is due almost exclusively to the removal of ubiquitin catalyzed by ubiquitin carboxyl-terminal hydrolases. The lack of inhibition of ubiquitin carboxyl-terminal hydrolases was directly confirmed by measuring the rate of hydrolysis of ubiquitin ethyl ester catalyzed by four different isozymes isolated from calf thymus (Table I).

The results presented here demonstrate that the ubiquitin adenylate analogue APU binds tightly and specifically to the free form of the ubiquitin activating enzyme, with no evidence that it interacts with the other enzymes involved in ATP- and ubiquitin-dependent protein degradation. The specificity attained is an important requirement for a useful inhibitor of the ubiquitination pathway, and it should prove a valuable in vitro inhibitor. The binding of the inhibitor is, however, competitive with ATP. The practical consequence of this becomes apparent if one considers that the K_m for ATP with the activating enzyme is about 10 μM and the physiological concentration of ATP in most cells is 2–3 mM. This would mean that in order to inhibit ubiquitin activation by 50% in a cell, it would be necessary to attain an APU concentration of 10 μM (90 μg/mL). It would probably be difficult to attain such concentrations with existing techniques for introduction of macromolecules into cells.

It should be possible to improve the affinity of this class of inhibitors. It has been calculated that the affinity of the activating enzyme for ubiquitin adenylate must approach 10^{-12} M. The binding constant (K_i) for APU is about 4×10^{-8} M. Analogues with altered geometry and/or length between the ubiquitin and the adenosine portion might be bound more tightly. As such, this general class of analogues may prove to be effective inhibitors of ubiquitin-dependent processes in vitro and in designing in vivo inhibitors.

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[2] This can be calculated as follows from the data given in Table I of Ferber and Ciechanover (1987). In 2 h 22% of the added ^{125}I-BSA (1 μg) is degraded and 40% of the added [^{3}H]arginyl-BSA (5 μg). Thus, under the same conditions the rate of ^{125}I-BSA degradation catalyzed by fraction II is about 0.1 μg/h while that of [^{3}H]arginyl-BSA is 1 μg/h.

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