The Mechanism of Ubiquitin Activating Enzyme

A KINETIC AND EQUILIBRIUM ANALYSIS*

Arthur L. Haas† and Irwin A. Rose§
From the Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111

The recently characterized ubiquitin activating enzyme catalyzes the first step in ubiquitin-protein isopeptide bond formation and as such may be central to a number of regulatory events in addition to its previously reported role in energy-dependent proteolysis. The present work substantiates the following sequence of reactions in ubiquitin activation: 1) initial formation of tightly enzyme-bound ubiquitin adenylate with PP, formation from ATP; 2) conversion of this intermediate to form AMP and a covalent enzyme-ubiquitin thiolester; and 3) activation of a second molecule of ubiquitin to give a ternary complex of one equivalent each of ubiquitin thiolester and tightly bound ubiquitin adenylate per subunit of enzyme. Isotope exchange kinetics at equilibrium demonstrate that substrate binding and product release are both strictly ordered, with ATP the leading substrate with respect to ubiquitin and PP, the leading product with respect to AMP. Equilibrium constants relating all resolved steps of the mechanism have been determined by direct measurement of ubiquitin adenylate and ubiquitin thiolester formation. The equilibrium constant for formation of enzyme-bound ubiquitin adenylate and PP, from bound substrates is 0.16. Ubiquitin distribution between the bound adenylate and the enzyme-ubiquitin thiolester plusbound AMP favors the latter by a factor of 2. Reactant dissociation constants were found to be 0.45 μM for ATP, 0.58 μM for ubiquitin, and 3.3 μM for PP.

Free enzyme shows an apparent binding of AMP, but the ubiquitin thiolester form of enzyme binds this mononucleotide with a dissociation constant of 0.027 μM. The effect of PP, and AMP on the distribution of bound intermediates suggests they may serve to regulate energy-dependent proteolysis and other processes requiring protein-ubiquitin conjugation by product inhibition of the first step toward isopeptide bond formation.

Accumulating evidence indicates that energy-dependent protein breakdown in reticulocytes, and probably all cells, proceeds through a multi-enzyme proteolytic system for which the initial covalent conjugation of ubiquitin to target proteins serves as an obligatory signal event for their degradation (1-6). Protein-ubiquitin conjugation involves the ATP-coupled condensation of a lysyl e-amino group on the target protein (4) with the carboxyl terminal glycine of ubiquitin to form an isopeptide bond (7) identical with that demonstrated for the histone conjugate A24 (8). In reticulocytes such conjugates are committed to degradation (6) with the subsequent liberation of free ubiquitin (4). In other cells ubiquitin conjugation to nucleosomal core histones 2A (8) and 2B (9) may serve to regulate chromatin structure during transcription (for a recent review see Ref. 10). Thus, ubiquitin ligation represents a novel form of protein modification that may function in multiple regulatory modes in addition to its role in protein breakdown.

The enzyme responsible for the activation of ubiquitin, purified to homogeneity (11), is a dimer comprised of 1 × 10² dalton subunits. The enzyme exhibits both ubiquitin-dependent ATP:PP, and ATP:AMP exchange (11, 12). In the presence of ATP a covalent enzyme-bound ubiquitin is formed having properties consistent with a thiolester between the carboxyl terminal glycine of the polypeptide and a sulfhydryl residue of the enzyme (7, 12). Conjugate bond formation proceeds from this ubiquitin thiolester, in the absence of ATP, by a subsequent enzyme of the system (13). Therefore, in addition to being the first enzyme of the degradative pathway, the ubiquitin activating enzyme probably represents the only energy-requiring step in proteolysis, although other fates of activated ubiquitin may make the ubiquitin activating enzyme central to a variety of cellular functions.

Ubiquitin activating enzyme catalyzes the biphasic ubiquitin-dependent formation of PP, from ATP. This process consists of a rapid pre-steady state release of two equivalents of PP, per subunit of enzyme followed by a significantly slower linear release of PP, the latter process being proportional to both the concentrations of enzyme and thiols such as dithiothreitol (13). The two equivalents of ATP hydrolyzed during the pre-steady state result in the formation of one equivalent each of covalently enzyme-bound ubiquitin thiolester and a second species of ubiquitin non-covalently bound to enzyme, ubiquitin adenylate (13). Knowing that the carboxyl-terminal glycine of ubiquitin is involved in the thiolester linkage to the enzyme (7), the ubiquitin adenylate is believed to be a mixed acyl phosphate anhydride between the carboxyl terminus of ubiquitin and AMP (13). A minimal mechanism for the formation of a ternary complex composed of the two forms of enzyme-bound ubiquitin has been proposed to consist of three steps (13):

\[ E_{\text{sub}} + ATP + Ub' = E_{\text{Mm}}^{\text{Ub}} + PP \] (1)

\[ E_{\text{Mm}}^{\text{Ub}} = E_{\text{ub}} + AMP \] (2)

\[ E_{\text{ub}} + ATP + Ub = E_{\text{sub}}^{\text{Ub}} + PP \] (3)

The product of step 3 has been isolated and shown to have the expected ratio of 2:1 for enzyme-bound ubiquitin:AMP.

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1 The abbreviations used are: Ub, ubiquitin; AMP-Ub, ubiquitin adenylate; NaDodSO₄, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.
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per subunit (13). The enzyme must possess an exceptional affinity for ubiquitin adenylate since, once formed, this intermediate does not measurably dissociate. The nucleotides explain the finding that ATP:PP exchange occurs in the absence of added AMP, probably entirely by way of step 3, but that ATP:AMP exchange requires PP, since the latter reaction must encompass both steps 1 and 2 (12).

The great stability of the enzyme-products complexes makes the ubiquitin activating enzyme inaccessible to a steady state kinetic examination. Isotope exchange at equilibrium can be used to quantitate the flux through the three steps and establish the order of reactant participation. Such studies also allow one to test the principal feature of the minimal model: the existence of two forms of chemically exchangeable enzyme-bound ubiquitin. As will be shown, it is additionally possible to determine the equilibrium constants for the steps of the scheme, when expanded to include all Michaelis complexes, by measuring the distribution of these two forms of enzyme-bound ubiquitin under a range of equilibrium conditions. Observation of unusually tight binding for AMP (or AMP) indicates that this mononucleotide may play an important role in regulating the rate of ubiquitin conjugate formation by determining the distribution of enzyme-bound forms in Step 2.

MATERIALS AND METHODS

Ubiquitin was purified to homogeneity from human erythrocytes by an extension of previous methods (14, 15). Concentrations of ubiquitin stock solutions were measured relative to a standard determined by amino acid analysis (5) and calculated based on a molecular weight of 3555. A portion of the purified ubiquitin was labeled with 125I by the chloramine-T method (3). The functional equivalence of native and iodinated ubiquitin for the activating enzyme has been demonstrated previously by the good agreement found in stoichiometry studies using 32P-ubiquitin, [35S]ATP, or [3H]ATP (13). Carrier-free Na[I] was obtained from Amersham; [35S]ATP, [3H]AMP, [2,8-3H]ATP, and Na[I]-PP were obtained from New England Nuclear. Reticulocyte-rich whole blood was obtained by phenylhydrazine induction of adult male rabbits (16) and used to prepare fraction II as previously described (1). Ubiquitin activating enzyme was purified to apparent homogeneity from fraction II by covalent affinity chromatography, adjusted to 3.1 M dithioreitol and 1 mg/ml of bovine serum albumin to enhance stability, then extensively dialyzed as described (13). The enzyme was divided into small aliquots and stored at -80°C. Although the enzyme retains full activity for at least 8 months when stored at -80°C in the presence of dithioreitol and bovine serum albumin, a slight loss of activity is observed with repeated freezing and thawing. Therefore, all experiments were performed with aliquots thawed only once. Activating enzyme was quantitated by the extent of ubiquitin [3H]adenylate formation in the presence of [3H]ATP and pyrophosphatase (13).

Isotope Exchange Assays—All isotope exchange assays were performed at 3° in a final volume of 50 ml containing 50 mM Tris-Cl, pH 7.6, 10 mM MgCl2, 0.1 mM dithioreitol, and 0.27 pmol of activating enzyme. Concentrations of ATP, AMP, PP, and ubiquitin were as described in the accompanying figure or table legends. Enzyme-bound ubiquitin thiol ester was determined using acid-precipitable radioactivity using [3H]ATP (13). Reactions of 50 ml of final volume containing 0.8 mg/ml of bovine serum albumin were initiated with enzyme then incubated 4 min and quenched by addition of 100 ml of ice-cold 16% trichloroacetic acid. After standing on ice for 10 min, the resulting pellicle was rinsed twice with cold 2% trichloroacetic acid, then dissolved in 10 mM dithiothreitol and 10 mM MgCl2. Nucleotide peaks were collected and quantitated by liquid scintillation counting. Data were corrected for a control to which enzyme had been introduced after addition of 4 M urea, 4% NaDodSO4-PAGE buffer and which was consistently less than 0.1% of total added radioactivity.

Enzyme-bound ubiquitin thiol ester was determined using 32P-labeled ubiquitin. Reactions of 20 ml of final volume were initiated with enzyme, then incubated 5 min and quenched by addition of 20 ml of 4 M urea, 4% NaDodSO4-PAGE buffer, 0.4% oxalate, and 0.1 M KI, preheated to 37°C. The enzyme-bound [32P]-ubiquitin was resolved by NaDodSO4-PAGE as described previously (13), except that the gel was dried and autoradiographed without staining. The band corresponding to enzyme was cut from the dried gel and radioactivity quantitated in an Abbott Autologic gamma counter. Data were corrected for a control to which enzyme had been introduced after addition of 4 M urea, 4% NaDodSO4-PAGE buffer and which was consistently less than 0.1% of total added radioactivity. The reproducibility of this method for quantifying ubiquitin thiol ester was such that independent replicates consistently agreed within 5% of their mean value.

RESULTS

Equilibrium Isotope Exchange Studies

AMP Dependence of ATP:PP and ATP:AMP Exchange—The ATP:PP, exchange rate at equilibrium exhibits an unusual response to changes in AMP concentration, as shown in Fig. 1. At either low or high concentrations, pyrophosphate exchange is independent of AMP. That no response of ATP:PP, exchange to AMP is observed at these extremes of concentration is consistent with the absence of an AMP requirement for this process (12). However, within a limited range of concentration an effect of AMP on the exchange rate is observed. The shape of the curve within this sensitive region of AMP concentration suggests a titration-like process. The inflection point defining the concentration of mononucleotide required for half maximal change in rate, K1/2, is inversely related to the concentration of PP, in the assay. The K1/2 value for AMP increases from 8 μM at a PP, concentration of 100 μM to a value for K1/2 of 20 μM at 16 μM PP, (Fig. 1). This effect of AMP is seen only in the pyrophosphate exchange reaction, since the AMP dependence on ATP:AMP exchange displays normal hyperbolic kinetics as shown by the linearity of a double reciprocal plot for such data in Fig. 2. At 10 μM PP, the maximal velocity of ATP:AMP exchange yields an enzyme turnover rate of 1.3 s-1 with a K1/2 for AMP of 100 μM.

The apparent partial inhibition by AMP in Fig. 1 cannot be due to competition by AMP for either ATP or PP, binding
since the ATP:PP, exchange rate does not continue to decrease at high concentrations of the mononucleotide. The latter observation also rules out formation of an abortive E-AMP complex as does the linearity of the reciprocal plot in Fig. 2 under similar conditions. However, the AMP dependence of ATP:PP, exchange is consistent with a shift between two parallel ATP:PP, exchange pathways that depends on binding of AMP to the enzyme. Such parallel ATP:PP, exchange pathways likely represent steps 1 and 3 of the proposed mechanism. A shift between these two steps would then depend on the position for the equilibrium represented by step 2 as influenced by AMP.

To test this directly, the extent of enzyme-bound 15N-ubiquitin thiolester formation was determined after resolution from free 15N-ubiquitin by NaDodSO4-PAGE (13). When 1.16 pmol of enzyme is incubated in the presence of 1 mM ATP, 10 mM MgCl2, 100 mM PP, and 1.2 mM 15N-ubiquitin, one finds an equivalent amount of labeled ubiquitin covalently bound to enzyme as thiolester at equilibrium after correction for a control sample (Experiment 1, Table I). When an identical incubation is conducted in the presence of 2 mM AMP, only 0.05 pmol (4.3%) of thiolester is formed. This effect of AMP on the equilibrium concentration of enzyme-bound ubiquitin thiolester is not observed if the nucleotide is added to the incubation after first quenching with urea-NaDodSO4, precluding any nonenzymic artifact. Therefore the equilibrium position of step 2 lies far in favor of thiolester formation at the level of AMP generated by the reaction and added nucleotide shifts the exchange process from step 3 to step 1. That the simultaneous presence of PP, is required for AMP to achieve this effect is shown when a similar experiment is conducted in the presence of inorganic pyrophosphatase to remove PP, generated in the reaction and allow quantitative formation of the ternary complex (13), Experiment 2 of Table I. In the absence of PP, AMP at 2 mM has no effect on the extent of ubiquitin thiolester formation. This observation, that with ubiquitin adenylate present on the enzyme AMP is unable to dislodge the ubiquitin thiolester, suggests that there is only one site for binding either AMP or ubiquitin adenylate.

**Table I**

*The Sequence and Distribution of Enzyme Intermediates*  

| Experiment | Radioactivity | Ubiquitin thiolester |
|------------|---------------|----------------------|
| Control | 30 | 0.79 |
| - AMP |  | 1.18 |
| + AMP (2 mM) | 222 | 0.84 |
| Quench, + AMP (2 mM) | 1670 | 0.81 |

The apparent maximum enzyme turnover rates for PP, and AMP exchange are 4.9 and 1.1 s⁻¹, respectively. The concentration of ATP required to obtain half-maximal velocity for PP, exchange is 36 µM, a value in good agreement with a K₅₀ of 38 µM for AMP exchange. The data in Fig. 3 alone do not reveal the sequence of substrate binding to activating enzyme. However, the data allow one to preclude the existence of any types of abortive E-ATP complexes.

**Ubiquitin Dependence of ATP:PP, and ATP:AMP Exchange**—The dependence of both PP, and AMP exchange on ubiquitin is shown in Fig. 4. For ATP:PP, exchange, the effect of ubiquitin was tested in the absence or presence of 1 mM AMP to shift the observed exchange segment from step 3 to step 1, respectively. Ubiquitin at concentrations above 2 µM (17 µg/ml) causes severe inhibition of pyrophosphate exchange by either step 1 or step 3. Similar inhibition by ubiquitin is observed for ATP:AMP exchange. Within the region of inhibition, increasing ubiquitin concentration causes the initial velocities of both PP, and AMP exchange to ap-
An expanded minimum mechanism including all Michaelis products from the enzyme in which the PPi formed in step PM complexes may be given by Scheme (not shown) allowing estimations for maximal turnover and K1/2 of product release from the enzyme. The exchange of PPi into ATP via step 5, (13), reflected in the equilibrium formation of ubiquitin adenylate. From the thiolester form of enzyme. Such determinations provide a direct measure of enzyme-bound ubiquitin adenylate since this intermediate is not measurably released from the enzyme (13). The dependence of total ubiquitin adenylate concentration, [AMP-Ub], as a function of the concentrations of reactants is given in linear form by Equation 4 (see "Appendix") for a derivation and definition of the complex term $\alpha$.

$$
\frac{[E]_p}{[\text{AMP}-\text{Ub}]} = \frac{1 + K_a}{K_a} + \left( \frac{K_1}{K_3} + \frac{K_2}{K_4} \cdot \frac{1}{[\text{ATP}]} \right) \frac{1}{[\text{Ub}]} \tag{4}
$$

When the ubiquitin dependence for formation of the adenylate intermediate is determined at fixed concentrations of ATP and the data plotted according to Equation 4, one obtains a set of lines having a constant intercept but slopes inversely related to [ATP] as predicted, Fig. 6. If the slopes of these lines are plotted versus the reciprocals of their respective ATP concentrations (Fig. 6, inset) K1 can be evaluated as the ratio of the slope:intercept of the secondary plot. One obtains a value of 0.45 $\mu M$ for K1, the dissociation constant for ATP from the thiolester form of enzyme. From the intercept values for the primary and secondary plots of Fig. 6, one may calculate a value of 0.58 $\mu M$ for the dissociation constant for ubiquitin, K2. In preliminary studies it was found that the data remained linear when plotted according to Equation 4 for ubiquitin concentrations up to 12 $\mu M$ at the ATP concentrations used in Fig. 6. Thus, the substrate inhibition by ubiquitin observed for isotope exchange in Fig. 4 is not reflected in the equilibrium formation of ubiquitin adenylate. This observation precludes schemes involving random binding of ATP and ubiquitin for which the initial binding of ubiquitin, rather than ATP, results in an abortive complex for isotope exchange.

**Equilibrium Constant Determinations for Individual Steps**

Based on previous studies of stoichiometry (13) and on the equilibrium isotope exchange experiments in the present work, an expanded minimum mechanism including all Michaelis complexes may be given by Scheme I for the ubiquitin activating enzyme. Supporting evidence for Scheme I is summarized under "Discussion." As is usually the case in all but the simplest mechanisms, Scheme I is too complex to allow evaluation of individual dissociation or equilibrium constants by kinetic methods alone. However, having identified the principle steps and their sequence, the constants identified in Scheme I could be determined graphically by measurement of ubiquitin adenylate and ubiquitin thiolester present over a range of equilibrium conditions.

**Determination of K1 and K2**—Ubiquitin adenylate concentration as measured by acid precipitation of ubiquitin [3H] adenylate (13) was used to determine the individual dissociation constants for ATP and ubiquitin from the ubiquitin thiolester form of enzyme. Such determinations provide a direct measure of enzyme-bound ubiquitin adenylate since this intermediate is not measurably released from the enzyme (13). The dependence of total ubiquitin adenylate concentration, [AMP-Ub], as a function of the concentrations of reactants is given in linear form by Equation 4 (see "Appendix") for a derivation and definition of the complex term $\alpha$.

$$
\frac{[E]_p}{[\text{AMP}-\text{Ub}]} = \frac{1 + K_a}{K_a} + \left( \frac{K_1}{K_3} + \frac{K_2}{K_4} \cdot \frac{1}{[\text{ATP}]} \right) \frac{1}{[\text{Ub}]} \tag{4}
$$

When the ubiquitin dependence for formation of the adenylate intermediate is determined at fixed concentrations of ATP and the data plotted according to Equation 4, one obtains a set of lines having a constant intercept but slopes inversely related to [ATP] as predicted, Fig. 6. If the slopes of these lines are plotted versus the reciprocals of their respective ATP concentrations (Fig. 6, inset) K1 can be evaluated as the ratio of the slope:intercept of the secondary plot. One obtains a value of 0.45 $\mu M$ for K1, the dissociation constant for ATP from the thiolester form of enzyme. From the intercept values for the primary and secondary plots of Fig. 6, one may calculate a value of 0.58 $\mu M$ for the dissociation constant for ubiquitin, K2. In preliminary studies it was found that the data remained linear when plotted according to Equation 4 for ubiquitin concentrations up to 12 $\mu M$ at the ATP concentrations used in Fig. 6. Thus, the substrate inhibition by ubiquitin observed for isotope exchange in Fig. 4 is not reflected in the equilibrium formation of ubiquitin adenylate. This observation precludes schemes involving random binding of ATP and ubiquitin for which the initial binding of ubiquitin, rather than ATP, results in an abortive complex for isotope exchange.

**Determination of K3 and K4**—At saturating ubiquitin, Equation 4 reduces to a form that can be rearranged to yield Equation 5 from which one may evaluate K3, the equilibrium constant for ubiquitin adenylate formation from enzyme.

$$
\frac{[E]_p}{[\text{AMP}-\text{Ub}]} = \frac{1 + K_a}{K_a} + \left( \frac{K_3}{K_3} \cdot \frac{1}{[\text{ATP}]} \right) \frac{1}{[\text{Ub}]} \tag{5}
$$

When the ubiquitin dependence for formation of the adenylate intermediate is determined at fixed concentrations of ATP and the data plotted according to Equation 4, one obtains a set of lines having a constant intercept but slopes inversely related to [ATP] as predicted, Fig. 6. If the slopes of these lines are plotted versus the reciprocals of their respective ATP concentrations (Fig. 6, inset) K3 can be evaluated as the ratio of the slope:intercept of the secondary plot. One obtains a value of 4.0 $\mu M$ for K3, the dissociation constant for ATP from the thiolester form of enzyme. From the intercept values for the primary and secondary plots of Fig. 6, one may calculate a value of 3.8 $\mu M$ for the dissociation constant for ATP, K4.

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*Scheme I neglects the required role of Mg2+ since the present studies did not bear directly on this question. However, by analogy to similar mechanisms it is reasonable to assume the actual substrates involved are ATP-$\text{Mg}^{2+}$ and PP-$\text{Mg}^{2+}$.
The Sequence and Distribution of Enzyme Intermediates

**Scheme 1**

**FIG. 6**. Determination of $K_{1}$ and $K_{3}'$ by ubiquitin adenylate formation. Data are plotted according to Equation 4 for ubiquitin [$\text{H}^{3}$]adenylate formation asayed as trichloroacetic acid precipitable radioactivity. Incubations contained 1.0 pmol of activating enzyme, 1.0 $\mu$m [PPi], the specified concentration of ubiquitin, and either 0.19 $\mu$m (O), 0.38 $\mu$m (X), or 0.76 $\mu$m (A) [3H]ATP (14400 cpm/pmol). Inset: replot of slope versus reciprocal of ATP concentration.

**FIG. 7** (left). Determination of $K_{2}'$ and $K_{4}'$ by ubiquitin adenylate formation. Data plotted according to Equation 5 for ubiquitin [$\text{H}^{3}$]adenylate formation assayed as trichloroacetic acid precipitable radioactivity. Incubations contained 1.0 pmol of activating enzyme, 1.0 $\mu$m PPi, the specified concentration of ubiquitin, and either 0.19 $\mu$m (O), 0.38 $\mu$m (X), or 0.76 $\mu$m (A) [3H]ATP (14400 cpm/pmol). As shown in Fig. 7, under the condition of [ubiquitin] $>$ $K_{2}'$, the dependence of PPi on the concentration of enzyme-bound ubiquitin adenylate shows the predicted linear form when plotted according to Equation 5. From the intercept of Fig. 7, $K_{3}'$ is directly determined at 0.16 while the ratio of slope:intercept yields a value of 3.2 $\mu$m for $K_{4}'$.

**FIG. 8** (center). Determination of $K_{5}'$. Data are plotted according to Equation 6 for the effect of AMP on the equilibrium formation of enzyme-bound [3H]-ubiquitin thiolester. Thiolester was quantitated by NaDodSO4-PAGE as in Table I. Incubations contained 1 mM ATP, 100 $\mu$m PPi, 1.2 $\mu$m [3H]-ubiquitin (4390 cpm/pmol), 0.73 pmol of enzyme, and the indicated concentration of AMP. Inset: direct plot of equilibrium thiolester formation versus concentration of AMP.

**FIG. 9** (right). Determination of $K_{6}$. Data are plotted according to Equation 7 for the effect of PPi on the equilibrium formation of enzyme-bound [3H]-ubiquitin thiolester. Incubations were as in the legend to Fig. 8 but with 100 $\mu$m AMP and the indicated concentrations of PPi. Inset: direct plot of equilibrium thiolester formation versus concentration of PPi.
The ratio of slope:intercept yields a value for $\beta E_{K_a}$ of 200 $\mu M$. As defined in the “Appendix,” $\beta E$ contains only known concentrations and experimentally determined equilibrium constants. Substituting these values into the expression for $\beta E_{K_a}$ can be calculated as 0.027 $\mu M$. In contrast, the apparent $K_{1/2}$ value for AMP with respect to ubiquitin thiolester formation is $-10$ $\mu M$ (Fig. 8, inset). This latter value is in good agreement with the $K_{1/2}$ of $8$ $\mu M$ determined from the reaction in ATP:PP exchange between steps 1 and 3 at the same concentration of PP (Fig. 1).

**Determination of $K_a$—** The equilibrium constant for ubiquitin transfer between adenylate and thiolester forms of enzyme, $K_a$, was evaluated in a similar manner by measuring the effect of PP on the concentration of thiolester at a constant level of AMP. The dependence of thiolester formation on PP, is described by Equation 7 (see “Appendix”) from which $K_a$ can be determined from

$$
\frac{[E_b]}{[E_{ub}]} = 1 + \frac{[AMP]}{K_a + [AMP]} + \frac{[AMP]}{K_{-a}K_a + [AMP]} \left( \frac{[PP]}{[PP]} \right)
$$

the ordinate intercept. At $[PP] > K_{-a}$, Equation 7 predicts the value for the complex term $\beta E$ to be independent of pyrophosphate and approach the value given by Fig. 8 determined at 100 $\mu M$ PP. That a plot of appropriate data according to Equation 7 is linear (Fig. 9) rather than concave up demonstrates the validity of the latter prediction. A value of $K_a$ equal to 2.0 was calculated from the ordinate intercept of Fig. 9 using a value for $\beta E_{K_a}$ of 200 $\mu M$ obtained from Fig. 8.

**Discussion**

A minimal model for the ubiquitin activating enzyme, given in steps 1–3, was proposed previously on the basis of stoichiometry studies that indicated the utilization of two equivalents of ATP per subunit of enzyme resulted in the formation of one equivalent each of enzyme-bound ubiquitin adenylate and ubiquitin thiolester (13). The model accounts for the observed properties of two ubiquitin-dependent exchange reactions: an ATP:PP exchange that occurred in the absence of added AMP and an ATP:AMP exchange that required the presence of PP (12). This work was initiated to provide additional evidence for the minimal model, establish in detail the sequence of interactions, and determine the thermodynamic and kinetic behavior of the intermediates.

From the kinetic data presented in this work the minimum mechanism for activating enzyme can be expanded to include all Michaelis complexes as shown in Scheme I. The strict order of substrate binding is indicated by the normal hyperbolic kinetics with varying ATP (Fig. 3), but inhibition by ubiquitin of exchanges that involve ATP (Fig. 4). Similarly, the ordered release of PP, and AMP follow from the normal hyperbolic kinetics for varying PP, in ATP:PP exchange but PP inhibition of ATP:AMP exchange (Fig. 5). The linearity of double reciprocal plots for isotope exchange studies in Figs. 2 and 5 provide no evidence for abortive complexes involving either AMP or ATP, respectively. While the substrate inhibition observed for the ubiquitin dependence on isotope exchange in Fig. 4 does not alone rule out formation of abortive enzyme-ubiquitin complexes, this possibility does not seem likely since ubiquitin at up to 12 $\mu M$ shows no similar inhibition of ubiquitin adenylate formation as noted earlier. By analogous reasoning the linearity of data for the effect of PP on the equilibrium concentrations of ubiquitin adenylate (Fig. 7) and ubiquitin thiolester (Fig. 9) preclude formation of any abortive complexes involving PP, that would not have been resolved by the concentration dependence of this reaction on isotope exchange between ATP and AMP (Fig. 5).

Scheme 1 is further substantiated by the observation that AMP affects the rate of ATP:PP exchange within a critical concentration range but is without effect above or below this region (Fig. 1). The effect of AMP on the rate of ATP:PP exchange is best explained by a shift of this exchange process from one occurring on enzyme forms involving PP to forms involving $E_{ub}$ by perturbation of the equilibrium position for enzyme-bound ubiquitin thiolester. This interpretation is demonstrated by three observations: 1) the transition between the two limiting AMP-independent rates of ATP:PP exchange is accompanied by a loss of enzyme-bound ubiquitin thiolester at high nucleotide concentration (Experiment 1, Table 1); 2) the effect of AMP concentration on the rate of ATP:PP exchange and on the extent of ubiquitin thiolester formation show good correspondence as judged by the nearly identical values for $K_{1/2}$ of 8 $\mu M$ (Fig. 1) and 10 $\mu M$ (Fig. 8, inset), respectively, for the two processes under the same conditions; and 3) the concentration of AMP giving half-maximal change in ATP:PP exchange and ubiquitin thiolester concentration in inhibition, is itself inversely related to the concentration of PP (Fig. 1). The point is a consequence of the individual sites for ubiquitin thiolester and ubiquitin adenylate shown from stoichiometry studies of the ternary complex, in addition to the extremely tight binding of the adenylate intermediate (13). While PP, would not be expected to participate directly in determining the equilibrium concentration of ubiquitin thiolester, it is required for the effect of AMP on this step to be observed (Experiment 2, Table 1) and does itself affect the equilibrium position for ubiquitin adenylate formation (Fig. 7). The influence of PP, on thiolester concentration reflects the mutually exclusive binding of AMP, ubiquitin adenylate, and ATP to a single site on $E_{ub}$. Thus, one would predict PP, to act in concert with AMP in defining the concentration of total ubiquitin thiolester.

Previously, ubiquitin thiolester was demonstrated as the immediate donor for protein-conjugate formation (13). That the concentration of AMP directly and of PP, indirectly, determine the equilibrium position for formation of this activated ubiquitin intermediate, suggests product inhibition as a possible regulatory mechanism for the rate of conjugate formation and ultimately protein degradation. The previous report by Hershko et al. (17) that AMP was a potent inhibitor of energy-dependent proteolysis in reticulocyte extracts is consistent with this hypothesis. With a dissociation constant of 0.027 $\mu M$, the AMP concentration of 10 to 100 $\mu M$ in cells (18, 19) seems sufficient totally to suppress ubiquitin thiolester formation and ultimately energy-dependent proteolysis. Concentrations of AMP much greater than that required for apparent saturation are necessary for inhibition of in vitro proteolysis (17). Assuming the rate-limiting step for this degradative pathway occurs subsequent to ubiquitin activation, the latter observation suggests PP, regulates the activating
enzyme by determining the $K_{1/2}$ at which AMP influences the distribution between forms involving $E_{5:Ub}$ and $E_{5:SN}$. Therefore, the low intracellular concentrations of PP, one might anticipate allow the enzyme to function in the presence of an intracellular AMP concentration that would otherwise cause complete inhibition. Since AMP and PP, are products of many key anabolic reactions, production inhibition by these metabolites at the level of ubiquitin activating enzyme may coordinate cell growth with proteolysis as has been noted in the decreased rate of protein degradation during liver regeneration (20, 21).

Factors that contribute to the high affinity of the enzyme for AMP may also be responsible for the observed tight binding of ubiquitin adenylate to the enzyme (13). There are indications that the enzyme is capable to exploit this inordinately tight association of ubiquitin adenylate to normalize the equilibrium involved in its formation and utilization from the standard free energies at 25 °C, pH 7.0, for hydrolysis of ATP to yield AMP + PP, ($-7.7$ kcal/mol; Ref. 22) and of acetyl adenylate to give AMP + acetate ($-13.3$ kcal/mol; Ref. 23) one might expect a value $8 \times 10^{-5}$ for $K'_3$, the equilibrium constant for ubiquitin adenylate formation. In contrast, the measured value for $K'_3$ is 0.16, representing a stabilization of the enzyme-bound intermediate by $-4.5$ kcal/mol. In a similar fashion, one may predict a value for the equilibrium constant of ubiquitin transfer to form thiolester, $K_e$, of $2 \times 10^3$ based on the standard free energies for hydrolysis of acetyl adenylate (given above) and acetyl CoA ($-7.5$ kcal/mol; Ref. 22). The actual determined value of $K_e$ is 2.0 (Table II), or a relative destabilization of this intermediate by $-5.4$ kcal/mol. The opposing directions and similar magnitudes of these thermodynamic differences suggest that the enzyme couples the highly favorable binding energy for ubiquitin adenylate to the unfavorable equilibrium constant for the formation of this intermediate. The thermodynamic advantage gained by increasing $K'_3$ would be achieved at the expense of diminishing the stability of the enzyme-bound ubiquitin thiolester. However, such an affect may itself serve a selective advantage by enabling regulation of the enzyme via product inhibition. One might test whether the binding energy of ubiquitin adenylate is coupled to the normalization of these equilibria since $K'_3$ and $K_e$ should be related to the observed dissociation constants for mononucleotides structurally related to AMP. One would predict the value of $K'_3$ to decrease and $K_e$ to increase with diminishing affinity for a series of such alternate substrates.

Ubiquitin adenylate formation represents but one example of a much broader class of enzymic reactions involving carboxylic activation by adenylation. The most pertinent analogy in the present context are the aminocetyl-tRNA synthetases which catalyze nucleotidytransfer between enzyme-bound ATP + amino acid to form aminocetyl adenylate + PP. How closely does the equilibrium constant for aminocetyl adenylate formation correspond to the chemically analogous step for ubiquitin adenylate formation defined by the constant $K'_3$ (or $K'_e$)? Such a constant has been measured only twice previously. For Escherichia coli methionyl-tRNA synthetase the equilibrium constant for nucleotidytransfer in the direction of methionyl adenylate formation has been determined kinetically as 1.5 at 25 °C, pH 7.6 (24), and has recently been confirmed by direct measurement using $^{32}P$ NMR (25). Kinetic studies of E. coli isoleucyl-tRNA synthetase yields a value 0.2 for the analogous nucleotidytransfer at 25 °C, pH 8.0 (26). The value for $K'_3$ of 0.16 shows surprisingly good agreement with these chemically similar but functionally diverse reactions. The correspondence in these values presents the intriguing alternatives of either convergent evolution toward a step of maximum catalytic efficiency or the divergent evolution of the ubiquitin activating enzyme from an ancestral aminocetyl-tRNA synthetase. In either case, it is likely that aminocetyl adenylate binding on the synthetases is also coupled to the stabilization of this intermediate. Experimentally the ubiquitin activating enzyme provides a better system for testing the validity of this hypothesis since the equilibria are more conveniently measured and ubiquitin transfer to form thiolester provides a good thermodynamic model for enzyme-bound aminocetyl adenylate transfer to tRNA.

The method of measuring ubiquitin [H]adenylate did not lend itself to direct determination of the individual constants comprising the upper reaction path in Scheme I. The high concentration of AMP required to shift the equilibrium position of enzyme-bound intermediates to the upper path would have led to experimental problems associated with isotope exchange and dilution. One might ask if occupancy of the thiolester site with ubiquitin influences the distribution of intermediates at the adenylate site. Three lines of evidence suggest that there is a minimal interaction of this type between the two ubiquitin sites: 1) the limiting rates of ATP-PP exchange do not differ greatly at low or high concentrations of AMP (Fig. 1); 2) the $K_{1/2}$ values of ATP for ATP-PP, and ATP-AMP exchange are comparable (Fig. 3); and 3) the estimated $K_{1/2}$ values of ubiquitin for ATP-AMP exchange and for ATP-PP, exchange in the absence and presence of $1 \text{mM AMP}$ show good agreement with values of 0.7, 1.2 and 0.6 $\mu M$, respectively. Therefore, it appears that the equilibrium and dissociation constants summarized in Table II approxi-

| Table II |
| --- |
| **Summary of constants** |
| Data at 37 °C, pH 7.6, for excess MgCl$_2$. |

| Step | $K$ | $k_1$ | $k_2$ |
| --- | --- | --- | --- |
| $E_{5:Ub}$ | $E_{5:Ub}$ | 0.45 $\mu M$ | 3.9 x $10^{-4}$ M$^{-1}$ s$^{-1}$ | 18 s$^{-1}$ |
| $E_{5:Ub}$ + ATP | $E_{5:Ub}$ + ATP | 0.58 $\mu M$ | 3.2 $\mu M$ | 1.8 x $10^{-3}$ s$^{-1}$ | 58 s$^{-1}$ |
| $E_{5:Ub}$ + Ub | $E_{5:Ub}$ + Ub | 0.16 | 2.0 |
| $E_{SO:Ub}$ + PP | $E_{SO:Ub}$ + PP | 2.0 |
| $E_{5:Ub}$ + AMP | $E_{5:Ub}$ + AMP | 0.027 $\mu M$ | 7.9 x $10^{-3}$ s$^{-1}$ | 21 s$^{-1}$ |
mate the analogous values for ubiquitin-adenylate formation on enzyme not having ubiquitin bound covalently at the thiolester site.

Knowledge of the equilibrium constants and isotope exchange rates allows one to calculate the rate constants for binding and dissociation of ATP, PP, and AMP. For example, one may calculate from the values given in Table II that under the conditions of Fig. 1 at low AMP and 100 μM PP, the concentration of $E_{S-\text{Ub}}'$ is 1.58 mM or 29% of total enzyme. The limiting rate of ATP:PP exchange, $v_0$, is 83 pmol/min under these conditions. Since $v_0 = k_{-1}[E_{S-\text{Ub}}']$, one may calculate a value of 18 s$^{-1}$ for $k_{-1}$, the unimolecular rate constant for dissociation of ATP from the ubiquitin thiolester form of enzyme. Given $K_{-1} = k_{-1}/k_1$, the bimolecular rate constant for binding of ATP to this enzyme intermediate, $k_1$, equals $3.9 \times 10^7$ M$^{-1}$ s$^{-1}$. A similar treatment yields values for the binding and dissociation of PP, to the thiolester form of enzyme of $1.8 \times 10^7$ M$^{-1}$ s$^{-1}$ and 58 s$^{-1}$, respectively. From the maximal turnover in ATP:AMP exchange (Fig. 2) one may determine analogous constants for AMP.

As summarized in Table II, the bimolecular association constants for ATP and PP show good agreement. This observation may indicate that a common conformational change in the enzyme limits the rate of ATP:PP binding. Such a conformation change would be consistent with the strictly ordered mechanism of Scheme I for which no evidence for abortive complexes was observed. The tighter binding of AMP relative to ATP results from a greater associative rate constant for AMP of $7.9 \times 10^7$ M$^{-1}$ s$^{-1}$ that approaches the diffusion limit. Although this rate constant for binding of AMP to $E_{S-\text{Ub}}$ is 20-fold greater than that for ATP, the respective dissociation rate constants for the two nucleotides to yield this enzyme form are nearly identical. The comparable values for $k_{-1}$ and $k_{-1}'$ may reflect a common rate-limiting conformational relaxation of the protein involved in dissociation of either nucleotide to yield $E_{S-\text{Ub}}$. Further, this suggests that the difference in nucleotide binding rates is due to the existence of two states of $E_{S-\text{Ub}}$ in an equilibrium ratio 20:1 favoring the species with which AMP binds relative to that with which ATP binds. These proposed conformational changes of the enzyme seem plausible on steric grounds in order to account for transfer of ubiquitin between two separate sites. The observation that AMP binds to $E_{S-\text{Ub}}$ but not $E_{S-Hb}$ with which ATP reacts is consistent with a conformation for free enzyme similar to the minor $E_{S-\text{Ub}}$ conformation.

Finally, it is important to note the remarkably high affinity of the enzyme displays for ATP and ubiquitin. An ATP dissociation constant, $K_{-1}$, of 0.45 μM is significantly lower than the usual intracellular concentration for ATP of 1–2 mm (27). However, this value for $K_{-1}$ is consistent with previous reports that ATP must fall to levels considerably less than 10% of its physiological concentration before a large effect on energy-dependent protein degradation is noted (17, 28–31). Similarly, the low dissociation constant for ubiquitin, $K_{-2}$, of 0.58 μM (5 μg/ml) indicates that the enzyme is probably saturated with regard to this substrate even at high steady state levels of conjugates since the intracellular concentration of total ubiquitin has been estimated as ≥12 μM in rabbit reticulocytes and human erythrocytes (14).

The present studies have substantiated an earlier minimal mechanism for the ubiquitin activating enzyme (13) and have demonstrated an expanded scheme to be a strictly ordered process. A role for regulation of protein degradation at the level of ubiquitin activation has been proposed involving product inhibition by AMP and PP. Future studies with purified components of this degradative pathway should allow one to test this hypothesis. Also, further thermodynamic binding studies should be valuable in assessing the importance of coupling ubiquitin adenylate binding energy to catalysis by this enzyme.

**APPENDIX**

For Scheme I rewritten as

$$
\begin{align*}
E_1 \xrightarrow{K_{-1}} & E_2 \xrightarrow{K_{-2}} E_3 \xrightarrow{K_3} E_4 \xrightarrow{K_4} E_5 \\
\text{ATP} & \xrightarrow{K_4} E_6 \\
\text{AMP} & \xrightarrow{K_6} E_7 \\
\text{PP} & \xrightarrow{K_7} E_8 \xrightarrow{K_8} E_9 \xrightarrow{K_7} E_{10} \xrightarrow{K_7} E_{11}
\end{align*}
$$

The following equilibrium and dissociation constants can be defined:

$$
\begin{align*}
K_1 &= \frac{[E_1][\text{ATP}]}{[E_3]} \\
K_2 &= \frac{[E_2][\text{Ub}]}{[E_3]} \\
K_3 &= \frac{[E_4]}{[E_5]} \\
K_4 &= \frac{[E_6][\text{PP}]}{[E_5]}
\end{align*}
$$

**I. Derivation of Expression for Total Ubiquitin Adenylate**—For $[\text{AMP}] = [E_0]$, where $[E_0]$ is the total concentration of enzyme, thiolester formation is essentially irreversible. Then the conservation equation for enzyme is given by

$$
[E_0] = [E_1] + [E_4] + [E_5] + [E_6] + [E_7]
$$

For total ubiquitin adenylate

$$
[\text{AMP-Ub}] = [E_{10}] + [E_{11}] = \frac{[E_0][\text{ATP}][\text{Ub}]K_{\alpha}(1 + K'_{-1}/[\text{PP}])}{K_{1}K_{2}}
$$

Expressing $[E_0]$ in terms of $[E_1]$ by the conservation equation and the relevant equilibria, then taking the reciprocal to linearize the equation, one obtains

$$
\frac{[E_0]}{[\text{AMP-Ub}]} = \frac{1 + K_{20}}{K_{1\alpha} + \frac{K'_{2}}{K_{2\alpha} + \frac{K'_{2}}{K_{2\alpha} + \frac{1}{[\text{ATP}]}[\text{Ub}]}]} \frac{1}{[\text{Ub}]}
$$

where $\alpha = 1 + K'_{-1}/[\text{PP}].$

**II. Derivation of Expression for Total Ubiquitin Thiolester**—For Scheme I, let $[E_0] = [E_{S-Hb}] + [E_{S-\text{Ub}}]$ where $[E_{S-Hb}]_r$ and $[E_{S-\text{Ub}}]_r$ are all forms of enzyme not containing an ubiquitin thiolester; $[E_{S-\text{Ub}}]_r$ is all forms of enzyme containing an ubiquitin thiolester; then

$$
[E_{S-\text{Ub}}]_r = [E_3] + [E_4] + [E_5] + [E_6] + [E_7]
$$

and $[E_{S-Hb}]_r$ can be expressed in terms of $[E_7]$ by substitution into its conservation equation. Similarly,

$$
[E_{S-\text{Ub}}]_r = [E_3] + [E_4] + [E_5] + [E_6] + [E_7]
$$

and $[E_{S-Hb}]_r$ can be expressed in terms of $[E_7]$ by substitution into its respective conservation equation. The resulting expressions for $[E_4]$ and $[E_5]$ can then be combined in the identity equation for $K_5$, $K_5 = [E_4]/[E_5]$, to yield an expression in $[E_{S-Hb}]_r$ and $[E_{S-\text{Ub}}]_r$. Recognizing that $[E_0] = [E_{S-Hb}]_r + [E_{S-\text{Ub}}]_r$ and rearranging, one obtains

$$
\frac{[E_0]}{[E_{S-\text{Ub}}]} = \frac{K_{-1} + \beta_1[\text{PP}]}{K_{1\alpha}K_{5}} \cdot [\text{AMP}] + \frac{\beta_2K_{-4} + [\text{AMP}]}{[E_{S-\text{Ub}}]}
$$

The Sequence and Distribution of Enzyme Intermediates
where
\[ \beta_1 = 1 + \frac{K_{-1}}{K_1} \cdot \frac{[ATP]}{[ATP][Ubang]} + \frac{K_4}{K_{-4}} \cdot \frac{K_{UB}[ATP]}{K_{UB}[ATP]} \]
\[ \beta_2 = 1 + \frac{ATP}{K_1} + \frac{[ATP][Ub]}{K_1} \cdot (1 + K_{-1} + K_{-1}^2) \cdot \frac{[PP]}{[PP]} \]

The above expression can be linearized by taking the reciprocal to yield
\[ \left( \frac{[E]_p}{[E_{i0}]_p} - 1 \right)^{-1} = \frac{K_{-1}K_1}{K_{-1} + \beta_2[PP]} + \frac{\beta_2[PP]}{K_{-1} + \beta_4[PP]} \cdot [AMP] \]

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