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The E2-25K Ubiquitin-associated (UBA) Domain Aids in Polyubiquitin Chain Synthesis and Linkage Specificity

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Abstract

E2-25K is an ubiquitin-conjugating enzyme with the ability to synthesize Lys48-linked polyubiquitin chains. E2-25K and its homologues represent the only known E2 enzymes which contain a C-terminal ubiquitin-associated (UBA) domain as well as the conserved catalytic ubiquitin-conjugating (UBC) domain. As an additional non-covalent binding surface for ubiquitin, the UBA domain must provide some functional specialization. We mapped the protein-protein interface involved in the E2-25K UBA/ubiquitin complex by solution nuclear magnetic resonance (NMR) spectroscopy and subsequently modeled the structure of the complex. Domain-domain interactions between the E2-25K catalytic UBC domain and the UBA domain do not induce significant structural changes in the UBA domain or alter the affinity of the UBA domain for ubiquitin. We determined that one of the roles of the C-terminal UBA domain, in the context of E2-25K, is to increase processivity in Lys48-linked polyubiquitin chain synthesis, possibly through increased binding to the ubiquitinated substrate. Additionally, we see evidence that the UBA domain directs specificity in polyubiquitin chain linkage.

Keywords

Ubiquitin; Ubiquitin-conjugating enzyme; E2-25K; UBA domain

1. Introduction

The ubiquitin-proteasome system is one of the primary means by which the cell degrades and recycles proteins. Protein molecules are tagged for interaction with the proteasome by covalent attachment of multiple linked units of the small protein ubiquitin. Conjugation of a target protein with ubiquitin requires a multi-step, multi-enzyme process. The first step is the ATP-dependent formation of a thioester bond between the C-terminal Gly76 residue of ubiquitin and the E1 ubiquitin-activating enzyme [1]. The ubiquitin thioester bond is transferred to a conserved catalytic cysteine residue of the E2 ubiquitin-conjugating enzyme. The E2 catalyzes the formation of an isopeptide bond between the ubiquitin C-terminal

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carboxylate group and the ϵ -amino group of a substrate lysine residue, either directly or through indirect transfer via an E3 ubiquitin ligase. Subsequent addition of ubiquitin molecules to the Lys48 residue of the conjugated ubiquitin builds a polyubiquitin (polyUb) chain that is recognized by the 19S proteasome subunit. Deubiquitinating enzymes remove and break down the polyUb chain while the target protein is degraded by the proteasome [2, 3].

E2-25K was initially identified [4] as a 25-kDa mammalian E2 conjugating enzyme with the ability to generate Lys48-linked polyUb chains, independent of the presence of protein substrate or E3 ligase. The significance of its unique C-terminal ubiquitin-associated (UBA) domain in the context of an E2 was not well understood, but was proposed to play a role in polyUb chain formation [5].

We examined the non-covalent binding of E2-25K to ubiquitin (Ub) by NMR chemical shift perturbation and subsequently modeled the E2-25K UBA domain/Ub complex using HADDOCK [6]. Results of polyUb chain synthesis assays point to a role for the UBA domain in polyUb chain elongation and linkage specificity.

2. Materials and Methods

2.1 Plasmids

The UBC domain (residues M1-S159) and UBA domain (residues G154-N200) of E2-25K were PCR amplified using full-length E2-25K in a pET30b expression vector as a template, and subcloned into pET28b for expression (to change antibiotic resistance from ampicillin to kanamycin). Clones were sequence verified (Functional Biosciences, Inc) and named UBC and UBA, respectively.

Wild-type ubiquitin (Ub) was PCR amplified (using a pET15b expression vector encoding ubiquitin as a template), and subcloned into pET28b for expression. Ubiquitin D77 and K48C mutants were created by site-directed mutagenesis. Clones were sequence verified (Functional Biosciences, Inc) and named Ub-D77 and Ub-K48C, respectively.

2.2 Protein expression and purification

The E2-25K, UBC domain, Ub, Ub-D77, and Ub-K48C plasmids were expressed and purified as previously described [7]. Fractions from a Ni²⁺-column (GE Healthcare) containing E2-25K were pooled and dialyzed against enterokinase buffer (20 mM Tris HCl, pH 7.4, 50 mM NaCl, 2 mM CaCl₂). Digestion with enterokinase was allowed to proceed overnight and protein was purified by ion-exchange chromatography on a Mono Q (GE Healthcare) column. Fractions containing digested E2-25K were pooled, dialyzed against assay buffer (20 mM Tris HCl, pH 8.0, 50 mM NaCl) and concentrated by ultra-filtration (Amicon) to approximately 1.6 mg/ml. Purified E2-25K UBC domain was dialyzed against assay buffer and concentrated by ultra-filtration to approximately 4.3 mg/ml. Purified Ub, Ub-K48C and Ub-D77 were dialyzed against 50 mM Tris-HCl, pH 8.0, 0.1 mM EDTA and 0.5 mM DTT and concentrated by ultra-filtration to approximately 10.8, 4.1 and 1.3 mg/ml, respectively.

2.3 Isotopic labeling

Labeled NMR sample proteins were prepared as described above except that transformed BL21(DE3) *E. coli* were grown at 37° C to an OD₆₀₀ of ~0.6 in M9 medium containing 35 µg/ml kanamycin and either 1 g/L ¹⁵NH₄Cl, or 2 g/L U-¹³C₆-glucose and 1 g/L ¹⁵NH₄Cl. The temperature was lowered to 18° C after induction and cultures were grown overnight.

Fractions from a Ni²⁺-column (GE Healthcare) containing E2-25K UBA domain were pooled and dialyzed against 20 mM Tris-HCl, pH 8.0, 50 mM NaCl before being applied to a HiTrap Q and SP column series (GE Healthcare) and eluted with 1 M NaCl. Purified E2-25K UBA domain was dialyzed against 50 mM sodium phosphate buffer, 100 mM NaCl, 0.1 mM BME, pH 7.0, and concentrated by ultra-filtration (Amicon) to ~6.5 mg/ml. Purified, digested E2-25K was dialyzed against 50 mM Na phosphate buffer, pH 6.5, and concentrated by ultra-filtration to ~12 mg/ml. Purified Ub was dialyzed against 50 mM sodium phosphate, pH 7.2, 100 mM NaCl, 0.1 mM BME, and concentrated by ultra-filtration to ~0.6mM. All final NMR samples contained 0.12 mM sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS), and 0.9% (v/v) NaN₃ in 90% H₂O/10% D₂O.

2.4 NMR Binding Experiments

Binding of ¹⁵N-labeled E2-25K UBA domain to ubiquitin was examined by solution NMR using ¹⁵N-HSQC chemical shift perturbation mapping [8]. Increasing amounts of unlabeled Ub (34.8 mM) to a maximum of 3.5 Ub/UBA were titrated into ¹⁵N-labeled E2-25K UBA domain (0.5mM). Specific changes in chemical shift values were used to identify amino acids in the UBA domain whose chemical environment was altered upon binding [9].

Titration of ¹³C, ¹⁵N-enriched full-length E2-25K (0.6 mM) with ubiquitin (34.8 mM) was performed as with the UBA domain, to a maximum molar ratio of 2.0:1 Ub/E2-25K.

Titration of ¹⁵N-ubiquitin (0.6mM) with unlabeled UBA was performed as before except that buffer pH was 7.2 and unlabeled UBA domain (2.5 mM) was added to a final molar ratio of 1.5 UBA/Ub. Backbone assignments of ubiquitin at pH 7.2 were completed using published assignments at pH 6.0 and 7.4 [10, 11].

2.5 NMR spectroscopy

NMR spectra were collected at 25° C on a Varian 800 MHz (18.7 T field) INOVA NMR spectrometer using a room temperature triple-resonance probe with triaxial pulsed field gradient capability. Pulse sequences were those provided in the Varian BioPack. ¹H chemical shifts were referenced using sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS) as an internal reference. ¹³C and ¹⁵N chemical shifts were referenced indirectly to DSS and liquid ammonia, respectively, using the appropriate frequency ratios [12]. NMR spectra were processed using NMRPipe [13]. NMRView [14] was used for visualization and chemical shift assignments of NMR data.

2.6 Modeling the E2-25K UBA Domain

The model of the E2-25K UBA domain was calculated using residual dipolar coupling (RDC) measurements. A ¹⁵N-labeled E2-25K UBA domain sample was aligned in the magnetic field (Varian 800 MHz) with a nonionic liquid crystalline medium made up of 5% C12E6 [*n*-dodecyl hexa(ethylene glycol)] and 5% *n*-hexanol [15] and gave a ²H splitting of 18 Hz at 30° C. RDCs were determined from aligned and unaligned samples using gNHSQCS3 (Varian Biopack) experiments with the phase cycling adjusted to observe the two proton components individually. Couplings were measured using in-house scripts written for NMRView [14] and the RDC values were analyzed using MODULE [16]. The resulting structure was validated using *MolProbity* [17].

2.7 Modeling the E2-25K UBA/Ubiquitin Complex

The complex structure model was calculated using the protein-protein docking approach, incorporating the chemical shift perturbation data from the titration analysis. The program HADDOCK [6] was used to perform docking between the UBA domain (RDC generated structure) and ubiquitin (PDB ID: 1UBQ) [18]. HADDOCK initially calculated one

thousand structures by rigid body minimization. The 200 lowest energy structures were selected for torsion angle dynamics and Cartesian dynamic calculations in water solvent. Three clusters were generated but only one contained the majority of the structures. The 10 lowest energy UBA/Ub complexes were selected and validated using MolProbity [17].

2.8 Polyubiquitin Chain Assay

The polyubiquitin chain synthesis assay was adapted from work performed by Piotrowski et al. [19]. The assay contained either 20 μ M E2-25K or E2-25K UBC domain along with 0.3 mM ubiquitin, 4 mM Mg-ATP Solution, 0.2 μ M E1 activating enzyme (BostonBiochem), 10 mM phosphocreatine, 0.6 unit/ml of inorganic phosphokinase and creatine phosphokinase, 10 mM phosphocreatine and 0.5 mM DTT. Assays were conducted at 37° C for 3 hours, and analyzed by SDS-PAGE.

3. Results

3.1 E2-25K UBA Domain Backbone Resonance Assignments and Structure Determination

Chemical shift assignments for the backbone ^1H , ^{13}C , and ^{15}N resonances of the UBA domain were completed using a two-dimensional ^1H - ^{15}N HSQC [8] spectrum and three-dimensional HNCACB, CBCACONH, and HNCB [20] spectra collected on $^{15}\text{N}/^{13}\text{C}$ -uniformly labeled protein (Figure 1, Table S1). Backbone amide chemical shifts were well resolved in the HSQC spectrum, with the exception of F174, K186, W188, and Q195.

Analyses of ^{13}C chemical shifts in protein structures have been used successfully as indicators of secondary structure [21]. Differences in $\text{C}\alpha$, $\text{C}\beta$ and CO chemical shifts from random coil values were calculated and the predicted secondary structural elements were compared with those reported in the crystal structure of E2-25K M172A (PDB ID: 3E46) [7]. Excellent agreement was found between the two (Figure S1).

The solution structure of the E2-25K UBA domain, calculated from RDCs, demonstrates that UBA is independently folded and maintains an overall structure in solution similar to that found in the context of the full-length protein crystal structure (RMSD = 1.56 Å). The most marked differences occur in the third helix of the domain (Figure 1, inset).

3.2 Binding of E2-25K UBA and Ubiquitin

When stoichiometric amounts of unlabeled ubiquitin were added to ^{15}N -labeled UBA, a limited number of ^1H and ^{15}N resonances (residues L169, M172, G173, L198) exhibited large chemical shift changes (Figure 2, Figure S2). The perturbed residues fall primarily in the MGF loop between helices 1 and 2 of the UBA domain, and in the adjacent face of helix 3 (Figure 3). Several additional peaks appeared upon titration, one of which was subsequently identified as F174. The chemical shift perturbation data demonstrate weak binding of ubiquitin by UBA, in fast exchange on the NMR time scale. Using the existing NMR data, the K_d for binding of ubiquitin was estimated to be approximately 1.2 ± 0.3 mM (Figure S3). Titration of ^{15}N -labeled full-length E2-25K with unlabeled ubiquitin likewise yielded K_d values of ~ 1 mM (data not shown), experimentally indistinguishable from UBA alone. Therefore, UBA is principally responsible for non-covalent Ub binding by E2-25K.

The same K_d was measured for titration of labeled ubiquitin with unlabeled E2-25K UBA domain and provided information on the residues in ubiquitin involved with binding (Figure 3). Perturbed Ub residues included L8, R42, I44, A46, K48, Q49, H68, L71, and R72 (Figure S4). This binding interface is consistent with that seen in other UBA domains binding Ub [24, 25].

3.3 E2-25K UBA Domain/Ubiquitin Complex Structure

The structure of the E2-25K UBA domain/Ub complex was modeled using the docking program HADDOCK [6]. This protein-protein docking approach utilizes the binding interface data from NMR chemical shift perturbations (Figure 3) as Ambiguous Interaction Restraints (AIRs) to drive the docking process and to calculate the structures of the complex. Residues utilized as AIRs included M172, G173, F174 and L198 from UBA, and L8, I44, A46, Q49, and L71 from Ub. The overall average RMSD for the final ten lowest energy UBA/Ub complex structures was 0.71 ± 0.4 Å for the C $^{\alpha}$ atoms.

The resulting complex structure (Figure 3) is stabilized primarily by hydrophobic interactions, but does contain hydrogen bonds. This heavily hydrophobic binding is seen for other UBA/Ub complexes [23, 26, 27]. The core of the complex is comprised of hydrophobic interactions between G173, F174 and L198 of the UBA domain and I44, A46 and G47 of Ub. The M172 sidechain of the UBA domain is positioned in a shallow pocket formed by I44, A46 and H68 of Ub. Other hydrophobic interactions of interest occur between the aliphatic region of residues E191 and E195 of the UBA domain and the sidechains of L8 and V70 of Ub. One hydrogen bond is formed between the sidechain of Ub R42 and the backbone carbonyl oxygen of UBA L198. It is important to point out that residues K48, Q49, L71, and R72 of Ub do not appear to be directly involved in binding to the UBA domain. It is more likely that chemical shift perturbation results from local structural changes induced on binding.

3.4 E2-25K Polyubiquitin Chain Assays

The role of the C-terminal UBA domain in polyubiquitin chain synthesis was examined by conducting a series of assays with different E2-25K and ubiquitin constructs. The assays contained no E3 ligase and no substrate other than ubiquitin itself. Assays comparing the abilities of the full-length E2-25K and the UBC domain to synthesize polyubiquitin chains from wild-type ubiquitin revealed that the UBA domain is required for efficient manufacture of chains larger than Ub₄ (Figure 4, Lanes 4 & 7).

Additional assays utilizing two modified ubiquitins (Ub-K48C and Ub-D77) in equimolar ratios, were designed to restrict the chain length to Ub₂ if only Lys48-linked chains are formed [19]. Under these conditions, SDS-PAGE analysis of chain synthesis demonstrated that both full-length E2-25K and UBC domain efficiently synthesized diubiquitin (Figure 4, Lanes 5 & 8). UBC domain, however, also synthesized chains larger than diubiquitin, suggesting that in the absence of the UBA domain, alternative linkages may be used. PolyUb chain assays in the presence of Ub-K48C alone confirmed that chains formed by full-length E2-25K are Lys48-linked (no chain synthesis is present), whereas UBC domain synthesizes polyUb with alternative linkages (Figure 4, Lanes 6 & 9). This indicates a possible role for the UBA domain in positioning the acceptor ubiquitin during chain synthesis.

4. Discussion

The primary contact surface for ubiquitin in most UBA domains is the MGF loop between helices 1 and 2 of the domain, and a leucine residue at the C-terminal end of helix 3 (residue L198 in E2-25K). The MGF loop, and several key residues which form the core of the domain, are highly conserved, but divergence in the remaining residues leads to differences in ubiquitin binding specificity and affinity. The binding of the E2-25K UBA domain to monomeric ubiquitin was initially reported by Raasi et al. [22] with a K_d of approximately 300–500 μM, which is consistent with the binding affinity reported here. Comparison of the protein-protein interface of E2-25K's UBA domain in complex with Ub reveals similarities

to that seen in several other UBA/Ub complexes [23 – 27]. Evidence for additional binding surfaces in E2-25K was not apparent from the NMR data (Figure S2). More recently the structures of E2-25K complexed with Ub and a mutant ubiquitin, Ubb⁺¹, were reported [28] which are highly similar to the complex presented here. Thus, E2-25K interaction with Ub occurs primarily through the UBA domain.

There are several studies which postulate a potential role for the UBA domain in E2-25K. Initial work by Haldeman *et al* [29] with truncated E2-25K (1–151) and E2-25K (1–153) suggested that the UBA domain was essential for formation of free, Lys48-linked polyubiquitin chains, but not for the normal UBC ligase activity. The crystal structure of E2-25K [7] subsequently revealed that residues 151 and 153 are located within an α -helix, suggesting that truncation at these residues could cause protein instability or improper folding. The addition of a GST-fusion tag to the truncated E2-25K constructs restored chain synthesis activity, possibly through stabilization of the protein or through dimerization via the GST. Later studies with a slightly longer, truncated E2-25K (1–155) demonstrated that chain synthesis was possible in the absence of the UBA domain [30]. Even though the difference in length was only 2 additional amino acids, this construct is less likely to disrupt the formation of helix 5. The UBC construct used in this work included residues 1–159, and demonstrated the ability to synthesize free polyubiquitin chains, although the length of the chain was limited in size. NMR, DSC, and ANS-binding fluorescence studies with this construct (data not shown) indicated that even though the UBC domain retains enzymatic activity and overall fold, the structure is less stable than that of full-length E2-25K. It would be reasonable to assume that previously studied, shorter constructs may not have been stably folded.

Studies of enzymatic activity with Ubc1, an E2-25K homolog in *Saccharomyces cerevisiae*, alluded to a possible role for the UBA domain in limiting ubiquitin chain length [5]. Truncated constructs of Ubc1 missing the UBA domain demonstrated synthesis of longer polyubiquitin chains than the full-length Ubc1. This is in contrast to the evidence presented here for enhancement of processivity in the presence of the UBA domain. However, unlike this work, the experiments with Ubc1 were conducted in the presence of both an E3 ligase and a substrate. These conditions may alter the effect of UBA/Ub binding. Alternatively, the Ubc1 protein, which has a much longer, flexible linker connecting the UBC and UBA domains, may function in a different manner from E2-25K. Future studies with E2-25K will explore the effect of E3 ligase partners on processivity.

Polyubiquitin chain synthesis assays of E2-25K in the absence of an E3 ligase (Figure 4) showed that deletion of the UBA domain does allow for the inefficient formation of alternative polyubiquitin chain linkages, indicating that the UBA domain helps direct Lys48-linked chain synthesis in the absence of an E3 partner. These data are consistent with a requirement for dimerization to direct chain linkage specificity in other E2 enzymes [31, 32]. This might suggest that the UBA domain of E2-25K mimics the presence of the non-catalytic E2 in a heterodimeric complex such as Mms2/Ubc13, which synthesizes free Lys63-linked polyubiquitin chains.

Hints of a more intriguing function for the UBA domain originated from studies of polyubiquitination of anaphase promoting complex (APC) targets [33]. UBA truncation mutants of both E2-25K and its yeast homolog Ubc1 were defective in binding to the ubiquitinated APC-substrate complex, resulting in decreased polyubiquitination of the target protein, but were not defective in E2~Ub thioester bond formation or in linkage specificity. This suggests that the UBA domain increases specificity for the monoubiquitinated or polyubiquitinated targets by providing an additional binding surface. The results presented

here are consistent with this idea, demonstrating that the UBA domain increases processivity through binding to the substrates (in this case ubiquitin and polyubiquitin).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

Ub	ubiquitin
UBC	ubiquitin-conjugating domain
UBA	ubiquitin-associated domain
NMR	nuclear magnetic resonance spectroscopy
CSI	chemical shift index
RDC	residual dipolar couplings

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Figure 1. Solution Structure of E2-25K UBA domain

^1H - ^{15}N HSQC spectrum of the UBA domain. Labels for residues G154 and F174 indicate location of backbone HN chemical shifts identified using other spectra; **Inset**) Comparison of NMR model (gray) with crystal structure (black) (PDB ID: 3E46).

Figure 2. Chemical Shift Perturbations

Overlay of the ^1H - ^{15}N HSQC spectra for residues M172, G173, F174, and L198 in the UBA domain for a) 0 equivalents, b) 2.0 equivalents and c) 3.5 equivalents of Ub added. Arrows indicate direction of shift as Ub concentration is increased.

Figure 3. Complex Structure of E2-25K UBA domain with Ub

Ribbon representation of the lowest energy complex structure, UBA shown on the left, monoUb shown on the right. Sidechains involved in binding are shown as sticks (UBA: M172, G173, F174, L198 ; Ub: R42, I44, K48, Q49, L71, R72). Surface model of the UBA domain with Ub-binding residues *highlighted in black*, and surface model of Ub with UBA-binding residues *highlighted in black* are shown below. Molecules have been rotated outward 90° to expose binding surfaces.

Figure 4. E2-25K Polyubiquitin Chain Assay

SDS-PAGE of Polyubiquitin Chain Assays. Lane 1 – LMW Marker, Lane 2 – E2-25K, Lane 3 – E2-25K UBC Domain, Lane 4 – Assay with E2-25K and wt Ub, Lane 5 – Assay with E2-25K and equimolar ratios of Ub-K48C and Ub-D77, Lane 6 – Assay with E2-25K and Ub-K48C only, Lane 7 – Assay with E2-25K UBC Domain and wt Ub, Lane 8 – Assay with E2-25K UBC Domain and equimolar ratios of Ub-K48C and Ub-D77, Lane 9 – Assay with E2-25K UBC Domain and Ub-K48C only.