Uncoupling Ubiquitin-Protein Conjugation from Ubiquitin-Dependent Proteolysis by Use of β,γ-Nonhydrolyzable ATP Analogues†

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ABSTRACT: Pathways of ubiquitin-dependent protein degradation have in common two requirements for ATP. Ubiquitin activation by the enzyme E1 is accompanied by ATP hydrolysis to yield AMP and PP_i, and during conjugate breakdown, the ubiquitin-dependent protease hydrolyzes ATP to ADP and P_i. We show here that either of two β,γ-nonhydrolyzable ATP analogues, 5'-adenylyl imidodiphosphate or 5'-adenylyl methylenediphosphate, can support ubiquitin–protein conjugation. With the ubiquitin-dependent protease, however, neither analogue could substitute for ATP. Thus, the substitution of a β,γ-nonhydrolyzable analogue for ATP offers a simple method to uncouple ubiquitin conjugation from proteolysis in crude systems. On the basis of pyrophosphate exchange kinetics, E1 has apparent K_m and V_{max} values that are similar for ATP and the analogues, but substrate inhibition by 5'-adenylyl methylenediphosphate made use of the β,γ-imido analogue preferable. In one application, β,γ-imido-ATP was used in combination with ubiquitin aldehyde (an inhibitor of ubiquitin–protein isopeptidases) to establish that several unfolded RNase A derivatives are recognized equally as ubiquitination substrates. This result extends an earlier study [Dunten, R. L., & Cohen, R. E. (1989) *J. Biol. Chem. 264*, 16739–16747] to show that conjugate yields, upon which relative ubiquitination rates were based, were not influenced by differential ubiquitin-dependent proteolysis. In a second application, ATP and β,γ-imido-ATP were compared in a pulse–chase experiment to investigate the contributions of ATP-dependent proteolysis and isopeptidase activities to conjugate stability.

Rapid protein degradation within normally growing eukaryotic cells has been characterized as predominantly nonlysosomal and energy-dependent (Goldberg & St. John, 1976; Hershko & Ciechanover, 1982). A major system for intracellular proteolysis involves the attachment of ubiquitin, a 76-residue polypeptide, as an intermediate step in the selection and subsequent degradation of protein substrates [for reviews, see Rechsteiner (1987), Hershko (1988), and Jentsch et al. (1990)]. The basic reactions of ubiquitin-mediated proteolysis are activation of the ubiquitin C-terminal carboxylate by the enzyme E1, transfer of the activated ubiquitin to a carrier/conjugating enzyme (E2), and subsequent covalent conjugation ("ubiquitination") of a protein target in a process that, for some substrates, requires E3, a ubiquitin–protein ligase (Hershko et al., 1983). Multiple ubiquitination of a protein usually precedes, and may be obligatory for, its ultimate degradation by the ubiquitin-dependent 26S protease [Hershko et al., 1984; Hough et al., 1987; Chau et al., 1989; Gregori et al., 1990; but see also Hershko and Heller (1985) and Haas et al. (1990)]. Both ubiquitin activation by E1 (Ciechanover et al., 1981, 1982) and proteolysis by the 26S protease (Hough et al., 1987; Ganoth et al., 1988; Armon et al., 1990) require ATP hydrolysis, and these reactions may account for much of the energy requirement of intracellular protein turnover.

Studies of ubiquitin–protein conjugation and proteolysis in vitro predominantly have employed the ubiquitin-depleted reticulocyte lysate ("fraction II") described by Hershko and his co-workers (Ciechanover et al., 1978; Hershko et al., 1983). The considerable complexity of this system is evident from the multitude of ubiquitin conjugation components (Hershko et al., 1983; Pickart & Rose, 1985; Pickart & Vella, 1988; Haas & Bright, 1988; Heller & Hershko, 1990), the multisubunit character of the 26S protease (Hough et al., 1987; Eytan et al., 1989; Driscoll & Goldberg, 1990), and numerous accessory enzymes that may be required for N-terminal processing of specific protein substrates (Ferber & Ciechanover, 1986, 1987; Gonda et al., 1989). Furthermore, many ubiquitin carboxy-terminal hydrolase/isopeptidase activities are present that can release ubiquitin from peptides and proteins and can disassemble multi-ubiquitin conjugates (Rose, 1988; Mayer & Wilkinson, 1989; Liu et al., 1989). The ubiquitin systems in yeast (Gonda et al., 1989; Jentsch et al., 1990) and plants (Hatfield & Vierstra, 1989; Sullivan & Vierstra, 1989) are similarly complex.

Conjugation assays that employ crude systems such as fraction II can be complicated by concurrent ubiquitination and degradation/disassembly reactions. Because products from these assays represent the net of synthesis and breakdown, the relative levels of conjugataes found with different substrates do not necessarily reflect their relative ubiquitination rates. Thus, when conjugate disassembly by ubiquitin–protein isopeptidases was inhibited with ubiquitin aldehyde, six different unfolded forms of RNase A yielded virtually identical conjugate patterns. In contrast, without ubiquitin aldehyde, RNase A conjugate levels varied dramatically (Dunten & Cohen, 1989). It was concluded that the six RNase A proteins were recognized equally for ubiquitination, although unequal rates of conjugate disassembly by isopeptidase(s) could obscure this. It still had to be assumed, however, that the product distributions were not influenced significantly by the ubiquitin-dependent protease.

The elimination of the ubiquitin-dependent protease activity from conjugation assays would be useful to reduce the complications of competing conjugate breakdown reactions. Thus far, no inhibitors specific for this protease have been described, and its physical removal may not be practical except from

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relatively well-characterized systems such as the rabbit reticulocyte lysate. Even with the reticulocyte system, the ubiquitination of one class of substrates, N^{α}-acetylated proteins, has depended upon the use of a crude cell extract (Mayer et al., 1989).

Potentially, a simple means to eliminate ubiquitin-dependent proteolysis from ubiquitin conjugation assays exploits the ATP requirements of these reactions. Ubiquitin activation by E1 generates AMP and PP_i (Haas et al., 1982) whereas proteolysis is accompanied by β–γ cleavage of ATP to release inorganic phosphate (Armon et al., 1990). It is already established that β,γ-nonhydrolyzable ATP analogues can function in E1-catalyzed pyrophosphate exchange reactions (Haas et al., 1983) but are incapable of supporting conjugate proteolysis (Hershko et al., 1984; Hough et al., 1986; Waxman et al., 1987). If these same analogues were to substitute for ATP in ubiquitin conjugation reactions, then a straightforward method would be available to uncouple ubiquitination from subsequent proteolysis.

We show here that both the β,γ-imido and β,γ-methylene analogues of ATP can support ubiquitin–protein conjugation. The kinetics of the ubiquitin activating enzyme, E1, are compared for ATP and its analogues. Applications of the β,γ-imido analogue are presented in which ubiquitination efficiencies of several RNase A substrates are compared, and the contributions of ATP-dependent proteolysis versus other disassembly and degradation reactions are examined in a pulse–chase study with ubiquitin–ox-RNase A[1] conjugates.

Experimental Procedures

Materials. Bovine ubiquitin, performic acid oxidized RNase A (ox-RNase A), bovine serum albumin (BSA), enzymes, and nucleotides were from Sigma Chemical Co. Proteins were radiolabeled with carrier-free $Na^{125}I$ (Amersham Corp.) by the chloramine-T method as described (Ciechanover et al., 1980). $Na_4[^{32}P]PP_i$ was obtained from Amersham, and [2,8-^{3}H]ATP (30 Ci/mmol) was from New England Nuclear. All RNase A derivatives other than ox-RNase A were available from an earlier study (Dunten & Cohen, 1989). Fraction II, a crude ubiquitin-depleted cell extract, was prepared from washed rabbit reticulocytes (Green Hectares, Oregon, WI) according to Hershko et al. (1983) and was stored at −80 °C in 0.5 mM ATP and 1 mM dithiothreitol (DTT). Typical preparations contained ~5 mg of protein/mL, as determined by the dye binding method of Bradford (1976) with chicken egg white lysozyme as a standard. Ubiquitin activating enzyme, E1, was purified according to Haas and Bright (1988), except that the E1 eluate from the ubiquitin affinity column was applied directly onto a Pharmacia Mono Q HR 5/10 anion-exchange column without the intermediate concentration and storage steps. Ubiquitin aldehyde was synthesized as described earlier (Dunten & Cohen, 1989).

ATP Depletion of Fraction II. Just before use, 100-μL aliquots of fraction II were depleted of ATP and DTT by centrifugation through two 3-mL columns of Sephadex G-25 (Penefsky, 1979) that had been equilibrated with 50 mM HEPES (Na^+), pH 7.2. Centrifugations were for 2 min at 700g and 4 °C. This method removed >99.9% of the ATP in which the fraction II had been stored, as determined by trace-labeling the fraction II with [2,8-^{3}H]ATP. Alternatively, enzymatic ATP depletion at 20 °C was accomplished with either hexokinase and glucose, or with glycerokinase and glycerol. After adjustment to 0.5 mM glucose or glycerol, either 0.2 unit of hexokinase or 0.04 unit of glycerokinase, respectively, was added for each microliter of fraction II. ATP was consumed completely within 20 min (data not shown).

Conjugation Assays. Conjugations of ^{125}I-ubiquitin to ox-RNase A and other RNase A derivatives were performed essentially as described earlier (Dunten & Cohen, 1989). Reactions contained 50 mM HEPES (Na^+), pH 7.2, 5 mM $MgCl_2$, 4 mM ATP or ATP analogue, and 2 mM DTT. Each 20-μL assay contained 50 pmol of ^{125}I-ubiquitin (~4×10^5 cpm), 8 μg of RNase A derivative, and 40–45 μg of fraction II protein which had been depleted of ATP as described above. Except where indicated otherwise, the fraction II was treated with 1.0 μM ubiquitin aldehyde for 5 min at 37 °C before addition to the conjugation mixture. After incubation at 37 °C, reactions were quenched with an equal volume of SDS–PAGE sample buffer (60 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.01% bromophenol blue, and 5% β-mercaptoethanol) and heated for 10 min at 95 °C. The conjugates were then separated by SDS–PAGE (Laemmli, 1970) on 12.5% polyacrylamide gels. The gels were stained with Coomassie Blue, destained, dried, and autoradiographed at −80 °C with Kodak X-Omat film and a DuPont Cronex Lightning Plus intensifying screen.

Degradation Assays. ATP-Depleted fraction II was assayed for ubiquitin- and ATP-dependent degradation of ^{125}I-ox-RNase A by the method of Hershko et al. (1983). The reactions contained 50 mM HEPES (Na^+), pH 7.2, 3 mM DTT, 5 mM $MgCl_2$, 2 mM ubiquitin, 4 mM ATP or analogue as indicated, and ATP-depleted fraction II (225 μg of protein). After a 5-min preincubation at 37 °C, 1.25 μg of ^{125}I-ox-RNase (2×10^5 cpm) was added to give a total volume of 62.5 μL. Over the course of 2 h at 37 °C, 25-μL aliquots were removed, mixed with 50 μL of 10% BSA as a carrier, and precipitated with 425 μL of 18% trichloroacetic acid. After centrifugation (5 min, 12000g), the pellets and supernatants were separated and counted in a Beckman Gamma 7000 γ-counter to determine the percent nonprecipitable radioactivity (i.e., degraded ^{125}I-ox-RNase A).

E1 Assays. Concentrations of active E1 were determined by the method of Rose and Warms (1987). Samples of 25 μL were treated with 10 mM iodoacetamide for 20 min at 37 °C in the dark, followed by DTT to consume excess alkylating agent. Samples then were adjusted to 50 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, 10 pmol of [2,8-^{3}H]ATP, 6.6 μM ubiquitin, and 0.04 unit of inorganic pyrophosphatase in a final volume of 70 μL. This mixture was incubated at 37 °C for 10 min, and then 10 μL of 10% BSA and 1 mL of cold 20% trichloroacetic acid were added, and the samples were chilled on ice for 10 min. After a 3-min centrifugation (12000g) at 4 °C, the pellets were washed twice with 1 mL of cold 3% trichloroacetic acid. Pellets were resuspended in 100 μL of 0.1 N NaOH, added to 5 mL of Beckman Ready Safe scintillation fluid, and counted in a Beckman LS 3133P liquid scintillation counter. The amount of E1 was calculated from the yield of ubiquitin [^{3}H]adenylate.

E1-Catalyzed pyrophosphate exchange into ATP was measured as described by Haas and Rose (1982). Assays were performed at 37 °C and contained 50 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 0.1 mM DTT, 1.2 μmol of ubiquitin, 0.625

[1] Abbreviations: ox-RNase A, performic acid oxidized ribonuclease A; BSA, bovine serum albumin; rcam, reduced and carboxamidomethylated; rcm, reduced and carboxymethylated; DTT, dithiothreitol, HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PP_i, inorganic pyrophosphate; AMPPNP, 5′-adenylyl imidodiphosphate; AMPPCP, 5′-adenylyl methylenediphosphate; Ub, ubiquitin; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

pmol of E1, 100 μM [^{32}P]PP_i [(1.2–1.5) × 10^6 cpm], and variable amounts of ATP or ATP analogue in a total of 62.5 μL. At 0 and 5 min, 25-μL aliquots were removed and combined with 250 μL of 5% trichloroacetic acid and then 300 μL of charcoal (10% w/v) in 2% trichloroacetic acid. The mixtures were centrifuged (5 min, 12000*g*), and the charcoal pellets were washed with 3 × 1 mL of 2% trichloroacetic acid; ^{32}P in the pellets was determined from Cerenkov radiation with a Beckman LS 3133P liquid scintillation counter. The PP_i/ATP exchange rates were calculated from the differences in radioactivity between 0- and 5-min reactions. Pilot assays were done to assure that the measured velocities were in a linear range.

Nonlinear least-squares regression analyses of kinetic data were performed with the program Enzfitter (Elsevier-Biosoft). The ATP and AMPPNP exchange data were fit to the Michaelis–Menten equation, whereas the AMPPCP data were fit to eq 1, where K_m and K_i are, respectively, the apparent

$$v = \frac{V_{max}[A]}{K_m + [A] + [A]^2/K_i} \quad (1)$$

Michaelis and inhibition constants for the inhibitory substrate A (Cleland, 1970). This equation describes a line in a Lineweaver–Burke plot which is linear at low substrate concentrations (i.e., high 1/[A]) and then curves asymptotically upward with higher [A].

Pulse–Chase Studies. Conjugation assays were performed with fraction II and ^{125}I-ox-RNase A as a substrate with combinations of ubiquitin aldehyde and ATP or AMPPNP. The basic assay mixture, in 100 μL, consisted of 50 mM HEPES (Na^+), pH 7.2, 2 mM DTT, 5 mM $MgCl_2$, 1 μg/mL leupeptin, 1.4 μg/mL pepstatin, 0.4 mM phenylmethanesulfonyl fluoride, 25 μg of ubiquitin, 365 μg of fraction II protein (where applicable, pretreated with ubiquitin aldehyde as described above), and 4 mM of either ATP or AMPPNP. Following a 5-min preincubation at 37 °C, ^{125}I-ox-RNase A (10 μg, 2 × 10^6 cpm) was added, and the incubations were continued. Aliquots of 20 μL were taken at 15 min, after which 240 μg of unlabeled ox-RNase A was added, and 20-μL aliquots were taken at 15, 60, and 120 min. Each aliquot was combined immediately with 20 μL of SDS–PAGE sample buffer and heated at 100 °C for 5 min. Following SDS–PAGE (12.5% acrylamide), the gel was stained with Coomassie Blue, dried, and autoradiographed. Ubiquitin conjugates with ^{125}I-ox-RNase A were quantified by γ-counting of bands excised from the dried gels.

Results

E1 Kinetics. Rose and his co-workers (Haas et al., 1982; Haas & Rose, 1982) have established that ubiquitin activation by the enzyme E1 involves adenylylation of the ubiquitin C-terminal carboxylate and the release of PP_i. Subsequent transfer of the ubiquitin moiety onto an E1 thiol to form enzyme-linked ubiquitin thiol ester is accompanied by AMP release, and a second round of ubiquitin adenylation can then proceed. Their experiments have supported the following minimal mechanism:

$$E_{SH} + ATP + Ub \rightleftharpoons E_{SH}^{AMP\text{-}Ub} + PP_i \quad (2)$$

$$E_{SH}^{AMP\text{-}Ub} \rightleftharpoons E_{S\text{-}Ub} + AMP \quad (3)$$

$$E_{S\text{-}Ub} + ATP + Ub \rightleftharpoons E_{S\text{-}Ub}^{AMP\text{-}Ub} + PP_i \quad (4)$$

Overall, ubiquitin activation is accompanied by ATP hydrolysis at the α,β-phosphodiester linkage. E1-Catalyzed pyrophosphate exchange into β,γ-nonhydrolyzable ATP analogues such as AMPPNP (5′-adenylyl imidodiphosphate) and AMPPCP (5′-adenylyl methylenediphosphate) has been reported (Haas et al., 1983), and is consistent with the above scheme. We have extended these results to better evaluate the potential of β,γ-nonhydrolyzable ATP analogues to substitute in ubiquitin–protein conjugation reactions.

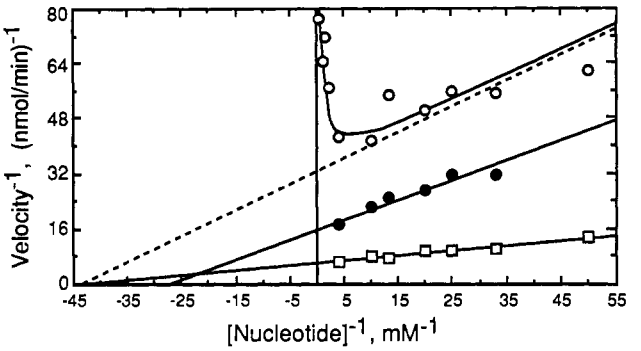

FIGURE 1: Lineweaver–Burk plot for E1-catalyzed PP_i exchange with ATP and β,γ-nonhydrolyzable ATP analogues. Steady-state velocities for [^{32}P]PP_i/nucleotide exchange were determined with 100 μM PP_i, 1.2 μM ubiquitin, and 0.625 pmol of E1 (see Experimental Procedures) as a function of ATP (□), AMPPNP (●), and AMPPCP (○) concentrations. The solid lines correspond to best fits of the data to the Michaelis–Menten equation (ATP and AMPPNP) or eq 1 (AMPPCP). The dashed line shows the exchange kinetics predicted for AMPPCP in the absence of substrate inhibition. Kinetic parameters are summarized in Table I.

Table I: Kinetic Parameters for E1 Catalysis of PP_i Exchange with ATP and β,γ-Nonhydrolyzable ATP Analogues[a]

nucleotide	apparent K_m (μM)	k_{cat} (s^{-1})	K_i (mM)
ATP	23.5 ± 2.7	10.8 ± 0.4	na[b]
	36[c]	4.9[c]	na
AMPPNP	36.7 ± 5.7	4.2 ± 0.6	na
AMPPCP	23.2 ± 13.5	2.0 ± 0.4	0.9 ± 0.6

[a] Parameters listed are nonlinear least-squares values and standard errors from fits to the data presented in Figure 1. [b] na, not applicable. [c] Taken from Haas and Rose (1982).

The utilization of ATP analogues by E1 was examined with the pyrophosphate exchange assay of Haas et al. (1982). E1-Catalyzed incorporation of [^{32}P]PP_i into ATP was investigated as a function of nucleotide concentration under conditions of saturating PP_i and with 1.2 μM ubiquitin, a level at which the exchange rate is maximal (Haas & Rose, 1982). In the absence of added AMP, the steady-state velocities reflect the reaction shown by eq 4. The results with ATP, AMPPNP, and AMPPCP are presented in Figure 1 in the form of a Lineweaver–Burk plot. For ATP and AMPPNP, K_m and V_{max} values were obtained by nonlinear fits to the Michaelis–Menten equation; these are reported in Table I. With AMPPCP, substantial substrate inhibition was observed, and to account for this, the data were fit by a modified equation (eq 1) that included an inhibition constant, K_i.

The apparent K_m's for exchange with the two β,γ-nonhydrolyzable analogues were within 50% of the values obtained for ATP in this study (23.5 μM) and previously [36 μM; from Haas and Rose (1982)]. With respect to the effects on V_{max} by substitutions of AMPPNP and AMPPCP for ATP, k_{cat} for PP_i exchange was reduced to 4 and 2 s^{-1} from 11 s^{-1}, respectively. Haas and Rose (1982) have reported a similar maximum PP_i/ATP exchange rate of 4.9 s^{-1}. In light of the substrate inhibition noted above for AMPPCP, our results are consistent with the observation made earlier (Haas et al., 1983) that 1 mM AMPPNP supports PP_i exchange at 17% of the rate with ATP and that the rate is slowed to 1.5% with

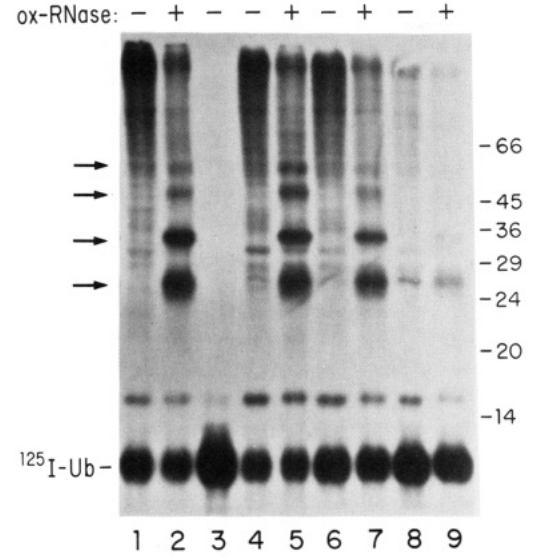

FIGURE 2: β,γ-Nonhydrolyzable analogues can substitute for ATP in ubiquitin–protein conjugation assays. Products from conjugation assays with ^{125}I-ubiquitin and either without (−) or with (+) ox-RNase A were visualized after SDS–PAGE and autoradiography. Migration positions of the major ^{125}I-ubiquitin–RNase A species, evident above the background of conjugates to endogenous fraction II proteins, are indicated by the arrows; positions of molecular mass standards ($M_r \times 10^{-3}$) are shown to the right. In lanes 1 and 2, fraction II was used without prior ATP depletion, whereas in lanes 3–9 endogenous ATP was removed by rapid gel filtration just before use. Reactions were supplemented with 4 mM ATP (lanes 1, 2, 4, and 5), AMPPNP (lanes 6 and 7), or AMPPCP (lanes 8 and 9).

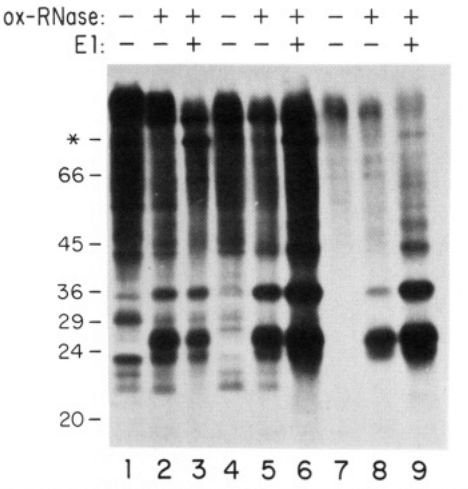

FIGURE 3: Ubiquitin activation with ATP analogues can become rate-limiting for conjugation in assays with fraction II. Conjugation reactions employed ^{125}I-ubiquitin and ATP-depleted fraction II, either without (−) or with (+) additional E1 (0.25 pmol per 25-μL assay). Ox-RNase A was added as a substrate where indicated. Nucleotide supplements were 4 mM ATP (lanes 1–3), AMPPNP (lanes 4–6), or AMPPCP (lanes 7–9). Molecular mass standards ($M_r \times 10^{-3}$) are shown on the left, and the migration position expected for an E1–ubiquitin adduct is shown by the asterisk. The lower portion of the gel contained no ^{125}I-ubiquitin–ox-RNase A conjugates and is not shown. Results of quantification from a similar experiment are given in the text.

AMPPCP. These data suggested that the ATP analogues should be capable of supporting ubiquitin–protein conjugation. This was tested with an ATP-depleted preparation of fraction II, as described below.

ATP Depletion of Fraction II. Fraction II is stabilized with 0.5 mM ATP during storage. In order to study ATP analogues in this system, it was necessary to thoroughly remove the ATP before use. Initially, we planned to deplete ATP from fraction II enzymatically with hexokinase and glucose. In this manner, the extent of ATP depletion could be regulated by the amount of glucose added to the reaction. The enzymatic method worked well to deplete ATP. This was apparent from the absence of ^{125}I-ubiquitin conjugates when assays were done using the depleted fraction II, and the complete restoration of conjugation upon addition of ATP (data not shown). Ubiquitin-dependent degradation, however, was consistently lower in the ATP-restored assay than it was when nondepleted fraction II was used. This loss of degradation activity was independent of the hexokinase source (yeast or rabbit) and persisted even when glycerokinase and glycerol were substituted as an alternative means for ATP depletion. Consequently, enzymatic depletion of ATP was abandoned and replaced by the method of rapid centrifugation through columns of Sephadex G-25 (Penefsky, 1979). Fraction II treated in this manner was incapable of either ubiquitin conjugation or ubiquitin-dependent degradation until ATP was added, whereupon full activity was restored (see below).

ATP Analogues Support Conjugation of ^{125}I-Ubiquitin to Oxidized RNase A. The ability of AMPPNP and AMPPCP to support ubiquitin conjugation to both endogenous fraction II proteins and ox-RNase A is demonstrated in Figure 2. Lane 1 shows ^{125}I-ubiquitin conjugates that are made to endogenous fraction II proteins, and in lane 2, conjugates formed specifically upon addition of ox-RNase A are apparent. Whereas no conjugates were made after ATP was depleted by rapid gel filtration (lane 3), ^{125}I-ubiquitin conjugation was restored upon ATP addition (lanes 4 and 5). Conjugation also was restored by AMPPNP, although at a slightly lower level (compare lanes 6 and 7 with lanes 4 and 5). With AMPPCP (lanes 8 and 9), conjugation is evident, but much less efficient.

Ubiquitin conjugation assays similar to those shown in Figure 2 were done with hexokinase and glucose included in the reactions. Under this condition, no conjugates formed from fraction II which contained only ATP, whereas with AMPPNP or AMPPCP conjugate levels were unaffected by hexokinase/glucose supplementation (not shown). Thus, the ubiquitin–protein conjugation observed with the β,γ-nonhydrolyzable analogues was not simply an artifact of ATP contamination.

The different levels of ubiquitin conjugation in assays with ATP, AMPPNP, and AMPPCP suggested that, in fraction II, ubiquitin activation by E1 with the ATP analogues must be limiting. Because the nucleotides were used at concentrations 100–200 times their apparent K_m values, the reduced conjugation observed with AMPPNP relative to ATP most likely reflects the difference in k_{cat} (see Table I). With AMPPCP, an even lower k_{cat} combined with substantial substrate inhibition could account for the very low conjugate levels seen in lanes 8 and 9 of Figure 2.

To test whether E1 was in fact rate-limiting, we compared the conjugation of ^{125}I-ubiquitin to ox-RNase A in fraction II with and without supplementation by purified E1 (Figure 3). Conjugate yields were increased by the inclusion of ubiquitin aldehyde to inhibit deubiquitination by ubiquitin–protein isopeptidases (Hershko & Rose, 1987; Dunten & Cohen, 1989). The addition to a 25-μL assay of 0.25 pmol of E1 [approximately 3 times the expected E1 content of fraction II (Haas & Bright, 1988)] greatly increased ubiquitin conjugation in reactions with AMPPNP and AMPPCP. By cutting and counting each gel lane, conjugation in a similar experiment with added ox-RNase A was quantified as the percent of the total ^{125}I-ubiquitin that was incorporated into higher molecular mass products. This value was 41% for the reaction containing ATP and was unchanged upon E1 sup-

plementation. With AMPPNP, 42% and 66% of the ^{125}I-ubiquitin were conjugated in reactions without and with additional E1, respectively. For AMPPCP, E1 supplementation increased the total conjugation from 19% to 31%. Thus, under conditions in which ubiquitin activation is slowed, in vitro conjugation assays using fraction II may be limited by the activity of E1. On the basis of the relative k_{cat} values for PP_i/nucleotide exchange with ATP and AMPPNP (see Table I), it appears that reduction of the ubiquitin activation rate by about half was sufficient to make this step limiting for conjugation in assays with fraction II.

In addition to the generally increased conjugation observed in assays with AMPPNP and AMPPCP, E1 supplementation also gave rise to a specific radiolabeled species of ~100 kDa (marked with the asterisk in Figure 3). With ATP as well as the two analogues, accumulation of this conjugate correlated with the addition of E1 (Figure 3, compare lanes 2 with 3, 5 with 6, and 8 with 9). We suspect that this species is a stable, monoubiquitin conjugate of E1 itself. Because the thiol ester linkage normally formed during ubiquitin activation (see eq 3) is not stable to heating with β-mercaptoethanol prior to SDS–PAGE, the ubiquitin–E1 adduct postulated here is likely to result from either conjugation or an intramolecular transfer of ubiquitin to generate an amide linkage. Presumably, some fraction of the E1 was damaged in a manner that allowed for its own ubiquitination. This result has precedence from studies of a thermolabile, mutated form of E1 (Finley et al., 1984).

Degradation of ^{125}I-Oxidized RNase A Is Not Supported by either AMPPNP or AMPPCP. Ubiquitin-dependent protein degradation requires ATP both for conjugation and for subsequent proteolysis. Studies of the ubiquitin-dependent protease have established that, for ^{125}I-labeled lysozyme and BSA, neither the β,γ-methylene nor the β,γ-imido analogues of ATP can substitute in degradation assays (Hershko et al., 1984; Hough et al., 1986; Waxman et al., 1987). To test the competence of our enzyme mixtures for degradation and the dependence of this reaction upon a hydrolyzable β,γ-phosphodiester linkage in ATP, degradation assays were performed with ^{125}I-labeled ox-RNase A as the substrate. These assays, which monitor the release of acid-soluble radioactivity from a radiolabeled protein, often contain an ATP-regenerating system. Because such a system could complicate some methods for ATP depletion and the use of ATP analogues, it was omitted, and the nucleotide concentration normally used was increased from 1 to 4 mM. To ensure that ubiquitin-dependent degradation activity was not lost irreversibly upon ATP removal, assays with either depleted or nondepleted fraction II, and supplemented with 4 mM ATP, AMPPNP, or AMPPCP, were compared (Table II). Without added ATP, degradation of ^{125}I-ox-RNase A was ~5% in 2 h. This rate reflects the contributions of "nonspecific" (i.e., ubiquitin and ATP-independent) proteases during the incubation. ATP, but neither one of the β,γ-nonhydrolyzable ATP analogues, significantly increased degradation above this background level. Control reactions in which ubiquitin was omitted showed that essentially all of the ATP-dependent proteolysis was ubiquitin-dependent as well.

Pulse-Chase Study of ^{125}I-Oxidized RNase A Ubiquitination. To study the fate of a substrate upon ubiquitination, we performed a pulse–chase experiment which compared the levels of conjugates in ATP- and AMPPNP-supplemented reactions. A 15-min ubiquitin conjugation reaction with ^{125}I-ox-RNase A, 30 μM ubiquitin, and 1.0 μM ubiquitin aldehyde generated the products shown in Figure 4A (ATP, lane 2; AMPPNP, lane 6). After 15 min, a 30-fold excess of unlabeled ox-RNase

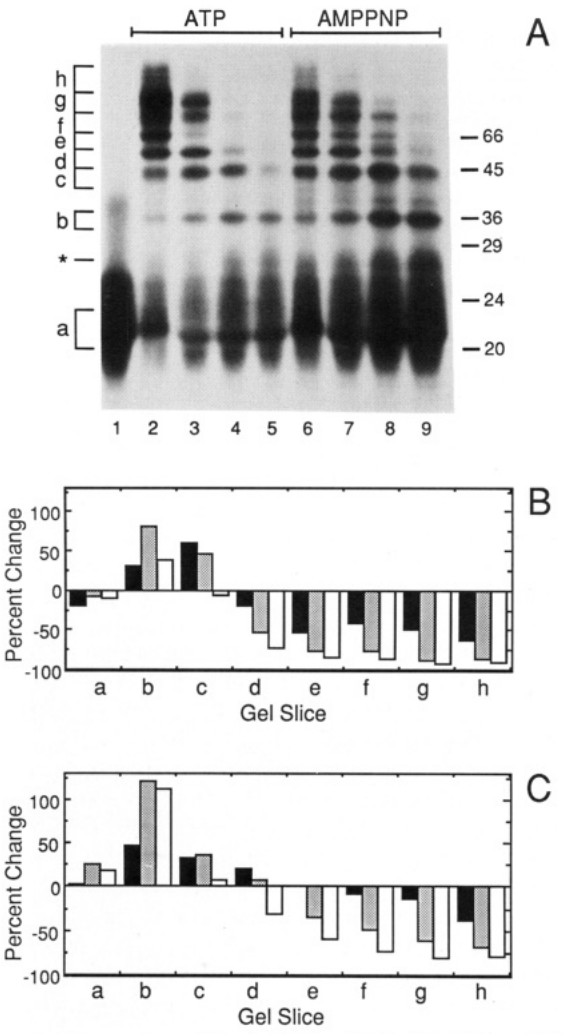

FIGURE 4: Comparison of the stabilities of ubiquitin–^{125}I-ox-RNase A conjugates in fraction II with ATP versus AMPPNP. Conjugation reactions containing ubiquitin, ubiquitin aldehyde, ^{125}I-ox-RNase A, and either ATP or AMPPNP were run for 15 min and then chased with unlabeled ox-RNase A as described under Experimental Procedures. Products were visualized after SDS–PAGE and autoradiography. (A) Lane 1 shows ^{125}I-ox-RNase A alone. Radiolabeled products from conjugation assays with ATP and AMPPNP are in lanes 2–5 and 6–9, respectively. Aliquots were taken at the end of the 15-min "pulse" (lanes 2 and 6), and then after 15 min (lanes 3 and 7), 60 min (lanes 4 and 8), and 120 min (lanes 5 and 9) into the chase period. Positions of protein standards ($M_r \times 10^{-3}$) are shown on the right, and bands excised for γ-counting are shown on the left (a–h). The asterisk shows the position of monoubiquitin–ox-RNase A, which overlaps with free ^{125}I-ox-RNase A and was not counted. (B) The amounts of the radiolabeled species from the ATP-supplemented reactions in panel A, bands a–h, are plotted relative to their levels after the 15-min pulse. The solid, stippled, and open bars correspond to 15, 60, and 120 min into the chase, respectively. (C) Data are presented as in (B), but for the AMPPNP-supplemented reactions.

A was added, and the mixtures were sampled at various times (ATP, lanes 3–5; AMPPNP, lanes 7–9). In parallel assays which lacked ubiquitin aldehyde, virtually all ^{125}I-conjugates were lost and reverted to free ^{125}I-ox-RNase A within 15 min of the chase period (data not shown). For the reactions that included ubiquitin aldehyde to inhibit isopeptidase activities, the disappearance of ^{125}I-labeled conjugates during the chase can be due only to the ubiquitin-dependent protease, nonspecific proteases, and ubiquitin aldehyde insensitive isopeptidases. Whereas the latter two activities can function in both assays, ubiquitin-dependent proteolysis requires ATP, as was demonstrated above. The differences in conjugate sta-

Table II: ATP Dependence of the Degradation of ^{125}I-Ox-RNase A in Reticulocyte Fraction II[a]

fraction II pretreatment	nucleotide supplementation[b]	% degradation of ^{125}I-ox-RNase A[c]
none	none	4.4 ± 1.0
	ATP	18.9 ± 0.7
	ATP	4.6 ± 0.5[d]
ATP-depleted[e]	none	4.8 ± 0.4
	ATP	17.3 ± 1.5
	ATP	5.1 ± 0.6[d]
	AMPPNP	6.0 ± 0.1
	AMPPCP	5.4 ± 0.6

[a] Assays were as described under Experimental Procedures and, except where indicated otherwise, contained 0.2 mM ubiquitin. [b] Where indicated, nucleotides were added to 4 mM. [c] Percent of the total radioactivity converted in 2 h to an acid-soluble form; values are the means and standard deviations of two experiments. [d] No ubiquitin was added. [e] By rapid gel filtration.

bilities in the two reactions therefore can be attributed specifically to the ubiquitin-dependent protease. Bands from the gel were excised as indicated in Figure 4A and counted. These data are presented in Figure 4B,C, where the radioactivity in each band, a through h, is expressed relative to its level immediately before the chase with unlabeled ox-RNase A. From these data, it is apparent that, even in the absence of ATP-dependent degradation, most high molecular weight ubiquitin-^{125}I-ox-RNase A conjugates (bands d–h) were lost during the course of the chase period. This loss, however, was much more rapid for the ATP-supplemented reaction, where over half of these conjugates had disappeared within 15 min; in contrast, these conjugates were reduced to similar levels only after 60 min in the reaction with AMPPNP (see Figure 4A, and compare Figure 4B with Figure 4C). Thus, ATP-dependent proteolysis can contribute substantially to the distribution and amounts of ubiquitin–protein conjugates in assays with fraction II.[2]

To distinguish the effects of possible ubiquitin aldehyde insensitive isopeptidase activities from other proteases, we compared the radioactivity in high (bands d–h) and low (bands b and c) molecular mass ^{125}I-conjugates as well as free ^{125}I-ox-RNase A (band a). After a 60-min chase, 46.7% of the total (b–h) ^{125}I-labeled conjugates were lost from the ATP-supplemented assay, whereas with AMPPNP only 4.4% were lost in the same period. After 120 min, 58.4% and 24.1% of these conjugates were lost in the ATP- and AMPPNP-supplemented reactions, respectively. With AMPPNP, most changes were due to isopeptidase activity and resulted in a redistribution of conjugates rather than a net loss. Thus, decreases of the high molecular mass conjugates were accompanied by compensating increases in radioactivity in bands a, b, and c, which correspond to intact ox-RNase A and di- and triubiquitinated conjugates, respectively. Although early in the chase period these species also accumulated in the ATP-supplemented reaction (see Figure 4B), their increase cannot account for the disappearance of the radioactivity from bands d–h. After 120 min, the "nonconserved" conjugate loss, where there was a net decrease of radioactivity from the entire gel lane, was 34.3% in the assay with ATP and 14% with AMPPNP. The disappearance of ^{125}I-labeled proteins in the assay with AMPPNP reflects nonspecific proteolysis of ^{125}I-ox-RNase A to fragments small enough to migrate out of the gel. The additional loss in the ATP-supplemented assay is the

[2] Although the fraction II preparations used in this study were quite consistent with respect to ubiquitin conjugating activities, the ATP-dependent proteolytic activity was variable and decreased upon storage.

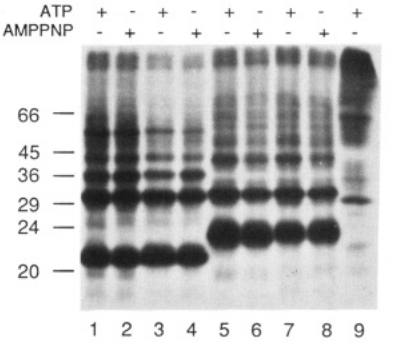

FIGURE 5: Unfolded derivatives of RNase A are ubiquitinated with equal efficiency, irrespective of methionine oxidation. RNase A derivatives, unfolded by complete disulfide reduction followed by carboxamidomethylation (rcam, lanes 1–4) or carboxymethylation (rcm, lanes 5–8) of the cysteines, were tested as ubiquitination substrates either without (lanes 1, 2, 5, and 6) or with (lanes 3, 4, 7, and 8) further modification to convert methionine residues to methionine sulfoxide. Reactions employed ^{125}I-ubiquitin, ATP-depleted fraction II, and ubiquitin aldehyde as described under Experimental Procedures; ATP (odd-numbered lanes) or AMPPNP (even-numbered lanes) were added to 4 mM. A control assay with no added RNase A substrate is shown in lane 9. Positions of molecular mass standards ($M_r \times 10^{-3}$) are to the left of the autoradiograph. The bottom of the gel contained no ^{125}I-ubiquitin–RNase A conjugates and is not shown. Note that the SDS–PAGE mobilities of the various ubiquitin–RNase A conjugates differ, and parallel the differences observed for the unconjugated RNase A derivatives (Dunten & Cohen, 1989).

result of ATP-dependent proteolysis. These data can be compared with the results summarized in Table I, where with ATP about ~18% of the ^{125}I-ox-RNase A was degraded in 2 h to acid-soluble counts. Presumably, degradation products that remain precipitable with 15% trichloroacetic acid are lost from the 12.5% polyacrylamide gel following SDS–PAGE.

AMPPNP Facilitates the Comparison of Substrates for Ubiquitin Conjugation by Fraction II. Recent work from this laboratory explored ubiquitin conjugation to six RNase A derivatives which differed with respect to modifications at their methionine and cysteine residues (Dunten & Cohen, 1989). In particular, methionine oxidation was investigated as a potential determinant for specific recognition by the ubiquitin–protein ligase. As visualized after SDS–PAGE, all of the RNase A derivatives showed similar levels of ubiquitin conjugation when isopeptidases in the fraction II were inactivated by ubiquitin aldehyde. The effects of oxidative damage were concluded to result from an altered protein conformation rather than the generation of new and specific recognition determinants. It was noted, however, that the conjugates observed actually represented the difference of ubiquitination and all degradation/disassembly reactions. Even with ubiquitin aldehyde to inhibit ubiquitin–protein isopeptidases (Hershko & Rose, 1987), results could have been biased due to differential conjugate degradation by the ubiquitin-dependent protease. It seemed possible to eliminate this ambiguity by application of a β,γ-nonhydrolyzable form of ATP.

Figure 5 shows that the levels of conjugates to the various RNase A derivatives were equivalent in reactions with either ATP or AMPPNP and in the presence of ubiquitin aldehyde (lanes 1–8). This result argues against the possibility that ubiquitination was enhanced specifically for proteins with methionine sulfoxide residues but was masked by subsequent ubiquitin/ATP-dependent degradation. Unfolded RNase A derivatives, with or without methionine sulfoxide moieties, are conjugated with equal efficiency. In parallel assays without ubiquitin aldehyde (not shown), relatively low product yields were obtained due to concomitant conjugate disassembly by isopeptidase activities. The profound differences with and

without ubiquitin aldehyde as compared to ATP versus AMPPNP indicate that the isopeptidases in fraction II were far more active on these conjugates than was the ubiquitin/ATP-dependent protease.

Discussion

We have shown that either of the two β,γ-nonhydrolyzable ATP analogues, AMPPNP or AMPPCP, can substitute for ATP in ubiquitin conjugation assays. Because neither analogue can support the activity of the 26S ubiquitin-dependent protease, their use affords a simple method to uncouple ubiquitination from subsequent degradation by the ubiquitin system. We have applied this method to demonstrate that, with several modified forms of RNase A as substrates and ubiquitin aldehyde to inhibit isopeptidases, the relative conjugate yields reflect rates of ubiquitination without complications from different rates of conjugate breakdown. This result extends our earlier work showing that RNase A unfolding, and not the availability of specific damaged residues such as methionine sulfoxide, provides the determinant(s) recognized for ubiquitination (Dunten & Cohen, 1989).

In a second application, the stabilities of ubiquitinated ^{125}I-ox-RNase A conjugates were examined in pulse–chase experiments with either ATP or AMPPNP. In this manner, the effects of de-ubiquitination by isopeptidases could be compared with ubiquitin-dependent proteolysis under conditions of continuing ubiquitination. The results confirmed that high molecular mass, multiply ubiquitinated conjugates are degraded preferentially by the ubiquitin-dependent protease (Hershko et al., 1984; Hough et al., 1986). This degradation was apparent above the background of concurrent conjugate disassembly by ubiquitin aldehyde insensitive isopeptidase(s), which also appeared to be most active with the higher order conjugates. However, whereas with AMPPNP low molecular mass conjugates (predominantly mono-, di-, and triubiquitinated- ^{125}I-ox-RNase A) accumulated and were relatively stable, the active ubiquitin-dependent protease in the ATP-supplemented reaction resulted in more rapid turnover of the entire spectrum of conjugates.

In their early ubiquitin–protein conjugation experiments, Hershko et al. (1980) had observed that multiply ubiquitinated products accumulate preferentially. They proposed that conjugation either is processive, where substrates remain enzyme-bound until after many rounds of ubiquitination, or is cooperative, where the ligation apparatus exhibits increasing affinity for substrates as more ubiquitins are attached. In our pulse–chase experiment with AMPPNP, despite continued ubiquitin conjugation during the chase period, we detect no further ubiquitination of the ^{125}I-labeled mono-, di-, or triubiquitinated species to higher mass products. This result, coupled with the fact that de novo ubiquitination of ox-RNase A yielded predominantly the higher mass conjugates (see Figure 4, lanes 2 and 6), favors a processive rather than cooperative mechanism. Ox-RNase A ubiquitination is primarily E3-mediated (Reiss et al., 1988), and properties of the E3 ubiquitin–protein ligase are consistent with processivity (Reiss et al., 1989).

That AMPPNP and AMPPCP can substitute for ATP during ubiquitin activation is consistent with, although it is not a necessary outcome of, the E1 mechanism (Haas et al., 1982, 1983). Aminoacyl-tRNA synthetases resemble E1 in that amino acid activation proceeds via an acyl adenylate to generate AMP and PP_i as the β,γ-cleavage products of ATP. Depending upon the particular synthetase, β,γ-methylene- and imido-ATP analogues either are substrates or inhibitors or have little effect (Papas & Case, 1970; Freist et al., 1980). Likewise, E1 enzymes from diverse sources may differ with respect to their nucleotide specificities, although the highly conserved nature of the ubiquitin system [see Jentsch et al. (1990) and references cited therein] suggests otherwise. In any case, the empirical test for the restoration of ubiquitin conjugation by ATP analogues in ATP-depleted extracts is straightforward, as we have demonstrated. We note, however, that for some substrates the use of ATP analogues could be complicated by other ATP-requiring steps in the ubiquitin pathway, such as tRNA aminoacylation (Ferber & Ciechanover, 1986, 1987).

PP_i exchange assays with purified rabbit reticulocyte E1 were done as a function of nucleotide concentration to compare ATP with the β,γ analogues. The results with ATP (K_m = 24 μM; k_{cat} = 11 s^{-1}) agree closely with previously reported values (Haas & Rose, 1982), and AMPPNP behaves similarly (K_m = 37 μM; k_{cat} = 4 s^{-1}). With AMPPCP, however, in addition to a further reduction of the k_{cat} to 2 s^{-1}, substantial substrate inhibition was observed (apparent $K_i \approx 0.9$ mM). We have not pursued this point but suspect that binding of AMPPCP to the PP_i site on E1 is responsible for the inhibition. If PP_i, with a fourth pK_a of ~8.2, binds to E1 as PP_i^{3-}, then a preference for AMPPCP at the same site may be due in part to the relatively high pK_a of its last ionization [~8.5, versus 7.7 and 7.1 for AMPPNP and ATP, respectively (Yount, 1975)]. However, because AMPPNP also is protonated under the conditions of the exchange assay (~50% protonated at pH 7.6), other structural differences among ATP forms with O, C, or N as the β,γ-bridging atom (Yount, 1975) must contribute to the exceptional behavior of AMPPCP.

The abilities of AMPPNP and AMPPCP to support ubiquitin–protein conjugation in ATP-depleted extracts were in accord with the PP_i exchange results reported here and previously (Haas et al., 1983). In assays with fraction II and 4 mM nucleotide, conjugate levels were slightly reduced with AMPPNP relative to ATP, and very much reduced with AMPPCP. Supplementation of the reaction with purified E1 increased conjugate yields to confirm that ubiquitin activation with the ATP analogues had been rate-limiting for ubiquitination. However, comparisons within pairs of otherwise identical assays showed that, apart from uniformly increased yields, conjugation patterns were affected little by additional E1 (see Figure 3). Possibly, a single E2 species was responsible for the bulk of the conjugation observed; alternatively, multiple E2's were involved, but were charged with ubiquitin from E1 with equal efficiency.

As with AMPPNP and AMPPCP, other ATP analogues such as adenosine 5′-(γ-thio)triphosphate (Haas et al., 1983) may serve to uncouple ubiquitin conjugation from degradation. Also, in analogy with our use of β,γ-nonhydrolyzable forms of ATP, α,β-nonhydrolyzable analogues may support conjugate proteolysis without further ubiquitination. The distinction of ubiquitin-mediated proteolysis from other ATP-dependent degradation pathways is another potential application. We have not explored these possibilities. The use of any analogue, however, will depend upon prior depletion of ATP from the assay. With reticulocyte fraction II, either enzymatic methods or rapid gel filtration could be used, and both ubiquitination and degradation activities were eliminated by these treatments. Full ubiquitination activity was restored upon ATP addition, but ATP-dependent proteolysis could be recovered only from the samples pretreated by gel filtration. Possibly, incubation with hexokinase/glucose or glycerokinase/glycerol removed small amounts of ATP which otherwise remained tightly bound to the 26S protease. Also, whereas samples were maintained at 4 °C during gel filtration, enzymatic ATP depletion involved

20-min incubations at 20 °C. Ganoth et al. (1988) have reported that, without ATP, the 26S protease can dissociate in subunits, two of which are highly thermolabile. The irreversible activity loss that we observe may relate to this property of the enzyme. In an attempt to stabilize the 26S complex, we included AMPPNP during the enzymatic ATP depletion step; even with this precaution, however, protease activity could not be restored upon subsequent addition of ATP (data not shown). Consequently, rapid gel filtration was used routinely to prepare fraction II for comparisons of assays with and without ATP-dependent proteolysis. Where such comparisons are unimportant and ATP-dependent degradation need not be restored, enzymatic ATP depletion also may be used.

We anticipate that, in future studies of ubiquitin-mediated degradation, β,γ-nonhydrolyzable ATP analogues will prove most useful where relatively complex or uncharacterized ubiquitination systems are employed. In particular, the detection of endogenous ubiquitination substrates that otherwise are rapidly degraded in cell extracts may benefit from the use of an analogue such as AMPPNP, possibly combined with ubiquitin aldehyde to inhibit most isopeptidases. Extremely short-lived proteins are of particular interest because they often are key regulators of cellular metabolism and development [see Hochstrasser and Varshavsky (1990) and references cited therein]. With β,γ-nonhydrolyzable ATP and ubiquitin aldehyde to limit subsequent breakdown, the identification and characterization of ubiquitinated forms of these proteins should be greatly facilitated.

ACKNOWLEDGMENTS

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Registry No. AMPPNP, 25612-73-1; AMPPCP, 3469-78-1; ATP, 56-65-5; proteinase, 9001-92-7; isopeptidase, 86480-67-3.

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Mechanism of Altered Renal Glutaminase Gene Expression in Response to Chronic Acidosis†

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ABSTRACT: Increased rat renal ammoniagenesis is sustained during chronic metabolic acidosis by the cell-specific induction of the regulatory enzymes of glutamine catabolism and of gluconeogenesis. A glutaminase-specific cDNA hybridizes to 6.0- and 3.4-kb mRNAs that are contained in total or poly(A)$^+$ RNA isolated from rat kidney. When translated in a rabbit reticulocyte lysate, each of the fractionated mRNAs produces the 72-kDa precursor of the mitochondrial glutaminase. The levels of both mRNAs are increased 5-fold within 1 day following onset of chronic acidosis and reach a maximum (8-fold) after 5 days. During recovery from chronic acidosis, the levels of the glutaminase mRNAs are returned to normal within 1 day. The observed changes in mRNA levels correlate with equivalent changes in the relative levels of translatable glutaminase mRNA. Nuclear run-on assays indicate that the rate of transcription of the renal phosphoenolpyruvate carboxykinase gene is increased and decreased in response to onset and recovery from chronic acidosis, respectively. In contrast, the rates of transcription of the glutaminase and β-actin genes are unaffected by alterations in acid-base balance. Thus, the increase in renal glutaminase activity during chronic acidosis results from an equivalent increase in the levels of total and translatable glutaminase mRNAs which apparently results from an increased stability of the glutaminase mRNA.

During normal acid-base balance, the rat kidney extracts very little, if any, of the plasma glutamine (Squires et al., 1976). Renal extraction and catabolism of plasma glutamine are increased rapidly following onset of metabolic acidosis (Hughey et al., 1980). The acute increase in renal ammoniagenesis results primarily from changes in the concentrations of metabolites and H^+ that regulate flux through the mitochondrial glutaminase, glutamate dehydrogenase, and α-ketoglutarate dehydrogenase (Tannen & Sastrasinh, 1984). During chronic acidosis, the initial changes in renal metabolites and plasma pH are largely compensated (Parry & Brosnan, 1978). The increased rat renal ammoniagenesis and gluconeogenesis are now sustained by the induction of glutaminase (Curthoys & Lowry, 1973), glutamate dehydrogenase (Wright & Kuepper, 1989), and phosphoenolpyruvate carboxykinase (Burch et al., 1978) that occur solely within the proximal convoluted segment of the nephron.

The increase in mitochondrial glutaminase activity is due to a gradual increase in the relative rate of glutaminase synthesis (Tong et al., 1986). The increased rate of synthesis reaches a plateau within 5 days that is 5-fold greater than normal. The apparent half-life of the glutaminase is unaltered during acidosis. As a result, the total renal glutaminase activity is increased approximately 5-fold after 7 days of chronic acidosis. The increase in the relative rate of glutaminase synthesis correlates with an increase in the relative level of translatable GA[1] mRNA (Tong et al., 1987). In vitro translation of rat renal poly(A)$^+$ RNA yields a 72-kDa peptide that is immunoprecipitated with anti-glutaminase IgG. Pulse-chase experiments with primary cultures of rat renal proximal tubular epithelial cells and in vitro processing studies (Perera et al., 1990) have established that the 72-kDa peptide is the cytosolic precursor of the 68- and 65-kDa peptides that constitute the mitochondrial glutaminase.

In this study, Northern blot analysis and nuclear transcription run-on assays were performed to further characterize the mechanism of glutaminase induction during chronic acidosis. The observed increase in the relative levels of translatable GA mRNA correlates with equivalent changes in the level of total GA mRNA. However, this increase occurs without increasing the rate of transcription of the GA gene. Thus, increased renal glutaminase activity that occurs in response to chronic acidosis apparently results from an increase in the stability of the GA mRNA.

Materials and Methods

[α-^{32}P]dCTP and [α-^{32}P]CTP (sp act. 3000 Ci/mmol), L-[^{35}S]methionine (sp act. 800 Ci/mmol), and Gene Screen were obtained from New England Nuclear Research. ^{14}C-Labeled protein molecular weight standards were purchased from Bethesda Research Laboratories. Glutamate dehydrogenase, restriction enzymes, and calf liver tRNA were products of Boehringer Mannheim. Oligolabeling kit and ACS solution were obtained from Pharmacia and Amersham, respectively. Oligo(dT)-cellulose type 2 was purchased from Collaborative Research, Inc. Low-melting-temperature aga-

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[1] Abbreviations: GA, glutaminase; PCK, phosphoenolpyruvate carboxykinase.

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