Supplementary Materials for:

An assay for 26S proteasome activity based on fluorescence anisotropy measurements of dye-labeled protein substrates

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Supplementary Table 1: Annotated sequences of the substrate proteins used in the degradation assays

Substrate	Annotated Amino Acid Sequence
Sic60^{PY}-Spectrin$^{12-16}$-SNAP-His$_6$	MEFTPSTPPPYSRGTRYLAQPSGNTSSSALMQGQKAPQKP SQNLVPVTPSTTKSFKNAPAPKLQISRDLEDETLWVEERLPL AQSADYGTNLQTVQLFMKKNQTLQNEILGHTPRVEDVLQR GQQLVEAAEIDCQDLEERLGHLQSSWDRLREAAAGRLQRL RDANEAQQYYLDADEAEAWIGEQELYVISDEIPKDEEGAIV MLKRHLRQQRAVEDYGRNIKQLASRAQGLLSAGHPEGEQI IRLQGQVDKHYAGLKDVAEERKRKLENMYHLFQLKRETD DLEQWISEKELVASSPEMGQDFDHVTLLRDKFRDFARETG AIGQERVDNVNAFIERLIDAGHSEAATIAEWKDGLNEMWA DLLELIDTRMQLLAASYDLHRYFYTGAEILGLIDEKHRELPE DVGLDASTAESFHRVHTAFERDVHLLGVQVQQFQDVATRL QTAYAGEKAEAIQNKEQEVSAAWQALLDACAGRRTQLVD TADKFRFFSMARDLLSWMESIIRQIETQERPRDVSSVELLM KYHQGINAEIETRSKNFSACLELGESLLQRQHQASEEIREKL QQVMSRRKEMNEKWEARWERLRMLLETGMDKDCEMKR TTLDSPLGKLELSGCEQGLHEIKLLGKGTSAADAVEVPAPA AVLGGPEPLMQATAWLNAYFHQPEAIEEFPVPALHHPVFQ QESFTRQVLWKLLKVVKFGEVISYQQLAALAGNPAATAAV KTALSGNPVPILIPCHRVVSSSGAVGGYEGGLAVKEWLLAH EGHRLGKPGPRLEVLFQGPTGHHHHHH*
UbL-SNAP-40-His$_6$	MVSLTFKNFKKEKVPLDLEPSNTILETKTKLAQSISCEESQI KLIYSGKVLQDSKTVSECGLKDGDQVVFMVSQKKVDGGS GGGSMDKDCEMKRTTLDSPLGKLELSGCEQGLHEIKLLGK GTSAADAVEVPAPAAVLGGPEPLMQATAWLNAYFHQPEAI EEFPVPALHHPVFQQESFTRQVLWKLLKVVKFGEVISYQQL AALAGNPAATAAVKTALSGNPVPILIPCHRVVSSSGAVGGY EGGLAVKEWLLAHEGHRLGKPGPRLRYQPLLRISQNCEAAI LRASQTRLNTISGRISPAETSHHHHHH*
UbL-SNAP-His$_6$	MVSLTFKNFKKEKVPLDLEPSNTILETKTKLAQSISCEESQI KLIYSGKVLQDSKTVSECGLKDGDQVVFMVSQKKSGSGGS GSGSMDKDCEMKRTTLDSPLGKLELSGCEQGLHEIKLLGK GTSAADAVEVPAPAAVLGGPEPLMQATAWLNAYFHQPEAI EEFPVPALHHPVFQQESFTRQVLWKLLKVVKFGEVISYQQL AALAGNPAATAAVKTALSGNPVPILIPCHRVVSSSGAVGGY EGGLAVKEWLLAHEGHRLGKPGPRASHHHHHH*

Supplementary Table 2: Decay rate constants of degradation with standard error of the mean for polyubiquitinated and UbL substrates in anisotropy and SDS-PAGE experiments

Method; **substrate type + condition**	**Decay rate constant (min^{-1})**	**Number of measurements**
SDS-PAGE; PolyUb substrate + ATP	0.28 ± 0.03	3
Anisotropy; PolyUb substrate + ATP	0.33 ± 0.03	8
Anisotropy; PolyUb substrate + ATPγS	0.006 ± 0.001	5
Anisotropy; PolyUb substrate + ADP	0.007 ± 0.003	3
Anisotropy; PolyUb substrate + 5 μM epoxomicin	0.011 ± 0.002	4
Anisotropy; PolyUb substrate + 10 μM epoxomicin	0.007 ± 0.001	3
Anisotropy; PolyUb substrate only	0.005 ± 0.001	3
Anisotropy; UbL-SNAP-40 + ATP	0.150 ± 0.002	3
Anisotropy; UbL-SNAP-40 ATP + 1 μM epoxomicin	0.0143 ± 0.0005	3
Anisotropy; UbL-SNAP (no USR) + ATP	-0.0025 ± 0.0002	3

Figure S1: *SDS-PAGE gel shows complete digestion of polyubiquitinated substrate by Proteinase K.*

SDS PAGE analysis of polyubiquitinated substrate incubated for 2 hours at 37 °C by itself or in the presence 200 μg/mL Proteinase K imaged by the Typhoon gel imager, detecting Alexa Fluor 546 fluorescence of the dye labeled substrate. Complete disappearance of the polyubiquitinated, high molecular weight substrate is seen only in the presence of 200 μg/mL Proteinase K.

Figure S2: *Monitoring degradation of Sic60^{PY}-Spectrin$^{12-16}$-His$_6$ by autoradiography.*

Sic60^{PY}-Spectrin$^{12-16}$-His$_6$ was synthesized by coupled *in vitro* transcription and translation in the presence of ^{35}S-methionine, polyubiquitinated and purified by ammonium sulfate precipitation and Ni-affinity chromatography as previously described [1]. The protein was then incubated with 50 nM purified yeast proteasome in the presence of 2 mM ATP and an ATP regenerating system at 30 ˚C. At the indicated times, aliquots were taken and analyzed by SDS-PAGE and electronic autoradiography.

Figure S3: *Degradation of the polyubiquitinated substrate by proteasome at different proteasome concentrations.*

Degradation of ~10 nM Sic60^{PY}-Spectrin$^{12-16}$-SNAP-His$_6$ by purified yeast proteasome at the indicated proteasome concentrations in the presence of 2 mM ATP or 2 mM ATPγS. Degradation of Alexa Fluor 546-labeled polyubiquitinated substrate was monitored by fluorescence anisotropy at room temperature.

Figure S4: *Conversion of measured anisotropy to the fraction of unreacted protein during the degradation of polyubiquitinated substrate.*

Plots showing the raw Anisotropy values versus time during the degradation of $Sic60^{PY}$-$Spectrin^{12-16}$-SNAP-His_6 by purified yeast proteasome from Figure 3 (left column) were converted to plots of the fraction of substrate remaining versus time (right column) using Equation 1 in the Methods Section. The decay rate constants (k_{deg}) obtained by fitting the plots to single exponential decays were compared between the left and right columns for the condition where proteasome and polyubiquitinated substrate were added in the presence of 2 mM ATP. k_{deg} was 0.28 ± 0.02 for the anisotropy versus time representation of the data and 0.26 ± 0.01 for the fraction of substrate remaining representation of the data. (**a**) Degradation of approximately 20 nM polyubiquitinated substrate in the presence of 2 mM ATP and 50 nM proteasome (blue squares), 50 nM proteasome + 5 μM epoxomicin (light blue squares) or no proteasome (black circles). (**b**) Degradation of approximately 20 nM polyubiquitinated substrate by 50 nM proteasome and 2 mM ATP (blue diamonds), 2 mM ADP (green circles) or 2 mM ATPγS (red circles). (**c**) Degradation of approximately 20 nM polyubiquitinated substrate in the presence of 2 mM ATP and 50 nM proteasome (blue diamonds), degradation of approximately 20 nM substrate lacking the polyubiquitin modification by 50 nM proteasome (red diamonds), and incubation of approximately 20 nM polyubiquitinated substrate without proteasome (black circles).

Figure S5: *Conversion of measured anisotropy to the fraction of unreacted protein during the degradation of the UbL substrate.*

Plots showing the raw Anisotropy values versus time during the degradation of UbL-substrates by purified yeast proteasome from Figure 4 (left column) were converted to plots of the fraction of substrate remaining versus time (right column) using Equation 1 in the Methods Section. The decay rate constants (k_{deg}) were compared between the left and right columns for the condition where proteasome and UbL-SNAP-40-His_6 substrate were added in the presence of 2 mM ATP. k_{deg} was 0.19 ± 0.03 for the anisotropy versus time representation of the data and 0.14 ± 0.02 for the fraction of substrate remaining representation of the data.

(**a**) Schematic representation of a model substrate that is targeted to the proteasome by a ubiquitin-like (UbL) domain from *S. cerevisiae* Rad23 protein. The substrate consists of the UbL domain at its N terminus, followed by the SNAP-tag and a 40 amino acid long unstructured region at the C terminus that functions as the proteasome initiation site (UbL-SNAP-40-His_6). (**b**) Degradation of UbL-SNAP-40-His_6 by purified yeast proteasome monitored by fluorescence anisotropy. The degradation reaction was monitored at room temperature over 20 minutes, taking an anisotropy readings every minute. Incubation of 20 nM substrate in the presence of 2 mM ATP and 50 nM proteasome (blue diamonds), of 20 nM substrate in the presence of 2 mM ATPγS and 50 nM proteasome (red circles), and of 20 nM substrate alone (black circles). (**c**) Degradation reaction of UbL substrate with (UbL-SNAP-40-His_6) and without (UbL-

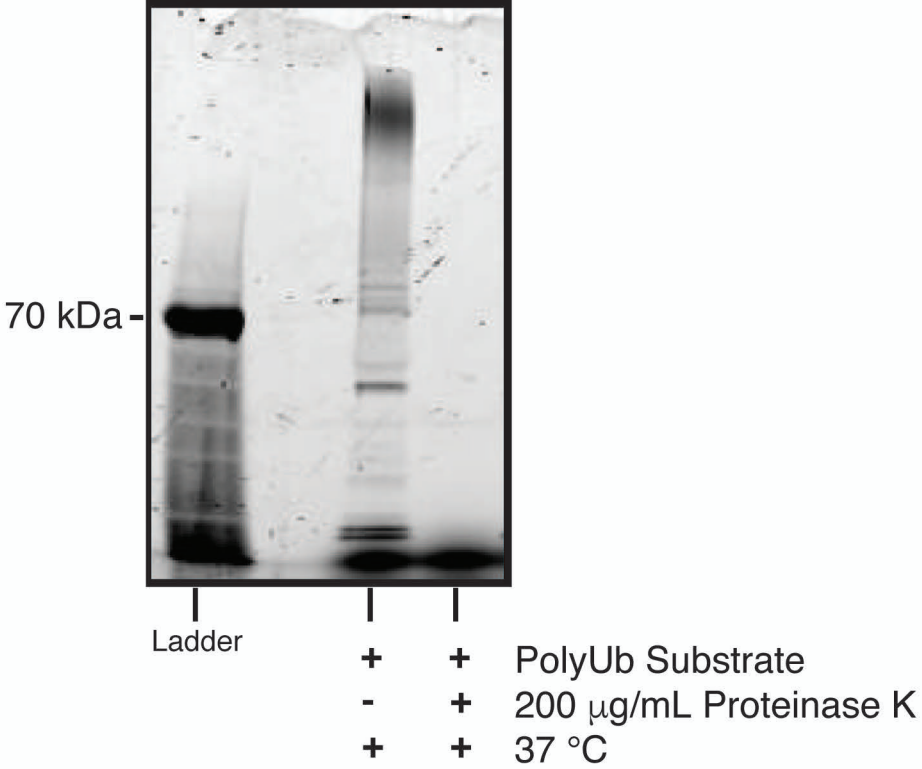
SNAP-His_6) a C-terminal unstructured region. Experiments are as in (b): 20 nM of UbL-SNAP-40-His_6 with 50 nM proteasome and 2 mM ATP (blue diamonds), 20 nM UbL-SNAP-His_6 with 50 nM proteasome and 2 mM ATP (pink diamonds), 20 nM substrate UbL-SNAP-40-His_6 alone (black circles), and 20 nM UbL-SNAP-His_6 alone (light purple circles).

Figure S6: *Degradation reaction of a UbL substrate in the presence of the proteasome inhibitor epoxomicin.*

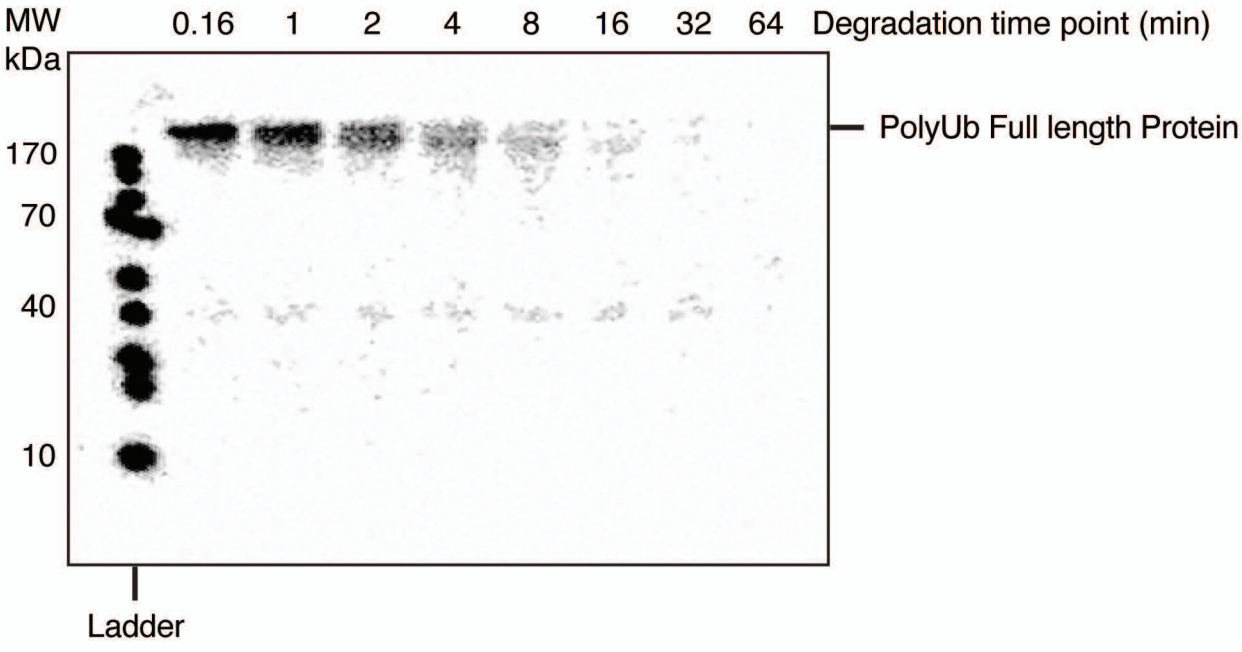
Degradation of UbL-SNAP-40-His_6 by purified yeast proteasome as monitored by fluorescence anisotropy measurements of Alexa Fluor 546-labeled protein. The degradation reaction was monitored at room temperature over 15 minutes by performing an anisotropy reading every minute. Degradation in the presence of ATP, and DMSO (0 μM epoxomicin; dark blue squares) or 1 μM epoxomicin (teal blue squares); incubation in the absence of proteasome (black circles).

References:

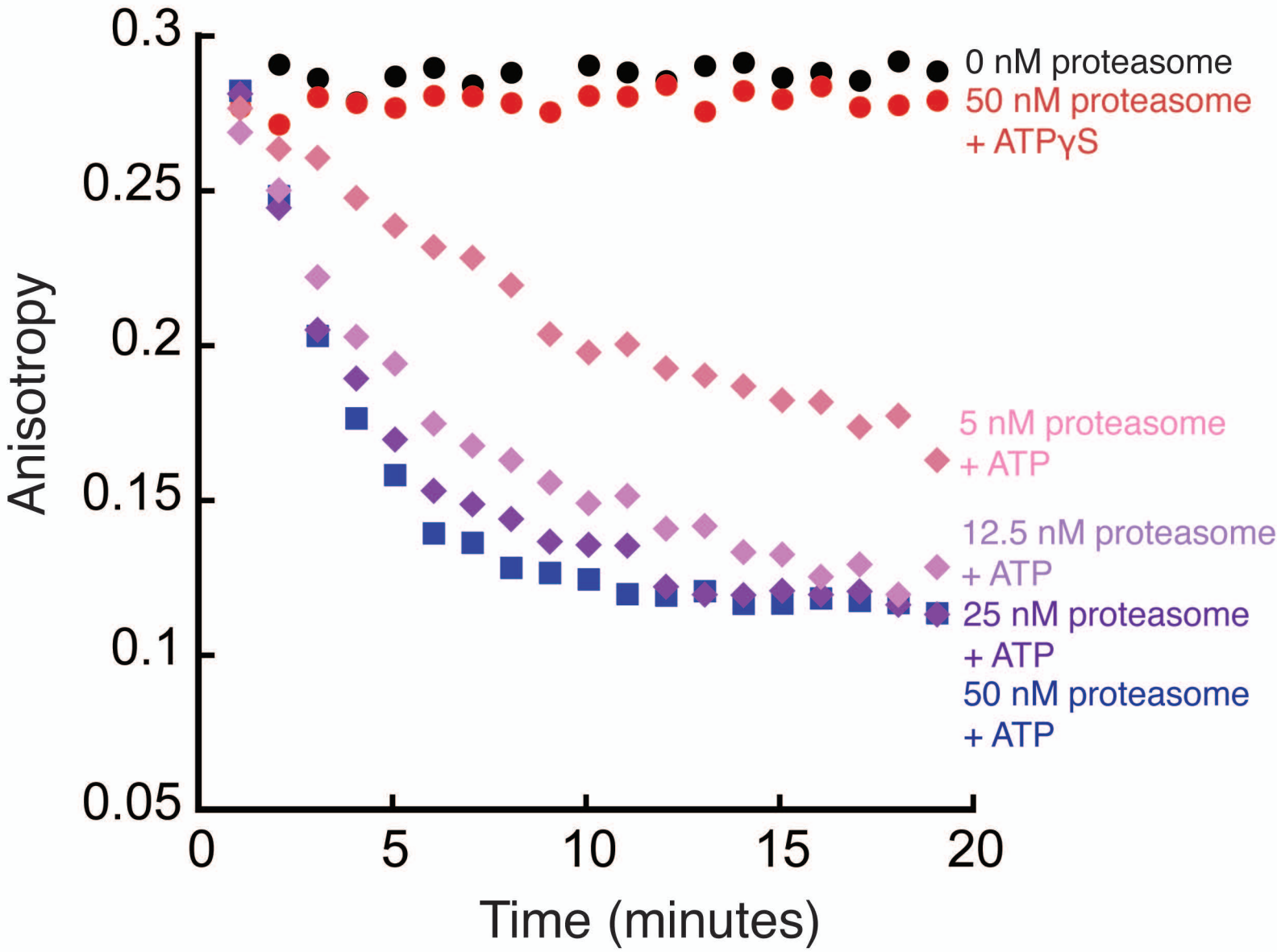
1. Kraut, D.A., et al., *Sequence- and species-dependence of proteasomal processivity.* ACS Chem Biol, 2012. **7**(8): p. 1444-53.



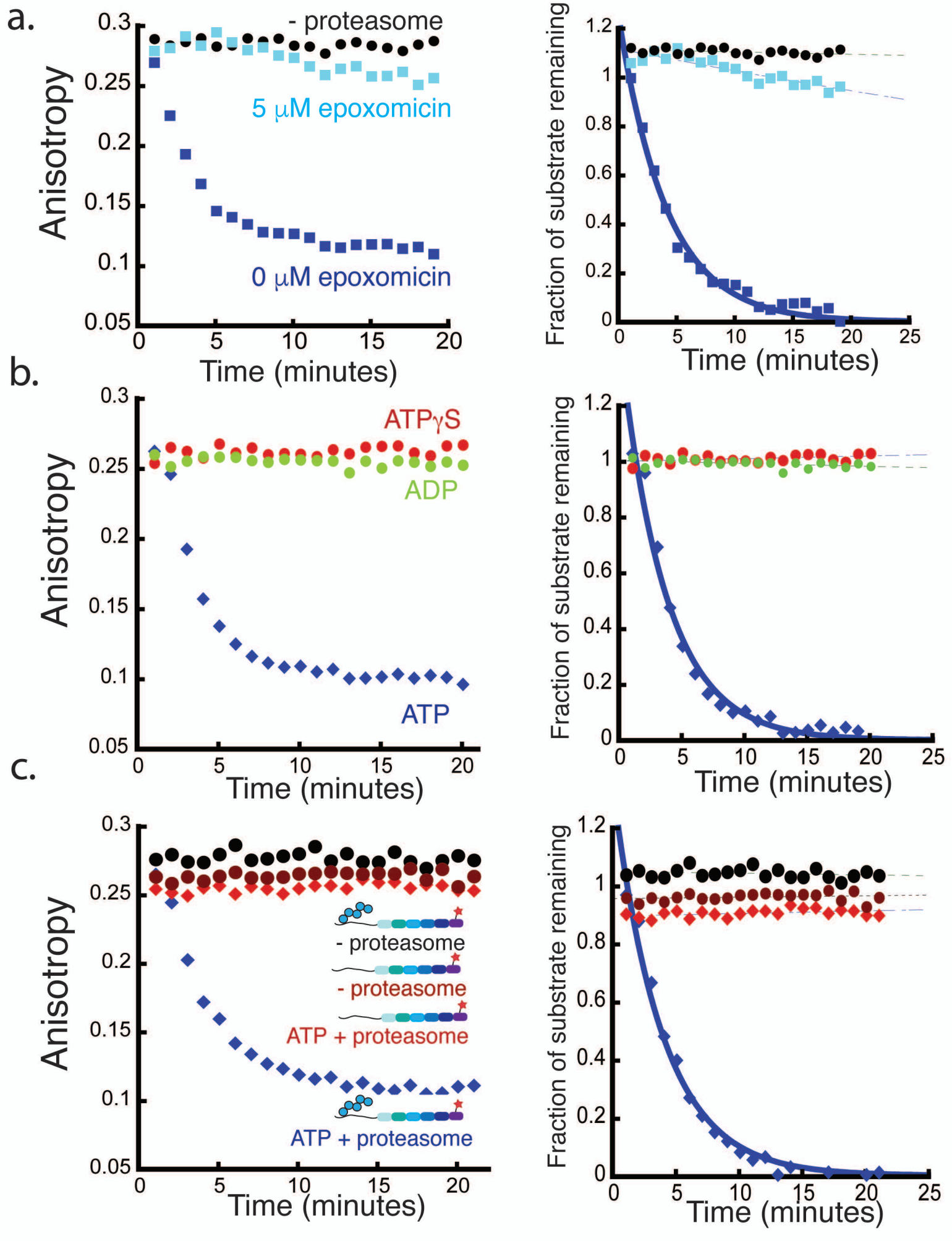
Bhattacharyya et al., Supplementary Figure 1



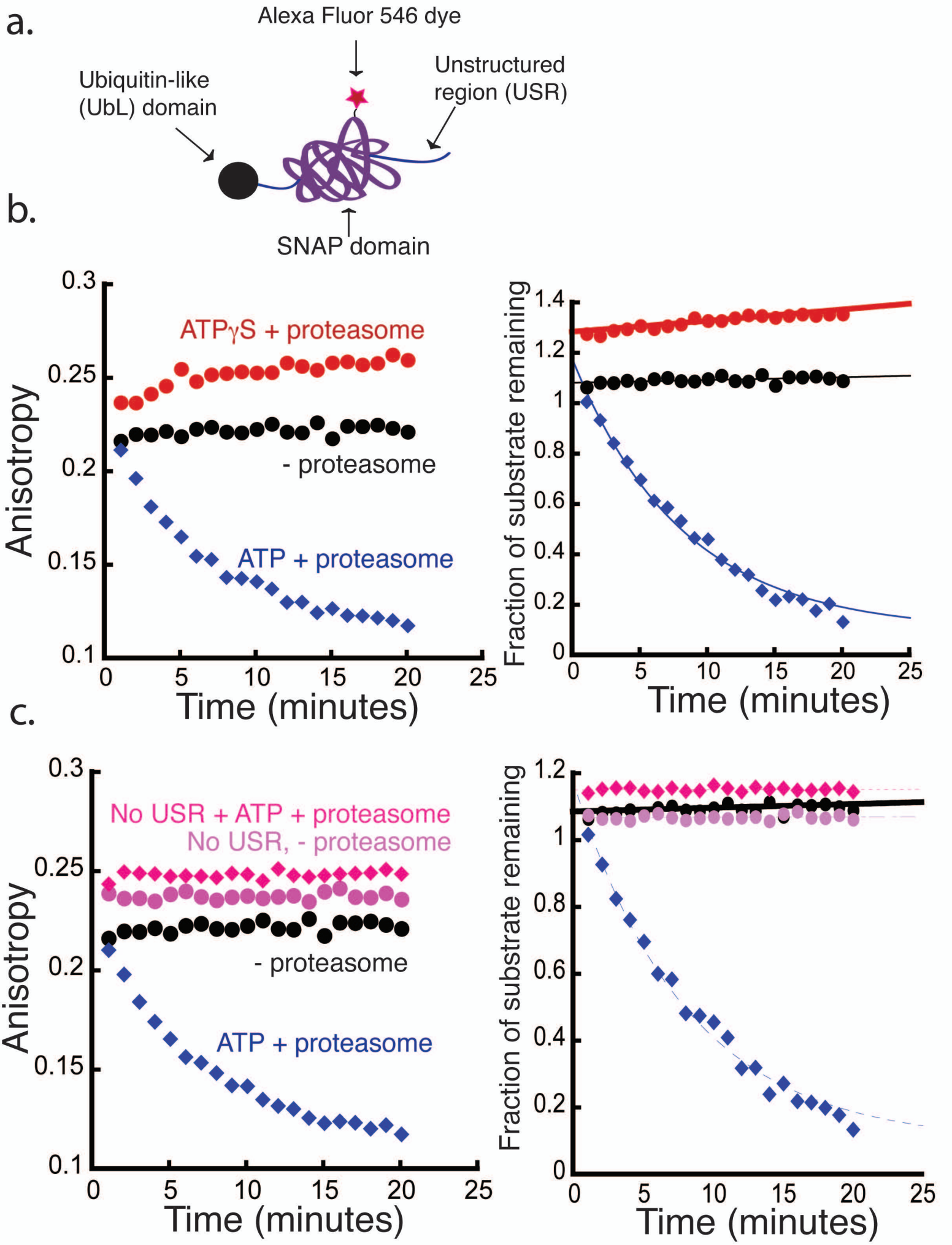
Bhattacharyya et al., Supplementary Figure 2



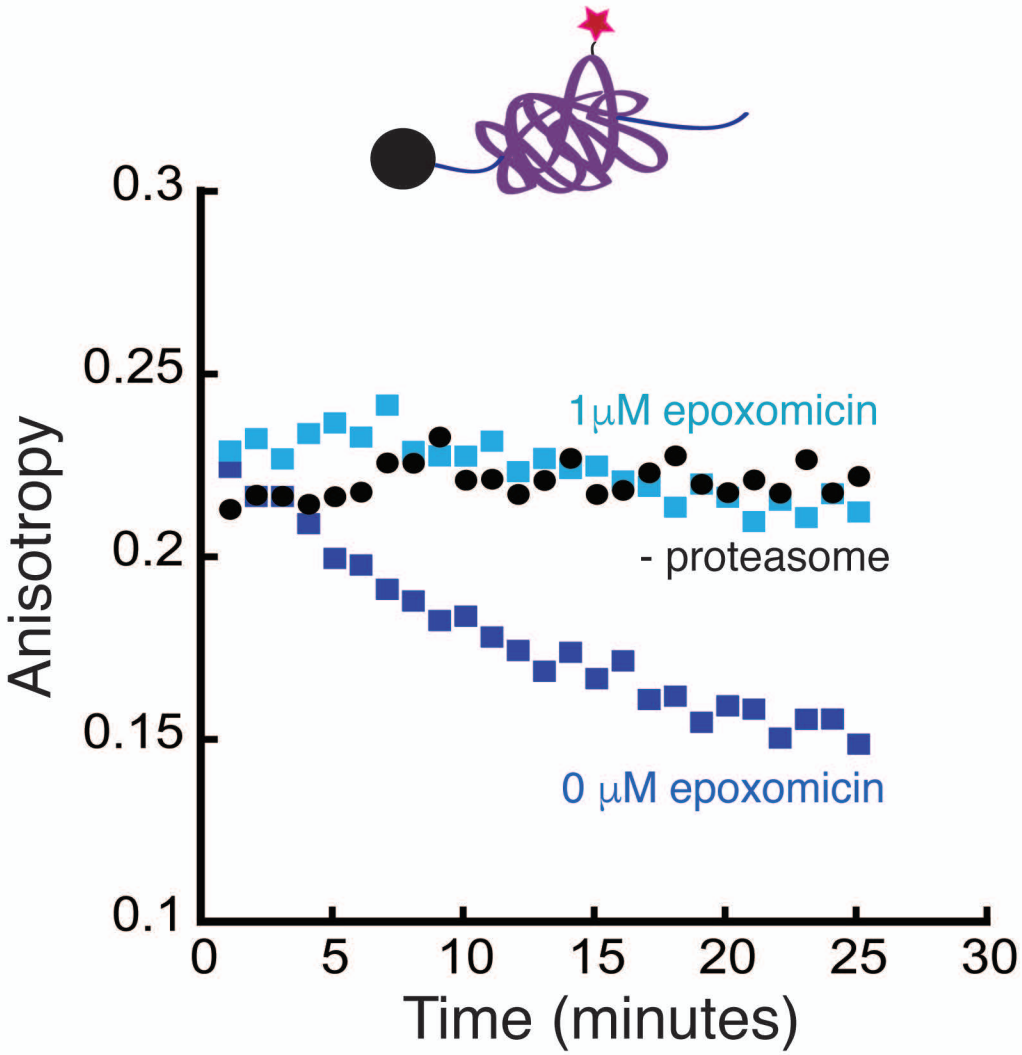
Bhattacharyya et al., Supplementary Figure 3



Bhattacharyya et al., Supplementary Figure 4



Bhattacharyya et al., Supplementary Figure 5



Bhattacharyya et al., Supplementary Figure 6