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**PROFESSOR:** So we have the five-step model. And what we're going to do-- this model was presented last time. And what we'll do is look at experiments that were designed to look at the denaturation, translocation, and degradation processes here.

So one question is, can we separate denaturation from translocation in experiments to learn about the rates of each process. And also, how can we examine the role of ATP? Because that's a question key question here-- how is ATP hydrolysis by ClpXP allowing this macromolecular machine to work?

And so we're going to begin with some experiments that involve a GFP substrate. So these are some studies of ClpXP activity with a substrate that has radio-label GFP ssrA. And so if we think about this substrate here, we have a radio-label-- bless you. We have green fluorescent protein.

And this has a particular fold. So we have a folded substrate that's fluorescent. And here we have our tag that will direct the GFP to the ClpXP degradation machine.

And so this substrate has been used to look at both degradation and unfolding. We'll get to the translocation issue in the second type of substrate we examine today. And so if we think about degradation, this is where the radio-label comes in. And if we think about unfolding, this is where the fluorescence comes in.

And so what we're going to look is at degradation and denaturation assays using this substrate. And so just as a reminder, for anyone not familiar with green fluorescent protein, I might just show you the barrel-like structure here and the chromophores in the interior. And so in order for GFP to fluoresce, it needs to have its proper fold.

And if it's denatured, that fluorescence emission is lost. So let's first look at a degradation assay. So this is experiment one.

So what is the experiment? We have GFP here. And it has this ssrA tag.

And we're going to incubate GFP with ATP and with ClpXP for some period of time. And then we're going to stop this reaction with a quench. And the quench will be acid.

And so if we think about this protein and this degradation process by ClpXP, what are possible products? So maybe there's some GFP ssrA that hasn't yet been degraded, depending on your time point. And we can imagine some of these short polypeptide fragments of seven to eight amino acids.

And so we have this radio-label. And what we want to do is track the radio-label. So here, we have radio-labeled protein. And here, we have radio-labeled peptides.

And so if we want to quantify how much degradation occurred, somehow, we need to separate these. And so what is a way we can do that here-- something simple. And so what you want to think about is just relative solubility under acidic conditions.

So if we have this large GFP that's folded, when that is treated with acid, GFP is going to precipitate. So this will be insoluble. And in contrast, these peptide fragments will be soluble, in most instances.

So as a result, we can take advantage of this differing solubility, effectively to centrifuge the mixture. And we can measure the radioactivity in the pellet and in the supernatant. And then we can quantify degradation here.

And so what are the results? So the result-- here, we can imagine a plot where we have percent of the substrate versus time. And these were conducted over the course of an hour.

And what's observed here-- for instance, we have reactions where the substrate was incubated with ClpXP and ATP. So we see that over time, there's a decrease in the percent of GFP ssrA. And from these data, we can get some degradation rate.

And we'll come back to that degradation rate in a little bit here. So what we see here is degradation. What happens if we add an inhibitor of the protease?

So in the introductory lecture, we talked about a number of different types of inhibitors. And so that experiment was done. And so here, if we take ClpXP plus ATP plus inhibitor, what we see is no degradation.

And the name of this inhibitor is DFP. And effectively, it covalently modifies the serine, in terms

of what was used. So what do we conclude from these data?

If the active site serine of ClpX is covalently modified with an inhibitor, which is diisopropyl fluorophosphate, we lose activity. So that serine is important. So what about unfolding or denaturation?

How can we get at that? So that will be experiment two. And in terms of thinking about denaturation, rather than the radio-label, we're going to think about the GFP.

And so imagine we have our folded GFP-- however we want to show that here-- that has this ssrA tag. So this is folded and fluorescent. So this gets denatured by ClpX of the machine to give us some unfolded polypeptide that has this ssrA tag-- unfolded and non-fluorescent.

And then what happens? This gets degraded. And we get these fragments.

And these fragments are also non-fluorescent. So effectively, s we can perform the exact same assay as we did an experiment one. But we'll look at fluorescence as a readout rather than quantifying radioactivity.

**STUDENT:** So If you take ssrA on any protein, would ClpXP break it down? [INAUDIBLE]

**PROFESSOR:** Yeah, so GFP probably isn't a native substrate of-- definitely not of E. coli ClpXP. What happens in a system that expresses GFP natively, I'm not sure. But yes, this has been a wonderful tool for experiments because many different protein substrates can be modified with this ssrA tag and directed to ClpXP.

This is just one example. So I think broadly, we can think that there are many, many possibilities for what can be delivered. Are there certain proteins that ClpXP just can't deal with? That's a possibility.

So the problem set for the upcoming week has a case where there's a disulfide bond, for instance, and asking what happens when we have some other types of structural features within a designed substrate. But for the purposes of this, yes, we can attach ssrA on to some protein that we can use to study the system and therefore do the experiments. And does that make sense, also, just thinking from the standpoint of what types of polypeptides might get directed to ClpXP in vivo? The ribosome could stall with many different types of proteins being synthesized there. So pretty versatile here.

So we're going to perform the same assay. But we're going to measure fluorescence rather than radioactivity. And so what is the result?

So here, we have fluorescence. And again, now, we have the percent of folded GFP-- 100. And again, we can we can imagine this going down zero to 60 minutes.

So here we have ClpXP. What happens if we have the inhibitor, for instance? What they found-- and I'll draw the inhibitor in a minute because I'm sure some of you are wondering. And here we have ClpX alone.

So how do we interpret these data? So if we have the full machinery-- ClpXP and ATP-- we see a loss in fluorescence over time, which indicates a loss in folded GFP. So the substrate is being denatured.

What about this case here when we only have ClpX present? And also, it won't have ATP. What's happening there?

**STUDENT:** Without ClpP, there's no actual degradation that goes on.

**PROFESSOR:** Do we need to see degradation in this assay? That's true, but what is this assay giving us a readout on? Just unfolding. So what do we learn from that? Rebecca?

**STUDENT:** ClpX needs to be found to ClpP to be in the correct confirmation to unfold.

**PROFESSOR:** Yes, yes. So this indicates that ClpX and ClpP need to be in complex in order to allow unfolding to occur. So thinking to the cellular environment, does not make sense? Yeah, I'm seeing nodding heads. Yes, right.

So we wouldn't want just ClpX to be able to bind and unfold anything it comes into contact with there. And in terms of this inhibitor, we're seeing that it's not unfolding very well. So this inhibitor is for the protease.

Just for that structure, effectively, what we have here-- you actually saw this in the lecture slides from last time. So this is DFP. And effectively, what it does is it will modify a serine side chain to give us this species here. And that will block proteolytic activity.

So how did these data compare? How does the denaturation and degradation data compare? And so we can look at what was done.

And effectively, what we want to ask is, how did the steady state kinetic data compare? And so steady state experiments were done, of course, with varying substrate. And the data were re-plotted.

And so those data are shown here. And what we're looking at on the y-axis is the loss of substrates-- so GFP ssrA versus the concentration of substrate. And what we see is that in circles, we have the fluorescence data. And in triangles, we have the data from radioactivity. So what does this analysis tell us?

**STUDENT:** The data set doesn't look that complete. But it looks like they're on about the same time scale.

**PROFESSOR:** They look very similar. We're getting the same steady state kinetic parameters for both analyses here. And yes, it might be nice to have more data. But that's just not available. So all of these data can be fit to the same kcat and km.

So what do these data tell us about a rate determining step, for instance? Not very much. And we also haven't yet thought about this issue of translocation. We're just seeing the unfolding step and seeing the degradation step in this assay here.

So we need some more information. So if we think about this, we have denaturation versus translocation degradation. And so far, we've been able to look at this and this. And our intuition tells us degradation by the protease should be very fast.

So can we learn something about translocation which we weren't able to see in these experiments here? And so that's what we want to focus on now because there was no readout on this step from experiments one and two here. So is it possible to separate denaturation and translocation with some strategically designed substrates?

**STUDENT:** From this experiment, can't we deduce that translocation step is much slower than denaturation?

**PROFESSOR:** Can we? How? Yeah, there's just no readout because this loss in fluorescence is just telling us that the protein is folded or unfolded. And the degradation is just telling us what happens in the protease chamber.

So what happens from that point-- unfolding to degradation-- in between, we don't know here. So what we need is a new set of substrates that are going to let us get at this and allow us to separate denaturation from translocation experimentally. And so what was the idea for doing

this?

The idea was to take some protein that's been studied and take that protein and a series of mutants of that protein that have also been studied. And the key here is that the mutants of the protein have varying instabilities-- so varying instabilities of the fold. And so you can imagine that there have been many studies of protein folding out there asking the consequences of making point mutations in a given protein fold on stability there.

And so that's exactly what was done. So what we need is a new set of substrates to probe effectively denaturation and translocation in more detail here. And the key question is, is it possible to separate denaturation from translocation?

And so what was done is to take an immunoglobulin-like domain from a protein found in striated muscle that has been the subject of many studies and mutants of this protein and to employ them in assays. So we're going to take a protein plus variants with varying stabilities and perform this assay and compare the data. And so here is the protein that was used as a model substrate.

So shown here, this is the titin I27 domain that has an ssrA tag attached. OK so if we take a look at this protein that has a beta sandwich fold, we see that there's a disulfide bond. There's a single tryptophan residue. And this is helpful because tryptophan residues have intrinsic fluorescence that's sensitive to the environment.

And we see it's buried in the inside here. So in a hydrophobic versus hydrophobic environment, the fluorescence will differ. And so we can use that as a readout of unfolding here.

And this is just an example of data from a prior study where this protein and various mutants of the protein like here, valine 11P Y9P were studied for stability of the fold. So guanidinium, we learned that's the denaturant in the folding section. So these various point mutations have different stabilities. And we can see that in these denaturation curves here.

So what was done in their experiments were very similar to what was done before. So we take this titin radio-labeled-- bless you. So this is experiment three with the ssrA tag. Incubate with ClpXP with ATP and asks what happened.

And in terms of these substrates, we have the wild-type, we have the mutants, as shown up here. And we have CM, you'll see in the data, which is chemically modified. And these

chemically modified variants are completely denatured-- we can consider them.

And so effectively, what was done here with cysteine modification, with iodoacetamide. So we saw that in discussions-- introductory discussions-- about unnatural amino acid incorporation. So the disulfide bond is completely disrupted here. The disulfide can be reduced, the cysteines modified, and we get an unfolded version here for that.

And here what do we find? So there's a number of different point mutants that are listed here. And we're just going to look at a few, in terms of what they found.

So in terms of degradation assay, which is how they did this readout, we're going to have the percent titin remaining. So again, using radioactivity in the supernatant or pellet versus time-- what did they find? So if we take a look at a selection of the data-- just put three examples-- here, what do we have?

Here, we have wild-type. Here, we have one of the mutants, B13P. And here, we have chemically modified wild-type. So what do these data tell us?

**STUDENT:** Degradation is faster. It's [INAUDIBLE]

**PROFESSOR:** Yeah. That's one thing we see here. So this chemically modified protein is denatured. And we see that the denatured protein is easier to degrade by ClpXP than the native protein.

We also see that the mutant is more rapidly degraded than the wild-type. So ClpXP is having an easier time with this one here too. So there's an apparent correlation here between the ease of unfolding and the ease of degradation.

A protein that's already unfolded or is relatively easy to fold is degraded more rapidly than the wild-type protein that has this beta sandwich fold here. If we think about the processes happening in each of these and we think back to that five-step model, what's happening? So here, we have denaturation plus translocation plus degradation.

And likewise, here, we have these three parameters as well. And in this case, we don't have denaturation. We just have the translocation and the degradation here.

**STUDENT:** Why are the rates linear here? And it was not linear in the previous one.

**PROFESSOR:** Just imagine this is-- well, one is a completely different substrate. The time frame, I haven't given here. Don't worry about that. We're just looking at that one part.

So here's the actual data from the report. And now, what we want to do is, using this whole family of substrates-- so the native I27 domain, the various point mutants, and these chemically modified forms, we want to look at the details of the steady state kinetic analysis. And we also want to look at what's going on with the ATPs.

So what is the rate of ATP hydrolysis. And how many ATPs are hydrolyzed? We know nothing about that yet, in terms of the data that's been presented so far.

So what we're going to do is take a look at this dataset and see what we learn here. So there's quite a bit of data in here. But we're just going to systematically work through.

So what do we see? Here, we have all of the different I27 domain-based substrates they used. And the table is divided basically in terms of whether or not the protein was chemically modified.

So on top, we have wild type. And then we have these four-- or sorry, five-- point mutants. And in this bottom half of the table, we have the chemically modified wild type and the chemically modified point mutants.

So these begin with a fold, and depending on the mutation here, there's differing stability of that fold. And here, we have unfolded variants because the disulfide was disrupted. So what are we looking at?

We have degradation, we have km, we have denaturation, and then we have the ATP S rate, and the number of ATPs per I27 domain degraded here. So the question is, what do we learn from each column of data? So if we take a look at these degradation rates here, what do we see?

So what happens amongst the proteins that are not chemically modified? And don't try to over-analyze it, just look for what are the obvious differences here. So what's the slowest?

Wild-type, right? Similar to what we saw here, and that makes sense because wild-type has the most stable fold, just based on what we saw here. And then what do we see for the mutants?

There's variability. And all of these values are greater. How do they compare to chemically modified variants? And what do we see here?

These are the fastest. And they're all pretty similar. So these data agree with what we drew up here.

What about the km values? So are these all similar or different? All pretty similar, yeah.

And why does that make sense? So that indicates that ClpX binds all of these substrates in a similar way. They all have the ssrA tag there.

So we can't attribute any changes we're seeing in rate to this km value here. What about these denaturation rates? So we don't have any values for the chemically modified forms because they're already denatured.

What do we see? We see the wild-type is more difficult to denature-- so the slower rate-- than these point mutations here. And you could imagine if you were the researcher going back and comparing these data to what's known about the relative stabilities of each fold from other data in the literature from studies like that guanidinium denaturation on the prior slide here.

So what about the data in these columns? What do we see? So here, we're looking at ATPase activity.

**STUDENT:** In that case, it's slower and less efficient for wild-type than chemically modified.

**PROFESSOR:** Yes. Yes. That's certainly the case. So, first, if we look at wild-type, and even for that matter, these single point variants, versus these chemically modified forms, we see that the wild-type has a value of about 150 per minute. And these are slightly higher.

We see these are on the order of about 600 per minute. So in a way, these fall into two groups-- the chemically modified forms defined one group. And this wild-type and single point mutants define another group here.

And the wild-type has the slowest ATPase right here. And then in terms of efficiency, as you mentioned-- maybe that's in terms of the number of ATPs degraded-- what do we see here? What is incredibly striking about these data?

We're seeing about 600 ATPs for I27 domain degraded for this wild-type that's a huge number of ATPs-- so 600. What do we see for these denatured variants? They're all around 115 ATPs per substrate consumed here.

So many, many ATPs are consumed here. Many ATPs are required to denature that native substrate. And it looks like many ATPs are required for translocation here. And if the substrate is less stable, what we see is that fewer ATPs are consumed.

So these are all filled in within your notes. And there's some additional details here. So these data indicate that the easier the protein unfolds, the faster it's degraded.

And just to reiterate, these denatured titins, we can think about ATP consumption as being indicative of that translocation event because they're already denatured. And for these native titin, the rate of ATP consumption is indicative of both the unfolding or denaturation process and the translocation process here for that. Here is just another way of plotting the data in the table, where they're just highlighting ATP hydrolysis and then the different types of substrates here.

So we see the rate's highest for denatured and that it decreases with increasing stability of the substrate to degradation by ClpXP. Another interesting thing they found in these studies is that the ATP is consumed very linearly with time. So if we look at ATP consumed versus the average denaturation time-- here, wild-type, and down in this region, the mutants.

So we have a linear relationship. And what came out of this is about 144 ATPs consumed per minute of unfolding from these experiments here. So what does this tell us about how ClpXP works-- how it works here?

So basically, this machine has been described as having a relentless try and try again mechanism here. And it's effectively explained in this cartoon. So ClpP is omitted, but imagine it's there. So what's happening?

We have some folded protein that's been condemned and has the ssrA tag attached. And so ClpXP needs to deal with it. There's the tag-mediated substrate binding. So the substrate binds, there can be ATP hydrolysis.

And that results in ClpX trying to unfold the protein. But frequently, the substrate can get released. And this cycle of binding and pulling can happen many, many times.

And that consumes a lot of ATPs here. And then at some point, there's going to be a successful unfolding event, which results in the polypeptide being translocated and entering the degradation chamber. So when thinking about a hard to denature substrate, you want to think about this substrate binding ClpX many times.

There might be multiple instances of binding and release before it's successfully denatured and before translocation occurs. And so that uses a lot of ATPs.

**STUDENT:** Does it all confine the substrate to many different places or just in one spot?

**PROFESSOR:** So the ssr tag is what's going to allow it to bind to the pore. And recall, for instance, there can be the adapter protein SspB to help ssrA tag proteins make their way to the pore. So think of it less as some undesirable protein-protein interaction than a failed attempt at unfolding by this ATPase here. So why might ClpX want to do this? When we think about the cell-- just some possibilities?

**STUDENT:** It would make sense, I guess, the more unstable the protein is, the easier it is to degrade it because proteins that are more unstable are already partially unfolded and are probably ones that need to be degraded.

**PROFESSOR:** Yeah, so that's one way to think about this. And then maybe another way to phrase that is perhaps this helps to avoid jamming the protease. If there's things that need to be degraded versus other things, if you have something that's very difficult to degrade, you don't want that to block the protease such that something unfolded can't be dealt with. Also, just dealing with a mixture, that maybe ClpXP likes to get rid of the substrates that are easiest to degrade first.

So is it energetically wasteful there-- just to think about. On one hand, it might seem like it. So many ATPs-- just think back to the TCA cycle, for instance, and how many ATPs you get from one cycle there versus 600 ATPs being consumed here for that wild-type titin domain. But this makes sense because in the cell, it does have to deal with many different types of substrates. And these substrates can have varying structure and varying stabilities.

So how does ClpX actually work? What's going on with this ATP hydrolysis? How are denaturation and translocation coupled?

And how do we even think about this translocation process? Effectively, we saw the cartoons, where it looked like ClpX was somehow pulling on this polypeptide. And so we'll close with some discussion about that, which we'll continue on Monday.

So effectively, we have our general paradigm of somehow having ATP hydrolysis leading to conformational change that provides some mechanical work. And so here in this system, conformational change in ClpX will drive unfolding and translocation.

And of course, the big question is how? And so a key observation that's not intuitive with this system and that we'll build upon in the first 10 minutes or so of Monday is the fact that ClpX is a homohexamer. We saw that when we went over the structure.

But this hexamer has some inherent asymmetry to it, despite the fact that each subunit is the same. So a key observation here-- ClpX is homohexamer. But it has inherent asymmetry.

And this asymmetry arises from nucleotide ATP binding. And the observation from a variety of studies is that ATP binds to some of the ClpX subunits but not others. OK And also it can bind to different subunits with different affinities, just as another detail.

And what we'll see is that this is also dynamic-- so just some subunits. So although we think about this as a homohexamer in terms of the ATP loading at different points, we don't have six ATPs bound. And where we'll begin on Monday is looking at individual ClpX subunits and then how the ClpX subunits work together and lessons learned from studies there. So effectively, this asymmetry is thought to be quite important, in terms of how ATP hydrolysis is allowing the movements and activity of the ATPase.