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Ubiquitin Adenylate: Structure and Role in Ubiquitin Activation†

Arthur L. Haas,‡ Jessie V. B. Warms, and Irwin A. Rose*

ABSTRACT: The acid precipitate of the ubiquitin activating enzyme after reaction with ATP and ubiquitin contains one enzyme equivalent of ubiquitin adenylate in which the carboxyl-terminal glycine of ubiquitin and AMP are in an acyl–phosphate linkage. The recovered ubiquitin adenylate has the catalytic properties proposed for it as a reaction intermediate. Thus, upon reaction with fresh enzyme in the absence of Mg^{2+} or ATP, the product complex, E-ubiquitin·AMP–ubiquitin, is formed. This complex is capable of generating ubiquitin–protein isopeptide derivatives when added to a reticulocyte fraction that catalyzes protein conjugation. This reproduces the effect previously shown to require ubiquitin, ATP, and Mg^{2+}. In the presence of activating enzyme, ubiquitin adenylate is converted to ATP and free ubiquitin in a step requiring PP_i and Mg^{2+}. On the basis of studies of [^{32}P]PP_i/nucleoside triphosphate exchange, the activating enzyme could be used to generate 2′-deoxy-AMP–, 2′-deoxy-IMP–, and 2′-deoxy-GMP–ubiquitin but not pyrimidine nucleotide–ubiquitin derivatives. The enzyme shows a modest preference for the *pro-S* diastereomers of adenosine 5′-*O*-(1-thiotriphosphate) and adenosine 5′-*O*-(2-thiotriphosphate). Inorganic phosphate, arsenate, methyl phosphate, and tripolyphosphate, but not nucleoside triphosphates, can serve as alternate substrates in place of PP_i in the reverse of ubiquitin adenylate formation. Therefore, the enzyme catalyzes the unusual reaction ATP + P_i ⇌ ADP + PP_i in the presence of ubiquitin.

A novel mechanism of protein modification has recently been described in which a low molecular weight polypeptide (M_r 8556), ubiquitin, is covalently conjugated to target proteins (Ciechanover et al., 1980; Hershko et al., 1980; Wilkinson et al., 1980). In the soluble fraction of reticulocytes (Ciechanover et al., 1980; Hershko et al., 1980, 1982) and other cells (Chin et al., 1982; Hershko et al., 1982), ubiquitin conjugation probably serves as the committed step of energy-dependent protein degradation (Haas & Rose, 1981). In the nucleus, ubiquitin conjugation to core histones 2A (Goldknopf & Busch, 1977) and 2B (Wu et al., 1981) may participate in regulating chromatin structure and subsequent transcription (Levinger & Varshavsky, 1982), as has been recently reviewed by Busch & Goldknopf (1981).

The enzyme responsible for the activation of ubiquitin for the subsequent formation of covalent conjugates with lysine ϵ-amino residues of target proteins is a dimer of 105-kilodalton subunits (Ciechanover et al., 1982; Haas et al., 1982). The steps catalyzed by the ubiquitin activating enzyme are known in some detail but may be condensed to three steps:

$$E_{SH} + ATP + Ub \rightleftharpoons E_{SH}^{AMP\text{-}Ub} + PP_i \quad (1)$$

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

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

The initial formation of a tightly bound ubiquitin adenylate (AMP–Ub)[1] in step 1 is followed by ubiquitin transfer to a thiol group of the same enzyme to form a covalent enzyme–ubiquitin thiol ester, with liberation of AMP (step 2). Activation of a second molecule of ubiquitin yields a stable ternary complex composed of 1 equiv each of ubiquitin adenylate and ubiquitin thiol ester per enzyme subunit (step 3). Stoichiometric evidence and kinetic evidence for this minimal mechanism have been presented previously (Haas et al., 1982; Haas & Rose, 1982). Ubiquitin-dependent ATP/PP_i and ATP/AMP equilibrium isotope exchange kinetics demonstrate that binding of substrates and release of products are strictly ordered, with ATP the leading substrate with respect to ubiquitin in both steps 1 and 3, and the PP_i formed in step 1 the leading product with respect to AMP (Haas & Rose, 1982).

In the absence of ATP, isopeptide bond formation may proceed from the enzyme–ubiquitin thiol ester complex in steps catalyzed by additional enzymes of the system (Haas et al., 1982). Since the carboxyl-terminal glycine of ubiquitin is involved in the thiol ester linkage to the enzyme (Hershko et al., 1981), the ubiquitin adenylate has been assumed to exist as a mixed acyl phosphate anhydride between the carboxyl

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‡ Present address: Department of Biochemistry, The Medical College of Wisconsin, Milwaukee, WI 53226.

[1] Abbreviations: Ub, ubiquitin; AMP–Ub, ubiquitin adenylate; BSA, bovine serum albumin; HPLC, high-performance liquid chromatography; etheno-ATP, 1,N^6-ethenoadenosine 5′-triphosphate; etheno-CTP, 3,N^4-ethenocytidine 5′-triphosphate; ATPαS, adenosine 5′-*O*-(1-thiotriphosphate); ATPβS, adenosine 5′-*O*-(2-thiotriphosphate); ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); $ADPCH_2P$, adenosine 5′-(β,γ-methylenetriphosphate); ADPNHP, 5′-adenylyl imidodiphosphate; Na$DodSO_4$, sodium dodecyl sulfate; DTT, dithiothreitol; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; NTP, nucleoside triphosphate; Cl_3CCOOH, trichloroacetic acid.

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terminus of ubiquitin and AMP (Ciechanover et al., 1981; Haas et al., 1982). The present work provides evidence for this structure by examination of ubiquitin adenylate liberated by acid from the activating enzyme. Ubiquitin adenylate is then shown to have the activities expected for a functional intermediate in the ubiquitin activating enzyme reaction.

Materials and Methods

Homogeneous human erythrocyte ubiquitin was that described previously (Haas & Rose, 1982), the single tyrosine of which was labeled with ^{125}I by the Chloramine-T method (Ciechanover et al., 1980). The quantitative equivalence of native and radioiodinated ubiquitin in reaction with the activating enzyme has been demonstrated elsewhere (Haas et al., 1982; Haas & Rose, 1982). Carrier-free $Na^{125}I$ was obtained from Amersham; $Na[^{32}P]P_i$, $Na_4[^{32}P]PP_i$, and [α-^{32}P]ATP were obtained from New England Nuclear; [2,8-^3H]ATP was obtained from ICN; [γ-^{32}P]ATP was synthesized enzymatically (Glynn & Chappell, 1964). Yeast inorganic pyrophosphatase, *Escherichia coli* alkaline phosphatase, soybean trypsin inhibitor, and trisodium tripolyphosphate were obtained from Sigma; yeast hexokinase, $ADPCH_2P$, and ADPNHP were obtained from P-L Biochemicals; TPCK–trypsin and snake venom phosphodiesterase were obtained from Worthington Biochemicals. Etheno-ATP, etheno-CTP, ATPαS (S_p and R_p), ATPβS (R_p), and ATPγS were generously supplied by Dr. Mildred Cohn. The sample of ATPβS (S_p) was the gift of Dr. Douglas Markham.

Reticulocyte-rich whole rabbit blood was obtained by phenylhydrazine induction of adult male rabbits and used to prepare fraction II which contains the enzymes for ubiquitin conjugation to proteins (Ciechanover et al., 1978). Ubiquitin activating enzyme was prepared from fraction II by covalent affinity chromatography and stored at −80 °C in small aliquots (Haas et al., 1982). Activating enzyme was quantitated by the amount of [^3H]AMP–Ub formed in the presence of [^3H]ATP and pyrophosphatase. The enzyme was substantially free of a newly described enzyme (Rose & Warms, 1983) capable of hydrolyzing AMP–Ub.

Preparation of AMP–Ub. Since AMP–Ub does not measurably dissociate from activating enzyme, this compound could only be produced in quantities stoichiometric to the enzyme on which it was generated (Haas et al., 1982). In general, the preparation of AMP–Ub was as follows. Activating enzyme (12 pmol) was incubated in a final volume of 0.45 mL containing 25 mM Tris-HCl, pH 7.6, 1 mM $MgCl_2$, 1.1 mg/mL BSA, 1.5 IU of inorganic pyrophosphatase, 1.3 μM ubiquitin, and 0.42 μM [^3H]ATP. After 3 min, 37 °C, 0.45 mL of 16% (w/v) trichloroacetic acid was added, and the sample was allowed to stand on ice 10 min and then centrifuged for 10 min at 15000*g*. The pellet containing AMP–Ub and unreacted ubiquitin was rinsed twice with 1 mL of ice-cold 2% trichloroacetic acid, dissolved in 0.2 mL of 0.1 M Tris-HCl, pH 7.6, and precipitated a second time by addition of 0.2 mL of 16% trichloroacetic acid. After the sample stood on ice 10 min, it was centrifuged for 10 min at 15000*g*. The resulting pellet was rinsed twice with 1 mL of ice-cold 2% trichloroacetic acid, dissolved in 0.1–0.2 mL of 0.1 M 2-(*N*-morpholino)ethanesulfonate, pH 6.0, and stored on ice where it was stable for several hours. Many experiments made use of ^{125}I-ubiquitin and/or [^3H]ATP which were assayed by using γ and scintillation counting with the latter isotope corrected for overlap by ^{125}I. The preparation is obviously contaminated by acid-insoluble components derived from the initial incubation. Its purity in terms of tritium is shown by monotonic kinetic behavior to completion in a number of experiments.

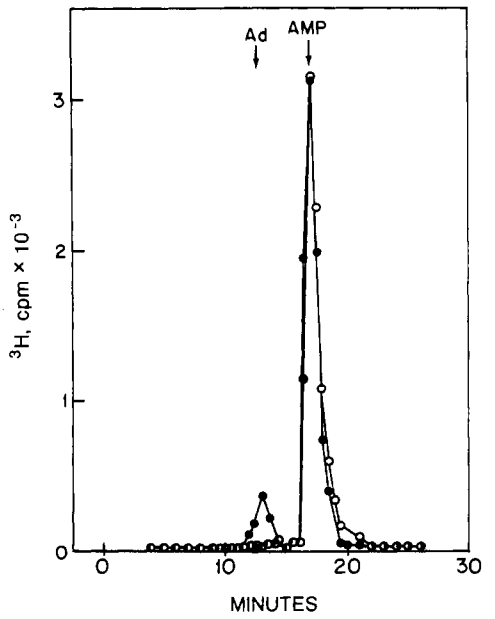

FIGURE 1: HPLC separation of the acid-soluble products generated by the action of trypsin on [^3H]AMP–Ub. Reaction of trypsin (7 IU) with [^3H]AMP–Ub (3.4 pmol, 14 300 cpm/pmol) in 0.1 mL of 0.1 M Tris-HCl, pH 7.6, and 1.7 mg/mol of BSA for 4 min, 37 °C, was quenched with 0.1 mL of ice-cold 16% Cl_3CCOOH, allowed to stand on ice for 10 min, and then centrifuged for 10 min at 15000*g*. Half of the supernatant was adjusted to pH 3.5 with potassium phosphate buffer to a final concentration of 0.14 M containing 10 mM tetrabutylammonium chloride in a final volume of 0.22 mL, and 0.1 mL was separated by HPLC (●). A second aliquot (○) was heated to 100 °C for 5 min before being similarly adjusted for HPLC analysis.

When ^{125}I-ubiquitin was used, suitable controls to distinguish it from its AMP derivative are included.

Ion-Pair Reverse-Phase High-Performance Liquid Chromatography. Labeled nucleotides were resolved by ion-pair reverse-phase HPLC on a Varian Model 5000 instrument equipped with a 4 × 250 mm Bio-Sil ODS-5S C_{18} reverse-phase column (Bio-Rad). Two separation systems were employed. In the experiment described in Figure 1, the column was equilibrated with 50 mM potassium phosphate buffer, pH 3.5, containing 10 mM tetrabutylammonium chloride. Samples of 0.1-mL volume containing 0.14 M potassium phosphate, pH 3.5, 10 mM tetrabutylammonium chloride, and 0.5 mM carrier AMP were applied and nucleotides eluted isocratically at a flow rate of 0.5 mL/min. In all other experiments, the column was equilibrated and eluted with 50 mM potassium phosphate, pH 6.0, containing 10 mM tetrabutylammonium chloride and 20% (v/v) acetonitrile at a flow rate of 1 mL/min. Samples comprising the trichloroacetic acid soluble fraction from incubations were neutralized with an equal volume of 0.6 M potassium phosphate, pH 9.0, containing 20 mM tetrabutylammonium chloride, 0.5 mM carrier AMP, and 0.5 mM ATP to yield a final pH of 6.0. Aliquots of 0.1 mL were applied to the HPLC. In both systems, the isocratic elution of nucleotides was monitored at 256 nm. Peaks of absorbance were collected, and radioactivity was quantitated by liquid scintillation counting.

Results and Discussion

Structure of AMP–Ub. Previously the nature of the chemical bond between AMP and ubiquitin in AMP–Ub was inferred by analogy to similar biochemical reactions and by the chemical stability of this intermediate. Thus, the observation that activating enzyme catalyzed both ubiquitin-dependent ATP/PP_i and ATP/AMP exchange reactions (Ciechanover et al., 1981, 1982) as well as the knowledge that

Table I: Effect of Various Hydrolytic Enzymes on the Stability of AMP-Ub[a]

	acid-insoluble label (pmol)			P_i extracted as Mo^{2+} complex (pmol)
	^3H	^{32}P	Δ	^{32}P
no addition	0.31	0.31		0
+phosphomonoesterase (81 mIU)	0.30	0.30	0	0.01
+phosphodiesterase (1 IU)	0.14	0.14	0.17	0.01
+both phosphatases	0.13	0.13	0.18	0.19
+trypsin (1.4 IU)	0	0	0.31	0.01
+trypsin, soybean trypsin inhibitor (150 BAEE units)	0.35	0.35	0	0

[a] Aliquots containing 0.32 pmol of [^3H,^{32}P]AMP-Ub (4500 cpm of ^3H and 7900 cpm of ^{125}I), prepared as described under Materials and Methods, were introduced into reactions of 50-μL final volume containing 0.1 M Tris-HCl, pH 7.6, and the specified additions and then incubated at 37 °C for 90 s. Reactions were quenched by addition of 0.2 mL of 16% Cl_3CCOOH, allowed to stand on ice for 10 min, and then centrifuged for 10 min at 15000*g*. [^{32}P]P_i in the supernatant was determined by isobutyl alcohol extraction (Berenblum & Chain, 1938). Pellet ^3H and ^{32}P radioactivities were determined after rinsing with cold 2% Cl_3CCOOH as described (Haas et al., 1982).

ubiquitin ultimately formed a thiol ester between a sulfhydryl group on the enzyme and the carboxyl terminus of ubiquitin (Hershko et al., 1981) strongly indicated a mixed-anhydride linkage between the phosphate of AMP and the carboxyl terminus of the polypeptide. That AMP–Ub is hydrolyzed rapidly at alkaline pH but slowly at acidic pH conforms to the expected chemical stability of an acyl phosphate anhydride (Haas et al., 1982).

Cleavage of the linking bond between AMP and ubiquitin, as measured by release of radiolabeled AMP from acid-insoluble ubiquitin, is given in Table I for incubations in the presence of enzymes having different hydrolytic specificities. *E. coli* alkaline phosphatase, a phosphomonoesterase, has no effect on AMP–Ub stability. The inability of the alkaline phosphatase to convert the 5′-^{32}P of the [^3H,^{32}P]AMP–Ub to [^{32}P]P_i shows that this phosphate is protected in the complex. If AMP–Ub is first hydrolyzed by brief incubation at pH 10, alkaline phosphatase completely liberates ^{32}P from AMP (not shown). Similar results were obtained at pH 8 during the slow, first-order, base-catalyzed hydrolysis of AMP–Ub. Mild treatment with snake venom phosphodiesterase caused simultaneous solubilization of ^3H and ^{32}P (Table I). Longer treatment gave complete solubilization.

Ubiquitin is remarkably resistant to most proteolytic enzymes (Schlesinger et al., 1975). However gentle treatment with trypsin inactivates ubiquitin, as measured by the loss in the ability to stimulate energy-dependent proteolysis (Wilkinson & Audhya, 1981), due to the cleavage of the carboxyl-terminal glycylglycine adjacent to arginine-74. The remaining polypeptide is resistant to further degradation (Wilkinson & Audhya, 1981). When a similar incubation is carried out with [^3H,^{32}P]AMP–Ub, both isotopes become acid soluble (Table I). This is completely blocked by pretreatment with soybean trypsin inhibitor, thereby excluding the possibility of a phosphodiesterase contaminant. The inference that AMP is attached to ubiquitin at its C-terminal glycine is supported, although differences in the conformation of ubiquitin and AMP–ubiquitin could result in different patterns of trypsin sensitivity. In an attempt to characterize the trypsin product from [^3H]AMP–ubiquitin, the acid-soluble radioactivity from the trypsin reaction was resolved by ion-pair reverse-phase HPLC. Most of the label coelutes with AMP (17 min) when the acid-soluble trypsin products are immediately subjected to HPLC separation (Figure 1). However, an earlier eluting peak (13 min) comprising ~10% of the total radioactivity is also observed. If the acid-soluble products are first heated to 100 °C for 5 min prior to HPLC separation, the peak eluted at 13 min is not observed (Figure 1). In ion-pair reverse-phase HPLC, nucleotides are separated by both charge and hydrophobicity. The peak occurring at 13 min may represent the surviving AMP–GlyGly, which at the pH of the separation (pH 3.5) would be electroneutral owing to the presence of the free α-amino group and thus be expected to elute more like adenosine (12.5 min) than AMP. Further characterization of the new peak for content of glycylglycine would have provided desirable confirmation of identical behavior of ubiquitin and AMP–ubiquitin toward trypsin but was prevented by the low yield of trypsin product.

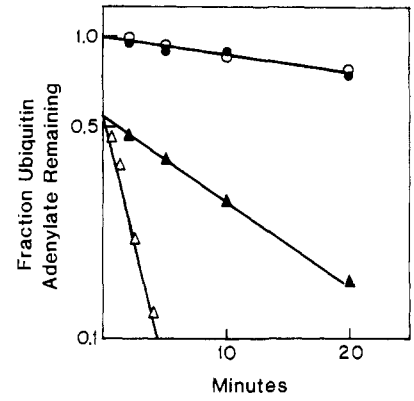

FIGURE 2: Reaction of AMP–Ub with activating enzyme. [^3H]-AMP–Ub (2.4 pmol, 14 400 cpm/pmol) was incubated in 0.3 mL of 0.1 M Tris-HCl, pH 7.6, 5 mM EDTA, 0.5 mg/mL BSA, 0.1 mM DTT, and ubiquitin activating enzyme as follows: no enzyme (●); 1.14 pmol of native enzyme (▲) [or (△) with 1 mM DTT present]; 1.14 pmol of iodoacetamide-treated enzyme (○). Cl_3CCOOH-insoluble counts in 50-μL samples were determined at the indicated times after addition of enzyme at 37 °C.

Reaction of AMP–Ub with Activating Enzyme. If the acid-liberated AMP–Ub is the true intermediate, in keeping with step 2 of the reaction scheme, one would expect the rapid release of one active-site equivalent of AMP upon addition of AMP–Ub to free enzyme, and in accord with step 3, the other product of the reaction should be the enzyme complex having ubiquitin and AMP in a 2 to 1 ratio. Shown in Figure 2 are time courses for release of [^3H]AMP from [^3H]AMP–Ub in the absence and presence of activating enzyme at pH 7.6, 37 °C. In the absence of activating enzyme, hydrolysis occurred with a half-time of 48 min in the experiment shown although values varied from 12 to 180 min depending on the preparation of activating enzyme from which the AMP–Ub was generated. This variability stems from differing degrees of trace contamination by an enzyme found to hydrolyze AMP–Ub which survives the acid denaturation of the activating enzyme (Rose & Warms, 1983).

In the presence of 0.19 pmol of activating enzyme and 0.1 mM dithiothreitol (DTT), the biphasic formation of acid-soluble tritium from [^3H]AMP–Ub is observed (Figure 2). The tritium is entirely accounted for as AMP (see Table II below). The extent to which [^3H]AMP is liberated in the initial rapid phase represents 46%, or 0.18 pmol, of the 0.42 pmol of [^3H]AMP–Ub initially present and agrees with the presence of active enzyme sites, assayed by the ability to form acid-insoluble [^3H]AMP–Ub. Additional studies demonstrate that this burst release of [^3H]AMP from AMP–Ub is directly

Table II: Trapping of [^{3}H]AMP-Ub as [^{3}H]ATP in the Presence of PP_i[a]

additions	pmol AMP-Ub	AMP	ATP
none	0.4	0	0
+E (0.08 pmol)	0.32	0.07	0
+E (0.16 pmol)	0.19	0.18	0
+E (0.08 pmol) + AMP	0.31	0.09	0
+E (0.08 pmol) + AMP + PP_i	0.31	0.10	0
+E (0.08 pmol) + AMP + PP_i + $MgCl_2$	0.02	0.20	0.19

[a] Reaction mixtures of 0.4 pmol of [^{3}H]AMP-Ub (14 400 cpm/pmol) were incubated 1 min, 37 °C, with the indicated additions in a final volume of 50 μL containing 0.1 M Tris-HCl, pH 7.6, 0.1 mM dithiothreitol, and 0.5 mg/mL BSA. Reactions were quenched with 50 μL of 16% (w/v) trichloroacetic acid, allowed to stand on ice for 10 min, and then centrifuged at 15000*g* for 10 min. Supernatants were analyzed by HPLC as described under Materials and Methods. When present, AMP, PP_i, and $MgCl_2$ were at 100 μM, 1 mM, and 1 mM, respectively. Activating enzyme, E, was added as noted.

proportional and approximately equal to the amount of activating enzyme present. The slow first-order release of [^{3}H]AMP from AMP-Ub following the burst is also enzyme catalyzed and is dependent on the DTT present, increasing from 0.065 to 0.38 min^{-1} in going from 0.1 to 1.0 mM DTT in the presence of 0.18 pmol of enzyme (Figure 2). DTT did not alter the size of the burst. As evident from Figure 2, more than 90% of the acid-insoluble tritium obtained by trichloroacetic acid precipitation of the functioning enzyme is active when tested with fresh enzyme.

Noteworthy is the fact that the rapid liberation of AMP from AMP-Ub and activating enzyme and the subsequent DTT-dependent rate did not require Mg^{2+} and were insensitive to the presence of 5 mM EDTA. This is in contrast with the ubiquitin-dependent biphasic utilization of ATP by activating enzyme (Haas et al., 1982) which requires Mg^{2+}. The burst release of [^{32}P]PP_i in this case corresponded to twice the amount of activating enzyme, as expected from steps 1 and 3. Additional slow hydrolysis of [γ-^{32}P]ATP depended on both enzyme and thiol concentrations. The effect of enzyme and DTT concentration on the rate of ATP reaction was interpreted as transfer of the ubiquitin moiety from the covalent enzyme–ubiquitin thiol ester to DTT via chemical transacylation. Supporting evidence for this interpretation was the observation that selective alkylation of the active-site thiol group with iodoacetamide blocked formation of the enzyme–ubiquitin thiol ester, greatly reduced the DTT-stimulated rate of ATP hydrolysis, and abolished ATP/AMP exchange which proceeds through the enzyme–ubiquitin thiol ester (Haas et al., 1982). Direct evidence for DTT–Ub formation has recently been reported (Rose & Warms, 1983).

Thus, the results of Figure 2 are consistent with the burst release of [^{3}H]AMP from AMP-Ub resulting from the formation of the covalent enzyme–ubiquitin thiol ester as in step 2 and the subsequent rate of AMP-Ub cleavage representing transacylation of this thiol ester to DTT. When the latter rates are corrected for spontaneous hydrolysis, first-order rate constants of 0.041 and 0.37 min^{-1} at 0.1 and 1.0 mM DTT were obtained. These agree well with independently determined turnover rates for [γ-^{32}P]ATP breakdown for the same amount of enzyme at saturating ubiquitin of 0.04 and 0.42 min^{-1} at 0.1 and 1.0 mM DTT, respectively. AMP-Ub was added to 0.19 pmol of iodoacetamide-modified activating enzyme to test this interpretation. As shown in Figure 2, no burst release of [^{3}H]AMP was observed nor was cleavage at

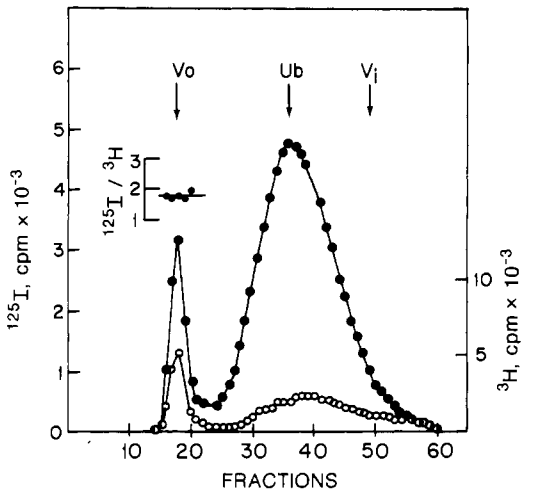

FIGURE 3: Reconstitution of ternary enzyme complex from AMP-Ub. Iodoacetamide-modified activating enzyme was used to generate [^{3}H]AMP-^{125}I-Ub (4916 cpm of ^{125}I and 14 400 cpm of ^{3}H per pmol). The double-labeled AMP-Ub (7.5 pmol) was treated with hexokinase and 2-deoxyglucose to remove any residual ATP and then added to 0.9 pmol of unmodified activating enzyme. After 1 min, 37 °C, the incubation was placed on a column (0.7 × 29 cm) of Sephadex G-75 equilibrated with 50 mM Tris-HCl, pH 7.6, containing 1 mg/mL BSA, and fractions of 0.23 mL were collected and counted: ^{125}I (●); tritium (○) (after correcting for the contribution of ^{125}I). Inset: ^{125}I/^{3}H represents the molar ratio of ubiquitin- to AMP-derived label present in the excluded volume.

0.1 mM DTT greater than that of the spontaneous rate of hydrolysis. That the iodoacetamide-modified enzyme was active was confirmed by formation of a stoichiometric amount of [^{3}H]AMP-Ub from [^{3}H]ATP and ubiquitin (not shown).

Reconstitution of Functional Ternary Enzyme Complex from AMP-Ub. The data of Figure 2 indicate that covalent enzyme bound ubiquitin thiol ester is formed on addition of AMP-Ub to activating enzyme. The enzyme-bound products were purified by gel-exclusion chromatography to determine if AMP-Ub produces a complete ternary complex (E_{S-Ub}^{AMP-Ub}). For characterization of products, [^{3}H]AMP-^{125}I-Ub was formed from ^{125}I-ubiquitin (4916 cpm/pmol) and [^{3}H]ATP (14 400 cpm/pmol) as described under Materials and Methods with the exception that the intermediate was generated on iodoacetamide-modified activating enzyme in order to avoid the complication of having acid-denatured enzyme–^{125}I-ubiquitin thiol ester in subsequent measurements. The double-labeled AMP-Ub (7.5 pmol) was treated with hexokinase and 2-deoxyglucose to remove any residual ATP followed by addition of 0.9 pmol of native activating enzyme. After 1 min at 37 °C, the incubation was resolved on a column of Sephadex G-75. As shown in Figure 3, both ^{125}I and ^{3}H label are associated with enzyme as indicated by their presence in the excluded volume (V_0) of the column where native activating enzyme elutes. Excess unbound [^{3}H]AMP-^{125}I-Ub coelutes with free ^{125}I-Ub. Neither isotope was found in the excluded volume when native enzyme was omitted (not shown). When iodoacetamide-modified enzyme is used, both labels elute in the excluded volume in molar equivalence to enzyme and in a ^{125}I:^{3}H molar ratio of 1, consistent with the inability of such a modified enzyme to form a ubiquitin thiol ester (Haas et al., 1982). This result also conforms to the previous observation that activating enzyme binds only 1 equiv of AMP-Ub per subunit (Haas et al., 1982; Haas & Rose, 1982).

Figure 4 shows that the ternary complex reconstituted from AMP-Ub and activating enzyme is functional in conjugate formation when added to reticulocyte lysate fraction II (lane 3). Conjugate formation results from enzyme-bound rather

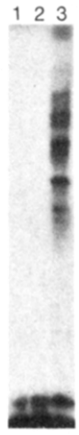

FIGURE 4: Complex formed from AMP–Ub and activating enzyme is active in conjugate formation. [^{3}H]AMP–^{125}I-Ub was generated from iodoacetamide-modified activating enzyme as in Figure 3 and treated with hexokinase and 2-deoxyglucose. (Lane 3) Double-labeled AMP–Ub (8.1 pmol) was in a final volume of 30 μL containing 0.1 M Tris-HCl, pH 7.6, 2 IU of hexokinase, 17 mM 2-deoxyglucose, 3 mM $MgCl_2$, and 4.0 pmol of activating enzyme for 1 min, 37 °C, followed by 140 μg of fraction II protein and the incubation continued for 2 min. The incubation was then quenched with sample buffer and analyzed by $NaDodSO_4$–polyacrylamide electrophoresis and autoradiography (Ciechanover et al., 1980). In lane 2, activating enzyme was added after the incubation was quenched. In lane 1, the incubation was quenched before addition of fraction II.

than free AMP–Ub as shown by the failure to form conjugates if the AMP–Ub is added to fraction II without first having been exposed to activating enzyme (lane 2, Figure 4). Although fraction II contains sufficient activating enzyme to support conjugate formation, a very active carboxyl-terminal ubiquitin hydrolase probably cleaves the AMP–Ub before it is able to react with activating enzyme (Rose & Warms, 1983). Labeled conjugates are not present in the AMP–Ub preparation (lane 1).

Trapping of AMP–Ub as ATP in the Presence of PP_i and Mg^{2+}. The minimum mechanism given in steps 1–3 predicts that AMP–Ub should be trapping as enzyme-bound ATP and ubiquitin by back-reaction in the presence of PP_i. Formation of [^{3}H]ATP trapped from [^{3}H]AMP–Ub on addition of various reactants is given in Table II and is compared to the liberation of [^{3}H]AMP generated by formation of the enzyme–ubiquitin thiol ester.

On addition of [^{3}H]AMP–Ub to enzyme, [^{3}H]AMP is rapidly liberated in equivalence to enzyme (Figure 2 and Table II). Addition of AMP (100 μM) to the reaction mixture has no effect on the formation of enzyme-bound thiol ester within the 1 min of incubation, indicating that AMP does not block the binding and subsequent reaction of AMP–Ub. PP_i (1 mM) without Mg^{2+} has no effect. However, when both PP_i (1 mM) and Mg^{2+} (1 mM) are added, half the [^{3}H]AMP–Ub was converted to [^{3}H]ATP, demonstrating that the divalent metal is required for reaction of AMP–Ub with PP_i, though not for ubiquitin transfer to E_{SH}. Formation of [^{3}H]AMP in excess of enzyme probably reflects a significant rate of exchange between enzyme-bound [^{3}H]AMP–Ub and the pool of unlabeled AMP in the presence of PP_i and Mg^{2+}. This conclusion is supported by other observations, not shown, that all [^{3}H]-AMP–Ub is converted to [^{3}H]ATP when incubated with iodoacetamide-modified activating enzyme, PP_i, and Mg^{2+}. When AMP was omitted from similar incubations with native enzyme, all ^{3}H of the [^{3}H]AMP–Ub was converted to [^{3}H]-ATP. This does not necessarily reflect the basic partition of E_{SH}^{AMP-Ub} between formation of ATP and E_{S-Ub} because at the concentration of enzyme (~2 nM) and AMP formed (~2 nM) the reverse process, $E_{S-Ub} + AMP \rightarrow E_{SH}^{AMP-Ub}$, would be rapid, leading to ATP, the product favored by equilibrium. Finally, free ubiquitin at a concentration of 30 μM failed to block the binding and subsequent reaction of [^{3}H]AMP–Ub as measured by production of both [^{3}H]ATP and [^{3}H]AMP (not shown).

Table III: Comparison of PP_i Exchange with Various Nucleoside Triphosphates[a]

NTP	$v_{o,XTP}/[E_o]$ (s^{-1})	$v_{o,XTP}/v_{o,ATP}$ (%)	$v_{o,XTP+ATP}/v_{o,ATP}$ (%)
ATP	6.5	100	
2′-deoxy-ATP	1.7	26	98
ITP	0.22	3.4	67
GTP	0.38	6.0	99
2′-deoxy-GTP	<0.005	<0.08	68
etheno-ATP	0.07	1.1	95
UTP	<0.005	<0.08	75
CTP	<0.005	<0.08	70
etheno-CTP	0.03	0.5	90
$ADPCH_2P$	0.1	1.5	83
ADPNHP	1.1	17	68

[a] Initial velocities for [^{32}P]PP_i exchange into various nucleoside triphosphates were determined at 37 °C as described by Haas & Rose (1982). Incubations in a final volume of 50 μL contained 0.1 M Tris-HCl, pH 7.6, 0.1 mM dithiothreitol, 10 mM $MgCl_2$, 0.1 mM [^{32}P]PP_i (8–12 cpm/pmol), 2.3 μM ubiquitin, 0.36 pmol of activating enzyme, and 1 mM nucleoside triphosphate in the absence ($v_{o,XTP}$) or presence of 1 mM ATP ($v_{o,XTP+ATP}$).

Table IV: Comparison of ATP and Phosphorothioate Analogues in PP_i Exchange[a]

	$V_m/[E_o]$ (s^{-1})	S_p/R_p	$K_{1/2}$ (mM)
ATP	7.4		0.036
ATPγS	7.7		0.088
ATPβS (S_p)	0.40	6.7	1.0
ATPβS (R_p)	0.06		1.0
ATPαS (S_p)	0.063	3.4	1.0
ATPαS (R_p)	0.018		0.059

[a] The kinetics of [^{32}P]PP_i exchange were determined as in Table III.

Specificity of Nucleotide–Ub Formation. The data in Table II for PP_i trapping of AMP–Ub verify the assumption that Mg^{2+}-dependent ATP/PP_i exchange depends on formation of enzyme-bound AMP–Ub (Ciechanover et al., 1981; Haas et al., 1982). A high degree of specificity for adenine nucleoside triphosphates in forming enzyme-bound nucleotide–Ub was observed by using NTP/[^{32}P]PP_i exchange (Tables III and IV). The ability of the nucleotides to inhibit ATP–PP_i exchange was examined (Table III) to distinguish between an unfavorable $K_{1/2}$ and poor catalysis. When 2′-deoxy-ATP is substituted for ATP, the decrease in rate demonstrates some tolerance for changes in the ribose ring although the effect is manifested principally as a less favorable $K_{1/2}$ since 2′-deoxy-ATP does not effectively compete with ATP. More profound differences in rate are observed with alterations in the purine moiety. The importance of the purine C-6 amino group in enzyme–nucleotide interactions is shown by the large decrease in rate when the C-6 carbonyl of ITP is substituted for this group. This appears to be a V_{max} effect since ITP competes with ATP in PP_i exchange, giving an estimated $K_{1/2}$ for ITP of ~80 μM, compared to 36 μM for ATP (Haas & Rose, 1982). Although ITP and GTP show comparable rates, introduction of an amino group at the C-2 carbon of the latter appears to weaken its affinity for the enzyme. The low rate for etheno-ATP suggests that introduction of this two-carbon bridge creates steric hindrance. Although it appears to bind more tightly than GTP, the rate with 2′-deoxy-GTP is insig-

nificant. No measurable rate was observed with UTP and CTP even though both compounds compete in ATP/PP_i exchange and thus presumably bind to enzyme. However, etheno-CTP supported a low rate of PP_i exchange, consistent with previous observations that this analogue frequently can substitute for ATP (Barrio et al., 1973). ADPNHP reacts at an appreciable rate. $ADPCH_2P$ is much less active as an AMP donor.

Adenosine phosphorothioates were examined as substrates in the presence of Mg^{2+}. The effect of nonbridge sulfur substitution on the stereospecificity of a number of ATP-utilizing enzymes has been reviewed recently (Cohn, 1982). Sulfur substitution for a nonbridge oxygen at either the α- or the β-phosphate of ATP creates a chiral center at phosphorus and therefore a total of four new diastereomers. Since Mg^{2+} readily complexes to oxygen but not to sulfur, the Mg^{2+} chelate to a given pair of nucleoside phosphorothioate diastereomers will possess different geometric configurations that many ATP-requiring enzymes are able to distinguish (Eckstein, 1980; Cohn, 1982). Table IV compares the kinetic parameters for PP_i exchange formation of ATP with the various adenosine phosphorothioates. ATPγS shows a value for $V_m/[E_0]$ comparable to that with ATP. However, PP_i fixation with ATPαS and ATPβS exhibits dramatically lower maximum rates, as is expected for substitution at the phosphorus atoms involved in bond breaking (Pimmer et al., 1976). A 6.7-fold kinetic discrimination favoring the S_p diastereomer of ATPβS is observed. For sulfur substitution at the α-phosphate, the preference also favors the S_p isomer but by only 3.4-fold. Since $K_{1/2}$ represents a complex term not directly related to substrate affinity, changes in the values for the various phosphorothioates are difficult to interpret.

When the specificity of the metal requirement for activating enzyme was examined by measuring the rate of ATP/PP_i exchange (10 mM metal present as the chloride salt), the order of reactivity expressed as the percent of that observed with Mg^{2+} was found to be the following: Mn^{2+}, 54%; Co^{2+}, 28%; Ca^{2+}, 4.5%; Ni^{2+}, 1.4%; Zn^{2+}, 0.3%; and Cd^{2+}, <0.06%. The inability of Cd^{2+} to support a measurable rate of PP_i exchange precluded testing for reversal of stereospecificity with the adenosine phosphorothioates (Jaffe & Cohn, 1979). Therefore, the results of Table IV do not allow one to distinguish whether the stereoselectivity exhibited by activating enzyme derives from specificity for a given metal–nucleotide coordination isomer or whether a cationic group within the nucleotide binding pocket participates in a specific interaction with a nonbridge oxygen, although by analogy to similar mechanisms, the former possibility appears more likely (Cohn, 1982).

Stimulation by P_i of Formation of PP_i from ATP. During the course of the present studies, P_i was observed to stimulate PP_i formation from ATP in a ubiquitin-requiring reaction of activating enzyme. The dependence on P_i is typically hyperbolic (inset to Figure 5) with a $K_{1/2}$ of 5 mM and a maximum turnover rate of 0.18 s^{-1}. Dependence on ubiquitin shows than P_i does not act by simply promoting a nonproductive ATPase activity of the enzyme. The P_i effect does not require formation of the covalent enzyme–ubiquitin thiol ester as seen by the activity of iodoacetamide-modified activating enzyme.

If activating enzyme is incubated in the presence of ATP, ubiquitin, inorganic pyrophosphatase, and [^{32}P]P_i, radioactivity becomes incorporated into an acid-soluble form that is readily adsorbed by charcoal. The latter result suggests that [^{32}P]P_i reacts with enzyme-bound AMP–Ub to yield [β-^{32}P]ADP. Microscopic reversibility predicts that ADP should be able to

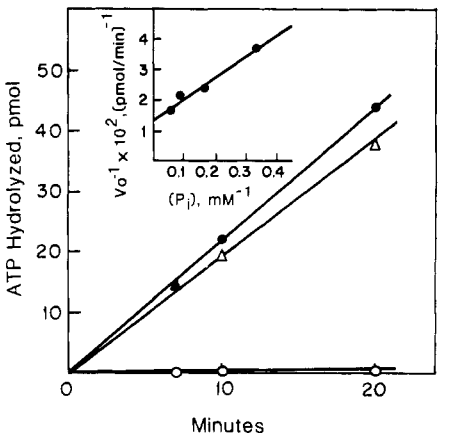

FIGURE 5: Stimulation by P_i of ubiquitin-dependent formation of PP_i from ATP. Native (●) and iodoacetamide-modified (△) activating enzymes, 1.4 pmol, were examined at 37 °C in incubations of 0.35 mL containing 50 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 0.1 mM DTT, 5 μM [γ-^{32}P]ATP (159 cpm/pmol), 1.5 IU of inorganic pyrophosphatase, 10 μM ubiquitin, and 10 mM P_i. At the indicated times, 50-μL samples were removed and assayed for [^{32}P]P_i (Berenblum & Chain, 1938). Omission of either ubiquitin or P_i shown by open circles (○). Inset: Double-reciprocal plot showing the rate of PP_i liberation from ATP as a function of [P_i].

Table V: Kinetic Parameters for Formal and Pseudoexchange Reactions Catalyzed by Activating Enzyme[a]

	$V_m/[E_0]$ (s^{-1})	$K_{1/2}$ (mM)
ATP + [^{32}P]PP_i ⇄ [^{32}P]ATP + PP_i	7.4	0.036 (ATP)
ADP + [^{32}P]PP_i → [^{32}P]ATP + P_i	0.73	0.005 (PP_i)
ADP + [^{32}P]PP_i → [^{32}P]ATP + P_i	0.73	0.17 (ADP)
ATP + [^{32}P]P_i → [^{32}P]ADP + PP_i	0.18	5 (P_i)
ADP + [^{32}P]P_i ⇄ [^{32}P]ADP + P_i	0.03	0.29 (P_i)

[a] Values for ATP/P_i exchange are from the inset to Figure 5. All other values for exchange reactions were determined as in Table III with the following exceptions: for ADP/PP_i exchange, varying concentrations of ADP were substituted for ATP, and for ADP/P_i exchange, ADP at a final concentration of 1 mM was substituted for ATP and the concentration of P_i varied.

replace ATP in E·AMP–Ub formation and therefore lead to ATP formation in the presence of PP_i. This is shown to occur at about 10% the rate of ATP/PP_i exchange (Table V). The stock solution of ADP used in these experiments gave a single peak on HPLC analysis corresponding to ADP. The effects on rate caused by substitution of ADP for ATP and P_i for PP_i appear to be cumulative (Table V). This action of P_i leading to ADP + PP_i would pose a threat to the ATP stability of cells that have P_i/PP_i ratios greater than 1000, the ratio of $K_{1/2}$ values found for PP_i and P_i (Table V). However, this would require that activating enzyme levels were much greater than are found in the reticulocyte since the rate constant at P_i saturation is only ~0.2 s^{-1}.

Other phosphate analogues tested as in Figure 5 for their ability to regenerate ubiquitin and enzyme from bound AMP–Ub were AsO_4^{2-}, methyl phosphate, and tripolyphosphate. AsO_4^{2-} and methyl phosphate were as active as P_i. Tripolyphosphate was much more active, probably as active as PP_i itself. Its reactivity could not have been due to contamination by PP_i which would have been removed by the pyrophosphatase present in the assay. ATP itself is unable to replace PP_i in the reaction with enzyme-bound AMP–Ub. This was tested in the presence of Zn^{2+} (150 μM) with Mg^{2+} (1 mM), which for certain amino acid activating enzymes and their cognate amino acid leads to the formation of diadenosine 1,4-tetraphosphate, Ap_4A (Plateau et al., 1982).

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Registry No. Ub, 60267-61-0; ATP, 56-65-5; 2′-deoxy-ATP, 1927-31-7; ITP, 132-06-9; GTP, 86-01-1; 2′-deoxy-GTP, 2564-35-4; etheno-ATP, 37482-17-0; UTP, 63-39-8; CTP, 65-47-4; etheno-CTP, 56405-86-8; $ADPCH_2P$, 3469-78-1; ADPNHP, 25612-73-1; ATPγS, 35094-46-3; ATPβS (S_p), 59261-36-8; ATPβS (R_p), 59261-35-7; ATPαS (S_p), 58976-48-0; ATPαS (R_p), 58976-49-1; PP_i, 14000-31-8; phosphate, 14265-44-2; arsenate, 15584-04-0; methyl phosphate, 512-56-1; tripolyphosphate, 14127-68-5.

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Effects of 2-Hydroxy-3-undecyl-1,4-naphthoquinone on Respiration of Electron Transport Particles and Mitochondria: Topographical Location of the Rieske Iron–Sulfur Protein and the Quinone Binding Site†

H. J. Harmon* and V. G. Struble

ABSTRACT: 2-Hydroxy-3-undecyl-1,4-naphthoquinone is a quinone analogue that inhibits mitochondrial respiration in the cytochrome b–c_1 region with an apparent K_i of 2.5×10^{-7} M. In electron transport particles, it prevents the reduction of cytochrome c_1 by succinate but not its oxidation by oxygen and prevents oxidation of cytochrome b but not its reduction by succinate. The analogue increases the amount of steady-state cytochrome b reduction in actively respiring particles. It inhibits oxidant-induced reduction of cytochrome b in the presence of antimycin. Inhibition of succinate oxidase activity in electron transport particles is independent of the pH of the suspending medium while at pH values above 8 with mitochondria, inhibition decreases. Since the apparent pK of the bound quinone is pH 6.6, the pH dependency of the inhibition is likely due to the pK of the Rieske iron–sulfur center (pH 8). The Rieske center and thus the quinone binding site are located on the cytoplasmic face of the inner membrane.

The second site of oxidative phosphorylation is associated with the ubiquinol–cytochrome c reductase portion of the mitochondrial electron transport chain. This complex, known as complex III (Hatefi et al., 1962), is also responsible for energy-linked proton translocation from the matrix to the cytoplasmic surface (Alexandre et al., 1980). Four electron carriers [two b-type cytochromes, cytochrome c_1, and the iron–sulfur center described by Rieske (Rieske et al., 1964a,b)] and ubiquinone are arranged in the membrane to allow vectorial proton translocation and generation of membrane potential. This arrangement is known as the protonmotive Q cycle (Mitchell, 1975; Bowyer & Trumpower, 1981).

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