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Flavodoxin, a new fluorescent substrate for monitoring proteolytic activity of FtsH lacking a robust unfolding activity

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Abstract

Escherichia coli FtsH, which belongs to the ATPases associated with diverse cellular activities (AAA) family, is an ATP-dependent and membrane-bound protease. FtsH degrades misassembled membrane proteins and a subset of cytoplasmic regulatory proteins. To elucidate the molecular mechanisms of the proteolysis, a system for precisely monitoring substrate degradation is required. We have exploited *E. coli* flavodoxin containing non-covalently bound flavin mononucleotide (FMN) as a model substrate for monitoring protein degradation. It was found that FtsH degrades FMN-free apo-flavodoxin but not holo-flavodoxin. However, degradation of a mutant flavodoxin carrying a substitution of Tyr94 to Asp with a lower affinity for FMN could be monitored by fluorimetry. This newly developed monitoring system will also be applicable for proteolysis by other ATP-dependent proteases.

Keywords: AAA family; ATP-dependent proteolysis; Flavodoxin; Fluorescence; FtsH

1. Introduction

All ATP-dependent proteases belong to the ATPases associated with diverse cellular activities plus (AAA^+) superfamily and share a conserved ATPase domain of 200–250 amino-acid residues (Lupas and Martin, 2002; Neuwald et al., 1999; Ogura and Wilkinson, 2001). AAA^+ proteases usually form barrel-shaped oligomers with a narrow central pore. It has been proposed that substrate proteins are unfolded and translocated for proteolysis, since the active sites are located in the protease chamber. The precise molecular mechanisms of substrate unfolding and threading are largely unknown.

Escherichia coli FtsH is a membrane-bound ATPases associated with diverse cellular activities (AAA) protease, which catalyzes proteolytic degradation of a set of cytoplasmic regulatory proteins and SsrA-tagged proteins, as well as misassembled membrane proteins (Akiyama et al., 2004; Ito and Akiyama, 2005).

To elucidate the molecular mechanisms of proteolysis, a precise monitoring system of substrate degradation needs to be established. Several AAA^+ proteases such as ClpAP, ClpXP and the proteasome exhibit strong enough unfolding activity to unfold and degrade tightly folded proteins such as green fluorescent protein (GFP), whose unfolding and degradation can be monitored by fluorimetry. GFP with recognition tags and substrate proteins fused to GFP have been successfully used to analyze proteolysis by these AAA^+ proteases (Kim et al., 2000; Navon and Goldberg, 2001; Singh et al., 2000; Weber-Ban et al., 1999). However, FtsH does not have a robust unfolding activity and cannot unfold and degrade GFP (Herman et al., 2003; Okuno et al., 2004). We have therefore used alternative model substrates such as Cy3-σ^{32} and fluorescently-labeled casein to monitor conformational changes and substrate degradation during the proteolytic reaction (Okuno et al., 2004). Although these model substrates have been useful in some experiments, their application has been limited. Therefore, we have sought more useful substrates.

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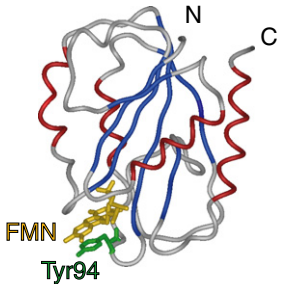

Fig. 1. Ribbon model of *E. coli* flavodoxin (PDB entry 1AG9; Hoover and Ludwig, 1997). Non-covalently bound FMN (yellow) and Tyr94 residue (green) are shown as sticks. α-Helices and β-sheets are colored red and blue, respectively.

It is well known that the fluorescence of flavin mononucleotide (FMN) is dramatically quenched upon binding to apo-flavodoxin. If FtsH were capable of unfolding and degrading a tagged holo-flavodoxin or the flavodoxin moiety fused to other substrate polypeptides, we would be able to monitor the degradation of holo-flavodoxin as a fluorescence increase, since non-covalently bound FMN would be released upon proteolysis. We chose *E. coli* flavodoxin as a candidate, based on four of its properties: (1) its lower thermal stability than GFP (Muralidhara and Wittung-Stafshede, 2004); (2) its well-characterized folding and unfolding properties (van Mierlo and Steensma, 2000); (3) the existence of a refined high-resolution crystal structure (Hoover and Ludwig, 1997; Fig. 1); and (4) the presence of a single folding domain in flavodoxin.

Here, we show that FtsH can degrade apo-flavodoxin, but not the more stable holo-flavodoxin. We constructed a mutant flavodoxin, carrying a substitution of Tyr94 to Asp, which has a lower affinity for FMN presumably due to the loss of the interactions between Tyr94 and the FMN moiety seen in the structure of the holo protein. Significant populations of both the apo- and holo-flavodoxin exist in solutions of the mutant flavodoxin. Degradation of flavodoxin(Y94D) in the presence of excess FMN could be monitored as a fluorescence increase.

2. Materials and methods

2.1. Strains and plasmids

DH5α [*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 deoR Δ(argF-lac)U169 ϕ80lacZΔM15*] and AR5771 [BL21(DE3) *sfhC21 ΔftsH3*::*kan ΔhflKC3*::*tet*] were used as host strains for construction of plasmids and for expression of genes cloned on plasmids. Cells were grown in L medium (10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl/liter, pH 7.4). Ampicillin (100 μg/ml) was added for growing strains carrying plasmid.

To construct the fusion gene coding for GST-flavodoxin, the *fldA* gene was amplified by PCR using a purified chromosome DNA as a template and inserted into the *Bam*HI–*Xho*I site of the multi-cloning site of pGEX-6p-1 (Amersham Biosciences) that is located downstream of the glutathione *S*-transferase gene. A mutation (Tyr94 to Asp) was introduced in the *fldA* gene using mutagenic oligonucleotides (5′-CGGCCGCTCGAGTTACGCTTCAATGG CAGCACGCAATTTACACATCGCGTTCTTTTCCAG- 3′ and 5′-GAGAGGGGATCCATGACTGACAAAATG-3′).

2.2. Protein preparation

FtsH was expressed in *E. coli* strain AR5771 and purified by the procedures described previously (Yamada-Inagawa et al., 2003). Purified FtsH was stored at −80 °C. GST fusion proteins were expressed in DH5α. Cells were grown at 30 °C and expression of GST-flavodoxin was induced by the addition of IPTG (1 mM) at 50 Klett units. After 3 h, cells were harvested by centrifugation at 4 °C, lysed by sonication, and centrifuged for 20 min at 10,000 rpm. The supernatant was loaded on a HiTrapQ column (Amersham Biosciences) in buffer A (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM DTT, and 10% glycerol) and eluted with a linear gradient from 150 mM to 1 M NaCl. Fractions containing GST fusion proteins were loaded on a GSTrap column (Amersham Biosciences) and eluted with buffer A containing 20 mM reduced glutathione. Pooled fractions were loaded on a Superdex 200 HR 10/30 column (Amersham Biosciences). The GST moiety of these GST fusion proteins was cleaved off by PreScission protease (Amersham Biosciences) to produce flavodoxin. These proteins contain an additional five amino-acid (GPLGS) sequence at the N-terminus that is not present in authentic flavodoxin. The reaction mixture was loaded on a Superdex 200 column in buffer A. Purified proteins were stored at −80 °C.

2.3. Preparation of holo- and apo-forms of flavodoxin

Purified flavodoxin was incubated with FMN for 1 h at 4 °C, and dialyzed against a buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, and 1 mM DTT) to prepare holo-flavodoxin. To prepare apo-flavodoxin, FMN was removed from holo-flavodoxin by adding 3% trichloroacetic acid (TCA) in 10 mM DTT, 3 mM EDTA solution on ice for 30 min (Genzor et al., 1996). Precipitated protein was collected by centrifugation at 4 °C and washed with TCA solution twice. White precipitated protein was dissolved with 300 mM Tris–HCl (pH 7.5) and purified by gel filtration using a Superdex 200 HR 10/30 column with buffer A.

2.4. Protein degradation assays

Purified flavodoxin proteins were incubated with FtsH at 27 °C in reaction buffer [50 mM Tris–HCl, pH 8.0, 5 mM $Mg(OAc)_2$, 25 μM $Zn(OAc)_2$, 1 mM DTT, and 0.1% NP-40] plus 3 mM ATP or ADP. At the indicated time points, an aliquot was removed from the reaction mixture, and the reaction was terminated by the addition of the sample buffer of electrophoresis. Samples were analyzed by SDS–polyacrylamide gel electrophoresis (PAGE) followed by