Mode of targeting to the proteasome determines GFP fate

Christopher Eric Bragança and Daniel Adam Kraut*
From the Department of Chemistry, Villanova University, Villanova, Pennsylvania, USA

The ubiquitin–proteasome system is the canonical pathway for protein degradation in eukaryotic cells. GFP is frequently used as a reporter in proteasomal degradation assays. However, there are multiple variants of GFP in use, and these variants have different intrinsic stabilities. Further, there are multiple means by which substrates are targeted to the proteasome, and these differences could also affect the proteasome’s ability to unfold and degrade substrates. Herein we investigate how the fate of GFP variants of differing intrinsic stabilities is determined by the mode of targeting to the proteasome. We compared two targeting systems: linear Ub4 degrons and the UBL domain from yeast Rad23, both of which are commonly used in degradation experiments. Surprisingly, the UBL degron allows for degradation of the most stable sGFP-containing substrates, whereas the Ub4 degron does not. Destabilizing the GFP by circular permutation allows degradation with either targeting signal, indicating that domain stability and mode of targeting combine to determine substrate fate. Difficult-to-unfold substrates are released and re-engaged multiple times, with removal of the degradation initiation region providing an alternative clipping pathway that precludes unfolding and degradation; the UBL degron favors degradation of even difficult-to-unfold substrates, whereas the Ub4 degron favors clipping. Finally, we show that the ubiquitin receptor Rpn13 is primarily responsible for the enhanced ability of the proteasome to degrade stable UBL-tagged substrates. Our results indicate that the choice of targeting method and reporter protein are critical to the design of protein degradation experiments.

The ubiquitin–proteasome system (UPS) is responsible for the bulk of protein degradation in eukaryotic cells. Proteins to be degraded are typically polyubiquitinated on one or more lysines by the action of E1, E2, and E3 enzymes. The polyubiquitin chain is recognized by one of three ubiquitin receptors on the 19S subunit of the proteasome, Rpn1, Rpn10, or Rpn13, or by ubiquitin shuttle proteins that bind to both polyubiquitin and one of the ubiquitin receptors. The substrate is then engaged by the ATP-dependent Rpt motor proteins at an unstructured initiation region and translocated into the 20S core particle’s central degradation chamber. The polyubiquitin chain passes by the deubiquitinase Rpn11 during translocation, which removes the chain, allowing ubiquitin to be recycled for additional rounds of protein targeting. Although recent advances have led to the understanding of many of the details of substrate recognition and unfolding, it remains unclear why three ubiquitin receptors (and multiple adaptors) are necessary or what controls whether a protein targeted to the proteasome by ubiquitination is successfully unfolded or escapes degradation.

GFP and its derivatives are some of the most commonly used reporters used to study the UPS both in vitro and in vivo (1). For example, N-end rule (Ub-X-GFP) and ubiquitin-fusion degradation (UbG76V-GFP) substrates (2) have been used to explore the effects of Gly-Ala repeats, Huntingtin protein fragments, and prions on global protein degradation (3–7), proteasome inhibitors in trypanosomes (8), and many other aspects of in vivo degradation. Other GFP-based systems have also been proposed for global monitoring of protein degradation in cells (9, 10). GFP is commonly fused to proteins of interest to determine whether and how fast they will be degraded in living cells or even to control their degradation (11–13). GFP has also been used extensively in in vitro investigations of proteasome activity and mechanism (14–19).

However, there are challenges to using GFP as a model substrate. GFP forms an exceptionally stable 11-stranded β-barrel. Extraction of a single β-strand from the barrel by ATP-dependent proteases leads to a still stable 10-stranded intermediate, and the original β-barrel can fully reform if pulling is not rapid enough (20). Indeed, GFP-containing fragments as an end point of degradation have been observed both in cells and in vitro (16); these partial-degradation events are potentially frequently missed because most experiments simply look at total fluorescence. On the other hand, circular permutants of GFP that unfold in a single step have been shown to be degraded by the bacterial ATP-dependent protease ClpXP without stalling (20). The proteasome has been suggested to be even more processive than ClpXP (21) and thus capable of degrading even difficult-to-unfold substrates like GFP without stalling (17). However, in some cases GFP unfolding and degradation in cells requires the unfoldase cdc48/p97 in addition to the proteasome’s own unfoldase activity (22–24). Thus, a better understanding of GFP unfolding by the proteasome will inform the interpretation of a diversity of experiments.

We recently showed that substrate ubiquitination can increase the proteasome’s unfolding ability and that proteasomal ubiquitin receptors mediate this increase, with Rpn13 playing the largest role (16, 25). Herein we set out to determine whether the degradation of GFP is affected by the mode of targeting to the proteasome, and, if so, which ubiquitin receptors are responsible. We used two previously established targeting systems, the linear Ub4 modification, with four nonhydrolyzable ubiquitins connected by short linkers, and the ubiquitin-like (UBL) domain from yeast Rad23, both attached to the N terminus. In both cases, addition of an unstructured initiation region
at the C terminus of a target protein has been shown to lead to efficient degradation by the proteasome (26). Rpn1 has been suggested to be the primary receptor for Rad23 (27), although Rpn10 and Rpn13 are thought to be the primary receptors for ubiquitinated substrates (28). Quite recently, the Matouschek laboratory used a circular permutant of GFP to show that indeed Rpn10 and to a lesser extent Rpn13 were the only receptors used by Ub4, whereas Rpn1 and Rpn13 were the receptors of choice for the UBL domain (18). However, because a relatively easy-to-unfold substrate was used, it remained unclear to what extent the proteasome’s unfolding ability is affected by the targeting mode or receptor choice. By using substrates containing GFP variants of different stabilities with WT and receptor-mutant proteasomes and using gel-based assays that can detect partial degradation, we show here that UBL-targeted substrates are degraded more efficiently than Ub4-targeted substrates and that this difference seems to be correlated with reliance on the Rpn13 receptor.

Results

sGFP is not degraded by the proteasome when targeted via a linear Ub4 signal

We first examined the ability of the proteasome to degrade a substrate consisting of superfolder GFP (sGFP) with a linear Ub4 tag on the N terminus and a C-terminal unstructured tail of 44 amino acids (including a terminal hexa-histidine tag), Ub4-sGFP-38-His6. The substrate was incubated with purified yeast proteasome, and the reaction mixture was run on an SDS-PAGE gel and visualized and quantified using the inherent fluorescence of GFP. When GFP is unfolded or degraded, it loses fluorescence. There was essentially no degradation of sGFP as determined by quantifying the total fluorescence (Fig. 1A, green). However, there was substantial “clipping” of the tail (Fig. 1A, red and blue) as has been seen previously with other GFP-containing substrates (16, 20). Clipping could occur because the unstructured tail is too short to initiate processive unfolding or because after the proteasome initiates degradation, it is unable to unfold sGFP, stalls, and eventually releases the substrate. Longer initiation regions improve degradation of some proteasomal substrates (18, 26), but we found replacing the tail with an 108-amino acid-long tail (Ub4-sGFP-102-His6) only modestly increased the rate and extent of complete degradation, and clipping remained the predominant outcome of degradation (Fig. 1, B, E, and F). On the other hand, replacement of sGFP with a circular permutant of GFP (in which the 8th β-strand is the C terminus instead of the 11th), cp8sGFP, led to rapid degradation of the substrate with very little clipping (Fig. 1C), consistent with the hypothesis that the proteasome stalls while trying to unfold the highly stable sGFP.

Additional experiments support the possibility that the linear Ub4 tag is capable of targeting proteins to the proteasome, but the ability to successfully unfold and degrade the substrates depends on their stabilities. First, we confirmed that the Ub4-sGFP substrate did bind to the proteasome, because it was able to inhibit the degradation of a conventionally ubiquitinated substrate (Fig. S1). As expected, clipping of the sGFP substrate and degradation of the cp8sGFP substrate were both greatly slowed by proteasome inhibitors (Fig. S2, A and B). However, the addition of free polyubiquitin chains slowed degradation of cp8sGFP but not clipping of sGFP, suggesting that clipping does not depend on ubiquitin (Fig. S2, C and D). We observed that clipping of sGFP could be mediated by either the 20S or 26S proteasome, because both free 20S core particle or doubly capped 26S proteasome (reconstituted by the addition of excess purified 19S particle to purified 20S core particle) clipped the sGFP substrate similarly. In contrast, the cp8sGFP substrate was clipped by 20S proteasome but degraded once any 19S particle was added (Fig. S3). Replacing sGFP with the less stable enhanced GFP (eGFP) led to intermediate levels of clipping and degradation (Fig. 1, D and F). In sum, although the linear Ub4 tag targets proteins to the proteasome, their unfolding and degradation depends on their stabilities. If substrates are too stable to be unfolded rapidly, they are instead clipped in a ubiquitin-independent manner, shortening and presumably inactivating the unstructured initiation site and thereby preventing retargeting.

Targeting via the UBL domain results in successful degradation of sGFP

Next, we replaced the Ub4 targeting sequence on sGFP with the UBL domain from yeast Rad23 (UBL-sGFP-102-His6). The proteasome was able to robustly degrade this substrate (as had been previously described (15)) with little clipping observed (Fig. 2, A and E). Degradation proceeded through a transient long fragment (blue curve), such that the rate of disappearance of full-length protein was much faster than the rate of disappearance of fluorescence (Fig. 2D). A shorter persistent fragment (purple curve) was also produced that presumably has an unstructured tail too short to serve as an initiation site. This fragment was likely produced directly from the full-length substrate, analogous to clipping of the Ub4-containing substrates via a proteasome-dependent but UBL-independent pathway (see Fig. 4 below). As with the Ub4-containing substrates, replacing sGFP with the less stable cp8sGFP (Fig. 2B) reduced the difference between full-length and fluorescence degradation rates (Fig. 2D) and reduced the extent of clipping (Fig. 2E) such that the cp8sGFP substrate was degraded without the detection of any intermediates or formation of clipped fragment. (We note that this substrate also ran as a thicker band on a gel, potentially obscuring detection of intermediates.) Only small differences were seen between eGFP and sGFP (Fig. 2C). In all cases, the extent of complete degradation of a given GFP protein was larger with a UBL tag then with a Ub4 tag, indicating that the UBL domain is better able to promote the unfolding and degradation of the substrate by the proteasome.

Rates and extents of degradation are dependent on ATPase rate

It had previously been shown that for the Ub4-cp8sGFP-38-His6 substrate, slowing proteasomal ATP hydrolysis with ATP/ATPγS mixtures led to a proportional decrease in GFP degradation (17). The authors concluded from this result that unlike bacterial ATP-dependent proteases, the proteasome is highly
processive and does not release GFP sufficiently for refolding to occur. That is, even at low ATPase rates, $k_{\text{refold}} \ll k_{\text{unfold2}}$ (Fig. 3A). However, as we have shown, this circularly permuted substrate is much easier for the proteasome to unfold than eGFP or sGFP and might indeed unfold catastrophically. We therefore set out to determine the effect of reducing ATP hydrolysis rates on unfolding and degradation of our substrates using ATPγS.

In contrast to previous results (which were conducted using mammalian proteasome, not yeast proteasome), we found that replacing even a small fraction of ATP with ATPγS led to a precipitous drop in degradation rates for Ub4-cp8sGFP-38-His6 (Fig. 3, B–D). At higher ATPγS concentrations, there was also a substantial increase in the extent of clipping (Fig. 3E). This result was surprising, because we had not anticipated the formation of a stable unfolding intermediate with the cp8sGFP substrate. However, this analysis assumes that replacing ATP with ATPγS will linearly decrease the ATPase rate, which to our knowledge had not been tested. Using a coupled lactate dehydrogenase/pyruvate kinase ATPase assay, we found that even a small fraction of ATPγS is able to dramatically decrease the ATPase activity of the proteasome, consistent with a cooperative ATPase mechanism (Fig. 3F). Similar results were seen using a malachite green assay (data not shown), indicating that the results were not due to ATPγS inhibition of pyruvate kinase. Thus, there is indeed a linear dependence of degradation rate on ATPase rate for the cp8sGFP substrate (Fig. 3G), consistent with a highly processive degradation mechanism for cp8sGFP, although important differences in terms of sensitivity to ATPγS are noted relative to mammalian proteasome. The increase in clipping seen at higher ATPγS concentrations indicates that slowing degradation sufficiently can cause the non-productive Ub-independent clipping pathway to become competitive with degradation even for this less stable substrate.

We next examined the degradation of the more stable sGFP-containing substrate, UBL-sGFP-102-His6. Replacing 25% of the ATP with ATPγS, which reduced the ATPase rate ~8-fold, reduced the initial rate of fluorescence loss by ~10-fold, approximately twice the effect seen with the cp8-sGFP substrate (an ~5-fold effect) (Fig. 3H versus Fig. 2A). Interestingly, the initial disappearance of full-length protein was only reduced by 1.6-fold, suggesting that formation of the longer clipped intermediate ($k_{\text{unfold1}}$) is less sensitive to ATP concentration than unfolding of the rest of GFP ($k_{\text{unfold2}}$). The extent of clipping was also doubled from ~10% to ~20%. Overall, these results indicate that although there is some ability for this more stable GFP to refold upon initial unfolding, even at low ATPase rates, processive degradation is the predominate...
pathway taken. Thus, at least when targeted via the UBL domain, the proteasome does indeed processively degrade even a highly stable protein with little stalling. For comparison, ClpXP requires only an ~10% drop in ATPase rates for an ~90% decrease in degradation rates of sGFP (20).

Partially degraded protein can be rebound and degraded

The larger GFP-containing fragment that appears and then decreases in intensity during degradation of UBL-targeted substrates (Fig. 2A) could represent transiently stalled protein bound to the proteasome before it has been degraded. Alternatively, it could indicate that partially degraded fragments can be released by the proteasome and then reacquired via the N-terminal proteasome-binding tag (Fig. 4A). If those partially degraded fragments retain long enough initiation regions at the C terminus of the protein, this would give the proteasome a second opportunity to attempt to unfold and degrade GFP (Fig. 4A; disengage/re-engage equilibrium). To distinguish between these possibilities, we set up a pulse-chase experiment using the UBL-sGFP-102-His6 substrate in the presence of 25% ATPγS to increase clipping (Fig. 3, D and H); an increase away from unfolding would increase clipping and thus the formation of no-longer engageable substrate fragments. Indeed, our kinetic model is able to reasonably fit all three data sets (Fig. S4, A–C). These results are also consistent with the ability of ATPγS to increase clipping (Fig. 3, D and H); an increase away from unfolding would increase clipping and thus the formation of no-longer engageable substrate fragments. Indeed, our kinetic model is able to reasonably fit the degradation of UBL-sGFP-102-His6 in the absence of ATPγS if 25% ATPγS slows the unfolding rate constant by ~5-fold and does not affect any other rate constants (Fig. S4D). Our results are consistent with previous experiments in which
ubiquitin-independent substrates created by fusing a stabilized target protein to Rpn10 were released and rebound rapidly during the degradation process (29). The ability to repeatedly release and rebind the substrate when degradation stalls might make it easier to degrade physiological substrates where the proteasome-binding element is distal to the initiation region (as opposed to many proteins where ubiquitination occurs on the initiation region, such that ubiquitin on the substrate would be removed prior to fragment release), and the ability of the proteasome to trim unstructured initiation sites may serve as a counterbalance, preventing the same substrate from being repeatedly fruitlessly engaged.

Rpn13 is primarily responsible for strong unfolding with UBL targeting

Ub₃ binds ~4 times more weakly to the proteasome than UBL (15, 19). However, decreasing the affinity 4-fold in our model has only a minor impact on simulated degradation, such that the proteasome should still largely degrade the substrate with minimal clipping. Given that a cp8sGFP substrate is degraded easily by either proteasome, there are likely also stability-linked differences in the engagement and unfolding rates. Ub₃ and UBL substrates might be positioned differently at the proteasome, affecting engagement and perhaps even unfolding ability. We were therefore curious to know whether one or more of the intrinsic proteasomal ubiquitin receptors were responsible for the proteasome’s ability to degrade hard-to-unfold substrates with UBL but not Ub₃ targeting. The Matoušek laboratory had previously shown that the linear Ub₃ degron (or more specifically the Ub₃-cp8sGFP-38-His₆ substrate) is recognized and degraded by both Rpn10 and Rpn13 (but not Rpn1), whereas the corresponding UBL substrate is degraded even if all three receptors are removed but requires Rpn1 or Rpn13 for maximal degradation efficiency (18). To determine the requirements for a more-difficult-to-unfold substrate, we used Ub₃- or UBL-eGFP-102-His₆, which is degraded very efficiently with the UBL signal and with moderate efficiency with the Ub₃ signal (Figs. 1D and 2B). When targeted via the Ub₃ signal, individual proteasome receptor mutants (Rpn1ΔUIM, Rpn10ΔUIM, and Rpn13-pru, each of which use point mutations to disrupt ubiquitin binding (25)) had modest effects, generally increasing the amount of clipping and, in the case of Rpn13-pru, reducing the rate of overall degradation (disappearance of fluorescence) (Fig. 5, A and B, and Fig. S5). Double mutants retaining only one receptor (Rpn1-only, Rpn10-only, and Rpn13-only) again had increased clipping but
only small effects on degradation rates, suggesting that all of the receptors are capable of mediating at least some degradation of this substrate, although none of the individual receptors are able to do so particularly efficiently. In contrast, targeting via the UBL domain was much more receptor-specific. Mutating either Rpn1 or Rpn13 modestly increased clipping and, in the case of Rpn13-pru, reduced overall degradation rates (Fig. 5, C and D). Surprisingly, mutating Rpn10 actually increased overall degradation rates, suggesting that binding of the UBL domain to Rpn10 might be non- or counterproductive. The effects of single-receptor–containing proteasomes were even more striking. Rpn13-only proteasome behaved essentially like WT, whereas Rpn1-only and Rpn10-only proteasome were almost incapable of unfolding and degrading GFP, with greatly reduced overall degradation rates and greatly elevated clipping. Indeed, these mutants essentially convert the well-degraded UBL substrate into the poorly degraded Ub4 substrate, indicating that Rpn13 is primarily responsible for the enhanced unfolding and degradation of UBL-containing substrates, although the combination of Rpn10 and Rpn1 is able to partially compensate for the loss of Rpn13’s ability to bind the UBL domain. We confirmed that the inability to degrade was not simply due to a lack of substrate binding, because the UBL-cp8sGFP-102-His6 substrate was still degraded by the Rpn1-only proteasome with an initial rate only 2–3-fold reduced from WT without any evidence of fragment formation (Fig. S6).

**Figure 4. Partially degraded protein can be rebound and degraded.** A, model for degradation of UBL-sGFP-102-His6 substrate. The substrate can be clipped, forming a short fragment, in a UBL-independent (but proteasome-dependent) process, or can be bound via the UBL and engaged, and then the tail is partly degraded to produce the longer fragment, which can either be unfolded and completely degraded or can alternatively be released and rebound repeatedly until it is degraded. Rate constants were derived from fitting the model to the data from C and D below (Fig. S4). For binding and release steps, the $K_a$ of 85 nM for UBL-sGFP-102-His6 (15) was used as the $K_D$, and $k_{bind}$ was set to 51 $\mu M^{-1} min^{-1}$ as determined for Ub4 from single-molecule data (30) (assuming $K_D$ differences are due to off-rate differences). Nondegradable rate constant was added to account for a fraction of substrate that remains intact throughout the experiment. Disengagement and re-engagement were explicitly modeled but fits were not sensitive to the exact numbers as long as the equilibrium constant was $\sim$0.1 (such that engagement was greatly favored). B, representative gels for degradation assays with 100 nM proteasome and 100 nM UBL-sGFP-102-His6 in which 5 $\mu M$ GST-UBLRad23 (or buffer) is added after 9 min or at the start of the assay. C and D, quantification of pulse-chase assays from Fig. 2A. Closed symbols show mock addition, and open symbols are with addition of GST-UBLRad23. Dashed lines show approximate times of addition.
**Discussion**

GFP is commonly used as a model substrate to study protein degradation. Here we show that both the ubiquitin binding tag (Ub₄ versus UBL) and the GFP variant’s stability are critically important for determining whether GFP is successfully unfolded and degraded or whether degradation is terminated unsuccessfully. UBL-targeted substrates with the same stability are degraded more easily than Ub₄-targeted substrates. This extra unfolding ability is mediated, at least in part, by the Rpn13 ubiquitin receptor.

Polyubiquitinated proteins have been shown to activate the proteasome via multiple mechanisms, including by opening the gate to the 20S core particle, increasing the ATPase activity of the proteasome, and increasing the proteasome’s ability to unfold substrates (16, 25, 31, 32). To our knowledge, no such effects have been reported for substrates containing UBL domains, but given their ability to bind to many of the same receptors that bind ubiquitin, UBL domains are likely to similarly mediate proteasomal activation. Because degradation of at least sGFP-containing substrates is limited by unfolding, activation via gate opening alone is unlikely to be responsible for the greater efficacy of UBL targeting.

We had previously proposed that binding of ubiquitinated proteins to Rpn13 might help activate the proteasome’s unfolding ability by shifting the conformation from the s1 substrate-binding state to s3-like substrate-processing states; in the kinetic model of Fig. 4A, this would correspond to increasing the initial engagement, degradation, and/or unfolding rates (16, 25). The same hypothesis could explain the UBL versus Ub₄ results we see, given that Ub₄ substrates rely primarily on Rpn10, whereas UBL substrates rely primarily on Rpn13 (18). Consistent with this hypothesis, release of polyubiquitin chains from Rpn13 has been suggested to slow multiple turnover degradation by the proteasome, suggesting that engagement of Rpn13 may keep the proteasome from returning to the s1 state for additional rounds of substrate binding (18). An alternative possibility is that differences between Ub₄ and UBL stem from the need to pull the proteasome-binding tag away from its receptor during translocation. Extracting a single UBL domain may be easier than extracting Ub₄, which might more easily rebinding during the process. However, the UBL domain binds ~4-fold more tightly to the proteasome than Ub₄ (15, 19), arguing against this model.

It has long been puzzling why so many ubiquitin receptors and shuttle proteins were needed for proteasomal function. Our results suggest that shuttle proteins like Rad23 may be used to bring harder-to-unfold proteins to the proteasome by tethering them to Rpn13 and activating an enhanced unfolding ability, whereas easier-to-unfold proteins are able to be degraded without this extra assistance by directly docking to Rpn10. Recently, it has been suggested that there is at least one additional ubiquitin or UBL receptor on the proteasome, because even a triple-receptor mutant can still degrade a UBL-cp8sGFP-102-His₅ substrate (18). Our results indicate that if indeed an additional ubiquitin or UBL receptor exists, it does not support unfolding of difficult-to-unfold substrates such as noncircularly permuted GFP.

Our results also indicate that UPS substrates where the two-component degron (proteasome-binding tag and unstructured

**Figure 5. Effects of proteasome receptor mutations on degradation of eGFP-containing substrates.** A, initial rates of degradation for Ub₄-eGFP-102-His₅ for either disappearance of full-length protein (red) or disappearance of fluorescence (green). Error bars are S.E. derived from curve-fitting four replicate experiments. B, percentage of full-length Ub₄-eGFP-102-His₅ that was clipped rather than being completely degraded. C and D, as in A and B, but for UBL-eGFP-102-His₅. Error bars are S.E. derived from curve-fitting four replicate experiments.

Mode of targeting to the proteasome determines GFP fate
initiation region) is split between different portions of the protein may be degraded more robustly than those where the initiation region and binding tag are near one another in the primary sequence. Separation allows partially degraded protein fragments to be retargeted to the proteasome, such that a lower processivity is required to get complete degradation of the substrate. However, this is balanced by the ability of the proteasome to trim unstructured initiation regions in a binding-tag-independent fashion (Fig. 4A), which may prevent futile cycles of attempted degradation. Indeed, "masked" initiation sites, which are only exposed when degradation is needed (33), may be important both for preventing premature degradation and for preventing premature destruction of the initiation site.

We have learned much about ATP-dependent proteases from studies of bacterial proteases such as ClpXP (34, 35), and much of what we have learned applies to the proteasome as well, but there are also important mechanistic differences. ClpXP stalls when trying to degrade GFP in low ATPase rate conditions because GFP partially unfolds and then refolds faster than the next pull can come. Our results (in agreement with previous results for the mammalian proteasome (17)) indicate that the proteasome does not have this same problem, and there is little refolding of a GFP intermediate that can occur. One possible explanation is that the proteasome grips the partially unfolded substrate more tightly, preventing the backsliding that would be required for refolding to occur. In agreement with this, we have found that the proteasome has a much higher unfolding ability (the ratio between unfolding and release rates) than bacterial ATP-dependent proteases (21) and that mammalian proteasomes has an even higher unfolding ability than yeast proteasome because of a slower release rate, despite a slower ATPase rate and unfolding rate (36). Given that the bacterial ATPases tend to run faster than the proteasome (ClpXP has an ATPase rate ~5–10-fold faster than that of the proteasome), it seems most likely that the higher unfolding ability of the proteasome, and its lower tendency to stall on GFP, is due to a slower release of partially degraded substrates.

Finally, these results have implications for the use of GFP as a tool in assays and screens that measure the global health of the UPS or that examine the fate of individual proteins. Given GFP’s high stability, it seems advisable to use a circular permute in cases where one simply wants to know whether an attached protein degrades, or how robust the UPS is for degradation of easy-to-unfold proteins. A more stable GFP such as sGFP could instead be used to look at the ability of the UPS to degrade more challenging substrates, and again, the nature of the targeting mechanism may play a role in determining how robust a proteasome response is required for degradation.

Experimental procedures

Constructs

Plasmids expressing Ub₄-sGFP-38-His₆, in pGEM3Zf⁺, Ub₄-cp8sGFP-38-His₆, in pGEM3Zf⁺, and UBL-sGFP-102-His₆, in pETDuet were kind gifts from the Matouschek laboratory. A plasmid expressing Ub₄-sGFP-102-His₆ was made by replacing the sGFP from UBL-sGFP-102-His₆, with the cp8sGFP from Ub₄-cp8sGFP-38-His₆ by restriction cloning. Plasmids expressing eGFP substrates were made by replacing sGFP with eGFP by restriction cloning. A plasmid expressing GST-UBLRad23 was made by inserting UBLRad23 from yeast into pDEST15. Plasmid sequences are available upon request.

Proteasome purification

Yeast (Saccharomyces cerevisiae) proteasome was purified from WT strain YYS40 or receptor mutant strains using a 3× FLAG-tagged copy of Rpn11 subunit of the 19S particle as described previously (16, 25). The cells were grown overnight in YEPD or selective medium before being transferred to YEPD medium for large-scale growth at 30 °C. The cells were harvested by centrifugation and resuspended in buffer containing 25 mM Tris, pH 7.5, 10 mM MgCl₂, 4 mM ATP, 1 mM DTT, 10% (v/v) glycerol, and a 2× ATP-regeneration system (ARS) consisting of 0.5 mg/ml creatine phosphokinase and 20 mM phosphocreatine. The cells were then lysed by homogenization, and the lysate was pH adjusted to 7.5 using 1 M Tris base before high speed centrifugation. The supernatant was supplemented with 5 mM ATP and 1× ARS, filtered, and incubated with anti-FLAG M2 affinity gel at 4 °C while rotating for 2 h. The resin was washed three times with 25 mM Tris, pH 7.5, 5 mM MgCl₂, 2 mM ATP, 1 mM DTT, and 10% (v/v) glycerol. The proteasome was eluted with 100 μg/ml 3× FLAG peptide. Peak elutions were pooled, and concentration was determined by Bradford assay. Proteasome was flash-frozen and stored at ~80 °C until use. To purify proteasome in the presence of ATPγS, the final wash and the elution buffer contained 2 mM ATPγS instead of ATP. ATPγS was purchased from Cayman Chemical, dissolved in double-distilled H₂O and 1 M Tris-Cl to a pH of ~8, and stored as 10 mM ATPγS at ~80 °C until use. To purify 19S particle, resin was washed three times with 25 mM Tris, pH 7.5, 500 mM NaCl, 5 mM MgCl₂, 2 mM ATP, 1 mM DTT, and 10% (v/v) glycerol and then once with 25 mM Tris, pH 7.5, 5 mM MgCl₂, 2 mM ATP, 1 mM DTT, and 10% (v/v) glycerol before elution. To purify 20S particle, strain yMDC11, containing a 3× FLAG-tagged copy of Pre1, was used; ATP, ARS, and MgCl₂ were omitted from buffers; and resin was washed three times with 25 mM Tris, pH 7.5, 500 mM NaCl, 1 mM DTT, and 10% (v/v) glycerol and then once with 25 mM Tris, pH 7.5, 1 mM DTT, and 10% (v/v) glycerol before elution.

Bacterial protein overexpression and purification

Ub₄-sGFP-38-His₆ and Ub₄-cp8sGFP-38-His₆ were transformed into Rosetta(DE3)pLysS cells, grown in LB medium at 37°C to an A₆₀₀ of 0.6, induced with 0.4 mM isopropyl β-D-thiogalactopyranoside at an A₆₀₀ of 0.6, and grown for 12–18 h. The cells were spun down, resuspended in lysis buffer (50 mM sodium phosphate, 300 mM NaCl, 10 mM imidazole, pH 8.0), lysed by high-pressure homogenization, bound to a nickel–nitrilotriacetic acid column, washed, and eluted with increasing concentrations of imidazole. The purest fluorescent fractions were pooled (as determined by SDS-PAGE), and the concentration...
was determined by absorbance at 280 nm. Proteins were flash-frozen and stored at −80 °C.

UBL-eGFP-102-His6 and Ub4-eGFP-102-His6 were purified identically, but they were transformed into BL21(DE3) cells and overexpressed in LB medium at 30 and 37 °C, respectively. UBL-cp8sGFP-102-His6 and Ub4-sGFP-102-His6 were purified identically, but they were transformed into BL21 (DE3) cells and overexpressed in autoinduction medium at 37 °C (37). GST-UBL-Rad23 was transformed into BL21AI cells, grown in LB medium at 37 °C to an OD600 of 0.6, and induced with 0.1% arabinose for 3 h. The cells were spun down, resuspended in PBS, lysed by high-pressure homogenization, and loaded onto a GSH–agarose column. After washing with PBS, the protein was eluted with GSH elution buffer (100 mM Tris-Cl, 100 mM NaCl, 10 mM GSH, 1 mM DTT pH 8.0) and then dialyzed against 25 mM HEPES, 5 mM MgCl2, 1 mM DTT, 10% (v/v) glycerol, pH 7.4. Concentration was determined using a Pierce 660 assay. Proteins were flash-frozen and stored at −80 °C. Neh2Dual-Barnase-DHFR substrate was expressed, purified, labeled, and ubiquitinated as described previously (25). Lys63-linked chains were enzymatically synthesized and purified as described previously (16).

Degradation assays

Degradation assays were conducted using 100 nM proteasome and 100 nM fluorescent substrate over a 2-h time course. The reactions were carried out at 30 °C in degradation buffer (50 mM Tris-Cl, 5 mM MgCl2, 5% (v/v) glycerol, 1 mM ATP, 10 mM creatine phosphate, 0.1 mg/ml creatine kinase, and 1% DMSO, pH 7.5). The reactions contained 1 mg/ml BSA to prevent nonspecific loss of fluorescence. For reconstitution experiments, 20S core particle was preincubated with 19S core particle in the presence of degradation buffer for 10 min at 30 °C before beginning the assay by the addition of substrate plus BSA. At designated time points, the samples were removed and placed into SDS-PAGE loading buffer to quench the reaction; the samples were not heated to prevent denaturation of GFP. SDS-PAGE gels were analyzed by fluorescence imaging on a Typhoon FLA 9500, and the resulting gel files were analyzed using ImageQuant (GE). The amount of full-length substrate at the initial 10-s time point was set to 100%, and the amount of clipped fragment at each time point was normalized to the full-length substrate at the initial 10-s time point (and any clipped fragment formed before the initial time point was subtracted such that curves started at 0% fragment). The total fluorescence was determined by adding the full-length and fragment amounts, because no other appreciable fluorescent bands were detected.

ATPase assays

Proteasome ATPase activity was measured using a coupled pyruvate kinase/lactate dehydrogenase assay in saturating ATP, which can be spectrophotometrically detected at 340 nm. The reactions contained 20 nM proteasome, 6.8 units/ml pyruvate kinase, 9.9 units/ml lactate dehydrogenase, 0.4 mM NADH, 2 mM phosphoenolpyruvate, 0.5 mM DTT, and various ATP/ATPγS ratios totaling 0.5 mM in a buffer consisting of 50 mM HEPES-KOH, pH 7.5, 50 mM KCl, and 5 mM MgCl2. The reaction was run at 30 °C in a 384-well plate with time points taken every 20 s for 20 min by a Bio-Rad Benchmark Plus UV-visible plate reader.

Native gels

Proteasome was reconstituted as for degradation assays, then run on a 3.5% native gel, and visualized using Suc-LLVY-AMC in the presence of 0.02% SDS (38).

Kinetic modeling

Kinetic modeling was carried out using COPASI software (39).

Data availability

All data not in the article will be shared upon request to D. A. K. (daniel.kraut@villanova.edu).

Acknowledgments—We thank Andreas Matouschek for sharing Ub2-cp8sGFP-38-His6, Ub4-sGFP-38-His6, and UBL-sGFP-102-His6 plasmids. We thank Aimee Eggler, David Smith, and members of the Kraut lab for helpful discussions and feedback.

Author contributions—C. E. B. data curation; C. E. B. and D. A. K. investigation; C. E. B. and D. A. K. writing-original draft; C. E. B. and D. A. K. writing-review and editing; D. A. K. conceptualization; D. A. K. formal analysis; D. A. K. supervision.

Funding and additional information—This material is based upon work supported by the Beckman Scholars Program and the National Science Foundation under Grants 1515229 and 1935596 (to D. A. K.). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Conflict of interest—The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: UPS, ubiquitin–proteasome system; UBL, ubiquitin-like domain; sGFP, superfolder GFP; eGFP, enhanced GFP; ATPase system; ARS, ATP-regeneration system.

References

1. Trauth, J., Scheffer, J., Hasenjäger, S., and Taxis, C. (2020) Strategies to investigate protein turnover with fluorescent protein reporters in eukaryotic organisms. AIMs Biophys. 7, 90–118 CrossRef Medline
2. Dantuma, N. P., Lindsten, K., Glas, R., Jellne, M., and Masucci, M. G. (2000) Short-lived green fluorescent proteins for quantifying ubiquitin/proteasome-dependent proteolysis in living cells. Nat. Biotechnol. 18, 538–543 CrossRef Medline
3. Heessen, S., Dantuma, N. P., Tessarz, P., Jellne, M., and Masucci, M. G. (2003) Inhibition of ubiquitin/proteasome-dependent proteolysis in Saccharomyces cerevisiae by a Gly-Ala repeat. FEBS Lett. 555, 397–404 CrossRef Medline
Mode of targeting to the proteasome determines GFP fate

1. Holmberg, C. I., Staniszewski, K. E., Mensah, K. N., Matouschek, A., and Morimoto, R. I. (2004) Inefficient degradation of truncated globular proteins by the proteasome. *EMBO J.* **23**, 4307–4318 CrossRef Medline

2. Maynard, C., J. Böttcher, C., Ortega, Z., Smith, R., Florea, B. I., Díaz-Hernández, M., Brundin, P., Overkleeft, H. S., Li, Y.-Y., Lucas, I. J., and Dantuma, N. P. (2009) Accumulation of ubiquitin conjugates in a polyglutamine disease model occurs without global ubiquitin/proteasome system impairment. *Proc. Natl. Acad. Sci. U.S.A.* **106**, 13986–13991 CrossRef Medline

3. McKinney, C., Goold, R., Andre, R., Devoy, A., Ortega, Z., Moonga, J., Linehan, J. M., Brandner, S., Lucas, J. J., Collinge, J., and Tabrizi, S. J. (2016) Prion-mediated neurodegeneration is associated with early impairment of the ubiquitin–proteasome system. *Acta Neuropathol.* **131**, 411–425 CrossRef Medline

4. Thibaudeau, T. A., Anderson, R. T., and Smith, D. M. (2018) A common mechanism of protease impairment by neurodegenerative disease-associated oligomers. *Nat. Commun.* **9**, 1097 CrossRef Medline

5. Moura, D. M. N., de Melo Neto, O. P., and Carrington, M. (2019) A new reporter cell line for studies with proteasome inhibitors in *Trypanosoma brucei*. *Mol. Biochem. Parasitol.* **227**, 15–18 CrossRef Medline

6. Bence, N. F., Sampat, R. M., and Kopito, R. R. (2001) Impairment of the ubiquitin–proteasome system by protein aggregation. *Science* **292**, 1552–1555 CrossRef Medline

7. Greussing, R., Unterlugger, H., Koziel, R., Maier, A. B., and I. Dür, D. (2006) Analysis of the hypoxia-sensing pathway in *Drosophila melanogaster*. *Biochem. J.* **393**, 471–480 CrossRef Medline

8. Khmelinskii, A., Keller, P. J., Bartosik, A., Meurer, M., Barry, J. D., Mardin, T. A., Anderson, R. T., and Smith, D. M. (2018) A common processing rate on substrate unfolding. *Nat. Biotechnol.* **30**, 708–714 CrossRef Medline

9. Wang, S., Tang, N. H., Lara-Gonzalez, P., Zhao, Z., Cheesemuth, D. K., Prevo, B., Chisholm, A. D., Desai, A., and Oegema, K. (2017) A toolkit for GFP-mediated tissue-specific protein degradation in *C. elegans*. Development **144**, 2694–2701 CrossRef Medline

10. Bashore, C., Dambacher, C. M., Goodall, E. A., Matsukiela, M. E., Lander, G. C., and Martin, A. (2015) Ubp6 deubiquitinase controls conformational dynamics and substrate degradation of the 26S proteasome. *Nat. Struct. Mol. Biol.* **22**, 712–716 CrossRef Medline

11. Yu, H., Kago, G., Yellman, C. M., and Matouschek, A. (2016) Ubiquitin-like domains can target to the proteasome but proteolysis requires a disorder region. *EMBO J.* **35**, 1522–1536 CrossRef Medline

12. Reichard, E. L., Chirico, G. G., Dewey, W. J., Narriss, N. D., Bard, K. E., Millas, N. E., and Kraut, D. A. (2016) Substrate ubiquitination controls the unfolding ability of the proteasome. *J. Biol. Chem.* **291**, 18547–18561 CrossRef Medline

13. Nager, A. R., Baker, T. A., and Sauer, R. T. (2011) Stepwise unfolding of a β barrel protein by the AAA+ ClpXP protease. *J. Mol. Biol.* **413**, 4–16 CrossRef Medline

14. Koodathungal, P., Jaffe, N. E., Kraut, D. A., Prakash, S., Fishbain, S., Her- man, C., and Matouschek, A. (2009) ATP-dependent proteases differ substantially in their ability to unfold globular proteins. *J. Biol. Chem.* **284**, 18674–18684 CrossRef Medline

15. Olszewski, M. W., Williams, C., Dong, K. C., and Martin, A. (2019) The Cde48 unfoldase prepares well-folded protein substrates for degradation by the 26S proteasome. *Commun. Biol.* **2**, 29 CrossRef Medline

16. Cundiff, M. D., Hurley, C. M., Wong, J. D., Boscia, J. A., Bashyal, A., Rosenberg, J., Reichard, E. L., Narriss, N. D., Brodbelt, I. S., and Kraut, D. A. (2019) Ubiquitin receptors are required for substrate-mediated activation of the proteasome’s unfolding ability. *Sci. Rep.* **9**, 14506 CrossRef Medline

17. Inobe, T., Fishbain, S., Prakash, S., and Matouschek, A. (2011) Defining the geometry of the two-component proteasome degron. *Nat. Chem. Biol.* **7**, 161–167 CrossRef Medline

18. Shi, Y., Chen, X., Elssasser, S., Stocks, B. B., Tian, G., Lee, B.-H., Shi, Y., Zhang, N., de Poot, S. A., Taeubing, F., Sun, S., Vannoy, J., Tarasov, S. G., Engen, J. R., Finley, D., et al. (2016) Rpn1 provides adjacent receptor sites for substrate binding and deubiquitination by the proteasome. *Science* **351**, aad421 CrossRef Medline

19. Grö, G. L., and Nathan, J. A. (2016) The recognition of ubiquitinated proteins by the proteasome. *Cell. Mol. Life Sci.* **73**, 3497–3506 CrossRef Medline

20. Henderson, A., Erales, I., Hoyt, M. A., and Coffino, P. (2011) Dependence of proteasome processing rate on substrate unfolding. *J. Biol. Chem.* **286**, 17495–17502 CrossRef Medline

21. Lu, Y., Lee, B.-H., King, R. W., Finley, D., and Kirschnier, M. W. (2015) Substrate degradation by the proteasome: a single-molecule kinetic analysis. *Science* **348**, 1250834 CrossRef Medline

22. Peth, A., Kushikin, N., Böss, M., and Goldberg, A. L. (2013) Ubiquitinated proteins activate the proteasomal ATPases by binding to Usp14 or Uch37 homologs. *J. Biol. Chem.* **288**, 7781–7790 CrossRef Medline

23. Peth, A., Besche, H. C., and Goldberg, A. L. (2009) Ubiquitinated proteins activate the proteasome by binding to Usp14/Ubp6, which causes 20S gate opening. *Mol. Cell* **36**, 794–804 CrossRef Medline

24. Tomita, T., Hübregtse, J. M., and Matouschek, A. (2010) A masked initiation region in retinoblastoma protein regulates its proteasomal degradation. *Nat. Commun.* **1**, 207 CrossRef Medline

25. Olivas, A. O., Baker, T. A., and Sauer, R. T. (2016) Mechanistic insights into bacterial AAA+ proteases and protein-remodelling machines. *Nat. Rev. Microbiol.* **14**, 33–44 CrossRef Medline

26. Olivas, A. O., Baker, T. A., and Sauer, R. T. (2018) Mechanical protein unfolding and degradation. *Annu. Rev. Physiol.* **80**, 413–429 CrossRef Medline

27. Kraut, D. A., Israeli, E., Schrader, E. K., Patil, A., Nakai, K., Nanavati, D., Inobe, T., and Matouschek, A. (2012) Sequence- and species-dependence of proteasomal processivity. *ACS Chem. Biol.* **7**, 1444–1453 CrossRef Medline

28. Studier, F. W. (2005) Protein production by auto-induction in high-density shaking cultures. *Protein Expr. Purif.* **41**, 207–234 CrossRef Medline

29. Elssasser, S., Schmidt, M., and Finley, D. (2005) Characterization of the proteasome using native gel electrophoresis. *Methods Enzymol.* **378**, 353–363 CrossRef Medline

30. Hoops, S., Sahle, S., Gauges, R., Lee, C., Pahle, J., Simus, N., Singhal, M., Xu, L., Mendes, P., and Kummer, U. (2006) COPASI: a Complex Pathway Simulator. *Bioinformatics* **22**, 3067–3074 CrossRef Medline