Mechanistic Study of Uba5 Enzyme and the Ufm1 Conjugation Pathway

Background: Human ubiquitin-like protein Ufm1 and its activating enzyme, Uba5, are involved in endoplasmic reticulum (ER) stress response. E1 enzymes activate ubiquitin or ubiquitin-like proteins (Ubl) via an adenylate intermediate and initiate the enzymatic cascade of Ubl conjugation to target proteins or lipids. Ubiquitin-fold modifier 1 (Ufm1) is activated by the E1 enzyme Uba5, and this pathway is proposed to play an important role in the endoplasmic reticulum (ER) stress response. However, the mechanisms of Ufm1 activation by Uba5 and subsequent transfer to the conjugating enzyme (E2), Ufc1, have not been studied in detail. In this work, we found that Uba5 activates Ufm1 via a two-step mechanism and formed a binary covalent complex of Uba5–Ufm1 thioester. This feature contrasts with the three-step mechanism and ternary complex formation in ubiquitin-activating enzyme Uba1. Uba5 displayed random ordered binding with Ufm1 and ATP, and its ATP-adenylate (PP₃) exchange activity was inhibited by both AMP and PP₃. Ufm1 activation and Uba5–Ufm1 thioester formation were stimulated in the presence of Ufc1. Furthermore, binding of ATP to Uba5–Ufm1 thioester was required for efficient transfer of Ufm1 from Uba5 to Ufc1 via transthiolation. Consistent with the two-step activation mechanism, the mechanism-based pan-E1 inhibitor, adenosine 5′-sulfamate (ADS), reacted with the Uba5–Ufm1 thioester and formed a covalent, tight-binding Ufm1-ADS adduct in the active site of Uba5, which prevented further substrate binding or catalysis. ADS was also shown to inhibit the Uba5 conjugation pathway in the HCT116 cells through formation of the Ufm1-ADS adduct. This suggests that further development of more selective Uba5 inhibitors could be useful in interrogating the roles of the Uba5 pathway in cells.

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Received for publication, April 14, 2014, and in revised form, June 20, 2014 Published, JBC Papers in Press, June 25, 2014, DOI 10.1074/jbc.M114.573972

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Background: Human ubiquitin-like protein Ufm1 and its activating enzyme, Uba5, are involved in endoplasmic reticulum (ER) stress response.

Results: The Uba5–Ufm1 conjugation pathway is characterized in both reconstituted systems and cellular settings.

Conclusion: Uba5 activates Ufm1 via a distinct two-step mechanism.

Significance: This study represents the first mechanistic characterization of Uba5, a homodimeric member of the E1 enzyme family.

E1 enzymes activate ubiquitin or ubiquitin-like proteins (Ubl) via an adenylate intermediate and initiate the enzymatic cascade of Ubl conjugation to target proteins or lipids. Ubiquitin-fold modifier 1 (Ufm1) is activated by the E1 enzyme Uba5, and this pathway is proposed to play an important role in the endoplasmic reticulum (ER) stress response. However, the mechanisms of Ufm1 activation by Uba5 and subsequent transfer to the conjugating enzyme (E2), Ufc1, have not been studied in detail. In this work, we found that Uba5 activates Ufm1 via a two-step mechanism and formed a binary covalent complex of Uba5–Ufm1 thioester. This feature contrasts with the three-step mechanism and ternary complex formation in ubiquitin-activating enzyme Uba1. Uba5 displayed random ordered binding with Ufm1 and ATP, and its ATP-adenylate (PP₃) exchange activity was inhibited by both AMP and PP₃. Ufm1 activation and Uba5–Ufm1 thioester formation were stimulated in the presence of Ufc1. Furthermore, binding of ATP to Uba5–Ufm1 thioester was required for efficient transfer of Ufm1 from Uba5 to Ufc1 via transthiolation. Consistent with the two-step activation mechanism, the mechanism-based pan-E1 inhibitor, adenosine 5′-sulfamate (ADS), reacted with the Uba5–Ufm1 thioester and formed a covalent, tight-binding Ufm1-ADS adduct in the active site of Uba5, which prevented further substrate binding or catalysis. ADS was also shown to inhibit the Uba5 conjugation pathway in the HCT116 cells through formation of the Ufm1-ADS adduct. This suggests that further development of more selective Uba5 inhibitors could be useful in interrogating the roles of the Uba5 pathway in cells.

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THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 289, NO. 33, pp. 22648 –22658, August 15, 2014

22648 JOURNAL OF BIOLOGICAL CHEMISTRY

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Several studies have linked Ufm1 to the cellular endoplasmic reticulum (ER) stress response pathways. Tatsumi et al. (17) first showed that an ER membrane protein, UFBP1 (also known as c2orf16 or DRRGK1) (19), and Ufl1 were co-localized on the ER and that UFBP1 was possibly a target protein for ufmylation. Although no endogenous UFBP1 ufmylation was detected, overexpression of both Ufm1 and UFBP1 in HEK293 cells led to apparent ufmylation at Lys-267 of UFBP1 (17). Interestingly, in a separate study, Ha et al. (14) demonstrated that one of the Ufm1-specific proteases, UfSP2, contained an N-terminal domain that interacted specifically with UFBP1 and was recruited to the ER in HeLa cells. Lemaire et al. (20) further showed that ER stresses induced expression of Ufm1, UFBP1, and Ufl1 in the pancreatic beta cell INS-1E. Genetic suppression of these proteins by siRNA sensitized INS-1E cells to ER stress and induced apoptosis (20). The connection between Ufm1 and the ER stress response was further demonstrated by a recent study that suggested that Ufm1 was a target for Xbp-1, a transcription factor essential in mediating ER stress response (21). mRNA levels of both Ufm1 and other pathway components were shown to be up-regulated in multiple cancer cell lines when they were treated with ER stress inducers such as thapsigargin, tunicamycin, or brefeldin A (21). Suppression of the Ufm1 pathway components by siRNA led to morphological changes of the ER network in U2OS cells (21). The Uba5-Ufm1 conjugation pathway was also shown to be implicated in the ER stress response in Caenorhabditis elegans (22). All these studies suggest that protein ufmylation is an important pathway that cells have evolved to maintain proper function of the ER and to mediate ER stress responses. In addition, Tatsumi et al. (23) showed that murine fetuses lacking Uba5 were not viable and developed severe anemia, suggesting that the Uba5-Ufm1 pathway might play an essential role in hematopoiesis during development.

Although the Ufm1 conjugation pathway has been implicated in important cellular functions, the mechanism of Ufm1 activation by Uba5 and its transfer has not been studied in detail. Here, we showed that, in contrast to “canonical” Ubl conjugation pathways, Ufm1 was activated by a two-step mechanism to form a binary, covalent complex of Uba5~Ufm1 thioester. In addition, we found that adenosine 5'-sulfamate (ADS) was a mechanism-based inhibitor of Uba5. ADS was shown to react with the Uba5~Ufm1 thioester to form a tight binding Ufm1-ADS adduct that occupied the adenylation site of Uba5 to prevent further substrate binding.

**EXPERIMENTAL PROCEDURES**

**Materials—**$[^{32}P]P_{i}$ (catalog no. NEX019) and $[^{32}P]ATP$ (catalog no. BLU003H) were purchased from PerkinElmer Life Sciences. Other chemicals were purchased from Sigma. Rabbit polyclonal anti-Uba5 (catalog no. 12093-1-AP) and anti-Ufc1 (catalog no. 15783-1-AP) antibodies were obtained from ProteinTech Group (Chicago). Rabbit monoclonal anti-Ufm1 (catalog no. 3463-1) antibody was obtained from Epitomics (Burlingame, CA). Rabbit monoclonal anti-adenosine sulfamate antibody was generated as described previously (24, 25). Untagged and N-terminal FLAG-tagged Ufm1 proteins were generated by gene synthesis and subcloning in a pDEST14 vector and were expressed in *Escherichia coli*. Other Ubl proteins were expressed in *E. coli* and purified as described previously (26). The concentrations of Ubl solutions were determined based on their calculated extinction coefficients at 280 nm. N-terminally His$_6$-tagged human Uba5 (and other E1s) and N-terminally glutathione S-transferase (GST)-tagged Uba5 fusion protein were expressed in S9 insect cells and purified as described (25). The purity of wild-type and C250A Uba5 was determined to be $\sim 90$ and $\sim 93\%$, respectively, based on SDS-PAGE/Coomassie staining and densitometry analysis. Ufc1 was expressed as an N-terminally His-tagged protein. Expressed proteins were purified by affinity or conventional chromatography using standard buffers. Concentrations of purified E1 and E2 proteins were determined using Coomassie Plus$^{TM}$ protein assay (Thermo Scientific, Waltham, MA, catalog no. 1856210) with bovine serum albumin (BSA) as the standard. Concentrations of purified Ufm1 were determined by its UV absorption at 280 nm ($\epsilon_{280nm} = 1.49 \text{ mm}^{-1} \text{cm}^{-1}$).

**ATP-$P_{i}$ Exchange Assay—**The ATP-$P_{i}$ exchange assays were carried out using a modified protocol developed by Bruzzese et al. (26). For titrations and $K_{1/2}$ determination, substrates were serially diluted 1 in 2 or 1 in 3 on a 96-well plate. The reaction mixtures containing 50 nM wild-type Uba5 or 100 nM C250A Uba5 were incubated at 37 °C in 1 × E1 Buffer (50 mM HEPES, 25 mM NaCl, 5 mM MgCl$_2$, 0.05% BSA, 0.01% Tween 20, and 1 mM tricarboxyethyl phosphate, pH 7.5) for 30 min. For Ufm1 titrations, 0.5 mM ATP and 40 µM PP$_i$ (50 cpmpmol $[^{32}P]PP_{i}$) were used. For ATP and PP$_i$ titrations, Ufm1 concentration was fixed at 25 µM for wild-type Uba5 and 0.2 µM for C250A Uba5 reactions. For AMP titrations, 50 µM ATP and either 5 or 250 µM PP$_i$ (50 cpmpmol $[^{32}P]PP_{i}$) were used. As described previously, in the ATP-PP$_i$ exchange assay $K_{1/2}$ was used instead of $K_{cat}$ (5). $K_{1/2}$ values were estimated by fitting the data using the Michaelis-Menten equation.

For $K_{cat}$ determinations, 25, 50, or 100 nM Uba5 was incubated at 30 °C for various time points in 1 × E1 Buffer with 50 µM Ufm1, 0.5 mM ATP, and 50 µM PP$_i$ (50 cpmpmol $[^{32}P]PP_{i}$). $K_{cat}$ values were calculated using rates of the reactions and an $[^{32}P]ATP$ standard curve.

For Uba5 inhibition studies, ADS was serially diluted into a 96-well plate in a mixture containing 40 nM Uba5, 100, or 1000 µM ATP, 50 µM PP$_i$ (50 cpmpmol $[^{32}P]PP_{i}$), and 25 µM Ufm1 in 1 × E1 Buffer. Reaction mixtures were incubated at 37 °C for 60 min.

To study time-dependent inhibition of Uba5 by ADS and to measure the rate of inactivation, ATP-PP$_i$ reactions were monitored over time at room temperature with varying concentrations of ADS in 1 × E1 Buffer containing 50 µM ATP.

**Homogeneous Time-resolved Fluorescence (HTRF)-based Uba5-Ufc1 Transthiolation Assay—**The HTRF assay was performed using a similar protocol previously developed for other E1 enzymes (24, 25, 27, 28). Biotinylated Ufc1 and FLAG-tagged Ufm1 were used for detection. The HTRF assay was used for reversibility studies. The inactive complex of Uba5-Ufm1-ADS was pre-formed by incubating 300 nM Uba5 with 1.5 µM FLAG-Ufm1, 50 µM ATP, 250 µM ADS in 1 × HEPES Buffer (5 mM MgCl$_2$, 0.05% BSA, 50 mM HEPES, pH 7.5) for 60 min at room temperature. Excess ADS was removed by passing sam-
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examples through a ZEBA desalting spin column (Thermo Scientific, catalog no. 89883) pre-equilibrated with 1× HEPES buffer. The flow-through of the spin column containing the Uba5-Ufm1-ADS complex was then diluted 1 in 600 into 1× HEPES buffer. The final reaction mixture contained 0.5 nM Uba5, 75 nM FLAG-Ufm1, 35 nM biotinylated-Ufc1, and 2 mM ATP in 1× HEPES buffer. Reaction mixtures were incubated at room temperature, and at various time points (between 0 and 240 min), HTRF signals were measured on a PHERAstar plus equipped with an HTRF optical module (BMG Labtech, Offenburg, Germany).

Thin Layer Chromatography (TLC) AMP Assay—The reaction mixtures contained 1 μM wild-type or C250A Uba5, 20 μM Ufm1, 50 μM ATP ([α-32P]ATP (1 μCi/nmol)), 200 μM ADS (where indicated), 5 mM MgCl2, in 1× HEPES buffer. Reactions were incubated at room temperature for 5 min before being quenched with 25 mM EDTA. To measure the rate of adenylate formation, similar conditions were used as above except that reactions were quenched at varying time points. The reaction mixtures were analyzed, and the signals on the TLC plates were quantified as described previously (27, 28).

Western Blot-based Thioester Assays—Rates of Uba5–Ufm1 formation were measured in reaction mixtures containing 1 μM Uba5, 40 μM untagged Ufm1, 400 μM ATP in 1× HEPES buffer. Reaction mixtures were incubated at room temperature and quenched with 4× lithium dodecyl sulfate sample loading buffer (Invitrogen, catalog no. NP0007) at various time points. The samples were then analyzed by SDS-PAGE under nonreducing conditions without boiling, transferred to a 0.2-μm Invitrolon™ PVDF membrane (Invitrogen, catalog no. H11003) at various time points. The samples were then analyzed by SDS-PAGE under nonreducing conditions without boiling, transferred to a 0.2-μm Invitrolon™ PVDF membrane (Invitrogen, catalog no. H11003) and probed with anti-Ufm1 antibody, and signal was quantified by Western blot analysis as described above using an in-house anti-ADS antibody as the primary antibody.

RESULTS

Uba5 Utilizes a Two-step Mechanism to Activate Ufm1—Ufm1 was previously shown to be activated by Uba5 (10). In this study, Uba5 was tested against a panel of Ubls for the ability to support ATP-PPi exchange (5, 26). Among the Ubls tested, Uba5 demonstrated measurable ATP-PPi, exchange activity with Ufm1 exclusively, and no activity was observed with SUMO2 as suggested by an earlier study (data not shown) (29).

The mechanism proposed for ubiquitin-activating enzyme Uba1 by Haas and Rose (5) predicts that 2 eq of ATP are hydrolyzed during the three-step activation cycle, producing 2 eq of AMP under the assay conditions, 1 eq from free AMP liberated in step 2 and 1 eq from hydrolysis of the ubiquitin adenylate generated in step 3 (Fig. 1A). Release of 2 eq of AMP has been experimentally confirmed for Uba1 and Uba6 in previous studies (27, 28). For Uba5, however, approximately 1 eq of AMP per monomer was produced in reactions with Ufm1 (Table 1). In addition, 1 eq of AMP release was detected either with C250A Uba5 mutant, which is not capable of catalyzing step 2, or in the presence of ADS (Fig. 1B), where a potential step 3 is prohibited by Ufm1–ADS adduct formation (see under “Discussion”). Together, these data suggest that unlike Uba1 or Uba6, Uba5 proceeds through a two-step activation mechanism where Uba5 binds 1 eq of ATP and 1 eq of Ufm1 to form a binary complex of Uba5–Ufm1 thioester.

Kinetic Analysis of Ufm1 Activation by Uba5—To gain a better understanding of the mechanism of Uba5, kinetic parameters were measured using Ufm1 in an ATP-PPi exchange assay (5, 26). First, the Ufm1 concentration dependence was analyzed by titrating Ufm1 into the assay mixture. Uba5 displays a random-ordered binding mechanism with Ufm1. With increasing concentrations of Ufm1, hyperbolic kinetics were obtained, and...
Mechanism of Ufm1 Activation and Conjugation

TABLE 1
Quantitation of AMP/adenylate release during Uba5-mediated Ufm1 activation

| Samples          | Ratio of AMP:Uba5 | No. of repeats |
|------------------|-------------------|---------------|
| WT Uba5          | 1.02 ± 0.29       | 13            |
| WT Uba5 + adenosine sulfamate | 0.96 ± 0.23       | 4             |
| C250A Uba5       | 0.94 ± 0.19       | 7             |
| Δ UFD Uba5       | 0.03 ± 0.05       | 4             |

no inhibition was observed at higher Ufm1 concentrations up to 300 μM (Fig. 2A). This observation is different from the pseudo-ordered and ordered-binding mechanism that was observed for other E1 enzymes such as Uba1 (5, 28, 30). An apparent Kᵢ₅₀ of 7.9 μM was obtained for Ufm1 by fitting the kinetic data over the concentration range tested (Table 2). The effects of ATP and PPᵢ on the ATP-PPᵢ exchange activity were also studied. ATP was first titrated into the ATP-PPᵢ exchange assay mixture, which resulted in a normal hyperbolic increase in activity with a Kₛₒ value of ~65 μM (Fig. 2B and Table 2). When PPᵢ was titrated into the reaction, at lower concentrations an apparent increase in activity was observed with increasing concentrations of PPᵢ. However, once the concentration surpassed 30 μM, PPᵢ became increasingly inhibitory. At 1 mM PPᵢ, the activity of Uba5 was inhibited more than 80% compared with the peak activity (Fig. 2C). The substrate inhibition observed for PPᵢ is reminiscent of the pseudo-ordered binding mechanism observed in the ubiquitin activation by Uba1 where a high concentration of ubiquitin, but not PPᵢ, inhibits the ATP-PPᵢ exchange activity. Further studies showed that the ATP concentration had no effect on potency of inhibition by PPᵢ, suggesting that ATP does not compete with PPᵢ for binding (data not shown). The estimated Kᵢ₅₀ for PPᵢ is determined to 6.2 μM when data from only the lower PPᵢ concentrations (<30 μM) are included for kinetic analysis (Table 2).

AMP Inhibits Uba5-dependent ATP-PPᵢ Exchange in a PPᵢ-dependent Manner—AMP was found to completely inhibit wild-type Uba5 with an estimated IC₅₀ of 4.0 ± 0.54 μM in the presence of 40 μM PPᵢ in the ATP-PPᵢ exchange assay (Fig. 2D). Because PPᵢ showed inhibitory effect on wild-type Uba5 at high concentrations, AMP inhibition was then examined at different concentrations of PPᵢ, to determine whether PPᵢ would affect the ability of AMP to inhibit ATP-PPᵢ exchange. The potency of AMP was found to be enhanced dramatically at higher concentrations of PPᵢ, as the IC₅₀ values decreased from 165 μM at 5 μM PPᵢ to 0.40 μM at 250 μM PPᵢ (Fig. 3). Smaller nucleotide analogs such as β-adenosine and adenosine 5’-monophosphoramide also showed enhanced potency at higher concentrations of PPᵢ; however, the effects were less prominent than AMP (8–16-fold change compared with 400-fold observed for AMP) (supplemental Fig. S1). A nonhydrolyzable ATP analog, AMP-PNP, showed weak inhibition, and PPᵢ did not affect its potency (supplemental Fig. S1).

Neither PPᵢ nor AMP Inhibits C250A Uba5-dependent ATP-PPᵢ Exchange Activity—Kinetic parameters were also measured for the catalytic cysteine mutant C250A Uba5 in ATP-PPᵢ exchange assays (Fig. 4, A–D). With the conserved cysteine mutated to alanine, the C250A mutant is predicted to be incompetent in forming Uba5~Ufm1 thioester (step 2) while retaining the ability to adenylate Ufm1 (step 1) (Fig. 1A). Interestingly, Ufm1 showed substrate inhibition at higher concentrations, suggesting a pseudo-ordered binding mechanism (Fig. 4A). Although ATP showed hyperbolic kinetics similar to the wild-type Uba5 (Fig. 4B), effects of PPᵢ and AMP are quite different. Unlike wild-type Uba5, C250A Uba5 was not inhibited at high concentrations of PPᵢ (Fig. 4C). In addition, AMP only weakly inhibits C250A Uba5’s ATP-PPᵢ activity (Fig. 4D). Higher concentrations of PPᵢ had no effect on the AMP inhibition of C250A Uba5 (data not shown). This observation suggests that similar to other E1 enzymes, AMP has very weak affinity to the apo-form of Uba5 (5). Therefore, inhibition of Uba5’s ATP-PPᵢ exchange activity by AMP is most likely due to a binding event subsequent to step 1, which alters the equilibrium between steps 1 and 2 (Fig. 1A).

Kinetic Analysis of Ufm1 Activation and Uba5-Ufc1 Transthiolation—The rate of Ufm1 adenylation formation (step 1) was measured by quantifying the amount of AMP released from hydrolysis of Ufm1 adenylate over time (Fig. 1A). Ufm1 adenylation was generated at a rate of 0.015 s⁻¹ (Table 3 and supplemental Fig. S2A), which is much slower than the rate of ubiquitin adenylation formation catalyzed by Uba1 (18.8 s⁻¹) or Uba6 (9.7 s⁻¹) (27, 28). The apparent rate of Uba5~Ufm1 thioester formation (step 2) was measured to be 0.021 s⁻¹ using a Western blotting-based method, which was also slower than Uba1 (4.3 s⁻¹) or Uba6 (1.0 s⁻¹) (Table 3 and supplemental Fig. S2B) (27, 28). The rate of Ufm1 adenylation formation (step 1) under the conditions tested appeared to be slightly slower than the rate of Uba5~Ufm1 thioester formation (step 2). However, the adenylation formation assays were performed with 50 μM ATP, which was below the estimated Kₛₒ (65 μM) determined in the ATP-PPᵢ exchange assays (Fig. 2B and Table 2). A saturating ATP concentration was not used in the adenylation reactions due to the decreasing sensitivity of AMP detection at higher ATP concentrations. Therefore, the maximal rate of adenylation may not have been achieved under the conditions tested.
The apparent rate for Uba5-Ufc1 transthiolation was also determined by incubating Ufc1 with pre-formed Uba5-Ufm1 and quantitating Ufm1 thioester by Western blot analysis (supplemental Fig. S2C). The observed transthiolation rate (0.12 ± 0.012 s⁻¹, Table 3) is much faster than either step 1 or 2, although it is still much slower compared with Uba1 (2.0 s⁻¹) or Uba6 (0.4 s⁻¹) (27, 28).

Ufc1 Inhibits ATP-PPi Exchange While Stimulating Formation of Uba5-Ufm1 Thioester—Ufc1 was shown to completely inhibit Uba5-dependent ATP-PPi exchange activity with an apparent IC₅₀ of 180 nM (supplemental Fig. S3). However, little inhibitory effect was observed when C250A Uba5 was used in the assay (supplemental Fig. S3), suggesting that inhibition by Ufc1 is probably due to a shift of equilibrium from step 1 to step 2 (Fig. 1A). When the overall rate of Ufc1-Ufm1 formation was determined in a fully reconstituted system containing Uba5, Ufm1, ATP-Mg²⁺, and Ufc1, we were surprised to find that, paradoxically, the observed rate (0.041 s⁻¹) was much faster than either step 1 (0.015 s⁻¹) or step 2 (0.021 s⁻¹) that was measured in the absence of Ufc1 under the same conditions. One explanation is that Ufc1 stimulates the rate of Uba5-Ufm1 formation. To test this hypothesis, the rate of Uba5-Ufm1 was determined in the presence of wild-type Ufc1 and was found to be 2-fold higher than that with no Ufc1 (Fig. 5). In addition, a catalytically inactive mutant of Ufc1 incapable of forming the Ufc1-Ufm1 thioester (C116A Ufc1) did not significantly affect the rate of Uba5-Ufm1 formation (Fig. 5), suggesting that Ufc1-Ufm1 might be responsible for the stimulatory effect. Indeed, purified Ufc1-Ufm1 was found to have much stronger stimulation than Ufc1 alone (~9-fold versus ~2-fold). Therefore, the presence of Ufc1 not only shifts the reaction equilibrium from step 1 to step 2 but also directly promotes the kinetics of Uba5-Ufm1 thioester formation.

Binding of ATP to Uba5-Ufm1 Thioester Is Required for Efficient Uba5-Ufc1 Transthiolation—For ubiquitin-activating enzyme Uba1, formation of a ternary complex containing both Uba1—ubiquitin thioester and ubiquitin adenylate has been shown to be sufficient for efficient E1-E2 transthiolation (31,
Pickart et al. (31) also found that occupancy of the adenylate site by ATP alone was adequate to stimulate transthiolation. Because Uba5 was shown to be unable to form a ternary complex, additional studies were designed to understand the mechanism of Uba5-Ufc1 transthiolation. First, Uba5 thioester was prepared by mixing Uba5 with Ufm1 and ATP-Mg<sup>2+</sup>. EDTA was then added to the mixture to chelate Mg<sup>2+</sup> and release any bound ATP. The resulting Uba5 thioester sample was then desalted to remove AMP, ATP, and Mg<sup>2+</sup>. When Ufc1 was added directly to the pre-formed, nucleotide-free Uba5 thioester sample, only very small amounts of Ufc1 thioester were formed (Fig. 6). However, when ATP-Mg<sup>2+</sup> was added together with Ufc1, Ufm1 was efficiently transferred from Uba5 to Ufc1 (Fig. 6). Uba5-Ufc1 transthiolation was also observed in the presence of ATP-CPP, a nonhydrolyzable ATP analog inactive in supporting ATP-PP<sub>i</sub> exchange (data not shown), albeit not as efficient as ATP (Fig. 6). When AMP was supplied with Uba5 thioester, only a small amount of Ufc1 was converted to a thioester form, suggesting that although Uba5 thioester does not appear to bind a second Ufm1 and ATP molecule to form a ternary complex as Uba1 does, the adenylate site of Uba5

![FIGURE 4. Substrate dependence in C250A Uba5 ATP-PP<sub>i</sub> exchange assay.](image)

**TABLE 3**

| Uba5 Reaction | Ufm1-AMP<sup>a</sup> (s<sup>-1</sup>) | Uba5−Ufm1 (s<sup>-1</sup>) | Ufc1−Ufm1 (s<sup>-1</sup>) |
|---------------|----------------------------------|-------------------------|-------------------------|
| Ufm1          | 0.015 ± 0.002                    | 0.021 ± 0.003           | 0.12 ± 0.012            |

*<sup>a</sup> The rates of Ufm1-AMP formation were performed at concentrations of ATP below K<sub>i</sub> due to limitations of the TLC AMP release assay.*

32). Pickart et al. (31) also found that occupancy of the adenylate site by ATP alone was adequate to stimulate transthiolation. Because Uba5 was shown to be unable to form a ternary complex, additional studies were designed to understand the mechanism of Uba5-Ufc1 transthiolation. First, Uba5−Ufm1 thioester was prepared by mixing Uba5 with Ufm1 and ATP-Mg<sup>2+</sup>. EDTA was then added to the mixture to chelate Mg<sup>2+</sup> and release any bound ATP. The resulting Uba5−Ufm1 thioester sample was then desalted to remove AMP, ATP, and Mg<sup>2+</sup>. When Ufc1 was added directly to the pre-formed, nucleotide-free Uba5−Ufm1 thioester sample, only very small amounts of Ufc1−Ufm1 thioester were formed (Fig. 6). However, when ATP-Mg<sup>2+</sup> was added together with Ufc1, Ufm1 was efficiently transferred from Uba5 to Ufc1 (Fig. 6). Uba5-Ufc1 transthiolation was also observed in the presence of ATP-CPP, a nonhydrolyzable ATP analog inactive in supporting ATP-PP<sub>i</sub> exchange (data not shown), albeit not as efficient as ATP (Fig. 6). When AMP was supplied with Uba5−Ufm1 thioester, only a small amount of Ufc1 was converted to a thioester form, suggesting that although Uba5 thioester does not appear to bind a second Ufm1 and ATP molecule to form a ternary complex as Uba1 does, the adenylate site of Uba5

![FIGURE 5. Ufc1 stimulates the rate of Uba5−Ufm1 thioester formation.](image)
Mechanism of Ufm1 Activation and Conjugation

Inhibition of Uba5 with ADS—MLN4924 and a pan-E1 inhibitor (Compound I, Fig. 1B) were previously identified as mechanism-based inhibitors against E1s, including Uba1 and NAE (Fig. 1B) (24). These compounds inhibited E1s by binding and reacting with E1~Ubl thioester to form a covalent Ubl-compound adduct that mimics Ubl adenylate and binds tightly at the E1 active site (24, 27, 28). To investigate whether ADS (Fig. 1B) could inhibit Uba5, the compound was titrated into reactions, and the activity of Uba5 was monitored by the ATP-PPi exchange assay. ADS was shown to inhibit Uba5 with an estimated IC_{50} of 13 μM (Fig. 7A). Inhibition of Uba5 by ADS was also found to be both ATP- and AMP-competitive (Fig. 7A and supplemental Fig. S4), which is consistent with the mechanism of inhibition proposed for other E1s (24, 27). Formation of the Ufm1-ADS adduct on Uba5 was detected by a Western blot-based assay using an in-house antibody specifically recognizing ADS (Fig. 7C) or by mass spectrometry ([M + H]^+ m/z calculated for Ufm1-ADS, 9124.4, and observed, 9123.4).

Inhibition of Uba5 by ADS appeared to be time-dependent and had a measurable rate of inactivation of 11 m^{-1} s^{-1} (Fig. 7B), which is more than 2000-fold slower than that with NAE (25,800 m^{-1} s^{-1}, supplemental Fig. S5). Time dependence of ADS inhibition, which is directly correlated with kinetics of Ufm1-ADS adduct formation, was probed directly by measuring the rate of Ufm1-ADS adduct formations in Western blot analysis using the anti-ADS antibody (Fig. 7C). As suggested in earlier studies (24, 27, 28), the Ufm1-ADS adduct was formed by binding of ADS to Uba5~Ufm1 thioester and subsequent nucleophilic attack on the thioester bond by the amino group of ADS. Therefore, the observed overall rate of Ufm1-ADS adduct formation was influenced by multiple kinetic steps leading to adduct formation, including Uba5~thioester formation, binding of ADS to Uba5~Ufm1, and the chemistry step where nucleophilic attack occurs. The observed rate of Ufm1-ADS formation (2.1 × 10^{-4} s^{-1}) is ~10-fold slower compared with the rate of Uba5~Ufm1 thioester formation under the same conditions, suggesting that Ufm1-ADS formation is the rate-limiting step under the assay conditions. In addition, the rate of Ufm1-ADS formation was also shown to be stimulated 4–5-fold by wild-type Ufc1 (Fig. 7C). Once formed, the Ufm1-ADS adduct appeared to bind very tightly with Uba5, and no recovery of activity was observed after 4 h (Fig. 7D).

Inhibition of Cellular Ufm1 Conjugation—The Ufm1 conjugation pathway has been linked with ER stress (20–22, 33). The ER membrane protein UFBP1 has been identified as a possible target protein for ufmylation in overexpression studies (17). In this study, HCT116 cell lysates were analyzed by SDS-PAGE and Western blot using an anti-Ufm1 antibody to detect ufmylated species. We found several distinct bands that represented potential ufmylated proteins besides Uba5~ and Ufc1~Ufm1 thioesters (Fig. 8A). To confirm the role of Uba5 and Ufc1 in protein ufmylation, siRNA oligonucleotides specific for Uba5 and Ufc1 were used to knock down the expression level of each protein, respectively. Successful knockdown of Uba5 and Ufc1 was achieved and resulted in the loss of several ufmylated bands (supplemental Fig. S6).

Because ADS inhibited Uba5 in biochemical assays, it was also studied in the cellular setting to see if the Uba5 pathway could be inhibited. HCT116 cells were treated with 10 μM ADS for 24 h before cells were harvested, lysed, and separated by SDS-PAGE. ADS was shown to inhibit both Uba5 and Ufc1 thioester in cells, which resulted in a significant reduction in ufmylated species (Fig. 8A). Immunoprecipitation studies using either anti-Ufm1 or anti-ADS antibody confirmed that Ufm1-ADS was produced in cells (Fig. 8B). Other Ubl-ADS adducts were also detected, which is consistent with its nonselective nature of E1 inhibition. Therefore, despite the slow rate of Ufm1-ADS adduct formation in a biochemical setting, ADS was able to inhibit the Uba5 pathway in cells.

DISCUSSION

There are eight E1 enzymes that have been identified in the human genome (3, 4). Based on the domain organization, these E1 enzymes can be further classified as canonical or noncanonical (4). The canonical E1s, including ubiquitin, NEDD8, and SUMO-activating enzymes, are either heterodimeric or monomeric with one functional and one inactive adenylate domain per enzyme molecule. A distinct catalytic cysteine domain exists in all canonical E1s, which hosts the conserved cysteine required for the E1~Ubl thioester formation. In addition, they also contain a ubiquitin-fold domain that is involved in recruiting the respective E2s (34, 35). Several earlier studies suggest that canonical E1s activate their cognate Ubls via a three-step mechanism (Fig. 1A) (5, 7). In the first step, E1 binds ATP and Ubl and forms a Ubl-adenylate intermediate, with PPi, as a byproduct. In the second step, the conserved cysteine residue in the catalytic cysteine domain reacts with the Ubl adenylate to form an E1~Ubl thioester. In the final step, the E1~Ubl thioester catalyzes another round of Ubl adenylation with ATP and forms a ternary complex. The E1-Ubl ternary complex has been shown to be kinetically competent to transfer Ubl to the conjugating enzyme (E2) via a transthiolation reaction (31). The crystallographic studies of ubiquitin and NEDD8-activating enzyme demonstrate that both the UFD domain and the catalytic cysteine domain are required for the ternary complex formation (34, 35). In addition, the UFD domain has also been shown to promote specific interaction between the cognate E1 and E2 pairs (32, 34). During the three-step acti-
vation process, two possible conformations, deemed open and closed, have been proposed for canonical E1s. The open conformation is present in apoE1s and has been shown to be responsible for binding ATP and Ubl in step 1 or 3 (35–38). However, step 2 implies a transition from the open conformation to a closed one where the 25–30-Å distance between the catalytic cysteine and the adenylation site needs to be bridged in order for the chemistry to take place. Recent x-ray structural studies of the SUMO-activating enzyme suggest that extensive structural reorganization around the catalytic cysteine domain likely occurs in step 2 (39). Our recent kinetic study also suggested that for canonical E1s such as Uba1 or NEDD8-activating enzyme, the rate-limiting step of Ubl activation is probably the E1 thioester formation (step 2) (27), which is consistent with the previous study by Haas and Rose (5).

**FIGURE 7.** Adenosine sulfamate is an ATP-competitive, slow-tight binding inhibitor of Uba5. A, ADS was titrated into WT Uba5 ATP-PPi reaction containing either 100 μM (●) or 1 mM ATP (□). B, Uba5 ATP-PPi exchange was used to monitor the time dependence of ADS inhibition over time. $k_{\text{obs}}$ values were determined by fitting the curves to the equation described above (top panel). The estimated rate of inactivation ($k_{\text{inact}}/K_i$) was determined by linear regression to be 11 m$^{-1}$s$^{-1}$ (bottom panel). C, Ufm1-ADS adduct formation was monitored and quantified over time in the presence (○) or absence (●) of WT Ufc1. $k_{\text{obs}}$ values were estimated from fitting curves to the equation, $Y = Ao \times (1 - \exp(-k_{\text{obs}} \times t))$. The estimated $k_{\text{obs}}$ for Ufm1-ADS adduct formation was $2.1 \times 10^4$ s$^{-1}$ and $9.0 \times 10^4$ s$^{-1}$ for the reaction without and with Ufc1, respectively. D, reversibility of Ufm1-ADS-inhibited Uba5 was measured in the Ufc1 HTRF transthiolation assay.
Mechanism of Ufm1 Activation and Conjugation

Unlike the five canonical E1s, the three noncanonical E1s, Uba5, ATG7, and Uba4, are predicted to be homodimeric proteins with no discernible catalytic cysteine domains. Instead, a conserved cysteine residue has been identified in noncanonical E1s via sequence alignment and functional studies (4). The conserved cysteine is thought to be responsible for forming E1–Ubl thioester after the adenylation step similar to their canonical counterpart. However, due to lack of a separate catalytic cysteine domain, it is not clear whether the noncanonical E1s undergo similar open-closed conformational changes in step 2. Various additional domains in these noncanonical E1s could also likely play a role in Ubl activation and E1-E2 transthiolation. For example, the recent structural studies of ATG7 suggest that the unique N-terminal domain of one monomer recruits the incoming E2 (ATG3) and positions the E2 to react (31). This study provides strong evidence for a two-step activation mechanism (Fig. 9) with formation of a binary complex of Uba5–Ufm1 thioester. The lack of ternary complex formation distinguishes Uba5 from the canonical E1s. The two-step activation mechanism is based on the observed one free AMP production per Uba5 in the TLC analysis. To rule out the possibility of an extremely stable Ufm1 adenylate formed by wild-type Uba5 after step 2, we boiled the sample and found that the amount of AMP production remained unchanged, suggesting that no additional Ufm1 adenylate, weakly or tightly bound, was formed (data not shown). The absence of step 3 was also supported by direct detection of 32P-labeled Ufm1 adenylate by SDS-PAGE in reactions containing Uba5, Ufm1, and [α-32P]ATP. For wild-type Uba5, we were only able to detect very weak Ufm1 adenylate formation compared with C250A Uba5 mutant, suggesting that no Uba5–Ufm1 thioester ternary complex was formed (supplemental Fig. S8). This observation is in direct contrast with canonical E1s such as Uba1 or NAE where strong Ubl adenylate formation was detected with both wild-type and Cys/Ala mutants (supplemental Fig. S8).

Uba5 also demonstrated distinct kinetic behaviors compared with canonical E1s. The apparent maximal turnover for ATP-PPi exchange activity of Uba5 was determined to be 0.090 s⁻¹ (Table 2), which is much slower than that determined for Uba1 (4.9 s⁻¹), NAE (0.49 s⁻¹), or Uba6 (0.83 s⁻¹) (5, 26, 28). Even with C250A Uba5 mutants where the stoichiometric amount of adenylate is likely present, the ATP-PPi exchange activity is not improved, suggesting intrinsically low reactivity of Uba5. The apparent rates for individual steps in Ufm1 activation and transfer to Ufc1 were also measured, and the rate-limiting step is most likely formation of Uba5–Ufm1 (Table 3). Ufc1 alone or Ufc1–Ufm1 thioester was shown to stimulate Uba5–Ufm1 thioester formation by 2–9-fold. In addition, Ufc1 is also shown to inhibit ATP-PPi exchange with K_{diss} of ~180 nM (supplemental Fig. S3), but had little effect on C250A Uba5 mutant. This result is consistent with binding of Ufc1 or Ufc1–Ufm1 thioester to Uba5–Ufm1 thioester and shifting the equilibrium to step 2, thus away from step 1 where ATP-PPi exchange occurs.

The most intriguing kinetic observation for Uba5 is the apparent inhibitory effect of both AMP and PPi in ATP-PPi exchange. For canonical E1s such as Uba1, increasing PPi concentration typically results in hyperbolic kinetics, and AMP titration leads to a shift of equilibrium between steps 1 and 3, both of which support ATP-PPi exchange reactions (5). For
Uba5 where there is no step 3, one would predict that PPi would have a similar hyperbolic kinetics and that AMP would have a stimulatory effect due to the shift of the equilibrium from step 2 to step 1. To the contrary, we saw an initial increase in activity at lower PPi concentrations, but the rate was inhibited significantly at higher concentrations (Fig. 2C). In addition, AMP completely inhibited the ATP-PPi exchange reaction, and inhibition was potentiated by PPi (Figs. 2D and 3). The inhibition by AMP was affected by increasing ATP concentrations (supplemental Fig. S7), suggesting that AMP could compete with ATP for apo-Uba5 binding. However, neither AMP nor PPi inhibits C250A Uba5 (Fig. 4, C and D), which implies that the binding event responsible for AMP or PPi inhibition could also take place after step 1. One can speculate that AMP and PPi might form a relatively stable complex with Uba5-PPi, S–Ufm1 thioester and shift the equilibrium away from step 1.

Several studies have linked Uba5 to ER stress response pathways (20–22, 33). UFBP1 (also known as C20orf116 or DDRGK1), an ER-localized protein, has been proposed to be a substrate for ufmylation. Previous studies in mammalian cells have demonstrated ufmylation of UFBP1 with overexpression of both UFBP1 and Ufm1 (17). However, we were not able to detect endogenous UFBP1-Ufm1 conjugation under a variety of conditions by Western blot analysis or co-immunoprecipitation using commercial or in-house anti-UFBP1 antibodies (data not shown). Our cellular analysis did reveal 4–5 ufmylated protein substrates, and the level of protein ufmylation seems to vary among different cell lines (supplemental Fig. S9). Knocking down the proposed E2 enzyme, Ufc1, decreases the intensity of all ufmylated substrates in Western blotting analysis (supplemental Fig. S6), suggesting that Ufc1 is the sole E2 that is required in the Uba5-Ufm1 conjugation pathway. Identities of the ufmylated substrates are under active investigation and will lead to better understanding of the role of Uba5 in ER stress response and other biological processes. In addition, we also show that ADS, a nonselective E1 inhibitor, suppresses Uba5 activity by forming a covalent Ufm1-ADS adduct in both reconstituted systems and cellular settings. Suppression of the Uba5–Ufm1 thioester level by ADS led to a decreased level of Ufc1 thioester and reduced Ufm1 conjugates (Fig. 8). However, ADS also suppresses other E1 conjugation pathways, which prevent its usage as a tool molecule to study Uba5 biology. A more Uba5-selective compound would help to elucidate the role of Uba5 and the biological consequences of Uba5 inhibition.

Acknowledgments—We thank J. Brownell and C. Tsu for helpful discussions.

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