Observing Protein Degradation by the PAN-20S Proteasome by Time-Resolved Neutron Scattering

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ABSTRACT  The proteasome is a key player of regulated protein degradation in all kingdoms of life. Although recent atomic structures have provided snapshots on a number of conformations, data on substrate states and populations during the active degradation process in solution remain scarce. Here, we use time-resolved small-angle neutron scattering of a deuterium-labeled GFPsrrA substrate and an unlabeled archaeal PAN-20S system to obtain direct structural information on substrate states during ATP-driven unfolding and subsequent proteolysis in solution. We find that native GFPsrrA structures are degraded in a biexponential process, which correlates strongly with ATP hydrolysis, the loss of fluorescence, and the buildup of small oligopeptide products. Our solution structural data support a model in which the substrate is directly translocated from PAN into the 20S proteolytic chamber, after a first, to our knowledge, successful unfolding process that represents a point of no return and thus prevents dissociation of the complex and the release of harmful, aggregation-prone products.

INTRODUCTION

The cellular proteome determines the specific function and behavior of a cell at any given time. Protein levels need to be tightly regulated throughout the cellular life cycle, during cell differentiation in multicellular organisms, and as a response to internal or external stimuli. Although the regulation of gene expression represents an early and efficient mechanism to adjust protein levels, controlled protein degradation allows the cell to remodel the existing proteome at later stages (1–4).

Controlled protein degradation inside cells is a complex process, involving networks of multiple partners in all three domains of life (5,6). Dysfunctional or superfluous proteins need to be identified, targeted, and destroyed with exquisite specificity by the cellular proteolytic machinery (3,7,8). In general, specific structural surface features, so-called degrons (tags, hydrophobic patches, and others), mark proteins for degradation; these marks are recognized by energy-dependent regulatory particles (RP) associated with core proteolytic particles (CP) (9). The proteasome represents the main intracellular proteolytic system in archaeal and eukaryotic cells; the endoproteolytic sites responsible for the trimming of target proteins are sequestered within the proteasomes and thus inaccessible to static techniques such as crystallography and cryogenic electron microscopy. The results allow us to propose a dynamic, regulated substrate-processing mechanism.

SIGNIFICANCE  Protein degradation by cells is an essential process for maintaining proteome homeostasis and for eliminating superfluous, dysfunctional, or otherwise potentially dangerous proteins. Although static structures of the large macromolecular complexes responsible for specific protein degradation have become increasingly available over the past years, the time course and kinetics of protein substrates during the process remain largely unknown. This study, combining protein substrate deuteration, solvent contrast variation, and time-resolved neutron scattering with online fluorescence, provides new insights into substrate states and populations as a function of time, inaccessible to static techniques such as crystallography and cryogenic electron microscopy. The results allow us to propose a dynamic, regulated substrate-processing mechanism.
Over the past decades, our understanding of the molecular mechanisms of protein degradation has improved significantly (9,12). In particular, single-molecule experiments have shed light on the relationships between substrate unfolding and translocation as well as their correlation with ATP hydrolysis (15): in parallel, structural studies of substrate-free RPs have revealed ATP-binding patterns as well as the associated conformational changes of the oligomeric complexes (16). Recently, cryogenic electron microscopy (cryo-EM) structures of substrate-engaged human (17) and yeast (18) 26S proteasome have revealed the correlations between ATP hydrolysis, coordinated conformational changes of the 19S RP, and substrate interaction and translocation from the RP into the CP. However, despite these tremendous advances, we are still lacking structural data from the unfolding machinery during the catalytic cycle in solution. In particular, structural information on native substrates, putative unfolded intermediate states, and proteolytic products remain scarce (19,20) as well as information on the respective populations during the catalytic process.

Here, we present a structural kinetics study by time-resolved small-angle neutron scattering (TR-SANS) that monitors the degradation of a protein substrate by the proteasome system from the extremophilic archaeon Methanocaldoccus jannaschii during the catalytic process in solution. The archaeal proteasome consists of a AAA+ ATPase RP called PAN and a 20S protease core. We were able to exploit temperature activation at 55°C of the hyperthermophilic system to trigger and fine-tune the rate of the degradation process and make it amenable to an in vitro structural study on the subminute timescale. As a substrate, we used ssrA-tagged green fluorescent protein (GFP) (21,22), which allowed obtaining complementary information on the unfolding process via online fluorescence measurements, conducted in parallel to the TR-SANS experiments (20).

Small-angle neutron scattering (SANS) allows monitoring structures and conformational changes of solubilized biomacromolecules or complexes (23,24). In contrast to its more common and widespread sister technique small-angle x-ray scattering, SANS is able to distinguish between different (isolated or interacting) proteins present in solution by means of selective macromolecular deuteration (25) and solvent contrast variation (H2O and D2O exchange) (26–28). In particular, at ~42% D2O, natural (i.e., hydrogenated) proteins are made “invisible,” and the SANS signal can be attributed almost exclusively to the deuterated proteins (29). This process is known as “contrast matching” and has been used in this work to mask the strong signal of the very large hydrogenated particles (PAN and 20S) and focus exclusively on the signal of the much smaller, deuterated GFP (d-GFP) substrate during the degradation process in solution.

With this experimental setup, we monitored the structures of the native GFP substrate and its degradation products throughout the unfolding and degradation process in the presence of the PAN-20S complex and quantified the respective populations at a time resolution of 30 s. We found that after a rapid initial exponential decay (characteristic time $\approx 1.5$ min) of natively folded GFP and the concomitant appearance of oligopeptide products, the degradation process continued at a slower rate (characteristic time $\approx 10$ min). The disappearance of native GFP structures, observed by SANS, correlated strongly with ATP hydrolysis, which displayed the same two distinct rates. GFP molecules aggregated strongly and instantly in the presence of PAN and ATP, whereas the addition of 20S prevented aggregation and triggered the emergence of small oligopeptide products. Importantly, our data strongly support a model in which the substrate, once successfully unfolded, is directly translocated from PAN into the tightly bound 20S core particle without intermediate release of unfolded, aggregation-prone substrates. Finally, our data are compatible with the hypothesis of a proteasome-substrate complex in which an elongated, unfolded intermediate substrate spans the PAN central channel and extends into the 20S catalytic chamber, potentially serving as a “tether” for the two enzymatic components until proteolysis is successfully completed. However, our SANS data also indicate that this intermediate stage, if it exists, must be very weakly populated throughout the degradation process.

### MATERIALS AND METHODS

#### Expression and purification of perdeuterated GFPssrA

**Plasmid construct**

GFPssrA is a variant (https://www.fphase.org/protein/alphagfp/) of the GFP from Aequorea victoria containing an A206K mutation to minimize dimerization, with its C-terminus fused to the 11-residue peptide ssrA (AANDELYQKICQGL). For the sake of simplicity, we mostly use the notation “GFP” for “GFPssrA” throughout the manuscript. The GFP gene was codon optimized for expression in Escherichia coli, synthesized, and cloned by Genecust Europe into a pET30a vector using Ndel and NotI cloning sites. GFPssrA amino acid sequence. MSKGEELFTGVPILVELDGDVGNGHKFVSVEGEQGDATYGLKTLKIFCTTGKLPVPWTLTRTFSGYVQCFSRYPDHMKRHDFDKSAMPEGYVQETISFKDNKNTLAEVKFEGDGTLVNRIELKGIDKEDGIGLHKLKLENYSHNYITADKQKNIGKANFK0RHeledGSVQLADHYQQTPIDGFPVLPDNDYHLYSTQKLSDKPNEKRDMMVHELLEVTAGAITHGMDLAAANDENNTAAGA

**Expression and purification**

d-GFP was expressed at the Deuteration Laboratory of the Institut Laue-Langevin (ILL) (Grenoble, France) in E. coli BL21 (DE3) cells. A high-cell-density fermentation process in D2O Enos minimal medium, with d8-glycerol (fully deuterated glycerol) as a carbon source and D2O as solvent (25,30), was used to grow bacteria at 30°C until the culture OD600 nm reached 11. Protein expression was induced by using 1 mM isopropyl-$\beta$-D-thio-galactoside until the OD600 nm reached 16.6. Bacteria were harvested by centrifugation at 3000 × g for 20 min at 4°C. Cells from 1 L of culture were resuspended in 50 mL of 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.1% Triton X-100 and supplemented with 0.25 mg/mL...
lysozyme (EUROMEDEX, Strasbourg, France), 0.05 mg/mL DNase I grade II (Roche, Basel, Switzerland), 0.2 mg/mL RNase (Roche), 1 mg/mL Pefabloc SC (Roche), and 0.01 mg/mL MgSO4.

Cells were lysed using a cell disruptor with four cycles at 18 kpsi at 7°C (Constant Systems, Kennesaw, GA), and the lysate was clarified by centrifugation at 10,000 x g for 1 h at 4°C. GFP was purified by organic extraction followed by Phenyl-Sepharose 6 Fast Flow (Sigma-Aldrich, St. Louis, MO) hydrophobic interaction chromatography as previously described (31).

The protein was loaded on a Superose 12 10/300 GL size exclusion column (GE Healthcare, Chicago, IL) and equilibrated in a 42% D2O buffer containing 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 10 mM MgCl2. The peak fractions were combined and concentrated at 50 mg/mL using Amicon Ultra-15 filtration units (3 kDa cutoff; Sigma-Aldrich). Protein absorbance at 280 nm was used to determine the concentration of the proteins, and purified proteins were stored at −80°C.

Expression and purification of hydrogenated PAN and 20S proteasome

Plasmid construct

The PAN gene from M. jannaschii (PAN) was optimized for expression in E. coli, synthesized, and cloned by GeneCust Europe into a pET30a vector using NdeI and Xhol cloning sites. The expressed protein contains a polyhistidine tag at its C-terminal end. The genes of the α- and β6-subunits of the 20S proteasome from M. jannaschii (20S) were also optimized for expression in E. coli, synthesized, and cloned by GeneCust Europe into a pETDuet-1 vector using, respectively, NcoI and AflII and NdeI and XhoI.

Expression of hydrogenated proteins

PAN and 20S were overexpressed in E. coli strain BL21 (DE3). Bacteria were grown in luria broth medium at 37°C with constant shaking (160 rpm) until the OD600 nm of the cultures reached 0.6. Protein expression was induced by using 1 mM isopropyl-β-D-thiogalactoside for 4 h at 37°C or overnight at 20°C for PAN and 20S, respectively. The cells were harvested by centrifugation at 3000 x g for 20 min at 4°C. Cells from a L of culture were resuspended in 50 mL of 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% Triton X-100, and 5 mM imidazole and supplemented with 0.25 mg/mL lysozyme (EUROMEDEX), 0.05 mg/mL DNase I grade II (Roche), 0.2 mg/mL RNase (Roche), 1 mg/mL Pefabloc SC (Roche), and 0.01 mg/mL MgSO4.

Cells were lysed using a cell disruptor with four cycles at 18 kpsi at 7°C (Constant Systems). The lysates were incubated for 20 min at 70°C (PAN) or 85°C (20S) and clarified by centrifugation at 10,000 x g for 1 h at 4°C. The clear lysates were filtered (0.2 μm) and loaded on a nickel column (GE Healthcare) and equilibrated in buffer composed of 20 mM Tris-HCl (pH 7.5), 10 mM MgCl2, and 100 mM NaCl and supplemented with 5 mM imidazole. After washing with equilibration buffer containing first 20 and, subsequently, 50 mM imidazole for five column volumes (CVs), bound proteins were eluted with a 10-CV linear imidazole gradient (50–400 mM imidazole in the equilibration buffer). PAN and 20S were eluted at ~150 and 200 mM imidazole, respectively, and dialyzed overnight against the buffer.

PAN was further purified by a RESOURCE Q anion exchange column (GE Healthcare) and equilibrated in the same buffer as before (20 mM Tris-HCl (pH 7.5), 10 mM MgCl2, and 100 mM NaCl). After washing for four CVs, bound proteins were eluted with a 15-CV linear NaCl gradient (100–300 mM NaCl). PAN was eluted at ~180 mM NaCl.

PAN and 20S were loaded on a Superose 6 Increase 10/300 GL size exclusion column (GE Healthcare) and equilibrated in a 42% D2O buffer containing 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 10 mM MgCl2. The peak fractions were combined and concentrated to 20 mg/mL (PAN) or 45 mg/mL (20S) by using Amicon Ultra-15 filtration units (50 kDa cutoff for PAN and 100 kDa cutoff for 20S, Sigma-Aldrich). Absorbance at 280 nm was used to determine protein concentration. The purified proteins were stored at −80°C.

Biochemical analyses

Expression and purification of hydrogenated proteins

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**Offline SANS of experimental conditions**

Because of the limited neutron flux and weak contrast, SANS experiments from dilute biomacromolecular solutions require sample volumes of several hundred microliters and protein concentrations of several milligrams per milliliter to yield a good signal (29). As a consequence, the SANS sample composition (Table S1) differed significantly from those typically used in biochemical assays (29,32,33). In particular, protein and ATP (100 mM) concentrations were much more elevated. To match the elevated ATP concentrations in the SANS samples, the MgCl2 concentrations in the buffers were also increased from 10 to 200 mM. We therefore investigated an array of temperatures and ATP concentrations by offline fluorescence spectroscopy (Fig. S1) to optimize the SANS experimental conditions under the requirement that each SANS curve (frame) would be collected in 30 s and that a complete experimental series would last 45 min.

**ATP and MgCl2 concentration assays**

Reaction mixtures were prepared with hydrogenated proteins (70 μM GFPssrA, 20 μM PAN, and 20 μM 20S) in an H2O buffer containing 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 10 mM MgCl2. The following conditions were measured: isolated GFP, GFP, and PAN or GFP, PAN, and 20S with 0 mM ATP and 10 mM MgCl2, 10 mM ATP and 20 mM MgCl2, 50 mM ATP and 100 mM MgCl2, and 100 mM ATP and 200 mM MgCl2. The denaturation of GFPssrA was monitored (λem = 400 nm and λem = 500 nm) by using a plate reader spectrophotometer (BioTek Synergy H4 Hybrid Multi-Mode Microplate Reader; BioTek Instruments, Winooski, VT). Fluorescence was measured over 1 h at 55°C with a time resolution of 40 s. Fluorescence curves as a function of time were generated by normalizing to the first measurement point, which was defined as 100%.
**Temperature assay**

Reaction mixtures were prepared with hydrogenated proteins (70 μM GFPssrA, 20 μM PAN, and 20 μM 20S) in an H2O working buffer containing 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 100 mM ATP, and 200 mM MgCl2. The following conditions were measured: isolated GFP, GFP, and PAN and GFP, PAN, and 20S at different temperatures (30, 40, 45, 50, 55, 60, and 65°C). The denaturation of the GFPssrA was monitored (λex = 400 nm and λem = 509 nm) using a plate reader spectrophotometer (BioTek Synergy H4 Hybrid Multi-Mode Microplate Reader; BioTek Instruments). Fluorescence was measured over 1 h at each temperature with a time resolution of 40 s. Fluorescence curves as a function of time were generated by normalization to the first measurement point, which was defined as 100%.

**ATPase activity assays**

**Offline ATP hydrolysis under SANS conditions**

The rate of ATP hydrolysis by PAN was measured offline in the same conditions as the SANS experiments. For this purpose, the amount of inorganic phosphate was recorded as a function of time. Reaction mixtures were prepared with hydrogenated proteins (70 μM GFPssrA, 20 μM PAN, and 20 μM 20S) in an H2O buffer containing 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 100 mM ATP, and 200 mM MgCl2. The following conditions were measured in triplicate: isolated PAN, PAN and GFP, PAN, 20S and GFP, isolated GFP, isolated 20S, and buffer. The samples were incubated at 55°C and monitored over 1 h with a 20 μL reaction mixture measured at each time point. The aliquots were incubated on ice to stop the reaction and diluted 150 times in the buffer. 4 μL were then supplemented with 36 μL of assay buffer and 200 μL of malachite green reagent (MAK113; Sigma-Aldrich). After 30 min of incubation at room temperature, absorbance at 630 nm was analyzed by using a plate reader spectrophotometer (BioTek Synergy H4 Hybrid Multi-Mode Microplate Reader; BioTek Instruments). A standard phosphate curve was used to estimate the amount of phosphate released during the hydrolysis of ATP. Phosphate concentrations were fitted with a biexponential process (Eq. 1) in which intensities were replaced by concentrations.

**ATP/ADP mixture**

To evaluate the inhibitory effect of ADP on the degradation reaction, we measured the rate of ATP hydrolysis by PAN in an ATP/ADP mixture in the presence of GFPssrA. Reaction mixtures were prepared with hydrogenated proteins (70 μM GFPssrA and 20 μM PAN) in an H2O buffer containing 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 200 mM MgCl2. The following conditions were measured in triplicate: 20 mM ATP, 80 mM ADP, and 20 mM ATP and 80 mM ADP. The samples were incubated at 55°C and monitored over 1 h with a 20 μL reaction mixture measured at each time point. The assay proceeded as described above.

**SANS data collection, reduction, and analysis**

SANS data sets (https://doi.ill.fr/10.5291/ILL-DATA.8-03-935) were recorded on the D22 diffractometer (http://www.ill.eu/instruments-support/instruments-groups/instruments/d22) at the European Neutron Source ILL in Grenoble, France.

Reaction mixtures containing 42% D2O (Table S1) were measured at a collimator or detector configuration of 2.0 or 2.8 m and allowed the matching of all hydrogenated proteins (hydrogenated PAN (h-PAN) and hydrogenated 20S (h-20S)). For the reaction mixture of d-GFP and h-PAN with 100 mM ATP, an additional independent measurement was performed at a collimator or detector configuration of 8.0 or 8.0 m to access lower q-values for a better characterization of the aggregates. 42% D2O was chosen because it corresponds to the experimental contrast match point of h-PAN determined in a previous study (20). It is in good agreement with its theoretical match point (40.9% D2O) calculated from its amino acid sequence (http://psicd.isi.ri.ac.uk/PsIdc/). The experimental contrast match point of h-20S was not determined in this study explicitly because its amino acid sequence predicts a theoretical contrast match point of 41.2% D2O virtually identical to the one of h-PAN.

For each sample (Table S2), 310 μL of the reaction mixture were prepared and pipetted into 1-mm path length quartz cells (Hellma 110-QS; Müllheim, Germany) just before transferring them to the thermostated sample rack already equilibrated at 55°C to start the reaction. 55°C was chosen as a compromise because of the practical limitations of the SANS experiments: at higher temperatures, PAN is more active (Fig. S1; S4), but the reaction would have been too fast for the 30-s frames, in particular in the first, rapid regime, and at lower temperatures, the reaction would have been too slow for the limited measurement time of the individual SANS experiments (45 min), in particular regarding the second, slower regime. The scattering curves were recorded with an individual exposure time of 30 s. An initial dead time of ~20 s was necessary to evacuate the experimental hatch and switch on the neutron beam. A neutron wavelength of 6 Å was used for data collection. A total of 90 individual scattering curves were recorded during the 45-min exposure times for each condition (apart from the control experiments (Fig. S2) in which only 45 were collected because of the limited beam time).

The corresponding buffers, empty cell, empty beam, and boron/cadmium were measured in the same collimator and detector setups to perform data reduction (correction for detector efficiency, electronic noise, and sample holder scattering). Data reduction and radial integration over a two-dimensional image were performed using standard ILL software LAMP (35), and the one-dimensional sample and buffer curves were presented in absolute units (cm⁻¹) calibrated against the direct beam. The scattering curves of the buffers were subtracted from the respective sample curves with the software PRIMUS from the ATSAS suite (36). The radii of gyration Rg (which report on the extension of a particle) and forward scattered intensities I(0) (which report on its molecular weight), as well as the respective errors, were extracted by using the Guinier approximation (37), i.e., a linear fit in an ln[I(q)] vs. q² plot, with the same program. The validity of the Guinier approximation (Rg × q² max < 1.3) was fulfilled in all cases apart from the d-GFP and h-PAN samples in which the aggregates occurred. In this latter case, the lowestmost three data points were used to extract a lower estimation of Rg and I(0).

The program OLIOMER (36) was used to fit the experimental scattering curves of GFP in the presence of PAN and 20S by using either two (natively folded GFP and decapetide product) or three components (natively folded GFP, decapetide product, and partially unfolded GFP intermediate). All fits were done in the same q-range (0.03–0.35 Å⁻¹). The form factor inputs (i.e., theoretical SANS curves) of each component in 42% D2O were calculated by the program CRYSON (38) and loaded into OLIOMGER. As a representative model of native GFP, we scored an ensemble of atomic models, including a flexible C-terminus, from previous work (39) and selected the model that fitted the d-GFP SANS data (Fig. 1 A) best at t = 0 min. As a representative decapetide product, the first 10 residues (N-terminus) of the same model were used. The partially unfolded intermediate was created from the native GFP model by deleting the flexible C-terminus and the first, connected β-barrel strand (up to P213) using PyMOL. Subsequently, unfolded peptide residues (1–28) and the first six connected ubiquitin residues from a blocked substrate solved by cryo-EM (18) were fused to P213 to yield a 247-residue GFP construct (Fig. 3, red model). The completely unfolded GFP (Fig. S10) was generated from native GFP by Coot (https://www2.mrc-lmb.cam.ac.uk/personal/pemsley/coot/).

Complementary to OLIGOMER, we estimated the number of independent GFP-derived species in solution (in the presence of PAN and 20S) by a singular value decomposition (SVD) analysis using the program BioXTAS RAW (40). Intensities were integrated over the whole q-range and for all 30-s frames during the 45-min experimental series (Fig. S13).
The time course of \(I(0)\) intensities, \(I(0, t)\), were fitted by using a biexponential function with the Igor Pro Software (https://www.wavemetrics.com):

\[
I(0, t) = I_0 + I_1[1 - \exp(-t/t_1)] + I_2[1 - \exp(-t/t_2)].
\] (1)

\(I_0\) is an initial intensity at \(t = 0\) min; \(I_1\) and \(I_2\) are the weight fractions of two different exponential decay processes with characteristic time rates \(t_1\) and \(t_2\). The same function was also used to fit the evolution of the radii of gyration \(R_g\) and the fluorescence intensities (Fig. 2) as well as the ATP hydrolysis (Fig. S3). Equation 1 was also used to describe the time

**FIGURE 1** Experimental SANS curves as a function of time. Shown are d-GFP alone (A), d-GFP in the presence of h-PAN (B), and d-GFP in the presence of h-PAN and h-20S (C) on a double logarithmic plot. Continuous lines display fits to the atomic Protein Data Bank model with CRYSON (38). Insets in (A) and (C) show the respective Guinier fits used to extract radii of gyration \(R_g\). GFP was perdeuterated in all cases and had a strong contrast in the 42% D\(_2\)O buffers. PAN and 20S were in their natural, hydrogenated state and effectively invisible in the 42% D\(_2\)O (and are therefore depicted as a transparent ribbon). All samples were measured at 55°C and in the presence of ATP. The arrows indicate the direction of the change of \(I(q)\) over time. In (C), the experimental points of the last curve (45 min) are connected by a line to facilitate their visualization at high angles. A total of 90 SANS frames of 30 s were recorded for all samples. For reasons of clarity, only a selection of frames is shown. Likewise, error bars are shown only for the first and last data set in case (B). Typical error bars of the data sets in cases (A) and (C) are shown in Fig. S12. The error bars shown represent statistical errors of the solvent-subtracted SANS intensities as obtained by the program PRIMUS. To see this figure in color, go online.
behavior of the natively folded GFP population from the OLIGOMER data (Fig. 3; Fig. S4). In this case, a biexponential fit did not converge, possibly because of the limited signal and noise of the original SANS data that limit the convergence of the OLIGOMER fits and/or because of the fact that the first SANS curve did not yield a 100% population of natively folded GFP. Indeed, because of the short experimental delay (20 s, see above) until neutron acquisition is started and averaged over 30 s, the reaction is already active. Therefore, a single exponential decay function ($t_2 = 0$) was used.
Online fluorescence measurement

In parallel to all TR-SANS measurements, we carried out online fluorescence measurements. To this end, a special setup with an in situ spectrophotometer (65000 pro; Ocean Optics, Largo, FL) was implemented on the D22 beamline at the ILL as reported previously (20). Fiber optics (Ingénierie Développement Instrument Laser; Idil Fibres Optiques, Lannion, France) were connected to the SANS quartz cell containing 310 μL of the reaction volume. The excitation was ensured by a laser diode at 405 nm, and the emission wavelengths were filtered with a passband filter centered on 500 nm with a full width at half maximum of 40 nm (Thorlabs, Newton, NJ), respectively. The fluorescence emission spectra (1 s) were recorded every 30 s, and the fluorescence data were registered as NEXUS file format. The decreases in the percentage of fluorescence (Table 1) were calculated with respect to time t = 0 min for each sample. The time course of the online fluorescence intensities was fitted with the python hd5py package (http://www.h5py.org) and fitted with a Gaussian function to determine the fluorescence intensity at 509 nm. The error of the fluorescence intensity was estimated based on the standard deviation of the fitting parameters. The decreases in the percentage of fluorescence (Table 1) were calculated with respect to time t = 0 min for each sample. The time course of the online fluorescence intensities was fitted by Eq. 1.

Mass spectrometry measurements

LC/electrospray ionization-TOF-MS analysis was carried out on d-GFP (6210; Agilent Technologies, Santa Clara, CA). The mass for d-GFP (28,956.80 Da) gave an estimate of 69% deuteriation (without the exchangeable H) because of the H2O-based mass spectrometry buffers and ~92% deuteration combined with the calculated mass (with deuteration of exchangeable H) in 42% D2O buffer. The Biological Scattering Tools website (http://pslcd.isis.rl.ac.uk/) was used for the calculation of the number of exchangeable H in each protein.

MALDI-TOF analysis was carried out on the reaction h-GFP, h-PAN, and h-20S with or without ATP (Autoflex; Bruker Daltonics, Billerica, MA). The reaction mixture was prepared as described in Table S1, with hydrogenated proteins in a final volume of 50 μL and incubated for 1 h at 55°C in an H2O buffer containing 20 mM Tris-HCl (pH 7.5) and 100 mM NaCl. The samples were diluted in the same buffer to deposit 7.5 ng of GFP for each sample on an SDS-PAGE plate. The PAN-20S complex degrades GFP at a biexponential rate that follows ATP consumption (21). Likewise, the PAN and 20S structures shown in this figure were exceptionally taken from the cryo-EM study of their eukaryotic homologs and are depicted as a transparent ribbon to indicate that their SANS signal was contrast matched by the solvent at 42% D2O. The continuous lines represent the fits with a single exponential decay function (Eq. 1 with t2 = 0). To see this figure in color, go online.

RESULTS

The PAN-20S complex degrades GFP at a biexponential rate that follows ATP consumption

In the absence of h-PAN and h-20S, ssrA-tagged d-GFP conserved its native structure for 45 min at 55°C: all SANS curves, acquired at 42% D2O (where hydrogenated proteins are invisible), were identical and corresponded to the native GFP structure (Fig. 1A). Both the radius of gyration RG and the forward scattered intensity I(0) remained constant over time (Fig. 2A; Table 1), demonstrating that neither the compactness of the protein (RG) nor its molecular weight (I(0)) were altered (23). This demonstrates that GFP did not unfold or form aggregates during the experiment (Fig. S5, left). Likewise, d-GFP conserved its native structure in the presence of h-20S and ATP (but in the absence of h-PAN) and in the presence of h-PAN (or
h-PAN and h-20S) but in the absence of ATP (Fig. S2). The moderate decrease of fluorescence at 509 nm (Fig. 2 A; Table 1) may be attributed to a diminished quantum efficiency of fluorescence at higher temperatures (33), to minor conformational changes (34), or to structural degradation caused by the strong ultraviolet illumination in the exposed sample area (Fig. S5, left).

The addition of (contrast-matched) h-PAN and ATP in a freshly prepared d-GFP sample induced significant changes in the SANS curves (Fig. 1 B): the radius of gyration $R_G$ and the $I(0)$ intensity increased by more than 10-fold and 1000-fold with respect to isolated d-GFP, whereas the fluorescence signal showed a considerable decrease (Fig. 2 B; Table 1). The evolution of all molecular parameters was compatible with the rapid appearance of GFP aggregates (Fig. S5, middle), with the average molecular weight and linear dimensions being 1000 and 10 times larger, respectively, than native GFP. The decrease of the fluorescence intensity could be described by a biexponential process (Eq. 1; Fig. 2 B) with a fast ($t_1$) and a slow ($t_2$) characteristic time constant (Table 1). The increase of $I(0)$ displayed a more complex behavior (e.g., initial sigmoidal lag time), probably because of the process of growing aggregates, and was not fitted by a biexponential function.

Addition of (contrast matched) h-20S (and ATP) to a freshly prepared h-PAN-d-GFP sample yielded SANS curves that could be fitted by the structure of natively folded d-GFP at low angles, whereas at high angles, the curves increasingly deviated from the native structure over time (Fig. 1 C). Both the $I(0)$ intensity and the fluorescence decreased rapidly over time in a biexponential process at identical fast ($t_1 \approx 1.5$ min) and slow ($t_2 \approx 10$ min) rates (Eq. 1; Fig. 2 C; Table 1). A single exponential process could not describe the decay in a satisfactory manner (Fig. S6). Thus, the SANS data indicate that GFP is efficiently degraded by 20S, leading to a progressive decrease

| TABLE 1  | Overview of Parameters Extracted from the Experimental Data |
|----------|-------------------------------------------------------------|
|          | GFP                              | GFP + PAN | GFP + PAN + 20S |
| SANS     |                                  |          |
| $R_G$,$t_0$ (Å) | 19.4 ± 1.1                  | 20.8 ± 0.7 | 19.7 ± 1.2 |
| $R_G$,$t_0$ (Å) | 20.7 ± 1.1                  | 195.9 ± 4.7 | 17.3 ± 4.8 |
| $R_G$ (%)  | +6.7                           | -842a    | -12.2 |
| $I(0)$,$t_0$ (cm$^{-1}$) | 0.132 ± 0.005          | 0.130 ± 0.003 | 0.127 ± 0.005 |
| $I(0)$,$t_0$ (cm$^{-1}$) | 0.136 ± 0.005          | 0.207 ± 1.21a | 0.033 ± 0.004 |
| $R_G$ (%)  | +3.0                           | +15,592a | -74 |
| $t_1$     | ND                             | NDb      | 1 min 17 s ± 27 s |
| $t_2$     | ND                             | NDb      | 11 min 29 s ± 4 min 43 s |
| ATP Hydrolysis   |                                  |          |
| $\Delta$ATP (%) | ND                           | -83      | -84 |
| $t_1$     | ND                             | 1 min 55 s ± 1 min 8 s | 1 min 22 s ± 41 s |
| $t_2$     | 8 min 10 s ± 2 min 45 s        | 10 min 25 s ± 5 min 31 s |
| Oligomer  |                                  |          |
| $\Delta$GFP$_{native}$ (%) | ND | ND | -87 |
| $t_1$     | ND                             | ND       | 4 min 55 s ± 41 s |
| $t_2$     | 141 min ± 136 minc            | 40 s ± 5 s | 1 min 23 s ± 8 s |
| Fluorescence |                                  |          |
| $Fluo_{final}$ (au) | 57.0 ± 0.9              | 50.7 ± 0.9 | 50.9 ± 0.6 |
| $Fluo_{initial}$ (au) | 35.6 ± 1.0               | 5.5 ± 0.4 | 7.3 ± 0.4 |
| $\Delta$Fluo (%) | -38                         | -89      | -86 |
| $t_1$     | 10 min 14 s ± 23 s            | 9 min 10 s ± 52 s |
| $t_2$     | ND                             | ND       | 10 min 14 s ± 23 s |

Relative changes of $R_G$, $I(0)$, fluorescence and populations of GFP, and ATP consumption are given. $R_G$, $I(0)$ and fluorescence at 509 nm (Fig. 2) were extracted from the SANS curves (Fig. 1) and from the online fluorescence measurements. The populations were determined by fitting the SANS curves with Oligomer (Fig. S6). ATP hydrolysis was measured using a standard assay (Fig. S3). The characteristic time constants $t_1$ and $t_2$ were fitted in all cases using Eq. 1, either with a biexponential function or a single exponential function (all Oligomer populations and fluorescence of isolated GFP). Errors represent standard deviations from the fits. au, arbitrary unit; ND, not determined.

$^a$ $R_G$ and $I(0)$ intensities of the aggregated sample ("GFP + PAN") are "apparent" values because the experimental $q$-range did not cover the Guinier range correctly ($R_G \times q_{max} > 1$). As a consequence, both values are underestimated and represent lower limits of the real values.

$^b$I(0) intensities of the aggregated samples display a sigmoidal behavior at short times. They can be fitted by a biexponential function only for $t > 2$ min (data not shown). Because the characteristic times include the growth process of the aggregates and can therefore not be compared directly with the characteristic times of the fluorescence decay or ATP hydrolysis, we did not attempt a quantitative analysis of the data.

$^c$The fluorescence decay of isolated GFP can be fitted by a linear function or a single exponential function (as done here). The addition of a second exponential function does not improve the fit significantly.
of the population of native GFP (Fig. S5, right). These findings were supported by a Western blot analysis (as well as by mass spectrometry results; see next section) using an anti-GFP primary antibody on the end product of the reaction, in which all native GFP disappeared in the presence of PAN, 20S, and ATP in conditions similar to SANS (Fig. S7).

Interestingly, ATP hydrolysis by the PAN-20S complex in the presence of GFP, measured offline in conditions identical to the SANS experiment (Fig. S3), followed the same biexponential decay process as the I(0) and fluorescence intensities, with identical fast and slow time constants (t₁ ≈ 1.5 min and t₂ ≈ 10 min; Table 1). This correlation indicates a direct relationship between ATP hydrolysis and the change in structural and functional parameters of the GFP substrate.

The GFP substrate is degraded into small oligopeptide products by the PAN-20S complex

The addition of 20S to the reaction volume efficiently prevented the appearance of GFP aggregates (Fig. 1 C; Fig. S5, right), which were observed in the presence of PAN alone (Fig. 1 B; Fig. S5, middle). As mentioned above, the SANS curve of d-GFP, in the presence of h-PAN, h-20S, and ATP, was in good agreement with the native GFP structure only at t = 0 min (Fig. 1 C, topmost red data points and fit), whereas the curves at subsequent times could no longer be fitted by native GFP alone over the entire q-range (Fig. 1 C, black data; Fig. S8). The scattering intensity of these curves decreased continuously over the reaction time at lower q-values, whereas it progressively increased at higher angles (q > 0.15 Å⁻¹) (Fig. 1 C, right black arrow).

The increase of I(q) at higher angles indicated the appearance of smaller reaction products that scatter homogeneously over a wider q-range (37,41). Fittingly, a mass spectrometry analysis of the reaction products revealed a distribution of oligopeptides in the range from 4 to 14 amino acids (Fig. S9). To verify whether the SANS data could be fitted with a combination of natively folded GFP and oligopeptide products, we generated an average structure of a decapeptide to represent the low molecular weight products. This crude approximation is justified by the limited statistics of the 30-s SANS data at higher angles, which make the curves insensitive to different conformations and moderate size variations of the oligopeptides. Furthermore, an SVD analysis with the program BioXXTAS RAW (40) suggested the presence of a maximum of two significant populations of different species (Fig. S13).

The SANS curves could be fitted well (0.8 < χ < 1.1) by a mixture of native GFP and the representative decapeptide by using the program OLIGOMER (Fig. S8, A–C; (36)); correspondingly, the time dependence of the populations of both species could be determined with good statistical precision (Table S3, left). The GFP population disappeared in a single exponential decay process (Fig. S4) to a final level of 13% (volume fraction) with a characteristic time of 5 min; concomitantly, oligopeptide products built up at the same rate to reach a final level of 87% (volume fraction). The rate of GFP proteolysis (5 min) lay between the slow and fast rates of the biexponential processes of ATP hydrolysis and fluorescence decay (Table 1).

Does a putative, elongated intermediate substrate state exist?

Recently, the structures of two eukaryotic homologs of the archaeal PAN-20S complex were solved by cryo-EM in the presence of blocked substrates (17,18). We combined information from the substrate-engaged structure of the yeast cryo-EM complex (18) and from single-molecule spectroscopy (42) to generate a bona fide structural model of a putative, partially unfolded GFP intermediate (see Materials and Methods). This model was added to the natively folded GFP structure and the decapeptide product to fit the TR-SANS data with the program OLIGOMER.

As in the case of the two-species fits, the three-species fits showed a rapid exponential decline of native GFP over the first 10 min, accompanied by an increase of the decapeptide population occurring at the same rate (Fig. 3; Fig. S8, D–F). The population of the putative intermediate, (partially) unfolded state could not be determined with high precision and remained below 5–10% throughout the experiment (Fig. 3; Table S1, right). In addition, the quality of the three-species fits was not better than that of the two-species fits, as judged by the χ-values (Fig. S8), and the populations of native GFP and decapeptide products did not change significantly between the fits. A significant population of a GFP intermediate with a high degree of unfolding is incompatible with the SANS curves at low q-values (Fig. S10). Together, our SANS data are compatible with the presence of a putative elongated and unfolded intermediate state as suggested by recent, substrate-engaged cryo-EM models but indicate also that such a state, if it exists in solution, must be very transient and weakly populated during the degradation reaction.

DISCUSSION

The time rates of the diminution of native GFP structures correlate with ATP hydrolysis

The experimental setup of the GFP-PAN-20S reaction allowed monitoring the disappearance of native GFP structures, the appearance of degradation products, and the decrease of fluorescence by TR-SANS and online fluorescence (Figs. 1 C, 2 C, and 3). The results could be compared with ATP hydrolysis assays, measured offline under identical conditions (Fig. S3), and revealed a strong correlation between the three experimental data sets (Fig. 4; Table 1).
Indeed, the slow rates measured for the disappearance of native GFP and for the fluorescence decay (Fig. 2) indicated that, if substrate is indeed released after such an attempt, it would have to be in a form ("destabilized GFP") that although not necessarily identical with native GFP, would have to be structurally very similar to it. In particular, it must not display any altered features (loose C-terminus, exposed hydrophobic patches, etc.) that would increase its propensity to aggregate (Fig. 5) and would therefore also have to differ from the (partially) unfolded intermediate state.

Previous biochemical assays (33) have demonstrated the dependence of ATP hydrolysis on the presence of substrate or the 20S particle, which were both shown to stimulate ATP consumption. These assays were conducted at ATP concentrations of ~1 mM, a value close to the \( K_m \) of ATP hydrolysis by PAN (0.5 mM) (43). Under these conditions, ATP hydrolysis is expected to be sensitive to modulations of the substrate or downstream effector concentrations. In our conditions, which were optimized for SANS experiments, the concentration of ATP was much higher (100 mM) to ensure the presence of sufficient ATP during the long reaction times as well as to counteract the inhibitory effect of accumulating ADP. Thus, we expect our assays to be much less sensitive to the modulation of the \( K_m \) or \( k_{cat} \) of ATP hydrolysis. Consequently, we did not observe any dependence of the characteristic time constants on the presence of substrate (Fig. S3).

**FIGURE 4** Comparison of characteristic time constants from different experimental data. Shown are the characteristic time constants \( t_1 \) and \( t_2 \) (Eq. 1) from fits of \( l(0) \) SANS intensities (indicative of molecular weight), fluorescence at 509 nm (indicative of the percentage of folded state), ATP consumption, and OLIGOMER populations (Table 1). All data refer to GFP in the presence of both PAN and 20S. In the case of OLIGOMER, a single exponential decay was sufficient to fit the data (Fig. 3); the corresponding characteristic time can be considered as an average of the \( t_1 \) and \( t_2 \) measured in the other experiments. The \( t_1 \) and \( t_2 \) error bars were obtained from fits of the experimental data by using Eq. 1 with the Igor Pro Software. To see this figure in color, go online.

Moreover, the loss of natively folded GFP structure, determined from the SANS data by OLIGOMER (~87%), was identical to the loss of fluorescence (~86%), indicating a direct relationship between the structural and functional parameters.

At the beginning of the reaction, ATP was rapidly hydrolyzed by PAN, and the disappearance of native GFP molecules and the fluorescence decay strictly followed the rate of ATP hydrolysis. After the first 10 min, ATP hydrolysis continued at a slower pace (Fig. S3), likely because of the inhibitory effect of accumulating ADP, which is known to compete with ATP for the PAN nucleotide-binding sites (16). In an offline control experiment, we observed that ATP hydrolysis was significantly slowed down in an ADP-ATP mixture with respect to ATP only, whereas at the same time, GFP unfolding was inhibited (Fig. S11). Indeed, the slow \( t_2 \) rates measured for the disappearance of native GFP and for the fluorescence decay (Fig. 2 C) correlated strongly with the slow rate measured for ATP consumption (Fig. 4; Table 1, light gray bars). Thus, the slowdown of ATP hydrolysis by the inhibitory effect of ADP resulted in a similar slowdown of GFP degradation at longer times.

**GFP is translocated directly from PAN to 20S in a processive degradation mechanism**

The comparison of the SANS data in the presence of PAN and 20S (Table 1; Fig. S5) shows that the GFP substrate is directly translocated from the unfoldase to the protease without the release of (partially) unfolded, aggregation-prone intermediate GFP states; GFP oligomers and/or aggregates were observed in the presence of PAN (Fig. 1 B) but were completely absent in the reaction mixture containing both PAN and 20S (Fig. 1 C). Because SANS intensities in the forward scattering direction, \( I(0) \), are proportional to the squared molecular weight of a given molecular species (23), they are exquisitely sensitive to the presence of oligomers and/or aggregates: a fraction as low as 0.1% of GFP released in the form of an aggregate 100 times its size (estimated from the “GFP and PAN” data; Table 1) would have resulted in a readily detectable 10% increase of \( I(0) \). We thus conclude that even though the PAN-20S complex is known to be short-lived in the presence of ATP in solution (44,45), a stoichiometric ratio as low as 1:1 (Table S1) is sufficient to keep both partners tightly associated, cause complete digestion of GFP, and prevent the release of aggregation-prone products.

Other studies have suggested that a successful degradation event is preceded by numerous unsuccessful unfolding attempts, followed by substrate release (12). Our SANS data indicate that, if substrate is indeed released after such attempts, it would have to be in a form (“destabilized GFP”) that although not necessarily identical with native GFP at an atomic level, would have to be structurally very similar to it. In particular, it must not display any altered features (loose C-terminus, exposed hydrophobic patches, etc.) that would increase its propensity to aggregate (Fig. 5) and would therefore also have to differ from the (partially) unfolded intermediate state.

In conclusion, our SANS data strongly support a model in which the translocation and proteolysis processes are spatially and temporarily coupled, with the unfolding intermediate substrate potentially serving as a “tether” for the PAN-20S complex during translocation, in agreement with recent cryo-EM structures of substrate-engaged eukaryotic proteasomes (17,18) and previous enzymatic studies using inhibitors and mutational analyses (34).
A putative, partially unfolded intermediate substrate state may exist in solution but would be weakly populated and transient

The TR-SANS data, recorded during the degradation reaction in solution, could be fitted satisfactorily using a distribution of only two species—native GFP and a representative decapeptide product (Fig. S8, A–C)—and the evolution of their respective populations during time could be determined with good precision (Fig. S4; Table S3). Inspired by recent cryo-EM studies of substrate-engaged eukaryotic 26S proteasomes using ubiquitinylated peptide substrates (17,18), we tested the existence of a third species representing a partially unfolded intermediate state (Fig. S8, D–F). The addition of this third species did not improve, but rather worsened, the quality of the SANS data fits and resulted in increased errors of the populations of native GFP. Minor populations (<5–10%) of the putative intermediate state were compatible with the SANS curves at individual time points throughout the reaction (Fig. 3). These time points were distributed stochastically with no specific pattern or correlation (e.g., a peak distribution during a specific time period). Another possibility would be a more fully unfolded intermediate state, displaying probably an ensemble of flexible conformations. Although such ensembles can in principle be generated and modeled to small-angle data (46,47), we believe that the statistics of our data, especially at higher angles (q > 0.2 Å⁻¹; e.g., Fig. S8), do not warrant such a sophisticated approach. We therefore estimated the impact of an ensemble by fitting a completely unfolded intermediate state as an upper case and found a strong disagreement with the experimental SANS data at low angles (Fig. S10). The direct fits of the SANS curves agree well with a model-free SVD analysis (Fig. S13), which suggested
a maximum of two non-negligible populations of independent species (i.e., native GFP and a representative oligopeptide population). In conclusion, whereas the SANS data do not strongly indicate the presence of a GFP state of a different shape than the native protein, they would be compatible with a very transient and weakly populated form of a partially unfolded and elongated intermediate substrate if it is structurally not too different from native GFP.

Results from force-induced, single-molecule unfolding of single and double (two fused) GFP molecules (15) or multidomain filamin A substrates (48) indicate that the bacterial ClpXP AAA+/protease complex unfolds individual protein domains in a cooperative manner within milliseconds before translocating them during several seconds. Both studies report ratios of \(\sim 1:3\) between translocation and dwell times (periods of up to 100 s during which ATP is being consumed by ClpXP in futile unfolding attempts). The low population of GFP substrates in the process of unfolding and their homogeneous spread over time, without a specific pattern (Fig. 3, red data points), are compatible with these current models that describe protein unfolding by AAA+ proteases as a stochastic process (12). Finally and importantly, the fast time constant \(t_1\) of 1.5 min observed by TR-SANS and online fluorescence (Table 1) agrees very well with the dwell times of \(\sim 100\) s from single-molecule experiments and would correspond to the half-life of a substrate unfolding process in solution.

CONCLUSIONS

A multitude of recent studies, including single molecule, cryo-EM, and NMR, have impressively increased our knowledge of the structure and molecular mechanisms of the proteasome complex. However, structural data on the substrate states during the degradation process remain scarce. The TR-SANS data of the GFPssrA-PAN-20S system presented here provide direct and selective structural information on the ensemble of GFP substrate states during the unfolding and proteolysis processes in solution.

The proteolysis of native GFP follows a biexponential process consisting of a first, fast reaction on the timescale of approximately 1 min and a second, slower reaction with a time constant of the order of 10 min. Both steps of the process correlate very strongly with the loss of fluorescence, measured online in parallel to SANS, and the consumption of ATP, as well as with the generation of small peptides (Fig. 4). The slowdown of the reaction rate after the first minutes is likely due to an inhibitory effect of accumulating ADP on the PAN unfoldase activity.

The comparison of SANS data in the presence of PAN alone and of PAN and 20S (Fig. 1, B and C, both in the presence of ATP) strongly suggest that the unfolding GFP substrate is directly and quickly translocated from PAN into the 20S catalytic chamber without intermediate release in solution and might serve as a “tether” for both partners, assuring that they stay associated during unfolding, translocation, and proteolysis (Fig. 5). The translocating substrate could function as a “safety mechanism” against the release of partially unfolded, and potentially harmful, aggregation-prone products into the cell.

Our results highlight the functional synergy between the regulatory protein AAA+ PAN and the catalytic particle 20S, which, together, build a high-processivity degradation machinery. Although several attempts of unfolding are conceivable, the first successful unfolding attempt marks a “point of no return” (Fig. 5) beyond which the system cannot release misfolded proteins in the cytosol because they would be toxic to the cell. These findings imply that the PAN-20S assembly must be closely regulated in vivo to avoid accumulation of denatured protein products. It is also conceivable that PAN is inactive in isolation in vivo.

Temperature activation of a hyperthermophilic archaeal system in combination with deuterium labeling and SANS contrast variation represents a promising route to gain insight into a number of other substrate-processing events. It may be used to study functional time-resolved processes of a variety of large macromolecular assemblies such as cytoskeletal molecular motors (myosin, kinesin, and dynein), RNA polymerase, DNA polymerase, chaperons, DNA repair machinery, or RNA degradation machinery. In this context, hyperthermophilic proteins remain interesting targets given their higher stability compared with the mesophilic homologs and the possibility they offer to tune the reaction rates to the acquisition times required by TR-SANS.

SUPPORTING MATERIAL

Supporting Material can be found online at https://doi.org/10.1016/j.bpj.2020.06.015.

AUTHOR CONTRIBUTIONS

E.M., A.M., and F.G. performed SANS experiments and analyzed data. E.M. performed wet lab experiments and analyzed data. J.C. and B.F. supervised wet lab experiments. G.K. and T.C. designed the project, experiments. M.M. and M.H. designed and performed deuteration experiments. N.C. performed SANS data reduction. F.G. designed the project, analyzed data, and wrote the manuscript together with E.M., B.F., and T.C. All authors reviewed, approved, and contributed to the final version of the manuscript.

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