Supporting Information

Dual unnatural amino acid incorporation and click-chemistry labeling to enable single-molecule FRET studies of p97 folding

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Experimental Methods

Site directed mutagenesis of p97

Site directed mutagenesis was performed using wildtype pET17b-p97-His_6 as a template. Three amber codon mutants were generated using a modified QuickChange procedure that employs offset oligonucleotides harboring the desired mutation.[1] The sequences of the primers were the following:

Q50TAG_F

5'-ATGGATGAATTGTAGTTGTTCCGAGGTTACACAGTGTTGCTGAAA-3'

Q50TAG_R

5'-ACCTCGGAACAACTACAATTCATCCATCTTGGGCTGGGACAAGGA-3'

F131TAG_F

5'-CTGGTAATCTCTAGGAGGTATACCTTAAGCCGTACTTCCTGGAAG-3'

F131TAG_R

5'-GTATACCTCCTAGAGATTACCAGTAATGCCTTCCACTGTGTCATC-3'

Q382TAG_F

5'-GGACGCTTAGAGATTCTTTAGATCCATACCAAGAACATGAAGCTG-3'

Q382TAG_R

5'-CTTGGTATGGATCTAAAGAATCTCTAAGCGTCCTGTAGCATCAGG-3'

K426TAG_F

5'-GCCATCCGCAAGTAGATGGATCTCATTGACCTAGAGGATGAGACC-3'

K426TAG_R

5'-AATGAGATCCATCTACTTGCGGATGGCTTGCAGAGCAGCCTCTGA-3'

K502TAG_F

5'-AGCACCCAGACTAGTTCCTGAAGTTTGGCATGACACCTTCCAAG -3'

K502TAG_R

5'-AACTTCAGGAACTAGTCTGGGTGCTCCACAGGATACTGGACCAG-3'

K658TAG_F

5'- GTTGCCATCCTCTAGGCTAACCTGCGCAAGTCCCCAGTTGCCAAG -3'

K658TAG_R

5'- GCGCAGGTTAGCCTAGAGGATGGCAACACGGGACTTCTCATCAGG -3'

All mutations were confirmed by DNA sequencing (GENEWIZ).

p97 protein expression and purification

E. coli BL21 (DE3) cells were transformed with pUltra-Ambrx and the mutant p97 plasmid prepared as described above. Cells were grown in Luria Broth (LB) medium containing 100 μg/mL ampicillin and 100 μg/mL spectinomycin at 37°C to an OD_{600} of 0.8 followed by induction with 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and the addition of the unnatural amino acid, *p*-azidomethylphenylalanine (2 mM). The expression and suppression was carried out overnight at 25°C. Cells were collected by centrifugation (3000 x g for 10 min), resuspended in 40 mL lysis buffer (50 mM HEPES, 150 mM KCl, 5 mM $MgCl_2$, 5% glycerol, 2 mM β-mercaptoethanol (BME), pH 7.4, one complete EDTA-free protease inhibitor cocktail tablets (Roche)), and lysed by single passage through an M110Y microfluidizer (Microfluidics). The lysate was clarified by centrifugation (118,834 x g, 45 min, 4°C) and the resulting supernatant was incubated for 1 hour at 4°C with TALON Metal

Affinity Resin (Clontech) in 50 mM HEPES, 150 mM KCl, 5 mM $MgCl_2$, 5% glycerol, pH 7.4. The resin and supernatant were then loaded into a 25 mL disposable column (Bio-Rad), washed with 10 column volumes of wash buffer (50 mM HEPES, 150 mM KCl, 5 mM $MgCl_2$, 5% glycerol, 2 mM BME, 10 mM imidazole, pH 7.4), followed by 10 column volumes of stringent wash buffer (50 mM HEPES, 150 mM KCl, 5 mM $MgCl_2$, 5% glycerol, 2 mM BME, 20 mM imidazole, pH 7.4), and eluted with elution buffer (50 mM HEPES, 150 mM KCl, 5 mM $MgCl_2$, 5% glycerol, 2 mM BME, 250 mM imidazole, pH 7.4). Fractions were analyzed by 12% SDS PAGE, and those containing p97 were pooled, dialyzed into storage buffer (20 mM HEPES, 150 mM KCl, 5 mM $MgCl_2$, 5% glycerol, 2 mM BME, pH 7.4), and concentrated with a 30 kDa Ultra-15 centrifugal filter (Amicon) to yield a solution of p97 protein at 5-10 mg/mL, which was aliquoted, frozen in liquid nitrogen, and stored at -80°C until needed. All purification steps were carried out at 4°C. The activity of the protein was checked using a standard malachite green ATPase activity assay as described.[2]

Malachite green assay

ATPase activity was measured in 50 mM Tris, 150 mM KCl, 2.5 mM $MgCl_2$, 10% glycerol, pH 7.4, with 1 μM p97 (hexamer), and initiated by addition of 2 mM ATP. A 10 μL aliquot of the reaction was added to 800 μL of malachite green solution (0.73 mM malachite green, 8.4 mM $(NH_4)_6Mo_7O_{24}$, 1.0 M HCl, 0.04% Tween 20) at 2 min, 5 min, 10 min, 15 min, and 20 min time points. After 1 min at 25°C, 100 μl of 34% sodium citrate was added to quench the reaction. The amount of inorganic phosphate was determined by measuring the OD_{660} on a GENESIS 10S VIS Spectrophotometer (Thermo Scientific), converting OD_{660} to amount of inorganic phosphate using a standard curve, and plotting this value as a function of time. The data were fit to a linear curve, where the slope indicated the ATP hydrolysis rate per minute.

ATPase assay in the presence of p47

p97 hexamer (1 μM) was suspended in 100 μL of assay buffer (50mM Tris, 150mM KCl, 2.5mM $MgCl_2$, 15% glycerol, pH 7.4) containing 3.3 μM p47 monomer, followed by 2mM ATP to initiate the reaction. Reactions were run at 37 °C. The rate of ATP hydrolysis was determined as in Malachite green assay section and the percent activity calculated and plotted.

Mass spectrometry

Purified p97 mutant protein containing dual unnatural amino acids was digested using Trypsin/Lys-C Mix (Promega) in two steps, since intact p97 has been shown to be resistant to trypsin digest.[3] First, 100 μg of protein was diluted in 200 μL of 50mM Tris, 6M urea, pH 8.0 buffer to denature the protein and then 5 μg of Trypsin/Lys-C Mix was added to the solution. The reaction mixture was incubated at 37 °C for 4 hours and then diluted with buffer minus urea to 1M urea. Diazo Biotin-DBCO (Click Chemistry Tools) (10 μg) was added to the diluted solution. The mixture was incubated overnight at 37°C to complete the protein digestion and biotin labeling. After incubation, High Capacity Streptavidin Agarose (Click Chemistry Tools) resin (100 μL slurry) was added to bind the biotin-labeled peptides and incubated at 25°C for 1 hour. After binding, the resin was washed 5 times with 100mM Tris, 150mM KCl, pH 7.4 buffer. Then the resin was incubated in 50mM Tris, 50mM KCl, 100mM $Na_2S_2O_4$, pH 7.4 (100 μL) solution for 1 hour at 25°C to release peptides from the resin by chemical cleavage. The resulting solution containing peptides were analyzed using MALDI (Scripps Center for Metabolomics and Mass Spectrometry).

Fluorescence Labeling and Purification

The protein was diluted into labeling buffer (50 mM Tris, 150 mM KCl, 1 mM $MgCl_2$, 5% glycerol, pH 7.4) to 2.5 μM (monomer). The diluted protein was mixed with Alexa Fluor 488 and Alexa Fluor 594 DIBO Alkyne dyes (Life Technologies), where the final concentration of each dye was 10 μM. The mixture was incubated at 25°C overnight. The resulting labeled protein was denatured using 6M urea and further purified using Ni-NTA resin and a spin column (Zymogen). The denaturation was done for the complete removal of truncated p97 mutants, which did not have the c-terminal His_6-tag yet were co-purified with full-length protein (with His_6-tag) in hexameric form, as intact D1 domain with D1-D2 linker is sufficient enough to form hexamers. The wash buffer for the Ni-NTA was identical with the labeling buffer with 6M urea. The protein was eluted in the same buffer without urea and with 250 mM imidazole. The protein eluate sample was buffer exchanged using an Amicon spin filter (3k MWCO) into the native labeling buffer containing 1 mM DTT. The purified protein's concentration and the labeling efficiency were measured using the UV-vis absorption spectrum after denaturation of the protein, utilizing native tryptophan in the protein and the absorption bands of the dyes (NanoDrop 2000c, Thermo Scientific).

Sample Preparation for smFRET

The labeled p97 was mixed with wild-type p97 (not labeled, c-terminal His_6-tag) in excess in labeling buffer containing 6M urea. The mixture was incubated at 25°C for 30 minutes for unfolding and disassembly of the hexameric p97. Following incubation, the solution was diluted in labeling buffer without urea to make the final concentration of urea < 0.5 M. The diluted solution was incubated at room temperature for at least 2 hours for refolding. Then, the diluted sample was further diluted for smFRET experiments in smFRET buffer (50mM Tris, 150mM KCl, 10mM $MgCl_2$, 2.5mM DTT, pH 7.4) where the final urea concentration ranged between 5 mM to 25 mM. There was no difference detected for smFRET signal due to the urea with the concentration within the given range.

Collection of smFRET Data and Analysis

The experiment was carried out in a confocal smFRET detection setup as explained previously.[4] The sample was placed on a Tween-20 coated chambered borosilicate glass slide (Thermo Scientific). The measurement buffer was 50 mM Tris, 150 mM KCl, 10 mM $MgCl_2$, 2.5 mM DTT, pH 7.4, with varying concentration of urea. The collected E_{FRET} data were analyzed using Origin (OriginLab).

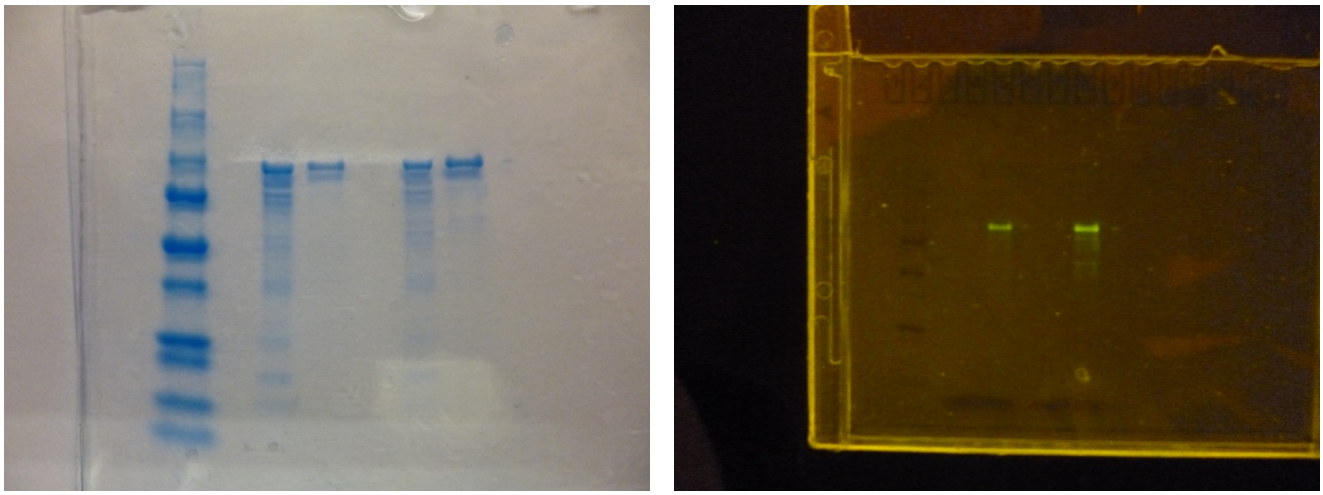

Figure S1. Original images of SDS-PAGE gel analysis for the p97 mutants and labeling. Left panel shows Coommassie stain of the gel while right panel shows fluorescence from the same gel visualized under blue light. From the left, the lanes are as follow: protein ladder (Precision Plus Protein Prestained Standards, Bio-Rad), blank, unlabeled F131Q382, labeled F131Q382, blank, unlabeled Q382K502, labeled Q382K502. All labeled samples were labeled with Alexa Fluor 488 DIBO Alkyne dyes.

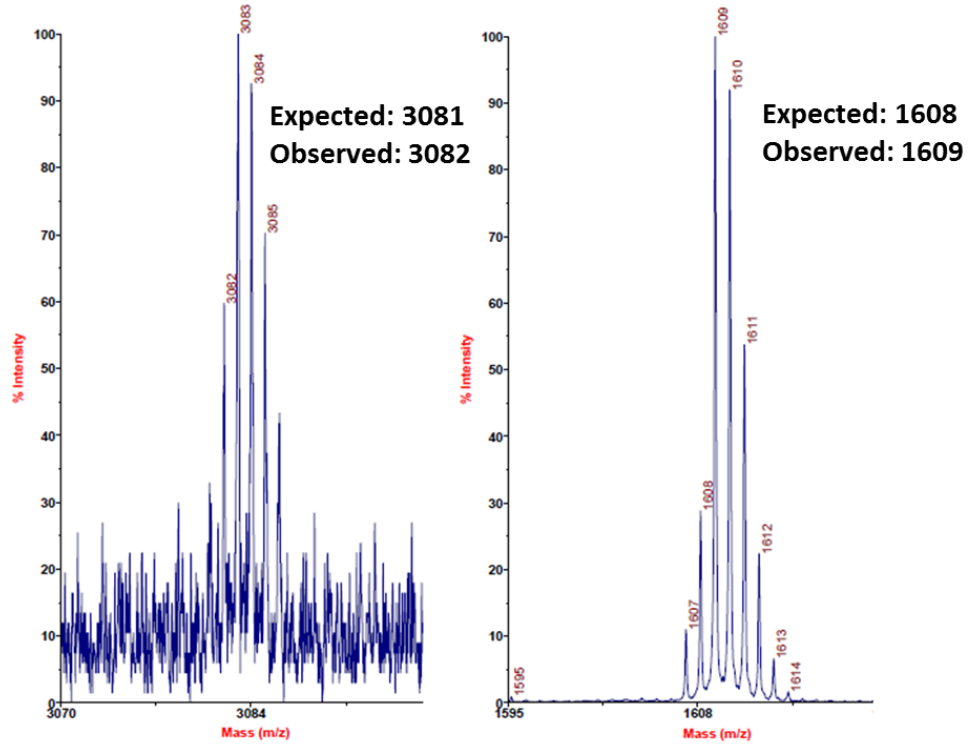

Figure S2. Mass spectrometry results for the Trypsin/Lys-C digested p97 mutant F131Q382. Left is peptide containing F131* (IHVLPIDDTVEGITGNL*EVYLK, * indicating position for unnatural amino acid incorporation), right is peptide containing Q382* (LEIL*IHTK). y-axis indicates the relative intensity of the signals, where the actual signal intensity would differ between left and right panel.

Table S1. Protein synthesis yield for each construct after HisTag purification

Construct	Yield (mg/1L LB)
Q50F131	11.5*
F131Q382	31.2
Q382K426	7.0*
Q382K502	31.0
K502K658	3.4*

* 1mM unnatural amino acid was used instead of 2mM described in the experimental methods.

Supplementary discussion: Fluorescence labeling of p97 provides access to other experimental observables useful for future studies of folding and assembly.

The reaction from unfolded monomeric p97 to folded hexamer is a complex reaction involving folding of domains and inter-domain and inter-monomer interactions. Using the labeling methodology reported in this paper, these various aspects can be probed in the future in detail. In addition to further smFRET experiments of the type described in this paper, other types of fluorescence experiments can also provide additional information – initial data sets for such data are provided for the reader's information below.

Polarization anisotropy

Polarization anisotropy reports on dye tumbling timescales, which are reflective of attached protein size. In the case of p97, the anisotropy can be used to observe changes in oligomerization state. An initial set of data are shown in Figure S3.

p97F131 (mono-label variant) labeled with Alexa Fluor 488 was denatured in 6M urea, then refolded by dialysis (Slid-A-Lyzer MINI dialysis device, Thermo Scientific) in 1000-fold excess volume native buffer (50 mM HEPES, 150 mM KCl, 10 mM $MgCl_2$, 5% glycerol, 2 mM DTT, pH 7.4) for over 2 hours at room temperature. The concentration of p97 was adjusted by adding varying amounts of denatured (6M urea) unlabeled wildtype p97 to the sample prior to refolding. The refolded sample was subjected to anisotropy measurement of the AF488 dye using a fluorimeter (PC1, ISS).

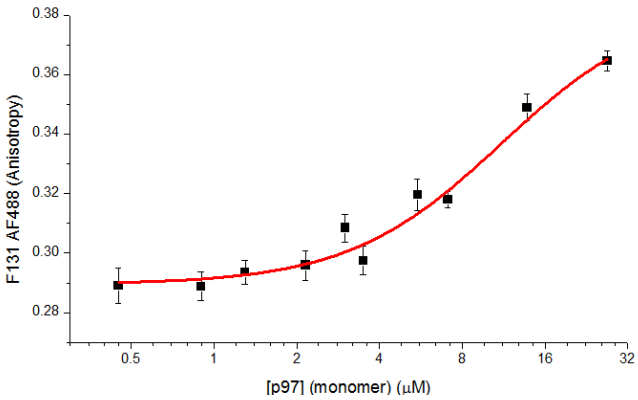

Figure S3. Anisotropy measurements using refolding conditions with increasing p97 concentration. Refolding monomer concentrations were adjusted using unlabeled wildtype p97. x-axis is on a log scale. The curve is a fit to a Hill equation.

As can be seen in Figure S3, samples with a monomer concentration above 10 μM (~0.9 mg/ml) showed higher anisotropy in comparison with lower-concentration samples. In addition, an ATPase activity assay at 10 μM p97 showed an over 20-fold activity per unit concentration of p97 in comparison with a 400 nM condition. These data are consistent with active hexameric p97 being assembled in the concentration range shown in Figure S3, though further experiments will need to be carried out to probe this issue in detail.

Intermonomer smFRET

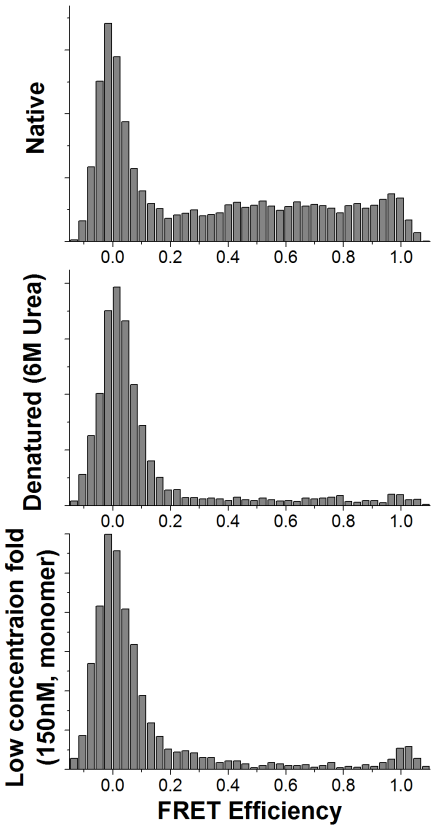

Figure S4 smFRET histograms for intermonomer FRET experiments.

Intermonomer smFRET experiments can provide more direct information on assembly. As in the anisotropy experiments above, mono-labeled proteins are used, but a mixture of donor and excess acceptor labeling was used. Inter-monomer smFRET would therefore be observed only when oligomerization occurs, but not with the monomer. Other than the labeling, the sample was prepared in the same way as described in the anisotropy experiment, where the protein was denatured in 6M urea, then folded back by dialysis for over 2 hours in room temperature. No wildtype p97 was added here. The sample was later diluted using a serial dilution into the smFRET buffer. We note that with the labeling ratio in this initial set of experiments (1:5 donor:acceptor, with about 30% unlabeled protein), we anticipate a variety of labeling isomers with various FRET efficiencies. Nonetheless, population density outside of the "zero peak" area will denote oligomerization. Consistent with this idea, the smFRET histogram in Figure S4 (top) shows a broad distribution extending from the zero peak up to 1. These data were collected on a sample diluted to single-molecule concentration (~200 pM) from a sample prepared at the high end of the concentration range (~30 μM) in Figure S4. These data therefore also indicate that hexamer dissociation is likely slow under native conditions. In contrast, when the sample was diluted using 6M urea, this non-zero population density largely disappears (Figure S4 middle), consistent with dissociation of hexamers under these denaturing conditions. A similar behavior as with the denaturing condition was observed with sample preparation at lower concentration (150nM, monomer concentration, Figure S4 bottom), consistent with monomeric protein at lower protein concentration.

Measuring folding kinetics

To further probe the timescale of p97 folding below the transition in Figure S3, ensemble experiments were carried out. Folding of p97F131 labeled with Alexa 488 was initiated by dilution from 6M urea, and the fluorescence intensity was monitored as a function of time. Interestingly, a relatively small but slow rise in the intensity was observed. These data are

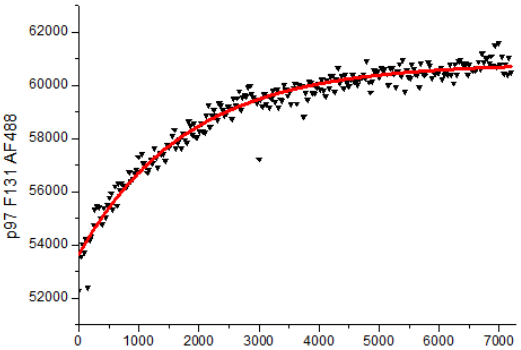

Figure S5. Folding kinetics followed for p97F131 labeled with AF488.

consistent with a de-quenching of the dye as a function of p97 folding. F131 is surrounded by multiple tyrosine residues (Y110, Y134, Y138, Y143, Y172) and tyrosine is a well-known quencher for AF488[5], which could collisionally quench the dye in the flexible unfolded state, but are spatially separated in the folded structure.

Supplementary References

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[2] T. P. Geladopoulos, T. G. Sotiroudis, A. E. Evangelopoulos, *Analytical biochemistry* **1991**, *192*, 112-116.

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