

Proteins Are Unfolded on the Surface of the ATPase Ring before Transport into the Proteasome

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Summary

The 19S component of the 26S proteasome contains six ATPase subunits. To clarify how they unfold and translocate proteins into the 20S proteasome for degradation, we studied the homologous archaeobacterial proteasome-regulatory ATPase complex PAN and the globular substrate GFP-SsrA. When we attached a small (Biotin) or large (Biotin-Avidin) moiety near its N terminus or a Biotin near its C terminus, GFP-SsrA was unfolded and degraded. However, attaching Avidin near its C terminus blocked passage through PAN and prevented GFP-SsrA degradation. Though not translocated, GFP-Avidin still underwent ATP-dependent unfolding. Moreover, it remained bound to PAN and inhibited further proteolysis. Therefore, (1) translocation and degradation of this substrate require threading through the ATPase in a C to N direction and (2) translocation does not cause but follows ATP-dependent unfolding, which occurs on the surface of the ATPase ring.

Introduction

In eukaryotic cells, most proteins are degraded by the 26S proteasome, which hydrolyzes ubiquitin-conjugated and certain nonubiquitinated proteins in an ATP-dependent manner (Coux et al., 1996; Voges et al., 1999). In this process, the proteins first bind to the 19S regulatory particle, which is believed to promote their translocation into the 20S core particle, where they are hydrolyzed to small peptides. The 19S complex contains six homologous ATPases in its base, adjacent to the 20S particle. These ATPases have been implicated in substrate recognition and in regulating the opening of the gate in the outer ring of the 20S particle (Kohler et al., 2001). They have also been proposed to catalyze protein unfolding, based largely on analogies to findings on the ATPase components in bacterial and archaeobacterial ATP-dependent proteases.

Although prokaryotes lack ubiquitin and 26S proteasomes, many contain 20S proteasomes, whose architecture and proteolytic mechanism resemble closely those of the 20S eukaryotic particles (Zwickl et al., 2000). Because of its greater simplicity, the archaeal 20S proteasome has proven very useful for structural (Lowe et al., 1995) and mechanistic studies (Akopian et al., 1997; Kisselev et al., 1998). These particles function in protein breakdown together with a 650 kDa ATPase complex,

termed PAN for proteasome-activating nucleotidase. When mixed with the archaeal 20S proteasome and ATP, PAN stimulates the degradation of proteins but not of small peptides, which by themselves can readily enter the 20S proteasome (Zwickl et al., 1999). PAN shares more than 40% identity with the six ATPases in the 19S complex and thus appears to be the evolutionary precursor to the 19S base and functioned before proteolysis became linked to ubiquitination in eukaryotes (Zwickl et al., 2000). These enzymes are all members of the AAA superfamily of multimeric ATPases, which also includes the ATP-dependent proteases, Lon and FtsH, and the regulatory components of the bacterial ATP-dependent proteases, ClpAP, ClpXP, and HslUV (Baumeister et al., 1998; Larsen and Finley, 1997). PAN appears to be composed of one or two hexameric rings surrounding a central pore (our unpublished data), and thus its general structure resembles that of other AAA family members. In the case of PAN and the other ATP-dependent proteases, it seems likely that protein substrates are translocated into the proteolytic component by transfer through this narrow opening in the center of the ATPase ring, and strong evidence in support of this conclusion is presented in this paper.

Like the base of the eukaryotic 19S particle (Braun et al., 1999; Strickland et al., 2000), PAN exhibits several chaperone-like properties including the ability to bind selectively unfolded polypeptides and prevent their aggregation (Benaroudj and Goldberg, 2000). In addition, PAN has been shown to catalyze the ATP-dependent unfolding of a globular protein and its translocation into the 20S proteasome for degradation (Benaroudj and Goldberg, 2000). A similar ability to unfold substrates and to translocate them into a neighboring proteolytic compartment (ClpP) has been demonstrated for the ClpA and ClpX ATPase complexes (Gottesman et al., 1997; Weber-Ban et al., 1999). Weber-Ban et al. (1999) introduced a convenient method for analyzing this unfolding activity by following the loss of fluorescence of the Green Fluorescent Protein (GFP), a single-domain, globular protein with no surface-exposed loops. Although wild-type GFP is not a substrate for ClpA or PAN, the addition of the 11 residue SsrA peptide to its C-terminal end allowed the ATP-dependent unfolding and degradation of GFP. In eubacteria, when a ribosome is stalled for the lack of a charged t-RNA, the SsrA peptide is incorporated into the nascent polypeptide chains from a specific t-RNA, and this modification targets proteins for degradation by ClpAX, FtsH, or other ATP-dependent proteases (Gottesman et al., 1998; Herman et al., 1998; Keiler et al., 1996). Although archaea lack a homologous SsrA tagging mechanism, attaching this 11 residue sequence onto the C terminus of GFP leads to its recognition by PAN, to ATP-dependent unfolding, and to translocation into the proteasome (Benaroudj and Goldberg, 2000).

The common feature believed to underlie the diverse functions of the AAA family of ATPases is their ability to undergo structural alterations through ATP-dependent conformational changes that cause unfolding of pro-

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teins or the disassembly of protein complexes. The specific mechanisms underlying these processes are unclear. One possibility is that the substrate is bound across the surface of the ATPase ring and that unfolding occurs on this surface through mechanical strain induced by ATP hydrolysis (Saibil, 2000). In such a model, translocation of the bound substrate into the 20S proteasome occurs after unfolding, either through passive diffusion or an active process powered by ATP-dependent conformational changes. Alternatively, unfolding may be a secondary consequence of an ATP-driven translocation process that pushes the substrate through the narrow opening in the center of the ATPase ring. In such a model, ATP-dependent pushing (or pulling) drives substrate translocation through this narrow pore, and this process could destabilize upstream globular domains, which might lead to global unfolding of the protein.

The present studies were undertaken to decide between these alternative models. If ATP-dependent substrate translocation involves passage through the central pore of the ATPase, one specific prediction is that attachment of a bulky ligand to the substrate should block its translocation into the proteasome and perhaps also prevent substrate unfolding if this process were a secondary consequence of translocation. We therefore engineered several modified forms of the model substrate GFP-SsrA, which enable us to attach either a small group (Biotin) or a bulky (Avidin) group at either its N or C terminus (Figure 1). Using PAN and these various GFP-SsrA derivatives, we investigated the chain of events leading to delivery of globular polypeptides into the 20S for degradation. Specifically, we asked (1) whether substrates are translocated through the central cavity of PAN; (2) whether translocation occurred exclusively in a specific direction, either from the C to the N terminus or in the N to C direction; and (3) if translocation were prevented, whether substrate unfolding could still occur. The present studies demonstrate that for PAN, and presumably for other AAA family members, substrate unfolding can take place on the surface of the ATPase complex and can be dissociated from its translocation, which proceeds in a unidirectional manner through the ATPase ring. In addition, these studies illustrate how if a polypeptide becomes part of a complex or an aggregate that cannot be dissociated by the proteasome, it may function as dominant inhibitor of protein breakdown, and such a mechanism may be contributing to the pathogenesis of certain neurodegenerative diseases (Sherman and Goldberg, 2001).

Results

Preparation of the Different GFP Species

To test whether the modification of either the C or N terminus or of both termini with a small (Biotin) or bulky (Avidin) group interferes with substrate translocation into the proteasome by PAN, we engineered several GFP variants starting with wild-type GFP with seven histidines at its N terminus or this gene with the 11 amino acid SsrA-tag at its C terminus (GFP-SsrA). Three additional mutations were made in this latter gene. One had a nonnative cysteine as its ninth amino acid (adjacent to the His-tag). A second contained a nonnative

cysteine inserted in the C terminus in place of an aspartic-acid residue, 6 amino acids before the SsrA-tag. The third variant contained both these additional cysteine residues (i.e., one at each terminus). After expression of these variants in *E. coli* and purification, the additional cysteines in these derivatives were selectively modified using an alkylating Biotin reagent (Iodoacetamido-Biotin). Although the wild-type GFP contains two cysteine residues at positions 48 and 70, which are necessary for fluorescence (our unpublished data), these native cysteines did not react with the Biotin reagent under these conditions (Figure 1).

Biotin was selected for protein modification for several reasons: (1) the Iodoacetamido-Biotin reacts specifically with thiol groups on cysteine residues, leading to its covalent attachment to the cysteine; (2) the Biotin used here contains eleven carbons in a highly flexible chain, and prior studies have shown that even larger modifications of proteins (e.g., FITC) do not block their degradation by the proteasome (Akopian et al., 1997); (3) the Biotin could be converted to a large globular moiety by addition of Avidin (about $62 \text{ \AA} \times 42 \text{ \AA}$; coordinates file 1SWC.PDB), and (4) the Avidin-Biotin complex is exceptionally stable ($K_A = 10^{-15}$) and is resistant to 4 M GndHCl and temperatures greater than 80°C . As shown in prior studies (Benaroudj and Goldberg, 2000), the intense fluorescence and unusual stability ($T_m > 65^\circ\text{C}$) of GFP made it possible to monitor both unfolding and degradation by measurement of fluorescence even at 45 or 55°C .

Characterization of the Different GFP Variants

Each of the purified GFP species behaved as a homogeneous polypeptide upon SDS-PAGE (Figure 1B, upper panel). Western blot analysis with an antibody against the Biotin moiety demonstrated that only the GFP species with the additional nonnative cysteine had been alkylated by the Biotin reagent. In addition, the Western blot analysis demonstrated that an anti-His-tag antibody could also be used for the detection of these substrates (Figure 1B). Each of these Biotin-derivatized proteins eluted as a single peak upon gel filtration chromatography (Figure 1C). The two forms, abbreviated Biotin-GFP (for the N-terminal derivative) and GFP-Biotin (for the C-terminal derivative) were incubated with Avidin (in a 1:1 molar ratio), and in parallel, similar amounts of Biotin-GFP and GFP-Biotin were incubated with buffer and served as controls. After mixing with Avidin, the Biotin-derivatized GFP molecules assumed a much higher molecular weight (Figure 1C, upper panel). These results confirm the quantitative formation of discrete complexes containing Biotin-GFP and Avidin, which were also eluted primarily as single peaks.

Introduction of Avidin near the C Terminus Prevents Degradation

In order to test if degradation proceeds in one direction exclusively either from the C to the N terminus or from the N to the C terminus, we used GFP-SsrA with Biotin or Biotin-Avidin on its N or C terminus (Figure 1). The four different GFP derivatives were incubated with PAN, 20S proteasomes, and ATP at 45°C . Addition of the Biotin moiety alone to either terminus did not interfere with

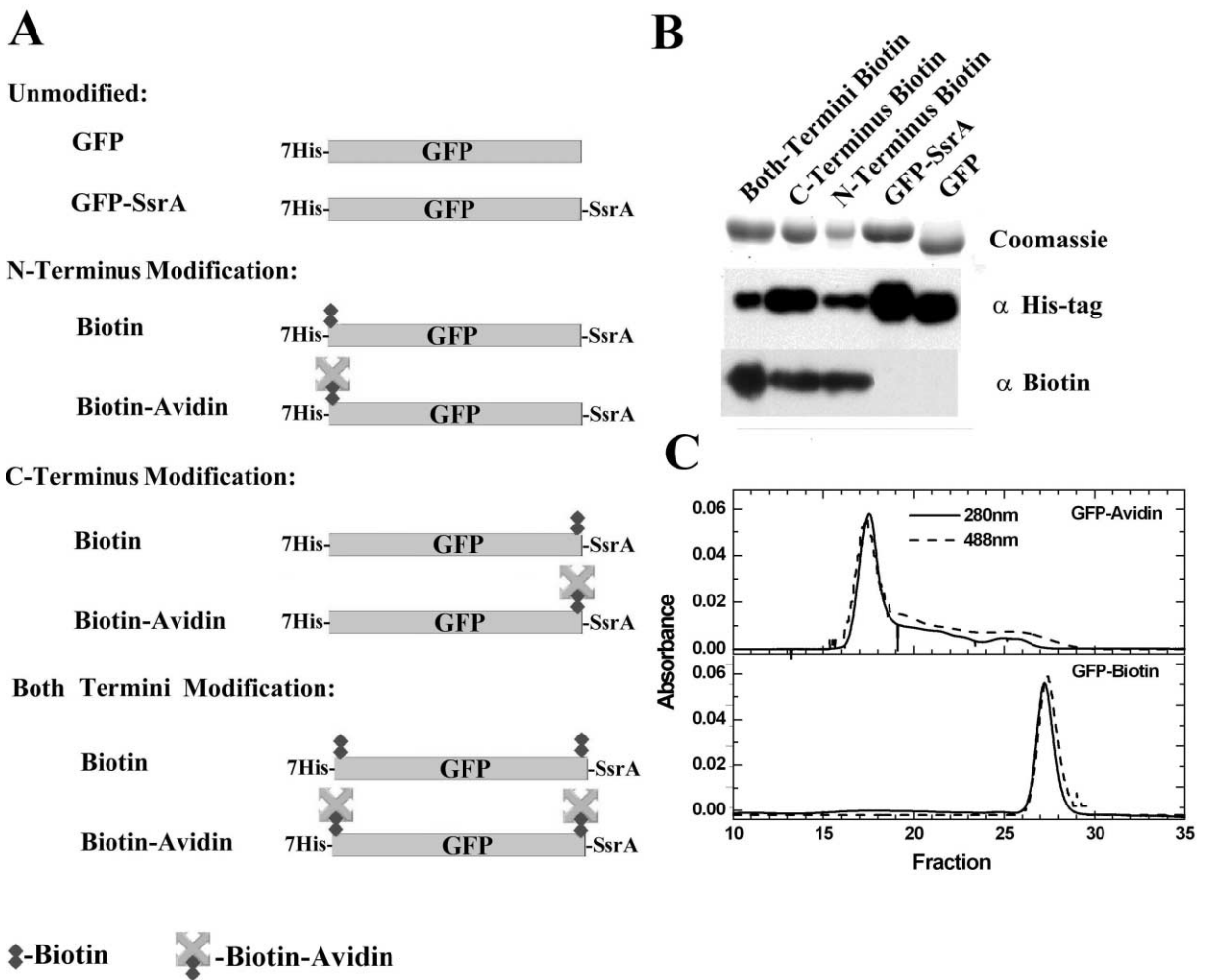


Figure 1. Substrates Engineered to Study Protein Translocation and Unfolding

(A) Graphic representation of the different GFP species used in this study. The light gray boxes represent the coding region of GFP. The 7 His at the N terminus represents the 7 His-tag, and the SsrA at the C terminus represents the 11 amino acid SsrA-tag. An additional nonnative cysteine was added as either the ninth amino acid (N-terminal modification) or 6 amino acids before the 11 amino acid SsrA-tag substituting for the wild-type aspartic residue (C-terminal modification).

(B) Analysis of the purity and the extent of the covalent attachment of Biotin by SDS-PAGE and Western blot analyses. Upper panel, Coomassie-stained 12.5% SDS gel of the different GFP species. The differences in length indicate the presence of the SsrA; middle panel, Western blot analysis of the different GFP species using an anti-His-tag antibody conjugated to HRP in an ECL reaction; lower panel, Western blot analysis of the different GFP revealed using anti-Biotin antibody conjugated to HRP in an ECL reaction.

(C) Size analysis of GFP-Biotin by itself (lower panel) and upon association with Avidin (upper panel), by gel filtration using a Superdex-200 10/30 column (Pharmacia). Similar elution patterns were obtained when Biotin-GFP (N-terminal) and Avidin-Biotin-GFP were chromatographed in a similar fusion.

The absorbance at 280 nm is depicted by a solid line, and the absorbance at 488 nm, which is a specific property of GFP, by a dashed line. Avidin by itself did not show any absorbance at 488 nm.

the degradation of GFP (Figure 2; Biotin-GFP, GFP-Biotin). Thus, the attachment of this small group permits normal transit into the 20S particle. Attaching the Avidin to the N terminus of the GFP-Biotin also did not interfere

with the degradation of the Biotin-GFP (Figure 2; Avidin-GFP). However, when the Avidin was attached to the Biotin on the C terminus, just before the 11 residue SsrA-tag, the GFP was not degraded (Figure 2; GFP-Avidin).

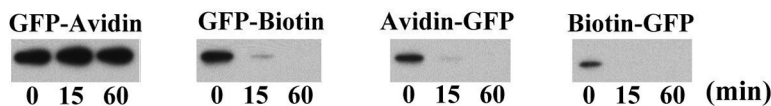
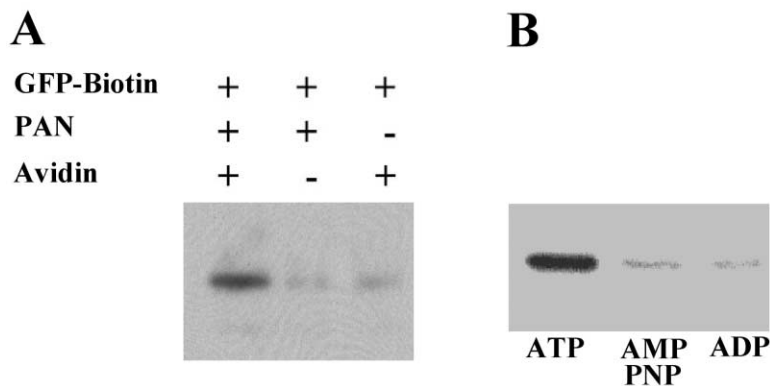


Figure 2. Degradation of GFP Is Prevented by the Presence of a Bulky (Avidin) C-Terminal Modification, but Not by Other Modifications

Biotin-GFP, Avidin-GFP, GFP-Biotin, and GFP-Avidin were incubated with PAN and

20S proteasomes in Buffer A containing 2 mM ATP at 45°C. The different GFP species were present at ten times the concentration of PAN, which was present at three times the molar concentration of 20S. Aliquots were removed at the times indicated, and the extent of degradation was assayed by Western blot analysis with an HRP-conjugated anti-His-tag antibody.



assayed with HRP-conjugated anti-His-tag antibody.

(B) The effect of different nucleotides on the association of GFP-Avidin with PAN. The immunoprecipitation was done as in (A) using 2 mM AMP-PNP (AMP) or ADP instead of ATP.

Apparently, the bulky Avidin group prevented protein translocation when it was attached to the C terminus, even though at the N terminus, it allowed normal translocation and degradation of the GFP. These findings argue that PAN catalyzes the entry of the GFP-SsrA into the proteasome exclusively in a C- to N-terminal direction.

Complex Formation between PAN and Nontranslocated Substrate

Two alternative possibilities can account for this prevention of degradation by the large globular moiety near the C-terminal SsrA-tag: (1) this bulky moiety might prevent the binding of the modified substrate to PAN, or (2) it might permit binding of the substrate to PAN but prevent its passage through PAN's central pore into the proteasome. We used immunoprecipitation in order to determine if the Avidin on the C terminus prevents the formation of a complex with PAN. These different substrates were incubated with PAN in the presence of ATP. We then immunoprecipitated PAN with an anti-PAN antibody from the reaction mixtures and scanned by Western blot for substrates that coimmunoprecipitated with PAN. The three substrates that were degraded in Figure 2 could not be coimmunoprecipitated with PAN, presumably because their association with the ATPase was short-lived, even though it allowed their unfolding and translocation. By contrast, when the substrate was attached to Avidin through its C terminus, the substrate could be coimmunoprecipitated with PAN (Figure 3A). Thus the only modification which prevented degradation led to the formation of a relatively stable complex between the substrate and PAN. These observations indicate that the presence of the bulky group does not prevent degradation by blocking entry into the 20S proteasome but blocks an earlier step involving PAN alone. The most likely explanation is that the C-terminal Avidin prevents passage of the substrate through the central opening in the ATPase ring.

To test if formation of this stable enzyme-substrate complex occurred as part of the ATP-dependent translocation mechanism, we carried out similar immunoprecipitation experiments after incubation of PAN and GFP-Avidin with either ADP, the nonhydrolysable ATP analog AMP-PNP, or ATP, which alone supports unfolding and

Figure 3. Association of GFP-Avidin with PAN, as Shown by Immunoprecipitation with anti-PAN Antibodies

(A) PAN was incubated with GFP-Biotin or GFP-Avidin for 30 min in buffer A containing 2 mM ATP at 45°C. Rabbit anti-PAN antibody was added to the reaction mixture and incubated for 15 min on ice. Twenty microliters of protein A beads (Pharmacia) were added with brief mixing. After 15 min on ice, the beads were washed three times with Buffer A containing 1% Tween. SDS-PAGE sample buffer was added to the aspirated beads. After boiling and brief centrifugation, an equal amount of the sample of the boiled sample buffer was loaded on to the SDS-PAGE gel. Coimmunoprecipitation of GFP with PAN was

degradation. When the immunoprecipitation was performed in the presence of ADP or AMP-PNP instead of ATP, the GFP-Avidin could not be coimmunoprecipitated with PAN (Figure 3B). Thus, ATP hydrolysis is necessary for the formation of this enzyme-substrate complex, and most likely the C-terminal Avidin blocks the process of ATP-dependent translocation through PAN.

Though Not Translocated, GFP-Avidin Was Unfolded by PAN

The capture of this substrate in a form that could not be translocated through PAN allowed us to test whether unfolding could precede translocation or only occurred as a consequence of its translocation through the central opening in PAN (i.e., by determining whether the GFP-Avidin bound to the surface of the PAN ring could be unfolded or not). To choose between these alternatives, we analyzed the unfolding of the different GFP derivatives by PAN by measuring the loss of the GFP fluorescence at 511 nm in the presence of ATP. In parallel, the degradation of these GFP derivatives was followed by measuring the irreversible loss of fluorescence when 20S proteasomes were also present. Under these conditions, the fluorescence loss was shown to be due to the complete hydrolysis of the GFP molecules, as assayed by SDS-PAGE (Figure 2). Upon incubation with PAN and ATP, the GFP-SsrA molecules with Biotin on either the N or C terminus were unfolded by PAN, and they were degraded when 20S proteasomes were also present, as shown by the further decrease in fluorescence (Figure 4). When Avidin was introduced on the N terminus, both unfolding and degradation occurred in a similar manner to the nonmodified GFP-SsrA (Figure 4), in accord with the finding obtained by SDS-PAGE (Figure 2).

By contrast, when the Avidin was introduced in close proximity to the SsrA-tag on the C terminus of GFP-SsrA, even though degradation did not occur with 20S proteasomes present, the GFP-Avidin could still be unfolded by PAN. Thus, PAN promoted the loss of the GFP-Avidin fluorescence, but there was no further decrease in fluorescence upon addition of the proteasomes, in accord with results obtained by SDS-PAGE (Figure 2).

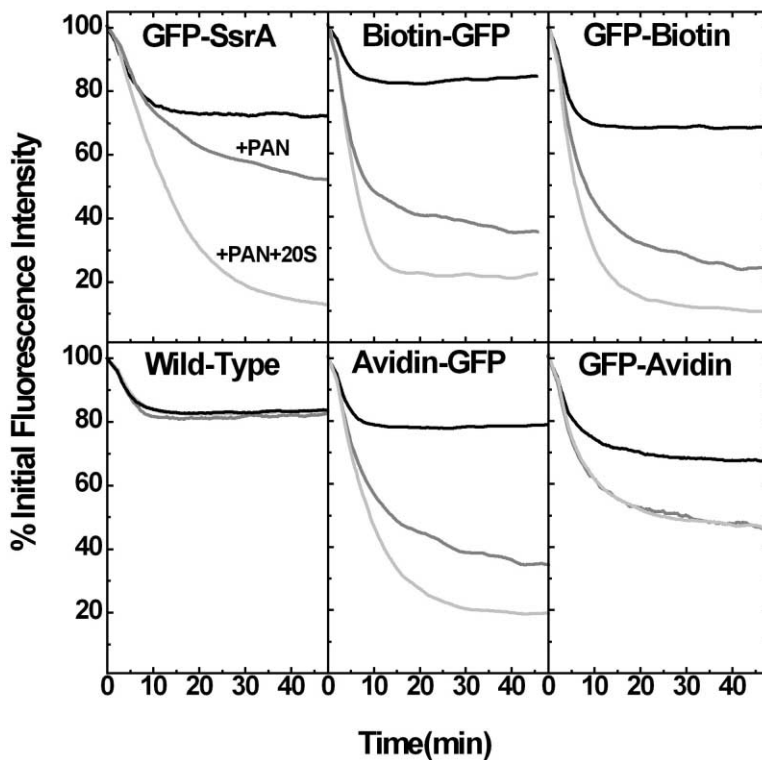


Figure 4. The Ability of PAN to Catalyze ATP-Dependent Unfolding and Degradation by 20S Proteasomes of the Different GFP Derivatives
The reactions were carried out as described in Figure 2, and fluorescence changes in the different reactions were measured at 511 nm in parallel. The black trace represents a reaction containing only the specific GFP variant in buffer A by itself. The gray trace represents a reaction with PAN present, and the light gray trace a reaction in which both PAN and 20S proteasome were present. The decrease of the fluorescence in this reaction mixture that does not contain PAN or proteasome is attributed to the nonspecific temperature quenching of fluorescence, and it reaches a plateau once the temperature in the cuvette reaches equilibrium.

The Nontranslocated GFP-Avidin Is Unfolded in a Stoichiometric Manner by PAN

Under these conditions, GFP was present in several-fold molar excess. So, PAN was acting catalytically in causing the unfolding and degradation of the Biotin-modified GFP-SsrA and the N-terminal Avidin-derivative. Although unfolding of the GFP-Avidin by PAN was clearly evident, the extent of the decrease in fluorescence was less than that with the other GFP variants (Figure 4). This result suggests that PAN catalyzes the unfolding of only a limited number of GFP-Avidin molecules. Thus, instead of acting catalytically, it appears to unfold GFP stoichiometrically, exactly as would have been predicted based on the findings that PAN could unfold the bound GFP-Avidin (Figure 4) and that the unfolded molecules remained associated with PAN in a stable complex (Figure 3).

Additional kinetic studies also supported this conclusion that the bulky adduct adjacent to the C terminus prevents threading of the unfolded GFP through the center of PAN into the 20S particle. When the GFP-Biotin or Avidin-GFP molecules were incubated with PAN, they were unfolded in a catalytic manner, since increasing the molar amount of PAN accelerated the unfolding reaction but did not change the total loss of fluorescence (Figure 5, left panel). In contrast, when GFP-Avidin was unfolded by PAN, the total amount of unfolding (loss of fluorescence) was much less and depended on PAN's concentration. Increasing the amount of PAN caused a further decrease in the fluorescence intensity, indicating that PAN acted stoichiometrically in unfolding a larger portion of the GFP-Avidin molecules (Figure 5, right panel). These observations suggest a "single round" unfolding of the GFP-Avidin in which the unfolded GFP remains

bound to PAN, because the bulky Avidin group prevented the translocation of the unfolded substrate through PAN's central pore.

The Nontranslocatable GFP-SsrA Behaves as a Dominant Inhibitor of Proteasome Function

This stoichiometric unfolding implies that GFP-Avidin, by forming a stable complex with PAN, behaves as a dominant inhibitor of proteolysis, since it caused only incomplete consumption of the GFP molecules. This finding predicts that this nontranslocatable substrate should also reduce or prevent the degradation of other substrates. To test this prediction, we used the Cyan Fluorescent Protein (CFP) tagged at its C terminus with the SsrA (CFP-SsrA). Its degradation by PAN-20S occurred in a similar manner as that of the GFP-SsrA and could be followed at a different wavelength (Ex = 430, Em = 480) (data not shown). After PAN and 20S proteasomes were preincubated with either GFP-SsrA, GFP-Biotin, or GFP-Avidin, and the reactions went to completion, CFP-SsrA was added as a fresh substrate. As shown in Figure 6, the degradation of CFP was rapid in the reactions pretreated with GFP-Biotin or GFP-SsrA. However, when the preincubation was with GFP-Avidin, which forms a stable complex with PAN, there was no significant degradation for the first 25 min. Thus, the GFP-Avidin indeed behaves as a dominant inhibitor of PAN function (although after longer incubations, the complex between the GFP-Avidin slowly dissociated, and PAN regained the ability to promote the degradation of CFP-SsrA [Figure 6]). These observations are of interest, since they suggest a possible mechanism by which a substrate in vivo may become an inhibitor of protea-

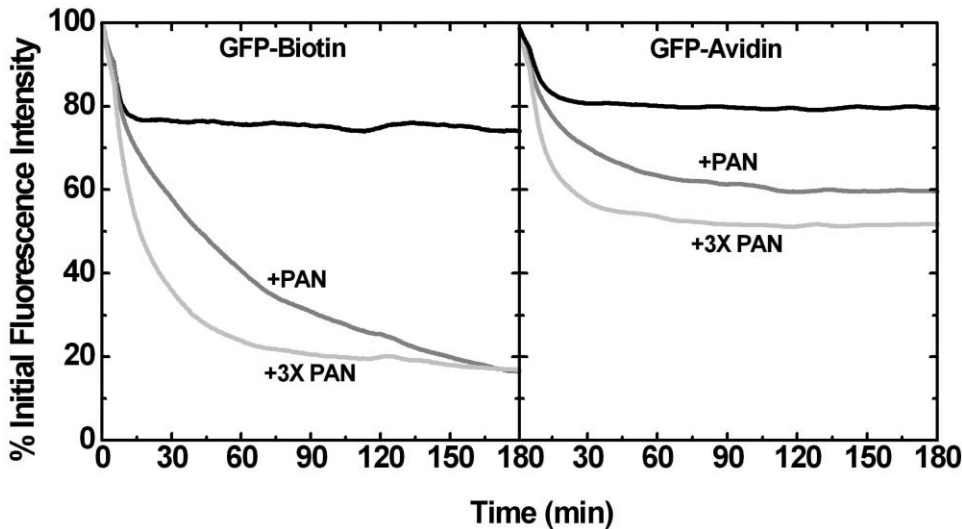


Figure 5. Increasing PAN Concentration Indicates Catalytic Unfolding of GFP-Biotin and Stoichiometric Unfolding of GFP-Avidin
These substrates were incubated alone (black traces) or in the presence of PAN in a molar ratio of 10:1 (light gray traces) or 30:1 molar ratio (gray traces). The unfolding and the degradation were assayed as in Figure 4.

some function and may even contribute to cellular pathology (see below).

Substrate Unfolding Occurs on the Surface of PAN

Since unfolding occurred when translocation was prevented, unfolding appears to take place on the surface of PAN before all but a few residues in the C-terminal SsrA-tag might enter the central pore. One alternative possible explanation of these findings might be that under these conditions, unfolding occurs because the N terminus of the GFP-Avidin might be partly translocated into PAN. To exclude this possibility, we prepared a GFP-SsrA mutant that contained two nonnative cysteines that were then modified by Iodoacetyl Biotin (Figure 1). We then derivatized both the C and the N termini with Avidin to prevent either end from translocating through

PAN. When the Biotin-GFP-Biotin and Avidin-GFP-Avidin were incubated with ATP and PAN or PAN plus 20S proteasomes (Figure 7), the two Biotin moieties did not prevent unfolding or degradation of GFP. When Avidin was attached to the two Biotin moieties, some of the GFP molecules could still be unfolded by PAN but not degraded by the PAN-20S complexes (i.e., there was no further decrease in fluorescence with 20S proteasomes present). This unfolding process was absolutely dependent on ATP (data not shown), as was the unfolding and degradation of all the other GFP-SsrA derivatives studied here. The degree of unfolding of the Avidin-GFP-Avidin was somewhat less than that of GFP-Avidin, presumably because the two bulky Avidin molecules may cause some steric hindrance and may also promote the formation of a large network of GFP-Avidin com-

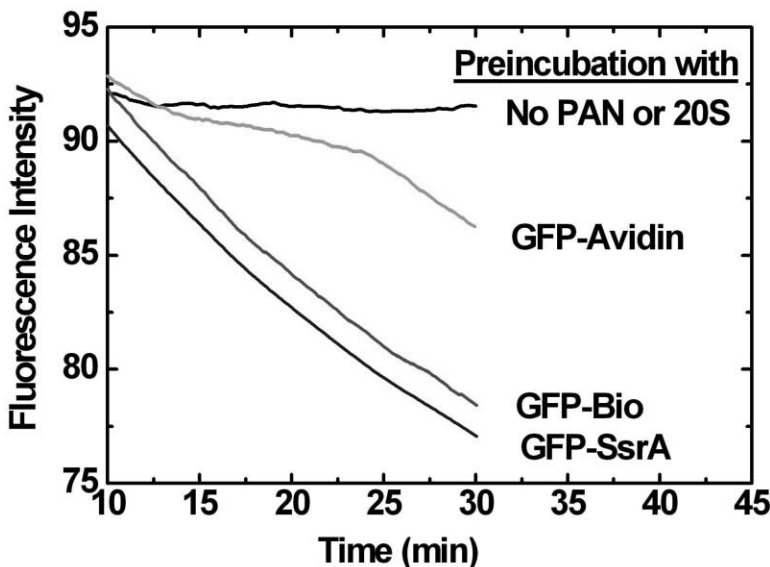


Figure 6. Preincubation of PAN-20S with GFP-Avidin Inhibits Its Ability to Degrade the Fresh Substrate, Cyan Fluorescence Protein (CFP-SsrA)

GFP-SsrA, GFP-Bio, and GFP-Avidin were preincubated with PAN and 20S as in Figure 4. After the loss of fluorescence from these substrates reached a plateau (50 min), CFP-SsrA was added at ten times the concentration of the GFP derivatives in the preincubation mixtures. To follow CFP-SsrA unfolding and degradation, the excitation wavelength was changed to 430 nm and the emission to 480 nm.

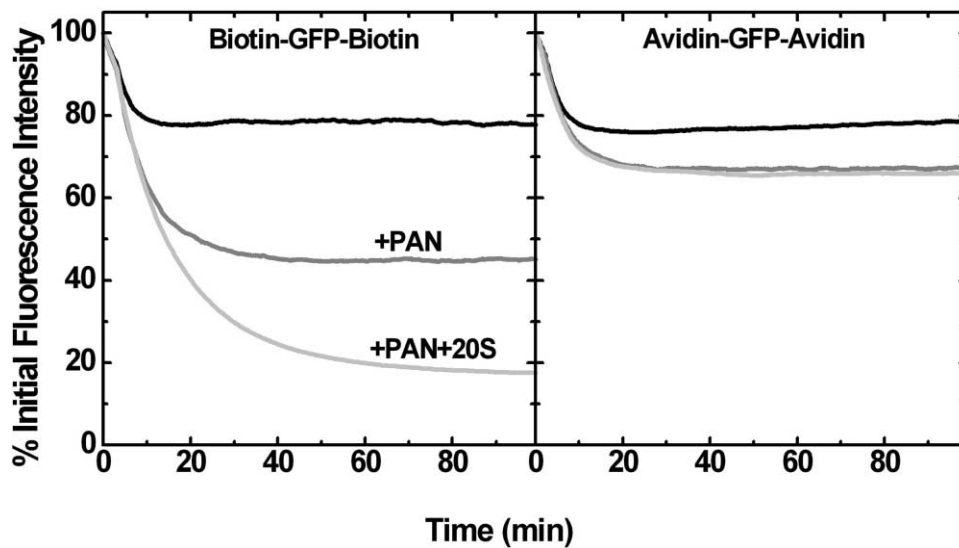


Figure 7. PAN Can Unfold GFP-SsrA Blocked on Both Termini by Bulky Groups (Avidin)

Fluorescence changes of Biotin-GFP-Biotin (left panel) and Avidin-GFP-Avidin (right panel) were assayed as in Figure 4. The black trace corresponds to GFP derivative alone, the gray trace with PAN present, and the light gray trace with both PAN and 20S proteasomes present.

plexes (because each Avidin has four valencies, and each GFP contains two Biotins) that is likely to reduce the fraction of the molecules that can interact with PAN. In addition, as was found for the GFP-Avidin, the doubly modified version was unfolded in a stoichiometric rather than a catalytic manner. In any case, some unfolding of this substrate did occur (Figure 7), even though it could not be translocated by either of its ends through PAN. This result clearly confirms that unfolding does not require threading of either terminus through PAN and occurs on the surface of the ATPase ring.

Discussion

A full understanding of the role of ATP in protein breakdown will require elucidation of the mechanisms by which the proteasome's regulatory ATPases bind protein substrates, unfold them, and promote their transfer into the 20S proteasome. In the present study, we have demonstrated that the translocation of the model substrate, GFP-SsrA, by the archaeal PAN complex occurs only in a single direction, C to N, and we have succeeded in dissociating the ATP-dependent degradative process into three sequential steps: unfolding, translocation, and hydrolysis. These findings were made possible by selective modification of the N or C terminus of the substrate with a small moiety (Biotin) or a large globular protein (Biotin-Avidin) (Figure 1). The Avidin adduct is much too large to pass through either the opening in the PAN ATPase, which in electron micrographs appears to be about 30 Å in diameter (our unpublished data), or the opening in the 20S α ring, whose diameter appears to be only 13 Å (Lowe et al., 1995).

All of the different GFP-SsrA derivatives generated here were degraded, with the exception of the GFP-SsrA linked to Avidin on its C terminus. This modification did not prevent degradation by blocking the protein's binding to the PAN complex. On the contrary, PAN not

only bound the GFP-Avidin (Figure 3A), but this association was much more stable than that between PAN and the other GFP-SsrA derivatives or with the unmodified substrate, none of which could be coimmunoprecipitated with PAN. Moreover, since the nondegradable GFP-Avidin was unfolded by PAN, the step blocked by the bulky C-terminal Avidin was the dissociation of the unfolded substrate from the ATPase complex. Because this failure of PAN to release the GFP-Avidin coincided with a blockage of its translocation into the proteasome, the normal release of the unfolded substrate must involve its translocation in a C to N direction through the central opening in the PAN ring.

Direct transfer of the unfolded substrate into the proteolytic compartment by threading through PAN is in accord with the mechanisms suggested for the other protease regulatory ATPase complexes, such as HslU and ClpA, which was shown by electron microscopy to bind substrates in its central opening (Ishikawa et al., 2001). Since polypeptide entry into the 20S proteasome is highly restricted (Baumeister et al., 1998; Larsen and Finley, 1997), a key role of PAN and the homologous ATPases in the eukaryotic 19S complex must be to facilitate substrate entry by direct injection into the central opening in the proteasome's α ring. Simply unfolding a substrate and releasing it into the cytosol might favor eventual entry into the proteasome, but it would also allow the released substrate either to reassume a globular structure or to aggregate, which could be quite deleterious to the cell.

The X-ray diffraction of the yeast 20S proteasome indicated that the channel for substrate entry in the α ring is normally in a closed state (Groll et al., 1997), which explains why these particles are inactive prior to their association with the PA700 (19S) or PA28 activating complexes (Whitby et al., 2000). Recent studies indicated that one of the six ATPases in the yeast 19S particle, Rpt2, functions to promote the opening of the

gate into the 20S proteasomes (Kohler et al., 2001). Unlike the eukaryotic 20S proteasome (Groll et al., 1997), the archaeal proteasome by itself can digest small peptides but not proteins (Zwickl et al., 1999), presumably because the channel in its α ring is in an open state (Lowe et al., 1995). However, recent studies indicate that the archaeal proteasomes also contain a functional gate that prevents the entry of unfolded proteins in the absence of the PAN ATPase (Benaroudj et al., personal communication). Accordingly, the sequences in the N terminus of the archaeal α subunits are homologous to those in the α subunits that comprise the gate in the yeast proteasome (Groll et al., 2000). Thus, in both PAN and the 19S complex, ATP-dependent opening of the gate is linked to ATP-dependent microinjection of protein substrates.

It is also noteworthy that ATP hydrolysis is required for the formation of the stable complex between PAN and GFP-Avidin. The nonhydrolysable analog, AMP-PNP, did not support complex formation (Figure 3B), even though AMP-PNP can support the association of PAN with the proteasome (Ng et al., personal communication) and can enhance the affinity of PAN for unfolded proteins in a similar way to ATP (Benaroudj and Goldberg, 2000). The formation of this stable PAN-GFP-Avidin complex thus differs from the initial association of GFP-SsrA with ClpA or ClpX (Singh et al., 2000), which only requires nucleotide binding, and where the subsequent ATP hydrolysis allows substrate unfolding and translocation. Because ATP hydrolysis by PAN is required for both GFP unfolding and translocation, it is very likely that the formation of this stable PAN-GFP-Avidin complex reflects an intermediate step in the normal process of substrate translocation. This requirement for ATP hydrolysis also suggests that the formation of this stable complex requires continual changes in PAN's conformation linked to nucleotide hydrolysis (perhaps to "push" or "pull" the substrate through the central pore), rather than a specific stable conformation induced by ATP or AMP-PNP binding.

Nontranslocatable Substrate Functions as Dominant Inhibitor of Proteolysis

Of particular interest was the unexpected finding that this nondegraded substrate (GFP-Avidin), by not dissociating from PAN, functioned as a dominant inhibitor of the unfolding and degradation of other substrate molecules, i.e., other GFP-SsrA or CFP-SsrA (Figure 6). This conversion of a substrate into an inhibitor suggests an interesting new model for understanding the mechanism underlying certain human neurodegenerative diseases, such as Huntington's disease, other poly-Q expansion diseases, and Parkinson's disease. These diseases are characterized by interneuronal inclusions in which a mutant or damaged protein accumulates in association with ubiquitin and proteasomes (Sherman and Goldberg, 2001). The present findings demonstrate that if a polypeptide has a sequence targeting it to the proteasome but is too large to be translocated (e.g., if it is linked to other proteins or is part of an aggregate), it may stably associate with the proteasome, block its function, and thus cause an inhibition of overall proteolysis. Furthermore, in related studies with a different protein that tends

to aggregate under these conditions (55°C), we have found that when targeted to the proteasome, it also became a dominant inhibitor of protein degradation. Very recently, a general inhibition of proteasomal degradation was actually reported in a model of Huntington's disease (Bence et al., 2001), when the cells expressed a poly-Q-containing polypeptide. Our results offer a molecular explanation for these findings. It will be of importance to test whether in various cases where abnormal proteins aggregate in the cytosol (e.g., in other diseases or in bacteria expressing abnormal proteins), the non-translocatable polypeptides function as dominant inhibitors of intracellular proteolysis.

GFP-SsrA Is Translocated and Degraded in a C to N Fashion

Because the narrow opening in the proteasome's α ring only permits entry of unfolded, linear polypeptides, proteins in principal may preferentially or exclusively enter the particle and be degraded in a C to N or N to C direction. Based upon the arrangement of active sites in the archaeal 20S, Huber and coworkers even suggested that its active site digests proteins in the C to N direction (Lowe et al., 1995). The present experiments clearly indicate that GFP-SsrA is translocated by PAN into the proteasome exclusively in a C to N manner (Figure 4). Recently, C to N translocation of substrates bearing a C-terminal SsrA-tag was also demonstrated for ClpAP (Reid et al., 2001). Similar polarity is also found in the processing by the mammalian proteasome of the transcription factor NF κ B, whose 50 kDa subunit (p50) is generated by the degradation of the C-terminal half of the 105 kDa precursor (p105) (Palombella et al., 1994).

These findings, however, do not rule out the possibility that the degradation of some other substrates occurs in the opposite N to C direction. Thus, it is likely that the directionality found here is not due to some inherent property of the ATPases but instead depends on the structure of the substrate; e.g., whether a specific terminus of the substrate is partially unfolded and accessible to associate with PAN or whether it contains a specific recognition sequence at either terminus (e.g., the SsrA-tag). Evidence in support of this latter alternative will be presented elsewhere (A.N. and A.G., unpublished data).

Recently Matouschek and colleagues (Lee et al., 2001) suggested that the ATPase component of the ATP-dependent proteases initiates unfolding by binding to one end of the substrate that contains a specific recognition sequence and that this end is then funneled into the central chamber of the proteolytic component. The efficiency of such a mechanism does not correlate with the spontaneous global unfolding rate of the protein or with its overall stability but instead on the ability of the ATPase to initiate a local unraveling of the terminal segment, which was proposed to trigger a cooperative unfolding of the adjacent domain of the protein. However, our finding that unfolding can occur in the absence of translocation does not support this mechanism. Instead, these results suggest an alternative interpretation of the findings of Lee et al., i.e., that a loose terminus might facilitate binding to the ATPase, which catalyzes global unfolding followed by vectorial transfer of the initially bound end of the protein into the 20S proteasome.

It is surprising that the attachment of Avidin to the N terminus of the GFP-SsrA did not prevent or even significantly reduce substrate release from PAN and its degradation by the proteasome. Presumably, this degradative process generates an Avidin-bound N-terminal fragment, whose length should correspond to the distance between the outer surface of the ATPase ring and the active sites in the central chamber of the 20S proteasome. Since proteolytic generation of such an Avidin-bound fragment did not form a stable complex with PAN or decrease degradation, this fragment (unlike the C-terminal Avidin) must efficiently dissociate from PAN, presumably without the fragment being translocated through the ATPase ring (due to the association with Avidin). Apparently, when the Avidin-GFP is degraded, the Avidin-bound N-terminal fragment dissociates efficiently from PAN and is replaced by a new substrate molecule containing the high-affinity SsrA-tag. Presumably, the dissociation of this N-terminal fragment is because it no longer contains the SsrA, which was removed by proteasomal degradation. Moreover, if true, this explanation implies (1) that the maintenance (like the formation) of the ATP-dependent complex between PAN and a nontranslocatable substrate (e.g., GFP-Avidin) requires a high-affinity sequence, such as SsrA, and (2) that even if a polypeptide extends through the openings in the ATPase and the α ring into the central chamber of the 20S particle, it can still dissociate rapidly from the particle if translocation is hindered (A.N. and A.G., unpublished data).

One other theoretical possibility is that the PAN ATPase dissociates this Biotinylated N-terminal peptide from the protecting Avidin moiety, which was not degraded during these incubations (data not shown). However, ATP-dependent dissociation of the Biotin-Avidin complex would appear unlikely in light of the very tight association between Biotin and Avidin and the failure of PAN to dissociate the C-terminal GFP-Avidin.

Unfolding Occurs on the Surface of the ATPase Ring

Several alternative mechanisms have been proposed to account for the capacity of the AAA family of ATPase involved in proteolysis to unfold globular substrates. The present demonstration that PAN can catalyze unfolding of the nontranslocatable substrates, GFP-Avidin and Avidin-GFP-Avidin, supports a mechanism in which unfolding occurs on the outer surface of the ATPase ring (Vale, 2000). One advantage of the ring architecture characteristic of the AAA family of ATPases is that it could potentially allow each subunit in the ring to switch through ATP hydrolysis between two conformations (e.g., tense and relaxed states), perhaps in a coordinated or cooperative manner. Once a protein is bound across the surface of the ring, the binding domains may undergo concerted, conformational changes, subjecting the bound protein to mechanical strain that may promote unfolding. Such unfolding on the surface of the ATPase appears analogous to the mechanism of the GroEL family of chaperonins (Saibil, 2000), where substrate proteins initially bind to the interior surface of GroEL and undergo ATP-hydrolysis-induced cycles of conformational change. In related studies, ATP binding

was shown to alter the conformation of other AAA family members such as CDC48 (Rouiller et al., 2000), and recently, using ANS as a fluorescence probe, we found that ATP binding increases the hydrophobic surface of PAN, which may contribute to the unfolding process (A.N. and A.G., unpublished data)

Our findings do not support alternative models for unfolding by these ATPases in which ATP-driven “threading” of the polypeptide through the narrow central opening in the ATPase ring causes the unfolding of a downstream globular domain. Although our findings indicate that the major loss of GFP-SsrA structure occurs on PAN’s surface, we followed only the main structural transition involved in loss of the specific tertiary conformation that allows GFP fluorescence. Therefore, the GFP-Avidin bound to PAN, while losing fluorescence, may retain some elements of its secondary or tertiary conformation. Thus, a more complex two-step model, combining elements from both, is also consistent with our data. While the primary unfolding event occurs on the surface of PAN and involves a major unfolding transition resulting in loss of GFP’s globular structure and its tight packing, the subsequent ATP-driven translocation of the bound end of the polypeptide through the ATPase pore may eliminate its residual structure. GFP was specifically chosen for this study because it is a tightly packed single domain protein that lacks any interdomain or surface-exposed loop that might serve as an initiation site to promote global unfolding. Therefore, it is highly unlikely that substrate unfolding occurs on the surface of PAN by some ATP-dependent partial translocation of an internal loop of the substrate. In addition, in a related study, we inserted a loosely folded polypeptide at the C terminus of GFP. The loose part of this fusion protein was degraded by the proteasome while leaving the GFP intact and active (A.N. and A.G., unpublished data). This result is also inconsistent with unfolding through some possible loop in the GFP.

Together, these findings indicate a three-step model for the degradation of globular proteins by the PAN-proteasome complex: (1) The substrate is bound to the ATPase either through recognition of a specific sequence, like the SsrA-tag, or perhaps through hydrophobic domains, which become exposed when proteins are damaged or fail to fold properly. (2) The major substrate unfolding transition occurs on the surface of the ATPase, probably through conformational changes in the ring subdomains driven by ATP hydrolysis. (3) The unfolded substrate is then actively injected through the ATPase ring into the proteasome. This threading through PAN’s central opening may catalyze the loss of residual local structures, which would facilitate passage through the channel in the proteasome’s outer ring and enhance susceptibility of the polypeptide to the proteolytic sites. It seems likely that the bacterial ATP-dependent proteases, ClpAP and HslUV, function through similar mechanisms as well as the six ATPases in the base of the eukaryotic 19S, where the initial binding of substrates may be via a polyubiquitin chain. The experimental approaches used here (i.e., preventing translocation by derivatizing with a large globular moieties) should allow testing of the generality of this mechanism and whether the other members of the AAA family (e.g., NSF) also

function by surface unfolding and translocation through the ATPase ring.

Experimental Procedures

Generation of the GFP Mutants

GFP_{uv} (Clontech) was amplified by PCR and cloned between the NdeI and XhoI sites of pET22B (Novagene) (pET22-GFP-SsrA). The primer corresponding to the N terminus was designed to add seven Histidines upstream of the second amino acid of the GFP, and the primer corresponding to the C terminus of the gene was designed to add in-frame, the 11 amino acid SsrA-tag followed by a stop codon. Several variants of the GFP-SsrA were prepared by point mutagenesis (Figure 1). In short, pET22-GFP-SsrA served as the phagemid for the generation of single-strand DNA. The plasmid was transformed into *E. Coli* strain (RZ1032). A single colony was selected and grown in liquid medium (LB) supplemented with Ampicillin. The culture was super infected with M13KO7 helper phage, grown overnight at 37°C. The bacteria were span down, and the phage was precipitated from the supernatant with NaCl-PEG solution. Single-strand DNA was purified using repeated phenol-chloroform extraction followed by ethanol precipitation. The single-strand DNA pellet was then resuspended in 50 μ l of water. An aliquot of the preparation was used as a template for oligonucleotide site-directed mutagenesis (Kunkel et al., 1987; Messing and Vieira, 1982) to generate the different GFP variants used in this study (Figure 1). The sequences of all the resulting plasmids were confirmed at the sequencing facility of the Department of Cell Biology at Harvard Medical School.

Primers

PCR Primers used to clone the GFP into pET22: Left, 5'-GCCATATG CATCACCATCACCATCACCATTCTACAAAGGGCGAGGAGCTGTG CACCGGGG-3'; Right, 5'-GCCTCGAGTTAGGCCGCTAGCGCGTA GTTCTCGTCTCGCGGCCGCTTATACAGCTCGTCCATGCCGA GAG-3'. Primer used to insert an additional nonnative Cysteine as the ninth amino acid (at the N terminus): 5'-CTCCTCGCCCTTT GTACAATGGTGATGGTG-3'. Primer used to substitute D242 to Cystein (at the C terminus): 5'-CCGCCTTATAGAGCTCGCACATGC CGAGAG-3'.

Protein Purifications

GFP Purification

The different GFP variants were purified similarly from *E. coli* BL21 (DE3). Freshly transformed bacteria were grown in LB medium (2 liters) for about 6 hr at 37°C with constant shaking until the OD of the culture reached 0.8 (at 600 nm). IPTG was added to a final concentration of 0.1 mM. The culture was grown for additional 2 hr, and the bacteria were recovered by centrifugation. The pellet was resuspended in 50 mM HEPES-KOH (pH 7.5) and sonicated for 3 min on ice. The sonicated lysate was then clarified by centrifugation. The clear lysate was filtered (0.2 μ m), and the GFP derivative was absorbed to Ni-NTA-agarose beads (Qiagen) followed by an anion exchange chromatography. The GFP eluted from the column (Source 15Q-Pharmacia) by a 0–0.5 M NaCl linear gradient as a sharp peak (data not shown). The peak fractions were combined, concentrated to about 5 mg/ml, and dialyzed against 10 mM HEPES-KOH (pH 7.5). Typically, the yields of the GFP preparations were between 10–40 mg depending on the specific mutant.

Purification of PAN

Recombinant PAN was purified from cells containing the plasmid pET22-PAN. A colony was picked from a freshly transformed *E. coli* BL21 (DE3) and served to inoculate LB-medium containing 150 μ g Ampicillin/ml. The culture was grown for about 6 hr at 37°C with constant shaking until the OD of the culture reached 0.8 (at 600 nm). IPTG was added to a final concentration of 0.1 mM. The culture was grown for additional 2 hr, and the bacteria were collected by centrifugation. The pellet was resuspended in Buffer A (50 mM HEPES-KOH [pH 7.5], 1 mM DTT, and 5 mM MgCl₂) and incubated for 10 min at 50°C. The lysate was then clarified by centrifugation. The clear lysate was filtered (0.2 μ m) and fractionated on a Sephacril 500 column (Pharmacia). The first protein peak was collected and dialyzed against buffer A followed by ion exchange chromatography.

PAN was eluted as a sharp peak with an NaCl gradient (at about 300 mM NaCl). The fractions containing PAN were pooled, concentrated to about 7 mg/ml, and dialyzed against Buffer A.

Fluorescence Assay of Unfolding and Degradation

Measurements of fluorescence were obtained at 45°C in Buffer A (50 mM HEPES-KOH [pH 7.5], 1 mM DTT, and 5 mM MgCl₂) containing 2 mM ATP. The excitation wavelength was 488 nm (with 4 nm band width), and the emission wavelength was 511 nm (with 4 nm band width). The volume of the unfolding and degradation reactions was 500 μ l. The unfolding and degradation reactions were performed in disposable cuvettes in a temperature-controlled rotating four-cell turret. The fluorescence signal was recorded with 4 s intervals on an Aminco fluorimeter. Unless stated otherwise, each reaction contained 0.1 μ M GFP and 0.01 μ M PAN complex (calculated from an approximate MW of 600 kDa) in the unfolding reactions and also 0.003 μ M 20S in the degradation experiments.

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