Role of the Zn$^{2+}$ Motif of E1 in SUMO Adenylation

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Post-translational modifications by ubiquitin-like proteins are among the most important mechanisms for regulating a wide variety of cellular functions. In these modifications an E1 enzyme activates each ubiquitin-like protein (Ubl) by adenylation of the Ubl C-terminal COOH group and then forms a thioester bond with the adenylated C-terminal COOH group of the Ubl. Previous x-ray crystallography studies revealed a conserved zinc motif in the SUMO and NEDD8 E1; however, the function of this Zn$^{2+}$ motif is unclear. In this study, using quantitative ATP:PP$i$ isotope exchange assays in combination with site-directed mutagenesis, we show that the conserved Zn$^{2+}$ motif in the SUMO E1 is important for SUMO adenylation and is critical for the E1 pseudo-ordered substrate binding mechanism. Furthermore, Zn$^{2+}$ motif mutants showed significantly reduced $k_{cat}$ values for ATP:PP$i$ isotope exchange assays, suggesting that the Zn$^{2+}$ motif is important in binding and preventing SUMO adenylation from dissociating from E1 before formation of the thioester conjugate. Because the Zn$^{2+}$ motif is located in a cross-over loop that is known to have conformational flexibility, the results described here suggest that this cross-over loop interacts with Ubl in the multistep, dynamic process of Ubl activation by E1s.

Post-translational modifications by ubiquitin-like proteins are among the most important mechanisms that regulate a wide variety of cellular functions (1). In these modifications a ubiquitin-like protein (Ubl) is first activated by an E1, known as the activation enzyme (2, 3). E1 catalyzes the adenylation of the Ubl C-terminal COOH group and then forms a thioester bond between the SH group of its active site Cys and the C-terminal COOH group of Ubl. The E1 for the small ubiquitin-like modifier (SUMO) is a tight heterodimer of two polypeptides known as SUMO activation enzyme 1 and 2 (SAE1 and SAE2), which is homologous to the N-terminal and C-terminal portions of the ubiquitin E1, respectively. Among the ubiquitin-like modifiers, SUMO is the most extensively studied, and its modification regulates many essential functions ranging from gene transcription, hormone response, DNA repair, and nuclear import (4–6).

Previous structural and biochemical studies identified several functionally important regions/domains in E1 (7–11). Ubl adenylation occurs on the E1 adenylation domain, which binds both Ubl and ATP. The ubiquitin E1 binds its two substrates, ATP and ubiquitin, in an “ordered” mechanism in which ATP is the leading substrate and ubiquitin is the trailing substrate (12, 13). Similarly, the NEDD8 E1 follows a “pseudo-ordered” mechanism in which ATP is the preferred leading substrate and NEDD8 is the preferred trailing substrate (14). After adenylation, Ubl is transferred to a Cys residue of E1, thereby forming a thioester bond with its cognate E1. To transfer Ubl from E1 to E2, the E2 enzyme is recruited to the E1–Ubl thioester conjugate by binding to multiple domains on E1, including the Cys domain (the domain containing the active Cys residue) and the ubiquitin-fold (UBF) domain (10, 11).

X-ray crystallography studies also revealed a conserved Zn$^{2+}$ motif located in a cross-over loop that connects the adenylation and UBF domains in the SUMO and NEDD8 E1 (8, 15). In the SUMO E1, the Zn$^{2+}$ motif is formed by Cys residues 158, 161, 441, and 444 of the SAE2 subunit through tetrahedral coordination to a Zn$^{2+}$ ion. The available data seem to indicate that this Zn$^{2+}$ motif may not play a significant role in E1 activity, as the x-ray structure did not reveal a direct interaction of SUMO with the Zn$^{2+}$ motif in a complex of E1 with SUMO and ATP (8) or with a SUMO adenylyl mimic (16). In addition, deletion of the C terminus of E1 (residues 442–640), which deleted one of the Zn-coordinating residues, Cys-444, did not appear to significantly impair the formation of the E1–SUMO thioester conjugate. However, quantitative enzyme kinetic analysis was not carried out to evaluate the activity of this mutant. Furthermore, mutation of the equivalent Zn$^{2+}$ motif Cys residues in the yeast E1 homologue did not significantly impair yeast growth (8). However, in the absence of genotoxic stress, the yeast growth assay may not be sufficiently sensitive to activity changes of the E1 enzyme, because SUMOylation is not directly related to replication.

Therefore, in this study we carried out quantitative enzyme kinetic analysis in combination with site-directed mutagenesis to investigate the role of the conserved Zn$^{2+}$ motif in the SUMO E1. Using quantitative ATP:PP$i$ isotope exchange assays, we have shown that SUMO adenylation follows a pseudo-ordered mechanism in which ATP is the preferred leading substrate and SUMO is the preferred trailing substrate. The Zn$^{2+}$ motif in the SUMO E1 plays a critical role in the pseudo-ordered binding mechanism and in SUMO adenylation. Because the Zn$^{2+}$ motif is distal from the ATP-binding site but close to the SUMO-binding site, these results suggest that the Zn$^{2+}$ motif is involved in binding SUMO during adenylation. Furthermore, quantitative analysis of the kinetics of the ATP:PP$i$ isotope exchange assays suggests that the Zn$^{2+}$ motif is important in interacting with SUMO during adenylation and preventing SUMO adenylation from dissociating from E1 before...
formation of the thioester conjugate. The Zn$^{2+}$ motif is located in a cross-over loop known to have conformational flexibility, and thus, it is likely to form key dynamic interactions with SUMO during the multi-step process of SUMO activation.

**EXPERIMENTAL PROCEDURES**

**Site-directed Mutagenesis and Protein Purification**—Vectors for expressing truncated SAE2 polypeptides were generated by creating a stop codon immediately after the intended C-terminal residue using the QuikChange mutagenesis protocol (Stratagene). Point mutants of SAE2 were also generated using the same mutagenesis kit, with different primer pairs for each mutation. All plasmid constructs were confirmed by DNA sequencing. Expression and purification of proteins were performed as described previously (11, 17).

Protein concentrations were determined as follows. The concentration of a SUMO sample was determined by both the Bradford assay (using bovine serum albumin as the standard) and amino acid analysis, and then the concentration obtained by amino acid analysis was used as the standard to obtain an adjustment factor for the Bradford assay determined concentration in future experiments. This was necessary because Bradford measurements, although reproducible, can result in systematic errors due to amino acid composition differences between different proteins. The concentrations of the wild-type and mutant E1 enzymes were equalized before each assay by running aliquots of all samples on SDS-PAGE followed by SimpleBlue staining (Invitrogen). Because the amount of impurities varied among samples for wild-type and various mutants of E1, the SAE2 band intensities of the wild-type and mutant E1 proteins were compared to adjust the concentrations. After the concentration adjustments with buffer, the above process was repeated until the SAE2 band intensities were equal. The effective concentrations of E1 were adjusted for the active percentage of E1, which is the percentage of wild-type E1 that can form the thioester conjugate with SUMO. The same expression and purification procedures yield consistent percentage of wild-type E1 that forms thioester conjugates with SUMO (~40%).

**In Vitro Conjugation Assays**—All conjugation assays were conducted in a mixture containing 50 mM Tris, pH 7.5, 5 mM MgCl$_2$, and an ATP regeneration system (2 mM ATP, 10 mM creatine phosphate, 3.5 units/ml creatine kinase, and 0.6 units/ml inorganic pyrophosphatase). Assays were incubated at 37 °C for the specified time before adding SDS loading buffer (with or without the reducing agent DTT) to terminate the reactions. Samples were resolved on NuPage SDS-PAGE gels (Invitrogen), and polypeptide bands were visualized by either SimpleBlue stain or Western blotting. For E1~SUMO thioester conjugate formation, 0.1 μM E1 and 4 μM SUMO-1 were incubated for 30 s or 5 min, and the modified SAE2 band was detected by Western blots using anti-SAE2 and anti-SUMO antibodies. For Ubc9~SUMO thioester complex formation, 20 μM SUMO and 10 μM Ubc9 were incubated in the presence of 0.05 μM E1 for 2 or 15 min, and the reaction products were visualized by SimpleBlue staining after gel electrophoresis. For RanGAP1~SUMO isopeptide formation, 15 μM SUMO and 15 μM RanGAP1 were incubated in the presence of 0.05 μM E1 and 1 μM Ubc9 for 30 s or 5 min, and the protein bands were visualized by SimpleBlue staining after gel electrophoresis.

**ATP:Pi Isotope Exchange Assays**—Radioactive isotope-based ATP:Pi exchange assays conditions were adapted from previous work by Haas and Burch (18). In a 50-μl reaction mixture containing 50 mM Tris, pH 7.5, 10 mM MgCl$_2$, 0.5 mM DTT, 1 mM ATP, 1 mM [$^{32}$P]Pi (PerkinElmer Life Sciences), 10 μM SUMO was incubated with 0.2 μM wild-type or mutant E1 proteins at 37 °C for 20 min before the reaction was quenched with 5% (w/v) trichloroacetic acid (0.5 ml) containing 4 mM carrier Pi. The $^{32}$P-incorporated ATP was absorbed to a 10% (w/v) slurry (0.3 ml) of activated charcoal (Sigma) in 2% trichloroacetic acid; the charcoal was rinsed twice with 1 ml of 2% trichloroacetic acid before Cerenkov counting. For the SUMO concentration-dependent exchange assay, all conditions were the same as above, except SUMO was added at increasing concentrations (0.1–800 μM). For data analysis, the results were normalized by setting the activities of wild-type E1 as 100%.

**RESULTS**

**Role of the Zn$^{2+}$ Motif in E1, as Revealed by Truncation Mutants**—We found by serendipity that deletion of residues 439–640 of the SAE2 subunit (referred as Δ439 in subsequent discussions) completely abolished the formation of the E1~SUMO thioester (Fig. 1A). This was unexpected, because previously published results indicated that deletion of amino acids 442–640 (Δ442) or 446–640 (Δ446) did not significantly impair formation of the E1~SUMO thioester (8), and Δ442 differ from Δ439 by only three amino acid residues. In addition, residue 436, which was shown to contact SUMO directly in the x-ray structure (8), is included in the Δ439 construct. Close inspection of the x-ray structure of the SUMO E1 suggested that truncating SAE2 at residue 439 should not affect the structural integrity of the remaining E1. In particular, the truncated SAE2 subunit still formed a tight non-covalent complex with SAE1, indicating that SAE2, particularly the adenylation domain that forms the extensive interaction with SAE1, maintained its structural integrity.

To confirm that the pronounced difference between Δ439 activity and the published Δ442 or Δ446 activity was not due to experimental procedures, we constructed another truncated version of E1 that terminated at Cys-444 (Δ445). This was unexpected, because the truncated mutant is consistent with previous reports that Δ442 and Δ446 could form a thioester with SUMO (8). As expected, neither Δ439 nor Δ445 could catalyze Ubc9~SUMO and RanGAP1~SUMO conjugates (Fig. 1B), confirming previous reports that the UBF domain, which was missing in these constructs, is critical for E2 recruitment but plays no role in the formation of the Ubl~E1 thioester conjugate (7, 8, 17). RanGAP1 is the first SUMO substrate identified, and its modification does not require an E3 ligase (19–22). These results clearly indicate that residues 439–441, which are within the Zn$^{2+}$ motif of the SAE2 subunit of E1 (Fig. 1C), play a crucial role in E1 function. In particular, in addition to the Zn-chelating Cys-441, the remaining two resi-
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![Figure 1](http://www.jbc.org/content/285/31/23734/F1)

**A** In vitro conjugation assays shown comparing activities of the wild-type (WT) E1 and the Δ439 and Δ445 mutants of E1 in forming SAE2–SUMO thioester conjugates. The assay mixture contained 0.1 μM E1 and 4 μM SUMO-1, which were co-incubated at 37 °C for 0.5 or 5 min before aliquots were taken and mixed with non-reducing gel-loading buffer. Another aliquot of identical volume was also taken after 5 min of reaction time to mixed with DTT-containing gel-loading buffer (lanes labeled as *) to hydrolyze the thioester conjugate. Shown is a Western blot using an anti-SAE2 antibody (red bands) and an anti-SUMO-1 antibody (green bands). B, in vitro conjugation assay compare activities of the wild-type and mutant E1s in forming the Ubc9–SUMO thioester and RanGAP1–SUMO isopeptide conjugates. For Ubc9–SUMO thioester formation, 20 μM SUMO and 10 μM Ubc9 were incubated in the presence of 0.05 μM E1 for 2 or 15 min (as indicated). For RanGAP1–SUMO isopeptide formation, SUMO and RanGAP1 were incubated with E1 and Ubc9 for 30 s or 5 min (as indicated). Shown are SDS-PAGE gels stained with SimpleBlue. All conjugation assays were conducted in a mixture containing 50 mM Tris, pH 7.5, 5 mM MgCl$_2$, and the ATP and ATP regeneration system (2 mM ATP, 10 mM creatine phosphate, 3.5 units/ml creatine kinase, and 0.6 units/ml inorganic pyrophosphatase). C, shown is a schematic of the domain structures of SAE2. All four Cys residues that form the Zn$^{2+}$ motif, along with the catalytic Cys-173, are indicated. In addition, residues within or surrounding the Zn$^{2+}$ motif that were tested by site-directed mutagenesis are also indicated. The truncation mutants are illustrated below the schematic. D, zoom-in of the Zn$^{2+}$ motif in the structure of the SUMO E1 in complex with SUMO, Mg$^{2+}$, and ATP. E1 is shown as the schematic, and bound SUMO at the adenylation domain is shown as the orange line ribbon with the N and C termini indicated. The residues of E1 near or within the Zn$^{2+}$ motif are indicated by black numbers, and the catalytic Cys-173 of E1 is also indicated. The SUMO residues adjacent to the Zn$^{2+}$ motif are shown with their side chains and indicated by red numbers. These residues are analogous to the ubiquitin residues identified by an Ala scanning mutagenesis as important for yeast growth.

Role of the Zn$^{2+}$ Motif in E1, as Revealed by Site-directed Mutagenesis—The dramatic difference between Δ439 and Δ442/Δ445 in formation of the E1–SUMO thioester conjugate suggested that residues 439 – 441 are pivotal for E1 activity. To elucidate the roles of these residues in E1 function, we constructed a series of SAE2 site-directed mutants for residues Asn-438 – Cys-444 (Fig. 2). We mutated Asn-438 and Asn-440 to Lys to create a significant change in electrostatic charge properties for these surface residues to investigate their roles in protein-protein interactions. We also generated Pro-439 → Ala and Asn-440 → Leu mutants to further investigate the role of Pro-439 and Asn-440 in E1 function by substituting with residues of different properties. In addition, we created double mutant Tyr-442 to Ala and Val-443 to Ala (Y442A/V443A) to investigate these two residues that immediately flank the Zn$^{2+}$ motif.

Finally, triple mutants that changed residues 439 – 441 to Ala (P439A/N440A/C441A) and residues 442 – 444 to Ala (Y442A/V443A/C444A) were made to confirm the difference in activity between the two deletion mutants, Δ442 (8) and Δ439, in the context of full-length E1. E1 mutants carrying each of the SAE2 mutations were expressed in *Escherichia coli* and purified using identical procedures as for wild-type E1 (8, 17). All mutant proteins contained a His tag and co-expressed with the untagged SAE1 subunit. Furthermore, the SAE1–SAE2 complex could be purified from these *E. coli* lysates using only the His tag on the SAE2 subunit, indicating that all mutated proteins maintained the necessary structural integrity to bind SAE1 and formed a tight complex with this subunit.

We first examined the thioester conjugation activity of these mutants. In vitro conjugation assays revealed that P439A, Y442A/V443A, N438K, N440K, N440L, C444A, and Y442A/V443A/C444A retained the ability to form thioester conjugates with SUMO (Fig. 2A). However, consistent with the Δ439 truncation mutant, the triple mutant P439A/N440A/C441A did not form a detectable SAE2–SUMO conjugate. As a control, an E1 mutant (C173S) lacking the Cys residue needed to form the E1–SUMO thioester bond and was also included in the assays. Most mutants, except N440K, showed significantly less SUMO–E1 formation and also displayed reduced formation of the E2 (Ubc9)–SUMO thioester conjugate (Fig. 2B) and RanGAP1–SUMO isopeptide formation (Fig. 2C). In addition, similar to wild-type E1, all of the site-directed mutants that contained an intact UBF domain were able to form detectable non-covalent complexes with Ubc9 on a non-denaturing polyacrylamide gel (data not shown).

Adenylation Activity of E1 Mutants—To address the mechanism by which the Zn$^{2+}$ motif participates in E1 activity, we investigated whether it is required for the first step of E1 catalysis, SUMO adenylation. SUMO adenylation requires binding of SUMO and ATP by the E1 adenylation domain, leading to ATP hydrolysis and the release of PPi. In the second step of E1 catalysis, SUMO is transferred to another active site in E1, Cys-173, forming a thioester bond with Cys-173, which results in the release of the covalently bound AMP. Both steps are reversibly.
ible. To examine SUMO adenylation, we adopted the E1-catalyzed ATP:PPi isotope exchange assay developed by Haas and Rose (12, 13), in which the E1-catalyzed exchange of ATP:PPi is measured by the increase in radioactive ATP from 32P-labeled PPi.

We compared the adenylation activities of the E1 mutants in an ATP:PPi exchange assay using 10 μM SUMO. The reaction time was chosen such that the ATP:PPi exchange level was linearly dependent on the exchange time for wild-type E1 such that the ATP:PPi exchange level reflected the initial exchange rates (supplemental Fig. 1). Using this assay, Δ445 retained more than 60% of the activity of wild-type E1, whereas Δ439 had almost no activity (Fig. 2D). This suggests the inability of this mutant to form the SUMO–E1 thioester conjugate is at least due to the loss of SUMO adenylation activity. Within experimental uncertainties, the C173S mutant had similar adenylation activity as wild-type E1, whereas Δ439 had almost no activity (Fig. 2D). This suggests the inability of this mutant to form the SUMO–E1 thioester conjugate is at least due to the loss of SUMO adenylation activity.

Within experimental uncertainties, the C173S mutant had similar adenylation activity as wild-type E1 (Fig. 2D), consistent with previous findings that the adenylation activity of the ubiquitin E1 is independent of E1–ubiquitin thioester formation (12, 13).

Among the site-directed mutants, the single mutants, N438K, P439A, and N440K, retained 40–60% of the wild-type E1 activity, and the double mutant, Y442A/V443A, also retained more than 40% of the wild-type E1 activity, indicating these residues play some role in adenylation. C444A had 17% of the wild-type enzyme activity, and one of the triple mutants, Y442A/V443A/C444A, had similar activity as C444A (~20% of wild-type activity), which is consistent with the critical role of the Zn2+ motif for E1 activity. Furthermore, the triple mutant, P439A/N440A/C441A, had almost no adenylation activity, similar to Δ439. Thus, the site-directed mutagenesis results are consistent with published data and the above mentioned activities of the deletion mutants in which Δ439 failed to form the SUMO–E1 thioester conjugate but Δ445 did. Similarly, the ~60% adenylation activity of Δ445, which contains an intact Zn2+ motif, also correlated with its ability to efficiently form the thioester conjugate with SUMO (Fig. 1). In addition, the reduction in activities of N438K, P439A, N440K, and Y442A/V443A suggests that other residues within and near the Zn2+ motif are also important.

**Importance of the Structural Integrity of the Zn2+ Motif—**To assess the importance of the structural integrity of the Zn2+ motif, we constructed a set of E1 mutants in which each of four cysteine residues of the Zn2+ motif (residues 158, 161, 441, and 444 of the SAE2 subunit) was replaced by an Ala. Each of these E1 mutants formed the E1–SUMO thioester conjugate within 5 min although with significantly reduced activities as compared with wild type (Fig. 3A). Similarly, the mutants could also stimulate SUMO conjugation to Ubc9 and RanGAP1 but with much less activity than the wild-type E1 (Fig. 3B).
To compare the adenylation activities quantitatively, ATP:PPi exchange assays were carried out for these mutants as discussed above. All Zn\(^{2+}\) motif mutants displayed measurable, but significantly reduced adenylation activities (Fig. 3). The slight differences among their activities in this assay could result from uncertainties arising from detection of much smaller amounts of radioactivity than that catalyzed by wild-type E1, although the variations among triplicate experiments were small. These results further confirmed the importance of the structural integrity of the Zn\(^{2+}\) motif for SUMO adenylation. Thus, taken together with the site-directed mutagenesis and Zn\(^{2+}\) motif studies, the complete loss of activity of P439A/N440A/C441A or Δ439 reflected a synergistic effect of losing the structural integrity and the contacting residues of the Zn\(^{2+}\) motif.

**Role of the Zn\(^{2+}\) Motif in SUMO Adenylation**

The adenylation reaction requires E1 to bind two substrates, SUMO and ATP. The ubiquitin and NEDD8 E1 enzymes use an ordered or pseudo-ordered mechanism to bind their two substrates; ATP is the leading substrate, and their cognate Ubl is the trailing substrate. The hallmark of the ordered or pseudo-ordered mechanism is inhibition of ATP:PPi exchange at high concentrations of ubiquitin or NEDD8, because binding a Ubl inhibits proper binding of ATP. We carried out a similar ATP:PPi exchange for the wild-type SUMO E1 and each of the mutants using increasing SUMO concentrations. The wild-type SUMO E1 displayed a similar pseudo-ordered substrate-binding mechanism (Fig. 4A); the rate of ATP:PPi exchange increased with increasing concentrations of SUMO up to \(5–10 \mu M\) and then decreased as the SUMO concentration increased. The E1 point mutants that displayed at least 40% of wild-type E1 adenylation activity (i.e. Δ445, N438K, P439A, N440K, and Y442A/V443A) showed a similar pseudo-ordered mechanism. However, the mutants that had reduced E1 activity also had lower maximum levels of ATP:PPi exchange at high concentrations of ubiquitin or NEDD8, because binding a Ubl inhibits proper binding of ATP. We carried out a similar ATP:PPi exchange for the wild-type SUMO E1 and each of the mutants using increasing SUMO concentrations. The wild-type SUMO E1 displayed a similar pseudo-ordered substrate binding mechanism (Fig. 4A); the rate of ATP:PPi exchange increased with increasing concentrations of SUMO up to \(5–10 \mu M\) and then decreased as the SUMO concentration increased. The E1 point mutants that displayed at least 40% of wild-type E1 adenylation activity (i.e. Δ445, N438K, P439A, N440K, and Y442A/V443A) showed a similar pseudo-ordered mechanism. However, the mutants that had reduced E1 activity also had lower maximum levels of ATP:PPi exchange (Fig. 4A), except for N438K, N438K displayed a slightly shifted maximum rate to higher SUMO concentration. All of these mutants contain all of the four Cys residues of the Zn\(^{2+}\) motif. These data suggest that some of these mutants have reduced interaction with SUMO in the forward reaction or with SUMO–AMP in the reverse reaction.

**FIGURE 3.** Structural integrity of the Zn\(^{2+}\) motif as assessed by site-directed mutagenesis of the four Cys residues. In vitro conjugation assays show the formation of the SAE2–SUMO thioester (A), Ubc9–SUMO thioester, and RanGAP1–SUMO isopeptide conjugate (B). Assay conditions and gel visualization methods are as described in Fig. 1A and B. Asterisks indicate samples mixed with DTT-containing gel-loading buffer to hydrolyze the thioester conjugate. C, SUMO adenylation activities of the Cys mutants as determined by ATP:PPi isotope exchange assays are shown. Details of the assay were the same as described for Fig. 2D. WT, wild type.

**FIGURE 4.** E1-catalyzed ATP:PPi isotope exchange assays at various SUMO concentrations to examine the effects of E1 mutations. A, shown is a line graph of ATP:PPi exchange, demonstrating that the SUMO E1 and the E1 mutants Δ445, N438K, P439A, N440K, and Y442A/V443A have a pseudo-ordered substrate-binding mechanism. WT, wild type. B, shown is a line graph of ATP:PPi exchange, demonstrating that the E1 mutants Δ439, Δ444A, P439A/N440A/C441A, and Y442A/V443A/C444A have a pseudo-ordered substrate-binding mechanism, and the ATP:PPi isotope exchange rates increase as SUMO concentrations rise. C, double-reciprocal plots of ATP:PPi isotope exchange rates versus SUMO concentrations for the wild-type and mutant E1s are shown. The plots were used to extract the \(k_{cat}\) and \(K_{1/2}\) values listed in Table 1.
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In contrast, the mutants that had 20% or less of wild-type E1 activity, such as Δ439, C444A, and the two triple mutants, were not inhibited by increasing SUMO concentrations (up to 800 μM) (Fig. 4B). Although individual mutants had different activities, all showed an ever-increasing exchange rate with increasing SUMO concentrations within the tested SUMO concentration range. All of these mutants lacked at least one of the Cys residues needed to coordinate Zn$^{2+}$ in the Zn$^{2+}$ motif. These results indicate that the mutants are severely deficient in binding SUMO, and thus, ATP binding was not inhibited over the SUMO concentration range used.

To quantitatively compare the kinetic properties of these E1 mutants, double-reciprocal plots over the SUMO concentration ranges before the onset of ATP binding inhibition were used to extract $K_{1/2}$ and/or $k_{cat}$ values (Fig. 4C) (18). The mutants that maintained the pseudo-ordered substrate binding mechanism had similar $K_{1/2}$ and $k_{cat}$ values as wild-type E1. However, mutants that lacked at least one Cys residue needed to coordinate Zn$^{2+}$ had much reduced $K_{1/2}$ and $k_{cat}$ values (Table 1). Because the forward and backward reactions were at equilibrium, the reduced $k_{cat}$ could result from a less than stoichiometric amount of SUMO—AMP bound to the mutant enzymes, for example, due to dissociation of the SUMO—AMP from the enzyme. The $K_{1/2}$ reflects both the internal rates and equilibrium constants for the formation of the ternary E1 complex (18).

### DISCUSSION

In this study we show that the SUMO E1 adenylation reaction, which involves SUMO and ATP, follows a pseudo-ordered substrate binding mechanism, preferring ATP as the leading substrate and SUMO as the trailing substrate. This is similar to the adenylation reactions involving the homologous NEDD8 and ubiquitin E1 enzymes, further illustrating the conservation of mechanisms used by activation enzymes for the family of ubiquitin-like modifiers.

This study has shown that the Zn$^{2+}$ motif of the SUMO E1, which is located in a cross-over loop connecting the structurally independent UBF and adenylation domains, is critical for SUMO activation. It is unlikely that the Zn$^{2+}$ motif is directly involved in catalysis of SUMO adenylation, because it is distal from the reaction center, and the large scale conformational changes in E1 demonstrated by previous and recent x-ray crystallographic studies would not bring the Zn$^{2+}$ motif into close proximity to the adenylation reaction center (8, 16). In addition, it is unlikely that the presence of the Zn$^{2+}$ motif is important for the large scale movement of the Cys domain to bring potential catalytic residues from the Cys domain to the adenylation center because amino acid substitutions of other residues in addition to the Cys of the Zn$^{2+}$ motif also significantly reduced adenylation activity.

The Zn$^{2+}$ motif is likely involved in direct binding of SUMO for adenylation. Site-directed mutations of the Cys residues that chelate the Zn$^{2+}$ ion disrupted the pseudo-ordered substrate binding mechanism of SUMO adenylation, indicating that structural integrity of the Zn$^{2+}$ motif is critical for binding SUMO for adenylation. In addition, site-directed as well as truncation mutations showed that residues surrounding the Zn$^{2+}$ motif, including residues 439, 440, 442, 443, are also important for SUMO adenylation. The Zn$^{2+}$ motif may also be involved in binding SUMO—AMP, as mutants that lacked one or two Cys residues in the Zn$^{2+}$ motif had significantly lower $k_{cat}$ values than wild-type E1, which may have resulted from dissociation of SUMO—AMP from the mutant enzymes. After adenylation, in the next step of SUMO activation, SUMO adenylate must dissociate from the ATP-binding site and the adenylation domain to form the thioester conjugate. Sufficient binding of E1 to SUMO—AMP is needed to prevent its loss before thioester formation.

A surface on SUMO that is close to the Zn$^{2+}$ motif could be responsible for interacting with the Zn$^{2+}$ motif. This surface of SUMO contains three residues (63, 69, and 70; Fig. 1D, labeled in red) that are equivalent, based on sequence alignment, to the critical surface residues (42, 48, and 49) in ubiquitin, revealed by a previous Ala-scanning mutagenesis (23). In that Ala-scanning mutagenesis, surface ubiquitin residues that are vital for functions in *Saccharomyces cerevisiae* were identified by temperature sensitivity. Only 16 of 63 surface ubiquitin residues were found to be important for yeast growth, and these 16 residues included a surface equivalent to the surface in SUMO that is proximal to the Zn$^{2+}$ motif. The potential importance of these SUMO residues is also suggested by their conservation among the three SUMO paralogues and/or conservation between the human and yeast SUMO-1 (24). The cross-over loop containing the Zn$^{2+}$ motif has significant conformational flexibility, as shown by comparison of the crystal structures of NEDD8 or SUMO E1 in complexes with their respective Ubl (8, 10, 16, 25). Movement of the Zn$^{2+}$ motif and/or SUMO/NEDD8 by a few angstroms would allow the direct interactions.

Direct interaction of the Zn$^{2+}$ motif of the SUMO or NEDD8 E1 with their respective Ubl was not observed in the crystal structures, including the recent structure of E1 in complex with a SUMO adenylate mimic (16). This suggests that the interaction is not of very high affinity and did not occur under crystallization conditions. The lack of the interaction in the crystal structures is responsible for the lack of SUMO or NEDD8 adenylation in the crystal structures with their respective E1 and ATP, although adenylation occurs rapidly in the presence of E1 and ATP under physiological conditions. Weak interactions can make dramatic contributions to binding and activity in the presence of other binding sites (11).

In conclusion, the studies described here have elucidated an important role of the cross-over loop of the SUMO E1 in SUMO adenylation. This loop undergoes conformational changes in the E1 catalytic cycle (10, 11). In this study we found that this loop is not only the flexible linker that connects struc-

### TABLE 1

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<td>0.046 ± 0.00067</td>
<td>0.016 ± 0.0042</td>
</tr>
<tr>
<td>Y442A/V443A/C444A</td>
<td>0.041 ± 0.025</td>
<td>0.2 ± 0.13</td>
</tr>
</tbody>
</table>
naturally independent domains of E1 but also directly participates in binding SUMO to activate it for modifications. Our findings also suggest that the cross-over loops in other E1s also play important roles in ubiquitin-like modifications. Although the ubiquitin E1 does not contain a Zn\textsuperscript{2+}/H11545 motif (26), the analogous cross-over loop may be involved in binding ubiquitin during ubiquitin activation. Because of the conserved structure and chemistry, the E1 enzymes across the Ubl family likely share similar enzymatic mechanisms, including the role of the cross-over loop in adenylation.

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REFERENCES
