Ubiquitin Manipulation by an E2 Conjugating Enzyme Using a Novel Covalent Intermediate*

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Nadine Merkley, Kathryn R. Barber, and Gary S. Shaw‡
From the Department of Biochemistry, The University of Western Ontario, London, Ontario N6A 5C1, Canada

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The ubiquitin-dependent proteolysis pathway controls the removal of damaged and misfolded proteins in the cell. One of the key steps in this pathway is the assembly of a polyubiquitin chain that ultimately targets a substrate for degradation (1, 2). This process involves tagging of a substrate protein with an arrangement of four to eight ubiquitin molecules linked in series through the C-terminal Gly76 of one ubiquitin molecule and the side-chain ε-NH2 from Lys48 of another (3). Once tagged, the polyubiquitinated protein is recognized by the 26 S proteasome, where it is degraded. The building of a polyubiquitin chain and subsequent labeling of a substrate protein is a complex process potentially involving the passage of ubiquitin through three different enzymes (1, 4, 5). Initially, ubiquitin is activated in an ATP-dependent step forming a high energy E1-ubiquitin thioester complex. Ubiquitin is then transferred to an E2 or ubiquitin-conjugating enzyme forming a thioester intermediate. E2 proteins have been demonstrated recently to bind to the ubiquitin-like domain of the E1 providing insight into the mechanism in which the thioester-bound ubiquitin is passed to the E2 (6–8). Two different E3 ligase proteins can catalyze the final passage of ubiquitin to the substrate. For RING E3 ligases, ubiquitin or a polyubiquitin chain is transferred directly from the E2 to the substrate, whereas HECT E3 ligases form an ubiquitin-E3 thioester prior to ubiquitin transfer to the substrate (5).

The E2 conjugating proteins are the key enzymes in this pathway because they are required to transfer ubiquitin either to the E3 ligase (HECT E3) or to the substrate (RING E3). All E2 proteins have a 150-residue catalytic domain that is structurally conserved throughout many species and contains the cysteine residue necessary for thioester formation with Gly76 of ubiquitin. Structures of E2 proteins show that the catalytic domain has an αβ-fold that is maintained upon complexation with either HECT (9) or RING (10, 11) E3 ligases. Details on the involvement of the E2 thioester in the polyubiquitin chain-building process or the mechanism for transfer of ubiquitin from an E2 protein to an E3 or substrate are less certain. The E2 proteins Ubc1 and Ubc3 (Cdc34) from Saccharomyces cerevisiae assemble polyubiquitin chains and exhibit autoubiquitination activities (12, 13), whereas the mammalian E2 protein E2-25K can assemble polyubiquitin chains in the absence of an E3 enzyme (14). Furthermore, Ubc1 has been shown to be important for the creation of polyubiquitin chains required for protein labeling and subsequent degradation (12).

The three-dimensional structure of Ubc1 from S. cerevisiae, determined by NMR spectroscopy, provides some insights into these biological activities (15). The structure shows that Ubc1 and its related class II conjugating enzymes form a unique two-domain protein having a typical αβ-fold catalytic domain connected via a flexible tether to a C-terminal UBA (ubiquitin-associated) domain. UBA domains, such as the one in Ubc1, are capable of interaction with mono- or polyubiquitin chains in a non-covalent fashion and may result in either inhibition of degradation or transfer enhancement via ubiquitin interaction (16–19). The E2 conjugating protein Ubc1 is a well positioned candidate to examine the transfer of ubiquitin from the ubiq-
ubiquitin-E2 thioester because of its ability to moderate and build polyubiquitin chains.

Mechanistic experiments that examine the role of the ubiquitin-E2 thioester in polyubiquitin chain assembly have been difficult because of the inherent instability of the thioester complex. To date the best details have been garnered from models derived from NMR chemical shift perturbation data for ubiquitin-E2 thioester intermediates (20). Attempts to stabilize this complex for more detailed structural and mechanistic experiments have met with limited success. To circumvent this we have created a novel disulfide-linked ubiquitin-Ubc1 complex that mimics the ubiquitin-E2 thioester intermediate. We show that this ubiquitin-Ubc1 complex can be purified in high amounts, is stable for long periods of time, and has similar structural characteristics to the ubiquitin-E2 thioester intermediate. We have used this complex and NMR spectroscopy to show that the UBA domain can bind ubiquitin in a non-covalent fashion even in the presence of an ubiquitin molecule covalently bound at the catalytic domain. These results provide a glimpse at the first step of polyubiquitin chain formation by Ubc1 and its related class II E2 enzymes.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification—Recombinant S. cerevisiae Ubc11–150, Ubc1, and ubiquitin (Ub) were overexpressed in Escherichia coli BL21(DE3)pLysS and purified as described previously (12). Both Ubc1 proteins carried K93R mutations, and ubiquitin contained a K48R mutation. All samples were prepared at pH 7.5 in 90% D2O. Isotopically labeled UbCys-[15N]Ubc1. NMR spectra were processed with NMRPipe (24) and analyzed with the software Prism 4. All protein concentrations were determined in triplicate by amino acid analysis.

RESULTS

The class II E2 enzyme Ubc1 from S. cerevisiae is a flexible two-domain protein comprising a canonical N-terminal catalytic domain and a C-terminal UBA domain (Fig. 1). The catalytic domain of Ubc1 contains four α-helices (α1, Lys5–Ala13; α2, Leu102–Leu113; α3, Ala17–Leu181; α4, Arg134–Leu147) and four β-strands (β1, Ile92–Leu94; β2, His124–Leu126; β3, Lys31–Val58; β4, Lys68–Gln70), and the fold is typical for E2 proteins (26). The catalytic cysteine (Cys88) used in the formation of the ubiquitin-E2 thioester intermediate is found in a relatively unstructured region below helix α2 and is the only cysteine in the protein. In Ubc1 and other class II E2 proteins, a C-terminal UBA domain consisting of three α-helices (α5, His170–Glu177; α6, Lys194–Arg196; α7, Asn197–Leu201) is present. Unlike the catalytic domain, the UBA domain is able to interact with ubiquitin in a non-covalent manner (15).

The ubiquitin-E2 thioester intermediate formed in the ubiquitin-mediated proteolysis pathway occurs between the C-terminal Gly76 carboxylate of ubiquitin and the side-chain thiol of Cys88. Based on a model of the Ubc1-ubiquitin thioester, the length of this linkage from Gly76 to Cys88 is ~4.8 Å. In attempting to stabilize the linkage between Ubc1 and ubiquitin we wanted to preserve this distance while increasing the stability of this junction to a significant extent. To accomplish this, we replaced the ubiquitin Gly76 with a cysteine residue (UbCys) to form a disulfide linkage between the side chains of Cys88 in ubiquitin and Cys88 in Ubc1. In this process the geometry of the ubiquitin-E2 thioester should be unaffected. Ubc1 is an ideal candidate to model this disulfide reaction because its sequence contains exclusively the catalytic cysteine preventing any multiple ubiquitin adducts or disulfide exchange between different sites from occurring. Furthermore, in UbCys the cysteine side chain, being at the C terminus of the protein, should be flexible enough to adopt a preferred conformation that may be dictated by interaction with Ubc1. In modeling studies, the span between the Ca atoms of Cys88 in UbCys and Cys88 in Ubc1 was ~4.9–5.5 Å, a distance similar to that observed in the native ubiquitin-E2 thioester.

Formation of a Stable, Covalent Ubiquitin-E2 “Intermediate”—A disulfide-linked complex between UbCys and Ubc1...
(UbCys–Ubc1) was formed by mixing fully reduced UbCys with an excess of fully reduced E2 protein in phosphate buffer containing a catalytic amount of Cu²⁺ as an oxidizing agent. In both cases the K93R mutant of Ubc1 and the K48R mutant of ubiquitin were used. These mutations are utilized to prevent autoubiquitination and polyubiquitin chain formation reactions from occurring, although these reactions were not a factor in our experiments. A disulfide complex between UbCys and a truncated version of Ubc1 lacking the C-terminal domain (Ubc1¹–¹⁵⁰) was also synthesized in addition to the complex utilizing the full-length Ubc1 protein containing the C-terminal UBA domain. Formation of the UbCys–E2 complex occurred for both Ubc1 and Ubc1¹–¹⁵⁰ over a period of 10 h at room temperature at which point the UbCys had been exhausted (Fig. 2A). In both experiments two products were observed, the UbCys–Ubc1 disulfide (or UbCys–Ubc1¹–¹⁵⁰) as the major species and a minor amount of UbCys–UbCys disulfide. Over the same time period and under identical conditions there was little difference between the rates or extent of formation for UbCys–Ubc1 compared with that of the truncated UbCys–Ubc1¹–¹⁵⁰ indicating that the C-terminal UBA domain in Ubc1 does not appear to enhance or inhibit formation of the disulfide. Both UbCys–Ubc1 and UbCys–Ubc1¹–¹⁵⁰ could be readily purified by size exclusion chromatography, and their identities were confirmed by mass spectrometry (UbCys–Ubc1 MWobs 33,270.7, MWcalc 33,274.2; UbCys–Ubc1¹–¹⁵⁰ MWobs 25,421.4, MWcalc 25,420.1). Because Ubc1 and Ubc1¹–¹⁵⁰ contain a single cysteine residue, which is at the catalytic site, the UbCys–Ubc1 (and UbCys–Ubc1¹–¹⁵⁰) disulfide must be formed using the catalytic site cysteine. The complexes were stable in solution for several weeks at room temperature. Formation of a disulfide-linked complex between UbCys and other E2 proteins was tested to assess the general applicability of this method. Both of the human E2 proteins, Ubc13 and UbcH7, formed stable disulfide complexes using the identical conditions for synthesis of UbCys–Ubc1. The sequence of Ubc13 has exclusively the catalytic cysteine and like Ubc1, the reaction afforded a single product, UbCys–Ubc13 (Fig. 2B) identified by mass spectrometry (UbCys–Ubc13 MWobs 26,180.8, MWcalc 26,180.2). A disulfide reaction between UbCys and UbcH7 yielded a single UbCys– UbcH7 disulfide, and multiubiquitin adducts were not observed by non-reducing gel electrophoresis (Fig. 2B), although UbcH7 has a catalytic cysteine residue and two additional cysteine residues.

UbCys–Ubc1 Mimics an Ubiquitin-E2 Thioester Intermediate—The instability of the ubiquitin-E2 thioester precluded three-dimensional structure determination of this species and limited characterization of the interface between the two proteins. NMR spectroscopy was used to identify residues in ubiquitin or Ubc1¹–¹⁵⁰ that decreased in intensity during formation of the thioester. However, the stability of the thioester (~1 h) did not allow sequence-specific assignment of ¹H-¹⁵N resonances in the Ub-E2 thioester complex thereby limiting this approach (27). Because UbCys–Ubc1 is orders of magnitude more stable than its thioester counterpart this has now allowed the ¹H, ¹³C, and ¹⁵N resonance ( backbone) assignment of the 33-kDa UbCys–Ubc1 complex using conventional triple resonance techniques. Fig. 3 shows the ¹H-¹⁵N HSQC spectrum of the UbCys–Ubc1 complex using ¹⁵N-labeled Ubc1 and unlabeled UbCys. In general the spectrum is similar to Ubc1 alone indicating that disulfide formation between UbCys and Ubc1 did not result in global conformational changes in Ubc1. In addition calculation of the Chemical Shift Index (CSI) using CA, CB, and C' shifts (28) indicated that there was little change in the secondary structure of Ubc1 upon formation of the disulfide complex.

Although the inter-residue distance between the active site Cys⁸⁸ in Ubc1 and the C-terminal Cys⁷⁶ in UbCys is similar to that in the thioester, it is possible that intermolecular contacts between the two proteins could be modified. A subtle difference also exists between the UbCys–Ubc1 disulfide and the Ub–Ubc1 thioester. Because of the difference in covalent linkage, the UbCys–Ubc1 disulfide possesses a free C-terminal carboxylate that would normally be used to form the thioester bond. Previously, the ubiquitin-Ubc1 interface for the Ubc1–¹¹⁰ thioester has been partially characterized by following changes in the ¹³C resonance line widths from the ¹⁵N HSQC spectra upon thioester formation (20). The protein-protein interface for the Ub–Ubc1–¹¹⁰ thioester complex was compared with that of UbCys–Ubc1¹–¹⁵⁰ using ¹⁵N-labeled UbCys to probe the similarity of the two interfaces for the two complexes. In the absence of Ubc1¹–¹⁵⁰, the ¹⁵N HSQC for UbCys is well resolved with most resonances in similar positions to those found in the wild-type protein (Fig. 4A). Upon formation of UbCys–Ubc1¹–¹⁵⁰ several resonances shift to new positions including Arg⁶⁸, Val⁷⁰, Leu⁷¹, Arg⁷², Leu⁷⁵, and Cys⁷⁶ in UbCys (Fig. 4B). These residues are essentially the same as those identified in the Ub–Ubc1¹–¹⁵⁰ thioester (Fig. 4C) (20) and are found in a tight cluster in the ¹⁵N HSQC spectra. Histograms of the chemical shift perturbations for both UbCys (Fig. 4D) and Ub (Fig. 4E) upon complex formation have similar patterns indicating that the same residues are affected in ubiquitin at the ubiquitin-E2 interface in both complexes. Minor variations between the histograms may be a result of the changes in experimental conditions (pH and ionic strength) upon sequential addition of the reaction mixture (E1 and ATP) to the Ub, E2 sample, which is required for the synthesis of the Ub–Ubc1¹–¹⁵⁰ thioester in situ (20). The UbCys–Ubc1¹–¹⁵⁰ on the other hand could be purified, and the pH and ionic strength was controlled. In addition, residual Ub correlations are present in the ¹⁵N HSQC spectrum of the thioester (Fig. 4C) as a result of incomplete thioester formation hindering the analysis of the chemical shift perturbations. Several of the Ub¹–¹⁵⁰ amide resonances in the UbCys–Ubc1 disulfide complex underwent chemical shift changes (residues Lys⁷⁴, Ser⁹¹, Cys⁸⁸, Trp⁹⁶, Ile¹⁰⁰, Ala¹⁰⁵, Ser¹¹⁵, and Asn¹¹⁹) or extensive line broadening (residues Ile⁸⁷, Leu⁸⁹, Ile⁹¹, and Leu⁹²) as a result of complex formation. These
are common to those reported to undergo a decrease in peak intensity in the Ub-Ubc1 thioester intermediate (27), indicating that the interacting surface in Ubc1 is similar to that of the thioester intermediate. The interacting residues in both Ubc1 and ubiquitin in the disulfide complex are similar to those observed in the thioester, indicating that the disulfide complex mimics the thioester intermediate, and a minimal influence on the E2 surface is created by the presence of a charged carboxylate group near the active site.

Two major advantages exist for the UbCys-Ubc1 disulfide complex. First, the complex contains no side products such as uncomplexed ubiquitin or E2 that can hamper data analysis. For example, in the UbCys-Ubc1 complex, resonances resulting from residues in the “tail” region of ubiquitin (Val70, Leu71, Arg72, and Leu73) are completely absent (Fig. 4B) from their original positions in UbCys (Fig. 4A). However in the Ub-Ubc11–150 thioester (Fig. 4C), remnants of these peaks exist because formation of the thioester only occurs to /H11011 90% completion.

Second, the stability of UbCys-Ubc11–150 has allowed backbone NMR assignments to be obtained for the UbCys component enabling identification of the new resonance positions in the UbCys-Ubc11–150 complex. This was not possible in the thioester due to hydrolysis of the Ub-Ubc11–150 thioester bond under aqueous conditions. Overall, the formation of the UbCys-Ubc11–150 complex not only mimics the Ub-Ubc11–150 thioester structure but also facilitates its analysis.

Ubc1 Coordinates Two Ubiquitin Molecules at Different Sites—In addition to the catalytic domain, Ubc1 has a C-terminal UBA domain that non-covalently binds ubiquitin (15). UBA domains bind mono- or polyubiquitin chains with a dissociation constant between 300 and 500 /H9262 M (29, 30). Ubc1 has the potential to coordinate two ubiquitin molecules simultaneously, suggesting that during the Ub-Ubc1 thioester formation process, it is possible that the UBA domain might guide or perturb the interaction of ubiquitin with Ubc1. Alternatively, the UBA domain could also interact with the thioester-bound ubiquitin once the bond is formed at Cys88 of Ubc1. Identification of either of these steps would provide insight into the first step of polyubiquitin chain formation. The UbCys-Ubc1 disulfide allowed us to examine the ubiquitin binding properties of the UBA domain of Ubc1 in the UbCys-Ubc1 disulfide complex. A similar investigation of the ubiquitin binding properties of the UBA in the Ub-Ubc1 thioester complex has not been possible because of the transient nature of the thioester conjugate.

We probed the UbCys-Ubc1 complex to determine whether this enzyme could coordinate two ubiquitin molecules concurrently. The UbCys-[15N]Ubc1 complex was titrated with unlabelled ubiquitin, and each addition was followed by 1H-15N

FIG. 3. 1H-15N HSQC spectrum of UbCys-Ubc1. The spectrum was collected at 600 MHz for a UbCys-Ubc1 complex containing [Val,Leu,ile-(61)-methyl-protonated-13C,15N]Ubc1 in 25 mM Tris, 1 mM EDTA, 250 mM NaCl at pH 7.5. Cross-peaks for the backbone amides are labeled with their one-letter amino acid code and position. Unassigned correlations (star) and minor impurities in the original Ubc1 spectrum (#) are indicated. Inset A is an expanded view of a crowded region. Pairs of side-chain amide resonances are connected with lines.
changes are assessed based on the nearest new peak to the original. Chemical shift changes were quantified using the equation

\[ \text{initio} \]

assignment of the residues of the thioester was not possible because of the instability of the thioester complex. As a result the chemical shift

\[
\text{[13C,15N]UbCys-Ubc11–150 (31736) for a number of af-}
\]

fected residues. This resulted in a dissociation constant of

\[ A \]

non-linear least-squares fitting (Fig. 6). Perturbations for thioester formation were reanalyzed from the data presented in Ref. 20. Ab initio assignment of the residues of the thioester was not possible because of the instability of the thioester complex. As a result the chemical shift changes are assessed based on the nearest new peak to the original. Chemical shift changes were quantified using the equation

\[ \Delta \delta = 0.5(\Delta \delta^3) + 0.125(\Delta \delta^3) \]

Bars marked with an asterisk are peaks that were perturbed by more than ±0.3 ppm in the \(^{31}N\) dimension or ±0.05 ppm in the \(^1H\) dimension.

HSQC spectroscopy. Fig. 5 shows a portion of the \(^1H,^{15}N\) HSQC spectrum of UbCys-[\(^{15}N\)]UbC1 in which resonances Gly180, Asn201, and Asn207 are perturbed upon ubiquitin binding (Fig. 5A). In addition, residues Glu177, Ser178, and Glu211 in the UBA domain of the UbCys-Ubc1 complex are strongly affected by ubiquitin binding (Fig. 5B). Several other residues (His170, Asp199, Asn203, Thr205, Ala206, Arg208, Ile209, and Leu214) in the UBA domain of the UbCys-Ubc1 complex underwent smaller but significant chemical shift changes. Overall the pattern of residues most affected in UbCys-Ubc1 upon interaction with ubiquitin, was specific to the UBA domain of UbC1. This is remarkably similar to that observed in UbC1 alone (Fig. 5, B and C). To quantify the interaction of ubiquitin with the UbCys-Ubc1 complex, the \(^15N\) chemical shift changes were analyzed by non-linear least-squares fitting (Fig. 6A) for a number of affected residues. This resulted in a dissociation constant of 259 ± 105 \(\mu M\). The dissociation constant calculated from \(^15N\) chemical shift changes for UbC1 titrated with Ub was 280 ± 69 \(\mu M\) using comparable residues. When the data for the ubiquitin interaction with UbCys-Ubc1 were normalized and compared with the interaction of ubiquitin with UbC1 alone, the curves were nearly superimposable, reinforcing the similarity of the strength and stoichiometries of the interactions (Fig. 6B).

**DISCUSSION**

Polyubiquitin chains linked through the C-terminal glycine of one ubiquitin to Lys48 of another are a required recognition motif for substrate degradation by the 26 S proteasome. The mechanism in which these polyubiquitin chains are assembled is unclear. Two possibilities exist in which either the polyubiquitin chain is constructed on the E2 protein and transferred to an E3 or substrate, or the chain is built on the target substrate. In both cases the ubiquitin-E2 thioester complex is a key intermediate in this process. The transient nature of this thioester species has not allowed its three-dimensional structure to be determined or permitted direct experimentation showing how the thioester participates in the assembly of polyubiquitin chains. To date, the best structural analysis of the ubiquitin-E2 thioester complex has been found from peak intensity changes in NMR experiments upon thioester formation. This has indicated that the surface on ubiquitin at the protein-protein interface encompasses residues Val70–Gly76 and Arg48 (27). In the current work, the ubiquitin-E2 disulfide is the first thioester intermediate synthesized that mimics this surface. In the disulfide the ubiquitin surface includes residues Arg48, Val70–Leu73, and Cys76, which are common not only to those in the thioester but also in the UBA domain interface. This indicates the ubiquitin-E2 disulfide is a suitable mimic of the transient ubiquitin-E2 thioester.

The ubiquitin-Ubc1 disulfide complex is straightforward to synthesize in high yields and may be purified from any remaining starting materials by size exclusion chromatography. The product is stable for several weeks at room temperature. This is a remarkable improvement from other attempts to stabilize the ubiquitin-E2 linkage. For example, mutation of the active site cysteine in the E2, HsUbc2b, to a serine residue has allowed formation of an ester linkage between ubiquitin and the E2 (31–33). Although the resulting complex could be purified, it was short-lived at pH 6.7 and unstable at alkaline pH impeding any attempts to model the association of the two proteins or to use the complex for biochemical characterization (31). However, chemical shift analysis revealed that residues Val70–Gly76 and Lys48 on ubiquitin were most significantly perturbed upon HsUbc2b binding, a similar observation to that obtained for the ubiquitin-Ubc1 thioester and the disulfide complex described here. An alternative procedure traps the thioester intermediate by reducing it to a hemithioacetal with sodium borohydride.

**Fig. 4.** UbCys–Ubc11–150 mimics the Ub–Ubc11–150 thioester intermediate. A–C, selected regions of \(^1H,^{15}N\) HSQC spectra for \[^{15}N\]UbCys (A), \[^{13}C,^{15}N\]UbCys–Ubc11–150 (B), and \[^{15}N\]UbCys–Ubc11–150 thioester (C). In spectra B perturbed residues are marked with a circle and include Arg48, Val70, Leu71, Arg72, Leu73, and Cys76, and in C, residues that decreased in intensity (Arg48, Val70, Leu71, Arg72, and Leu73) are marked with a square (see “Results” for details). All spectra were collected at pH 7.5 and 35 °C. Histograms of the chemical shift perturbations in: UbCys upon disulfide formation (D) and Ub upon thioester formation (E). Perturbations for thioester formation were reanalyzed from the data presented in Ref. 20. A

Ab
This approach was successful in stabilizing the complex between ubiquitin and an ubiquitin hydrolase for several days allowing for structural characterization by NMR spectroscopy (34). Our method for mimicking the ubiquitin-E2 thioester by forming a stable disulfide complex with *S. cerevisiae* Ubc1, human Ubc13, and UbcH7 E2 proteins can be used for mechanistic or structural studies with ubiquitin and other ubiquitin-conjugating enzymes. Approximately 20% of E2 conjugating enzymes contain exclusively the catalytic cysteine residue (35), and this procedure can be used as described in this work. For other conjugating enzymes such as UbcH7, which contains multiple cysteine residues, site-directed mutagenesis may be required to mutate the non-catalytic cysteine residues. In addition, the generality of this procedure illustrates that the disulfide complex designed could be used to investigate the intermediates between ubiquitin and other ubiquitin pathway enzymes, for example, E1 enzymes or HECT E3 ligases.

A unique feature of Ubc1 is that it is a flexible two-domain protein that consists of catalytic and UBA domains, representative of several other class II E2 proteins. Upon titration of UbCys-Ubc1 with Ub, the UBA domain is able to non-covalently bind a second ubiquitin molecule. Therefore, Ubc1 has the ability to bind an ubiquitin molecule as a thioester at the active site of the catalytic domain and a second ubiquitin at the C-terminal UBA domain. The ability of Ubc1 to interact with two ubiquitin proteins may explain how Ubc1 and its related class II E2 proteins can build or manipulate polyubiquitin chains. The presence of a UBA domain affects the chain-building properties of two homologous class II E2 proteins *in vitro*, Ubc1 and E2-25K. Ubc1 undergoes autoubiquitination by assembling polyubiquitin chains at Lys93 near the Cys88 active site (12). Polyubiquitin chains comprising up to 10–12 ubiquitin molecules are assembled in the absence of the UBA domain. Shorter chains averaging only four ubiquitin molecules are built when full-length Ubc1 is utilized. A flexible tether (22 amino acids) links the catalytic and UBA domains in Ubc1. This tether is long enough to allow the UBA domain to reach the thioester-bound ubiquitin thereby either aiding or interfering with thioester formation or polyubiquitin chain formation. However, our data show that the UBA domain does not interfere with disulfide formation. Furthermore, the affinity of the UBA domain for ubiquitin is not affected by the presence of a covalently attached ubiquitin at the catalytic site. This indicates that any interaction between the thioester-bound ubiquitin and the UBA domain is either very weak or does not occur. It is possible that the UBA domain may perturb the chain elongation step by potentially interacting with the growing thioester-bound polyubiquitin chain upon assembly of longer polyubiquitin chains at the thioester or on Lys83. A related class II E2 protein, E2-25K, has a flexible two-domain structure similar to Ubc1 and does not autoubiquitinate itself. Instead unanchored polyubiquitin chains are detected *in vitro* (14). Deletion of the UBA domain in E2-25K results in termination of polyubiquitin chain formation, although ubiquitin-E2 thioester formation proceeds (36). Both of these observations are consistent with our observations that the UBA domain in Ubc1 does not affect initial thioester formation and must be involved in construction of polyubiquitin chains. Further structural characterization of the ubiquitin-Ubc1 disulfide should
provide some insight into how the UBA domain influences polyubiquitin chain assembly by Ubc1 and other related class II E2 proteins.

From a mechanistic perspective, the ability of Ubc1 to bind two ubiquitin molecules may allow the thioester-bound acceptor ubiquitin (Gly76) to be in close proximity to the UBA-bound donor ubiquitin, which contains the nucleophilic side-chain ε-NH₂ of Lys⁴⁸. Recently, mechanisms for the construction of polyubiquitin chains have been proposed in which the acceptor ubiquitin is near the donor ubiquitin in other E2 complexes. Assembly of Lys⁶³-linked polyubiquitin chains by the canonical E2 (Ubc13) proceeds through a heterodimer of Ubc13 with an inert E2 variant (Mms2) that non-covalently binds ubiquitin (37). A thioester formed between Ubc13 and ubiquitin accepts a polyubiquitin chain assembly by Ubc1 and other related class II E2 proteins.

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