

Lecture 33  
5/7/04

**Proteasome:**

interacting proteins (“lids”)

AAA<sup>+</sup> type ATPases – unfold and translocate protein substrate

Hexameric rings

Remember- proteasome itself is made up of heptameric rings- not symmetry related to lids

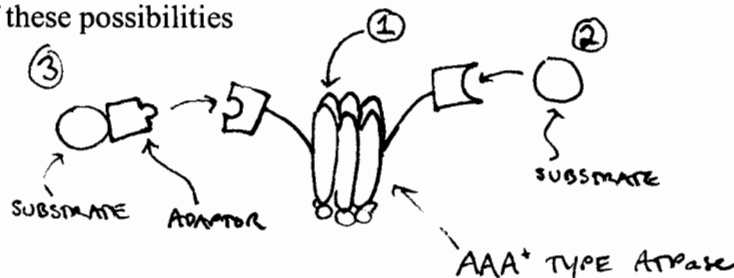
SPECIFICITY (possibilities for interaction with substrate protein)

1) Direct binding to AAA<sup>+</sup> domain

2) 2<sup>nd</sup> domain attached to the AAA<sup>+</sup> domain binds substrate

3) substrate binds to an adapter protein that then binds to the AAA<sup>+</sup> domain

cartoon of these possibilities



Function of ATPase domain

- 1) is it involved in unfolding?
- 2) Does it denature by mechanical force? One big force, many small forces? How is this force generated? Is it associated with a conformational change related to ATP binding energy or ATP hydrolysis energy? [This problem should remind you of the role of GTP in EF-Tu or EF-G.]
- 3) Is translocation ATP dependent? (yes.) Is denaturation coupled to translocation?
- 4) Is the amount of ATP used related to protein stability?

Examples:

ClpXClpP- well studied system (bacteria)

Structure- ClpP is the proteasome

2 kinds of “lids” -> ClpA and ClpX

ClpA- 2 AAA<sup>+</sup> domains

ClpX – 1 AAA<sup>+</sup> domain

Aside: ClpB (structurally homologous to ClpA and ClpX) is involved in pulling apart protein aggregates, interacts with DNAK/DNAJ and not with ClpP.

ClpA, ClpX- different lids, lead to different substrate specificities?

In eucaryotic systems: 19S machine= regulatory complex-RC (AAA<sup>+</sup> ATPase domain)  
PLUS between 15-20 additional proteins (Dynamic interactions)

20S = proteasome and the 19S = RC ; the 20S proteasome and the 19S RC = 26S machine.

The eucaryotic system is orders of magnitude more complicated than the bacterial system.

Exception to the rule that all lids use AAA+ ATPases: Another example of a lid to the human proteasome is P28 (11S) = 2<sup>nd</sup> type of lid (euk)

Heptameric rings > 2 different but homologous subunits

No ATP required or ubiquitin required to observe protein degradation.

WORKING MODEL: See page 14 handout 4a

Tools: Study of the rate of unfolding – **Fluorescence**- (buried W, or GFP). A W is the only useful naturally occurring amino acid. Its fluorescence changes dramatically from hydrophobic interior of a protein to solvent exposure. It increases on entering a hydrophobic environment.

Study of the rate of proteolysis- <sup>35</sup>S labeling of the protein to be degraded.

EXPERIMENTS:

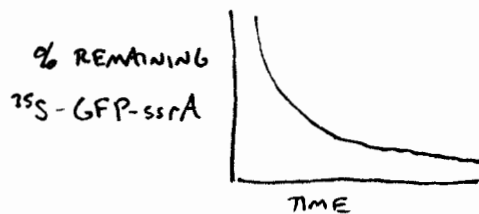
GFP-ssrA, Mol Cell (2000) 5, 639-48 where GFP is green fluorescent protein (see T & D) and then monitor proteolysis using the following protocol.

<sup>35</sup>S labeled GFP (green fluorescent protein) w/ ssrA degradation tag (11 amino acid sequence at C-terminus of GFP. GFP is from jellyfish and is therefore not natural a substrate of bacterial ClpXClpP, ssrA tag targets it to this proteasome)

Quench reaction w/ acid, protein precipitates, peptides are soluble

Compare radioactivity in soluble supernatant vs the precipitated fractions

Plot of remaining <sup>35</sup>S-GFP-ssrA vs. time



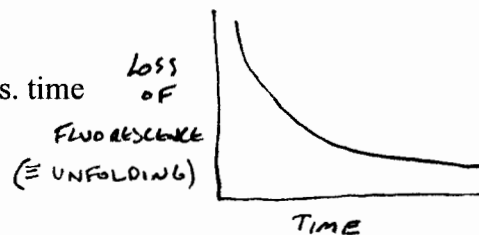
Decreases over time as the protein is degraded

Rate constant is  $4 \text{ min}^{-1}$

Control: treat ClpP w/ isopropylfluorophosphate inhibitor which modifies the active site serine and hence blocks protein degradation

Monitor unfolding

Plot loss of fluorescence at given  $\lambda$  vs. time



Absorption of GFP decreases at a defined wavelength as GFP unfolds in presence of both ClpX and ClpP

Rate constant for unfolding = rate constant for formation of peptides

If the experiment is carried out with ClpX alone, no change in fluorescence is observed, no unfolding- demonstrates importance of ClpXClpP interactions in this process

How important is protein stability to the unfolding process and to the amount of energy required for this process?

How many ATPs are needed?

Titan-ssrA, Cell (2003) 114, 511-28

Is protein stability related to # of ATP needed to unfold/translocate?

Substrate= titan-ssrA 127 amino acid protein targeted to ClpXClpP proteasome w/ ssrA-tag

Single buried tryptophan (W)

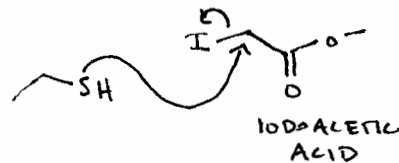
Monitor unfolding through W fluorescence changes (decrease in fluorescence)

See handout 4c

Tested the following analogues of titan

- 1) global mutations of titan that affect stability
- 2) mutations for stability near degradation tag (local stability)
- 3) soluble denatured titan – iodoacetic acid to modify the two cysteines

reaction with iodoacetic acid.



Measure ATP hydrolysis needed for unfolding –both the amount and rate of this process  
Compare with rate of unfolding of titan analogues

Conclusions: depending on the substrate- either denaturation or translocation can be the rate determining step (related to stability of protein)

Next time: how many ATPs are needed? (we will see that the amount is correlated to protein stability)