Journal of Structural Biology 168 (2009) 267–277

Contents lists available at ScienceDirect

Journal of Structural Biology

journal homepage: www.elsevier.com/locate/yjsbi

Studying chaperone–proteases using a real-time approach based on FRET

Kristina Kolygo, Namit Ranjan, Wolfgang Kress, Frank Striebel, Kaspar Hollenstein [1], Kai Neelsen [1], Miriam Steiner [1], Heike Summer, Eilika Weber-Ban *

ETH Zürich, Institute of Molecular Biology & Biophysics, CH-8093 Zürich, Switzerland

ARTICLE INFO

Article history:
Received 28 January 2009
Received in revised form 24 June 2009
Accepted 6 July 2009
Available online 8 July 2009

Keywords:
FRET
Real-time
ClpXP
ClpAP
PAN
20S
AAA
ATPase
Proteasome
Protease
Chaperone

ABSTRACT

Chaperone–proteases are responsible for the processive breakdown of proteins in eukaryotic, archaeal and bacterial cells. They are composed of a cylinder-shaped protease lined on the interior with proteolytic sites and of ATPase rings that bind to the apical sides of the protease to control substrate entry. We present a real-time FRET-based method for probing the reaction cycle of chaperone–proteases, which consists of substrate unfolding, translocation into the protease and degradation. Using this system we show that the two alternative bacterial ClpAP and ClpXP complexes share the same mechanism: after initial tag recognition, fast unfolding of substrate occurs coinciding with threading through the chaperone. Subsequent slow substrate translocation into the protease chamber leads to formation of a transient compact substrate intermediate presumably close to the chaperone–protease interface. Our data for ClpX and ClpA support the mechanical unfolding mode of action proposed for these chaperones. The general applicability of the designed FRET system is demonstrated here using in addition an archaeal PAN-proteasome complex as model for the more complex eukaryotic proteasome.

1. Introduction

Energy-dependent protein degradation is crucial for maintaining normal function in all living cells (Wickner et al., 1999). This process requires unraveling of the protein's tertiary structure (Weber-Ban et al., 1999; Maupin-Furlow et al., 2000; Benaroudj et al., 2001; Lee et al., 2001) followed by processive proteolytic events yielding small peptide products that can be further processed by downstream peptidases. The molecular entities carrying out this demanding task are chaperone–proteases, large barrel-shaped complexes that sequester the proteolytic active sites from bulk solution inside a central cavity (Groll et al., 2005). Substrates can only enter the cavity through distal narrow pores, the access to which is controlled by ring-shaped ATPases (Hoskins et al., 2001; Groll and Huber, 2003; Sauer et al., 2004). While different such complexes exist in bacterial, archaeal and eukaryotic cells or cellular compartments, they all share the basic two-component blueprint (Lupas et al., 1997a; Pickart and Cohen, 2004; Inobe and Matouschek, 2008). The ATPase rings belong to the AAA+ (ATPases associated with various cellular activities) protein family (Ogura and Wilkinson, 2001; Hanson and Whiteheart, 2005) characterized by the AAA-module responsible for nucleotide binding and hydrolysis (Erzberger and Berger, 2006) and for communicating conformational changes in response to the nucleotide state (Lupas and Martin, 2002; Martin et al., 2007). The role of the ATPase rings is to recruit substrate proteins and to unfold and translocate them into the core cylinder for degradation (Sauer et al., 2004).

Eubacteria contain energy-dependent, two-component chaperone–protease complexes (e.g. ClpAP, ClpXP, HslUV) that represent the simplest architecture among chaperone–proteases from all kingdoms of life (Hoskins et al., 2001). Their proteolytic core cylinders are homooligomeric and consist of only two stacked rings (Horwich et al., 1999; Wickner et al., 1999) that are flanked by single-ring chaperone partners.

In eukaryotes, the 26S proteasome is responsible for the degradation of polyubiquitinated proteins (Baumeister and Lupas, 1997; Larsen and Finley, 1997). Its 20S core cylinder is composed of four stacked rings made from two different types of subunits, the β-type subunits forming the two central rings and the α-type subunits forming the two outer rings (Groll and Huber, 2003). It is capped on both ends by the 19S complex containing an AAA ATPase ring. Archaeal 20S proteasomes are of the same built, but with simpler

* Corresponding author. Address: Institute of Molecular Biology & Biophysics, ETH Zurich, Schafmattstrasse 20, CH-8093 Zurich, Switzerland. Fax: +41 44 633 1229.
E-mail address: eilika@mol.biol.ethz.ch (E. Weber-Ban).
[1] These authors contributed equally to this work.

1047-8477/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.jsb.2009.07.003

subunit composition (Lupas et al., 1997b). They interact with the AAA ATPase PAN, a homologue of the eukaryotic proteasomal ATPases (Zwickl et al., 1999; Benaroudj and Goldberg, 2000; Wilson et al., 2000). The archaeal PAN-20S complex thus serves as an excellent model system for the more complex eukaryotic 26S proteasome.

Chaperone–proteases not only maintain the housekeeping turnover of cellular proteins, but also play an important role in post-translational quality control and were shown to carry out regulatory functions (Bukau et al., 2006; Frees et al., 2007). Due to their enormous importance for all cells, research groups are working on elucidating their roles and mechanisms of action.

Here, we present an *in vitro* system based on fluorescence resonance energy transfer (FRET) that allows real-time detection of the main reaction steps catalyzed by chaperone–proteases. The strong dependence of FRET efficiency on the distance between donor and acceptor fluorophores (Stryer, 1978) can be used to follow the dynamic behavior of molecules in solution. FRET reports on structural changes within a range of 20–90 Å (Van Der Meer et al., 1994) and has been successfully employed to study the kinetics of protein (Lipman et al., 2003) and RNA folding (Murchie et al., 1998) as well as the dynamics of protein–protein interactions (Rye, 2001; Motojima et al., 2004; Ueno et al., 2004). We make use of the distance-dependence of FRET by engineering model substrates reporting in real-time on their folding state, arrival in the proteolytic chamber and cleavage throughout the chaperone–protease reaction cycle.

We introduced cysteine residues into the model substrate and the protease itself by site-directed mutagenesis and covalently modified them with thiol-reactive fluorophores forming a FRET donor–acceptor pair. The FRET assay has the advantage over other methods that the labeled model substrate can be observed without interference from other macromolecules. This approach is applicable to various chaperone–protease systems, as we demonstrate by following these activities *in vitro* in the *Escherichia coli* chaperone–proteases ClpAP and ClpXP as well as the archaeal PAN-proteasome complex.

2. Methods

2.1. DNA constructs

Cys-substituted variants of the λ repressor N-terminal domain (residues 1–92) carrying the ssrA-tag were generated by oligonucleotide-directed mutagenesis (Kunkel et al., 1987). Residues in λ repressor variants are numbered omitting the first methionine. ClpA, ClpP and ClpPi overexpressing plasmids were donated by Arthur Horwich. The ClpX overexpressing vector was kindly provided by Michael Maurizi. ClpX was then recloned via NdeI/BamHI sites into a modified version of the pProEX vector (Invitrogen), in which the EheI site was replaced by an NdeI site, resulting in a cleavable N-terminal His_6-tag. *Methanosarcina acetivorans* proteasomal α- and β-subunits were cloned into the pETDuet vector (Novagen). The α-subunit was inserted into multiple cloning site I using NcoI/BamHI restriction sites and the β-subunit was inserted into multiple cloning site II via NdeI/BglII sites. *M. acetivorans* PAN was cloned via NdeI/BamHI restriction sites into pET20b(+) (Novagen).

2.2. Protein preparation

ClpA, ClpP, ClpPi and Rep variants were expressed in *E. coli* BL21 (DE3) cells at 37 °C; ClpX was expressed at 30 °C. ClpA was purified as described (Kress et al., 2007). ClpPi was purified using anion-exchange chromatography (Q Sepharose) followed by gel filtration (Superdex 200). ClpP was purified as described (Maurizi et al., 1990). Variants of λ repressor N-terminal domain were purified using ion-exchange chromatography (DEAE-Sephacel, Heparin–Sepharose) followed by gel filtration (Superdex 75). ClpX was expressed with an N-terminal cleavable His_6-tag and was purified using Ni-affinity chromatography. After digestion with tobacco etch virus protease (TEV, Invitrogen), untagged ClpX was separated from TEV and the cleaved His_6-tag by Ni-affinity chromatography and stored in 50 mM Hepes pH 7.5, 300 mM KCl, 10% glycerol, 1 mM DTT, 2 mM $MgCl_2$.

Methanosarcina acetivorans PAN was overexpressed in Rosetta (DE3) cells at 15 °C. *M. acetivorans* α- and β-subunits of the 20S proteasome (CP) were overexpressed from pETDuet vector (Novagen) in BL21 (DE3) cells at 25 °C. PAN was purified using ion-exchange chromatography (Q Sepharose), ammonium sulfate precipitation and dialysis against 50 mM Hepes, pH 7.5, 300 mM KCl, 10% glycerol, 50 mM $MgSO_4$, 1 mM DTT. The 20S particle was purified using ion-exchange (Q Sepharose) and hydrophobic interaction chromatography (Phenyl Sepharose) followed by gel-filtration (Superdex 200). All chromatographic materials were purchased from GE Healthcare.

Protein concentrations were determined by absorbance measurements at 280 nm as described (Gill and von Hippel, 1989). Unless indicated otherwise, protein concentrations refer to the SspB dimer, ClpA, ClpX or PAN hexamers, ClpP 14mer and 20S proteasome 28mer. All reactions were performed at 23 °C.

2.3. Preparation of fluorescently labeled molecules for FRET measurements

Cysteine containing variants of λ repressor (Rep9C79C, Rep92C, Rep4C13C, Rep77C92C) previously reduced with DTT, were exchanged into 20 mM potassium phosphate buffer (pH 7.2), 2 mM EDTA using a PD-10 gel filtration column (GE Healthcare). Rep9C79C and Rep4C13C (20 μM) were labeled with the donor fluorophore 5-((((2-iodoacetyl) amino) ethyl) amino) naphthalene-1-sulfonic acid (1, 5- IAEDANS, Invitrogen) for 12 min at 23 °C in the dark with 5- or 40-fold molar excess of the label over the protein, respectively. Rep77C92C (20 μM) was incubated in the dark for 1.5 h at RT with 10-fold molar excess of donor dye over the protein. The reactions were terminated by addition of 10-fold molar excess of reduced glutathione (GSH) over the label. Removal of free label and exchange to 50 mM Hepes (pH 8), 5% glycerol, 2 mM EDTA (supplemented with 50 mM KCl for Rep9C79C and Rep77C92C) was performed via several rounds of dilution and concentration using a stirred Amicon ultrafiltration cell (Millipore) followed by several passes over PD-10 gel filtration columns. Anion-exchange chromatography on a MonoQ column (8 ml; GE Healthcare) was used to separate singly-donor-labeled molecules from unlabeled and doubly-labeled molecules. The labeling mixture was eluted with a linear gradient from 50 to 320 mM KCl over 23 column volumes (cv) for Rep9C79C, 0–175 mM KCl over 30 cv for Rep4C13C and 50–240 mM KCl over 35 cv for Rep77C92C. Singly-donor-labeled Rep9C79C eluted at ~137 mM KCl, singly-donor-labeled Rep4C13C at ~45 mM KCl, singly-donor-labeled Rep77C92C at ~200 mM KCl. Doubly-donor-labeled molecules elute later in the gradient. Half of the singly-donor-labeled material was exchanged into 50 mM Hepes (pH 7.5), 15% glycerol, 2 mM EDTA, 300 mM KCl buffer (buffer S) using a PD-10 gel filtration column or dialysis and was used as a donor-only control in the FRET measurements. The second half of the singly-donor-labeled material was exchanged into 50 mM Hepes buffer (pH 7.2), 5% glycerol, 2 mM EDTA, 50 mM KCl and labeled with the acceptor fluorophore (Fluorescein-5-maleimide, Perbio Science) for 3 h at RT in the dark with 25-fold molar excess of label over the protein (20 μM). The reaction was terminated by addition of 10-fold molar excess of GSH over the label. Removal of free label was performed according to the procedure described above for labeling with donor