Recognition of Misfolding Proteins by PA700, the Regulatory Subcomplex of the 26 S Proteasome*

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The 26 S proteasome is a large proteolytic complex that catalyzes the degradation of both native and misfolded proteins. These proteins are known to interact with PA700, the regulatory subcomplex of the 26 S proteasome, via a covalently attached polyubiquitin chain. Here we provide evidence for an additional ubiquitin-independent mode of substrate recognition by PA700. PA700 prevents the aggregation of three incompletely folded, nonubiquitinated substrates: the ΔF-508 mutant form of cystic fibrosis transmembrane regulator, nucleotide binding domain 1, insulin B chain, and citrate synthase. This function does not require ATP hydrolysis. The stoichiometry required for this function, the effect of PA700 on the lag phase of aggregation, and the temporal specificity of PA700 in this process all indicate that PA700 interacts with a subpopulation of non-native conformations that is either particularly aggregation-prone or nucleates misassociation reactions. The inhibition of off-pathway self-association reactions is also reflected in the ability of PA700 to promote refolding of citrate synthase. These results provide evidence that, in addition to binding polyubiquitin chains, PA700 contains a site(s) that recognizes and interacts with misfolded or partially denatured polypeptides. This feature supplies an additional level of substrate specificity to the 26 S proteasome and a means by which substrates are maintained in a soluble state until refolding or degradation is complete.

The 26 S proteasome is a large proteolytic machine that participates in nearly all of the multiple roles played by intracellular protein degradation in cellular function (reviewed in Refs. 1–3). For example, the proteasome catalyzes both the constitutive turnover of the bulk of intracellular proteins and the conditional degradation of specific proteins that regulate various cellular processes. The 26 S proteasome is also a component of the quality control machinery that selectively degrades proteins with abnormal structures. Most known protein substrates of the 26 S proteasome are covalently modified with a polyubiquitin chain as a prerequisite for their proteolysis (1, 2). This requirement seems to have evolved, at least in part, because of the unique structure of the 26 S proteasome.

The 26 S proteasome is composed of two 700,000-dalton subcomplexes, the 20 S proteasome and PA700 (1, 2, 4). The 20 S proteasome, the catalytic core of the complex, is a cylindrical particle consisting of four stacked heptameric rings that contain a hollow center in which all six of the catalytic sites are located (5, 6). This topology excludes both folded and aggregated substrates from contact with, and hydrolysis by, the active sites. The role of initial substrate recognition and binding by the complex is relegated to PA700, a 20-subunit regulatory complex (7–9). Because the 26 S proteasome typically requires polyubiquitinated protein substrates for degradation, the polyubiquitin chain binding site on PA700 (10–12) is an important determinant of substrate specificity. In addition to binding protein substrates, PA700 also regulates the access of substrates to the active sites of the proteasome. ATP-dependent binding of PA700 to the outer rings of the proteasome probably induces a conformational change in the proteasome that creates narrow pores in the center of the outer rings, thereby exposing the interior catalytic sites to the exterior (2, 3, 13, 14). This effect explains, in part, the PA700-dependent activation of the proteasome’s hydrolysis of short nonubiquitinated peptides, which can freely cross the pores without need for interaction with or structural modification by PA700 (8). In contrast, the PA700-dependent opening of pores in the proteasome does not explain fully how PA700 promotes the degradation of ubiquitinated proteins with native or partially folded structures because such proteins are too large to traverse the pores (15). Therefore, it is commonly assumed that as an essential feature of 26 S proteasome-catalyzed proteolysis, PA700 must unfold the bound, ubiquitinated proteins and processively translocate them through the pores to the catalytic sites of the proteasome. This model would explain the known ATP requirement for degradation of ubiquitinated proteins by the assembled 26 S proteasome, because it predicts that substrate unfolding and/or translocation are coupled to ATP hydrolysis by one or more of the six AAA protein subunits of PA700 (7, 13, 14, 16). This attractive model, however, has little direct experimental support and, with the exception of ATPase activity, the properties of PA700 required for such activities are poorly defined.

To mediate unfolding and/or translocation, PA700 must contain a binding site for the non-native structures characteristic of both the products of the unfolding reaction and the sub-

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The abbreviations used are: PA700, proteasome activator, 700 kDa; CFTR, cystic fibrosis transmembrane conductance regulator; NBD1, nucleotide binding domain 1; ΔF-NBD1, ΔF508 mutant N-terminal nucleotide binding domain of CFTR; PA28, proteasome activator, 28 kDa; CS, citrate synthase; ATPγS, adenosine 5’-O-(thiotriphosphate); GdnHCl, guanidinium hydrochloride; NEM, N-ethylmaleimide.
strates for the translocation reaction. Prexist lent for this from studies of the bacterial Clp protease system, which, like the proteasome, has occluded active sites and capping ATPases ClpA and ClpX. These AAA ATPases are known to mediate molecular chaperone and unfoldase activities (17–20). To gain insight into the recognition of protein substrates by PA700 we have analyzed interactions of PA700 with several model proteins in native and non-native conformations. Our data demonstrate that PA700 can directly interact with non-native structures of nonubiquitinated proteins and suggest models for how such interactions can participate in the degradation or refolding of protein substrates.

EXPERIMENTAL PROCEDURES

Materials—PA700 (21), latent 20 S proteasome, PA28, and modulator were purified from bovine red blood cells as described previously (8, 22, 23). “Lid” and “base” subcomplexes of PA700 were identified during the purification of PA700 from bovine red blood cells and are essentially equivalent to their counterparts in yeast (24). Lid was purified as an isolated complex, whereas, the base was purified bound to 20 S proteasome. Details of the purification and characterization of these complexes will be published elsewhere.4 ΔF508 mutant CFTR-NBD1 from residues 404–589 was expressed with a six-histidine tag and purified as described previously (25). Porcine heart citrate synthase was purchased from Sigma; it was dialyzed into TE buffer (50 mM Tris-HCl, pH 8.0, 2 mM EDTA), concentrated to 4.65 mg/ml, and stored at −20 °C in aliquots. Porcine insulin was from Calbiochem.

Aggregation of ΔF-NBD1—Aggregation of ΔF-NBD1 was performed as described previously (26) at 2 μM final protein concentration in Buffer R (100 mM Tris-HCl, pH 7.4, 0.585 mM L-arginine hydrochloride, 2 mM EDTA, 10 mM dithiothreitol, and 25 mM guanidine hydrochloride (GdnHCl)). Aggregation was initiated by rapid dilution from 6 M GdnHCl, while vortexing into a solution of 37 °C Buffer R, and was monitored by the increase in turbidity at 400 nm. 26 S proteasome, PA700, or 20 S proteasome were present at 100 nM prior to the addition of ΔF-NBD1 unless otherwise noted.

Assembly of 26 S proteasome—The 26 S proteasome was assembled in an ATP-dependent reaction from purified 20 S proteasome and PA700 components, as described previously (8). Assembly conditions contained 2-fold molar excess of 20 S proteasome with respect to PA700. Less than 10% of the PA700 was unbound to the proteasome after the assembly reaction.

Reductive Aggregation of Insulin—A 2 mM stock solution of porcine insulin was prepared in 0.1 M potassium phosphate buffer, pH 7.6. Aggregation assays were performed by a modification of previously reported methods (27, 28). Reactions were performed at 37 °C in a final volume of 250 μl containing 45 mM Tris-HCl, pH 7.8, 4 mM reduced glutathione, 0.4 mM oxidized glutathione, 160 μM insulin, and concentrations of various proteins as indicated for specific experiments. Aggregation was monitored continuously as an increase in turbidity at 650 nm. In independent experiments, the length of the lag prior to initial detection of turbidity with insulin alone varied typically from about 300 s to about 1100 s. However, within a given experiment, repetitions varied by less than 10%. All reported results were obtained in at least three to four independent experiments.

Thermal Aggregation of Citrate Synthase—Thermal aggregation of citrate synthase (CS) was carried out by adding 150 mM (monomer) CS to a solution of 40 mM HEPES-KOH, pH 7.5, heated to 43 °C (29). Aggregation was followed by light scattering at 500 nm in a PTI fluorometer at right angles with 2 nm excitation and 4 nm emission slit widths. PA700 was present before the addition of CS unless stated otherwise.

Reactivation of Citrate Synthase—The reactivation of citrate synthase was monitored in a multi-step assay as described previously (30). In the first step, native CS was denatured in 6 M GdnHCl, 50 mM Tris-HCl, pH 7.9, 2 mM dithiothreitol for 1 h at room temperature. Reactivation of denatured CS was then initiated by dilution into a reactivation buffer containing 100 mM Tris-HCl, pH 7.9, 2 mM dithiothreitol, 10 mM KCl and allowed to continue at room temperature. Reactivating ATP (2 mM) also included 5 mM MgCl2. The activity of the reactivated CS was then assayed spectrophotometrically according to the method of Srere and Kosicki (31) at various time points with freshly prepared oxaloacetic acid and acetyl-CoA. Experiments

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RESULTS

PA700, the Regulatory Component of the 26 S Proteasome, Inhibits Aggregation of Misfolding ΔF-NBD1—To understand better the cellular and molecular mechanisms involved in the recognition and processing of misfolded proteins, we studied the interaction of the 26 S proteasome with ΔF-NBD1. ΔF-NBD1 is the nucleotide binding domain of CFTR that contains the common cystic fibrosis disease-causing mutation responsible for impaired folding of the full-length CFTR protein (25, 32–34). In refolding experiments, less ΔF-NBD1 reaches the native state than does wild-type NBD1. A certain fraction, instead, forms large aggregates of misfolded, mutant protein (25, 26, 34). In the cell this misfolding protein is recognized by molecular chaperones (35, 36) and degraded by the ubiquitin-proteasome pathway (37, 38). Therefore, ΔF-NBD1 aggregation provides a useful and physiologically relevant in vitro model system for studying interactions of unfolded or misfolded proteins with components of the cellular quality control machinery. As shown in Fig. 1A, the 26 S proteasome completely inhibited aggregation of ΔF-NBD1. The lack of turbidity observed in done at single time points were assayed after a 45-min reactivation. The slope of the initial rate during the linear portion of the progress curve was plotted relative to the activity of mock-treated native CS. During the time course of the activity assay, which is short relative to the time course of reactivation, no further reactivation occurred. To ensure uniformity in initiating the reactivation, denatured CS was added to the reactivation mixture while vortexing. The fraction of citrate synthase that could be reactivated was reproducible within an experiment but varied between 25 and 75% among different citrate synthase preparations. Each experiment was repeated multiple times with consistent results obtained with multiple preparations.

FIG. 1. Components of the proteasomal machinery inhibit CFTR ΔF-NBD1 aggregation. GdnHCl-denatured ΔF-NBD1 was diluted into Buffer R under conditions (2 μM ΔF-NBD1, 37 °C) where folding to the native state is disfavored and ΔF-NBD1 aggregates. A, aggregation in the absence or presence of 100 nM 26 S proteasome (+26 S). B, Aggregation of ΔF-NBD1, ΔF-NBD1 with 100 nM 20 S proteasome (+20 S), or ΔF-NBD1 with 100 nM PA700 (+PA700).
the presence of the 26 S proteasome was not due to the degradation of ΔF-NBD1 into small, soluble peptides (data not shown). To determine whether either subcomplex of the 26 S proteasome was sufficient to inhibit aggregation, 20 S proteasome and PA700 were assayed individually for their ability to inhibit the aggregation of ΔF-NBD1. The 20 S proteasome did not affect aggregation, whereas PA700 inhibited aggregation as effectively as the intact 26 S proteasome (Fig. 1B). These data demonstrate that the inhibitory activity of the 26 S proteasome can be attributed to PA700.

PA700 Inhibits the Aggregation of Other Misfolding Proteins—To determine whether the ability of PA700 to interact with the non-native conformation of ΔF-NBD1 is a general property, analogous experiments were conducted with two additional proteins whose misfolding can be initiated under different conditions. Insulin is composed of two polypeptide chains, A and B, linked by two disulfide bonds. Upon reduction of the disulfides, the chains separate, and the B chain aggregates (27, 28). As shown in Fig. 2, PA700 inhibited this aggregation as effectively as the 26 S proteasome, whereas the 20 S proteasome had no inhibitory effect.

To extend further the generality of this effect, we tested the ability of PA700 to inhibit the aggregation of thermally denatured citrate synthase, a protein commonly utilized for studying chaperone activity (29, 30, 39). Native citrate synthase is a homodimer; when heated to 43 °C, the population of partially folded conformations of citrate synthase increases, leading to aggregation. As with the other tested proteins, citrate synthase aggregation was inhibited by PA700 at low molar ratios (Fig. 3). PA700 increased the lag period of citrate synthase aggregation in a manner similar to its effect on insulin. Together, these data demonstrate that PA700 interacts with non-native forms of a variety of proteins.

The ability of PA700 to inhibit aggregation is specific to PA700 and is not a property of either of two related multiprotein proteasome regulator complexes. First, a protein complex, termed “modulator,” consisting of two of the ATPase subunits found in PA700 and a third novel protein not found in PA700, enhances PA700-dependent activation of the proteasome (21) but was not effective at inhibiting the aggregation of insulin (Fig. 2B). Second, the proteasome activator PA28 (23), which is known to associate with the 20 S proteasome, did not inhibit the aggregation of insulin (Fig. 2B). In addition, PA700 that has been heat-treated for 15 min at 75 °C is no longer effective in retarding the aggregation of insulin (data not shown). These data provide further evidence for specificity of the PA700-dependent inhibition of aggregation.

The extent of inhibition of insulin aggregation was directly proportional to the concentration of PA700; but surprisingly, inhibition occurred at PA700 concentrations 1000-fold less than those of the substrate insulin. Furthermore, PA700 increased the lag time of insulin aggregation in a concentration-dependent manner (Fig. 2C). In contrast, simple reduction of the insulin concentration in the absence of PA700 reduced the rate of aggregation but did not have a significant effect on the lag phase in this concentration range (data not shown). These results suggest that PA700 interacts with a subpopulation or with a transient intermediate of misfolding insulin which is particularly prone to aggregation (see "Discussion").

PA700 Promotes Refolding of Misfolding Citrate Synthase—In addition to inhibiting aggregation of misfolding proteins, many molecular chaperones promote refolding of these proteins to native structures. Therefore, we tested the ability of PA700 to promote reactivation of chemically denatured citrate synthase. Native citrate synthase was first denatured with 6 M guanidinium hydrochloride, and then refolding was initiated by rapid dilution into a nondenaturing buffer. The restoration of native structure was monitored by the recovery of enzymatic activity. Under these conditions, denatured citrate synthase refolds poorly in the absence of chaperones (Fig. 4 and Refs. 29, 30, and 39). However, in the presence of PA700, enzymatic activity was recovered in both a time-dependent and PA700 concentration-dependent fashion (Fig. 4). These effects were achieved at the same low molar ratios required for inhibition of aggregation. Thus, by inhibiting aggregation of misfolded citrate synthase, PA700 promotes its refolding.

PA700 Does Not Depend on ATP to Inhibit Aggregation or Promote Refolding of Misfolded Proteins—Many well characterized molecular chaperones utilize ATP to modulate the rate

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**Fig. 2.** **PA700 inhibits the aggregation of insulin.** Aggregation was initiated by dilution of native insulin (160 μM) into reducing buffer. A, aggregation of insulin alone, insulin with 300 nM 20 S proteasome (+20 S), insulin with 300 nM 20 S, 160 nM PA700-assembled 26 S proteasome (+26 S), or insulin with 160 nM PA700 (+PA700). B, aggregation of insulin alone, insulin with 300 nM modulator (+Modulator), or insulin with 300 nM PA28 (+PA28). C, aggregation of insulin with no PA700, 50 nM PA700 (3200:1), 100 nM PA700 (1600:1), 200 nM PA700 (800:1), or 400 nM PA700 (400:1).
with N-ethylmaleimide (NEM), which abolishes its ATPase activity (16). NEM-treated PA700 effectively inhibited the aggregation of insulin (Fig. 5D) and promoted the reactivation of citrate synthase (Fig. 5E). These data indicate that the interaction of PA700 with non-native protein substrates does not depend upon ATP.

**PA700 Recognizes a Subset of Non-native Substrate Conformations**—The substoichiometric PA700:substrate ratios required for inhibition of aggregation and promotion of refolding and the progressively increased delay in onset of aggregation in the presence of PA700 suggest that PA700 interacts with a subpopulation of non-native conformations. To test this possibility further, PA700 was added to either the citrate synthase aggregation assay or reactivation assay after each process had begun. As shown in Fig. 6, PA700 remained effective at inhibiting the thermal aggregation of initially folded citrate synthase when added 20 s after the initiation of misfolding. However, PA700 was progressively less effective at inhibiting aggregation when addition was delayed for longer times. These results cannot be explained by simple stoichiometric binding of PA700 because of the large excess of unfolded monomer and, thus, imply that PA700 may act on a species critical for initiation of aggregation. Furthermore, once citrate synthase aggregates were formed, PA700 could not disassemble and solubilize them (data not shown). Likewise, PA700 promoted the reactivation of citrate synthase when present at the start of reactivation, but not when added 2 min later. In sum, these results suggest that PA700 acts on a non-native conformation not yet fully committed to off-pathway aggregation.

The **Base Subcomplex of PA700, Containing ATPase Subunits, Inhibits Aggregation of Misfolded Proteins**—PA700 is composed of 20 distinct subunits, including six AAA-ATPases (16, 21, 47, 48), at least one subunit that binds ubiquitin chains (10–12), and an isopeptidase (49). The functions of the remaining subunits are unknown. To determine which PA700 subunits are responsible for the effects on inhibition of protein aggregation we repeated some of the experiments described above using subcomplexes of PA700 essentially equivalent to the base and lid complexes described for yeast PA700 (24). The bovine base (in a complex with the 20 S proteasome) inhibited aggregation as effectively, if not more effectively, as intact PA700 when compared on a molar basis (Fig. 7A). The base contains all the ATPases of PA700 and two other subunits (data not shown). In contrast, lid, which contains the polyubiquitin chain binding subunit, inhibited aggregation of insulin very poorly (Fig. 7B). Similar effects of base and lid were observed with inhibition of citrate synthase aggregation (data not shown). These results suggest that the various functions of PA700 described here are localized to the base component of PA700.

**Discussion**

This work demonstrates that PA700, the regulatory complex of the 26 S proteasome, recognizes non-native conformations of proteins. PA700 inhibited the aggregation of three misfolding proteins and promoted the refolding of a denatured protein to the native state. These newly discovered functions of PA700 are defining features of molecular chaperones (50–53) and, in combination with the previously established function of PA700 in regulating the degradation of ubiquitinated proteins, place PA700 in a unique and central position for determining the fate of protein substrates as part of the quality control machinery of the cell. Like known chaperones, PA700 could bind to non-native proteins, thereby inhibiting their aggregation and permitting refolding subsequent to their release (Fig. 8). Alternatively, by inhibiting the competing aggregation reaction, PA700 could promote entry of non-native proteins into the degradation process.

**Fig. 3.** PA700 inhibits the thermal aggregation of citrate synthase. Native citrate synthase (150 nM) was heated to 43°C either alone or in the presence of 0.9 nM PA700 (160:1), 1.9 nM PA700 (80:1), or 5 nM PA700 (30:1). a.u., arbitrary units.

**Fig. 4.** PA700 reactivates citrate synthase. GdnHCl-denatured citrate synthase was diluted into refolding conditions, and the formation of active, native protein was monitored.

A. time course of CS reactivation in the absence (open squares) or presence (closed circles) of 300 nM PA700. 

B. CS reactivation at 45 min with increasing concentrations of PA700.
pathway, as has been reported for a known molecular chaperone (54). The possible role of PA700 as a molecular chaperone in the cell may be analogous to known chaperone functions played by ClpA and ClpX, the regulatory subcomplexes of prokaryotic ATP-regulated proteases (55). In addition, PA700 recently has been shown to play a nonproteolytic role in nucleotide excision repair in yeast, perhaps by exerting a chaperone-like activity that disassembles or structurally rearranges the repair complex (56).

Several features of the effects of PA700 reported here are atypical of many well characterized molecular chaperones and provide insights regarding their possible physiological significance. PA700 inhibited the aggregation of misfolded proteins at very low PA700:protein substrate ratios, whereas other chaperones normally require near stoichiometric ratios for function. For example, 30–100 times more Hsc70 was required to achieve the same degree of inhibition accomplished by PA700 in the assays reported here (data not shown and Ref. 26). These results indicate that PA700 interacts with a subpopulation of proteins in a conformation prone to aggregation. Thus, to in-

Fig. 5. ATP is not required for PA700 inhibition of aggregation or the reactivation of CS. A, ΔF-NBD1 (2 μM) was aggregated at 37 °C as in Fig. 1. Aggregation was either with no PA700, 100 nM PA700 (+PA700), or 100 nM PA700, 2 mM ATP (+PA700 (+ATP)). B, insulin (160 μM) was aggregated under reducing conditions as in Fig. 2A. Reactions contained insulin, insulin + 160 nM PA700 (+PA700), or insulin + 3.6 mM MgCl2, 800 μM ATP, 160 nM PA700 (+PA700 (+ATP)). C, CS (150 nM) thermal aggregation was carried out with CS, CS + 7.5 nM PA700 (+PA700), CS + 200 μM ATP, 7.5 nM PA700 (+PA700 (+ATP)), or CS + 200 μM ADP, 7.5 nM PA700 (+PA700 (+ADP)). MgCl2 (1 mM) was present in all reactions. D, insulin (160 μM) aggregation was initiated with no additions, with 215 nM PA700 (+PA700) or with 215 nM NEM-treated PA700 (+PA700NEM). E, Gdn-HCl-denatured CS (150 nM) was reactivated either alone or with the addition of 2 mM ATP; 2 mM ATP; 300 nM PA700; 300 nM PA700, 2 mM ATP; 300 nM NEM-treated PA700, 2 mM ATP; or 300 nM PA700, 2 mM ATP. All reactions contained 5 mM MgCl2. Bars represent the average of three experiments.
hibit aggregation effectively, PA700 need not bind each substrate molecule, but only those disposed to off-pathway associations. Additionally, order-of-addition experiments suggest that PA700 likely recognizes a conformation early in the misfolding process. These features are consistent with the observation that PA700 lengthens the lag phase for aggregation of both insulin (Fig. 2C) and citrate synthase (Fig. 3) in a concentration-dependent manner. The lag phase observed in each of these aggregation processes is likely due to the formation of a specific conformation or nucleus prone to self-association that PA700 may recognize and interact with.

Finally, PA700 does not require ATP either to inhibit aggregation or to promote refolding. Many previously characterized molecular chaperones utilize ATP hydrolysis as a timing mechanism to coordinate the rate of substrate binding and release with the rate of substrate folding, rather than utilizing the hydrolytic energy to promote an otherwise unfavorable reaction. Therefore, the current results may indicate that the known ATP requirement for protein degradation by the 26 S proteasome supports energy-dependent unfolding or processive translocation of unfolded substrates to the proteasome active sites by PA700.

While this manuscript was in preparation, Braun et al. (57) reported that the 26 S proteasome inhibited the aggregation and promoted the refolding of citrate synthase, one of the substrates used in the current study. Both of these activities were localized to the base of PA700. Although the results of the two studies are in general agreement, they differ in several important ways. First, much higher protein:substrate ratios were required for the effects reported by Braun et al. (57) than for those needed here. Second, ATP promoted a small stimulation of inhibition in their experiments. Third, consistent with the low stoichiometry or possibly the dynamic nature of the complex, we were unable to detect significant amounts of association of either insulin or citrate synthase with PA700 during density gradient centrifugation (data not shown). The basis for these differences is unclear but may have significant consequences for interpretation of the mechanisms in which these processes are involved (see below).

What are the physiological implications of the current results? Regardless of whether PA700, either alone or as part of the 26 S proteasome, functions independently as a molecular chaperone in the cell, the novel activities described here are directly relevant to its established function in the degradation of ubiquitinated proteins. For example, PA700 must contain a binding site for non-native protein structures as an essential feature for its role in proteolysis because non-native structures are the products of unfolding reactions and the substrates for translocation reactions that are part of the putative proteolytic mechanism. The potential function of this site for both protein folding and protein degradation is illustrated in the model proposed in Fig. 8. In this model, PA700 may bind to: (i) native ubiquitinated proteins (via recognition of polyubiquitin chains), (ii) non-native nonubiquitinated proteins (via recognition of non-native protein structure), or (iii) non-native ubiquitinated proteins (via recognition of both polyubiquitin chains and non-native protein structure). These multiple recognition and binding modes would allow PA700 to serve a central editing function that determines the final fate of substrates. For example, the known isopeptidase activity of PA700 may pro-
mote the removal of polyubiquitin chains from both native and non-native proteins (49). Inappropriately ubiquitinated native proteins, thus, could be recovered directly, whereas release of the non-native conformer would be less likely due to interactions with the non-native binding site. Alternatively, PA700 may promote the unfolding of bound native or partially folded, ubiquitinated proteins as a prerequisite for their degradation. This process requires a site for binding the product of the unfolding reaction, and the current data provide strong evidence for the existence of such a site on PA700. Finally, as discussed above, PA700 may contribute directly to the recognition and disposition of non-native nonubiquitinated proteins. Elucidation of the relative affinities and coupling between the polyubiquitin and non-native protein binding sites and isopeptidase active site of PA700, depicted in the thermodynamic cycle shown in Fig. 8, will be required for understanding the molecular details of how the 26 S proteasome determines the ultimate fate of protein substrates.

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FIG. 8. Model for the interactions of the 26 S proteasome with protein substrates. Established (black lines) and hypothetical (gray lines) actions of the 26 S proteasome with protein substrates are depicted. Pathways denoted with heavy arrows represent reactions directly related to the studies in the current report. The model distinguishes four different levels through which proteins are processed. At the first level, protein structure is converted between native and non-native forms, either of which may be modified further by ubiquitination. At the second level, the PA700 subcomplex of the 26 S proteasome can recognize and interact with each of the protein forms except native nonubiquitinated proteins, for which it has very low affinity. Otherwise, it can bind to the polyubiquitin chain on either the native or non-native protein or with lower affinity to the non-native, nonubiquitinated protein via a second site that recognizes features of non-native structure, as described in the text. Assuming the two sites are energetically coupled, the non-native ubiquitinated substrate would be the most likely to bind the PA700 subcomplex. At the third level, the substrate protein is edited by one or more of the activities of the PA700 subcomplex. The relative outcome of these activities is determined at the fourth level, substrate fate, and includes: recovery of the native substrate as a consequence of isopeptidase activity, degradation as a consequence of the action of ATP-dependent protease activity, or as described in this text, refolding of the non-native structure to a native structure. The model also depicts the formation of aggregates, which is the fate of non-native structures that escape recognition and editing by the 26 S proteasome.

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