Chemoenzymatic Synthesis of Bifunctional Polyubiquitin Substrates for Monitoring Ubiquitin Chain Remodeling

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Supporting information

Table of Contents

I. Materials

All chemicals were purchased from Sigma-Aldrich unless otherwise specified.

II. Cloning and Protein Expression[1-3]

Ubiquitin variants in which specific lysine residues are substituted with cysteine (Ub-KxC-D77; where x represents the position of native lysine residues) were constructed by introducing cysteine mutations at specified sites in the DNA sequence using splice overlap extension. Primers containing the TGC mutation were inserted at the desired codon position. D77 was encoded in the reverse primer to afford two constructs, which were ligated into a pET22b vector (Novagen).

The Ub variants were expressed and purified from Rosetta™ 2(DE3)pLysS cells (Novagen). Media (2×YT, 1L) containing ampicillin (100μg/mL) was inoculated with a starter culture (1 mL, OD_{600}=0.8), grown to OD_{600}=0.6 at 37°C while shaking at 225 rpm, then induced with isopropyl β-D-1-thiogalactopyranoside (IPTG, 0.4 mM). Induction was allowed to proceed for a total of 4h at 37°C. Cells were harvested by centrifugation (4°C, 8,000xg) for 30 min and the pellet was resuspended in lysis buffer (25 mL of 50 mM Tris pH 7.5, 0.5 mM EDTA, 1 mM EGTA, 0.02% v/v IGEPAL®, 1 mM PMSF, 1 mM DTT). Cells were lysed by sonication and the lysate was clarified by centrifugation (8,000xg). Perchloric acid (70%, 0.19 mL) was added dropwise to the soluble layer and stirred for 20 min to precipitate impurities. After centrifuging (8,000xg), the supernatant was exchanged into Ub buffer A (50 mM NH4OAc pH 4.4, 1 mM EDTA, 1 mM DTT) via dialysis. The lysate was then batch bound to SP Sepharose Fast Flow resin (10 mL, GE Healthcare) for 1 h. The resin was then poured into an empty gravity column (BioRad) and washed with buffer A, 5% buffer B (50 mM NH_4OAc pH 4.4, 1 mM

EDTA, 1 mM DTT, 1M NaCl), and 10% buffer B. The Ub variants were then eluted with 25% buffer B. Fractions containing Ub (monitored by SDS-PAGE) were exchanged into H_2O and lyophilized.

III. Polymerization of Synthetic Ub Polymers[3]

Ub polymerization reactions contained Ub-KxC-allylamine (2 mM), lithium acyl phosphinate (LAP) (0.5 mM) in a NaOAc buffer (250 mM) at pH 5 (total reaction volume is 2 mL). Samples were placed on ice and irradiated with 365 nM light for 30 minutes using an OmniCure series 1500 light source placed 15 cm above the sample. Polymerization reactions were combined and purified using a Superdex 75 HiLoad size exclusion column at a flow rate of 0.2 mL/min, collecting 2.5 mL fractions over 0.7 column volumes.

IV. Expression and Purification of Yuh1 (adapted from Hill *et. al.*)[4]

Yuh1 in pET-3a (Plasmid 18895) was supplied by Addgene and expressed in Rosetta™ 2(DE3)pLysS cells (Novagen) using the previously described procedures. Luria-Bertani (LB) broth (1 L) containing ampicillin (100 μg/mL) was inoculated with a starter culture (1 mL, OD_{600}=0.8) while shaking at 225 rpm at 37°C. Once the cultures reached an OD_{600} of 0.8, cells were induced with IPTG (1mM) at 18°C and induction was allowed to proceed overnight. Cells were harvested by centrifugation (4°C, 8,000xg, 30 min) and the pellet was resuspended in lysis buffer (20mM sodium phosphate buffer, 0.5 NaCl, pH 7.4). Lysis was induced by sonication and the resulting lysate was clarified by centrifugation (4°C, 30,000xg). Yuh1 was then purified via ammonium sulfate precipitation: impurities were removed by precipitation at 40% $(NH_4)_2SO_4$ and Yuh1 was precipitated at 60% $(NH_4)_2SO_4$. The pellet was resuspended in 25 mM Tris pH 8 and

dialyzed into 50 mM HEPES pH 6.8, 25 mM NaCl, 1 mM DTT. The protein was further purified via anion exchange using a MonoQ column and eluting with a 0-100% gradient of buffer B (25 mM Tris pH 8, 1M NaCl) in buffer A (25 mM Tris pH 8). Fractions containing Yuh1 were buffer exchanged via an Amicon 3.5 kDa MW cut-off filter into 50 mM Hepes pH 8, 0.5 mM EDTA, and 10% glycerol. The protein solution was snap frozen, and stored at -80°C. The concentration of Yuh1 (26.4 kDa) was determined using the calculated extinction coefficient (ε) of 27,310 $cm^{-1}M^{-1}$.

V. Synthesis of (+)-Biotinyl-3,6-dioxaoctanediamine (Amine-biotin)

Synthesis was adapted from a published patent.[5] A solution of biotin (0.9 g, 3.69 mmol, 1.5 eq), HATU (1.2 g, 3.16 mmol, 1.3 eq) and *N,N*-diisopropylethylamine (DIPEA) (0.85 mL, 4.90 mmol, 2 eq) in dry dimethylformamide (DMF, 15 mL) was stirred for 30 min at 0°C before a solution of *tert*-butyl-N-(2-(2-(2-aminoethoxy)ethoxy)ethyl)carbamate (0.6 g, 2.4 mmol, 1 eq) in DMF (2 mL) was added dropwise. The solution developed a yellow color and was allowed to stir overnight at room temperature. Water was then added to the reaction flask and DMF was removed *in vacuo*. The crude reaction was purified by column chromatography using a mixture of methanol and dichloromethane. Upon removal of the solvent *in vacuo*, a yellow solid was obtained which was further purified by HPLC using a C18 prep column (Phenomenex) and eluting with 50-100% acetonitrile in water over 15 minutes. Fractions were then concentrated to furnish Boc-protected amine-biotin as a white solid (550 mg, 48% yield). To then remove the Boc group, Boc-amine-biotin (300 mg, 0.63 mmole) was stirred in TFA (5 mL) for 1.5 h at room temperature. TFA was removed *in vacuo* to afford amine-biotin (AB) (230 mg, quant) as a crystalline solid. ^{1}H NMR (300 MHz, MeOD) δ 4.49

(dd, J = 4.8, 7.8 Hz, 1H), 4.30 (dd, J = 4.5, 7.8, 1H), 3.66-3.71 (m, 5H), 3.56 (t, J = 6 Hz, 2 H), 3.34-3.39 (m, 3H), 3.17-3.24 (m, 1H), 3.12 (t, J = 3.3Hz, 2H), 2.92 (dd, J = 4.8, 13 Hz, 1H), 2.70 (d, J = 13, 1H), 2.22 (t, J = 7.5, 2H), 1.60-1.72 (m, 4H), 1.45 (q, J = 7.5, 2H). (**Figure S1**) HRMS (ES) calculated for $C_{16}H_{30}N_4O_4S$ $[M+H]^+$ 375.2061, observed 375.2067.

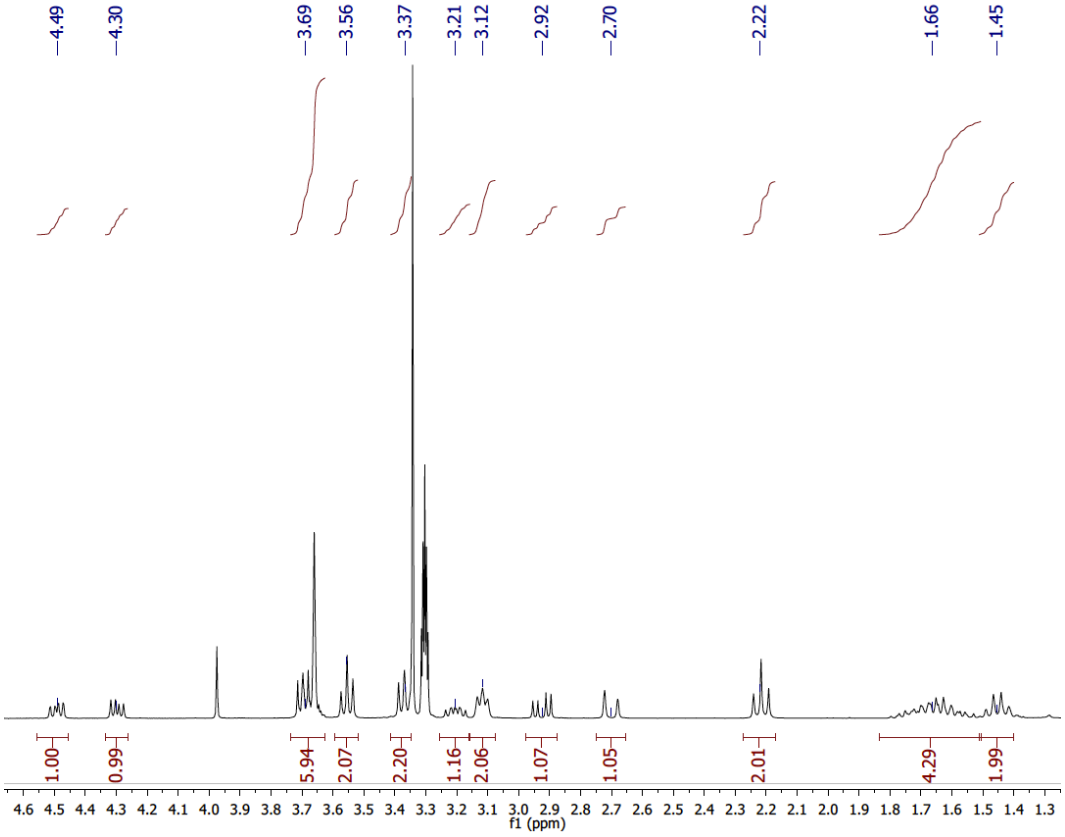

Figure S1. [1]HNMR spectrum of Amine-biotin.

VI. Transamidation of Ub-D77 and Ub-AA with Amine-biotin

For the enzyme comparison, Ub-D77 (0.75 mM) was incubated with AB (250mM, pH 10.4) and Yuh1 (150 nM, 500 nM, or 2.5 μM) overnight at room temperature. For the Ub-AA comparison experiment, Ub-D77 or UB-AA (0.75mM) were incubated with AB

(250 mM, pH 10.4) and Yuh1 (500 nM) for 1.5 hours at room temperature. (**Figure S2)** Reactions were quenched with 10% (v/v) acetic acid, 5 mM iodoacetic acid, and applied to a cation exchange HPLC column (TosoBioscience TSK SP-NPR). The substrate was separated from the products using a linear gradient of 0-0.4 M NaCl in 25 mM ammonium acetate pH 4.4 over 15 min. Derivatives of Ub were detected at 214 nm and the corresponding peaks integrated using the Waters HPLC Empower™ software. Product formation was confirmed by Fourier-transform ion cyclotron (FT-ICR) MS analysis.

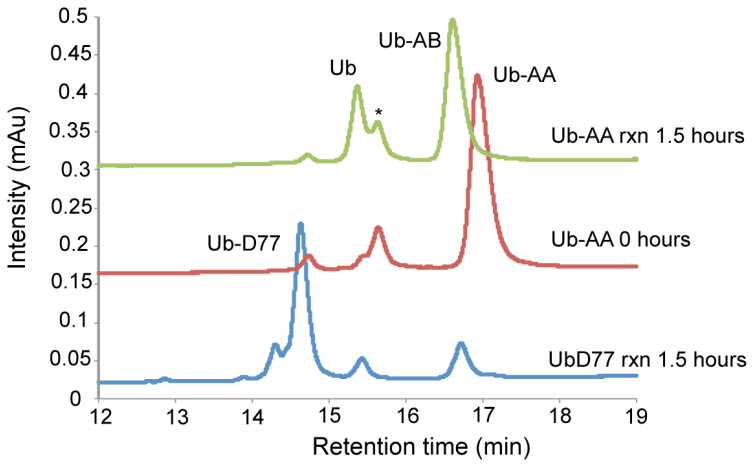

Figure S2. HPLC traces showing the conversion of Ub-D77 or Ub-AA into Ub-AB after 1.5 hours at room temperature. * indicates contaminant found in Ub-AA stock solution.

VII. Labeling of Ub-K63C-D77 with TAMRA (adapted from Wolberger et.al.[6])

To synthesize TAMRA-Ub-D77, Ub-K63C-D77 (350 μM) was incubated in buffer [10 mM sodium phosphate pH 7.4, 150 mM NaCl, 3 mM KCl, and 3.5 mM tris-(2-carboxyethyl)phosphine (TCEP)] for 15 min at room temperature. Tetramethylrhodamine (TAMRA) iodoacetamide (3.5 mM, Anaspec) dissolved in DMSO was added and the mixture was incubated at room temperature in the dark for 2 h. The reaction was

quenched with excess β-mercaptoethanol and dialyzed into Ub buffer A (see above). The protein solution was then bound to SP Sepharose Fast Flow resin (15 mL) for 1 h and washed with 5% Ub buffer B (300 mL). Protein was eluted with 25% buffer B (30 mL). MALDI analysis indicated nearly complete conversion of Ub-K63-D77 to TAMRA-labeled Ub-D77 (**Figure S3**).

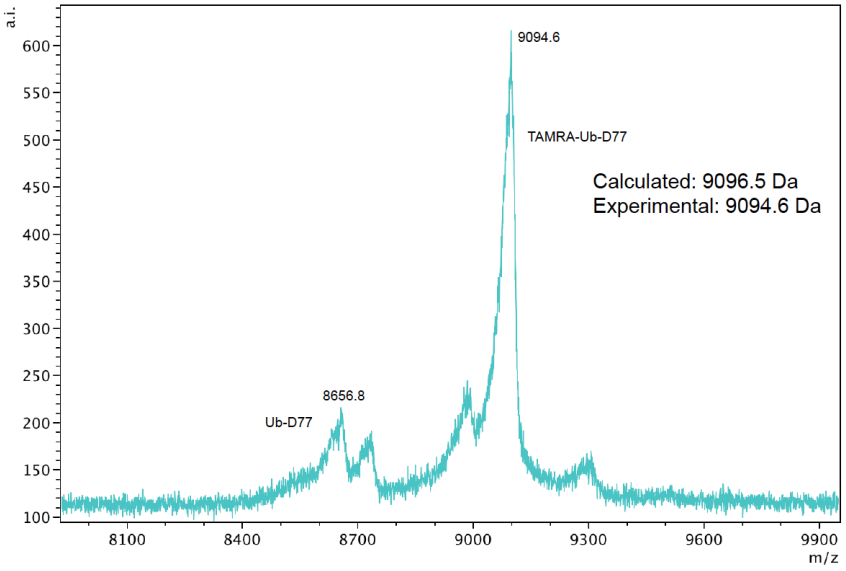

Figure S3. MALDI trace of TAMRA-labeled Ub-D77.

VIII. Yuh1-Catalyzed Hydrolysis and Transamidation of TAMRA-Ub-D77

Because the absorbance of Ub at 280 nm is low, concentrations of TAMRA-Ub- D77 were determined using the extinction coefficient of TAMRA (ε = 80,000 $cm^{-1}M^{-1}$ at 548nm, recommended by AnaSpec). Yuh1-catalyzed hydrolysis at pH 7.5 was performed at 25°C in a buffer containing HEPES pH 7.5 (50 mM) and EDTA (1 mM). Reactions were initiated upon addition of Yuh1 (5 nM) and were quenched with 10% (v/v) acetic acid, 5 mM iodoacetic acid, and applied to a cation exchange HPLC column (TosoBioscience TSK SP-NPR). The substrate was separated from the products using a

linear gradient of 0.15-0.35 M NaCl in 25 mM ammonium acetate pH 4.4 over 12 min. Derivatives of TAMRA-Ub were detected at 548 nm and the corresponding peaks integrated using the Waters HPLC Empower™ software. For comparison, the peaks were also integrated manually using Excel 2011. The results of the two methods are congruent. With regards to hydrolysis at alkaline pH (pH 10.4), a CAPS buffer (50 mM) was used. Reactions were initiated upon addition of Yuh1 (9.5 nM) and were quenched as described above and analyzed via cation exchange chromatography using a 15 min linear gradient of 0.15-0.35 M NaCl in 25 mM ammonium acetate pH 4.4. Transamidation reactions were performed at 25°C with AB (250 mM, pH 10.4) and Yuh1 (135 nM). Reactions were quenched as described above, analyzed via cation exchange chromatography, and quantified at wavelength 548 nm. Product formation was confirmed by FT-ICR MS analysis (**Figure S4**).

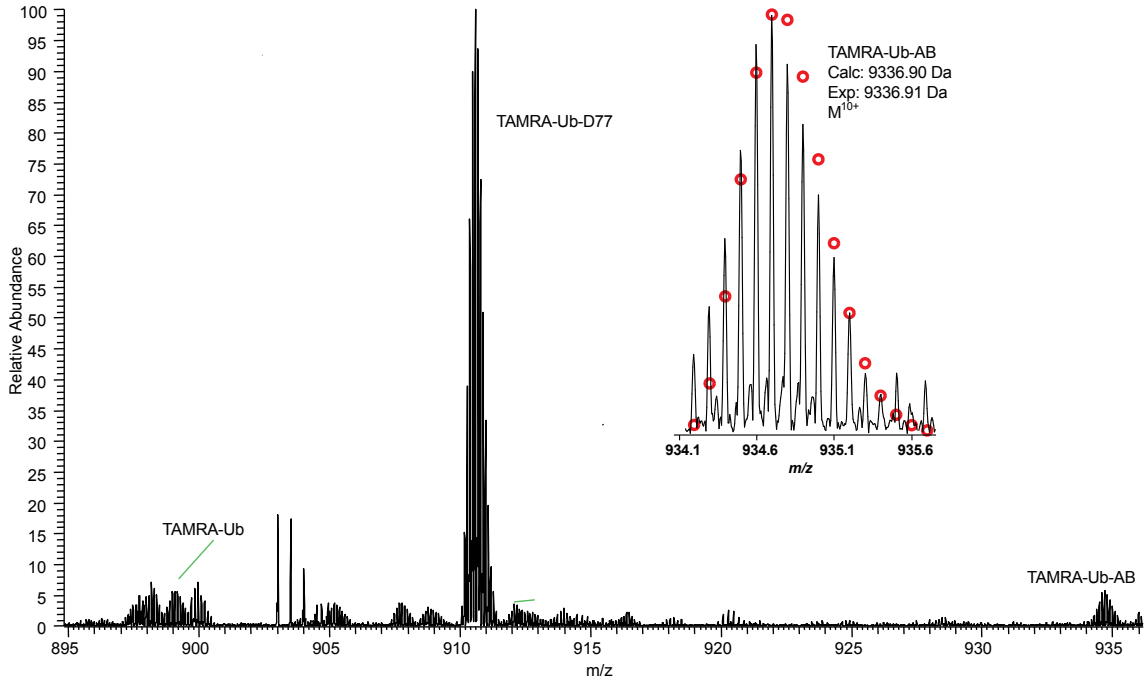

Figure S4. MS analysis of TAMRA-Ub-AB transamidation reaction (all peaks are in the M^{10+} ionization state. The isotopic distribution of aminolysis product (TAMRA-Ub-AB) in a single charge state (z=10 or M^{10+}) is shown on the right. Red circles correspond to the theoretical distribution of isotopic abundance. Calc'd: calculated most abundant MW. Expt'l: experimental most abundant MW.

To examine the kinetics of Yuh1-catalyzed hydrolysis and transamidation, a standard curve was first used to establish a correlation between the area of TAMRA-Ub-D77 and concentration (**Figure S5**). Given the absorbance at 548 nm is due to TAMRA, the same standard curve was used to determine concentrations of TAMRA-Ub-AB. Initial rates were then measured, which showed a linear relationship between concentration and time (**Figure S6**). Using the initial rates over a range of TAMRA-Ub-D77 concentrations the kinetic parameters, K_m and k_{cat}, were determined. Fits to equation 1 were obtained using the OriginLab software (**Figures S7-S9**).

$$\upsilon = \frac{k_{cat}[E]_{tot}[S]}{[S] + K_M} \qquad (1)$$

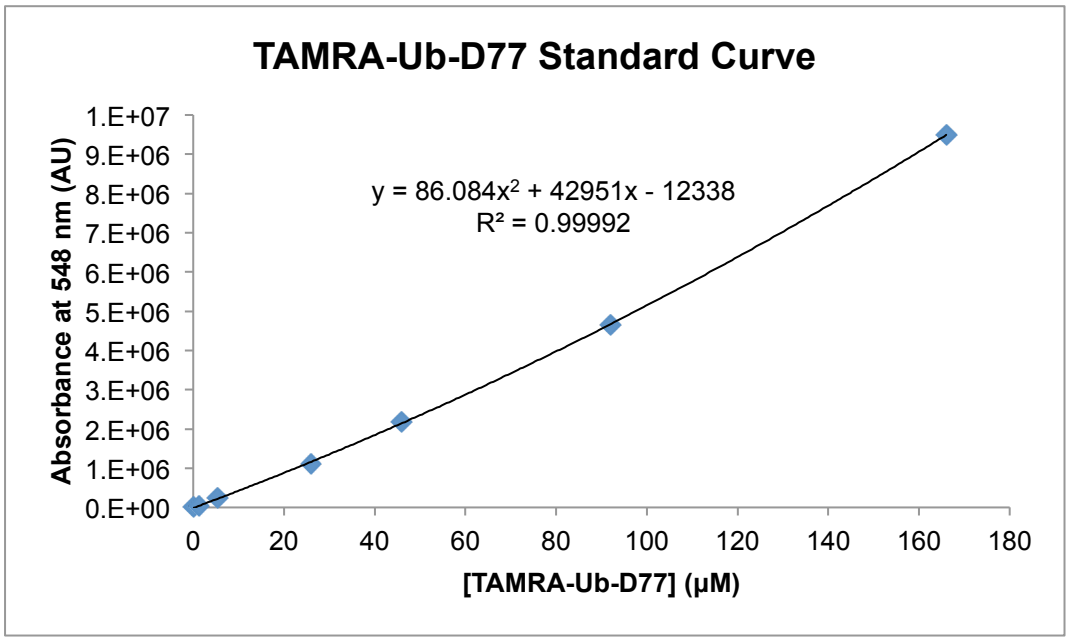

Figure S5. Standard curve generated with TAMRA-Ub-D77 used for kinetic assays. Line was fit to a polynomial equation.

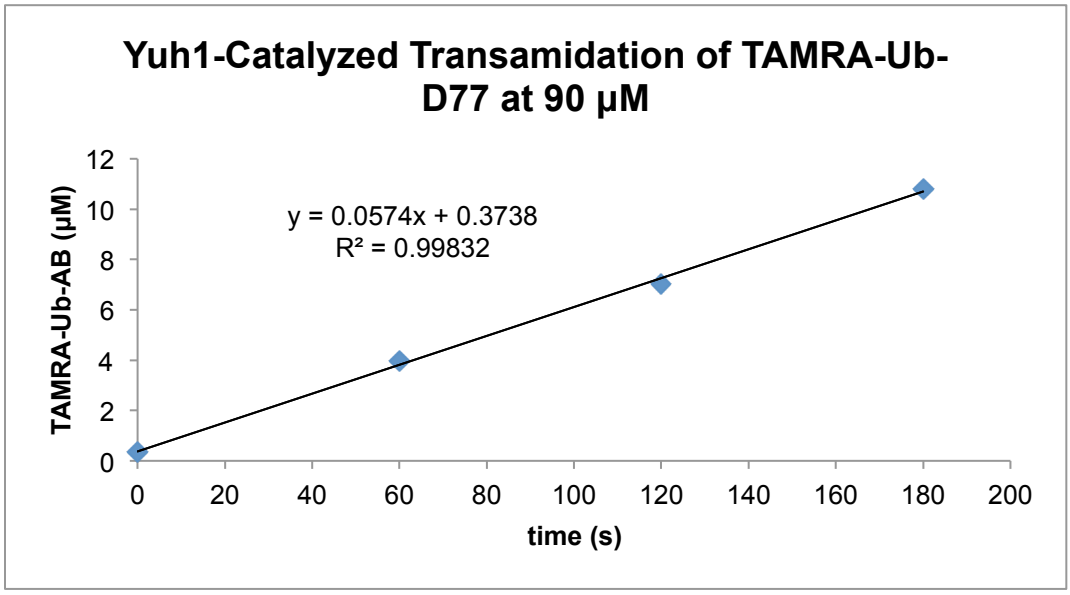

Figure S6. Representative curve of Yuh1-mediated TAMRA-Ub-AB formation as a function of time. Line was fit to a linear equation.

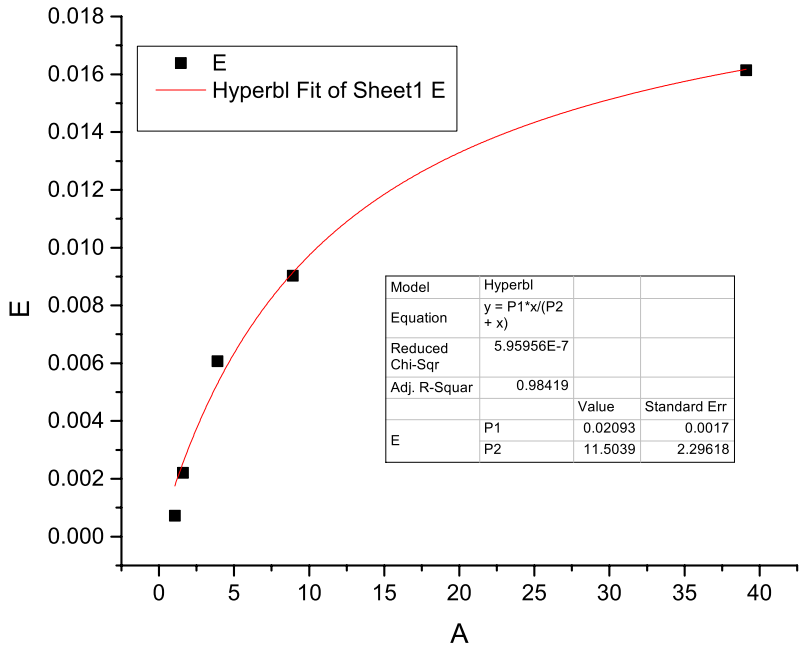

Figure S7. Yuh1-catalyzed hydrolysis of TAMRA-Ub-D77 at pH 7.5. Non-linear regression analysis affords P1, which corresponds to V_{max}(µM•s^{-1}), and P2, which corresponds to K_M (µM). The y-axis (E) is displayed as the initial rate (µM•s^{-1}) and the x-axis (A) is the concentration of TAMRA-Ub-D77 (µM).

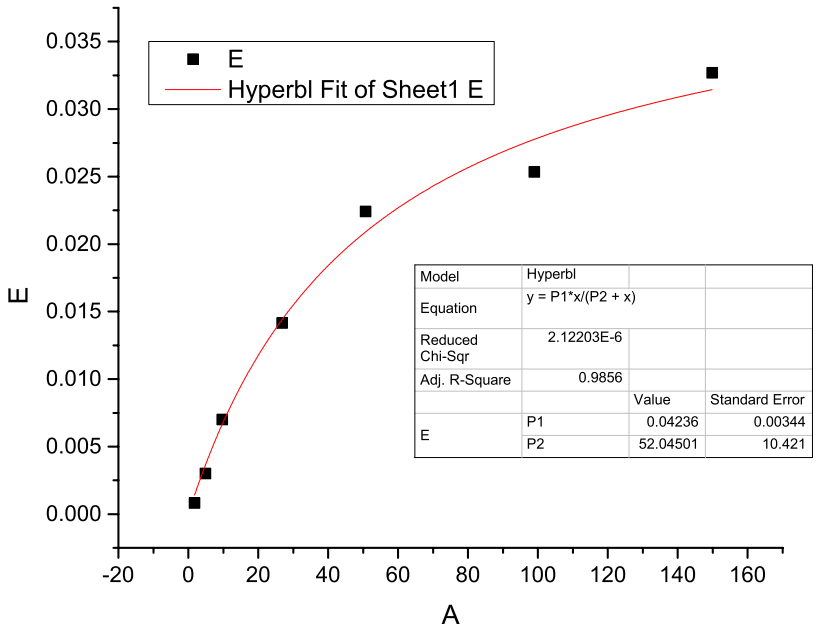

Figure S8. Yuh1-catalyzed hydrolysis of TAMRA-Ub-D77 at pH 10.4. Non-linear regression analysis affords P1, which corresponds to V_{max}(μM•s^{-1}), and P2, which corresponds to K_M (μM). The y-axis (E) is displayed as the initial rate (μM•s^{-1}) and the x-axis (A) is the concentration of TAMRA-Ub-D77 (μM).

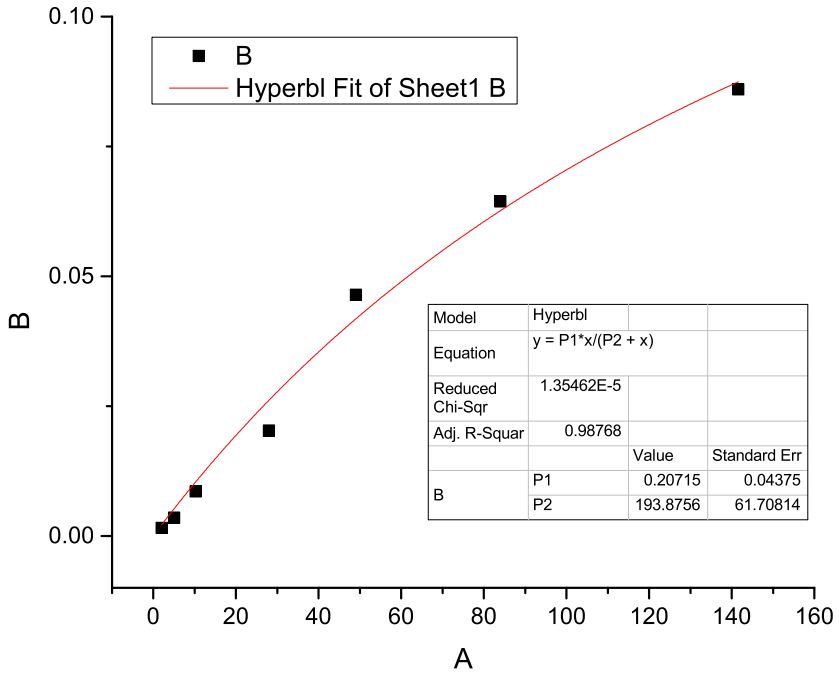

Figure S9. Yuh1-catalyzed transamidation of TAMRA-Ub-D77 in the presence of amine-biotin (250 mM). Rate of aminolysis product formation was monitored. Non-linear regression analysis affords P1, which corresponds to V_{max}(μM•s^{-1}), and P2, which corresponds to K_M (μM). The y-axis (B) is displayed as the initial rate (μM•s^{-1}) and the x-axis (A) is the concentration of TAMRA-Ub-D77 (μM).

Michaelis Menten fit of aminolysis was confirmed using the Lineweaver-Burk plot

(**Figure S10**)

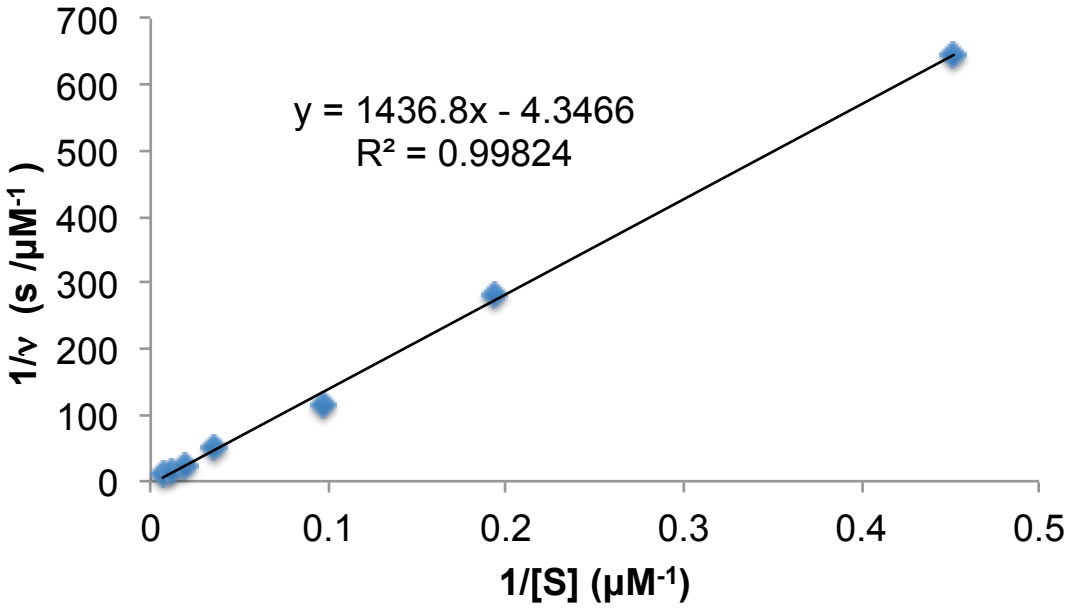

Figure S10. Line-weaver Burk plot of Yuh1-catalyzed transamidation of TAMRA-Ub-D77 in the presence of amine-biotin (250 mM) with 135 nM Yuh1 where rate of aminolysis product formation was monitored. The calculated k_{cat}/K_m is 5.2×10^3 $M^{-1}s^{-1}$, which is in agreement with the non-linear regression fit.

IX. Yuh1-Catalyzed Transamidation with Di-Ub as a Substrate

48-linked di-Ub (0.75 mM) harboring an allylamine moiety on the C-terminus was incubated with AB (250 mM, pH 10.4) and Yuh1 (150 nM) at room temperature overnight. As a control, Yuh1 was inactivated with *N*-ethyl maleimide (100 mM) and buffer exchanged into HEPES pH 7.5 prior to addition to the reaction mixture containing di-Ub and AB. Reactions were separated using a 15% SDS-PAGE gel, transferred to low fluorescence PVDF membrane for 2 hours at 75V, and analyzed using TAMRA-streptavidin (Invitrogen). Fluorescence was detected with a green laser (532 nm) and a 580±15 nm filter on a Typhoon imager FLA 9500 (GE Healthcare). Data were processed using ImageJ software[7].

X. Yuh1-Catalyzed Site-Specific Modification of 63-Linked Tetra-Ub

The 63-linked tetramer (0.7 mM), obtained via thiol-ene coupling, was incubated with AB (250 mM, pH 10.4) and Yuh1 (300 nM) at room temperature overnight. The reaction was quenched upon addition of 10% (v/v) acetic acid and buffer exchanged into Hepes pH 7.5 (50 mM). The Yuh1-treated tetramers were reduced with 20 molar excess TCEP and mixed with Cy5 maleimide (Amersham). The conjugation was allowed to proceed overnight at 4°C. The dually functionalized tetramers were purified away from excess dye and Yuh1 using a SP Sepharose Fast Flow gravity column as described above. The tetramers were buffer exchanged into water and stored for future use.

XI. Control Experiments for AMSH-Catalyzed Tetra-Ub Chain Diassembly

All AMSH DUB assays were performed in DUB buffer: 50 mM HEPES, 30 mM KCl, 2 mM DTT pH 7.5.

63-linked tetramer (10 μM) was incubated with AMSH (600 nM) for 1 h at 37°C. To assess the effects of oxygen-scavenging agents such as glucose oxidase and protocatechuate dioxygenase as well as radical-scavenging systems, e.g., Trolox, on the activity of AMSH, control experiments contained the latter and either the glucose oxidase or protocatechuate dioxygenase scavenging systems[8]. Reactions were quenched with loading dye and visualized by silver staining (**Figure S11**).

To test if AMSH was capable of degrading chains with the fluorophore attached, Cy5-labeled 63-linked chains (5 μM) were incubated with AMSH (600 nM) for 1 h at 37°C and visualized using fluorescence imaging (Typhoon) (**Figure S12**).

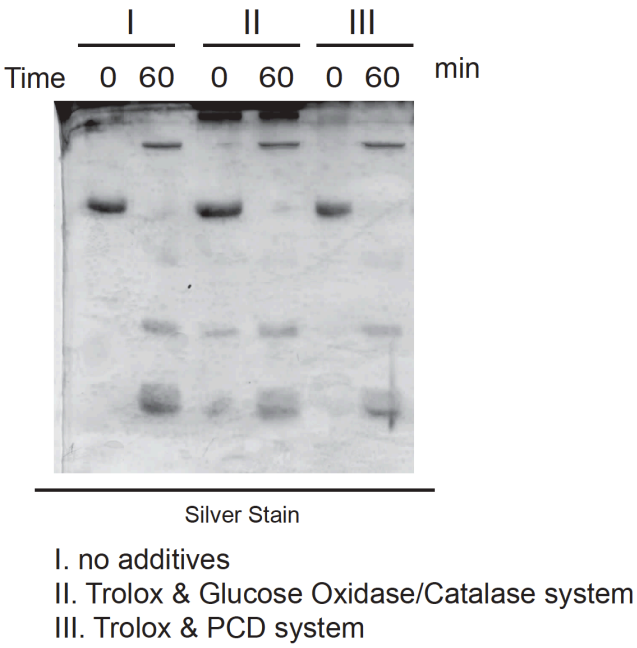

Figure S11. AMSH activity in the presence of oxygen- and radical-scavenging systems. AMSH (600 nM) was incubated with 63-linked tetramers (10 μM) for 1 h at 37°C in the presence of the indicated oxygen scavenging systems and Trolox (1 mM). These conditions mimic those used for single-molecule fluoresence experiments.

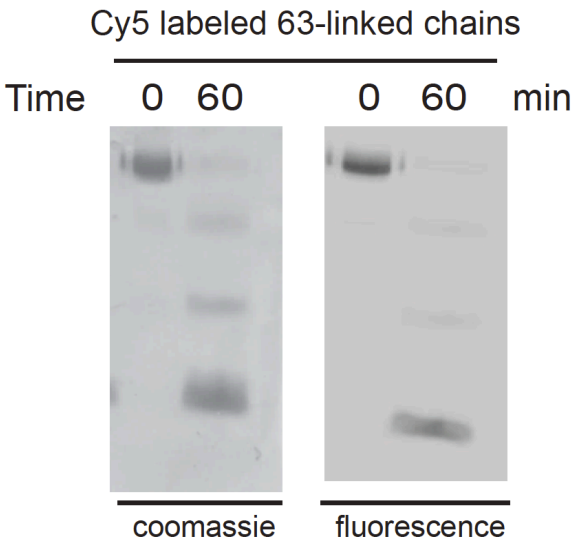

Figure S12. AMSH activity in the presence of Cy5 fluorophore on position 63 of the N-terminal subunit of the tetra-Ub chain. AMSH (600 nM) was incubated with Cy5-labeled 63-linked tetramers (5 μM) for 1 h at 37°C. Reactions were quenched with Laemmli buffer (sans dye) and visualized using coomassie staining and fluorescence.

XII. Single Molecule Experiments

Single molecules of Ub or Ub oligomers were visualized using either a custom-built TIRF microscope with a prism-based illumination scheme[9] or a custom-built TIRF microscope with a micromirror illumination scheme[10].

TAMRA-Ub-AB was imaged on quartz slides that were first derivitized with PEG and PEG-biotin (LaysanBio) prior to coating with streptavidin as previously described[10]. TAMRA-Ub-AB was diluted in phosphate buffered saline (PBS) prior to application to the sample chamber and imaging was carried out in PBS with the addition of a glucose oxidase scavenging system[8]. TAMRA-Ub molecules were visualized using the prism-based microscope by laser excitation at 532nm (10.3mW, Coherent) and with a 60x objective (1.2 NA Nikon). Fluorescence light was passed through a DualView apparatus

(Photometric DV2; 630dcxr, Chroma; HQ575/40m, Chroma) before being imaged by a EMCCD camera (Andor). Data were collected using MetaMorph control software and analyzed using ImageJ[7] and custom MatLab software.

For imaging 63-linked chains and performing the DUB activity assay, the micromirror TIRF microscope was used. Glass slides were derivitized with PEG and PEG-biotin and coated with streptavidin as previously described[10]. 63-linked tetra-Ub chains were then applied to the slide and imaged in PBS buffer containing DTT (1mM), Trolox (1 mM) and either the glucose oxidase or protocatechuate dioxygenase scavenging systems[8]. Control experiments were treated with Yuh1 (300 nM) for 2 h prior to addition to slides. NEM-inactivated Yuh1 (300 nM) was also added in the second control. Single molecules were visualized by laser excitation at 633nm (180μW, JDSU) and through a 60x objective (1.45 NA, Olympus). Fluorescence light was passed through a laser line filter (Z633/10X, Chroma) and a home-built dualview apparatus (536dcxr, Chroma; LP02-633RS-25, Semrock) before being imaged by an EMCCD camera (Andor). Data were collected using custom LabView control software and analyzed using custom MatLab software.

For the DUB activity assay, Cy5-labeled, tetra-Ub-AB was immobilized on the glass surface and to this was added AMSH (600 nM) in imaging buffer (50 mM HEPES, 30 mM KCl, 2 mM DTT pH 7.5, 1mM Trolox, and protocatechuate dioxygenase scavenging system). Immediately following addition of AMSH, time-lapse images were acquired with a 1 s exposure every 30 s for 15 min. Images were collected and analyzed with MatLab using custom software. Loss of Cy5 fluorescence was compared between

experiments with active and 1,10-phenanthroline-inactivated AMSH to assess activity on single immobilized 63-linked chains.

XIII. References

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